JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. **2009,** *57,* 5219–5226 **5219**
DOI:10.1021/jf900259m

Identifying Peach and Plum Polyphenols with Chemopreventive Potential against Estrogen-Independent Breast Cancer Cells

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Our objective was to evaluate the cancer suppression activity of extracts from a commercial variety of yellow-fleshed peach 'Rich Lady' (RL) and a red-fleshed plum 'Black Splendor' (BS) and identify the phenolic fractions that may possess potential as chemopreventive and/or chemotherapeutic natural compounds. The peach RL extract effectively inhibited the proliferation of the estrogen-independent MDA-MB-435 breast cancer cell line. The concentration to inhibit 50% of cell proliferation (IC_{50}) was ~42 mg/L for this cell line compared to an IC_{50} of ~130 and ~515 mg/L for the noncancerous breast cell line MCF-10A and the estrogen-dependent breast cancer cell line MCF-7, respectively. Similarly, BS extracts showed greater effects on MDA-MB-435 cells as compared to the other breast cancer or the normal breast cell lines. In general, BS extracts were less effective than RL extracts. Within all RL and BS fractions, fraction 3 (F3, flavonoids) and fraction 4 (F4, procyanidins) were more potent than fraction 1 (F1, phenolic acids) and fraction 2 (F2, anthocyanins) against the three cell lines. The order of potency of RL fractions against MDA-MB-435 was $F_3 \sim F_4 > F_1 > F_2$. The antiproliferative activity of pure compounds identified in F_3 and F_1 confirmed that quercetin 3β-glucoside is the bioactive compound in F_3, with the same level of toxicity on the estrogen-independent MDA-MB-435 breast cancer and breast epithelial MCF-10A cells (IC_{50} = 1.9 ± 0.2 and 1.8 ± 0.3, respectively). However, we confirmed that phenolic acids present in F_1: chlorogenic and neo-chlorogenic acids have potential as chemopreventive dietary compounds because of the relatively high growth inhibition exerted on the estrogen-independent MDA-MB-435 breast cancer cell line and low toxicity exerted in the normal MCF-10A cells.

KEYWORDS: Breast cancer; peaches; plums; chlorogenic acid; neo-chlorogenic acid

INTRODUCTION

According to the World Health Organization (WHO), cancer is one of the top 10 leading causes of death around the world and, among adult women, breast cancer accounts for 16% of cancer deaths (*1*). In the U.S., breast cancer is the second leading cause of cancer-related deaths and continues to be the most diagnosed type of cancer. The American Cancer Society has estimated that there will be 182 460 new cases of invasive breast cancer and 67 770 new cases of *in situ* breast cancer among women in the U.S. during 2008 (*2*). Most deaths from breast cancer are the result of distant metastasis rather than primary tumor burden, and chemotherapy is based on the use of nonspecific cytotoxic agents that kill cells indiscriminately.

Commercial varieties of peaches and plums have been previously quantified for their antioxidant activity and level of phenolic compounds (*3*). A dietary increase of antioxidant defense capacity has been considered a reasonable way to prevent reactive oxygen species (ROS)-mediated carcinogenicity. Epidemiological and human studies indicate that a lower risk of cancer is related to antioxidant-rich diets (*4*). Plant polyphenols have shown potential as natural anticarcinogenic compounds that according to their mechanisms of action may have potential as cancer-blocking and/or cancer-suppressing agents. Cancer-blocking agents act during the initiation stage protecting cellular targets by scavenging ROS and other oxidative species, enhancing carcinogen detoxification, modifying the carcinogen uptake and metabolism, and enhancing DNA repair. Cancer-suppressing agents inhibit the promotion and progression stages after the formation of pre-neoplastic cells by interfering with cell-cycle regulation (cyclin-dependent proteins), regulation of signal transduction pathways (MAP kinase, TGF-β serine−threonine kinase signaling, and β-catenin pathways), and transcription (NF-kB activation) and inducing tumor cell apoptosis (programmed cell death) (*5*). However, while most flavonoids/phenolics are considered safe, the consumption of dietary supplements, in which doses exeed those realistically biodisponible through a diet rich in fruits and vegetables, may be toxic. This has been supported by

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 Published on Web 05/28/2009

studies showing the pro-oxidant properties of flavonoids that may cause mitochondrial toxicity and interactions with drug-metabolizing enzymes (*6*, *7*).

Plant polyphenols, in general, may target one or more of the molecular pathways in cancer cells with only low toxicity in normal cells. Peaches and plums, in particular, contain a mixture of phytochemicals that include phenolic acids, flavonols, anthocyanins, procyanidins, and carotenoids (*8*). Several *in vivo* and *in vitro* studies have shown that these groups of phenolic compounds found in plant extracts have potential in chemoprevention and chemotherapy of breast cancer (*9*). Our goal was to study the antiproliferative activity of phenolics present in peaches (*Prunus persica*) and plums (*Prunus salicina*) in terms of high chemopreventive potential and low toxicity in normal cells and identify their bioactive compounds.

MATERIALS AND METHODS

Plant Material. Commercial varieties of yellow-fleshed peach 'Rich Lady' (RL) and red-fleshed plum 'Black Splendor' (BS) grown in California were collected at a mature firm stage and stored at 2−4 °C until use (less than 5 days). Upon arrival at Texas A&M University, the fruits were frozen after removal of the stones and kept at −20 °C until use.

Extraction of Phenolic Compounds. A total of 100 g of frozen fruit (flesh plus skin) was blended with 300 mL of methanol and left at 4 °C overnight. Extracts were filtered through Whatman #1 filter paper and methanol-evaporated at 45 °C using a rotavapor (Büchi, Switzerland). The remaining aqueous extracts were frozen at −80 °C and freeze-dried (FTS Systems, Inc., Stone Ridge, NY) at −50 °C and 200 mmHg of pressure. These extracts, designated as "crude extract", were further used for the cell-culture assays by applying different doses based on total phenolic content measured spectrophotometrically by the Folin−Ciocalteu method (*10*). The crude extract was redissolved in cell-specific culture medium and sterile-filtered for the cell growth proliferation assay. Complete solubility was confirmed by comparing the high-performance liquid chromatography (HPLC) profile of the methanolic extract before evaporation and the redissolved dry extract in the culture medium.

