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Comparison of Antiproliferative and Antioxidant Properties among Nineteen Apple Cultivars

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***Abstract*. Aqueous ethanol extracts prepared from 19 apple (*Malus* ×*domestica* Borkh.) cultivars were studied to explore their antiproliferative activity. Half of them showed strong inhibition on proliferation of human leukemic HL-60 cells, while the others were weak. Total polyphenols, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, and total anthocyanins were measured and the results indicated that the antiproliferative activity was more strongly correlated to the polyphenols and radical scavenging activity than to the anthocyanin content. Several polyphenols in 'Jonathan' were identified and quantified by high-performance liquid chromatography (HPLC) analysis. Among those compounds found during HPLC, catechin and epicatechin seemed partially responsible for HL-60 antiproliferation. A careful examination on parentage of the apple cultivars tested revealed that 'Jonathan' and its progeny showed high antiproliferation toward HL-60. This is the first observation about the relationship between antiproliferative activity and parentage of apples, and the information would be useful to create new apple cultivars that posses more anticancer potential.**

Apples (*Malus* ×*domestica* Borkh.) are recently indicated to have many health-promoting activities, especially anticancer, antiradical, and antioxidant activities—most of these activities are believed to be due to their polyphenolic ingredients (Boyer and Liu, 2004; Eberhardt et al., 2000). On the other hand, many apple cultivars have historically been developed to achieve high production, better taste and flavor, longer shelf life, and reduction of labor. The health-promoting activities of apples can be influenced by their chemical components, which are the products of genetic interpretation; thus, the effect of apple cultivars on biological activities and mode of genetic inheritance of the activities were set as the object of our interest. To investigate these, we examined antiproliferative and antiradical activities of 19 apple cultivars, including the important ancestors in apple breeding and the economically important progeny. Antiproliferative activity was studied using human leukemia HL-60 cells and antiradical activity was examined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging system.

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Materials and Methods

Reagents. 2-Amino-2-hydroxymethyl-1,3-propandiol (Tris), 2,6-di-*tert*-butyl-4-methylphenol (BHT), DPPH, Folin-Ciocalteu reagent, catechin, chlorogenic acid, epicatechin, gallic acid, kaempferol, phloretin, quercetin, and quercitrin were purchased from Wako Pure Chemicals Industry (Osaka, Japan). Phlorizdin dihydrate was from MP Biomedicals, Inc. (Irvine, Calif.).

Apple extracts. All apple cultivars were maintained in the field of the Department of Apple Research, National Institute of Fruit Tree Science (Iwate, Japan). Apples were harvested ripen (suitable for picking) on September to November 2001 from the same growing block. The harvested dates for each cultivar are listed in Table 1. In total, 300 g of fresh fruit (two to three fruit from each cultivar) were homogenized in 300 mL of ethanol. The ethanol extract was filtrated, concentrated *in vacuo* to remove ethanol, and dissolved in distilled water to give 100 mL of apple extract, which was used as stock solution (the concentration was 3 g fresh fruit equivalent/mL H_2O).

Cells. HL-60 cells were obtained from the Riken Gene Bank (Tsukuba, Japan), and were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS). HL-60 cells in log phase (about 10^6 cells/mL) were diluted to 1.2×10^5 cells/mL and preincubated for 18 h in 24-well plates (about 2×10^5 cells/mL).

Cell proliferation assay. The level of cell proliferation was measured by using alamar Blue (Biosource International, Lewisville, Texas), an oxidation-reduction indicator. The level of proliferation was measured for HL-60 cells grown in 96-well microtiter plates. Triplicate plates were prepared. To each well 5×10^3 cells/100 μL of HL-60 cell suspension was added, grown for 24 h, and then mixed with 100 μL of medium containing serial dilution of samples to be assayed. Usually 200 μL of filter-sterilized apple extract (1/10 diluted from the stock solution) was mixed with 600 μL of culture medium, and 4-fold serial dilution was made in microtiter plates. Water-insoluble standard samples were dissolved in DMSO (dimethylsulfoxide), and 8 μL of the solution was added to 1 mL of the medium, then 4-fold serial dilution were made in the microtiter plates, so that the final DMSO concentration did not exceed 0.4% (v/v). After 3 d of incubation, 20 μL of alamar Blue was aseptically added to each well, and incubated for 6 or 24 h. Cellular proliferation (% of untreated positive control) was calculated with Eq. [1]:

$$\text{Proliferation } (\%) = \frac{\{A_{570} - A_{595}\}\beta \text{ sample dilution} - \{A_{570} - A_{595}\}\beta \text{ blank}}{\{A_{570} - A_{595}\}\beta \text{ untreated positive control} - \{A_{570} - A_{595}\}\beta \text{ blank}} \times 100$$

where A_{570} and A_{590} are the absorbance at 570 nm and 595 nm, respectively.

