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In vitro absorption and antiproliferative activities of monomeric and polymeric anthocyanin fractions from açai fruit (*Euterpe oleracea* Mart.)

Lisbeth A. Pacheco-Palencia, Susanne U. Mertens-Talcott, Stephen T. Talcott *

Department of Nutrition and Food Science, Texas A&M University, College Station, TX 77843, USA

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ABSTRACT

Anthocyanins are among the most important and widely consumed natural pigments in foods, and have attracted increased attention as natural food colourants and potent bioactive agents. However, anthocyanins are generally unstable and may undergo chemical changes that include oxidative and polymerisation reactions during processing and storage. The role of anthocyanin polymerisation reactions on *in vitro* intestinal absorption and anti-cancer properties has not been assessed. This study investigated the chemical composition, antioxidant properties, antiproliferative activity, and *in vitro* absorption of monomeric and polymeric anthocyanin fractions from açai fruit (*Euterpe oleracea* Mart.). Cyanidin-3-rutinoside (58.5 ± 4.6%) and cyanidin-3-glucoside (41.5 ± 1.1%) were the predominant compounds found in monomeric fractions, while a mixture of anthocyanin adducts were found in polymeric fractions and characterised using HPLC–ESI-MS^n analyses. Monomeric fractions (0.5–100 μg cyanidin-3-glucoside equivalents/ml) inhibited HT-29 colon cancer cell proliferation by up to 95.2% while polymeric anthocyanin fractions (0.5–100 μg cyanidin-3-glucoside equivalents/ml) induced up to 92.3% inhibition. *In vitro* absorption trials using Caco-2 intestinal cell monolayers demonstrated that cyanidin-3-glucoside and cyanidin-3-rutinoside were similarly transported from the apical to the basolateral side of the cell monolayers (0.5–4.9% efficiency), while no polymeric anthocyanins were transported following incubation for up to 2 h. The addition of polymeric anthocyanin fractions also decreased monomeric anthocyanin transport by up to 40.3 ± 2.8%. Results from this study provide novel information regarding the relative size, absorption, and bioactive properties of anthocyanin monomers and polymer adducts.

1. Introduction

Anthocyanins are considered one of the largest and most important plant pigments and are responsible for most red, blue, and purple hues in fruits, vegetables, and flowers (Kong, Chia, Goh, Chia, & Brouillard, 2003). Nutritional interest in anthocyanins is based on their considerable daily intake (180–215 mg/day in the United States), which is significantly higher than the estimated intake of other flavonoids (23 mg/day), including quercetin, kaempferol, myricetin, apigenin, and luteolin (Galvano et al., 2004). Yet numerous studies have reported the relatively low absorption (generally <1.0%) of anthocyanins from rich dietary fruit sources, in both animal and human trials (Bub, Watzl, Heeb, Rechkemmer, & Briviba, 2001; Felgines et al., 2002; Matsumoto et al., 2001; Miyazawa, Nakagawa, Kudo, Muraishi, & Someya, 1999). Anthocyanins were also shown to be absorbed mostly without change in their glycosylated form directly into the bloodstream (Cao, Muccitelli, Sanchez-Moreno, & Prior, 2001) with peak plasma concentrations observed within 1–2 h of ingestion (Matsumoto et al., 2001). However, anthocyanins can also be metabolized *in vivo* to modified forms, including methylated, glucuronated, and sulfo-conjugated anthocyanin metabolites, which have been reported in urine and blood plasma (Felgines et al., 2003).