Fractionation of Phenolic Compounds. The crude extract was fractionated into phenolic acids (F_1), anthocyanins (F_2), flavonols (F_3), and procyanidins/polymeric anthocyanins (F_4) by solid-phase extraction using C18 cartridges as reported by Oszmianski et al. (*11*) (**Figure 1**). Briefly, the aqueous extract was adjusted to pH 7.0 with 5 N NaOH. A total of 50 mL of extract was loaded in SEP Pack C18 cartridge (55−105 μm, Waters Corp., Milford, MA) previously conditioned to pH 7.0 with 50 mL of 100% methanol and 50 mL of nanopure water (pH 7.0). The neutral phenolics were absorbed in the cartridge, while the phenolic acids were not. The cartridge was washed with 50 mL of water (pH 7.0). The water from the wash was combined with the phenolics that were not adsorbed in the cartridge and adjusted to pH 2.0. This mixture of compounds was loaded into a second cartridge previously conditioned at pH 2.0 with 50 mL of 100% methanol and 50 mL of nanopure water at pH 2.0. Phenolic acids bound to the matrix of the second cartridge (F_1) were later eluted with 50 mL of 100% methanol. After the pH was adjusted to 2.0 in the first cartridge, elution of anthocyanins was accomplished by passing 50 mL of 16% acetonitrile at pH 2.0 (F_2). The flavonols were eluted using 50 mL of 100% ethyl acetate (F_3) and the anthocyanin polymers using 50 mL of 100% methanol (F_4). Fractions eluted with low boiling point solvents (F_1, F_3, and F_4) were completely evaporated at 45 °C using a speed vac (Savant SC100). The F_2 was evaporated using a rotavapor (Büchi, Switzerland), followed by freeze-drying (FTS Systems, Inc., Stone Ridge, NY) at −50 °C and 200 mmHg of pressure. All fractions were kept under nitrogen gas at −20 °C for use in cell culture.

HPLC−Diode Array Detector (DAD) Analysis. The RL and BS crude extracts as well as their fractions were analyzed using a Waters (Milford, MA) HPLC system. An Atlantis C_{18} 5 μm, 4.6 × 150 mm column and a 4.6 × 20 mm guard column was used for the separation of phenolic compounds. The mobile phase was composed of solvent A (nanopure water adjusted to pH 2.3 with 2 N HCl) and solvent B (acetonitrile HPLC grade). The elution was as follows: isocratic conditions from 0 to 5 min with 85% A and 15% B, gradient conditions from minutes 5 to 30 starting with 85% A and ending with 0% and starting with 15% B and ending with 100%, and then isocratic conditions from minutes 30 to 35 with 0% A and 100% B (*12*). The mobile phase was used at a flow rate of 1 mL/min, and 20 μL of sample previously dissolved in MeOH and filtered through a 0.2 μm PTFE filter was injected. Identification of phenolic compounds was performed by a comparison of retention times and UV−vis spectral data of the chromatographic profiles, with standards obtained from Sigma-Aldrich (St Louis, MO).

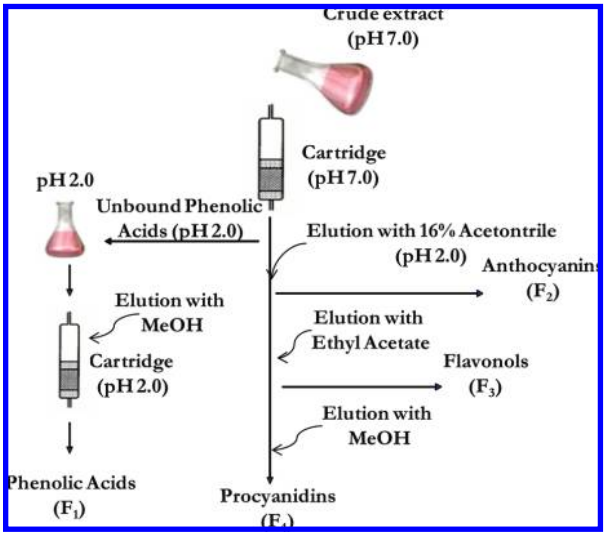

Figure 1. Scheme of fractionation used for RL and BS crude extracts.

Cell Lines. Cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown as recommended by the ATCC. MCF-7, the estrogen-positive human breast cancer cell line, was cultured using Dulbecco's modified Eagle's medium (DMEM), high glucose, L-glutamine, 25 nM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pyridoxine hydrochloride, without sodium pyruvate, and without phenol red (Invitrogen Corp., Grand Island, NY), supplemented with 5 mL of insulin (1 mg/mL), 10% (v/v) fetal bovine serum (FBS), and 1% penicillin−streptomycin antibiotic mix. MDA-MB-453, the estrogen-negative human breast cancer cell line, was cultured using DMEM with L-glutamine, 4.5 g/L glucose, and without sodium pyruvate, supplemented with 10% (v/v) FBS and 1% penicillin−streptomycin antibiotic mix. MCF-10A, the breast epithelial cells, were cultured in DMEM/F12, supplemented with 5% (v/v) FBS, 1% penicillin−streptomycin antibiotic mix, 1 mL of epidermal growth factor (EGF) (10 μg/mL), 5 mL of insulin (1 mg/mL), 250 μL of hydrocortisone (1 mg/mL), and 20 μL of cholera toxin. Culture media were supplied by Invitrogen (Gibco, Invitrogen Corp., Grand Island, NY). Cells were maintained at 37 °C with a humidified 5% CO_2 atmosphere.

Cell Proliferation. Cell growth and the concentration of phenolic compounds to inhibit the cell growth by 50% (IC_{50}) was assessed using an electronic counter (Z1 Series, Beckman Coulter, Inc.). Briefly, the number of viable cells was quantified by removing culture medium and rinsing with trypsin solution (Gibco, Invitrogen Corp., Grand Island, NY), followed by the addition of 0.2 mL of trypsin solution to the plate, which remained at 37 °C until the cells detached. After detachment, 0.8 mL of fresh culture medium was added to stop trypsination and total volume was recovered for cell counting. Cells were seeded (40 000/well in a 6-well plate) and incubated for 24 h to allow for cell attachment before exposure to varying concentrations of extracts. A pretreatment number of cells (0 time value) was established, and medium was replaced containing the crude extracts or fractions at different concentrations. Total phenolics were quantified by the Folin−Ciocalteau method (*10*), and doses were based on their phenolic content and expressed as milligrams of chlorogenic acid equivalent per liter. Fresh medium along with compounds was replaced every 48 h. Crude phenolics extracts, F_1 and F_2, were redissolved in the culture medium and sterile-filtered before use, whereas F_3 and F_4 were dissolved in ethanol and added to the culture medium in the ratio 1:1000 (ethanol/culture medium). Each experiment was carried out in triplicate, and results were expressed as means ± standard deviation (SD). The difference in number of cells between the final and 0 time represents net growth.

