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Cytoprotective effect of fruit extracts associated with antioxidant activity against ultraviolet rays

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Abstract

Antioxidant activity in the extracts of Redfield apple flesh, Pione grape berry skin, and Ishiji satsuma mandarin skin was evaluated by a superoxide dismutase (SOD) assay and hemolysis of red blood cell assay. Although SOD activity was higher in both the Redfield apple flesh and Pione grape berry skin, hemolysis of the red blood cells was delayed most in the Redfield apple flesh, followed by the Pione grape berry skin and Ishiji satsuma mandarin skin. The protective effects of extracts from these fruits against ultraviolet rays were also examined using human skin dermal fibroblasts. The Redfield flesh extract increased the percentage of cell viability against ultraviolet rays compared to the untreated control. The participation of polyphenolics in Redfield apples is discussed.

Keywords: Antioxidant activity; Apples; Ultraviolet rays

1. Introduction

Plant-derived antioxidants scavenge free-radicals, which are associated with cancer or heart disease in the human body (Ames, Shigena, & Hagen, 1993), and are found in abundance in fruit. For example, polyphenolics are powerful antioxidants, and apple and grape berry fruit contain large amounts of phenolic compounds (Hamauzu & Iijima, 1999; Kohno, Yamada, Mitsuta, Mizuta, & Yoshikawa, 1991). In addition to polyphenolics, carotenoids, flavonoids, and vitamins also have antioxidant activity, and *Citrus* fruits are rich in these latter three antioxidants (Kondo, Katayama, & Uchino, 2005). However, these antioxidants are found in different concentrations and in different kinds of fruit and cultivars, and their antioxidant activities vary (Kondo, Tsuda, Muto, Nakatani, 2002). Therefore, the abilities of different fruit in preventing the diseases caused by free-radicals may also vary. In previous reports (Kondo, Tsuda, Muto, Nakatani, 2002; Kondo, Tsuda, Muto, Ueda, 2002), we investigated free-radical scavenging activities in different kinds of apples and *Citrus* fruits, and found that Redfield apples, Ishiji satsuma mandarins, and Pione grapes had high antioxidant activities. In general, fruit skin contains a higher concentration of antioxidant substances than the flesh of the fruit (Awad, de Jagar, van der plas, & van der krol, 2001). However, the skin of *Citrus* and grape berries generally are not edible in Japan, and the Redfield apple, which is classified as a cider apple, is not edible because of its bitter taste. The natural antioxidants such as polyphenolics in apples and berries and carotenoids in *Citrus* without impurities from synthesis may be used as safe antioxidant substances. In this study, we investigated the utilization of fruit-derived antioxidants to protect against ultraviolet (UV) rays.

2. Materials and method

2.1. Plant materials

Redfield apples [*Malus sylvestris* (L.) Mill. *var. domestica* (Borkh) Mansf.] and Pione grape berries (*Vitis* spp.) were selected from three randomly chosen 15-year-old

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trees, all growing in an open field at the Prefectural University of Hiroshima. In addition, Ishiji satsuma mandarins (*Citrus unshiu* Marc.) were harvested from three 13-year-old trees growing in an open field at Hiroshima Prefectural Agriculture Research Center.

2.2. Hemolysis of red blood cell assay

Samples of 0.5 g flesh weight (FW) were extracted with 13.7 M ethanol. After filtration, the extract was made up to a constant volume of 10 ml with 13.7 M ethanol. This assay analyses the ability of antioxidants in samples to retard the hemolysis of red blood cells induced by a peroxyl radical generator, AAPH. Sheep blood cells (Nippon Bio-Supp. Center, Tokyo) were used for this experiment. Phosphate buffered saline (PBS) of 10 mM consisting of a phosphate buffer solution (pH 7.2) and 0.85% (w/v) sodium chloride was used. An underlayer of red blood cells centrifuged at $3000g_n$ for 10 min was suspended by adding an equal amount of PBS and adjusting to an absorbance of 1.0 with distilled water. The reaction mixture contained 0.29 ml PBS, 0.2 ml 0.5 M AAPH, 0.5 ml suspended red blood cells, and 10 μl sample solution in a final volume of 1 ml. The sample solution, suspended red blood cells, and PBS were preheated at 37 °C for 5 min. The reaction was initiated by the addition of AAPH. Absorbance was recorded every 15 min at 550 nm with a spectrophotometer (Hitachi, U-2001). The untreated control consisted of suspended blood cells, AAPH, and PBS. PBS (0.2 ml) was added instead of AAPH as a blank. Absorbance of the blank was subtracted from each sample absorbance, and the rate of hemolysis calculated after each measurement.