Total polyphenol analysis. The total phenolics were determined by Folin-Ciocalteu reagent primarily according to the method described in the literature (Prior et al., 1998; Slinkard and Singleton, 1977), that was modified to use 96-well microtiterplate. To 20 μL of 1/100 diluted sample from the stock solutions or 50, 40, 30, 20, and 10 $mg \cdot L^{-1}$ and a 0-blank of standard series from gallic acid solutions in 96-well microtiter plates were added 100 μL of 1/100 diluted Folin-Ciocalteu stock reagent,

followed after 5 min by the addition of 80 µL of 7.5% (w/v) Na_2CO_3 solution. After 1 h at room temperature, a microplate reader (Benchmark Plus, BioRad Laboratories) measured the absorbance at 765 nm. The results were expressed as milligrams of gallic acid equivalent per gram fresh fruit.

Total anthocyanin analysis. The total anthocyanin was estimated by a pH differential method (Cheng and Breen, 1991). Absorbance was measured at 510 and 700 nm in the mixture of 1/10 diluted stock solutions and buffers of pH 1.0 and 4.5, using $A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$ and molar extinction coefficient of cyanidin-3-glucoside of 29,600. The results were expressed as micrograms of cyanidin-3-glucoside equivalent/g fresh fruit.

DPPH radical scavenging activity. The scavenging activity of apple extracts against DPPH radical was measured according to the method described previously (Yamaguchi et al., 1998). Each 0.05 mL of 1/100 diluted stock solutions of apple was added to 1.95 mL of 100 mM Tris-HCl buffer (pH 7.4) and 3.0 mL of 100 µM DPPH in ethanol, and the mixture was kept at 25ºC under dark for 20 min. The absorbance at 517 nm was measured. Deionized water was used as blank experiment, and BHT (5 µg·mL^{-1}) was used as positive control. The scavenging activity of DPPH radical (%) was calculated with Eq. [2]:

$$\text{Scavenging activity (\%)} = \frac{A_{517\,\text{DPPH Blank}} - A_{517\,\text{DPPH Sample}}}{A_{517\,\text{DPPH Blank}}} \times 100$$

Each assay contains extract derived from 1.5 mg fruit.

Identification and quantification of polyphenols. Identification of polyphenols was performed by HPLC according to the method modified from literature (Lattanzio et al., 2001). A HPLC (L-7100; Hitachi) equipped with a UV detector, an autosampler, and an integrator was used. The analysis was performed on a Cadenza CD-C18 (150 mm × 4.6 mm i.d.) (Imtakt Corporation, Kyoto, Japan) with the solvent flow 1.0 mL·min^{-1}. Mobile phase A was 5.0% acetic acid and B was methanol. The binary linear gradient method was used as follows: 1) linear increase in B from 5% to 15% from 0 to 20 min, 2) linear increase in B from 15% to 40% from 20 to 30 min, and 3) linear increase in B from 40% to 99% from 30 to 50 min. After washing the column with 99% methanol in 5.0% acetic acid, the program turned to initial condition and the system was re-equilibrated for 10 min. The peaks were compared with those of the apple polyphenols described in the literature (Lattanzio et al., 2001; Lee et al., 2003; Tsao et al., 2003; van der Sluis et al. 2001), and the amounts were calculated from standard curves obtained from authentic samples. The antiproliferative activities of these polyphenols were also examined according to the method described above.

Results and Discussion

Our group had been interested in anticancer activity of various fruit earlier (Yoshizawa et al., 2000, 2004). In this paper, the important ancestors in apple breeding program and their progeny were examined for their antiproliferative and antiradical activities. Figure 1 summarizes the antiproliferative activity of 19 apple cultivars based on their ED_{50} (50% effective dose) values. Eleven cultivars strongly inhibited the cellular proliferation of HL-60 with ED_{50} values of <20.0 mg fresh fruit equivalent per well of microtiter plate. Table 1 summarizes the DPPH radical scavenging activity of apple cultivars, and indicates 14 cultivars showed potent radical scavenging activity, whereas 'Akane', 'Kitaro', 'Kotaro', 'Mutsu (bagging), and 'Sansa' had only weak activity.

