Polyphenols of selected peach and plum genotypes reduce cell viability and inhibit proliferation of breast cancer cells while not affecting normal cells

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A B S T R A C T

Polyphenolic extracts and fractions of selected peach and plum genotypes were evaluated for cell viability and antiproliferation activity *in vitro* against an estrogen independent MDA-MB-435 and estrogen dependent MCF-7 breast cancer cell lines and one non-cancerous breast line MCF-10A. All extracts showed a phenolic dose-dependent cytotoxic effect against MDA-MB-435, weak activity against MCF-7 and small or no activity against MCF-10A. Genotype phenolic profiles showed varying degrees of polyphenolic mixtures. Fractionation of peach BY00P6653 extracts gave 4 fractions, with fraction F-I (caffeic acid derivatives) showing a strong activity against MDA-MB-435 followed by fraction F-II (anthocyanins). Induced-apoptosis by F-I on MDA-MB-435 was confirmed by Tunnel nuclear staining of cells with apoptotic DNA fragmentation (0–100 μg/mL) with no effects in normal cells (0–200 μg/mL). Selected stone fruit genotypes can be added to the list of fruits with cytotoxic effects against breast cancer cells while not affecting normal cells.

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1. Introduction

Multiple factors contribute to the development of human breast cancer; however, environmental factors, especially diet, appear to have a great effect. Studies have shown that women in industrialized countries consuming a high-fat diet are more likely to have breast cancer than women in populations that consume a low-fat diet like in Asian countries (Parkin, 2004; Pasqualini, 2004). Fruit and vegetable consumption provide an array of bioactive phytochemicals which can help lower cancer risk (Hertog et al., 1995).

The anticancer activity of many fruit extracts have been studied using *in vitro* cell line model systems. For example, a flavonol fraction from cranberry inhibited proliferation of the estrogen independent MDA-MB-435 human breast tumour cell line by blocking cell cycle progression and inducing apoptosis (Ferguson, Kurowska, Freeman, Chambers, & Koropatnick, 2004). Ripe fruits of *Solanum nigrum* inhibited cell growth and induced cell death by apoptosis in estrogen dependent MCF-7 human breast cancer cell (Son et al., 2003). Phenolics from *Terminalia chebulia* fruits

decreased cell viability, inhibited cell proliferation, and induced cell death of MCF-7 breast cancer cell (Saleem, Husheem, Harkonen & Pihlaja, 2002). Red wine flavonoids show selective cytotoxicity in breast cancer cells and a marginal effect on normal cells (Hakimuddin, Paliyath & Meckling, 2004) while whole apple extract inhibits mammary cancer growth *in vivo* (Liu, Liu & Chen, 2005). However, little information has been published related to the antitumour properties of peach and plum fruit. Previous studies have shown that plum extracts suppressed growth and induced apoptosis on human hepatoma cell line (HepG2) (Ramos, Alia, Bravo & Goya, 2005) and HT-29 and MCF-7 cell lines (Olsson, Gustavsson, Andersson, Nilsson, & Duan, 2004). Peach extracts in *in vivo* studies reduce micronuclei induction in bone marrow cells by 43–50% confirming its protective effect (Edenharder, Krieg, Kottgen & Platt, 2003). More recently, prune extracts (*Prunus domestica* L.) were shown to suppress proliferation and induce apoptosis in Caco-2 colon cancer cells (Fujii, Ikami, Xu & Ikeda, 2006).

In general, these different plant species have compounds that are active against cancer proliferation by blocking cell cycle progression and/ or inducing apoptosis which is directly associated to DNA fragmentation. Selective cytotoxicity against cancer cells while not affecting normal cell is a desired feature of these bioactive compounds. Many phenolics have been reported in stone

fruits such as anthocyanins, hydroxycinnamates, flavan 3-ols and flavonols, predominantly chlorogenic acid, neo-chlorogenic acid, catechin, epicatechin, and quercetin 3-rutinoside (Kim, Chun, Kim, Moon, & Lee, 2003; Tomas-Barberan et al., 2001). Many of these phenolics act as antioxidants (Kim et al., 2003), antimutagenics (Miyasawa & Hisama, 2003), and anticarcinogenics (Kamei et al., 1995).

In the present study we examined whether selected peach (*Prunus persica*) and plum (*Prunus salicina*) genotype extracts and phenolic fractions affected cell viability and proliferation on estrogen dependent MCF-7 and estrogen independent MDA-MB-435 human cancer cell lines compared to a non-cancerous breast cell line MCF-10A. In addition, we identified the bioactive phenolic compounds and showed evidence of apoptosis induction in the cytotoxic effect.

2. Materials and methods

2.1. Chemicals, standards and reagents

The anthocyanin standards cyanidin-3-glucoside and cyanidin-3-galactoside were purchased from Polyphenols Laboratories AS (Sandness, Norway). Phenolic standards such chlorogenic acid, caffeic acid, quercetin, rutin and Folin–Ciocalteu reagent, sodium carbonate (Na_2CO_3), Trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH) MTT, acetonitrile, methanol, DMEM, and other chemicals such as sodium hydroxide, potassium hydroxide were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Extraction of phenolic compounds

The peach and plum varieties were from the breeding programs at Texas A&M University (College Station, TX, USA) and the USDA (Parlier, CA and Byron, GA, USA). Fruits from four peach varieties (Flameprince, BY00P4555, BY00P6653, and BY98P5369) and three plum varieties (Byrongold, Black Splendor, and Burgundy) were extracted and analyzed. One-hundred g of fresh tissue (flesh plus skin) was homogenized with 300 mL of methanol. Tubes were capped and stored overnight at 2–4 °C and then centrifuged at 29,000g for 20 min at 2 °C (Mod. J10-6, Beckman Instruments Inc., Fullerton, CA, USA). The supernatant was evaporated in a rotavapor (Büchi, Flawil, Switzerland) at 40 °C under 240 mbar pressure until dryness and re-suspended in nanopure water. Samples were frozen at −80 °C and freeze-dried (FTS® Systems, Inc., Stone Ridge, NY, USA) at −50 °C and 200 µHg of pressure. These samples were denominated "crude extract" and were used for further fractionation of the phenolic compounds.

