

Submitted to the *Food and Chemical Toxicology*  
Original Submission on May 12, 2008  
Revised and Resubmitted October 14, 2008

**A Comprehensive Study of Anthocyanin-Containing Extracts from Selected Blackberry  
Cultivars: Extraction Methods, Stability, Anticancer Properties and Mechanisms**

J. Dai <sup>a</sup> A. Gupte <sup>a</sup>, L. Gates <sup>a</sup>, and R. J. Mumper <sup>b,\*</sup>

<sup>a</sup>Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky,  
Lexington, Kentucky 40536-0082, USA;

<sup>b</sup>Division of Molecular Pharmaceutics, School of Pharmacy, University of North Carolina at  
Chapel Hill,  
Chapel Hill, North Carolina 27599-7360, USA

\* Corresponding author:

Russell J. Mumper, Ph.D.  
John A. McNeill Distinguished Professor  
Director, Center for Nanotechnology in Drug Delivery  
Division of Molecular Pharmaceutics  
UNC Eshelman School of Pharmacy, CB # 7360  
University of North Carolina at Chapel Hill  
Chapel Hill, North Carolina 27599-7360  
E-mail: [mumper@email.unc.edu](mailto:mumper@email.unc.edu)  
Phone: (919) 966-1271  
Fax: (919) 966-0197

*Running title:* Composition, Bioactivity of Berry Extracts

*Keywords:* Blackberry extracts; Extraction; Stability; Anticancer; Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)

## Abstract

The purpose of these studies was to investigate and compare the composition, stability, antioxidant and anticancer properties and mechanisms of anthocyanin-containing blackberry extracts (ACEs) from selected cultivars and using different extraction methods. ACEs were analyzed for total anthocyanins and phenolics content, polymeric color, and total antioxidant capacity (TAC). The influence of water content in the extraction system was evaluated. A 90-day stability study of the extract and a 48-h stability study of the extract in biologically relevant buffers were completed. The cytotoxic effects of ACEs on HT-29, MCF-7, and HL-60 cells were determined.  $H_2O_2$  production in culture medium was measured and intracellular ROS levels were quantified. As compared to powder-derived ACEs, puree-derived ACEs contained similar amounts of anthocyanins, but greater levels of phenolics, increased TAC, significantly enhanced production of  $H_2O_2$ , and significantly enhanced cytotoxicity in all cell lines. Catalase could not protect cells from ACE-induced cell death. Cyanidin 3-glucoside exerted anticancer effect by acting synergistically or additively with other active components in the extracts. These data suggest that anthocyanins and non-anthocyanin phenolics in ACEs act synergistically or additively in producing anti-cancer effects. These studies also provide essential information for the development of fruit-derived ACEs as potential Botanical Drug Products.

## 1. Introduction

In addition to the protective effects of endogenous antioxidant defenses, the consumption of dietary antioxidants appears to be of great importance in counteracting oxidative stress-associated chronic diseases. Dietary phenolics have captured increasing attention in recent years because of their known antioxidant properties (Manach et al., 2004). It has been stated that dietary phenolics are multifunctional antioxidants either acting as reducing agents, hydrogen-donating antioxidants, and/or single oxygen (free-radical) quenchers (Rice-Evans et al., 1996). Among the many phenolic compounds, anthocyanins have been given a great amount of attention since they possess potent antioxidant activity (Rice-Evans et al., 1995; Seeram and Nair, 2002). Various anthocyanin-containing extracts from plants and fruits have been shown to reduce the oxidative stress-associated inflammatory diseases and cancer (reviewed in Nichenametla et al., 2006; Prior and Wu, 2006), and there may be synergistic or additive biological effects due to unique combination of anthocyanins and phenolics in the extracts prepared from different fruit samples or by different extraction methods (Bagchi et al., 2004; Zhao et al., 2004).

In spite of the accumulated evidence of their potential usefulness in the treatment and prevention of oxidative stress-related diseases, dietary bioactive constituents have been promoted as health-promoting products or nutrition supplements instead of drug products. However, the FDA's Guidance Document on "Botanical Drug Products" (June 2004) paved the way for the development of registered drug products derived from natural sources. These products may contain multiple chemical constituents, often comprising phenolic phytochemicals including phenolic acids, non-flavonoids, and flavonoids including anthocyanins. In many cases, the active constituents in a botanical drug product are not identified, nor are their biological activity well

characterized. The first FDA-registered botanical drug product, Veregen™, was approved in 2006 to treat genital warts and is a topical cream containing a mixture of catechins and other components from the water extract of green tea leaves. There are now many additional naturally-derived products in clinical development under the Botanical Drug Product registration pathway to treat, for examples, cancer and other inflammatory-mediated diseases. As development activity increases in this area, it is of critical importance to investigate methods of extraction, extract stability, and factors which may influence and/or elucidate mechanisms of action.

Blackberries are a species of fruit belonging to the subgenus *Eubatus* in the genus *Rubus*, and are very complex in terms of genetic background, growth characteristics, and number of species. It has been shown that blackberries contain higher amount of anthocyanins and other antioxidants than other fruits (Halvorsen et al., 2006; Moyer et al., 2002; Pantelidis, 2007). Blackberry extracts have been shown to have various bioactivities including protecting against endothelial dysfunction and vascular failure *in-vitro* (Serraino et al., 2003), attenuating the injury caused by LPS-induced endotoxic shock in rats (Sautebin et al., 2004) and exhibiting cytotoxic effects on human oral, prostate (Seeram et al., 2006), lung (Feng et al., 2004) cancer cells. In our previous report, we showed that an anthocyanin-containing extract (ACE) from Hull cultivar grown in Kentucky inhibited HT-29 colon cancer cell growth and reduce Lipid A-induced Interleukin-12 release from murine dendritic cells (Dai et al., 2007).

Although the mechanisms behind the bioactivities of anthocyanins and phenolics involve many pathways (reviewed in Hou, 2003; Nichenametla et al., 2006), the most remarkable aspect of their activities may be their ability to act as either antioxidants or prooxidants in some biological environments. The anti-inflammatory and chemopreventive properties of dietary phenolics are generally believed to be related to their antioxidant properties. For example, Elisia

*et al.* showed that a purified cyanidin 3-glucoside extract from blackberry protected Caco-2 cells from peroxy radical-induced apoptosis (Elisia and Kitts, 2008). On the other hand, dietary phenolics may be subject to redox cycling generating reactive oxygen species (ROS) and free radicals under defined conditions (Galati and O'Brien, 2004). Importantly, the prooxidant action of phenolics may be an important mechanism for their anticancer and apoptosis-inducing properties (Hadi et al., 2000). For example, cyanidin-3-rutinoside was shown to induce the accumulation of peroxides, which were involved in the induction of apoptosis in HL-60 cells (Feng et al., 2007). Dietary phenolics were also shown to cause oxidative strand breakage in DNA in the presence of transition metal ions such as copper (Hadi et al., 2007), generate substantial amounts of H<sub>2</sub>O<sub>2</sub> in the commonly used media (Lee et al., 2005), and subsequently exerted antiproliferative effects on cancer cells.

It has been shown that the phenolic content and/or profile as well as antioxidant activity of blackberries varies between cultivars (Siriwoharn et al., 2004). Moreover, different extraction methods may introduce different combinations of anthocyanins and phenolics in the extract resulting in different bioactivities. The purpose of these present studies was to extend our previous studies on Hull blackberry extract to compare ACEs prepared from three blackberry cultivars grown in Kentucky (Hull, Black Satin and Chester). Two different blackberry materials, puree and powder (freeze-dried puree), were used to obtain the ACEs. The total phenolic and anthocyanin content, polymeric color, and total antioxidant capacity (TAC) in the ACEs were compared. In addition, the effect of the water-to-ethanol ratio of the extraction system was investigated on the properties of the obtained ACEs. To investigate the anticancer properties of ACEs, the growth effects of the powder and puree-derived ACEs on a panel of human cell lines was evaluated and in selected studies compared directly to the dominant anthocyanin in the

extracts, cyanidin 3-glucoside. Possible mechanisms of cytotoxicity were evaluated including  $H_2O_2$ , ROS generation, and the role of copper. In addition, the ability of catalase to quench these effects was investigated. A final aim of the present studies was to investigate the stability of these extracts stored alone as a function of time, temperature, and light, and when incubated in biologically relevant buffers.

## 2. Materials and methods

### 2.1. *Materials*

Hull, Chester, and Black Satin blackberries were grown at WindStone Farms (Paris, Kentucky). Catalase, hydrogen peroxide solution (30%), dimethyl sulfoxide (DMSO), 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt (ABTS), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, formic acid (ACS, >96%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Folin-Ciocalteu phenol reagent, gallic acid (98% purity) and trichloroacetic acid were purchased from Sigma (St. Louis, MO). Hydrogen chloride ~1.25 mol/l in ethanol was purchased from Fluka (St. Gallen, Switzerland). USP grade Ethanol (Absolute–200 proof) was purchased from AAPER Alcohol and Chemical Co. (Shelbyville, KY). HPLC grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). Cyanidin 3-glucoside (Kuromanin chloride, HPLC grade) was purchased from Indofine Chemical Company (Hillsborough, NJ). 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CH<sub>2</sub>DCFDA) was purchased from Invitrogen, Inc. (Carlsbad, CA).

### 2.2. *Preparation of anthocyanin-containing extracts (ACE)*

The Hull blackberries were harvested in July 2005 and July 2006, and the Chester and Black Satin blackberries were harvested in July 2006. The seeds and skin of the berries were removed using a Langsenkamp type 161 Colossal Pulper as previously described (Dai et al., 2007). The blackberry puree was stored frozen at -20 °C until processed for these present studies.

The frozen blackberry puree was lyophilized in a VirTis Model AD2 Lyophilizer and ground into a free-flowing purple powder. ACEs were obtained from blackberry powder or puree as previously reported (Dai et al., 2007). Briefly, an aliquot of blackberry powder (1 g) or blackberry puree (10 g) was treated under sonication for 30 min with 25 ml of extraction solvent of ethanol containing 0.01% HCl (v/v). The supernatants were collected after filtration and dried by rotary evaporation at 40 °C. The dried extract was

resuspended in deionized water and filtered through a 20-25  $\mu\text{m}$  filter paper and lyophilized to obtain dried ACE. Dried ACE was then redissolved in deionized water as a stock ACE solution (140 mg/ml) and stored at  $-80\text{ }^{\circ}\text{C}$  for further characterization and cell-based studies.

Black Satin puree was used to prepare various ACEs with different solvent combinations of water and ethanol/0.01% HCl to investigate the solvent influence on the properties of ACEs. Briefly, 10 g of puree was mixed with 25 ml of extraction solvent. The water to ethanol/0.01% HCl (v/v) ratios were as follows: 0/100, 10/90, 25/75, 50/50, 75/25, 90/10, and 100/0. ACEs were then obtained following the same extraction procedure as described above.

### 2.3. *Monomeric anthocyanins and polymeric color measurement*

Monomeric anthocyanin content was determined using the pH-differential method of Giusti and Wrolstad (Giusti and Wrolstad, 2001). Total anthocyanin content was calculated using an extinction coefficient of  $26900\text{ l cm}^{-1}\text{ mg}^{-1}$  and a molecular weight of 449.2 g/mol of cyanidin 3-glucoside (dominant anthocyanin). Color density and polymeric color were calculated using absorption at 420, 510 and 700 nm with and without bisulfite treatment. The percentage of polymeric color was determined by the ratio of polymerized color to color density.