It is very natural for us to speculate that the antiproliferative and DPPH radical scavenging activities due to the polyphenolic components, since much literature reported the anticancer and antiradical effect of polyphenolic compounds. Thus, total polyphenols and total anthocyanins were measured by the methods described in Materials and Methods, and the relationships between antiproliferative activity and contents of polyphenols and anthocyanins are summarized in Fig. 2a and b, respectively. The results showed that the antiproliferative effect was more negatively correlated to the total polyphenols (R = –0.66) than to total anthocyanins (R = –0.33), whereas no significant correlation was found between DPPH antiradical activity and total polyphenols (R = –0.07) and total anthocyanins (R = 0.01). However, there was a significant relationship (R = –0.26) between antiproliferative activity and DPPH antiradical activity, as indicated in Fig. 2c, suggesting the involvement of other unidentified compounds on antiproliferative and antiradical activities. Fruit color had no effect on antiproliferative and antiradical activities.

According to the antiproliferative activity, the apple cultivars were classified as those with strong (EC_{50} < 3 mg), medium (EC_{50} was 3 to 5 mg), and weak activity (EC_{50} > 5 mg). The results summarized in Table 2 demonstrated that 'Jonathan' and the most of its progeny were classified as the cultivars with strong activity.

A close examination of parentage relationships of 'Jonathan' revealed a characteristic feature. As indicated in Table 2, 'Jonathan' is the parent of 'Akane', 'Tsugaru', 'Jonagold', 'Himekami', and 'Hokuto' and is the grand-

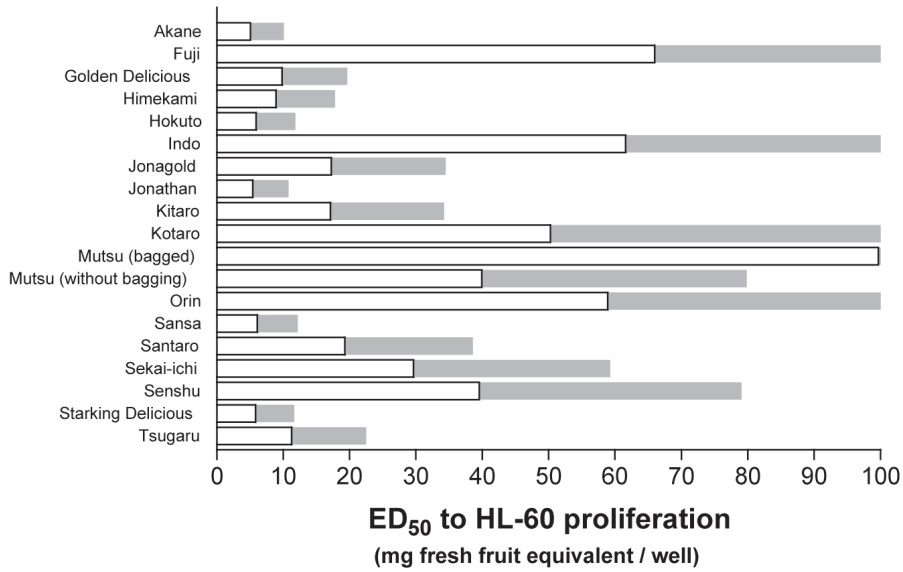

Fig. 1. ED_{50} values of 19 apple cultivars on HL-60 antiproliferation.

Table 1. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and harvest time of apple cultivars.

Apple cultivar	DPPH radical scavenging activity (%)	Harvest
1 Akane	37.0	Late September
2 Fuji	52.0	Early November
3 Golden Delicious	52.4	Mid-October
4 Himekami	51.7	Late September
5 Hokuto	49.2	late October
6 Indo	52.0	Mid-November
7 Jona gold	51.4	Mid-October
8 Jonathan	51.2	Mid-October
9 Kitaro	21.2	Mid-October
10 Kotaro	33.1	Late October
11 Mutsu (bagged)	24.1	Late October
12 Mutsu (without bagging)	48.8	Late October
13 Orin	46.0	Late October
14 Sansa	39.6	Early September
15 Santaro	52.4	Late September
16 Sekai-ichi	48.2	Mid-October
17 Senshu	52.0	Early October
18 Star king Delicious	43.2	Mid-October
19 Tsugaru	45.4	Mid-September

parent of 'Santaro', 'Kitaro', 'Kotaro', and 'Sansa'. 'Jonathan' itself and its next generation of hybrids were classified as the strong group according to their antiproliferative activities. Other progeny of 'Jonathan' also had medium and strong activities, with the exception of 'Kotaro'. A difference of antiproliferative activity was found between 'Kotaro' and 'Kitaro', although they have same parent, i.e., 'Fuji' and 'Hatsuaki'. The inconsistency can be explained as results of the highly heterozygous nature of the cultivated apples. It is well known that there is a considerably wide range of expression of most characteristics in apple seedlings, and the difference in fruit color, i.e., 'Kitaro' is yellow and 'Kotaro' is red, also seemed to be the result of heterozygous nature.