2.3. Fractionation of the phenolic compounds

The fractionation of the phenolic compounds was based on the procedure described by Oszmianski et al. (1988) (Fig. 1(I)). Three grams of freeze dried sample were mixed thoroughly in 200 mL of nanopure water and adjusted to pH 7.0 with 5 M NaOH. The extract was loaded in SEP Pack C18 cartridge 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 wash water 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 100% methanol and 50 mL nanopure water pH 2.0. Phenolic acid compounds bounded to the matrix of the second cartridge and were later eluted with 50 mL 100% methanol (F-I). After adjusting the pH to 2.0 in the first cartridge, elution of anthocyanins monomers, procyanidins and catechins was accomplished by passing 50 mL of 16% acetonitrile at pH 2.0 (F-II). The flavonols were eluted using 50 mL 100% ethyl acetate (F-III) and the anthocyanin polymers using 50 mL of 100% methanol (F-IV).

2.4. Total phenolics and HPLC phenolic profiles

Total phenolics were quantified in the different genotypes and the fractions by the Folin–Ciocalteu method (Cevallos-Casals & Cisneros-Zevallos, 2003). A 0.05 g freeze-dried sample was mixed with 5 mL of methanol in a screw-cap tube using a vortex mixer. A 0.5 mL aliquot of samples were taken from the prepared sample and diluted with 8 mL of nanopure water. At the same time, a blank containing 0.5 mL of methanol was equally diluted and analyzed. Each sample and blank were combined with 0.5 mL of 0.25 N Folin–Ciocalteu reagent, and allowed to react for 3 min before the addition of 1 mL 0.5 M Na_2CO_3. The reaction mixture was incubated for 2 h at room temperature and measurements of absorbance at 725 nm were taken. The spectrophotometer was set to zero absorbance using the blank. Measurements were taken in a quartz cuvette. The concentration of total phenolics was estimated from a chlorogenic acid standard curve and expressed as chlorogenic acid equivalent.

The identification of the phenolic profiles present in the crude extracts and fractions was performed by using HPLC-PDA analysis with the following characteristics: binary Waters 515 HPLC (Milford, MA, USA) pump system, Waters 717 plus auto sampler automated gradient controller, SP8792 temperature controller and Waters 996 Photodiode array detector. For peak integration Millenium32 software from Waters was used. Phenolics were separated by an Atlantis™ C18 5 µm, 4.6 mm × 150 mm column and a 4.6 mm × 20 mm guard column using a gradient elution at a flow rate of 1 mL min^{-1} for 30 min. The mobile phase consisted of acidified nanopure water at pH 2.3 with HCl (A) and acetonitrile HPLC grade (B). The elution was isocratic conditions from 0 to 5 min with 85% A and 15% B. Gradient from 5 to 30 min began with 85% A and 15% B and ended with 0% A and 100% B. followed by isocratic conditions from 30 to 35 min with 0% A and 100% B to re-equilibrate the column. Column temperature was maintained at 35 °C. The injection volume was 10 µL. The identification of peaks was based on retention time and the spectra of external standards. Caffeic acid derivative was identified after basic hydrolysis of fraction I. Fraction I sample was hydrolyzed with NaOH 4 M for 16 h after being flushed with N_2. Hydrolysis was stopped with HCl 6 M until sample changed colour. A sample was injected into the HPLC. The concentration of phenolic compounds was determined from standard curves constructed for individual compounds by injecting different concentrations of corresponding standards.

