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Methyl jasmonate enhances antioxidant activity and flavonoid content in blackberries (*Rubus* sp.) and promotes antiproliferation of human cancer cells

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Abstract

The effects of preharvest methyl jasmonate (MJ) application on fruit quality, antioxidant activity and flavonoid content in blackberries (*Rubus* sp.) were determined. Anticancer activity against human lung A549 cells and HL-60 leukemia cells was also evaluated. Three blackberry cultivars (Chester Thornless, Hull Thornless and Triple Crown) were used in these experiments. Blackberries treated with MJ (0.01 and 0.1 mM) had higher soluble solids content, and lower titratable acids than untreated fruit as well as enhanced content of flavonoids and increased antioxidant capacity. Extracts of treated fruit showed enhanced inhibition of A549 cell and HL-60 cell proliferation and induced the apoptosis of HL-60 cells. Cultivar Hull Thornless had higher soluble solids and lower titratable acids compared to cv. Chester Thornless and Triple Crown. On the basis of fresh weight of fruit, Hull Thornless also had significantly higher anthocyanin, total phenolic content, antioxidant and antiproliferation activity than other two cultivars.
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Keywords: Antioxidant activity; Antiproliferation; Blackberries; Methyl jasmonate; *Rubus* sp.

1. Introduction

Jasmonic acid (JA) and methyl jasmonate (MJ) (Fig. 1) occur widely in plants. Their biosynthesis starts with linolenic acid and proceeds through a number of stages involving lipoxidation, cyclization and β-oxidation (Creelman & Mullet, 1997). There are two chiral carbon atoms in the methyl jasmonate molecule; each can have either the *R*- or *S*-absolute configuration, so that there are four potential isomers. Methyl (+)-epijasmonate, (3*R*, 7*S*)-(+)-methyl 3-oxo-2-[2-(*Z*)-pentenyl] cyclopentane-1-acetate, has the strongest odour of the isomers, demonstrating the importance of molecular shape in fitting receptors and activating the sensory response. JA/MJ, which are endogenous phytohormones, are potent elicitors or signaling agents and play key roles in plant growth and affecting a great diversity of physiological and biochemical processes (Creelman & Mullet, 1997). Upon exposure to stresses such as wounding and pathogens, plants produce JA/MJ and induce a proteinase inhibitor (Farmer & Ryan, 1990). MJ has been shown to stimulate secondary metabolites such as stilbene in leaves and berries of grapevine plants (Larrondo et al., 2003); to enhance anthocyanin accumulation in soybean seedling (Franceschi & Grimes, 1991), peach shoots (Saniewski, Miyamoto, & Ueda, 1998), apple fruit (Kondo, Tsukada, Niimi, & Seto, 2001) and strawberry ripening (Pérez, Sanz, Olías, & Olías, 1997); to increase β-carotene accumulation in ripening tomatoes (Saniewski & Czapski, 1983); and to

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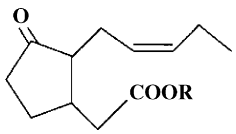

Fig. 1. Chemical structure of jasmonates. Jasmonic acid (R = H), methyl jasmonate ($R = CH_3$).

increase synthesis of alkaloids, terpenoids and phenolics in *Nicotiana* species, *Hyoscyamus muticus,* and Norway spruce stems and sweet basil (Keinänen, Oldham, & Baldwin, 2001; Kim, Chen, Wang, & Rajapakse, 2006; Martin, Tholl, Gershenzon, & Bohlmann, 2002; Singh, Gavrieli, Oakey, & Curtis, 1998).

Blackberries are a good source of natural antioxidants (Wang, Cao, & Prior, 1996). In addition to vitamins and minerals, extracts of blackberries are also rich in secondary metabolites such as anthocyanins and phenolic acids (Wang & Lin, 2000). Blackberries have been shown to exhibit strong antioxidant capacity in comparison to many other fruits (Wang et al., 1996). Our previous study also showed blackberries have a high oxygen radical absorbance activity against peroxyl radical ($ROO^{\cdot}$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), and singlet oxygen ($^1O_2$) (Wang & Jiao, 2000; Wang & Lin, 2000). Blackberry fruit extracts or a natural product derived from blackberries, cyanidin 3-glucoside, has been shown to exhibit chemopreventive and chemotherapeutic activity (Ding et al., 2006; Feng et al., 2004). However, no information is available on the effects of preharvest application of methyl jasmonate on blackberry fruit quality, antioxidant capacity and their secondary metabolites. The purpose of this study was to determine the effect of MJ on the induction of secondary metabolites which are beneficial to human health and to investigate if MJ could enhance the antioxidant and therapeutic activities of blackberries. The effects of MJ on inhibition of cell proliferation and induction of apoptosis in human cancer cells were also examined.

2. Materials and methods

2.1. Cell lines and reagents

The JB6 P^+ mouse epidermal cell line, stably transfected with the AP-1-or NF-κB luciferase reporter plasmid (JB6/AP/κB) (Li, Westergaard, Ghosh, & Colburn, 1997), was cultured in Eagle's Minimal Essential Medium (EMEM) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin–streptomycin. Human lung carcinomatous cell line A549 and HL-60 human leukemia cells were obtained from American Type Culture Collection (Manassas, VA 20108) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 50 U/ml pencillin and 50 μg/ml streptomycin in 75 cm^2 T-flasks. The cells were grown at 37 °C, 85% humidity and 5% CO_2 atmosphere.