### 2.4. *Total phenolic measurement*

Total phenolic content was estimated using the Folin-Ciocalteu method for total phenols (Singleton and Rossi, 1965). Briefly, diluted samples were mixed with Folin-Ciocalteu reagent (0.1 N) and then treated with saturated sodium carbonate to maintain the reaction pH at 10. The absorbance was measured at 765 nm with a Beckman DU800 UV-Visible Spectrophotometer after incubation for 2 h at room temperature. Total phenols were calculated as gallic acid equivalent based on the standard curve with gallic acid standard prepared in corresponding vehicles.

### 2.5. Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay for the extracts was carried out using a Beckman DU640B UV-Vis Spectrophotometer in the kinetic mode following procedures described by (Re et al., 1999).  $\text{ABTS}^{\bullet+}$  was produced by reacting 7 mM ABTS with 2.5 mM potassium persulfate for 16 h in the dark at room temperature. The  $\text{ABTS}^{\bullet+}$  solution was diluted with ethanol or water to an absorbance of 0.70 ( $\pm$  0.02) at 734 nm and equilibrated at 30 °C. Twenty (20)  $\mu\text{l}$  of ACE samples or Trolox standards in ethanol were added to 980  $\mu\text{l}$  of diluted  $\text{ABTS}^{\bullet+}$  solution, such that each final sample produced between 20-80% inhibition of the blank absorbance. The absorbance readings were taken continuously for 7 min at 734 nm at 30 °C. The standard curve was generated based on the percentage of inhibition of the blank absorbance by Trolox at 7 min versus Trolox concentration. The total antioxidant capacity of samples was calculated as Trolox equivalent (TE) based on the percentage of inhibition of the blank absorbance by samples at 7 min.

### 2.6. HPLC assay for anthocyanins

HPLC analysis was performed using a X-Bridge<sup>TM</sup> C18 column (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ ) (Waters Corp. Milford, MA) equipped with an X-Bridge<sup>TM</sup> C18 guard column and a Thermoquest HPLC system with a UV6000LP photodiode array detector. The mobile phase was comprised of solvent A, 10% formic acid (pH 1.37), and Solvent B, 100% acetonitrile. The elution profile was 100% to 91% A for 0.5 min, 91% A (isocratic) for 0.5-4 min, 91% A to 87% A (linear gradient) for 4-10 min, 87% A to 70% A (linear gradient) for 10-20 min, 70% A to 0% A (linear gradient) for 20-21 min, 0% A (isocratic) for 21-24 min, 0% A to 100% A (linear gradient) for 24-25 min, and 100% A (isocratic) for 25-30 min. The flow rate was 1.0 ml/min, and the detection wavelength was 524 nm. Each determination was run in triplicate. Quantification of cyanidin 3-glucoside in ACEs was carried out using the external standard method.

### 2.7. *90-day stability study of ACE under different storage conditions*

A freshly prepared ACE solution derived from powder of Hull blackberries (2005) was filled into polypropylene microcentrifuge tubes (1.5 ml) and kept in the following controlled conditions:  $-80\text{ }^{\circ}\text{C}$  freezer,  $2\text{-}8\text{ }^{\circ}\text{C}$  stability chamber,  $25\text{ }^{\circ}\text{C}$  stability chamber (60% humidity). On days 3, 10, 17, 24, 31, 45 and 90, three samples at each storage condition were withdrawn and analyzed for pH, osmolality, cyanidin 3-glucoside content, total anthocyanin content, total phenolics, and polymeric color. TAC was measured at day 24, 45 and 90. The stability of ACE stored in a temperature-controlled room ( $22\text{-}28\text{ }^{\circ}\text{C}$ , 38% humidity) with white light (fluorescent light, 1929 lx) was also investigated using the same procedure over a period of 45 days.

### 2.8. *48-h stability study of ACE in biologically relevant buffers at $25\text{ }^{\circ}\text{C}$ and $37\text{ }^{\circ}\text{C}$*

ACE stock solution derived from powder of Hull blackberries (2005) was diluted to a final concentration of 2 mg/ml with (1) pH 1.0 buffer (214.6 mM NaCl, 8.7 mM KCl); (2) pH 7.4 phosphate buffered saline (PBS: 30.3 mM  $\text{Na}_2\text{HPO}_4$ , 8.7 mM  $\text{KH}_2\text{PO}_4$ , 0.9% NaCl); (3) pH 7.4 PBS with 10% Fetal Bovine Serum (FBS) (ATCC, Rockville, MD); (4) RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS in 50-ml screw-capped polypropylene centrifugation tubes (25 ml each). ACE in buffer (1), (2), and (3) were kept at  $25 \pm 0.5\text{ }^{\circ}\text{C}$ . Another sample of ACE in buffer (3) and (4) were kept in  $37 \pm 0.5\text{ }^{\circ}\text{C}$ . At time 0, 10 min, 30 min, 2, 6, 12, 24, 48 h after mixing with each buffer, an aliquot of sample was withdrawn and anthocyanin degradation in buffers (pH 7.4) was stopped by adding 36.5% hydrochloric acid (1/100, v/v). To avoid protein interference in the total phenolics measurement, protein was removed by trichloroacetic acid precipitation method before the Folin-Ciocalteu assay. Briefly, samples were mixed with 20% TCA and kept in  $4\text{ }^{\circ}\text{C}$  for 0.5 h. Then samples were centrifuged at 13,000 rpm for 15 min. at  $4\text{ }^{\circ}\text{C}$  and the supernatant was collected for the analysis of total phenol content.

## 2.9. Cell culture

The human colorectal cancer cell line HT-29, human ovarian cancer cell lines MCF-7 (her2 negative and ER+), human leukemia cell line, HL-60, were purchased from American Type Cell Culture Collection (ATCC, Rockville, MD). HT-29 cells were grown in modified McCoy's 5A medium (ATCC, Rockville, MD). All other cells were grown in RPMI-1640 medium (Invitrogen; Carlsbad, CA). All media were supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS. Cells were maintained at 37°C in a humidified 5% CO<sub>2</sub> incubator.

## 2.10. Cell viability assay

The adherent HT-29, MCF-7 and the suspension HL-60 cells were seeded at an initial concentration of  $3 \times 10^4$  cells/well in 96-well plates. After 24 h, cells were treated with the ACE stock solution (140 mg/ml) with final doses ranging from 0.084 to 11.2 mg/ml. Vehicle controls were the normal media with the corresponding pH adjusted with 2.5 N HCl for each treated group. HL-60 cells were also treated with cyanidin 3-glucoside (in 0.1% DMSO) with final doses ranging from 10.4 to 62.2 µg/ml. After 48 h of treatment, medium was removed and cell viability was measured for all cells using the MTT assay. Briefly, cells were incubated with MTT (0.5 mg/ml) in fresh medium in the dark at 37 °C for 4 h. Next, supernatant was removed and 200 µl of DMSO was added to each well. Plates were read at 570 nm using Biotek Synergy 2 Microplate Reader. Cell viability was calculated using Equation 1:

$$\% \text{ cell viability} = \text{ABS}_t / \text{ABS}_{\text{Ctrl}} \times 100\% \quad (\text{Equation 1})$$

where  $\text{ABS}_t$  is the absorbance of cells treated with HBE and  $\text{ABS}_{\text{Ctrl}}$  is the absorbance of corresponding vehicle control.

### 2.11. Determination of intracellular ROS in HL-60 cells

5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CH<sub>2</sub>DCFDA) was used as an indicator of intracellular ROS generation. HL-60 cells ( $3 \times 10^4$  cells/well) were loaded with 100  $\mu$ M CH<sub>2</sub>DCFDA (dissolved in DMSO) for 30 min at 37 °C. The cells were washed twice with fresh PBS to remove excess CH<sub>2</sub>DCFDA. Non-CH<sub>2</sub>DCFDA-loaded cells were used as negative controls. The fluorescence of control and sample wells was recorded at excitation  $485 \pm 20$  nm, emission  $528 \pm 20$  nm with the microplate reader every 10 min for 1.5 h. Data are reported as percentage increase of DCF fluorescence associated with the CH<sub>2</sub>DCFDA-loaded cells compared to that of corresponding non-CH<sub>2</sub>DCFDA-loaded cells (negative controls).

### 2.12. H<sub>2</sub>O<sub>2</sub> generation in medium

The ability of sample to produce H<sub>2</sub>O<sub>2</sub> was assessed in both cell culture medium and cell culture medium with HL-60 cells. The samples analyzed were cyanidin 3-glucoside, puree and powder-derived ACEs from Black Satin at various concentrations. The effect of catalase (100 U/ml) addition as well as 10% FBS in the medium was also determined at time points up to 48 h. At each time point, an aliquot was removed and H<sub>2</sub>O<sub>2</sub> concentration was measured using a Bioxytech H<sub>2</sub>O<sub>2</sub>-560 Kit (Oxis International, Foster City, CA) according to the instructions from the manufacturer. Briefly, 1 volume of sample was mixed with 10 volumes of ferrous ion-xylene orange working reagent and the absorbance was measured at 1 h at 560 nm using the microplate reader. Appropriate controls were used to subtract the possible interference of the corresponding vehicles. H<sub>2</sub>O<sub>2</sub> concentrations were calculated based on the standard curve of authentic H<sub>2</sub>O<sub>2</sub> at concentrations ranging from 0 to 50  $\mu$ M.

### 2.13. Statistical analysis

All values of each assay other than cell culture studies were based on independent triplicate samples of ACEs and calculated as the mean  $\pm$  standard error (SE). In cell culture studies, each experimental

group consisted of  $n = 3$  and data were expressed as mean  $\pm$  standard error (SE) of three independent experiments. Statistical analysis was performed using one way ANOVA followed by Dunnett's or Bonferroni's Multiple Comparison test ( $\alpha \leq 0.05$ ) with GraphPad 5.0 (GraphPad Software Inc.; San Diego, CA).