2.5. Antioxidant activity

Antioxidant activity was quantified by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical method (Cevallos-Casals & Cisneros-Zevallos, 2003). A 0.05 g of freeze-dried sample was mixed with 5 mL of methanol in a screw-cap tube using a vortex mixer. Before running the reaction, the spectrophotometer was blanked with methanol, and DPPH was diluted with methanol from a mother solution to reach an absorbance of 1.1 AU at 515 nm. 150 µL of sample was combined with 2850 µL of the DPPH solution. A blank was simultaneously prepared with 150 µL methanol. Samples and blank were left to react for 24 h. Measurements of absorbance were taken with a quartz cuvette at 515 nm. Antioxidant activity was estimated as equivalents of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) by comparison to a standard curve.
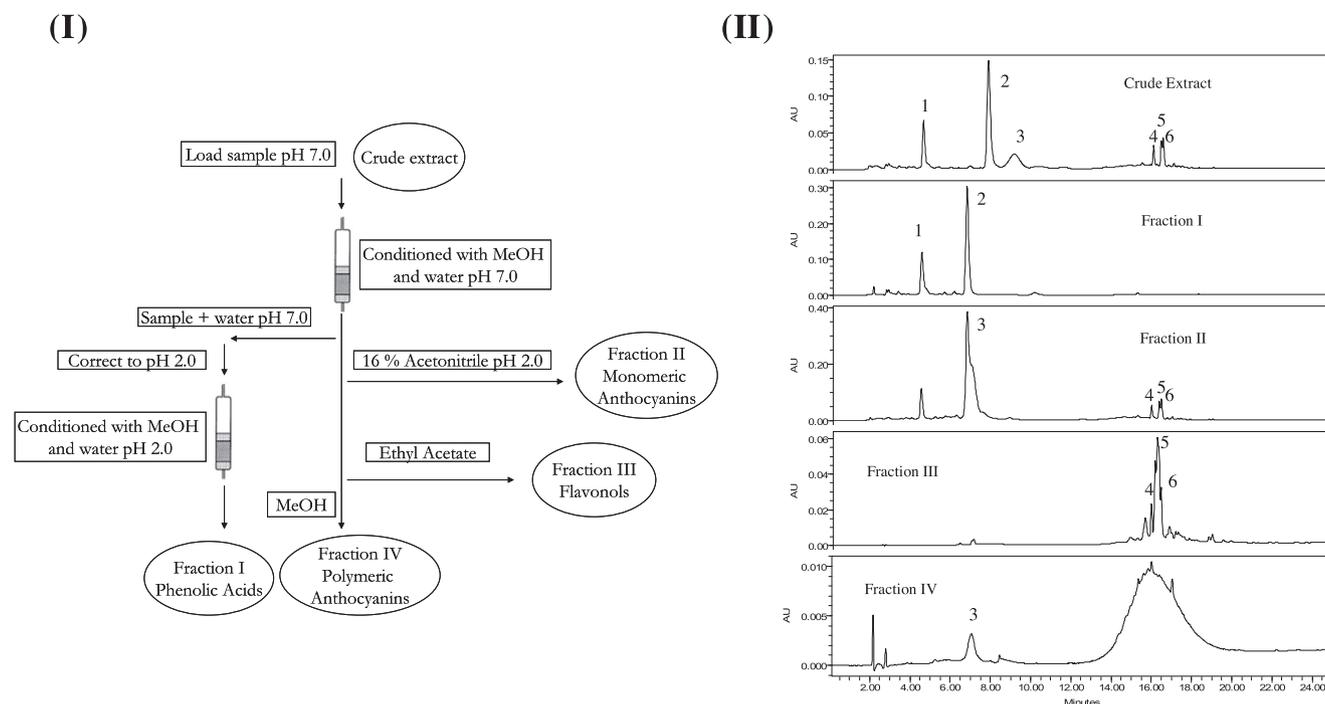
(I)　　　　　　　　　　　　　　　　　　　　**(II)**

Fig. 1. Scheme of phenolic fractionation used for peach BY00P6653 genotype (I). Typical HPLC-PDA phenolic chromatograms of the crude extract and fractions of peach BY00P6653 at 280 nm (II). Peaks correspond to caffeic acid derivative (1), chlorogenic acid (2), cyanidin-3-glucoside (3), quercetin derivative 1 (4), quercetin derivative 2 (5) and quercetin derivative 3 (6).

2.6. Cell lines and cell culture

MCF-7, the estrogen-positive human breast cancer was cultured in Petri dishes using Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% (v/v) stripped fetal bovine serum (FBS) high glucose, L-glutamine, 25 nM HEPES buffer, pyridoxine hydrochloride, without sodium pyruvate and without phenol red (Gibco™ Invitrogen Corp., Grand Island, NY, USA) at 37°C in a 5% CO_2 atmosphere. MDA-MB-453, the estrogen-negative human breast cancer was cultured in Petri dishes using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% strippicin- penicillin antibiotic mix, with L-glutamine, 4.5 g/L glucose and without sodium pyruvate (Bio-whittaker™ Cambrex Bioscience Walkersville Inc., Walkersville, MD, USA) at 37 °C in a 5% CO_2 atmosphere. MCF-10A, the non-cancerous breast cell line was cultured in Petri dishes using Dulbecco's modified Eagle's medium/F12 (DME/F12) supplemented with 5% (v/v) fetal bovine serum (FBS), 1% strippicin- penicillin antibiotic mix, 1 mL EGF (10 μg/ml), 5 mL insulin (1 mg/mL), 250 μl hydrocortisone (1 mg/mL), and 20 μL Cholera toxin (Gibco™, Invitrogen Corp., Grand Island, NY, USA) at 37 °C in a 5% CO_2 atmosphere.

2.7. Cell viability assay

Antiproliferation was measured in the presence and absence of treatments by using MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] or methyl thiazol tetrazolium assay (Mosmann, 1983) based on its conversion to MTT-formazan. Cells were seeded in 96-well microtitre plates at a density of 7000 cells per well. After 12–24 h period of incubation to allow cell attachment, cells were exposed to varying concentrations of peach and plum extracts for 24 h. The concentrations used for all the genotypes and fractions were based on total phenolic content and expressed as μg of chlorogenic acid/mL. After the appropriate incubation period, 100 μL of MTT (5 mg/mL) was added per well and incubated for 1 h at 37 °C. After incubation, MTT was aspirated and 100 μL of dimethyl sulfoxide (DMSO) was added to lyse the cells and dissolve the blue formazan crystals. Cell viability was calculated according to the following equation.

$$\text{Cell viability } (\%) = [OD_1 - OD_3/OD_2 - OD_3] \times 100 \qquad (1)$$

where OD is the optical density of cell culture with sample (OD_1), without sample (OD_2) and of the medium (OD_3) measured by spectrophotometer at 570 nm. The IC_{50} value (phenolic concentration needed to reduce proliferation in 50%) was determined from a linear equation passing through two points in each curve that were within the 50% reduction values.