EMEM and DMEM were obtained from Whittaker Biosciences (Walkersville, MD). EDTA (ethylenediaminetetracetic acid, disodium salt, dihydrate-Na_2 EDTA · $2H_2O$), FBS, penicillin–streptomycin, L-glutamine, and trypsin were purchased from Life Technologies Inc. (Gaithersburg, MD). Gallic acid, MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5,-diphenyl tetrazolium bromide] solution, 2-propanol, bis-benzimide Hoechst 33258 and SDS were purchased from Sigma Chemical Co. (St. Louis, MO). 6-Hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and fluorescein were purchased from Aldrich (Milwaukee, WI). 2,2′ Azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA Inc. (Richmond, VA). Cyanidin 3-glucoside was purchased from Indofinehemical Co. Inc. (Hillsborough, NJ).

2.2. Plant material and field experiments

Ten-year-old thornless blackberry (cv. Chester Thornless, Hull Thornless, and Triple Crown Thornless) used in this study were grown at the Henry A. Wallace-Beltsville Agricultural Research Center. Three sets of four primocanes per cultivar were randomly selected and the treatments were designated: (1) control; (2) MJ 0.01 mM; (3) MJ 0.1 mM. MJ at 0.01–0.1 mM plus 0.05% Tween-20 was applied as a foliage-berry spray to runoff when berries were in early light pink stage, and spraying was applied for two more times at four-day intervals. The control primocanes were also sprayed with 0.05% Tween-20. Three 100 g undamaged berries were randomly selected around 0900 h from each treatment and each cultivar four weeks after experiments and were used for chemical analyses. The field experiments were carried out at same location over two growing seasons.

2.3. Soluble solids content (SSC), and titratable acid (TA)

Soluble solids content (SSC) of the blackberry fruit juice was determined at 20 °C on a Bausch and Lomb refractometer. Titratable acid (TA) was determined by diluting each 5 ml aliquot of blackberry juice to 100 ml with distilled water, then titrating to pH 8.2 using 0.1 N NaOH. Acidity was expressed as mg citric acid/100 ml juice.

2.4. Total anthocyanin and total phenolic content

Blackberries were extracted with 80% acetone containing 0.2% formic acid using a Polytron (Brinkmann Instruments Inc., Westbury, NY). The homogenized samples from the acetone extracts were then centrifuged at 14,000*g* for 20 min at 4 °C. The supernatants were transferred to vials, stored at −80 °C, and later used for determination of total anthocyanins, soluble phenolics and ORAC analysis.

Total anthocyanin contents in blackberry extract were determined by using pH differential method (Cheng & Breen, 1991). Absorbance was measured using a Shimadzu

Spectrophotometer (Shimadzu UV-160) (Shimadzu Scientific Instruments Inc., Colmbia, MD) at 510 nm and 700 nm in buffers at pH 1.0 and 4.5, using $A = [(A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}]$ with a molar extinction coefficient of cyaniding 3-glucoside (29,600) for blackberry fruit. Results were expressed as milligrams of cyaniding 3-glucoside equivalents, in the strawberry extract, per 100 gram of fresh weight basis.

Total soluble phenolics in the fruit extract were determined with Folin-Ciocalteu reagent by the method of Slinkard and Singleton (1977) using gallic acid as a standard. Results were expressed as milligrams gallic acid equivalents (GAE), in the blackberry extract, per 100 gram fresh weight.

2.5. HPLC analysis of blackberry anthocyanins and phenolic compounds

High performance liquid chromatography (HPLC) was used to separate and determine individual anthocyanins and phenolic compounds in blackberry fruit samples. Five grams of each fruit sample were extracted twice with 15 ml of acetone using a Polytron (Brinkmann Instruments Inc., Westbury, NY) for 1 min. Extracts (30 mL) were combined and concentrated to 1 ml using a Buchler Evapomix (Buchler Instruments, Fort Lee, NJ, USA) in a water bath at 35 °C. The concentrated sample was dissolved in 10 ml of acidified water (3% v/v of formic acid) and then passed through a C_{18} Sep-Pak cartridge (Waters Corporation, Milford, MA), which was previously activated with methanol followed by water and 3% aqueous formic acid. Anthocyanins and other phenolics were adsorbed onto the column while sugars, acids, and other water-soluble compounds were eluted with 10 ml of 3% aqueous formic acid. The anthocyanins and other phenolics were then recovered with 2.0 ml of acidified methanol containing 3% formic acid. The methanolic extract was passed through a 0.45-μm membrane filter (Millipore, MSI, Westboro, MA) and 20 μl of it was analyzed by HPLC. The samples were analyzed using a Waters HPLC system equipped with two pumps (600E system Controller; Waters) coupled with a photodiode array detector (Waters 990 Series). Samples were injected at ambient temperature (20 °C) onto a reverse phase NOVA-PAK C_{18} column (150 × 3.9 mm, particle size 4 μm) (Waters Corporation, Milford, MA) with a guard column (NOVA-PAK C_{18}, 20 × 3.9 mm, particle size 4 μm) (Sentry guard universal holder) (Waters Corporation, Milford, MA). The flow rate was 1 ml/min, with a gradient profile consisting of A with the following proportions (v/v) of B: 0 min, 5%, 1–15 min, 5–15% B; 15–20 min, 15–25% B; 20–25 min, 25% B; 30–35 min, 25–100% B and isocratic mixture for 5 min before returning to the initial conditions. The wavelengths of detection were set at 320, 350, and 510 nm. Scanning between 240 and 550 nm was performed and data were collected by the Waters 990 3D chromatography data system. Retention times and spectra were compared to pure standards. Results were expressed as microgram per gram fresh weight of phenolic acid or flavanol or expressed as microgram per gram fresh weight of cyanidin 3-glucoside.