### 3. Results

#### 3.1 *Extraction and characterization of ACEs*

In these studies, an ultrasound-assisted acidified ethanol extraction method was used for the preparation of ACEs. Total anthocyanin and phenolics content, polymeric color and total antioxidant activity of puree-derived or powder-derived ACE from all Hull, Chester, and Black Satin cultivars are shown in Table 1. Using the same extraction method, it was found that the total anthocyanin and phenolic content, polymeric color and TAC were comparable between cultivars of the same harvest year (2006). As compared to Hull (2006), puree-derived ACE from Hull (2005) had 27% less total anthocyanins content and significantly increased polymeric color. Interestingly, using the same extraction process, ACEs derived from the puree contained about two-fold greater amounts of phenolics than the extract derived from powder. However, the total anthocyanins content in puree and powder-derived ACEs was about the same. Moreover, the TAC values of puree ACEs increased by about two to three-fold as compared to those of powder-derived ACEs. A significant positive correlation was found between total phenolics and TAC values ( $r^2 = 0.9832$ ). In addition, the percentage of polymeric color in puree ACEs was higher than that in powder ACEs indicating that more polymeric browning products were extracted from blackberry puree than powder. It must be noted that since the puree contains around 90% (w/w) water, the actual extraction solvent was not 100% ethanol/0.01 HCl but 71.4% ethanol/0.01 HCl. Puree ACEs were of interest since the time consuming and expensive lyophilization step was avoided in its preparation. <Insert Table 1 here>

To investigate the effect of the extraction solvents with different water to ethanol ratios on the properties of the extracts, a series of ACEs were prepared from Black Satin puree using a solvent system with 0 to 100% water content. As shown in Fig. 1, the ACEs obtained using

solvents with 0 to 50% water content contained about the same amount of total anthocyanins and TAC. An increasing trend of total phenolics and percentage of polymeric color was observed but there was no significant difference in this range. On the other hand, in ACEs prepared using solvent systems with water content above 50%, total anthocyanin, total phenolics content, and TAC decreased while the percentage of polymeric color significantly increased as compared to those in ACE prepared using solvent systems with water content below 50%. A strong positive correlation was found between TAC and total phenolics ( $r^2 = 0.8057$ ) as well as between TAC and total anthocyanins content ( $r^2 = 0.8746$ ). Total anthocyanins content was also found to be negatively correlated with the percentage of polymeric color ( $r^2 = 0.9646$ ). (Insert Fig. 1 here)

### 3.2 *Stability of ACE under various storage conditions*

The major anthocyanins in powder-derived ACE from Hull blackberry were identified in our previous report (Dai et al., 2007). The chromatograms resulting from the developed HPLC method showed a fast and selective separation of the five major blackberry anthocyanins, 1-5, which account for 99% of total anthocyanins in the ACE (Fig. 2). The chromatogram fingerprint of anthocyanins was found to be the same for the ACEs prepared from Hull, Chester and Black Satin (data not shown). In these studies, powder-derived ACE from Hull blackberry was used to investigate the influence of storage conditions on the stability of anthocyanins and phenolics in ACEs. Cyanidin 3-glucoside (peak 1) was the dominant anthocyanin (71% of the total anthocyanins) in ACEs. The HPLC method was validated with external standard cyanidin 3-glucoside and demonstrated linearity within the range of 4.7-14.2 mg/l ( $r^2 = 0.9984$  and an RSD = 1.15%). The intra and inter-day variations were 1.0 and 2.0%, respectively. The recovery percentage was in the range of 98.6-100.4%. (Insert Fig. 2 here)

The change of cyanidin 3-glucoside concentration in ACE at different storage conditions over 90 days is shown in Fig. 3. No significant change of cyanidin 3-glucoside concentration was observed at  $-80\text{ }^{\circ}\text{C}$  over 90 days. However, with increasing storage temperature ( $4\text{ }^{\circ}\text{C}$  and  $25\text{ }^{\circ}\text{C}$ ), a decrease in cyanidin 3-glucoside was observed at each time point. The retention percentage of the other four major anthocyanins were also calculated based on the percentage of peak area at each time point compared with the corresponding peak area at day 0 in the HPLC chromatogram (data not shown). Their degradation profiles showed the same trend as cyanidin 3-glucoside. Moreover, the stability (most stable to least stable) followed as: cyanidin 3-glucoside (peak 1) = cyanidin-3-arabinoside (peak 2) > cyanidin-3-xyloside (peak 3) = cyanidin-3-dioxalylglucoside (peak 5) > cyanidin-3-malonylglucoside (peak 4). The results were in accordance with the previous report that the aliphatic acyl anthocyanins, particularly malonic acid, are more labile under an acidic environment as compared to other anthocyanins (Francis, 1989). (Insert Fig. 3 here)

The pH of ACE samples increased slightly from day 0 to day 90 from  $1.95 \pm 0.01$  to  $2.04 \pm 0.01$ ,  $2.12 \pm 0.00$ ,  $2.23 \pm 0.01$  when stored at  $-80\text{ }^{\circ}\text{C}$ ,  $4\text{ }^{\circ}\text{C}$  and  $25\text{ }^{\circ}\text{C}$ , respectively. There was no significant change in the osmolality of the ACE samples observed in any of the storage conditions over 90 days. As shown in Table 2, with increasing storage temperature, total anthocyanins content, total phenolics and TAC values decreased over time whereas the percentage of polymeric color increased. A similar trend was also observed for all other time-points to day 90 (data not shown). Moreover, it was found that there was no significant difference for all the parameters measured between samples stored in the  $25\text{ }^{\circ}\text{C}$  stability chamber and the temperature-controlled room ( $22\text{-}28\text{ }^{\circ}\text{C}$ , 38% humidity, white light) over a period of 45 days (data not shown), which indicated that white light may not have a significant effect on the

degradation of anthocyanins and phenolics in ACE. It was also observed that frozen storage of ACE for up to 90 days caused about 10% loss of total phenolics and TAC, which was in accordance with the previous report by Srivastava *et al.* (Srivastava et al., 2007). Taken together, temperature and time contributed to the loss of anthocyanins, total phenolics and antioxidant activity in ACE, while the effect of light was insignificant. <Insert Table 2 here>

### 3.3 Stability of ACE in biologically relevant buffers at 25 and 37 °C

The degradation profiles of ACE anthocyanins and phenolics in biological buffers at 25 °C and 37 °C are shown in Fig. 4. The final pH of after adding ACE (2 mg/ml) to all media and buffers was pH 7.2, except for the pH 1.0 buffer which was maintained at pH 1.0. As expected, elevated pH and/or temperature accelerated anthocyanins degradation (Fig. 4A). Total phenolics content was also decreased with increasing temperature (Fig. 4B). As a control, ACE was diluted in pH 1.0 buffer and it did not show any significant loss of total anthocyanins and phenolics content at 25 °C over 48 h. There was no significant decrease of total phenolics content observed for up to 24 h in samples with PBS and 10% FBS in PBS buffer at 25 °C. The lowest amount of total phenolics content retained after 48 h was in samples with RPMI 1640 medium at 37 °C, about 53.8% of that at time 0. <Insert Fig. 4 here>

Total anthocyanins content decreased much more rapidly than total phenolics content in biological buffers. The anthocyanin degradation curves were fitted using nonlinear regression of first-order kinetics. The estimated half-lives of samples with PBS, 10% FBS in PBS at 25 °C, 10% FBS in PBS at 37 °C, and RPMI 1640 medium at 37 °C were 32.7, 12.6, 5.0 and 6.2 h with  $r^2$  of 0.9757, 0.9780, 0.9907 and 0.9850, respectively.

### 3.4 *Cytotoxic effects of ACEs and cyanidin 3-glucoside on human cancer cell lines*

Powder and puree-derived ACEs from Black Satin cultivar were evaluated for their cytotoxic properties on human colon cancer (HT-29, Fig. 5A), ovarian cancer (MCF-7, Fig. 5B) and leukemia (HL-60, Fig. 5C) cell lines. It was found that leukemia cells were most sensitive to ACEs among the cell lines tested. On the other hand, the cytotoxic effect of puree-derived ACEs was more profound than that of powder-derived ACEs in all three cell lines tested. The cytotoxic effect of cyanidin 3-glucoside on HL-60 cells is shown in Fig. 5D. The cell viability of HL-60 cells was decreased from 99.5% to 45.9% in a dose-dependent manner after 48 h of exposure with cyanidin 3-glucoside in a concentration range of 10.4-62.2  $\mu\text{g/ml}$ . *<Insert Fig. 5 here>*

### 3.5 *H<sub>2</sub>O<sub>2</sub> generation by ACEs and cyanidin 3-glucoside in RPMI 1640 medium*

H<sub>2</sub>O<sub>2</sub> production by ACEs at 1.4 mg/ml with or without catalase in RPMI 1640 medium with 10% FBS over time is illustrated in Fig. 6. Detailed results with respect to cyanidin 3-glucoside, ACEs at various concentrations in medium with or without 10% FBS, as well as in the presence of HL-60 cells are presented in Table 3. When authentic H<sub>2</sub>O<sub>2</sub> was incubated with RPMI 1640 medium, its concentration decreased over time. Hence, the H<sub>2</sub>O<sub>2</sub> level in the RPMI 1640 medium was determined by the rate of its generation and decomposition. Puree and powder-derived ACE as well as cyanidin 3-glucoside produced H<sub>2</sub>O<sub>2</sub> in a dose-dependent manner in the RPMI 1640 medium, and the puree-derived ACE generated more H<sub>2</sub>O<sub>2</sub> than powder-derived ACE at the same concentration. Catalase (100 U/ml) depleted the H<sub>2</sub>O<sub>2</sub> in the medium at all concentrations tested for authentic H<sub>2</sub>O<sub>2</sub>, as well as puree and powder-derived ACEs. *<Insert Fig. 6 here>*

Our results also showed that the rate of H<sub>2</sub>O<sub>2</sub> generation by puree-derived extract was inversely correlated to the extract concentration. For example, the level of H<sub>2</sub>O<sub>2</sub> peaked at 2 h

with 0.7 mg/ml of puree-derived ACE but not until 48 h with 1.4 mg/ml of puree-derived ACE in RPMI1640 medium with 10% FBS. Since the pH of the medium decreased from pH 7.2 to 6.5 with increasing concentration of ACE from 0.14 to 7 mg/ml, it is possible that the active components in ACE that produce  $H_2O_2$  was stabilized at relatively lower pH and the rate of  $H_2O_2$  generation was reduced. On the other hand, powder-derived extract produced  $H_2O_2$  at a lower rate and extent than the powder-derived extract and it did not reach a plateau until 48 h for all concentrations tested. Further, when puree-derived ACEs incubated with RPMI 1640 medium without FBS, the  $H_2O_2$  level was 1.95 and 2.46-fold higher than those when incubated with RPMI 1640 medium with 10% FBS at 6 h and 48 h, respectively. Similar results were also found with powder-derived ACE as well as cyanidin 3-glucoside. These results indicated that FBS significantly inhibited the generation of  $H_2O_2$  by ACEs. A similar phenomenon was observed by Lapidot *et al.* using apple extracts in medium containing fetal calf serum; and it was suggested that the  $H_2O_2$  might be decomposed by the residual enzymatic activity in the serum (Lapidot *et al.*, 2002).

We also measured the  $H_2O_2$  level in the medium in the presence of HL-60 cells. It was found that when authentic  $H_2O_2$  (100  $\mu$ M) was added to the medium with cells,  $H_2O_2$  was not detected in the medium at 2 h. In contrast, 90% of the added  $H_2O_2$  was detected when it was incubated with medium without cells. These data suggested that the rate of  $H_2O_2$  diffusion into cells was much greater than its rate of decomposition in the medium. With extract derived from puree at 1.4 mg/ml, the concentration of  $H_2O_2$  at 2 h in the medium was 6.3-fold lower in the presence of cells as compared to the absence of cells suggesting the rapid cell entry of either the generated  $H_2O_2$  or the component in the extract responsible for the generated  $H_2O_2$ .