2.8. Antiproliferation assay

Cell growth determination was performed using an electronic counter (Z1 Coulter). Ten thousand cells per well were seeded in 6-well plate and incubated for 24 h to allow cell attachment before exposure to varying concentrations of extracts. After the appropriate incubation period, the medium was aspirated and the cells rinsed with 1 mL of PBS (phosphate saline buffer). After incubation period of 5 min with 200 μL of trypsin, 800 μL of medium were added and the mix was transferred to a vial previously filled with 19 mL of isotone II solution. Two readings were taken from each replication. The results were expressed as number of cells.

2.9. Terminal Deoxynucleotidyl Transferase-mediated dUTP nick and labeling (TUNEL) assay

To detect apoptotic cells, in situ ending of labeling of the 3-OH end of the DNA fragments generated by apoptosis was performed using the terminal transferase, recombinant kit (Roche, Penzberg, Germany). Cells were grown in culture chamber slides (Falcon®, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and treated with fraction I for 12 h. The cells were washed in 500 μL of

phosphate-buffer saline PBS (0.138 M NaCl, 0.0027 M KCl at pH 7.4) and fixed by addition of 300 μL of 4% paraformaldehyde for 15 min at 4 °C. They were then washed twice in fresh PBS for 5 min. Cells were permeablized by 0.2% Triton X-100 solution in PBS for 5 min., washed 5 times for 5 min in fresh PBS and stored at 4 °C until further analyses. Then the slides were covered by 200 μL of equilibration buffer (CoCl₂ diluted in TE buffer) for 10 min. Two hundred microlitres of Terminal Deoxynucleotidyl Transferase (TdT) reaction mix were added to the slide and incubated at 37 °C for 60 min for the end-labeling reaction to occur. The reaction was ended by adding SSC sodium chloride-sodium citrate buffer to the slide for 15 min. The slides were washed 5 times for 5 min in fresh PBS. After washing, the slides were mounted with Vectashield mounting medium for fluorescence with 4′, 6-diamidino-2-phenylindole (DAPI) staining (Vector Laboratories, Inc., Burlingame) and examined with a fluorescent microscope.

2.10. Statistical analysis

Quantitative data represent mean values with the respective standard deviation values corresponding to 3 replicates. SPSS software (SPSS Inc. 2002) was used for all specific statistical analysis applying ANOVA and Duncan's test ($p \leqslant 0.05$).

3. Results and discussion

3.1. Total phenolics and antioxidant activity

Results showed a broad range of phenolic content per g of freeze-dried sample (~6.5-fold) and antioxidant activity (~5.5-fold) for the selected peach and plum varieties studied (Table 1). The phenolic content of peach varieties followed the order BY00P6653 > BY98P5369 > Flameprince > BY00P4555, while for plum varieties the order was Black Splendor > Burgundy > Byrongold. The antioxidant activity (AOX) of peach and plum varieties followed similar order as phenolic content, however in peaches, Flameprince ∼ BY00P4555 and in plums, Burgundy ∼ Byrongold. In general, red-fleshed varieties showed higher phenolic and antioxidant activity than yellow- or white-fleshed varieties. Interestingly, the specific AOX (expressed on phenolic basis) which is the antioxidant activity of the mixture of phenolics present in each type of fruit was similar for all varieties (~1098 μg Trolox/mg phenolics) suggesting similar radical scavenging properties. In previous work it was shown that in different peach and plum varieties the specific AOX tended to be similar between plum varieties (average ~750 and range of 300–1400 μg Trolox/mg phenolics) and of a broader range values between peach varieties (average ~800 and range of 250–2200 μg Trolox/mg phenolics) (Vizzotto, Cisneros-Zevallos, Byrne, Ramming, & Okie, 2007).

3.2. Cell viability and IC_{50} values of extracts

Results showed that peach and plum crude extracts had cytotoxic effects on both breast cancer cell lines and this effect was concentration dependent (Fig. 2(I)). The crude extracts dramatically reduced cell viability of the estrogen independent MDA-MB-435 cell line at phenolic concentrations of ⩽100 μg/mL after an incubation period of 24 h (Fig. 2(IA)). Calculated IC_{50} values for the crude extracts on the MDA-MB-435 cell line showed two distinct groups, one for the red-fleshed BY00P6653 peach and Burgundy plum fruits (~32.5 μg/mL) and another for the other varieties studied (~75 μg/mL) (Table 1).

The crude extracts showed a biphasic effect on cell viability of the estrogen dependent MCF-7 cell line (Fig. 2(IB)). There was a slight increase in cell viability at low phenolic concentrations and a reduction in cell viability at higher concentrations. Calculated IC_{50} values for the crude extracts on the MCF-7 cell line were higher than those of the MDA-MB-435 cell line and very similar among each of the selected varieties studied (~435 μg/mL). Only the Burgundy plum and BY00P4555 peach were not cytotoxic to the estrogen dependent MCF-7 breast cancer cell line (Table 1). In preliminary experiments the estrogen dependent MCF-7 cells were treated with estrogen and no further reduction in cell viability was observed when exposed to peach and plum crude extracts, suggesting phenolics did not interfere with estrogen receptor activity (data not shown).

The fruit crude extracts were not cytotoxic to the normal breast MCF-10A cell line (Fig. 2(IC)) and with most crude extract treatments there was an increase in cell viability. The only exceptions were a reduction in cell viability caused by Byrongold plum (~25%) and BY00P6653 peach (~50%) extracts at very high phenolic concentrations (~500 μg/mL), however, within the range 0–375 μg/mL these genotype extracts were not cytotoxic. These results indicate that the peach and plum varieties preferentially reduced cell viability and were cytotoxic to the MDA-MB-435 cell line which is a very aggressive and invasive breast cancer cell line as well as the less invasive MCF-7 cell line, while not affecting the normal breast cell line MCF-10A. Most breast cancers are heterogeneous and consist of estrogen dependent and independent cancer cells. Extracts that may inhibit both cancer cell lines and have little or no affect on normal cells would be of great interest.