2.6. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out using a high-throughput instrument platform consisting of a robotic eight-channel liquid handling system and a microplate fluorescence reader which a FL800 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT) was used with fluorescence filters for an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm. The plate reader was controlled by software KC4 3.0 (revision 29). Sample dilution was accomplished by a Precision 2000 automatic pipetting system managed by precision power software (version 1.0) (Bio-Tek Instruments Inc., Winooski, VT). The ORAC values were determined by calculating the net area under the curve (AUC) of the standards and samples (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). The standard curve was obtained by plotting 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) concentrations against the average net AUC of the two measurements for each concentration. Final ORAC values were calculated using the regression equation between Trolox concentration and the net AUC and were expressed as micromole Trolox equivalents per gram of fresh weight (Huang et al., 2002).

2.7. Inhibition of cancer cell proliferation

Sample preparation for assay of cell proliferation and cell apoptosis, blackberry fruit extracts were prepared by mixing 100 g of fruit tissue with 100 ml of distilled de-ionized H_2O and blended at high speed. The blended homogenates were strained, centrifuged at 6000*g* at 4 °C for 20 min and the supernatants were filtered. The supernatants were transferred to vials, and stored at −80 °C until analysis.

Two cell lines, human lung cancer A549 cells, and HL-60 human leukemia cells, were cultured as described above. Subcultures were carried out every 2–3 days using a 0.25% trypsin and 0.02% EDTA solution. Briefly, A549 and HL-60 cells were plated in their growth medium at a density of 1×10^4 cells/well in 96-flat bottomed well cell culture plates and incubated at 37 °C. Twenty-four hours after plating, 0.33 μL of indicated doses of blackberry fruit extracts (50 and 100 μg/ml) was added to each well (except for control wells). Following 48 h incubation, 10 μl MTT solution was added in each well to form formazan salt crystals and the plates were further incubated for 4 h. Then 100 μL solubilization solution (10% SDS in 0.01 M HCl) were added, and the plate was incubated overnight at 37 °C. The amount of formazan produced was proportional to the number of viable cells (Mosmann, 1983). After incubation, the MTT-formazan was solubilized in 2-propanol and the optical density was measured at a wavelength of 575 nm

and a reference wavelength of 690 nm using a Microplate Spectrophotometer (Spectra MAX™ 250, Molecular Devices Inc., Sunnyvale, CA), where higher OD values indicated more cell proliferation. Proliferation was expressed as a percentage of cell growth in wells that received no extract. Cyanidin 3-glucoside (50 μM) was used as a positive control. Data are expressed as means ± SD of three samples.

2.8. Induction of apoptosis in cancer cells

HL-60 human leukemia cells and JB6 P^+ mouse epidermal cell line were used in this study. To test whether blackberry extracts possessed any apoptotic induction on cancer cells, HL-60 cells (1×10^4 cells/well in 24-flat bottom well) and JB6P^+ (5×10^5 cells/well in 24-flat bottom well) were treated with blackberry extracts (50 and 100 μg/ml) for 18 h. Cells were assessed for typical apoptotic morphology by staining with 10 μmol/l bis-benzimide Hoechst 33258 fluorochrome (Molecular Probes) for 30 min. Apoptotic cells were counted with a fluorescence microscope, and photographed using a digital video camera (Pixera, Los Gatos, CA). Approximately 200–400 cells per group were assessed in randomly selected fields to avoid experimental bias.

2.9. Statistical analysis

Data presented were the means ± SD values. All statistical analyses were performed with NCSS Statistical Analysis System (Statistical Analysis and Graphics, Kaysville, UT, USA) (NCSS, 2007). One- and two-way analysis of variance (ANOVA) was used to compare the means. Differences were considered significant at $p \leqslant 0.05$.

Correlations were determined between antioxidant activity (ORAC), total phenolics, total anthocyanins, individual flavonoid with antiproliferation activity (cell viability and MTT uptake), and apoptosis in three cultivars of blackberries by using NCSS Statistical Analysis System (NCSS, 2007). Results are reported as R^2 values.

3. Results and discussion

The effects of preharvest application of MJ on fruit SSC, and TA were significant in three cultivars of blackberry (Chester Thornless, Hull Thornless and Triple Crown). Compared to the control, SSC was higher in fruit treated with MJ. Fruit treated with MJ had lower TA and higher SSC/TA than untreated-control (Table 1). Blackberries treated with 0.1 mM MJ had higher SSC, ratio of SSC/TA and lower TA than those treated with 0.01 mM MJ. The SSC were in the range of 7.9–12.9%, the TA was 0.38–0.85% and the ratio of SSC/TA was from 9.29 to 33.95. Among the cultivars, Hull Thornless had the greatest SSC, and the highest ratio of SSC/TA. Triple Crown had the most TA (Table 1). The general flavour selection criteria for blackberries have been high sweetness and high acidity. All three cultivars tested had good and pleasing flavours, suggesting that there are many combinations of SSC and TA that confer good flavour.