Information regarding the absorption of anthocyanin glycosides is widely available, yet the literature is devoid of information concerning the absorption or mere presence of anthocyanin polymers in cell culture or biological systems. This gap in knowledge is surprising, since a preponderance of studies exist that show that anthocyanin polymerisation reactions readily occur during processing and storage of anthocyanin-containing fruits and vegetables. Studies on the absorption of structurally similar compounds, such as flavanols, have revealed high absorption levels for monomers that decrease as the degree of polymerisation increases (Shoji et al., 2006). Scalbert et al. (2000) reported (+)-catechin, procyanidin dimer B3, and procyanidin trimer C2 were transported through the human intestinal epithelial Caco-2 cell monolayer yet polymers with a higher degree of polymerisation were not. Additional studies using LC–MS techniques have suggested that procyanidin dimers were absorbed *in vitro* (Spencer et al., 2001) and also into the bloodstream in both animals (Baba, Osakabe, Natsume, & Terao, 2002) and humans (Sano et al.,

* Corresponding author. Tel.: +1 979 862 4056; fax: +1 979 458 3704.
E-mail address: stalcott@tamu.edu (S.T. Talcott).

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2003; Shoji et al., 2006). However, the limits of polyphenolic absorption in relation to the degree of polymerisation are still unclear.

Açai fruit (*Euterpe oleracea* Mart.) was selected as an anthocyanin source for these trials, mainly due to its inherently high polymeric anthocyanin content, commonly between 25% and 45% in fresh açai fruit pulp (Pacheco-Palencia, Hawken, & Talcott, 2007a, 2007b), and its high anthocyanin polymerisation rates during storage, reaching polymeric anthocyanin contents of 70–80% in 8 days at 35 °C (Pacheco-Palencia et al., 2007a). Thus, a few days of storage may result in significant variations in anthocyanin composition in açai fruit juices, potentially leading to changes in anthocyanin bioactive and absorption properties. These studies were conducted in order to evaluate the influence of anthocyanin monomer and polymers on the *in vitro* intestinal absorption and antiproliferative activity of anthocyanins from açai fruit and to determine a dose–response for anthocyanin forms potentially absorbed by the human body. Caco-2 cell monolayers were used as *in vitro* models for intestinal absorption, while antiproliferative activities were evaluated in HT-29 human colon adenocarcinoma cells. Results from these investigations are aimed at promoting postharvest, processing, and storage conditions that retain absorbable and bioactive anthocyanin forms in juices, beverages, and other anthocyanins-containing food products.

2. Materials and methods

2.1. Anthocyanin extracts

Pasteurized açai pulp (*Euterpe oleracea*) was donated by the Bossa Nova Beverage Group (Los Angeles, CA) and shipped frozen to the Department of Nutrition and Food Science at Texas A&M University. Prior to anthocyanin isolation, açai pulp was clarified according to a previously described procedure (Pacheco-Palencia et al., 2007b) to remove lipids and insoluble solids. Clarified açai pulp was then loaded onto activated C18 Sep-Pak 6 cc cartridges (Waters Corporation, Milford, MA) and eluted sequentially with water and ethyl acetate to remove sugars, organic acids, metals, proteins, phenolic acids, and other flavonoids as previously described (Pacheco-Palencia et al., 2007a). Anthocyanins were recovered with acidified methanol (0.01% HCl) and redissolved in a 0.1 M citric acid buffer (pH 3.0) following solvent evaporation under vacuum (<40 °C). The resulting total anthocyanin isolate was adsorbed onto a second Sep-Pak cartridge and monomeric anthocyanins eluted with an alkaline borate solution (0.1 N, pH 9.0), until a colourless eluent was obtained. These monomers were immediately diluted with 4N HCl to favour anthocyanin equilibrium toward the more stable flavylium ion form, loaded onto a third Sep-Pak cartridge, and recovered with methanol (0.01% HCl). The remaining polymeric anthocyanins, retained onto the second cartridge, were eluted with acidified methanol (0.01% HCl). Both monomeric and polymeric fractions were concentrated under vacuum at <40 °C until complete solvent removal and reconstituted in a 0.1 M citric acid buffer (pH 3.0). Anthocyanin monomer and polymer fractions were standardised to a final concentration of ~2500 mg cyanidin-3-glucoside equivalents/l, based on their OD at 510 nm in a pH 1.0 buffer (Pacheco-Palencia et al., 2007b). Anthocyanin isolates were held at −20 °C for 1 day and sterile-filtered through 0.20 μm filters prior use in cell culture experiments.