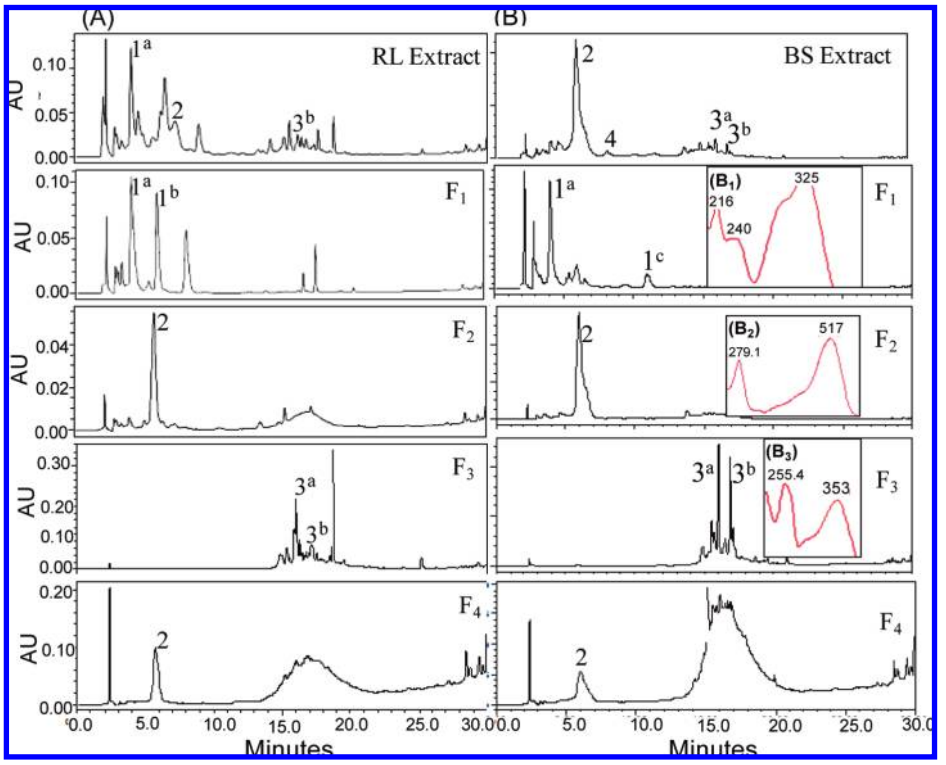

Figure 2. Chromatograms at 280 nm of (A) RL and (B) BS extracts and the four fractions obtained using solid-phase extraction with a C_{18} cartridge. (1^a) neo-chlorogenic acid, (1^b) chlorogenic acid, (1^c) caffeoylquinic acid derivative, (2) cyanidin 3β-glucoside, (3^a) quercetin 3β-rutinoside, (3^b) quercetin 3β-glucoside, and (4) catechin derivative. UV−vis spectrum of standards: (B_1) chlorogenic and neo-chlorogenic acid, (B_2) cyanidin 3β-glucoside, and (B_3) quercetin 3β-glucoside and quercetin 3β-rutinoside.

Statistical Analysis. Quantitative data represent mean values with the respective SD or standard error of the mean (SEM) corresponding to three or more replicates. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS, version 15.0 (SPSS, Inc., Chicago, IL). Posthoc Tukey pairwise comparisons were used ($p < 0.05$ and $p < 0.01$).

RESULTS AND DISCUSSION

HPLC−DAD Analysis of Phenolic Compounds in RL and BS Extracts and Fractions. The RL and BS crude extracts and fractions were analyzed by HPLC−DAD. Identification of phenolic compounds was performed at 280 nm by a comparison of retention times and UV−vis spectral data with known standards (**Figure 2**).

The chromatographic profile of RL extract (**Figure 2A**) indicated the presence of neo-chlorogenic acid (peak 1^a, RT = 4 min), cyanidin 3β-glucoside (peak 2), and quercetin 3β-glucoside (peak 3^b, RT = 16.2 min). The amount of neo-chlorogenic acid represents ~3.57% of the total phenolics extracted from RL. We were unable to quantify chlorogenic acid on the crude RL phenolic extract because it coeluted with cyanidin 3β-glucoside. Chromatograms of RL fractions showed that the main compounds in F_1 were neo-chlorogenic acid (peak 1^a, RT = 4 min) and chlorogenic acid (peak 1^b, RT = 7 min). F_2 contained cyanidin 3β-glucoside (peak 2, RT = 6.8 min). In F_3, flavonols with the typical glycosylation at position 3 were found (*13*) between 15 and 17 min. We identified quercetin 3β-rutinoside (peak 3^a, RT = 15.9 min) and quercetin 3β-glucoside (peak 3^b, RT = 16.3 min). F_4 was composed of procyanidins and traces of cyanidin 3β-glucoside (peak 2).

Chromatograms of BS extract (**Figure 2B**) indicated the presence of cyanidin 3β-glucoside (peak 2) and the flavonols quercetin 3β-rutinoside (peak 3^a) and quercetin 3β-glucoside (peak 3^b), and on the basis of spectral characteristics, a catechin derivative was also found (peak 4, RT = 8 min). The BS fractions presented profiles similar to those found for RL fractions: F_1 contained neo-chlorogenic acid (peak 1^a) and a caffeoylquinic acid derivative with the same UV spectra though different retention time (peak 1_c, RT = 11 min) of chlorogenic acid, and other minor compounds with the characteristic spectra of hydroxycinnamic acid derivatives were detected as well. The F_2, F_3, and F_4 of BS had the same phenolic profiles as those found in the RL fractions.

In general, our findings regarding the phenolic profile of RL and BS extracts and fractions agree with those previously reported for California peach and plum varieties (*8*). Among them, peaches contain mainly the phenolic acids, chlorogenic and neo-chlorogenic acids; the flavan-3-ols, catechin, catechin derivatives, and procyanidins; the flavonols, quercetin and quercetin derivatives; and small amounts of anthocyanins, cyanidin 3β-glucoside and cyanidin 3β-rutinoside (*8*). The red-fleshed plum varieties contain the same pattern of phenolics combined with high amounts of anthocyanins (*3*, *8*). Moreover, the study on California RL peach using HPLC−mass spectrometry (MS) analysis (*8*) confirmed the structure of the main phenolics identified in the F_1, F_2, and F_3 of the present study. These phenolics have been the subject of many health-related claims supported by *in vitro* and *in vivo* studies and related to their properties as antioxidants and anticarcinogenic dietary compounds (*14*−*16*).