2.3. Assay of SOD

The 5 g FW samples were homogenised in 30 ml 0.1 M potassium phosphate buffer (pH 7.5) with 0.1 mM ethylenediamine-*N,N,N′,N′*-tetraacetic acid disodium salt dihydrate (EDTA). The samples were purified by a Sephadex G25 PD-10 column (Amersham Pharmacia Biotech., Uppsala, Sweden) with EDTA, hypxanthine, potassium phosphate buffer (pH 7.2), SOD, WST-1, and xanthin oxidase. The analyses using a microplate reader (Tecan, Mannedorf, Austria) were carried out according to the method described previously (Kondo et al., 2005).

2.4. Cell viability assay

Human skin dermal fibroblasts DUMS-16 were kindly supplied by Dr. Masayoshi Namba of Okayama University. Dulbecco's modified Eagles's minimum essential medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen Japan (Tokyo). The cells were grown to the logarithmic phase in DMEM-10% FBS, and were seeded at densities of 0.4–5 (×) 10^4 cells/2.0 cm^2 in a 24-well microplate so as to proliferate to the subconfluent state upon UV irradiation.

The cytoprotective effects of the fruit-derived extract solution against UV cell injuries were examined. The fruit-extracted solution was added to human skin fibroblasts at 18 h after seeding. Fruit samples (1 g FW) were homogenized in 13.7 M ethanol, filtrated, and lyophilized. The solution was diluted in 0.04%, 0.07%, and 0.1% concentration with distilled water. The fruit-extracted solution (200 μl) was mixed with DMEM-10% FBS, which was gently homogenized with a potter-type teflon homogenizer and then poured into the top of the cell layer in a 2 cm^2 well. The control cells received the culture medium without the fruit-extracted solution, and were similarly treated. The cells were then irradiated with a Spectronics UVB transilluminator EBF-260 (1.0 mW/cm^2 unless otherwise described) at a dose of 45 mJ/cm^2 and with a Yamashita Denso UVA irradiator Hypercure 200 at a dose of 28.92 J/cm^2 under moistening conditions so as to prevent the cells from drying. After aspiration and rinsing with Ca^{2+}, Mg^{2+} – free phosphate buffered saline (PBS), the cells were further cultivated in DMEM-10% FBS, followed by the cell viability assay.

UV-irradiated or control cells underwent aspiration of the medium and received 300 μl of phenol red-free DMEM-10% FBS and 30 μl of solution of the formazan-forming redox indicator WST-1 (Wako, Osaka), as previously described (Hayashi, Takeshita, Nagao, Nikaido, & Miwa, 2001). After 3 h incubation, the medium that was transferred into a 96-well microplate and cell viability was measured with a Bio-Rad microplate photometer model 3550 (Tokyo).

2.5. Measurement of polyphenolics

Fruit samples (5 g FW) were homogenized with 40 μg of 2′,4′,6′-trihydroxyacetophenone monohydrate as the internal standard in 30 ml 13.7 M ethanol. The extract was filtered and evaporated to the aqueous phase in vacuo, then the aqueous phase was adjusted to pH 2.5 using 0.1 M phosphoric acid. Polyphenolics were then extracted with ethyl acetate, with the solvent removed in vacuo, and the sample redissolved in 10 ml distilled water. Polyphenolics were measured using high performance liquid chromatography (HPLC) (Gulliver series; JASCO, Tokyo) equipped with a ODS column: mobile phase of methanol with 10 mM acetic acid (0–14 min, 0.5–6 M methanol; 14–25 min, 6–8.6 M; 25–35 min, 8.6–13.6 M; 35–55 min, 13.6–0.5 M); flow rate of 0.7 ml min^{-1}; detector = UV 280 nm. Total phenolics were analyzed with the Folin–Ciocalteau reagent according to a previous report (Kondo, Tsuda, Muto, Ueda, 2002).

2.6. Statistical analysis

In Fig. 1, the data were subjected to regression analysis. In Figs. 2 and 3, the data were subjected to analysis of variance procedures (ANOVA) and separated by standard deviation (SD) and by LSD, $p \leqslant 0.05$ (SAS, Cary, USA).