2.2. Chemical analyses

Polyphenolic isolates were analysed by reversed phase HPLC and mass spectrometric analyses using a Thermo Finnigan LCQ Deca XP Max MSn ion trap mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA). Separations were conducted using an Acclaim 120 5 μm 120 Å (4.6 × 250 mm) column (Dionex Corporation, Sunnyvale, CA). Mobile phases consisted of 0.5% formic acid in water (phase A) and 0.5% formic acid in methanol (phase B) run at 0.6 ml/min. Polyphenolics were separated with a gradient elution programme in which phase B changed from 5% to 10% in 3 min, from 10% to 30% in the following 17 min, from 30% to 50% in the following 20 min, from 50% to 70% in the following 15 min, and from 70% to 100% in the following 10 min. Initial conditions were then restored and held constant for 20 min prior each injection. Ionization was conducted in the negative ion mode under the following conditions: sheath gas (N_2), 60 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 3.5 kV; capillary temperature, 250 °C; capillary voltage, 1.5 V; tube lens offset, 0 V. Identification and quantitation of anthocyanins was based on their spectral characteristics, retention time, and mass spectrometric properties, as compared to authentic cyanidin-3-glucoside and cyanidin-3-rutinoside standards (Sigma Chemical Co., St. Louis, MO). Antioxidant capacity was determined by the oxygen radical absorbance capacity assay using a BMG Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission), as described previously (Pacheco-Palencia et al., 2007b). Results were expressed in μmol Trolox equivalents (TE)/ml. Total soluble phenolic contents were determined by the Folin–Ciocalteu assay, as a measure of the metal ion reducing ability of anthocyanin frac-

Table 1
HPLC–ESI-MS^n of monomeric and polymeric anthocyanin fractions from açai fruit.

Retention time (min)	Anthocyanin	$[M-H]^-$ (m/z)	MS/MS (m/z)[1]
Monomeric anthocyanin fractions			
29.7	Cyanidin-3-glucoside	447.0	285.1, 257.1, 183.0
31.5	Cyanidin-3-rutinoside	593.1	285.1, 257.1, 183.0
34.1	Pelargonidin-3-glucoside	431.0	269.0, 225.0, 183.1
Polymeric anthocyanin fractions			
19.1	Cyanidin glycoside adduct	611.0	593.0, 449.1, 431.1, 285.2, 257.1
20.7	Pelargonidin glycoside adduct	721.1	685.1, 524.0, 431.1, 269.1, 225.1
21.8	Cyanidin glycoside adduct	883.0	721.1, 685.1, 524.0, 431.1, 269.1 225.1
22.5	Cyanidin glycoside adduct	865.1	685.0, 431.1, 285.0, 257.0, 183.1
23.4	Cyanidin glycoside adduct	611.0	593.0, 449.1, 285.2, 257.1
26.8	Cyanidin glycoside adduct	611.1	593.0, 449.1, 285.2, 257.1
28.7	Pelargonidin glycoside adduct	793.0	595.0, 449.1, 431.1, 269.0, 225.1
29.5	Cyanidin-3-glucoside	447.0	285.1, 257.1, 183.0
31.5	Cyanidin-3-rutinoside	593.1	285.1, 257.1, 183.0
34.1	Pelargonidin-3-glucoside	431.0	269.0, 225.0, 183.1
35.6	Peonidin-3-glucoside	461.0	299.1, 284.1, 240.0

tions, as previously noted (Pacheco-Palencia, Mertens-Talcott, & Talcott, 2008).