Cell Proliferation. The yields of RL and BS extracts after methanol extraction were 5.8 ± 0.8 and 5.6 ± 0.5 g of crude phenolic extract/100 g of fruit, respectively. The RL and BS extracts exerted cell growth inhibition against both cell lines, the estrogen-dependent MCF-7 and the estrogen-independent MDA-MB-435 (**Figure 3**). Within the range of 0−35 mg of chlorogenic acid equivalent/L, RL extracts suppressed the proliferation of MDA-MB-435 cells (**Figure 3C**), whereas the MCF-7 estrogen receptor positive cells were several times more resistant

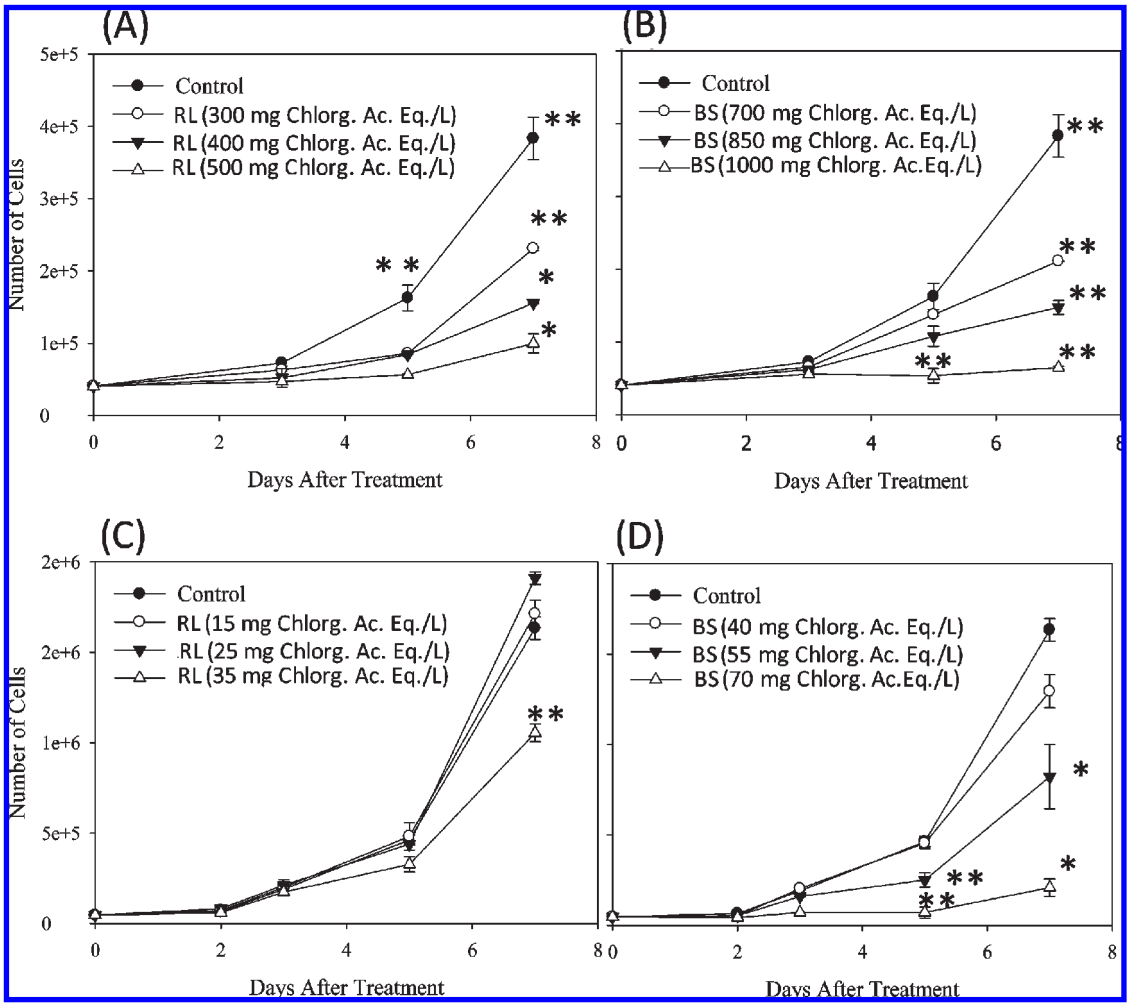

Figure 3. Cell proliferation of MCF-7 estrogen receptor positive breast cancer cells treated with (A) RL and (B) BS crude phenolic extracts and MDA-MB-435 estrogen receptor negative breast cancer cells treated with (C) RL and (D) BS crude phenolic extracts. Cells were treated with different doses of extracts, and cell growth was assessed every 2 days up to day 7. Values are the means ± SEM ($n = 3$). Asterisks indicate a significant difference between treatments at the same incubation time (days): (*) $p \leq 0.05$ and (**) $p \leq 0.01$.

to RL extracts (active concentrations in the range of 0−500 mg of chlorogenic acid equivalent/L) (**Figure 3A**). A similar pattern of dose- and time-dependent cell growth suppression in both cell lines was found for BS extracts (panels **B** and **D** of **Figure 3**), although the dose range was higher than the active dose range found for RL (0−70 and 0−1000 mg of chlorogenic acid equivalent/L for MDA-MB-435 and MCF-7, respectively).

Several studies have reported the association of fruit and vegetable consumption with reduced cancer risk (*9*). Edible plants, because of their safety and the fact that they are not perceived as "medicine", are increasingly being considered as sources of natural anticancer compounds. This is supported by evidence that many non-nutrient compounds in plant foods are effective inhibitors of human breast cancer cell proliferation *in vitro*. The antiproliferative activity of fruit extracts as sources of dietary phytochemicals has been previously reported for several cancer cell lines, and most of the studies have attributed their enhanced antiproliferative activity to interactions of individual phytochemicals, suggesting a synergistic or additive effect (*17*). However, most of these studies fail to assess the toxicity of these natural compounds in normal cells.

Cell-Growth-Suppressive Activity of RL and BS Crude Phenolic Extracts against Epithelial MCF-10A Breast Cells. To evaluate the potential toxicity of RL and BS extracts to nontumor cells, we included the MCF-10A breast epithelial cells to our study (**Figure 4**). At 35, 70, and 140 mg of chlorogenic acid equivalent/L, RL suppressed the proliferation of MCF-10A by 11, 23, and 53%, respectively. The BS extracts also inhibited the growth of MCF-10A, although to a lesser degree. Within the range of 55−220 mg of chlorogenic acid equivalent/L, BS extracts suppressed the proliferation of MCF-10A by 5−28%.