In comparison to the puree derived extract, a relatively small concentration of H<sub>2</sub>O<sub>2</sub> (< 10 μM) was detected after the addition of cyanidin 3-glucoside with HL-60 cells over a period of 2 to 6 h. <Insert Table 3 here>

### 3.6 *Effect of ACEs and cyanidin 3-glucoside on intracellular ROS level in HL-60 cells*

Intracellular ROS level in HL-60 cells after treatment of cyanidin 3-glucoside and ACEs for 1 h is shown on Fig. 7. It was found the intracellular ROS level peaked at 1 h after treatment of puree-derived ACE but remained the same for either powder-derived ACE or cyanidin 3-glucoside over 1.5 h (data not shown). As shown in Fig. 7, the baseline intracellular ROS level in HL-60 cells (Veh.) was increased by  $71.8 \pm 2.7\%$  (mean  $\pm$  SE) as compared to non-CDCFDA loaded cells. Authentic H<sub>2</sub>O<sub>2</sub> (98 μM) significantly increased the fluorescence intensity by about 1104.4%. As expected, catalase (500 U/ml) decreased the increase of fluorescence intensity caused by H<sub>2</sub>O<sub>2</sub> (98 μM) to the baseline level. Puree-derived ACE increased the intracellular ROS level dose-dependently from  $69.3 \pm 16.2\%$  to  $322.2 \pm 23.1\%$  in concentrations ranging from 0.14 to 2.80 mg/ml. However, no significant difference was found in intracellular ROS levels for powder-derived ACE treatment groups as compared to the control group. Cyanidin 3-glucoside (20 and 60 μg/ml) slightly reduced the intracellular ROS level though the difference was insignificant as compared to the control group. <Insert Fig. 7 here>

### 3.7 *Effect of H<sub>2</sub>O<sub>2</sub> produced by ACEs in the medium on cytotoxic properties of ACEs in HL-60 cells*

To evaluate the influence of H<sub>2</sub>O<sub>2</sub> produced by ACEs in the medium on their cytotoxic properties in HL-60 cells, cells were pretreated with catalase and compared to the ACE-induced cytotoxicity with non-catalase treatment groups (Fig. 8). Authentic H<sub>2</sub>O<sub>2</sub> (98 μM) reduced HL-60 cell viability to  $8.8 \pm 0.5\%$ . Catalase (500 U/ml) rescued the H<sub>2</sub>O<sub>2</sub>-induced cell death to  $92.1 \pm 1.3\%$ . This demonstrated that addition of exogenous catalase almost completely abolished H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity on HL-60 cells in our experiment conditions. Interestingly, in spite of the fact that puree-derived ACE produced more H<sub>2</sub>O<sub>2</sub> in the medium than powder-derived ACE, the cytotoxicity induced by puree-derived ACE was not affected by catalase, whereas catalase partially protected the HL-60 cells from powder-derived ACE-induced cytotoxicity. There was no significant difference in cell viability between catalase and non-catalase treatment groups for puree-derived ACE at all concentrations tested. In contrast, the cytotoxicity resulting from powder-derived ACE was significantly recovered by catalase from  $48.7 \pm 4.0\%$  to  $77.2 \pm 1.8\%$ , and  $28.8 \pm 2.7\%$  to  $52.3 \pm 1.5\%$  at ACE concentrations of 2.1 and 2.8 mg/ml, respectively. However, at high concentration (5.6 mg/ml), there was no significant difference in cell viability between catalase and non-catalase treatment groups. (Insert Fig. 8 here)

#### 4. Discussion

In these studies, we provide a comprehensive evaluation of ACEs from Kentucky-grown blackberries including extraction methods, stability, anticancer activity and its possible mechanisms of action. Solvent extraction of phenolic compounds from fresh, dried, or freeze-dried fruit materials has been the most common method in fruit sample preparation. The solvents generally used are methanol, ethanol, acetone, water, and their mixtures. To obtain high yield of anthocyanins in the extract, solvents are usually mildly acidified to facilitate liberation and solubilization of anthocyanins from the fruit tissue and to stabilize anthocyanins as well. The relative recovery efficiency between solvents varies with different plant materials. For example, Metivier *et al.* found that methanol was 20% more effective than ethanol and 73% more effective than water in recovering anthocyanins from grape pomace (Metivier et al., 1980). However, Giusti *et al.* found that water was more effective in recovering anthocyanins from purple corn waste than acidified water and ethanol (Jing and Giusti, 2007). Besides the solvent system, other factors such as temperature and time are important. It has been reported that elevated temperature improves extraction efficiency due to enhanced solubility and diffusion rate of compounds into the solvent. However, high temperature accelerates anthocyanin degradation in the extraction process. A few extraction technologies such as pressurized liquid extraction (Ju and Howard, 2003) have been developed to enable rapid extraction of anthocyanins and other phenolics at high temperature ( $> 50$  °C) and were found to be successful in retarding anthocyanin degradation during processing. It was also found that ultrasound-assisted solvent extraction was more efficient to extract anthocyanins than conventional solvent extraction due to the strong disruption of the fruit tissue under ultrasonic acoustic cavitation (Chen et al., 2007).

In these studies, we used an ultrasound-assisted ethanol extraction method to prepare anthocyanin-containing extract from blackberries. The starting materials used for extraction were either blackberry puree or freeze-dried puree which was ground into powder. Because the puree contains around 90% w/w water, 25 ml of solvent was used to extract 10 gram of puree (corresponding ~1 gram of powder) to keep the solid-to-liquid ratio at 1:2.5. It was found that the ACEs from Hull, Black Satin and Chester contained similar amount of total anthocyanins and total phenolics. However, ACEs from puree contained 2-3 fold greater total phenolics than those from powder while the anthocyanins content was about the same. This suggested that the water content inside the puree contribute to the difference of total phenolics in ACEs extracted from blackberry puree and powder. To further evaluate the influence of the water content in the extraction system on the property of the extracts, various ACEs were prepared from puree of Black Satin cultivar with solvent systems comprising increased water content in ethanol/0.01% HCl. It was found that total anthocyanins and total phenolics contents were similar in the ACEs obtained using the solvent systems ranging from 0 to 50% water, indicating that this range of water to ethanol was preferable. A sharp increase of the percentage of polymeric color in ACE prepared with 75% water was observed, which suggested that water content greater than 50% led to increased anthocyanin degradation and the degradation and/or condensation products contributed significantly to the percentage of polymeric color. These data may suggest that in the extraction system using high water content, some of the polyphenol oxidase activity was retained and contributed to the condensation of anthocyanin and other phenolics during extraction.

It is well-known that anthocyanins and other phenolics are prone to degradation (Matsufuji et al., 2007; Srivastava et al., 2007; Xiong et al., 2006). Understanding the mechanism and extent of degradation are critical in the development of Botanical Drug Products. Anthocyanins are very

stable under acidic conditions; however, under normal processing and storage conditions readily convert to colorless derivatives and subsequently to insoluble brown pigments (e.g., increased polymeric color). The mechanisms of anthocyanins degradation involve hydrolysis, oxidation and condensation with other polyphenols. For example, the degradation of cyanidin 3-glucoside in aqueous solution begins with hydrolysis of glucosidic bonds and the opening of the pyrylium ring upon heating (Sadilova et al., 2007) or by anthocyanase (Zhang et al., 2001). They are further degraded and produce protocatechuic acid and phloroglucinaldehyde. Cyanidin 3-glucoside can also be co-oxidized with other phenolics such as chlorogenic acid by polyphenol oxidase with the formation of *o*-quinones that generate polymerized products by quinone-phenol reactions (Kader et al., 1998). The most important factors that influence anthocyanin degradation are pH and heat (Sadilova et al., 2007). Thus, the stability of anthocyanins and total phenolics was examined in extract stock solution as well as in biologically relevant buffers at various storage temperatures. It was found that anthocyanin degradation in aqueous solution followed first-order kinetics. Anthocyanins concentrated in extract stock solution exhibited relatively high stability over time. The half-life of cyanidin 3-glucoside in the extract stock solution having a pH of 2 at 25 °C was 63.8 days ( $r^2 = 0.9949$ ).

To assess the stability of ACEs in biologically relevant environment, the ACE was diluted with biologically relevant buffers to the  $EC_{50}$  concentrations obtained in the cytotoxicity studies. It was found that when ACE was diluted with buffers at physiological pH, the anthocyanins degradation rate increased substantially (Fig. 4A). Further, our results showed that FBS contributed significantly to the acceleration of anthocyanins degradation. The half-life of total anthocyanins in samples with 10% FBS in PBS was 2.6-fold shorter than that with PBS at 25 °C, although the mechanism behind it remains unclear. When the temperature was increased from 25

°C to 37 °C, the half life of total anthocyanins in samples with 10% FBS in PBS decreased by 2.5-fold. In this experiment, precipitation was not observed under any condition and the percentage of polymeric color remained the same after 48 h (data not shown).

Our studies demonstrated that ACEs are cytotoxic to human colon (HT-29), ovarian (MCF-7) cancer, and leukemia (HL-60) cells, with different degrees of potency (Fig. 5A, 5B, 5C). Based on the  $EC_{50}$  values, puree-derived ACE was 4-fold more potent than powder-derived ACE in HL-60 cells. Moreover, the HL-60 cells showed much greater sensitivity to ACEs than MCF-7 and HT-29 cells since the  $EC_{50}$  of puree-derived ACE was 5.7 and 3.3-fold lower for HL-60 than that for MCF-7 and HT-29 cells, respectively. A number of dietary phenolics, such as quercetin, which was also found in blackberries (Mertz et al., 2007), have been shown to produce  $H_2O_2$  in generally-used medium including RPMI 1640 medium and contribute to the cytotoxicity of cancer cells (reviewed in Halliwell, 2008). In addition,  $H_2O_2$  was shown to be an important regulator in HL-60 cells growth (Hachiya and Akashi, 2005). Therefore, we investigated the  $H_2O_2$  generation by puree and powder-derived ACEs in medium and its influence on HL-60 cell growth. A detailed examination of  $H_2O_2$  production by ACEs over time in RPMI 1640 medium with or without HL-60 cells is provided in Table 3. Our results showed that both puree and powder-derived ACEs produced levels of  $H_2O_2$  ( $>10 \mu M$ ) which caused cytotoxicity in HL-60 cells when incubated with RPMI 1640 medium within 48 h. Interestingly, the cytotoxic activity of puree-derived ACE was not blocked by exogenous catalase. On the other hand, the intracellular ROS level was also found to increase with puree-derived ACE, which may induce cytotoxicity in HL-60 cells. Since it is well-known that  $H_2O_2$  diffuses freely through cell membranes while catalase is membrane-impermeable, the results suggested that the cytotoxic activity of puree-derived ACE was not due to the  $H_2O_2$  generated in the medium, but due to

some active components in ACE which were quickly taken up by the cells and subsequently induced cytotoxicity by generating ROS inside the cells and/or by other mechanisms. However, as for powder-derived ACE, its growth inhibition activity was partially abolished by the catalase. Since intracellular ROS level in HL-60 cells remained the same after treatment of powder-derived ACE for 1 hour (Fig. 7) and H<sub>2</sub>O<sub>2</sub> concentration in medium did not exceed 10 μM in the first 6 h of incubation (Table 3), it is apparent that the cytotoxic activity through ROS mechanism by powder-derived ACE was relatively delayed. The difference of anticancer mechanism between puree and powder-derived ACEs in HL-60 cells may be attributed to the different major active components that contribute to their cytotoxic activity resulting in different anticancer potency in HL-60 cells, and similarly in HT-29 and MCF-7 cells. Since we showed that puree-derived ACE contained 2-fold greater amount of total phenolics than powder-derived ACE, it was possible that the elevated antiproliferative potency of puree-derived ACE was due to the higher level of phenolics. It is noteworthy that we also investigated the effect of copper on the H<sub>2</sub>O<sub>2</sub> generation by ACEs in medium. It was found that H<sub>2</sub>O<sub>2</sub> production by ACEs did not increase with increasing concentration of cupric sulfate from 0 to 100 μM in the RPMI 1640 medium (data not shown), which suggests that the H<sub>2</sub>O<sub>2</sub> generation by ACEs was likely not copper-mediated.