3.3. Cell viability, IC_{50} values and antiproliferation activity of phenolic fractions of the BY00P6653 peach

Fractionation of BY00P6653 peach extracts allowed the separation of phenolic compounds into different families including phenolic acids (F-I), anthocyanins (F-II), other flavonoids (F-III), and polymerized compounds (F-IV). The phenolic content and AOX

Table 1
Phenolic content, antioxidant activity and cell viability effects of different peach and plum genotype extracts.[a]

Genotypes	Fresh colour	Phenolic content[b]	Antioxidant activity[c]	Specific AOX[d]	IC₅₀ (μg chlorogenic acid equivalent/ml)		
					MDA-MB-435	MCF-7	MCF-10A
BY00P4555	White	7962.0 ± 251.7f	9964.5 ± 542.4d	1.25a	73.9 ± 9.4	–	–
Flame prince	Yellow	10584.8 ± 275.0e	11453.9 ± 510.3d	1.08ab	70.0 ± 3.8	471.9 ± 8.4	–
BY98P5369	Red	21281.0 ± 1047.0d	23609.4 ± 1552.0c	1.11ab	82.9 ± 1.8	378.9 ± 23.7	–
BY00P6653	Red	52323.6 ± 229.7a	55560.6 ± 2839.3a	1.06ab	29.6 ± 0.9	473.5 ± 11.1	⩾500
Byrongold	Yellow	21898.2 ± 879.7d	22249.8 ± 1428.2c	1.02b	72.6 ± 2.0	422.2 ± 40.0	–
Burgundy	Red	23732.9 ± 822.0c	24741.5 ± 994.1c	1.04ab	35.5 ± 4.8	–	–
Black Splendor	Red	28989.2 ± 455.3b	32826.1 ± 476.6b	1.13ab	76.1 ± 0.3	428.1 ± 11.2	–

[a] Data indicates mean ± SD of three replicates.
[b] Phenolics = μg chlorogenic acid eq/g freeze-dried extract.
[c] Antioxidant activity = μg Trolox eq/g freeze-dried extract.
[d] Specific antioxidant activity = μg Trolox eq/μg chlorogenic acid eq. Means in each column followed by different letters are statistically different by Duncan's test ($p \leqslant 0.05$).
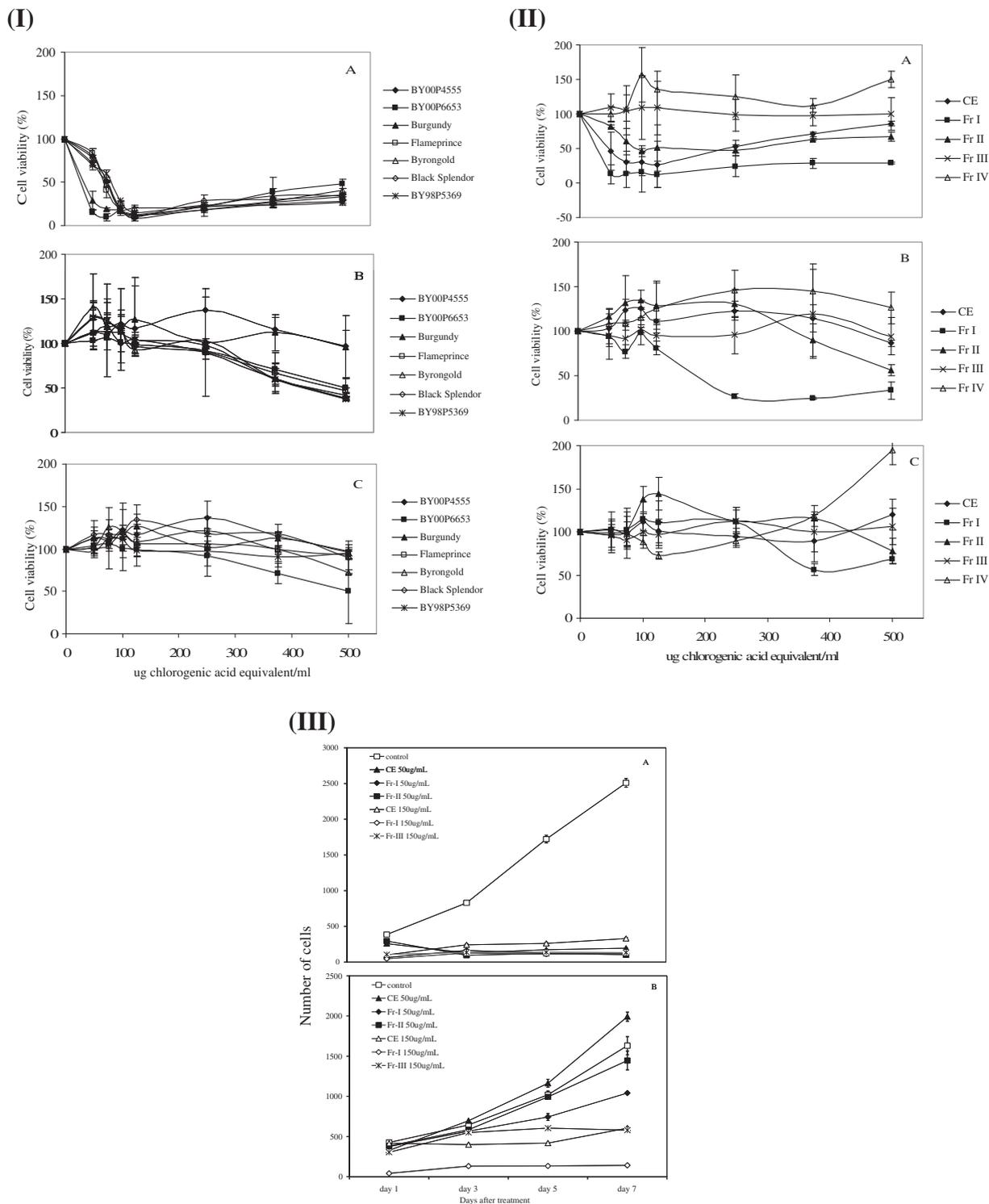
Fig. 2. Effects of peach and plum extracts (I) and phenolic fractions of the BY00P6653 peach(II) on cell viability of estrogen independent MDA-MB-435 breast cancer (A), estrogen dependent MCF-7 breast cancer (B), and MCF-10A normal breast (C) cell lines in a period of 24 h. Antiproliferation effects of crude extracts, F-I and F-II from the BY00P6653 peach (III) on estrogen independent MDA-MB-435 (A), and estrogen dependent MCF-7 (B) breast cancer cell lines in a period of 7 d. Data are means of three replicates ±SD. Phenolic concentrations of crude extracts or fractions are expressed as µg chlorogenic acid equivalents/mL.