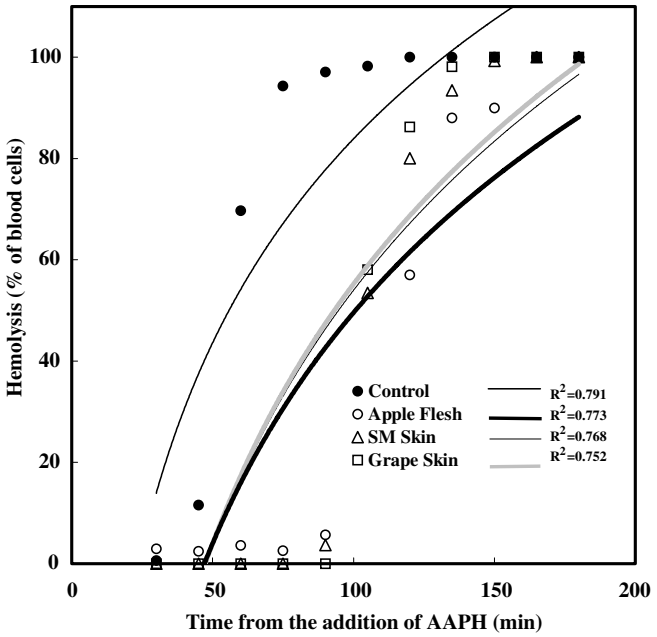

Fig. 1. Effects of extract from Redfield apple flesh, Ishiji satsuma mandarin skin, and Pione grape berry skin on the hemolysis of red blood cells induced by a peroxyl radical generator, AAPH. Data are the means of three replications.

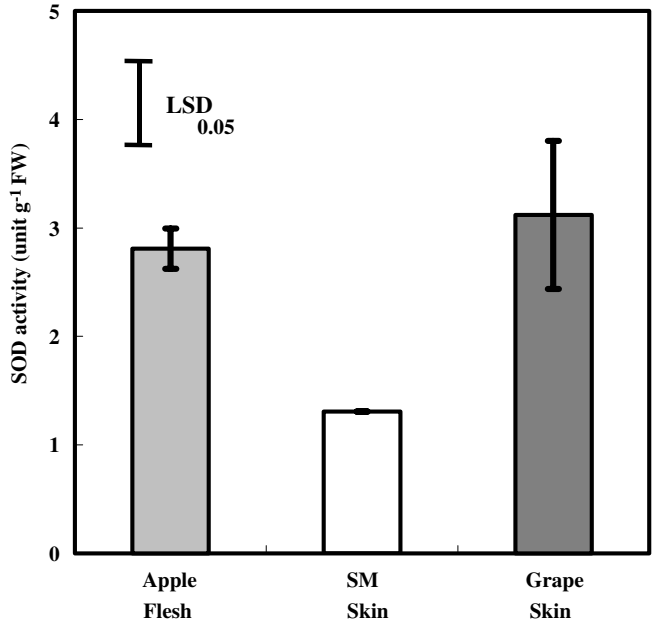

Fig. 2. SOD activity in Redfield apple flesh, Ishiji satsuma mandarin skin (SM), and Pione grape berry skin. Data are the means of three replications.

The data in Table 1 were subjected to ANOVA and separated by LSD as well as shown in Figs. 2 and 3.

3. Results and discussion

The level of total polyphenolics, including accumulated anthocyanin, in the flesh of Redfield apples was three to six times higher than in other apple cultivars. In addition, there were only slight differences in the concentrations of polyphenolic in the skin and flesh of these apples (Kondo, Tsuda, Muto, Nakatani, 2002). Therefore, we only used the flesh of Redfield apples in our study. The hemolysis of red blood cells was delayed most in the extracts from Redfield apples, followed by the extracts of the Ishiji satsuma mandarin and Pione grape berry skin (Fig. 1). In contrast, the SOD activities in the flesh of Redfield apples and in the skin of Pione grape berries were higher than that in the skin of the Ishiji satsuma mandarin (Fig. 2). SOD catalyzes the dismutation of O_2^- to O_2 and H_2O_2; therefore, Redfield and Pione have higher scavenging activity against O_2^- compared with Ishiji. Although the total polyphenolic concentrations in the skin of Ishiji satsuma mandarin were around 21 mmol/kg fresh weight (FW) at harvest, those in the flesh of the Redfield apples and in the skin of the Pione grape berries were around 27 and 46 mmol/kg FW, respectively (Table 1). The addition of (+)-catechin and (−)-epicatechin to scavenge O_2^- and DPPH radicals were more effective than ascorbic acid and β-cryptoxanthin (Kondo, Yoshikawa, & Katayama, 2004). Although the skin of Ishiji satsuma mandarin is rich in ascorbic acid and β-cryptoxanthin, the polyphenolic concentrations are lower than in the apples and grape berries. Therefore, the component of antioxidant substances may be caused by SOD activities and hemolysis of red blood cells in the three types of fruit. Furthermore, free-radical scavenging activity also depends on the kind of polyphenolics (Yanagida, 1997). Phloridzin has almost no antioxidant activity, but (+)-catechin, (−)-epicatechin, and chlorogenic acid have strong antioxidant activity (Kondo et al., 2004; Lee, Kim, Kim, Lee, & Lee, 2003). In fact, in Redfield apple extracts, which delayed longest the hemolysis of red blood cells, the concentrations of chlorogenic acid, (+)-catechin, and (−)-epicatechin were higher than in Isiji satsuma mandarin and Pione grape berry skin extracts (Table 1). However, the polyphenolic concentration of each type of fruit was lower compared to the levels in total phenolic concentration, because several phenolics in apples were present as glycosides (Lee et al., 2003).

