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Antiproliferative and Antioxidant Properties of Crabapple Juices

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Juices prepared from 42 crabapples (*Malus* spp.) were studied attempting to explore their antiproliferative activity. Most of the juices examined showed strong inhibition of the proliferation of HL-60 human leukemia cells. The amounts of total phenolics and total anthocyanins, as well as DPPH radical scavenging activity were measured. The antiproliferative effect was more correlated to the amount of polyphenol than of anthocyanins. Generally speaking, the stronger was the DPPH radical scavenging activity, the higher was the antiproliferation shown of HL-60. The colors and sizes of fruit and the colors of flower were not strongly correlated to antiproliferative activity, although all of the middle size (1 to 3 cm in diameter) fruits examined showed strong HL-60 antiproliferative and DPPH radical scavenging activities. The results suggested that utilizing crabapple juice as a processed food and beverage contributes to the maintenance of good health.

Keywords: crabapple, HL-60, antiproliferative activity, antioxidant activity, DPPH.

Introduction

Crabapple is one of the ornamental trees widely grown in gardens and parks in Europe and North America, and the number of cultivars is now over 700 with great diversity in colors and shapes of the flower and fruit, some cultivars producing a large amount of fruits. Although most crabapple fruits are edible, very little has been reported on their biological activities in human. We herein report their antiproliferative activity toward human leukemic HL-60 cells in relation to their antioxidative properties.

Materials & Methods

Reagents 2-Amino-2-hydroxymethyl-1,3-propandiol (Tris), 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene, BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, and gallic acid were purchased from Wako Pure Chemicals Industry (Osaka, Japan).

Preparation of crabapple juices All crabapple fruits were harvested from September to November 2000 from collections at the Division of Apple Research, National Institute of Fruit Tree Science (Morioka, Japan). Approximately 300 g of fruits were homogenized in 300 ml of ethanol. The ethanol extract was filtered, concentrated *in vacuo* to remove ethanol, and dissolved in distilled water to give 100 ml of crabapple juice, which was used as stock solution. An apple cultivar Fuji, which is commonly available in local markets, was also extracted in the same manner as a reference sample.

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Cells HL-60, obtained from the Riken Gene Bank (Tsukuba, Japan), was maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS). HL-60 in log phase (approximately 10^6 cells/ml) were diluted to 1.2×10^5 cells/ml and pre-incubated for 18 hr in 96-well plates (approximately 2×10^5 cells/ml).

Cell proliferation assay Each stock solution above mentioned was aseptically diluted with RPMI 1640 medium containing 10% FBS to make 4-fold serial dilution of fruit extract sample. The level of proliferation was measured for HL-60 grown in 96-well microtiter plates. To each well 5×10^3 cells/100 µl HL-60 suspension was added, grown for 24 h, and then mixed with 100 µl of the medium containing a serial dilution of the fruit extract to be assayed. Doses of fruit extract were usually 30, 7.5, 1.9, and 0.47 mg fresh fruit equivalent/well. After 3 days of incubation, 20 µl of alamar Blue™ (Biosource International, Lewisville, TX, USA), an oxidation-reduction indicator, was aseptically added to each well, and incubated for 6 h or 24 h. Cellular proliferation (% of untreated control) was calculated with the following equation:

$$\text{Proliferation (\%)} = \frac{\begin{array}{c}[(A_{570} - A_{595}) \text{ of test agent dilution}] \\ - [(A_{570} - A_{595}) \text{ of blank}]\end{array}}{\begin{array}{c}[(A_{570} - A_{595}) \text{ of untreated positive growth control}] \\ - [(A_{570} - A_{595}) \text{ of blank}]\end{array}} \times 100$$

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

Total phenolic analysis The total phenolics was determined by Folin-Ciocalteu reagent primarily according to

the literature method (Prior *et al.*, 1998, Slinkard and Singleton, 1977), that was modified to use a 96-well microtiter plate. To 20 µl of 1/1000 diluted sample from crabapple stock solutions or 50, 40, 30, 20, and 10 mg/l 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, the absorbance at 765 nm was measured by a microplate-reader (Benchmark Plus, BioRad Laboratories). The results were expressed as milligrams of gallic acid equivalent/g fresh fruit.