MJ treatments significantly enhanced total anthocyanin and total phenolic content in blackberry fruit. Increased MJ concentration from 0.01 to 0.1 mM resulted in an increase in flavonoid content and antioxidant capacity (expressed as ORAC values) in fruit of Chester Thornless, Hull Thornless and Triple Crown (Table 1). On the basis of fresh weight of fruit, Hull Thornless had significantly higher anthocyanin, total phenolic content and ORAC

Table 1
Effect of methyl jasmonate (MJ) treatment on antioxidant capacity (ORAC), total phenolics, total anthocyanins, soluble solids content (SSC), and titratable acids (TA) in fruit of three cultivars (Chester Thornless, Hull Thornless and Triple Crown) of blackberry[a]

Cultivar	Treatment	ORAC[b] (μmol TE/g fw)	Total phenolics[c] (mg/100 g fw)	Total anthocyanin[d] (mg/100 g fw)	SSC (%)	TA (%)	Ratio SSC/TA
Chester Thornless	Control	28.0 ± 1.1	180.2 ± 2.6	94.2 ± 0.9	9.0 ± 0.2	0.80 ± 0.3	11.25
	0.01 mM	32.2 ± 2.9	215.8 ± 2.5	164.2 ± 1.9	10.2 ± 0.3	0.56 ± 0.2	18.21
	0.1 mM	35.9 ± 1.2	249.5 ± 7.5	196.4 ± 2.4	12.5 ± 0.1	0.40 ± 0.2	31.25
Hull Thornless	Control	30.9 ± 2.1	210.6 ± 2.4	99.6 ± 0.9	9.5 ± 0.2	0.55 ± 0.3	17.27
	0.01 mM	31.8 ± 1.1	265.2 ± 9.4	189.5 ± 3.2	10.7 ± 0.4	0.47 ± 0.1	22.77
	0.1 mM	38.1 ± 0.3	317.2 ± 7.8	221.6 ± 2.8	12.9 ± 0.3	0.38 ± 0.4	33.95
Triple Crown	Control	28.0 ± 2.3	189.2 ± 5.2	100.4 ± 1.1	7.9 ± 0.2	0.85 ± 0.3	9.29
	0.01 mM	31.8 ± 0.0	241.8 ± 6.8	181.2 ± 2.1	10.5 ± 0.3	0.61 ± 0.3	17.21
	0.1 mM	33.4 ± 3.5	261.4 ± 5.3	216.9 ± 1.9	11.2 ± 0.2	0.53 ± 0.2	21.13
Significance[e]							
Cultivar (*C*)		*	*	ns	*	*	*
Treatment (*T*)		*	*	*	*	*	*
C × *T*		*	*	*	*	*	*

[a] Data expressed as means ± SD.
[b] Data expressed as micromoles of Trolox equivalents per gram of fresh weight.
[c] Data expressed as milligrams of gallic acid (GAE) equivalents per gram of fresh weight.
[d] Data expressed as milligrams of cyanidin 3-glucoside equivalents per 100 gram of fresh weight.
[e] *, ns, significant or non-significant at $p \leqslant 0.05$.

values than Chester Thornless and Triple Crown (Table 1). The ORAC values were closely correlated to the total phenolics and total anthocyanins with $R^2 = 0.8021$ and 0.7957, respectively.

HPLC analysis of blackberry fruit extract showed that ellagic acid, quercetin 3-glucoside, quercetin 3-rhamnoside, kaempferol 3-glucoside, kaempferol derivative, cyanidin 3-glucoside, pelargonidin 3-glucoside, cyanidin 3-xyloside, cyanidin 3-glucoside derivative, and dicarboxylic acid acylated of cyanidin 3-glucoside derivative were present in significant amounts (except that Chester Thornless contained no kaempferol derivative and Chester Thornless and Hull Thornless contained no pelargonidin 3-glucoside) (Tables 2 and 3). Among the three cultivars, Hull Thornless had higher amounts of anthocyanins and phenolic compounds than the other two cultivars (Tables 2 and 3). Blackberries treated with MJ had significantly higher fruit flavonoids. Increased MJ concentration from 0.01 to 0.1 mM significantly increased flavonoid contents in fruit (Tables 2 and 3). Comparing the control-untreated fruit and MJ-treated fruit, there was no qualitative change of the main phenolic compounds present in all three cultivars, but there were significant quantitative differences. The HPLC analysis of blackberry flavonoids showed that cyanidin 3-glucoside

Table 2
Effect of methyl jasmonate (MJ) treatment on content of ellagic acid, quercetin 3-glucoside, quercetin 3-rhamoside, kaempferol 3-glcoside, and kaempferol derivative (micrograms per gram of fresh weight) in fruit of three cultivars (Chester Thornless, Hull Thornless and Triple Crown) of blackberry[a]