'Golden Delicious' was developed from a chance seedling, perhaps from 'Grimes Golden', and a relationship between 'Golden Delicious' and 'Jonathan' has not been clear. 'Golden Delicious' is the parent of 'Tsugaru', 'Jonagold', 'Mutsu' (bagged and no bagging), 'Orin', and 'Sekai-ichi' and is the grandparent of 'Santaro', 'Kitaro', 'Kotaro', 'Sansa', and 'Senshu'. 'Golden Delicious' also had strong activity, although most of its progeny only possessed weak activity. In contrast to 'Jonathan', the antiproliferative activity of 'Golden Delicious' seemed not to be inherited to its progeny.

Similarly, 'Indo' is the parent of 'Mutsu' and 'Orin' and is the grandparent of 'Senshu'. Although the parentage of 'Indo' has not been known, so we cannot discuss its low activity, all of its progeny also showed weak activity. 'Delicious', which is also an important breeding ancestor, is the parent of 'Fuji' and 'Sekai-ichi' and the origin of 'Starking Delicious', although it is not examined in this report. 'Starking Delicious', which is a bud mutation of 'Delicious', showed strong antiproliferative activity, whereas other 'Delicious' progenies had weak antiproliferative activity.

'Hokuto' had been reported to be a result of the cross 'Fuji' × 'Mutsu' (Yamada et al., 1987); however, recent findings suggested that 'Mutsu' could not be the pollen parent based on the *S*-glycoprotein profiles (Sassa et al., 1994) as well as the self-incompatible genotyping by allele-specific PCR (Sakurai et al., 1997). Furthermore, the comparison of *S*-glycoprotein profiles of 'Hokuto' with its parents suggested that 'Jonathan' was the true pollen parent of 'Hokuto' (Sassa et al., 1994). Its strong antiproliferative activity may be another evidence to support the parentage of 'Hokuto'.

Bagging is an operation to induce fruit coloration for green skin in 'Mutsu' (Fukuda, 1994), and the effect is not really relevant to the breeding aspects. However, from our results, bagging 'Mutsu' decreased antiproliferation of HL-60 and DPPH radical scavenging activity.

To specify polyphenols involving antiproliferative activity in 'Jonathan', its extract was analyzed by HPLC and the results are shown in Table 3. The comparison with standard compounds revealed that catechin, chlorogenic acid, epicatechin, phlorizdin, and quercetin were in 'Jonathan', and their EC_{50} values for HL-60 were as shown. The antiproliferative activity of 'Jonathan' seemed partially due to catechin and epicatechin, but should be an integrated effect of not only the ones found on HPLC but unidentified compounds, such as procyanidins. Kaempferol, phloretin, and quercitrin were reported as common apple polyphenol compounds (Boyer and Liu, 2004), and some of them showed activity to HL-60; however, these were not detected from 'Jonathan'. The composition of procyanidins and other polyphenols in 'Jonathan' are currently being investigated.

In conclusion, the genetic characteristics of 'Jonathan' had an intimate relationship toward the antiproliferative property, and most of 'Jonathan' relatives (eight of nine cultivars) tested in this study showed strong or medium activity. This is the first observation of antiproliferative activity related to the parentage of apples, and the information will be useful to create new apple cultivars with anticancer potential.