was determined for each fraction and the specific AOX was calculated (Table 2) Interestingly, there was no difference among the specific AOX for each fraction (~1122 µg Trolox/mg phenolics) suggesting the mixtures of phenolics in each fraction have similar radical scavenging properties. The red-fleshed peach BY00P6653 extract was selected for fractionation due to its potent effect on both the estrogen independent MDA-MB-435 and MCF-7 cells while having little effect on the MCF-10A cells. According to our

results only the crude extract (CE) and fractions F-I and F-II showed a dose dependent response in reducing in cell viability of the MDA-MB-435 cancer cell line, while fractions F-III and F-IV showed no cytotoxic effects (Fig. 2(IIA)). Furthermore, F-IV slightly increased cell viability at concentrations >100 µg/mL. The IC_{50} values followed the order F-I < CE < F-II (Table 2).

For the estrogen dependent MCF-7 cancer cell line, once again F-I and F-II showed a reduction in cell viability, however, this

cytotoxic effect was observed at higher phenolic concentrations compared to MDA-MB-435 cells (Fig. 2(IIB)). Other fractions were not cytotoxic to this cancer cell line and even enhanced cell viability (CE, F-III and F-IV). The IC$_{50}$ values followed the order F-I $\ll$ F-II (Table 2).

For the MCF-10A normal breast cell line, the different fractions studied did not have cytotoxic effects and there was even an increase in cell viability for F-II and F-IV (Fig. 2(IIC)). The only exception was fraction F-I which showed a cytotoxic effect at very high phenolic concentrations ($\sim$50% at 375 µg/mL).

To determine if the selected crude extract and fractions F-I and F-II inhibited cancer cell growth, we assayed the antiproliferative activities counting cells for both cancer cell lines during an incubation period of 7 d (Fig. 2(III)). Growth of the MDA-MB-435 cell line was completely inhibited by the crude extract and both fractions at phenolic concentrations of 50 and 150 µg/mL. However, growth inhibition of the MCF-7 cell line depended on the fraction and amount of phenolics used. Fraction F-I showed a greater effect compared to the other treatments and at 150 µg/mL there was complete growth inhibition. In contrast, the crude extract promoted cell growth of MCF-7 cells at a phenolic concentration of 50 µg/mL, confirming previous results in the present study of an enhanced cell viability for some crude extracts (Fig. 2(I)) and fractions at the lower phenolic concentration range (Fig. 2(II)). Some compounds may have a dose dependent effect on the cell growth of cancer cells, stimulating cell growth at low dose by altering the cell cycle components, while inducing cell cycle block and apoptotic cell death at high dose (Yuri et al., 2006).

In the present study there was no relationship between the specific AOX of the crude extracts or the phenolic fractions and the cell viability or antiproliferation activity (Tables 1 and 2). Other studies with fruit extracts have reported similar lack of relationship between AOX and the inhibition of proliferation on HepG2 human liver-cancer cells (Sun, Chu, Wu & Liu, 2002), HT-29 colon cancer cells and MCF-7 breast cancer cells (Olsson et al., 2004). This has

very important implications since it would be misleading to use AOX for the screening or selection of fruits and varieties or its use as a maturity index (Brovelli & Cisneros-Zevallos, 2007) when targeting anticancer properties. More appropriate would be the use of anticancer bioassays or screening for the bioactive compounds.

3.4. HPLC-DAD analysis of peach and plum genotypes and BY00P6653 fractions

Phenolic identification and quantification of the genotypes were performed at 280 nm to verify presence of general phenolics and at 520 nm for anthocyanins. Comparison of retention times and UV–visible spectral data with known standards revealed the presence of a caffeic acid derivative, chlorogenic acid, cyanidin-3-glucoside, cyanidin-3-galactoside and three derivatives of quercetin (Table 3). A typical chromatogram profile of the peach BY00P6653 crude extract is shown in Fig. 1(II). The peach BY00P6653 showed higher concentrations of individual phenolics compared to other genotypes with exception of cyanidin-3-galactoside which is found in higher concentration on Black Splendor (Table 3). Phenolic acids such as chlorogenic acid, flavonols such as quercetin-3-glucoside, -3-galactoside, -3-rhamnoside, -3-xyloside, -3-rutinoside, and quercetin pentosyl-pentoside and anthocyanins such as cyanidin-3-glucoside, -3-galactoside, and -3-rutinoside, as well as -3-acetylglucoside have been previously reported in stone fruits (Tomás-Barberán et al., 2001; Kim et al., 2003).