Results showed that the MCF-7 estrogen receptor positive cell line was more resistant to RL and BS phenolics (IC_{50} = 515 and 925 ± 70 mg of chlorogenic acid equivalent/L, respectively) than MDA-MB-435, suggesting that these compounds may be acting through some alternative mechanisms than those reported for phytoestrogens that have shown to inhibit the growth of MCF-7 *in vitro* and *in vivo*. In contrast, the noncancer MCF-10A cells were more resistant to the extract-mediated growth suppression than MDA-MB-435 cells. For example, at concentrations that RL and BS extracts suppressed the growth of MDA-MB-435 cells by 50% (42 and 54 mg/L, respectively), RL and BS extracts inhibited the growth of MCF-10A by ~15 and ~6%, respectively, demonstrating the preferential suppression of these extracts on the growth of MDA-MB-435 estrogen receptor negative breast carcinoma cells (**Table 1**).

The low concentration of RL and BS extracts (25 and 40 mg of chlorogenic acid equivalent/L, respectively) seems to increase the net cell growth; this phenomenon is known as mitohormesis. It is hypothesized that a low-dose challenge with a toxin may trigger

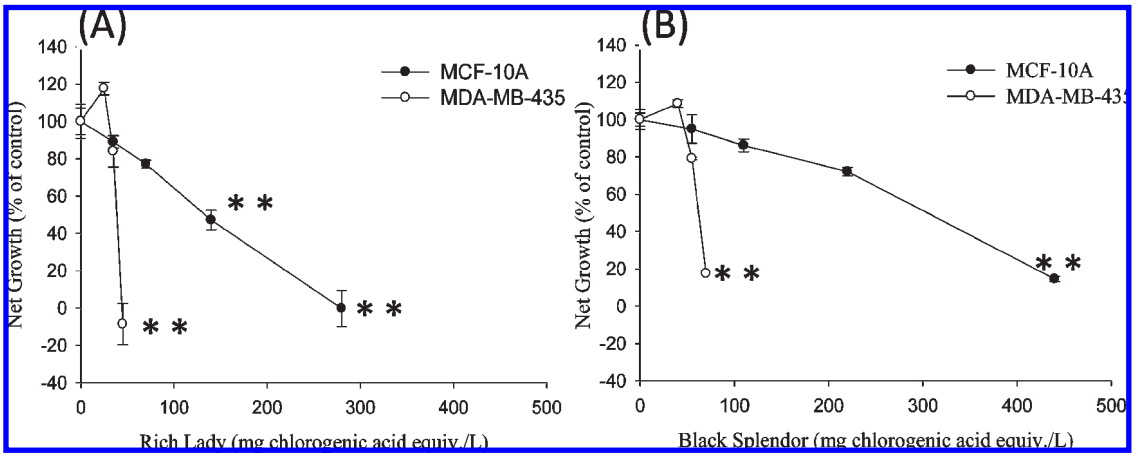

Figure 4. Representative evaluation of the concentration-dependent impact of crude phenolics extracts from (A) peach RL and (B) plum BS on the net growth of human MDA-MB-435 breast cancer cells and the epithelial MCF-10A breast cells. Cells were incubated with extracts, and net growth was measured at 3 days. Values are mean ± SEM (n = 3). Asterisks indicate a significant difference compared to the untreated control: (**) $p \leq 0.01$.

Table 1. IC_{50} Values of Crude Phenolic Extracts Obtained from RL Peaches and BS Plums for Growth Suppression of Different Breast Cell Lines

	IC_{50} (mg of chlorogenic acid equivalent/L)	
cell line	RL	BS
MCF-7	515[a]	925[b] ± 70
MDA-MB-435	42[b] ± 4	54[b] ± 8
MCF-10A	130[b] ± 36	223[b] ± 103

[a] Average of two determinations. [b] Average of three or more independent determinations ± SD.

certain repair mechanisms in the cell, which once initiated, not only neutralize the effect of the toxin but also repair other defects not caused by the toxin, thus promoting cell growth (*18*).

Cell-Growth-Suppressive Activity of RL and BS Phenolic Fractions. To identify the specific compounds with chemopreventive and chemotherapeutic potential for breast cancer treatment, the four fractions obtained by solid-phase extraction were tested for their cell growth inhibition activity. The yields of F_1, F_2, F_3, and F_4 for RL were 54 ± 11, 73 ± 23, 40 ± 10, and 12 ± 6 mg of phenolic fraction/100 g of fruit fresh weight, whereas the yields for BS were 30 ± 18, 200 ± 6, 67 ± 15, and 61 ± 11 mg of phenolic fraction/100 g of fruit. The RL fractions exerted a dose-dependent cell growth inhibition with the order of potency against MDA-MB-435 of $F_4 \geq F_3 > F_1 > F_2$.

The active growth inhibition doses for F_3 and F_4 were from 5 to 20 mg of chlorogenic acid equivalent/L, whereas F_1 and F_2 were less potent, with an active dose range from 40 to 80 mg of chlorogenic acid equivalent/L (**Figure 5A**). A similar pattern was observed for BS fractions; however, F_2 (anthocyanin-rich fraction) appears more potent than F_1 (phenolic-acid-rich fraction) (**Figure 5B**). Although cyanidin 3β-glucoside was identified in both F_2 and F_4, the relatively higher potency of F_4 may be attributed to the presence of procyanidins, which, in combination with cyanidin 3β-glucoside, might have potentiated the human breast cancer antiproliferative activity (*17*, *19*). The estrogen-dependent MCF-7 cells were more resistant to RL and BS fractions with active growth inhibition doses reaching levels as high as ~100−150 mg/L (F_3 and F_4) or ~300−500 mg/L (F_1 and F_2) (data not shown). This may indicate that the chemopreventive effect of RL and BS polyphenols in breast cancer is not mediated by modulation of estrogen nuclear receptors, as reported for phytoestrogens (*20*).

We further evaluated the chemopreventive potential of RL and BS fractions by assessing their dose-dependent cell growth inhibition on the breast epithelial MCF-10A cells (panels **C** and **D** of **Figure 5**). We found that F_3 (flavonol) and F_4 (procyanidins) were the most potent in inhibiting the growth of MCF-10A within the same range of active concentrations for MDA-MB-435 (5−20 mg/L). However, MCF-10A were more resistant to F_1 (phenolic acids) and F_2 (anthocyanins), as reflected for their higher IC_{50} values (**Table 2**). These results suggest that phenolic acids present in F_1, mainly chlorogenic and neo-chlorogenic acids (*8*), may have potential as chemopreventive and chemotherapeutic natural compounds against metastasis cancer because they preferentially target the MDA-MB-435 cancer cells over the noncancer breast epithelial cells (*5*).