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

DPPH radical scavenging activity The scavenging activity of crabapple juices against DPPH radical was measured according to the method described previously (Yamaguchi *et al.*, 1998). Each 0.05 ml of 1/100 diluted crabapple stock solutions 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 25°C in the dark for 20 min. The absorbance at 517 nm was measured. Deionized water was used as blank experiment, and BHT (5 µg/ml) was used as positive control. The scavenging activity of DPPH radical (%) was calculated with the equation $(A_{517,\,blank} - A_{517,\,sample})/A_{517,blank} \times 100\%$. Each assay contained juice derived from 1.5 mg fruit.

Results and Discussion

The antiproliferative and DPPH radical scavenging activities of fruit extracts from 42 crabapples tested in this study are shown in Table 1. All samples had EC_{50} lower than 15 mg fruit equivalent per well. Twenty-five cultivars showed strong activity with EC_{50} of less than 2 mg fruit equivalent per well, moderate activity was found in 15 cultivars with EC_{50} from 2 to 5 mg, and 2 cultivars, namely Gorgeous and Neville copeman, had only weak activity with EC_{50} of more than 10 mg equivalent per well. In contrast, Fuji, an apple cultivar used as reference, only showed its EC_{50} at 66 mg fruit equivalent per well, more than four times weaker, so we considered that Fuji had almost no activity compared to crabapples.

The total phenolics and the total anthocyanins were measured using Folin-Ciocalteu reagent and pH differentiating method, respectively. The relationships between those data and HL-60 antiproliferative activity are shown in Figures 1 and 2. The antiproliferative activity was more strongly correlated with the total phenolics ($r = -0.41$) than the total anthocyanins ($r = -0.19$). Fuji contained only a small amount of total phenolics and anthocyanins: total phenolics was 6.38 mg/g fruit and total anthocyanins was 1.5 µg/g fruit.

Table 1. HL-60 antiproliferative and DPPH radical scavenging activities of crabapple cultivars.

No.	Cultivar name	EC_{50} for HL-60 (mg fruit equivalent/well)	DPPH radical scavenging activity (%)
1	Adam's crab	1.08	76.3
2	Arnold crab	2.92	74.7
3	Atrosanguiner	2.76	71.3
4	Beverly crab	4.56	75.3
5	Chuisihaitang	1.11	21.3
6	Donald wyman crab 81078HT	0.96	67.6
7	Eley purple crab	3.02	55.4
8	Golden hornet	3.06	47.9
9	Gorgeous	14.59	10.8
10	Hopa crab B	0.97	71.3
11	Hubeihaitang 83051	3.84	73.1
12	Hubeihaitang 84078	4.01	60.3
13	Indian magic crab	1.22	13.8
14	Indian summer crab 81082HT	0.97	0.0
15	Jack crab	2.64	76.4
16	Jay darling (Mink)	0.97	66.7
17	Kanazawazairai	1.32	71.7
18	*M.floribunda* 88071	0.96	53.2
19	*M.hupehensis* Rehd.	0.96	23.3
20	Makamik crab	3.23	65.3
21	Mandschurica-2	4.56	70.3
22	Maoshanjingzi	1.30	63.7
23	Mary potter crab	1.08	70.7
24	Miyamakaidou	1.09	71.0
25	Morioka guuhatsu RS-3	2.16	71.0
26	Morioka guuhatsu RS-4	1.09	52.9
27	Morioka guuhatsu RS-5	2.55	66.2
28	Neville copeman	11.45	40.9
29	Nikkouzumi	0.97	67.3
30	NY 11894	0.97	67.2
31	NY 49-23	0.96	47.7
32	Ormiston roy crab	0.96	67.2
33	Red bud crab	1.10	76.4
34	Red splendor crab	1.14	42.0
35	Robinson crab 81094	1.08	63.5
36	Robusta No. 5	0.97	67.7
37	Sargent crab	2.64	76.4
38	Sentinel crab	0.96	77.3
39	Shirobana-Robusta	0.97	67.3
40	Snowdrift crab	5.19	76.4
41	Xifuhaitang	3.74	16.5
42	Yantaishaguo	1.24	65.0