UV rays generate free-radicals in cells and result in cell death. UV-A required a dose 1800 times stronger than UV-B to cause cell death (Hayashi, Kayasuga, Nagao, & Miwa, 2000). The effects of UV-A in inducing cell death are 120–600 times smaller than UV-B on the ground, because the amount of UV-A that reaches the ground is 3–15 times more than UV-B. Therefore, in our study, the dose of UV-A was 640 times higher than that of UV-B, and the effects of extract from the fruit on cell death was compared between UV-A and UV-B treatment. The extracts from the skin of Ishiji satsuma mandarin and Pione grapes did not have significantly different effects on cell viabilities compared with the control (Fig. 3). In contrast, the extracts from the Redfield apple flesh significantly increased cell viability. That is, only the concentrations of 0.07% and 0.1% of the extracts had an effect on both UV-A and UV-B.

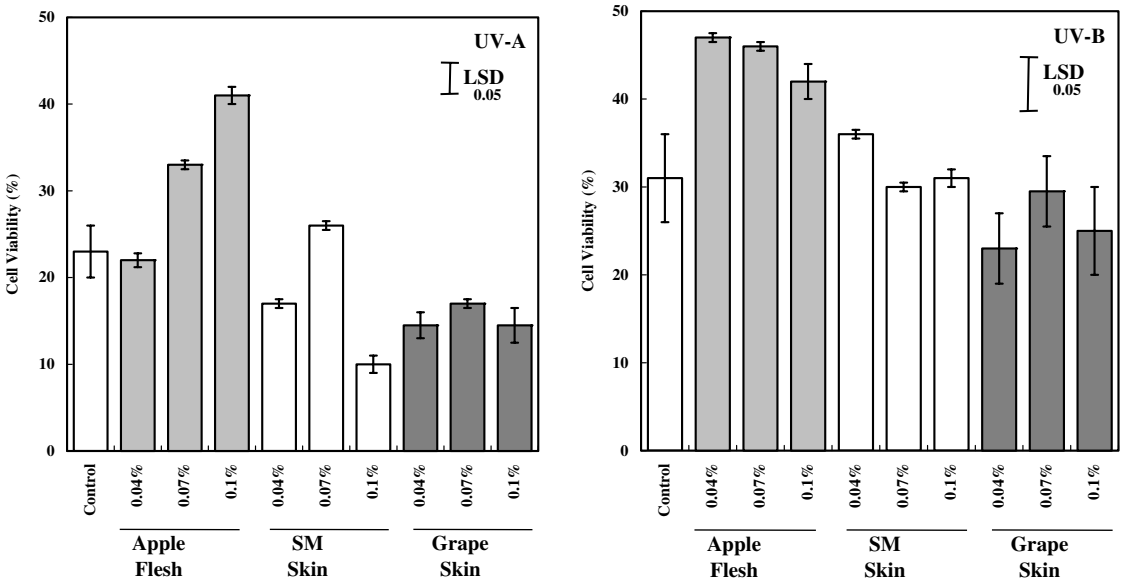

Fig. 3. Effects of extract from Redfield apple flesh, Ishiji satsuma mandarin skin (SM), and Pione grape berry skin on viability of human skin dermal fibroblasts DUMS-16 cells which affected by ultraviolet A and B. Data are the means of three replications.

Table 1
Polyphenolic compositions in Redfield apple flesh, Ishiji satsuma mandarin skin, and Pione grape berry skin

	Chlorogenic acid ($\mu mol\ kg^{-1}$ FW)	(+)-Catechin ($\mu mol\ kg^{-1}$ FW)	(−)-Epicatechin ($\mu mol\ kg^{-1}$ FW)	Phloridzin ($\mu mol\ kg^{-1}$ FW)	Total phenolics ($mmol\ kg^{-1}$ FW)
Red field					
Apple flesh	361	293	1480	99.1	26.7
Ishiji					
Satsuma mandarin skin	95.1	43.7	36.6	90.0	20.9
Pione					
Grape berry skin	6.69	58.5	47.8	72.4	45.9
LSD (0.05)	15.2	11.3	12.5	16.3	2.25

Data are the means of three replications.