Most studies that have reported the growth inhibitory effect of plant extracts against different cancer cell lines and identified the most active fraction of polyphenols have found compounds other than phenolic acids, such as anthocyanins and flavonols, as the most active (*21*). However, most of these studies failed to assess the growth inhibitory effect of the fractions on noncancerous cells. Furthermore, even some studies show that these phenolic acids (i.e., chlorogenic acid and caffeoylquinic acid derivatives) exert a dose-dependent inhibition of cell proliferation, as reported for sweet potato leaf extract (*22*). The antiproliferative activity of phenolic acids has been inconsistent and highly dependent upon the cell line.

Cell-Growth-Suppressive Activity of Pure Compounds Present in F_1 and F_3. The main compounds found in F_1 and F_3, the phenolic acids chlorogenic and neo-chlorogenic acids, and the favonols quercetin 3β-glucoside and quercetin 3β-rutinoside, were obtained as pure standards from Sigma-Aldrich (St Louis, MO) and tested for their growth-suppressive activity against the estrogen-dependent MCF-7 breast cancer cells, the estrogen-independent MDA-MB-435 breast cancer cells, and the noncancerous MCF-10A cells (**Figure 6**). The most potent inhibitor of cell proliferation was quercetin 3β-glucoside, with an order of potency: MDA-MB-435 ~ MCF-10A > MCF-7. The active dose range for cell growth suppression for MDA-MB-435 and MCF-10A was 2−3 mg/L, whereas the active dose range for MCF-7 was relatively high (~10−25 mg/L, data not shown). Even though quercetin 3β-glucoside exerted the highest cell growth suppression on the estrogen-independent MDA-MB-435, it also exerted the same level of growth inhibition on the noncancerous cells MCF-10A. The toxicity of quercetin was previously reported (*23*) and seems to be related to an oxidative stress effect. In general, the cytotoxicity of polyphenols has been related to ROS formation, which increases upon a decrease in the potential of phenoxyl radical/phenol redox couple (0.54 and 0.33 for chlorogenic acid

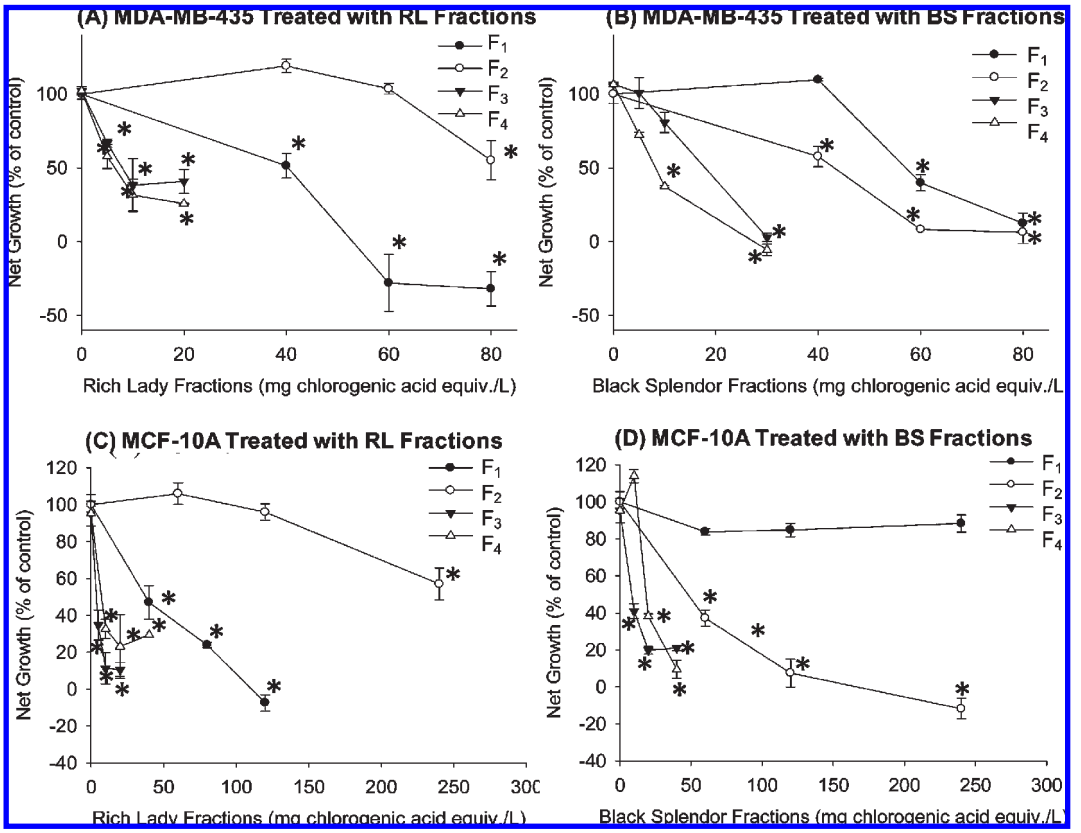

Figure 5. Representative evaluation of the concentration-dependent impact of phenolic fractions from RL and BS on the net growth of (A and B) human MDA-MB-435 breast cancer cells and (C and D) epithelial MCF-10A breast cells. Cells were incubated with fractions, and net growth was measured at 3 days. Values are mean ± SEM (n = 3). An asterisk indicates a significant difference compared to the untreated control: (∗) $p \leq 0.05$.

Table 2. IC_{50} Values of Phenolics Fractions Obtained from Rich Lady Peaches (RL) and Black Splendor Plums (BS) for Growth Suppression of Different Breast Cell Lines

	IC_{50} (mg of chlorogenic acid equivalent/L)					
	MCF-7		MDA-MB-435		MCF-10A	
cell line	RL	BS	RL	BS	RL	BS
F_1	~210	~250	30[a] ± 10	62[a] ± 9	43[a] ± 8	>240[b]
F_2	~600	~220	85[a] ± 9	56[a] ± 14	223[a] ± 38	47[a] ± 18
F_3	~110	~200	10[a] ± 3	16[a] ± 2	4.5[a] ± 0.7	13[a] ± 3
F_4	~140	~180	10[a] ± 9	12[a] ± 5	6[a] ± 3	16[a] ± 6

[a] Average of three or more independent determinations ± SD. [b] Average of two determinations.

and quercetin, respectively) (*24*). In addition, it has been reported that polyphenol cytotoxicity increases with an increase in their lipophilicity (octanol/water distribution coefficient, log D). This emphasizes the potential intracellular accumulation and higher cytoxicity of quercetin versus chlorogenic acid (log D = 2.74 and −2.36, respectively) (*24*). In contrast, the chemically related quercetin 3β-rutinoside did not exert growth inhibition on any of the cell lines tested (**Figure 6**). On the other hand, the phenolic acids chlorogenic and neo-chlorogenic acids induced growth suppression on the estrogen receptor negative MD-MB-435 cells, with no effect on the growth of breast epithelial MCF-10A cells up to the highest dose tested (60 mg/L) (**Figure 6**).