DPPH radical scavenging activity was measured by spectrophotometry, and the result in relation to HL-60 antiproliferation is shown in Figure 3. Since a weak correlation ($r = -0.26$) was observed between the antiproliferative and DPPH radical scavenging activities, it is natural to consider that the antiproliferative principles and the antioxidant principles were partially overlapped, and that common substances, presumably polyphenolics were involved in these activities. However, the weakness of correlation suggested the involvement of other uncharacterized substances. The correlation of the total phenolics and the DPPH radical scavenging activity was –0.37, suggesting that a variety of antioxidant principles exists in crabapples. The colors and

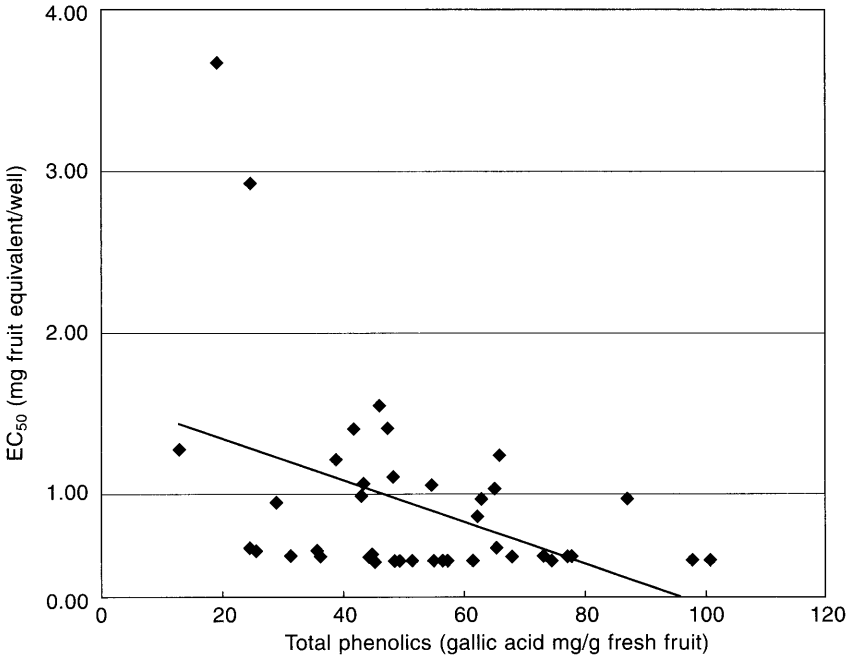

Fig. 1. Relationship between total phenolics and HL-60 antiproliferation. $r = -0.41$.

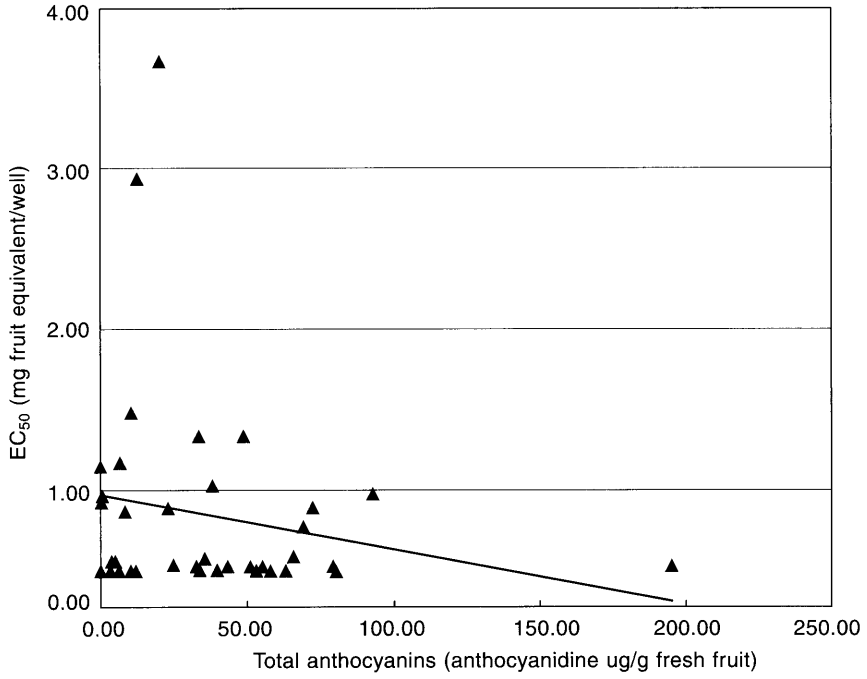

Fig. 2. Relationship between total anthocyanins and HL-60 antiproliferation. $r = -0.19$.