Cultivar	Treatment	Ellagic acid[b]	Quercetin 3-glucoside[c]	Quercetin 3-rhamnoside[c]	Kaempferol derivative[c]	Kaempferol 3-glucoside[c]
Chester Thornless	Control	49.6 ± 3.9	30.3 ± 1.8	7.5 ± 0.2		6.6 ± 0.7
	0.01 mM	82.5 ± 3.7	38.3 ± 2.1	15.9 ± 0.7		10.1 ± 1.1
	0.1 mM	121.1 ± 8.9	59.7 ± 2.9	19.2 ± 0.4		14.8 ± 1.2
Hull Thornless	Control	65.0 ± 3.8	28.2 ± 1.4	9.3 ± 0.3	4.8 ± 0.2	9.5 ± 0.2
	0.01 mM	74.8 ± 2.7	48.3 ± 3.8	20.5 ± 1.2	6.5 ± 0.2	13.9 ± 0.9
	0.1 mM	132.2 ± 7.8	75.6 ± 4.2	31.4 ± 1.7	6.0 ± 0.2	17.8 ± 1.2
Triple Crown	Control	53.8 ± 2.1	29.3 ± 1.1	12.0 ± 1.1	4.7 ± 0.1	8.3 ± 0.5
	0.01 mM	44.6 ± 3.2	40.4 ± 2.7	19.5 ± 1.2	4.9 ± 0.1	13.6 ± 0.8
	0.1 mM	76.8 ± 4.2	51.0 ± 2.8	25.4 ± 3.2	5.1 ± 0.2	16.6 ± 1.2
Significance [d]						
Cultivar (*C*)		*	ns	*	*	*
Treatment (*T*)		*	*	*	ns	ns
C × *T*		*	*	*	ns	ns

[a] Data expressed as means ± SD.
[b] Data expressed as micrograms of ellagic acid equivalents per gram of fresh weight.
[c] Data expressed as micrograms of quercetin 3-glucoside equivalents per gram of fresh weight.
[d] *, ns, significant or non-significant at $p \leqslant 0.05$.

Table 3
Effect of methyl jasmonate (MJ) treatment on content of cyanidin 3-glucoside, pelargonidin 3-glucoside, cyanidin 3-xyloside, cyanidin 3-glucoside derivative, and dicarboxylic acid acylated of cyanidin 3-glucoside derivative in fruit of three cultivars (Chester Thornless, Hull Thornless and Triple Crown) of blackberry[a]

Cultivar	Treatment	Cyanidin 3-glucoside[b]	Pelargonidin 3-glucoside[b]	Cyanidin 3-xyloside[b]	Cyanidin 3-glucoside derivative[b]	Dicarboylic acid acylated of cyanidin 3-glucoside derivative[b]
Chester Thornless	Control	161.1 ± 2.1		15.2 ± 0.3	10.9 ± 2.1	91.2 ± 0.7
	0.01 mM	647.6 ± 4.3		40.9 ± 1.6	32.2 ± 4.3	109.8 ± 0.6
	0.1 mM	952.5 ± 5.4		60.3 ± 1.8	35.1 ± 5.4	152.9 ± 0.4
Hull Thornless	Control	424.6 ± 3.7		17.4 ± 0.6	15.6 ± 3.7	95.6 ± 3.2
	0.01 mM	1095.2 ± 7.6		54.1 ± 1.2	55.5 ± 7.6	161.8 ± 2.8
	0.1 mM	1343.4 ± 6.8		80.3 ± 2.2	66.2 ± 6.8	195.5 ± 4.5
Triple Crown	Control	114.9 ± 3.6	57.5 ± 2.5	14.3 ± 1.0	9.4 ± 3.6	88.9 ± 1.7
	0.01 mM	558.4 ± 7.3	50.7 ± 3.2	21.6 ± 0.9	36.9 ± 7.3	161.8 ± 2.3
	0.1 mM	743.2 ± 5.8	55.6 ± 2.7	35.5 ± 1.4	42.9 ± 5.8	153.2 ± 1.8
Significance[c]						
Cultivar (*C*)		*	*	ns	ns	ns
Treatment (*T*)		*	ns	*	*	*
C × *T*		*	ns	*	*	*

[a] Data expressed as means ± SD.
[b] Data expressed as micrograms of cyanidin 3-glucoside equivalents per gram of fresh weight.
[c] *, ns, significant or non-significant at $p \leqslant 0.05$.

was the predominant anthocyanin in Chester Thornless, Hull Thornless, and Triple Crown with 161.1, 424.6 and 114.9 μg/g fresh wt, respectively (for control untreated fruit) (Table 3). Upon treatment with 0.1 mM MJ, cyanidin 3-glucoside were 5.91-, 3.16- and 6.47-fold higher than those of the untreated control for Chester Thornless, Hull Thornless, and Triple Crown, respectively.

It has been reported that the antioxidant properties of berry fruit can be influenced by various external factors including environmental conditions. Growing temperature during preharvest season affected the antioxidant capacity of strawberries (*Fragaria* × *ananassa* Duch.). Higher day/night temperature condition (30/22 °C) resulted in greater production of phenolic acids, flavonols, and anthocyanins and on antioxidant capacities against peroxyl radical, superoxide radical, hydrogen peroxide, hydroxyl radical, and singlet oxygen, than lower day/night temperature condition (18/12 °C) in Earliglow and Kent strawberry cultivars (Wang & Zheng, 2001). This suggests that antioxidant activity and secondary metabolites may be manipulated by the modification of environmental conditions, such as preharvest application of MJ. This suggests that modification of environmental conditions such as preharvest application of MJ might also be possible to alternate antioxidant activity and secondary metabolites. MJ has been demonstrated to enhance anthocyanin accumulation in soybean seedling (Franceschi & Grimes, 1991), peach shoots (Saniewski et al., 1998), apple fruit (Kondo et al., 2001) and strawberry ripening (Pérez et al., 1997) and to stimulate β-carotene accumulation in ripening tomatoes (Saniewski & Czapski, 1983).