Even though both chlorogenic and neo-chlorogenic acids, when analyzed for their scavenging activity on superoxide anion radicals and their inhibitory effect against oxidation of methyl linoleate, showed almost the same values (*25*), they differ in their growth-suppressive activity against MDA-MB-435 breast cancer cells (IC_{50} = 17 and 10 mg/L for chlorogenic and neo-chorogenic acids, respectively) (**Table 3**). This indicates a structure−activity relationship that may identify neo-chlorogenic acid as a more potent bioactive compound against metastatic cancer. The order of sensitivity of MDA-MB-435 cells to the tested compounds follows quercetin 3β-glucoside > neo-chlorogenic acid > chlorogenic acid. It is interesting to find that chlorogenic and neo-chlorogenic acids, which are widely distributed in fruits and vegetables, may have potential as chemopreventive and chemotherapeutic natural compounds, because they preferentially reduce the number of MDA-MB-435 breast cancer cells over the normal MCF-10A breast epithelial cells. Chlorogenic acid is found in many types of fruit (0.5−2 g of hydroxycinnamic acids/kg of fresh weight) (*26*), in coffee (70−350 mg of chlorogenic acid/cup) (*27*), and in traditional Chinese medicinal herbs (*28*). Daily intake in coffee drinkers might provide ~0.5−1.0 g of chlorogenic acid, and bioavailability studies indicate that approximately 33% is absorbed in the small intestine and enters into the blood circulation (*29*). The unabsorbed chlorogenic acid would reach the colon, where its bioavailability largely depends upon its metabolism by the gut microflora (*30*). An investigation on the absorption of chlorogenic acid in rats showed that a maximal concentration of 17.73 ± 2.76 mg/L was reached in plasma after oral administration of 600 mg/kg (*28*). These studies provide insights for potential chemopreventive effects of chlorogenic and neo-chlorogenic acids *in vivo*.

In conclusion, peaches and plums contain a mixture of phenolic compounds that preferentially inhibit the growth of the estrogen-independent MDA-MB-435 breast cancer cells over either the estrogen-dependent MCF-7 breast cancer cells or the breast epithelial MCF-10A cells. In general, RL extracts were more effective than BS extract in inhibiting the proliferation of the three cell lines, with the order of sensitivity being MDA-MB-435

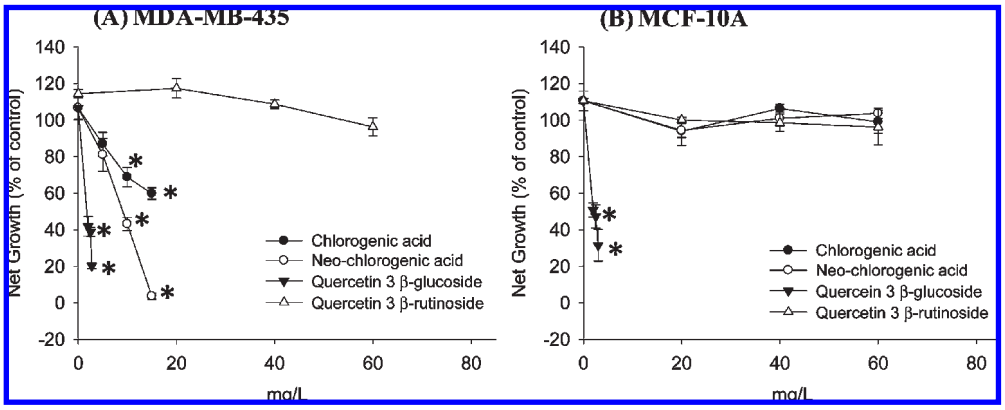

Figure 6. Representative curves showing the concentration-dependent growth suppression of the phenolic acids chlorogenic acid and neo-chlorogenic acid and the flavonols quercetin 3β-glucoside and quercetin 3β-rutinoside on the (A) estrogen-independent MDA-MB-435 breast cancer cells and (B) epithelial MCF-10A breast cells. Cells were incubated with standards dissolved in DMSO, and net growth was measured at 3 days. Values are mean ± SEM (n = 3). An asterisk indicates a significant difference compared to the untreated control: (*) $p \leq 0.05$.

Table 3. IC_{50} Values of Pure Compounds Present in F_1 and F_3 of Peach RL for Growth Suppression of Different Breast Cell Lines

	IC_{50} (mg/L)		
compound	MDA-MB-435	MCF-7	MCF-10A
chlorogenic acid	17[a] ± 4	>60[a]	>60[b]
neo-chlorogenic acid	10[a] ± 3	>60[a]	>60[b]
quercetin 3β-glucoside	1.9[a] ± 0.2	~23[a]	1.8[b] ± 0.3
quercetin 3β-rutinoside	>60[b]	>60[a]	>60[b]

[a] Average of two determinations. [b] Average of three or more independent determinations ± SD.

> MCF-10A > MCF-7. We found that F_1, which represents ~30 and ~8% (mg/100 mg) of the total phenolic fraction extracted from RL and BS, respectively, may possess chemopreventive and chemotherapeutic potential. The identification of active compounds with chemopreventive potential may lead to the development of natural supplements that exert low or no toxicity to normal cells. The main compounds identified in F_1 from RL chlorogenic and neo-chlorogenic acids did not inhibit the growth of the noncancerous cells up to 60 mg/L (~5−6-fold difference in IC_{50} compared to MDA-MB-435). These results have important clinical implications because chlorogenic and neo-chlorogenic acids are widespread among food plants, including vegetables and fruits, and constitute an integral part of the human diet.

LITERATURE CITED

(1) World Health Organization (WHO). World Health Statistics 2008. WHO, Geneva Switzerland, **2008**, http://www.who.int/whosis/whostat/2008/en/index.html (accessed on March 13, **2009**).

(2) American Cancer Society. American Cancer Society Cancer Facts and Figures 2008; http://www.cancer.org/downloads/STT/2008CAFFfinalsecured.pdf (accessed on March 13, **2009**).

(3) Vizzotto, M.; Cisneros-Zevallos, L.; Byrne, D. H.; Ramming, D. W.; Okie, W. R. Large variation found in the phytochemical and antioxidant activity of peach and plum germplasm. *J. Am. Soc. Hortic. Sci.* **2007**, *132* (3), 334–340.

(4) Block, G.; Patterson, B.; Subar, A. Fruit, vegetables, and cancer prevention: A review of the epidemiological evidence. *Nutr. Cancer* **1992**, *18* (1), 1–29.