Our previous report (Hayashi et al., 2000) showed that the application of UV-A and UV-B was caused by the production of free-radicals in the nucleus. Therefore, it is considered that the increase of cell viability by the extract from Redfield apples against UV rays may be associated with the scavenging of free-radicals. This can be supported by the fact that the hemolysis of red blood cells was delayed most during the extraction from the Redfield apples, although the SOD activities between the Redfield apples and Pione grape berries did not differ. Apple fruit contains a great deal of condensed apple tannin. Condensed apple tannin is composed of only (−)-epicatechin and (+)-catechin (Guyot, Doco, Souquet, Moutounet, & Drilleau, 1997), and does not contain polymerized gallo catechin and gallic acid, which are found in grape berries (Kohno et al., 1991; Yanagida, 1997). In general, polyphenolics are not easily soluble in water, but the degree of dissolution of condensed apple tannin is high (Yanagida, 1997). Thus, the differences in the dissolution of polyphenolics between apples and grape berries may have influenced the results of the cell viability assay. A characteristic such as polyphenolic compounds in apples may retard the activities of free-radicals and may be connected to the increase of cell viability against UV. Free-radicals cause UV rays to damage DNA, and then cause dermatitis (Hayashi et al., 2001). UV rays therefore increase the production of melanin (Tsukamoto, 2005). Thus, the utilization of the extract from Redfield apples in cosmetics is expected to protect people from UV rays.

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References

Ames, B., Shigena, M. K., & Hagen, T. M. (1993). Oxidants, antioxidants and the degenerative diseases of aging. *Proceeding of National Academic Science USA, 90*, 7915–7922.

Awad, M. A., de Jagar, A., van der plas, L. H., & van der krol, A. R. (2001). Flavonoid and chlorogenic acid changes in peel of 'Elstar' and 'Jonagold' apples during development and ripening. *Scientia Horticultura, 90*, 69–83.

Guyot, S., Doco, T., Souquet, J. M., Moutounet, M., & Drilleau, J. F. (1997). Characterization of highly polymerized procyanidins in cider apple (*Malus Sylvestris* var. Kermerrien) skin and pulp. *Phytochemistry, 44*, 351–357.

Hamauzu, Y., & Iijima, E. (1999). Polyphenolic composition and antioxidative activity of apple flesh extracts. *Journal of Japanese Society for Food Science Technology, 46*, 645–651.

Hayashi, S., Kayasuga, A., Nagao, N., & Miwa, N. (2000). System for development of skin protect ants against ultraviolet ray. *Fragrance Journal, 8*, 81–86.

Hayashi, S., Takeshita, H., Nagao, N., Nikaido, O., & Miwa, N. (2001). The relationship between UV-B screening and cytoprotection by microcorpuscular ZnO or ascorbate against DNA photodamage and membrane injuries in keratinocytes by oxidative stress. *Journal of Photochemistry and Photobiology, 64*, 27–35.

Kohno, M., Yamada, M., Mitsuta, K., Mizuta, y., & Yoshikawa, T. (1991). Spin-trapping studies on the reaction of iron complexes with peroxides and the effects of water-soluble antioxidants. *Bulletin of the Chemical Society of Japan, 64*, 1447–1453.

Kondo, S., Katayama, R., & Uchino, K. (2005). Antioxidant activity in meiwa kumquat as affected by environmental and growing factors. *Environmental and Experimental Botany, 54*, 60–68.

Kondo, S., Tsuda, K., Muto, N., & Nakatani, S. (2002). Changes in antioxidant activity during fruit development in citrus fruit. *Horticulture Research, 1*, 63–66.

Kondo, S., Tsuda, K., Muto, N., & Ueda, J. (2002). Antioxidative activity of apple skin or flesh extracts associated with fruit development on selected apple cultivars. *Scientia Horticulturae, 96*, 177–185.

Kondo, S., Yoshikawa, H., & Katayama, R. (2004). Antioxidant activity in astringent and non-astringent persimmons. *Journal of Horticultural Science and Biotechnology, 79*, 390–394.

Lee, K. W., Kim, Y. J., Kim, D. O., Lee, H. J., & Lee, C. Y. (2003). Major phenolics in apple and their contribution to the total antioxidant capacity. *Journal of Agricultural Food Chemistry, 51*, 6516–6520.

Tsukamoto, K. (2005). Mechanism of a liver spot. *MB Derma, 98*, 9–15.

Yanagida, A. (1997). Antioxidativity and other functions of apple polyphenols. *Fragrance Journal, 4*, 63–69.