Cyanidin-based anthocyanins were the dominant anthocyanins in the blackberry extract and cyanidin 3-glucoside was the major anthocyanin (71%). Cyanidin 3-glucoside has been found to possess chemopreventive and chemotherapeutic activities. Chen *et al.* showed that cyanidin 3-glucoside purified from Black rice (*Oryza. sativa* L. indica) inhibited human breast cancer cell HS578T growth via G2/M arrest and it also improved the cytotoxicity of doxorubicin at non-toxic concentrations (Chen et al., 2005). Ding *et al.* showed that cyanidin 3-glucoside not only

protected JB6 cells from ultraviolet B-induced insult but also can inhibit the proliferation, migration and invasion of A549 lung tumor cells (Ding et al., 2006). Cyanidin (Hou et al., 2003) and cyanidin-3-rutinoside (Feng et al., 2007) were also found to increase the intracellular ROS level which may involve induction of apoptosis in HL-60 cells. Therefore, we were interested in assessing the possible role of cyanidin 3-glucoside in ACEs in the cytotoxic property of ACE in HL-60 cells. The antiproliferative effect of pure cyanidin 3-glucoside was examined on HL-60 cells (Fig. 5D) as well as its ability to spontaneously generate H<sub>2</sub>O<sub>2</sub> in RPMI 1640 medium (Table 3) and intracellular ROS in HL-60 cells (Fig. 6). It was found that cyanidin 3-glucoside exhibited antiproliferative activity in a concentration range of 10.4-62.2 µg/ml. However, ACEs caused cytotoxicity in HL-60 cells with much lower anthocyanin concentrations. For example, the anthocyanins concentration in powder and puree-derived ACEs at EC<sub>50</sub> were 3.6 and 14.5 µg/ml, respectively (of which 71% was cyanidin 3-glucoside). However, pure cyanidin 3-glucoside caused little or no cytotoxic effects on HL-60 cells at these concentrations. These data suggested that cyanidin 3-glucoside in ACEs may act additively or synergistic with other active components in inhibition of cell growth, but that a significant part of the cytotoxicity could not be explained by anthocyanins alone. Moreover, it was found that the concentrations of cyanidin 3-glucoside which induced cytotoxicity in HL-60 cells produced non-toxic levels of H<sub>2</sub>O<sub>2</sub> in the RPMI 1640 medium with 10% FBS with little or no increase in the intracellular ROS in HL-60 cells. These results suggested that the active components that produced H<sub>2</sub>O<sub>2</sub>, increased intracellular ROS, and cytotoxicity with puree-derived ACE were components predominantly other than cyanidin 3-glucoside. Related, McDougall *et al.* compared the antiproliferative effectiveness of a series of polyphenol-rich berry extract on human cervical (Hela) and colon (Caco-2) cancer cells and their polyphenol compositions and they suggested that phenolics that

responsible for the antiproliferative activity of berry extracts was due to other constituents such as ellagitannins and procyanidins more than anthocyanins (McDougall et al., 2008). Importantly, it has been shown that ellagitannins are the major phenolic class than anthocyanins in blackberry species (Mertz et al., 2007). Identification of the active components in ACEs is currently ongoing in our lab.

In conclusion, these studies demonstrated that ACEs from blackberry cultivars of Hull, Black Satin and Chester grown in Kentucky have potent antioxidant and anticancer properties. Using the same extraction method, total anthocyanins and phenolics contents as well as total antioxidant capacity by TEAC method were comparable in ACEs derived from these cultivars. Extraction solvent with different water to ethanol ratio greatly influenced the yield of total anthocyanins and phenolics in ACEs. In general, puree-derived ACEs contained similar amount of total anthocyanins but higher amount of total phenolics with higher total antioxidant capacity and were more potent in their antiproliferative activity in cancer cells as compared to powder-derived ACEs. Furthermore, our studies suggested that the antiproliferative activity of puree-derived ACE was not due to the  $H_2O_2$  produced in the medium, but some active components in ACE which were quickly taken up by the cells to induce cytotoxicity in the cells before they produced toxic levels of  $H_2O_2$  in the medium. On the other hand, the antiproliferative activity of powder-derived ACE was partially related to the toxicity of  $H_2O_2$  generated in medium since catalase was partially effective in reducing extract-mediated cytotoxicity. Moreover, our studies suggested cyanidin 3-glucoside itself contributed little to the antiproliferative activity of ACEs and it suggested that cyanidin 3-glucoside may act synergistically or additively with other active components in ACE to cause cytotoxicity in cancer cells. Together with the stability studies of ACE, these findings warrant further investigation into the anticancer effects of blackberries in

both *in-vitro* and *in-vivo* models and provide essential information for the development of Botanical Drug Products from ACEs derived from blackberries and other fruits.

**Conflict of interest statement**

Dr. Mumper owns stock in the company, Four Tigers LLC, that provided funding to support these studies, in-part.

**Acknowledgements**

The authors would like to thank WindStone Farms in Paris, KY for providing the blackberry puree used in these studies and Kentucky Tobacco Research and Development Center at the University of Kentucky for providing funding to support these studies, in-part.

**Table 1.****Composition and Characterization of Blackberry Extracts <sup>a</sup>**

Raw Material	Cultivar (year)	Level (mg/g of DBE)		Polymeric color (%)	TAC (μmol TE/g DBE)
		Total Anthocyanins <sup>b</sup>	Total Phenolics <sup>c</sup>		
Puree	Hull (2005)	5.34 ± 0.19	24.07 ± 2.70	12.99 ± 2.50	171.43 ± 15.67
	Hull (2006)	7.54 ± 0.14	25.78 ± 0.56	7.20 ± 0.28	186.65 ± 0.63
	Chester (2006)	7.95 ± 0.15	25.31 ± 1.80	4.89 ± 0.33	186.75 ± 10.49
	Black Satin (2006)	7.16 ± 0.22	22.91 ± 1.24	3.27 ± 0.15	158.52 ± 6.90
Powder	Hull (2005)	4.51 ± 0.50	12.00 ± 0.77	1.97 ± 1.97	64.40 ± 0.60
	Chester (2006)	7.88 ± 0.23	14.61 ± 1.48	0.94 ± 0.94	78.02 ± 6.07
	Black Satin (2006)	7.67 ± 0.51	14.81 ± 1.59	1.11 ± 0.75	71.68 ± 9.18

<sup>a</sup> The extraction was repeated at least two times. All assays were carried out in triplicate. Data are expressed as the mean ± SE.

<sup>b</sup> Total anthocyanins are expressed as cyanidin 3-glucoside equivalent.

<sup>c</sup> Total phenolics are expressed as gallic acid equivalent.

**Table 2.****Effect of Storage Conditions on Several Parameters of the Hull Blackberry Extract after 90 Days <sup>a</sup>**

Parameters	Day 0	Day 90		
		-80 °C	4 °C	25 °C
		Level / (% of Retention)		
Cyanidin 3-glucoside (mg/l)	600 ± 4.3 (100%)	589 ± 8.2 (98.3%)	539 ± 3.8 (89.8%)	230 ± 0.9 (38.4%)
Total Phenolics (mg/l) <sup>b</sup>	1899 ± 18.1 (100%)	1680 ± 6.9 (88.5%)	1696 ± 6.8 (89.3%)	1537 ± 16.2 (80.1%)
Total Anthocyanins (mg/l) <sup>c</sup>	710 ± 22.3 (100%)	684 ± 15.8 (96.3%)	621 ± 8.3 (87.5%)	261 ± 4.7 (36.8%)
Polymeric Color (%)	0.12 ± 0.12 NA	1.43 ± 0.11 NA	2.61 ± 0.88 NA	13.96 ± 1.11 NA
TAC (µmol/l) <sup>d</sup>	8.9 ± 0.13 (100%)	8.0 ± 0.27 (89.2%)	7.8 ± 0.11 (86.4%)	7.3 ± 0.09 (81.2%)

<sup>a</sup> Data are expressed as mean ± SE ( $n = 3$ ).

<sup>b</sup> Total anthocyanins are expressed as mg of cyanidin 3-glucoside equivalent/l of extract.

<sup>c</sup> Total phenolics are expressed as mg of gallic acid equivalent/l of extract.

<sup>d</sup> TAC are expressed as µmol of trolox equivalent/l of extract.

% of Retention was calculated based on the day 0 concentration as 100%.

NA: Not applicable.

**Table 3.**  
**H<sub>2</sub>O<sub>2</sub> Generation by ACEs and Cyanidin 3-glucoside (C-3-G) in Medium at 37°C <sup>a</sup>**