Different antioxidant compounds have different antioxidant abilities to scavenge various reactive oxygen species (Wang & Jiao, 2000). Blackberries had a high content of antioxidants, and cyanidin 3-glucoside was the most prevalent anthocyanin, contributing the most antioxidant activity in blackberries (Ding et al., 2006). Anthocyanins and phenolics are secondary plant metabolites. They protect the plant against damaging photodynamic reactions by quenching singlet oxygen species (Lewis, 1993). Anthocyanins are probably the largest group of phenolic compounds in the human diet and have been used for several therapeutic purposes including the treatment of diabetic retinopathy, fibrocystic disease, and vision disorders (Leonardi, 1993; Scharrer & Ober, 1981). Anthocyanins also have the potential to serve as radiation-protective agents, vasotonic agents, chemoprotective agents (Wang, Cao, & Prior, 1997), and decrease the fragility of capillaries, inhibit blood platelet aggregation, and strengthen the collagen matrix of connective tissues (Morazzoni & Bombardelli, 1996). Cyanidin belongs to the group of anthocyanins and has the typical C6–C3–C6 structure. In plants, the cyanidin is bound to a sugar molecule to form cyanidin 3-glucoside. Cyanidin has antioxidant and radical-scavenging activities. These actions may protect our cells against oxidative damage and reduce the risk of cancer and hearth disease. Cyanidin 3-glucoside suppresses nitric oxide production for the prevention of nitric oxide mediated inflammatory diseases (Tsuda, Horio, Uchida, Aoki, & Osawa, 2003).

Uncontrolled cell division is a primary key in the progression of a cancer tumor. Aberrations in mitotic regulatory pathways controlling cell proliferation are necessary for the establishment of all tumors. Deregulation of cell proliferation together with suppressed apoptosis is the minimal common platform for all cancer evolution and progression (Evan & Vousden, 2001). We found proliferation was inhibited in a dose-dependent in human lung cancer A549 cell and human promyelocytic leukemia HL-60 cell after exposure to the blackberry extracts (50 and 100 μg/ml) (Fig. 2). Among the three blackberry cultivars, Hull Thornless showed relatively potent antiproliferative activities on A549 cell growth and HL-60 cells. Pre-harvest application of MJ (0.01 and 0.1 mM) significantly enhanced the inhibition of proliferation A549 cells and HL-60 cells (Fig. 2). This may be due to the presence of increased content of antioxidants and other secondary

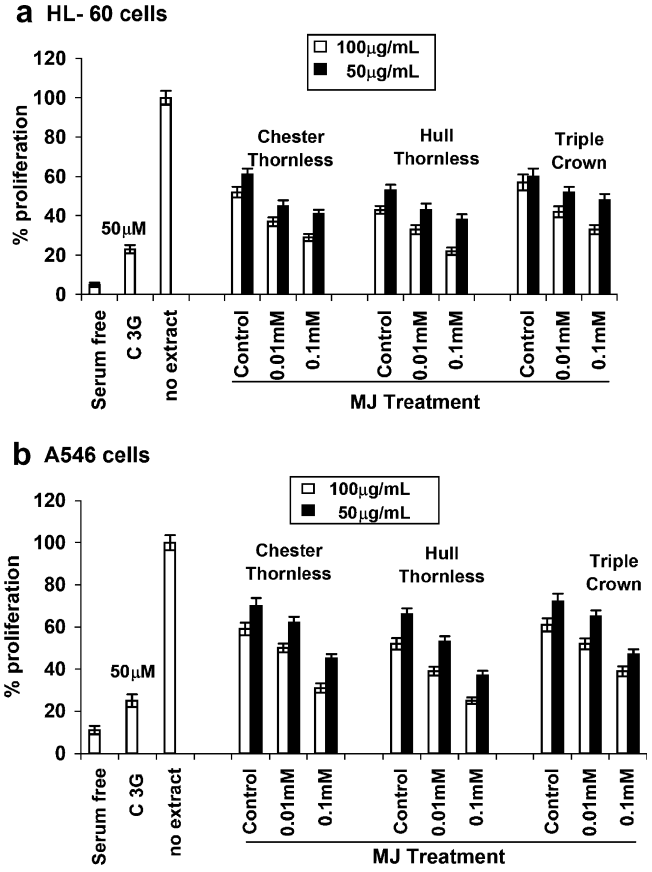

Fig. 2. Inhibition of proliferation of cancer cells by blackberry fruit extracts (a) HL-60 human leukemia cells and (b) human lung cancer cells, A549 (1×10^4), were incubated with different concentrations of three cultivars (Chester Thornless, Hull Thornless, Triple Crown) of blackberry fruit extracts (50 and 100 μg/ml) for 48 h and cell proliferation was determined by the MTT assay. Proliferation was expressed as a percentage of cell growth in wells that received no extract. Cyanidin 3-glucoside (C 3G) at 50 μM was used as positive control. Data are expressed as means ± SD of three samples.

metabolites in MJ treated blackberries. These results suggest that the chemopreventive effects of fresh blackberries may be through its antioxidant properties by blocking reactive oxygen species. Dietary freeze-dried strawberries were shown to effectively inhibit *N*-nitrosomethylbenzylamine-indueced tumorigensis in the rat esophagus (Evan & Vousden, 2001). Several other fruits have also shown inhibition of $HepG_2$ human liver cancer cell proliferation (Carlton et al., 2001; Meyers, Watkins, Pritts, & Liu, 2003; Sun, Chu, Wu, & Liu, 2002).