2.3. Cell proliferation

HT-29 human colon adenocarcinoma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA), cultured in Dulbecco's modified Eagle's medium (1X) (DMEM) containing 5% fetal bovine serum, 1% non-essential amino acids, 100 units/ml penicillin G, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B, and 10 mM sodium pyruvate (Gibco BRL Life Technology, Grand Island, NY). Cells were incubated at 37 °C at 5% CO_2, and used between passages 10 and 20. Cells were seeded at a density 2×10^4 cells/well, into 24-well tissue culture plates. Following incubation for 24 h, the growth medium was replaced with 500 μl of media (pH 7.4) containing different concentrations of standardised anthocyanin extracts (from 0.5 to 100 μg cyanidin-3-glucoside equivalents/ml). Cell numbers were determined following incubation for 48 h, using a Beckman Coulter Particle Counter (Fullerton, CA). Cell numbers were expressed as a percentage of the untreated

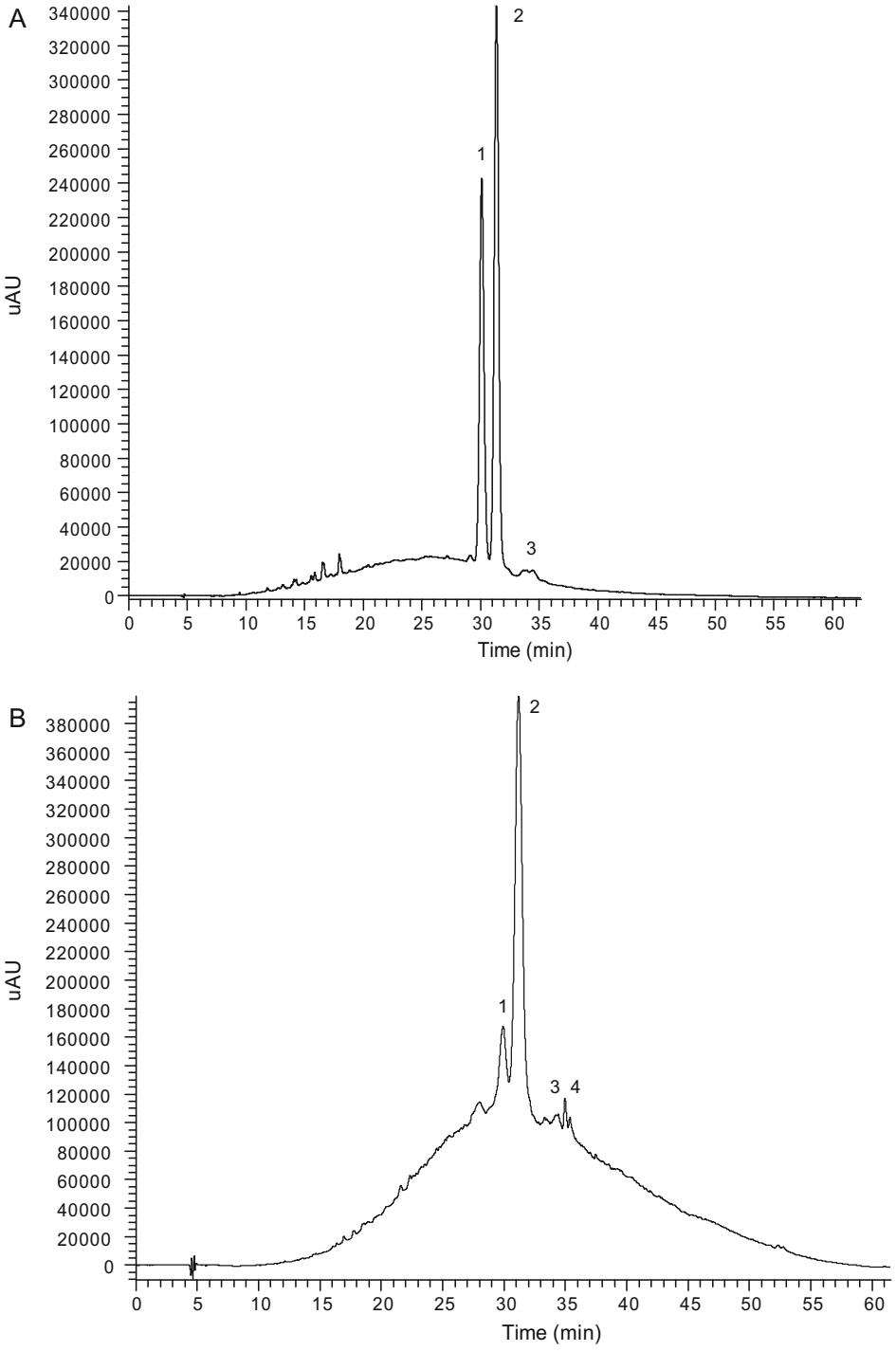

Fig. 1. HPLC chromatogram (520 nm) of anthocyanin monomer extracts (A) and anthocyanin polymer extracts (B). Peak assignments: 1. cyanidin-3-glucoside; 2. cyanidin-3-rutinoside; 3. pelargonidin-3-glucoside and 4. peonidin-3-glucoside.

control and the anthocyanin extract concentration at which cell proliferation was inhibited by 50% (IC_{50}) was calculated by linear regression analyses for each anthocyanin fraction.

2.4. Transepithelial transport model

Caco-2 colon carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (1X) high glucose (DMEM) containing 10% fetal bovine serum, 1% non-essential amino acids, 100 units/ml penicillin G, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B, and 10 mM sodium pyruvate (all chemicals supplied by Sigma–Aldrich Co., St. Louis, MO). Cells between passages 5 and 10 were seeded in 12-mm transparent polyester cell culture insert well plates (Transwell, Corning Costar Corp., Cambridge, MA) at 1.0×10^5 cells per insert, with 0.5 ml of growth medium in the apical side and 1.5 ml in the basolateral side. Cells were grown and differentiated to confluent monolayers for 21 days, as previously described (Hidalgo, Raub, & Borchardt, 1989). Transepithelial electrical resistance (TEER) values were monitored with an EndOhm Volt ohmmeter equipped with a STX-2 electrode (World Precision Instruments Inc., Sarasota, FL). Monolayers with TEER values >450 Ω cm^2 after correction for the resistance in control wells