	Medium	Concentration	H <sub>2</sub> O <sub>2</sub> level (μM) at various times (h)					
			1	2	6	24	48	
ACEpuree (mg/ml)	RPMI 1640 10% FBS	0.14	5.33 ± 0.12	5.09 ± 1.00	4.42 ± 0.40	6.57 ± 0.25	6.74 ± 0.37	
		0.70	8.98 ± 0.24	27.45 ± 0.83	20.85 ± 0.61	11.82 ± 0.26	9.97 ± 0.37	
		1.4	11.48 ± 0.28 (ND) <sup>#</sup>	33.00 ± 1.76 (ND) <sup>#</sup>	41.97 ± 1.89 (ND) <sup>#</sup>	63.70 ± 2.89 (ND) <sup>#</sup>	61.13 ± 1.20 (ND) <sup>#</sup>	
		7.0	9.06 ± 0.22	21.98 ± 1.56	52.74 ± 2.00	145.41 ± 3.98	171.91 ± 1.15	
	RPMI 1640 10% FBS HL60 cells	0.70	2.33 ± 0.19 (ND) <sup>#</sup>	4.24 ± 0.22 (ND) <sup>#</sup>	3.10 ± 0.79 (ND) <sup>#</sup>			
		1.4	1.96 ± 0.23 (ND) <sup>#</sup>	5.22 ± 0.20 (ND) <sup>#</sup>	4.32 ± 0.24 (ND) <sup>#</sup>			
		2.8	3.26 ± 0.25 (ND) <sup>#</sup>	6.54 ± 0.18 (ND) <sup>#</sup>	ND (ND) <sup>#</sup>			
	RPMI 1640	1.4			81.70 ± 2.66		150.46 ± 1.22	
	ACEpowder (mg/ml)	RPMI 1640 10% FBS	0.14	1.30 ± 0.25	1.09 ± 0.30	1.50 ± 0.40	2.05 ± 0.31	3.01 ± 0.29
			0.70	ND	1.92 ± 0.26	4.93 ± 0.73	6.09 ± 0.41	10.92 ± 0.39
1.4			ND (ND) <sup>#</sup>	3.63 ± 0.29 (ND) <sup>#</sup>	8.59 ± 1.12 (ND) <sup>#</sup>	15.18 ± 0.63 (ND) <sup>#</sup>	19.39 ± 1.21 (ND) <sup>#</sup>	
7.0			ND	3.42 ± 0.21	6.74 ± 0.52	11.28 ± 0.65	28.69 ± 1.09	
RPMI 1640 10% FBS HL60 cells		1.4	1.40 ± 0.10 (ND) <sup>#</sup>	1.54 ± 0.17 (ND) <sup>#</sup>	ND (ND) <sup>#</sup>			
		2.8	2.02 ± 0.28 (ND) <sup>#</sup>	1.68 ± 0.17 (ND) <sup>#</sup>	1.43 ± 0.14 (ND) <sup>#</sup>			
RPMI 1640		1.4			9.68 ± 0.36		33.36 ± 1.20	
C-3-G (μg/ml)		RPMI 1640 10% FBS	20.7		ND	ND	ND	
	62.2			2.97 ± 0.16	2.73 ± 0.14	1.90 ± 0.28		
	RPMI 1640	20.7		1.26 ± 0.10	5.90 ± 0.15	12.79 ± 0.31		
		62.2		3.09 ± 0.18	9.04 ± 0.18	19.39 ± 0.32		
	RPMI 1640 10% FBS HL60 cells	20.7		ND				
		62.2		ND				
H <sub>2</sub> O <sub>2</sub> (μM)	RPMI 1640 10% FBS	50	46.26 ± 1.16 (ND) <sup>#</sup>	44.24 ± 1.92 (ND) <sup>#</sup>	35.29 ± 0.90 (ND) <sup>#</sup>	17.65 ± 0.33 (ND) <sup>#</sup>	12.49 ± 0.25 (ND) <sup>#</sup>	
	RPMI 1640 10% FBS HL60 cells	100		ND				

<sup>a</sup> Data are expressed as mean ± SE (*n* = 6).

<sup>#</sup> With catalase (100 U/ml).

ND: Not detected.

## Figure Captions

**Fig. 1.** The water content in the extraction solvent on the properties of ACEs: 10 g of Black Satin puree was mixed with 25 ml of extraction solvent. The water to ethanol/0.01% HCl ratios (v/v) were as follows: 0/100, 10/90, 25/75, 50/50, 75/25, 90/10, and 100/0. Various ACEs were obtained and analyzed for total anthocyanins (●), total phenolics (□), percentage of polymeric color (▲), and total antioxidant capacity (○). Results were normalized to the corresponding values of each parameter of ACE prepared using 100% ethanol/0.01% HCl. Data are presented as the mean ± SE of three independent extractions.

**Fig. 2.** HPLC chromatogram of anthocyanins in: cyanidin 3-glucoside (peak 1), cyanidin-3-arabinoside (peak 2), cyanidin-3-xyloside (peak 3), cyanidin-3-malonylglucoside (peak 4), and cyanidin-3-dioxalylglucoside (peak 5).

**Fig. 3.** Changes in cyanidin 3-glucoside concentration in powder-derived ACE from Hull blackberry under different storage conditions over 90 days: -80 °C (■); 2-8 °C (Δ); temperature-controlled room (22-28 °C) with white light (▼); 25 °C (◇).

**Fig. 4.** The effects of biologically relevant buffers on the stability of anthocyanins and phenolics in powder-derived ACE from Hull blackberry at 25 °C and 37 °C. ACE stock solutions were diluted to a final concentration of 2 mg/ml in (1) pH 1.0 buffer at 25 °C (●); (2) pH 7.4 PBS at 25 °C (□); (3) pH 7.4 PBS with 10% FBS at 25 °C (▲); (4) pH 7.4 PBS with 10% FBS at 37 °C (○); (4) RPMI 1640 medium supplemented with 10% FBS at 37 °C (▼) and incubated for 48 h. At each time point, an aliquot was withdrawn, and measured for total anthocyanins content (A) and total phenolics content (B). Data are presented as the mean ± SE of three independent experiments.

**Fig. 5.** The cytotoxic effects of Black Satin ACEs and Cn-3-G on human cancer cell lines. Cells were exposed to Black Satin puree and powder-derived ACE (A, B, C), or Cn-3-G (D) for 48 h and cell viability was measured by MTT assay. Data in A-D are representative of three independent experiments and data are presented as the mean ± SE ( $n = 3$ ). Curves were fit by nonlinear regression using GraphPad Prism. Half maximum effective concentration ( $EC_{50}$ ) is reported as the mean ± SE ( $n = 3$ ) in the figure legend.

**Fig. 6.**  $H_2O_2$  generation by Black Satin ACEs in RPMI 1640 medium with 10% FBS over time. Authentic  $H_2O_2$  (50  $\mu$ M), puree and powder-derived ACE (1.4 mg/ml) with or without catalase (100 U/ml) were incubated with RPMI 1640 medium with 10% FBS at 37°C for 48 h. At each time point, an aliquot was taken out and measured for  $H_2O_2$  concentration. Data are presented as the mean ± SE ( $n = 6$ ).

**Fig. 7.** The effects of Black Satin ACEs and Cn-3-G on the intracellular ROS level in HL-60 cells. CH<sub>2</sub>DCFDA-loaded and non-CH<sub>2</sub>DCFDA loaded cells were incubated with either ACEs, or cyanidin 3-glucoside (Cn-3-G), or media alone (vehicle). H<sub>2</sub>O<sub>2</sub> (98 μM), H<sub>2</sub>O<sub>2</sub> (98 μM) plus catalase (500 U/ml) treatment groups were used as controls. The results are reported as the percentage increase fluorescence associated with the CH<sub>2</sub>DCFDA-loaded cells compared to that of corresponding non-CH<sub>2</sub>DCFDA-loaded cells. Data are presented as the mean ± SE of three independent experiments. <sup>b</sup>  $P \leq 0.01$ , <sup>c</sup>  $P \leq 0.001$  compared to the vehicle group.

**Fig. 8.** The effects of H<sub>2</sub>O<sub>2</sub> generated by ACEs in medium on cytotoxic properties of ACEs in HL-60 cells. Cells were treated with catalase (500 U/ml) (■) or PBS (□), and concomitantly with H<sub>2</sub>O<sub>2</sub> (98 μM) or puree and powder-derived ACEs from Black Satin for 48 h and cell viability was measured. Data are presented as the mean ± SE of three independent experiments. <sup>b</sup>  $P \leq 0.01$ , <sup>c</sup>  $P \leq 0.001$  compared to the non-catalase treatment group with corresponding H<sub>2</sub>O<sub>2</sub> or ACE concentration.