According to the HPLC chromatogram, the main compounds in F-I are hydroxycinnamic acid derivatives including chlorogenic acid and a caffeic acid derivative which is likely neo-chlorogenic acid. After basic hydrolysis the two peaks disappear and a peak identified as caffeic acid appears. This fraction was the most potent at inhibiting proliferation of the estrogen dependent MDA-MB-435 and estrogen independent MCF-7 breast cancer lines. Chlorogenic acid and caffeic acid derivatives have been reported to have anti-carcinogenic activity on hepatic, HL-60, U937 and jurkat cells

Table 2
Phenolic content, antioxidant activity, specific antioxidant activity and cell viability of phenolic fractions from peach BY00P6653 genotype.[a]

Fractions	Total phenolics[b]	Antioxidant activity[c]	Specific AOX[d]	IC$_{50}$ (µg chlorogenic acid equivalent/ml)		
				MDA-MB-435	MCF-7	MCF-10A
CE	53510.0 ± 5718.1a	64465.3 ± 685.0a	1.21 ± 0.10a	47.2 ± 5.8	–	–
F-I	11609.2 ± 4276.8bc	11860.1 ± 3239.8c	1.04 ± 0.74a	28.6 ± 0.2	191.4 ± 4.2	–
F-II	24815.8 ± 1526.2b	26938.7 ± 103.2b	1.09 ± 0.50a	96.7 ± 5.6	>500	–
F-III	9175.8 ± 1820.8c	9065.0 ± 507.6c	1.01 ± 0.18a	–	–	–
F-IV	8797.5 ± 491.4c	11106.5 ± 1492.8c	1.26 ± 0.70a	–	–	–

[a] Data indicates mean ± SD of three replicates.
[b] Phenolics = µg chlorogenic acid eq/g freeze-dried fraction extract.
[c] Antioxidant activity = µg Trolox eq/g freeze-dried fraction extract.
[d] Specific antioxidant activity = µg Trolox eq/µg chlorogenic acid eq. Means in each column followed by different letters are statistically different by Duncan's test ($p \leqslant 0.05$).

Table 3
Quantification of phenolic compounds present in peach and plum genotypes using HPLC-DAD.[a]

Genotypes	Caffeic acid derivative[b]	Chlorogenic acid	Cyanidin-3-glucoside	Cyanidin-3-galactoside	Quercetin derivative1	Quercetin derivative2	Quercetin derivative3
BY00P4555	175.1 ± 7b	532.9 ± 20c	nd	nd	nd	nd	nd
Flameprince	188.9 ± 6b	794.0 ± 35c	nd	nd	nd	nd	nd
BY98P5369	194.1 ± 7b	1489.2 ± 181b	3554.9 ± 147c	nd	nd	nd	nd
BY00P6653	571.3 ± 37a	3431.2 ± 124a	10116.7 ± 622a	1232.9 ± 76b	678.3 ± 37a	28.6 ± 29a	395.6 ± 34a
Byrongold	140.4 ± 15bc	1467.6 ± 105b	nd	nd	nd	nd	nd
Burgundy	179.5 ± 53b	1995.1 ± 585b	3516.8 ± 535c	806.9 ± 117c	55.2 ± 13c	100.5 ± 18b	69.2 ± 7b
Black Splendor	94.9 ± 49c	3774.9 ± 427a	5530.3 ± 781b	1729.1 ± 141a	107.8 ± 8b	116.3 ± 15b	50.7 ± 2b

[a] Data represents the means of three replicates ± standard deviation and expressed as µg phenolics/g of freeze dried extract. Derivative compounds were quantified using caffeic acid and quercetin standards.
[b] Caffeic acid derivative is tentatively identified as neo-chlorogenic acid. nd = not detected. Means within each column followed by different letters are significantly different at $p \leqslant 0.05$ with Duncan's test.

(Park & Schoene, 2003; Tanaka et al., 1990) and apoptosis-inducing activity on leukemia cells (Yu, G, L, R.; Rahmani, & P.; Grant, 2002; Bandyopadhyay et al., 2004). Fraction F-II contains anthocyanins with cyanidin-3-glucoside the most abundant combined with small amounts of flavonols (Fig. 1(II)). Although catechins were expected to be in this fraction these compounds were not in sufficient concentrations to be identified. Comparison of retention times and UV–visible spectral data with known standards revealed the presence of cyanidin-3-glucoside (peak 3) and cyanidin-3-galactoside (small peak visible only at 520 nm). The HPLC analysis of F-III indicated that this fraction is composed of quercetin derivative compounds (Fig. 1(II)). However, the presence of other flavonols cannot be discarded. Flavonols have high affinity for ethyl

acetate due to their hydrophobic characteristic (Krasteva, Nikolova, Danchev & Nikolov, 2004). Polymer compounds, mainly polymerized anthocyanins, were the compounds present in F-IV according to the HPLC analysis (Fig. 1(II)).