sizes of fruit and the colors of flower were examined in relation to antiproliferative and DPPH radical scavenging activities. The colors of fruit were divided into two categories, yellow and red, and the sizes were characterized as less than 1 cm, between 1 and 3 cm, and over 3 cm in diameter. Flower colors were categorized as white, pink, and red. The flower with white petals inside and red outside was called a white flower. These parameters were not necessarily correlated to antiproliferative and DPPH radical scavenging activities, however, all of the middle size (1–3 cm in diameter, $n = 13$) fruits showed strong activities on HL-60 and DPPH.

Crabapples have been widely planted as ornamental trees in many western countries, some of which have a high production of fruits, but the fruits have rarely been utilized. On the other hand, apples are known to be abundant in

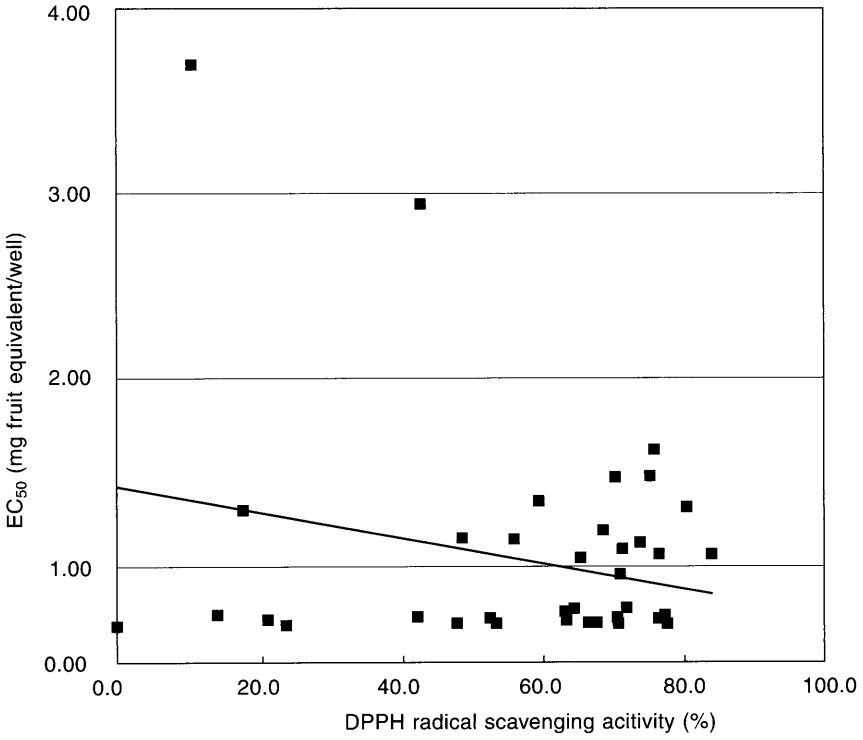

Fig. 3. Relationship between DPPH radical scavenging activity and HL-60 antiproliferation. $r = -0.26$.

vitamin C, diet fiber, and antioxidants, suggesting that crabapples, because they come from the same genus *Malus*, would also be a rich source of health promoting factors. The crabapple fruit has been reported to contain ascorbic acid, and to be rich in phenolics such as condensed tannins (procyanidins), chlorogenic acid and epicatechin, but very little is known about its biological activities (Loughrin *et al.*, 1996) especially to human. As shown in Table 1, strong HL-60 antiproliferative activity was found in most crabapples tested, while Fuji, a typical commercial apple, showed almost no activity. The correlation with the amount of phenolics and the DPPH radical scavenging activity suggested that the effect on HL-60 is probably due to antioxidative polyphenols, and the quantitative analysis of several phenolic compounds in crabapples is currently ongoing.

Although crabapple cultivars had been developed mainly for their visual beauty and the taste of the fruit has not previously been of much interest, the activities found in this study would suggest crabapple could be used as a processed food and beverage which would contribute to the maintenance of good health.

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