Blackberry extract induced human leukemia HL-60 cell apoptosis (Fig. 3a). Cancer cell apoptosis is a novel target for chemoprevention study (Sun et al., 2002). We found that blackberry extracts induced the apoptosis of human HL-60 leukemia cells in a dose-dependent manner (Fig. 3a). These data suggest that the treatment of HL-60 cells with blackberry extract causes cancer cell death by an apoptotic mechanism. Several fractions in the extract might be responsible for the induction of apoptosis, but

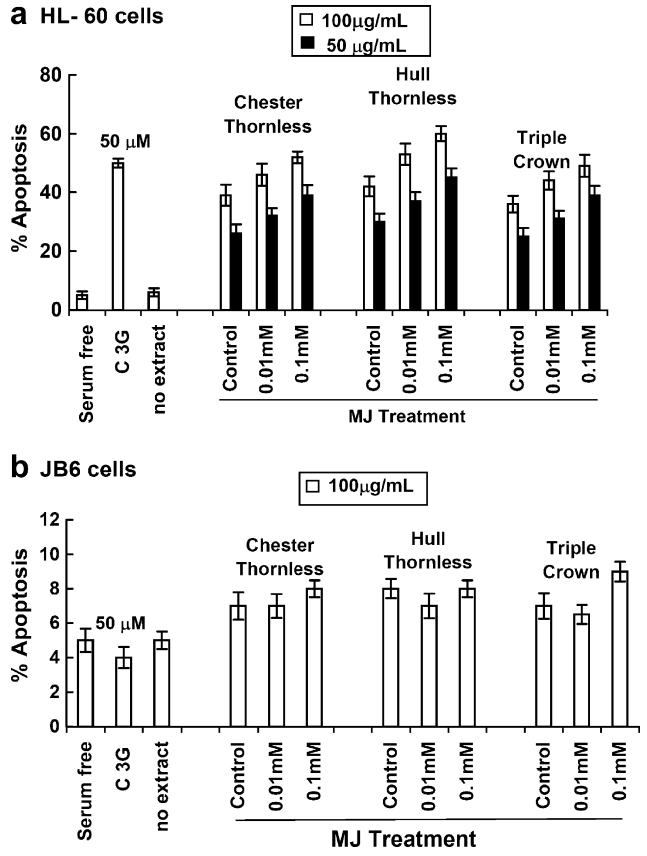

Fig. 3. Blackberry fruit extracts induced (a) human leukemia HL-60 cells apoptosis but (b) JB6 P^+ mouse epidermal cells were resistant to blackberry fruit extracts induced apoptosis. Human leukemia HL-60 cells and JB6 cells were treated with indicated doses of three cultivars of blackberry (Chester Thornless, Hull Thornless, Triple Crown) fruit extracts (50 and 100 μg/ml) for 18 h. The percent apoptotic cells were calculated by determining the number of cells with nuclear morphology change divided by the total number of cells. Cyanidin 3-glucoside (C 3G) at 50 μM was used as positive control. Data are expressed as means ± SD of three samples.

the possibility that antioxidant property of the extract may enhance the apoptosis of cancer cells should not be ruled out. We have found that the most dominant anthocyanin, cyanidin 3-glucoside, in blackberries possessed chemopreventive and chemotherapeutic activity (Ding et al., 2006). In cultured JB6 cells, cyanidin 3-glucoside was able to scavenge ultraviolet B (UVB)-induced $^{\cdot}OH$ and ($O_2^{\cdot-}$) radicals. In vivo studies indicated that cyanidin 3-glucoside treatment decreased the number of nonmalignant and malignant skin tumors per mouse induced by 12-*O*-tetradecanolyphorbol-13-acetate (TPA) in 7,12-dimethylbenz (*a*) anthracene (DMBA)-initiated mouse skin (Ding et al., 2006). Pretreatment of JB6 cells with cyanidin 3-glucoside inhibited UVB- and TPA-induced transactivation of nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1) and expression of cyclooxygenase-2 (COX-2) and tumor necrosis factor α (TNFα). Cyanidin 3-glucoside also blocked TPA-induced neoplastic transformation in JB6 cells. In addition, cyanidin 3-glucoside inhibited proliferation of a human lung carcinoma cell line, A549. Animal studies showed that cyanidin 3-glucoside reduced the size of A549 tumor xenograft growth and significantly inhibited metastasis in nude mice. Mechanistic studies indicated that cyanidin 3-glucoside inhibited migration and invasion of A549 tumor cells (Ding et al., 2006). Mulberry anthocyanins, cyanidin 3-rutinoside and cyanidin 3-glucoside, also exhibited an inhibitory effect on the migration and invasion of human lung cancer cell lines (Chen et al., 2006). This suggests that cyanidin could decrease the in vitro invasiveness of cancer cells. Therefore, cyanidin could be of great value in cancer therapy.