The specific phenolic profile of each genotype crude extract could have determined the cytotoxic effectiveness against the cancer cells through synergistic, additive or antagonistic effects of the polyphenolic mixtures. For example, the phenolic profiles (ratios of caffeic acid derivative:chlorogenic acid:anthocyanins:quercetin derivatives) of BY00P6653 (1:6:17:2) and Burgundy (1:11:19:4) extracts were similar and these fruits showed higher cytotoxic properties (Fig. 2(I)). On the other hand, phenolic profiles of BY98P5369 (1:7:18:nd), Black Splendor (1:40:58:18), BY00P4555 (1:3:nd:nd), Byrongold (1:10:nd:nd) and Flameprince (1:4:nd:nd) were different and these fruits showed lower cytotoxic effectiveness. More work is needed to prove this hypothesis.
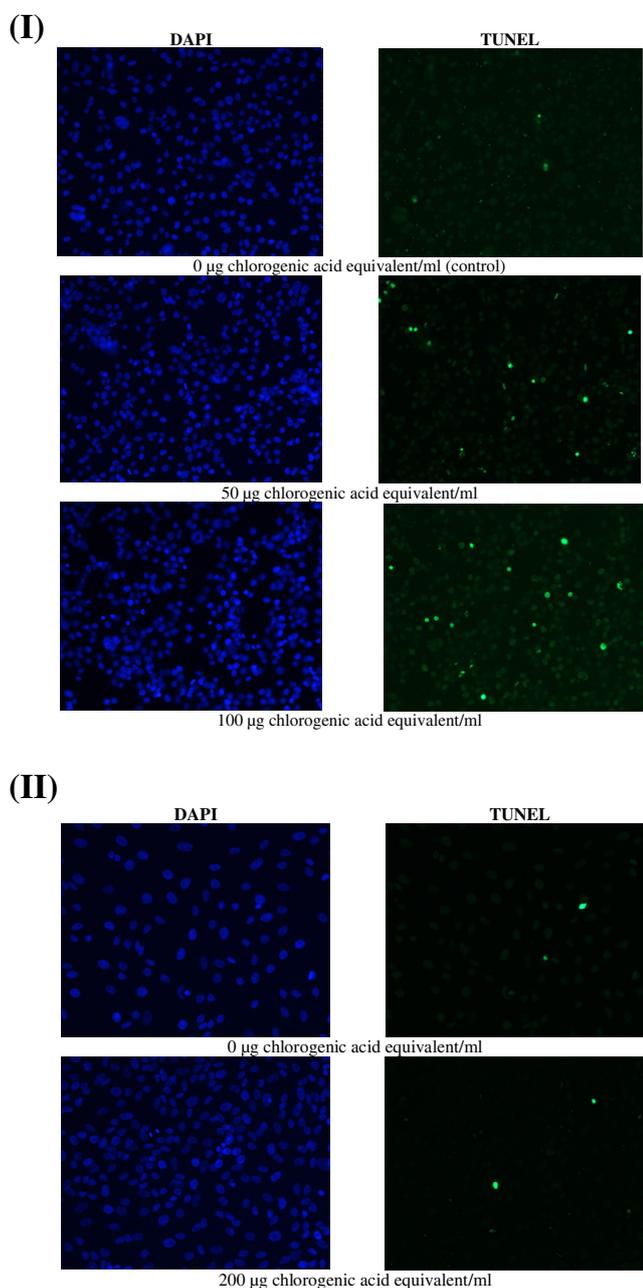
3.5. Apoptosis identified by TUNEL assay

During apoptosis, the chromatin condenses against the nuclear envelope and the DNA inside the nucleus becomes fragmented. Terminal Deoxynucleotidyl Transferase (TdT) when used with a fluorescent marker allows detection of cells with apoptotic DNA fragmentation. TdT is an enzyme that catalyzes the repetitive addition of dUTPs (Alexa 488) to the 3-OH ends of a DNA fragment. In TUNEL analysis, the fluorescently conjugated dUTP are added to the 3-OH groups of the DNA fragments making the apoptotic cells visible by fluorescent microscopy (Jewell & Mastro, 2002).

After incubation with 50 μg chlorogenic acid/mL of F-I for 12 h, TUNEL assay was performed to visualize apoptosis in MDA-MB-435 estrogen receptor-negative cells (Fig. 3(I)) and MCF-10A normal cells (Fig. 3(II)). Control or untreated cells were assayed as well. Control MDA-MB-435 cells did not show a positive TUNEL reaction, whereas the cells treated with F-I (50 and 100 μg/mL) incorporated the labeled nucleotide into the fragmented DNA (Fig. 3(I)). On the other hand MCF-10A cell did not show a positive reaction neither for treated cells (200 μg/mL) nor for the control cells (Fig. 3(II)).

4. Conclusions

In summary, polyphenolic extracts from peach and plum genotypes showed selective cytotoxicity against the estrogen independent MDA-MB-435 breast cancer cells compared to the estrogen dependent MCF-7 cells and small or no effects on MCF-10A non-cancerous breast cell line. Polyphenolic profiles for the selected stone fruit genotypes had varying degrees of phenolic mixtures. Chlorogenic acid and a caffeic acid derivative present in F-I fraction of the BY00P6653 peach genotype was the most effective among the phenolic fractions in reducing cell viability of both cancer cell lines, with little effect on the normal cells. Furthermore, F-I showed antiproliferative activity in both cancer cell lines. The Tunel assay confirmed that F-I induced apoptotic cell DNA fragmentation in MDA-MB-435 cancer cells while not affecting normal cells. This study shows that selected stone fruits can be added to the list of fruits with cytotoxic properties against breast cancer cells while not affecting normal cells; however, further work is needed to elucidate the molecular mechanism involved in induced apoptosis by stone fruit polyphenols.

Fig. 3. TUNEL nuclear staining on estrogen independent MDA-MB-435 breast cancer (I) and MCF-10A normal breast (II) cell lines. Cells were treated with varying concentrations of total phenolics present in F-I from peach BY00P6653 and incubated for 12 h. Cells were washed in phosphate-buffer saline, fixed and permeablized and subjected to TUNEL nuclear staining, mounted and viewed by fluorescence microscopy.

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