were used for transport experiments. To insure monolayer integrity, TEER values were also measured at the conclusion of transport experiments and data was only collected from monolayers with corrected TEER values >350 Ω cm^2. Prior transport experiments, growth media was replaced by Hank's balanced salt solution (HBSS, Fischer Scientific, Pittsburgh, PA) containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid solution (MES, Gibco BRL Life Technology, Grand Island, NY) adjusted to pH 6.0 in the apical side, and HBSS containing *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] buffer solution (1 M) (HEPES, Gibco BRL Life Technology, Grand Island, NY) adjusted to pH 7.4 in the basolateral side, creating a pH gradient similar to the small intestine environment. Anthocyanin fractions were diluted in HBSS adjusted to pH 6.0 (50 and 500 μg cyanidin-3-glucoside equivalents/ml) and loaded into the apical side of the cell monolayers. Sample aliquots (200 μl) were taken from the basolateral compartment at 0.5, 1.0, 1.5, and 2.0 h, immediately acidified with a known volume of 4 N HCl, kept frozen (−20 °C), and analyzed within 1 week. Samples were finally filtered through 0.45 μm PTFE membranes (Whatman, Florham Park, NJ) and injected directly into the HPLC–ESI-MS system.

2.5. Statistical analyses

Data were analysed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL). Mean separations were conducted by post hoc LSD ($p < 0.05$) pairwise comparisons. A significance level of 0.05 was also used for parametric correlations and linear regression analyses.

3. Results and discussion

3.1. Composition of anthocyanin fractions

Anthocyanins present in monomeric and polymeric fractions from açai fruit were normalised to equivalent total anthocyanin contents (2500 ± 100 mg cyanidin-3-glucoside equivalents/l) and characterised by HPLC–ESI-MS^n analyses. Mass fragmentation patterns for anthocyanins in both fractions are shown in Table 1. Cyanidin-3-rutinoside (1397.7 ± 64.3 mg/l) and cyanidin-3-glucoside (992.8 ± 10.9 mg/l) were predominant in monomeric fractions, along with trace amounts of pelargonidin-3-glucoside (Fig. 1). Polymeric anthocyanin fractions were characterised by the presence of pelargonidin and cyanidin adducts, which eluted as a large, unresolved peak in the reversed phase HPLC chromatograms, with strong UV absorption ~520 nm (Fig. 1), responsible for the characteristic dark red colour observed in these fractions.