Fig. 1

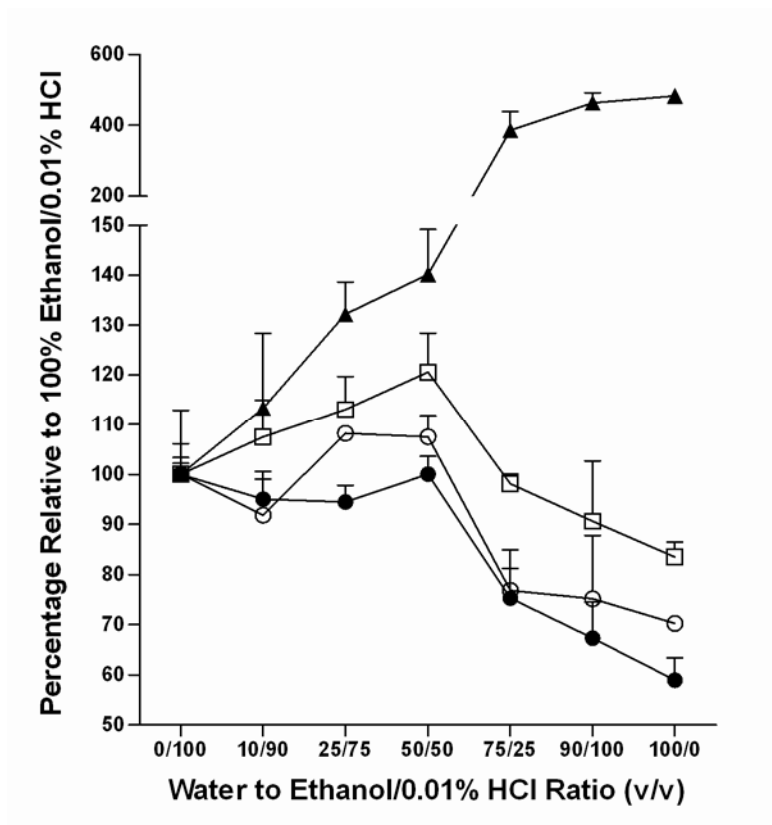


Fig. 2

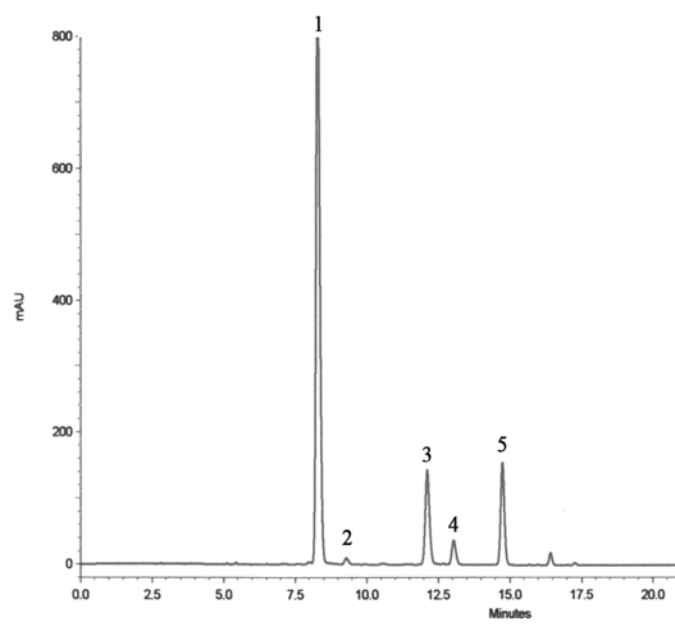


Fig. 3

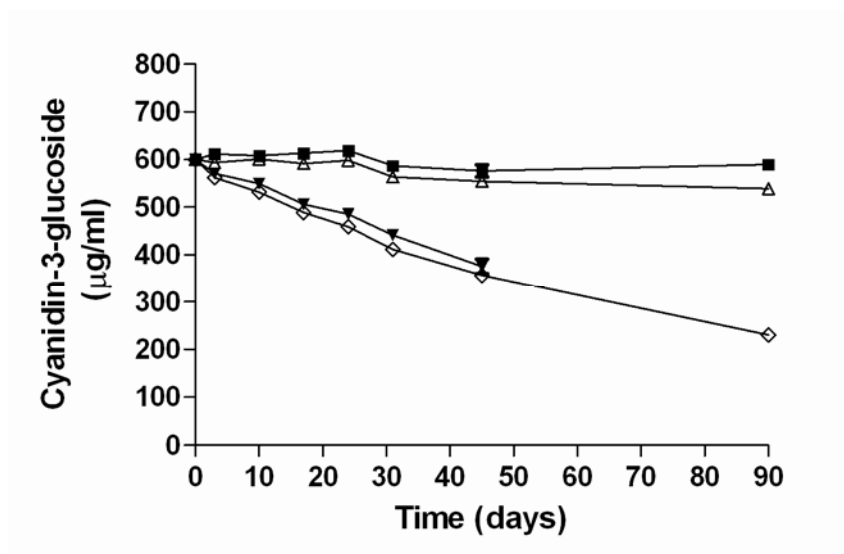


Fig. 4

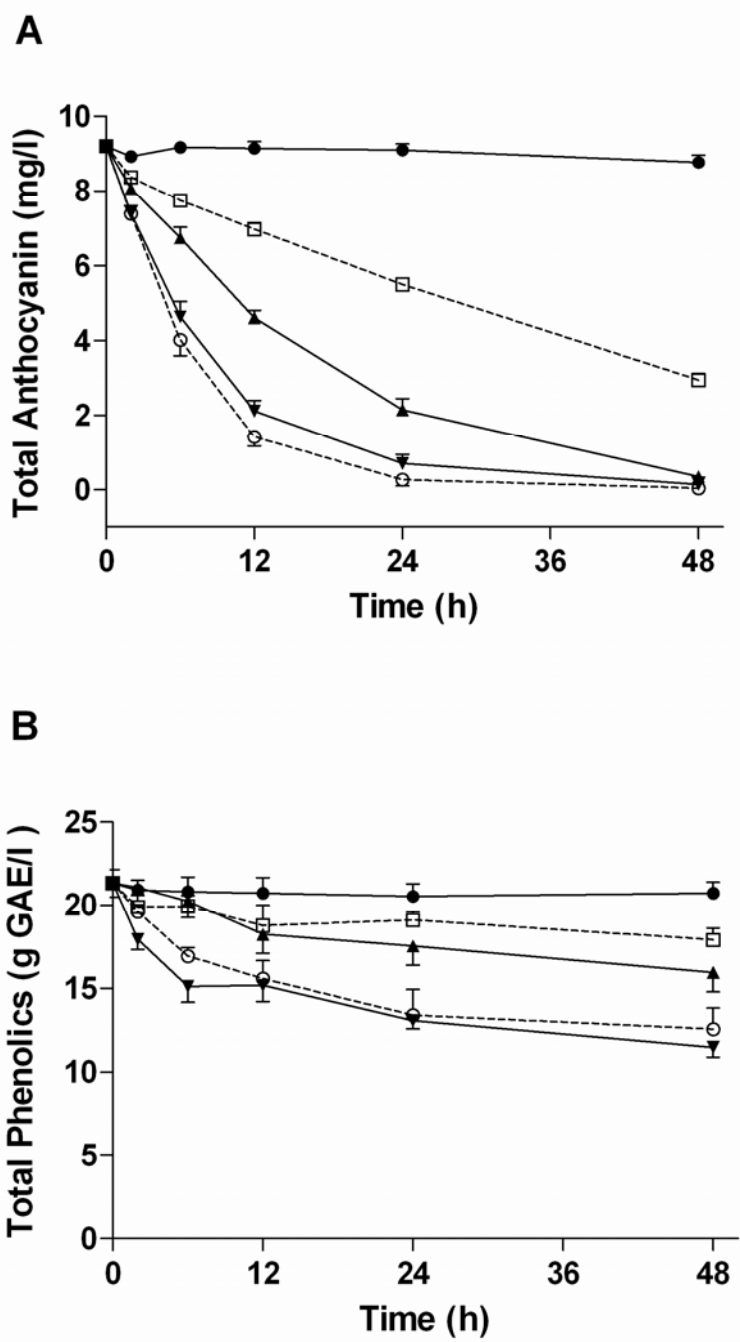


Fig. 5

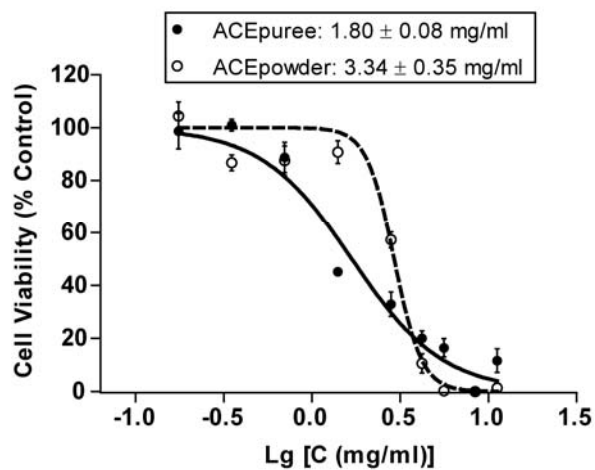
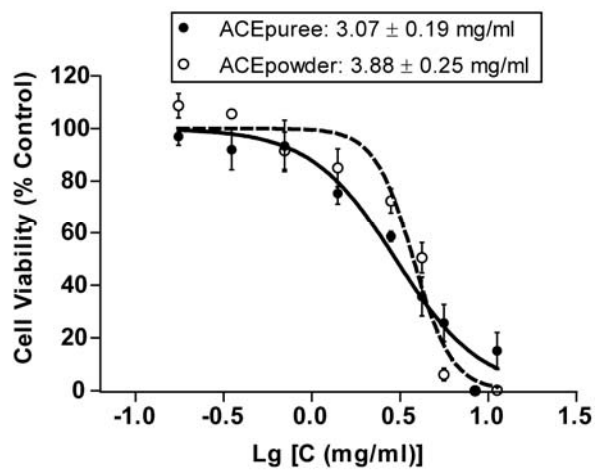
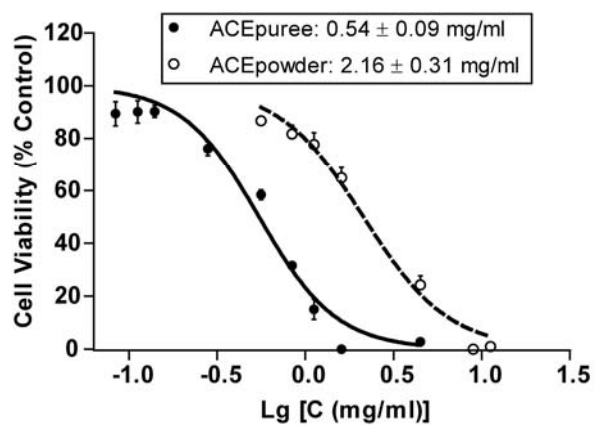
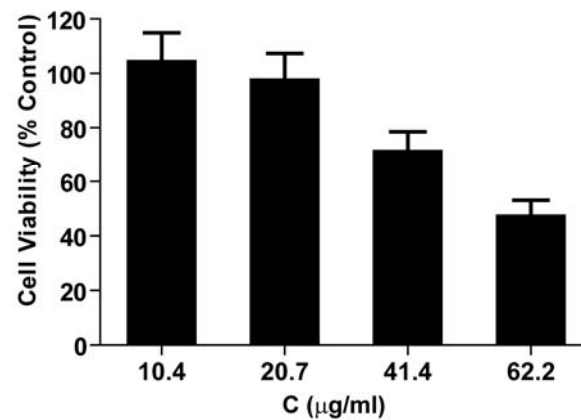
**A HT-29****B MCF-7****C HL-60****D HL-60**

Fig. 6

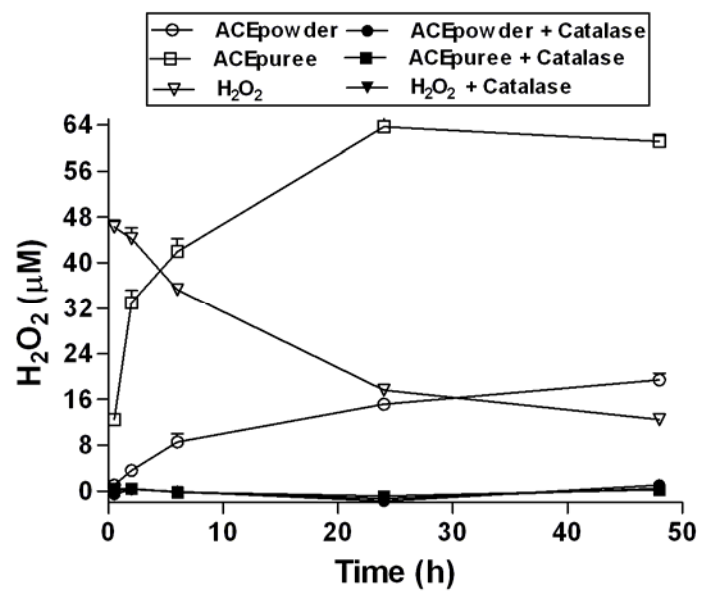


Fig. 7

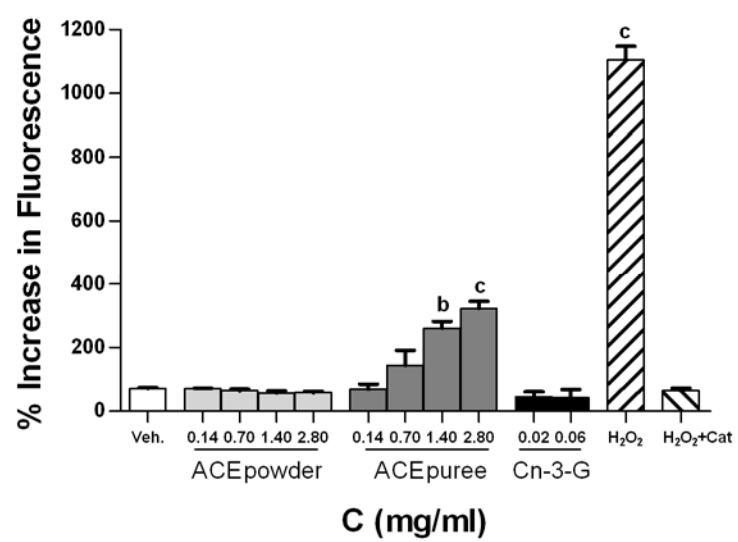
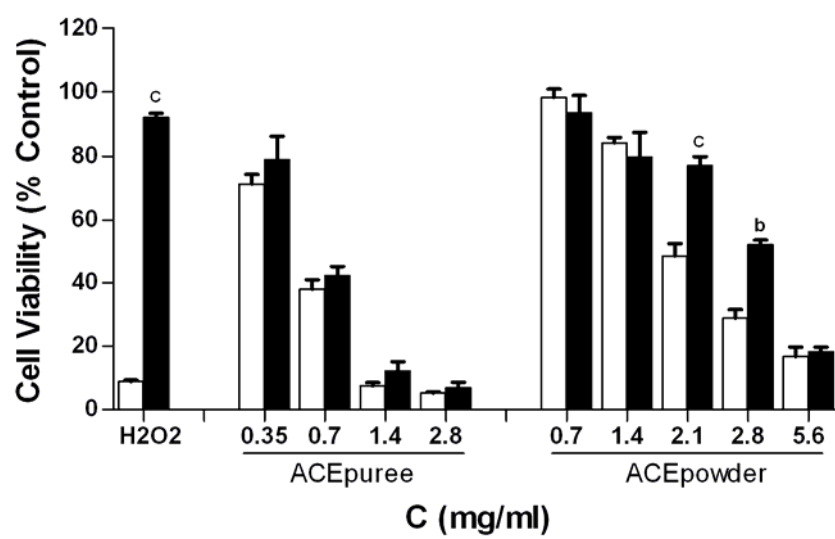