(5) Russo, G. L. Ins and outs of dietary phytochemicals in cancer chemoprevention. *Biochem. Pharmacol.* **2007**, *74* (4), 533–544.

(6) Galati, G.; O'Brien, P. J. Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. *Free Radical Biol. Med.* **2004**, *37* (3), 287–303.

(7) Tsuji, P. A.; Walle, T. Cytotoxic effects of the dietary flavones chrysin and apigenin in a normal trout liver cell line. *Chem.-Biol. Interact.* **2008**, *171* (1), 37–44.

(8) Tomas-Barberan, F. A.; Gil, M. I.; Cremin, P.; Waterhouse, A. L.; Hess-Pierce, B.; Kader, A. A. HPLC−DAD−ESIMS analysis of phenolic compounds in nectarines, peaches, and plums. *J. Agric. Food Chem.* **2001**, *49* (10), 4748–4760.

(9) Thomasset, S. C.; Berry, D. P.; Garcea, G.; Marczylo, T.; Steward, W. P.; Gescher, A. J. Dietary polyphenolic phytochemicals—Promising cancer chemopreventive agents in humans? A review of their clinical properties. *Int. J. Cancer* **2007**, *120* (3), 451–458.

(10) Swain, T.; Hillis, W. The phenolic constituents of *Prinus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **1959**, *10*, 63–68.

(11) Oszmianski, J.; Ramos, T.; Bourzeix, M. Fractionation of phenolic compounds in red wine. *Am. J. Enol. Vitic.* **1988**, *39* (3), 259–262.

(12) Pedreschi, R.; Cisneros-Zevallos, L. Phenolic profiles of Andean purple corn (*Zea mays* L.). *Food Chem.* **2007**, *100* (3), 956–963.

(13) Markham, K. R. *Techniques of Flavonoid Identification*; Academic Press: New York, 1982.

(14) Dechsupa, S.; Kothan, S.; Vergote, J.; Leger, G.; Martineau, A.; Berangeo, S.; Kosanlavit, R.; Moretti, J. L.; Mankhetkorn, S. Quercetin, siamois 1 and siamois 2 induce apoptosis in human breast cancer MDA-MB-435 cells xenograft in vivo. *Cancer Biol. Ther.* **2007**, *6* (1), 56–61.

(15) Granado-Serrano, A. B.; Martin, M. A.; Izquierdo-Pulido, M.; Goya, L.; Bravo, L.; Ramos, S. Molecular mechanisms of (−)-epicatechin and chlorogenic acid on the regulation of the apoptotic and survival/proliferation pathways in a human hepatoma cell line. *J. Agric. Food Chem.* **2007**, *55* (5), 2020–2027.

(16) Hou, D. X. Potential mechanisms of cancer chemoprevention by anthocyanins. *Curr. Mol. Med.* **2003**, *3* (2), 149–159.

(17) Seeram, N. P.; Adams, L. S.; Hardy, M. L.; Heber, D. Total cranberry extract versus its phytochemical constituents: Antiproliferative and synergistic effects against human tumor cell lines. *J. Agric. Food Chem.* **2004**, *52* (9), 2512–2517.

(18) Tapia, P. C. Sublethal mitochondrial stress with an attendant stoichiometric augmentation of reactive oxygen species may precipitate many of the beneficial alterations in cellular physiology produced by caloric restriction, intermittent fasting, exercise and dietary phytonutrients: "Mitohormesis" for health and vitality. *Med. Hypotheses* **2006**, *66* (4), 832–843.

(19) Faria, A.; Calhau, C.; de Freitas, V.; Mateus, N. Procyanidins as antioxidants and tumor cell growth modulators. *J. Agric. Food Chem.* **2006**, *54* (6), 2392–2397.

(20) Xiao, C. W.; Wood, C.; Gilani, C. S. Nuclear receptors: Potential biomarkers for assessing physiological functions of soy proteins and phytoestrogens. *J. AOAC Int.* **2006**, *89* (4), 1207–1214.

(21) Yi, W. G.; Fischer, J.; Krewer, G.; Akoh, C. C. Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. *J. Agric. Food Chem.* **2005**, *53* (18), 7320–7329.

(22) Kurata, R.; Adachi, M.; Yamakawa, O.; Yoshimoto, M. Growth suppression of human cancer cells by polyphenolics from

sweetpotato (*Ipomoea batatas* L.) leaves. *J. Agric. Food Chem.* **2007**, *55* (1), 185–190.

(23) Dickancaite, E.; Nemeikaite, A.; Kalvelyte, A.; Cenas, N. Prooxidant character of flavonoid cytotoxicity: Structure−activity relationships. *Biochem. Mol. Biol. Int.* **1998**, *45* (5), 923–930.

(24) Nemeikaite-Ceniene, A.; Imbrasaite, A.; Sergediene, E.; Cenas, N. Quantitative structure−activity relationships in prooxidant cytotoxicity of polyphenols: Role of potential of phenoxyl radical/phenol redox couple. *Arch. Biochem. Biophys.* **2005**, *441* (2), 182–190.

(25) Nakatani, N.; Kayano, S.; Kikuzaki, H.; Sumino, K.; Katagiri, K.; Mitani, T. Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (*Prunus domestica* L.). *J. Agric. Food Chem.* **2000**, *48* (11), 5512–5516.

(26) Macheix, J.; Fleuriet, A.; Billot, J. *Fruit Phenolics*; CRC Press: Boca Raton, FL, 1990.

(27) Clifford, M. N. Chlorogenic acids and other cinnamates—Nature, occurrence and dietary burden. *J. Sci. Food Agric.* **1999**, *79* (3), 362–372.

(28) Ren, J.; Jiang, X.; Li, C. R. Investigation on the absorption kinetics of chlorogenic acid in rats by HPLC. *Arch. Pharmacal Res.* **2007**, *30* (7), 911–916.

(29) Olthof, M. R.; Hollman, P. C.; Katan, M. B. Chlorogenic acid and caffeic acid are absorbed in humans. *J. Nutr.* **2001**, *131* (1), 66–71.

(30) Gonthier, M. P.; Verny, M. A.; Besson, C.; Remesy, C.; Scalbert, A. Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. *J. Nutr.* **2003**, *133* (6), 1853–1859.

Received January 22, 2009. Revised manuscript received April 23, 2009. Accepted May 11, 2009. This material is based upon work supported by the Cooperative State Research, Education, and Extension Service, U.S. Department of Agriculture under Agreement No. 2006-34402-17121, Designing Foods for Health through the Vegetable & Fruit Improvement Center.