Compared with non-neoplastic cells, cancer cells constitutively generate large but tolerable amounts of ROS that apparently function as signaling molecules in the mitogen-activated protein kinases (MAPKs) pathway to constantly activate redox-sensitive transcription factors and responsive genes. These gene products are involved in the survival of cancer cells as well as their proliferation (Kawagoe, Kawagoe, & Sano, 2001). From these perspectives, Toyokuni, Okamoto, Yodoi, and Hiai (1995) proposed the concept of persistent oxidative stress in cancer cells. Reducing oxidative stress may suppress the proliferation of tumor cells and enhance cancer cell apoptosis (Lee et al., 2002; Shacter, Williams, Hinson, Sentürker, & Lee, 2000; Sun, Hail, & Lotan, 2004). The inductive effect of blackberry extracts on the apoptosis of human leukemia HL-60 cells may partially be due to its antioxidant properties by perturbing the favorable redox condition in cancer cells. In contrast, the extracts did not cause apoptosis in non-tumor JB6 cells (Fig. 3b).

There were negative correlations between cell proliferation of lung epithelial cancer cell line, A594 and HL-60 human leukemia cells, *vs.* antioxidant activity (ORAC) ranging from $R^2 = -0.6228$ to -0.9247 (Table 4). A positive correlation coefficient was also found between apoptosis activity of HL-60 human leukemia cancer cell and activities of antioxidants ranging from $R^2 = 0.7080$ to

Table 4
Correlations (R^2) among human cancer cell proliferation, and cancer cell apoptosis against antioxidant activities (orac), total phenolics, total anthocyanins and individual flavonoid from fruit extracts of three blackberry cultivars (Chester Thornless, Hull Thornless and Triple Crown)

	% Cancer cell proliferation (HL-60 cells)		% Cancer cell proliferation (A546 cells)		% Cancer cell apoptosis (HL-60 cells)	
	Fruit extract (100 μg/ml)	Fruit extract (50 μg/ml)	Fruit extract (100 μg/ml)	Fruit extract (50 μg/ml)	Fruit extract (100 μg/ml)	Fruit extract (50 μg/ml)
ORAC	−0.8802	−0.8265	−0.8939	−0.8874	0.8444	0.8943
Total phenolics	−0.8279	−0.7378	−0.8402	−0.8361	0.8967	0.9078
Total anthocyanins	−0.7840	−0.7233	−0.7364	−0.7722	0.7568	0.8157
Ellagic acid	−0.7133	−0.7276	−0.8100	−0.7771	0.7080	0.7219
Quercetin 3-glucoside	−0.8121	−0.7357	−0.9172	−0.9263	0.8833	0.9067
Quercetin 3-rhamnoside	−0.7039	−0.6228	−0.7179	−0.7787	0.7529	0.8236
Kaempferol 3-glucoside	−0.7765	−0.6631	−0.8001	−0.8303	0.7638	0.8726
Cyanidin 3-glucoside	−0.9247	−0.9223	−0.9022	−0.8440	0.9835	0.9080
Cyanidin 3-xyloside	−0.8347	−0.8752	−0.8843	−0.8371	0.9200	0.8385
Cyanidin 3-glucoside derivative	−0.7929	−0.7556	−0.7370	−0.7280	0.8871	0.8199
Dicarboylic acid acylated of cyanidin 3-glucoside derivative	−0.7148	−0.6273	−0.7253	−.7134	0.7883	0.7786

0.9835 (Table 4). The positive correlation values (R^2) between cancer cell apoptosis and ORAC, total phenolics, total anthocyanins and individual flavonoid (ellagic acid, quercetin 3-glucoside, quercetin 3-rhamnoside, kaempferol 3-glucoside, cyanidin 3-glucoside, cyanidin 3-xyloside, cyanidin 3-glucoside derivative and dicarboxylic acid acylated of cyanidin 3-glucoside derivative) were 0.8444, 0.8967, 0.7568, 0.7080, 0.8833, 0.7529, 0.7638, 0.9835, 0.9200, 0.8871 and 0.7883, respectively, for 100 μg/ml of blackberry fruit extracts (Table 4). This indicates that there is a significant relationship between antioxidant activity, antioxidant content and anticancer activity in blackberries. Early studies reported that natural antioxidants from fruits or vegetables could inhibit cancer cell growth and induce apoptosis (Agarwal, Singh, & Agarwal, 2002; Eberhardt, Lee, & Liu, 2000; Lu et al., 1998). It has been proposed that the consumption of whole fruits may provide the antioxidant balance needed to quench ROS, which has been implicated in tumorigenesis (Cao, Booth, Sadowski, & Prior, 1998).

4. Conclusion

Collectively, the data presented here indicate that blackberries could be an excellent food source for scavenging oxygen species in the human diet. Different cultivars of blackberries contains different amount of flavonoids with potent antioxidant properties. Preharvest application of MJ significantly enhanced blackberry fruit quality. Blackberries treated with MJ had higher soluble solids content, and lower titratable acid than untreated fruit. MJ treatment also significantly enhanced the content of flavonoids, antioxidant capacities and the inhibition A549 cell and HL-60 cell proliferation and also induced apoptosis in HL-60 cells. Their inhibitory activities had varying magnitudes because of the differences in blackberry cultivars and different concentrations of MJ treatment. The inhibitory effect of cancer cell proliferation and induction of apoptosis may be the result of the synergistic combination of the polyphenols in the berries. The results from this study indicate that there is a potential to select certain genotypes for health promoting properties as components in blackberry cultivars are different. Furthermore, the application of a naturally occurring compound such as MJ could enhance the secondary metabolites. Dietary supplementation with various fruits and vegetables including blackberries is beneficial to human health.

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