Fig. 8



## References

- Bagchi, D., Sen, C. K., Bagchi, M., Atalay, M., 2004. Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula. *Biochemistry (Mosc)* 69, 75-80, 71 p preceding 75.
- Chen, F., Sun, Y., Zhao, G., Liao, X., Hu, X., Wu, J., Wang, Z., 2007. Optimization of ultrasound-assisted extraction of anthocyanins in red raspberries and identification of anthocyanins in extract using high-performance liquid chromatography-mass spectrometry. *Ultrason. Sonochem.* 14, 767-778.
- Chen, P. N., Chu, S. C., Chiou, H. L., Chiang, C. L., Yang, S. F., Hsieh, Y. S., 2005. Cyanidin 3-glucoside and peonidin 3-glucoside inhibit tumor cell growth and induce apoptosis in vitro and suppress tumor growth in vivo. *Nutr. Cancer* 53, 232-243.
- Dai, J., Patel, J. D., Mumper, R. J., 2007. Characterization of blackberry extract and its antiproliferative and anti-inflammatory properties. *J. Med. Food* 10, 258-265.
- Ding, M., Feng, R., Wang, S. Y., Bowman, L., Lu, Y., Qian, Y., Castranova, V., Jiang, B. H., Shi, X., 2006. Cyanidin-3-glucoside, a natural product derived from blackberry, exhibits chemopreventive and chemotherapeutic activity. *J. Biol. Chem.* 281, 17359-17368.
- Elisia, I., Kitts, D. D., 2008. Anthocyanins inhibit peroxy radical-induced apoptosis in Caco-2 cells. *Mol. Cell Biochem.* 312, 139-145.
- Feng, R., Bowman, L. L., Lu, Y., Leonard, S. S., Shi, X., Jiang, B. H., Castranova, V., Vallyathan, V., Ding, M., 2004. Blackberry extracts inhibit activating protein 1 activation and cell transformation by perturbing the mitogenic signaling pathway. *Nutr. Cancer* 50, 80-89.
- Feng, R., Ni, H. M., Wang, S. Y., Tourkova, I. L., Shurin, M. R., Harada, H., Yin, X. M., 2007. Cyanidin-3-rutinoside, a natural polyphenol antioxidant, selectively kills leukemic cells by induction of oxidative stress. *J. Biol. Chem.* 282, 13468-13476.
- Francis, F. J., 1989. Food Colorants: Anthocyanins. *Crit. Rev. Food Sci. Nutr.* 28, 273-314.

- Galati, G., O'Brien, P. J., 2004. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radic. Biol. Med.* 37, 287-303.
- Giusti, M. M., Wrolstad, R. E., 2001. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In: R. E. Wrolstad, T. E. Acree, H. An, E. A. Decker, M. H. Penner, D. S. Reid, S. J. Schwartz, C. F. Shoemaker, P. Sporns (Eds.), *Current Protocols in Food Analytical Chemistry*. John Wiley & Sons, NY, pp. F1.2.1-F1.2.13.
- Hachiya, M., Akashi, M., 2005. Catalase regulates cell growth in HL60 human promyelocytic cells: evidence for growth regulation by H<sub>2</sub>O<sub>2</sub>. *Radiat. Res.* 163, 271-282.
- Hadi, S. M., Asad, S. F., Singh, S., Ahmad, A., 2000. Putative mechanism for anticancer and apoptosis-inducing properties of plant-derived polyphenolic compounds. *IUBMB Life* 50, 167-171.
- Hadi, S. M., Bhat, S. H., Azmi, A. S., Hanif, S., Shamim, U., Ullah, M. F., 2007. Oxidative breakage of cellular DNA by plant polyphenols: a putative mechanism for anticancer properties. *Semin. Cancer Biol.* 17, 370-376.
- Halliwell, B., 2008. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? *Arch. Biochem. Biophys.* 476, 107-112.
- Halvorsen, B. L., Carlsen, M. H., Phillips, K. M., Bohn, S. K., Holte, K., Jacobs, D. R., Jr., Blomhoff, R., 2006. Content of redox-active compounds (ie, antioxidants) in foods consumed in the United States. *Am. J. Clin. Nutr.* 84, 95-135.
- Hou, D. X., 2003. Potential mechanisms of cancer chemoprevention by anthocyanins. *Curr. Mol. Med.* 3, 149-159.
- Hou, D. X., Ose, T., Lin, S., Harazoro, K., Imamura, I., Kubo, M., Uto, T., Terahara, N., Yoshimoto, M., Fujii, M., 2003. Anthocyanidins induce apoptosis in human promyelocytic leukemia cells: structure-activity relationship and mechanisms involved. *Int. J. Oncol.* 23, 705-712.
- Jing, P., Giusti, M. M., 2007. Effects of extraction conditions on improving the yield and quality of an anthocyanin-rich purple corn (*Zea mays* L.) color extract. *J. Food Sci.* 72, C363-368.

- Ju, Z. Y., Howard, L. R., 2003. Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. *J. Agric. Food Chem.* 51, 5207-5213.
- Kader, F., Haluk, J. P., Nicolas, J. P., Metche, M., 1998. Degradation of Cyanidin 3-glucoside by Blueberry Polyphenol Oxidase: Kinetic Studies and Mechanisms. *J. Agric. Food Chem.* 46, 3060-3065.
- Lapidot, T., Walker, M. D., Kanner, J., 2002. Can apple antioxidants inhibit tumor cell proliferation? Generation of H<sub>2</sub>O<sub>2</sub> during interaction of phenolic compounds with cell culture media. *J. Agric. Food Chem.* 50, 3156-3160.
- Lee, K. W., Hur, H. J., Lee, H. J., Lee, C. Y., 2005. Antiproliferative effects of dietary phenolic substances and hydrogen peroxide. *J. Agric. Food Chem.* 53, 1990-1995.
- Manach, C., Scalbert, A., Morand, C., Remesy, C., Jimenez, L., 2004. Polyphenols: food sources and bioavailability. *Am. J Clin. Nutr.* 79, 727-747.
- Matsufuji, H., Kido, H., Misawa, H., Yaguchi, J., Otsuki, T., Chino, M., Takeda, M., Yamagata, K., 2007. Stability to light, heat, and hydrogen peroxide at different pH values and DPPH radical scavenging activity of acylated anthocyanins from red radish extract. *J. Agric. Food Chem.* 55, 3692-3701.
- McDougall, G. J., Ross, H. A., Ikeji, M., Stewart, D., 2008. Berry Extracts Exert Different Antiproliferative Effects against Cervical and Colon Cancer Cells Grown in Vitro. *J. Agric. Food Chem.* 56, 3016-3023.
- Mertz, C., Cheynier, V., Gunata, Z., Brat, P., 2007. Analysis of phenolic compounds in two blackberry species (*Rubus glaucus* and *Rubus adenotrichus*) by high-performance liquid chromatography with diode array detection and electrospray ion trap mass spectrometry. *J. Agric. Food Chem.* 55, 8616-8624.
- Metivier, R. P., Francis, F. J., Clydesdale, F. M., 1980. Solvent extraction of anthocyanins from wine pomace. *J. Food Sci.* 45, 1099-1100.

- Moyer, R. A., Hummer, K. E., Finn, C. E., Frei, B., Wrolstad, R. E., 2002. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: vaccinium, rubus, and ribes. *J. Agric. Food Chem.* 50, 519-525.
- Nichenametla, S. N., Taruscio, T. G., Barney, D. L., Exon, J. H., 2006. A review of the effects and mechanisms of polyphenolics in cancer. *Crit. Rev. Food Sci. Nutr.* 46, 161-183.
- Pantelidis, G. E., Vasilakakis, M., Manganaris G. A., Diamantidis Gr., 2007. Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, gooseberries and Cornelian cherries. *Food Chem.* 102, 777 - 783.
- Prior, R. L., Wu, X., 2006. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radic. Res.* 40, 1014-1028.
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 26, 1231-1237.
- Rice-Evans, C. A., Miller, N. J., Bolwell, P. G., Bramley, P. M., Pridham, J. B., 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radic. Res.* 22, 375-383.
- Rice-Evans, C. A., Miller, N. J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* 20, 933-956.
- Sadilova, E., Carle, R., Stintzing, F. C., 2007. Thermal degradation of anthocyanins and its impact on color and in vitro antioxidant capacity. *Mol. Nutr. Food Res.* 51, 1461-1471.
- Sautebin, L., Rossi, A., Serraino, I., Dugo, P., Di Paola, R., Mondello, L., Genovese, T., Britti, D., Peli, A., Dugo, G., Caputi, A. P., Cuzzocrea, S., 2004. Effect of anthocyanins contained in a blackberry extract on the circulatory failure and multiple organ dysfunction caused by endotoxin in the rat. *Planta Med.* 70, 745-752.
- Seeram, N. P., Adams, L. S., Zhang, Y., Lee, R., Sand, D., Scheuller, H. S., Heber, D., 2006. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* 54, 9329-9339.

- Seeram, N. P., Nair, M. G., 2002. Inhibition of lipid peroxidation and structure-activity-related studies of the dietary constituents anthocyanins, anthocyanidins, and catechins. *J. Agric. Food Chem.* 50, 5308-5312.
- Serraino, I., Dugo, L., Dugo, P., Mondello, L., Mazzon, E., Dugo, G., Caputi, A. P., Cuzzocrea, S., 2003. Protective effects of cyanidin-3-O-glucoside from blackberry extract against peroxynitrite-induced endothelial dysfunction and vascular failure. *Life Sci.* 73, 1097-1114.
- Singleton, V. L., Rossi, J. A. J., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16, 144-158.
- Siriwoharn, T., Wrolstad, R. E., Finn, C. E., Pereira, C. B., 2004. Influence of cultivar, maturity, and sampling on blackberry (*Rubus L. Hybrids*) anthocyanins, polyphenolics, and antioxidant properties. *J. Agric. Food Chem.* 52, 8021-8030.
- Srivastava, A., Akoh, C. C., Yi, W., Fischer, J., Krewer, G., 2007. Effect of storage conditions on the biological activity of phenolic compounds of blueberry extract packed in glass bottles. *J. Agric. Food Chem.* 55, 2705-2713.
- Xiong, S., Melton, L. D., Easteal, A. J., Siew, D., 2006. Stability and antioxidant activity of black currant anthocyanins in solution and encapsulated in glucan gel. *J. Agric. Food Chem.* 54, 6201-6208.
- Zhang, Z., Pang, X., Ji, Z., Jiang, Y., 2001. Role of anthocyanin degradation in litchi pericarp browning. *Food Chem.* 75, 217-221.
- Zhao, C., Giusti, M. M., Malik, M., Moyer, M. P., Magnuson, B. A., 2004. Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth. *J. Agric. Food Chem.* 52, 6122-6128.