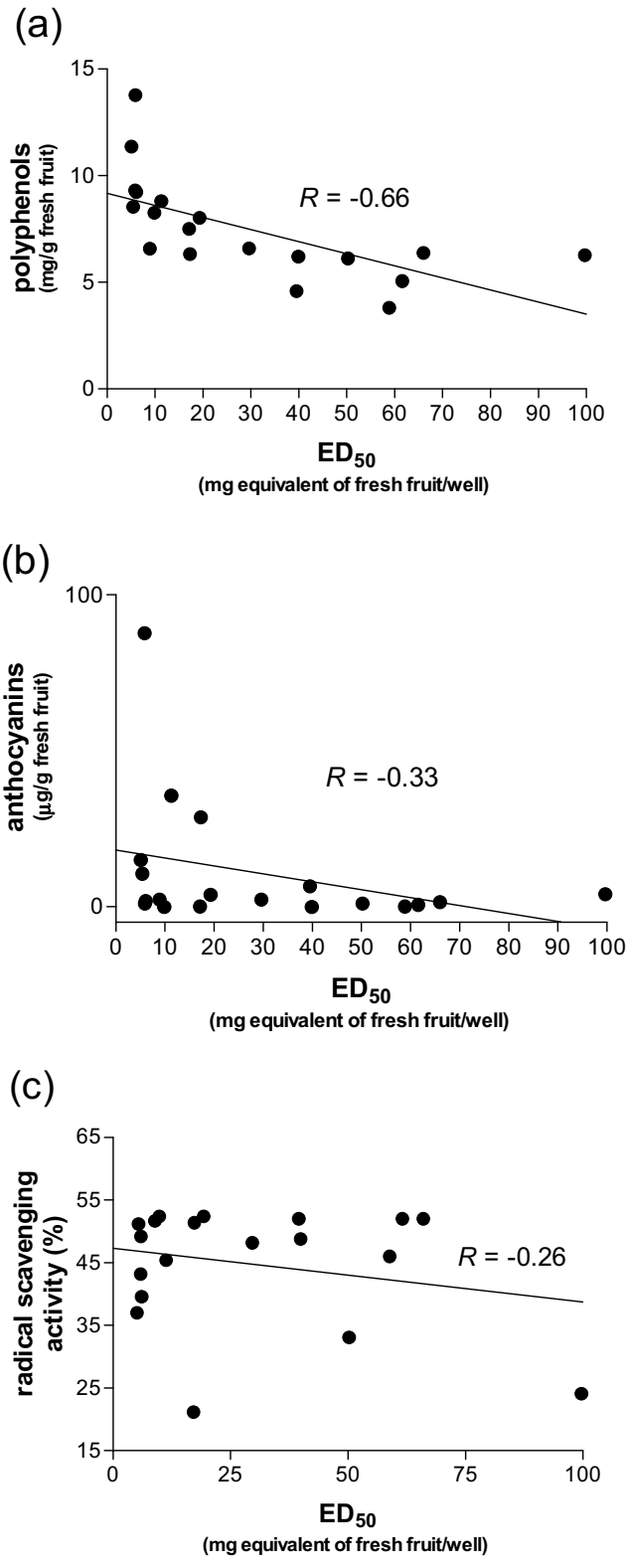

Fig. 2. Relationship between total polyphenols and antiproliferative activity.

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Table 2. Relationship between parentage and HL-60 antiproliferation.

No	Fruit color	Name	HL-60[z]	Seed parent	Pollen parent
8	Red	Jonathan	S	Unknown	
3	Yellow	Golden Delicious	S	Unknown	
6	Yellow	Indo	W	Unknown	
1	Red	Akane	S	Jonathan	Worcester Pearmain
19	Red	Tsugaru	S	Golden Delicious	Jonathan
7	Red	Jonagold	M	Golden Delicious	Jonathan
4	Red	Himekami	S	Fuji (Ralls Janet × Delicious)	Jonathan
5	Red	Hokuto	S	Fuji (Ralls Janet × Delicious)	Jonathan
15	Red	Santaro	M	Hatsuaki (Jonathan × Golden Delicious)	Starking Delicious
9	Yellow	Kitaro	M	Fuji (Ralls Janet × Delicious)	Hatsuaki (Jonathan × Golden Delicious)
10	Red	Kotaro	W	Fuji (Ralls Janet × Delicious)	Hatsuaki (Jonathan × Golden Delicious)
14	Red	Sansa	S	Gala (Kidd's Orange Red × Golden Delicious)	Akane (Jonathan × Worcester Pearmain)
11	Red	Mutsu (bagged)	W	Golden Delicious	Indo
12	Yellow	Mutsu (without bagging)	W	Golden Delicious	Indo
13	Yellow	Orin	W	Golden Delicious	Indo
16	Red	Sekai-ichi	W	Delicious	Golden Delicious
2	Red	Fuji	W	Ralls Janet	Delicious
17	Red	Senshu	W	Toko (Golden Delicious × Indo)	Fuji (Ralls Janet × Delicious)
18	Red	Starking Delicious	S	bud mutation of Delicious	

[z]S = strong activity (EC_{50} < 3 mg), M = medium activity (EC_{50} was 3 to 5 mg), W = weak activity (EC_{50} > 5 mg).

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Table 3. The amounts and the antiproliferative effects to HL-60 of polyphenols in 'Jonathan'.

Compound	Amount in 'Jonathan' (μg·g^{-1} fresh fruit)	EC_{50} (μM)
Catechin	64	42.8
Chlorogenic acid	202	80.2
Epicatechin	787	15.5
Kaempferol	ND[z]	72.1
Phloretin	ND	400
Phlorizdin	10	<500
Quercetin	Trace	9.2
Quercitrin	ND	<500

[z]ND = not detected.

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