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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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ABSTRACT

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.

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

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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 29,000*g* 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.