Cyanidin adducts were identified by negative ion signals at *m/z* 611.0, 883.0, and 865.1, yielding common fragments at *m/z* 285.2 and 257.1, corresponding to the fragmentation pattern of cyanidin glycosides. Likewise, pelargonidin adducts were characterised by negative ion signals at *m/z* 721.1 and 793.0 and common fragments at *m/z* 431.1, 269.1, and 225.1, corresponding to the fragmentation of pelargonidin glycosides (Table 1). Compared to the monomeric anthocyanin isolate, lower concentrations of cyanidin-3-rutinoside (332.8 ± 24.4 mg/l), cyanidin-3-glucoside (84.6 ± 5.9 mg/l), and traces of pelargonidin-3-glucoside and peonidin-3-glucoside were also detected in polymeric anthocyanin fractions. Polymeric anthocyanin fractions contained higher proportions of cyanidin-3-rutinoside (79.7 ± 5.8%) and relatively smaller amounts of cyanidin-3-glucoside (20.3 ± 1.4%) when compared to monomeric fractions at 58.5 ± 4.6% cyanidin-3-rutinoside and 41.5 ± 1.1% cyanidin-3-glucoside. Variations in anthocyanin composition were hypothesised to be important contributing factors to differences in absorption and bioactive properties between monomeric and polymeric anthocyanin fractions. Thus, mixtures containing varying proportions of monomers and polymers (75/25 and 50/50 monomer/polymer ratios) were also evaluated in order to assess the influence of polymeric anthocyanins on the absorption and bioactive properties of monomeric anthocyanin glycosides.

Total antioxidant capacity of anthocyanin fractions and fraction mixtures were also related to total anthocyanin contents. Both anthocyanin fractions were comparable in terms of antioxidant capacity, with respective values of 256.9 ± 12.6 μmol TE/ml for monomeric fractions and 206.8 ± 6.7 μmol TE/ml for polymeric fractions. Comparable reducing capacities were also observed for both monomeric and polymeric anthocyanin fractions in the Folin–Ciocalteu assay, ranging from 5220 to 6577 mg gallic acid equivalents/ml. Analogous observations have been reported in anthocyanin extracts from *Hibiscus sabdariffa*, where degradation of monomeric anthocyanins to brown polymeric forms resulted in a <10% change in antioxidant capacity (Tsai & Huang, 2004).

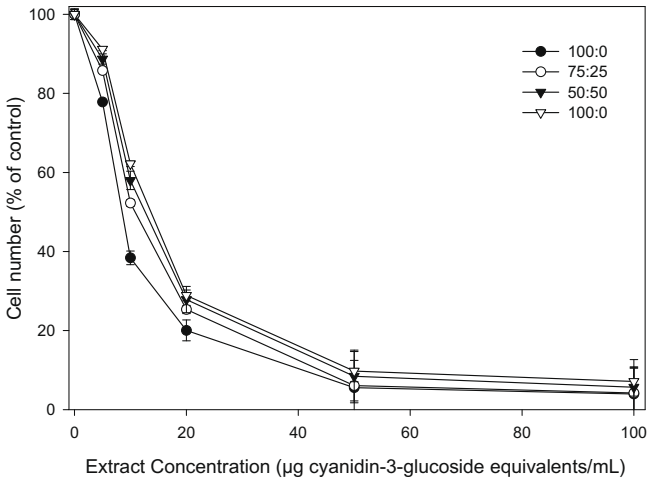

Fig. 2. Percent changes in total HT-29 cell numbers expressed as a ratio to control cells following treatment of cells with açai fruit anthocyanin mixtures adjusted to different concentrations (μg cyanidin-3-glucoside equivalents/ml) for 48 h. Legends denote monomeric to polymeric anthocyanin ratios. Error bars represent the standard error of the mean ($n = 6$).

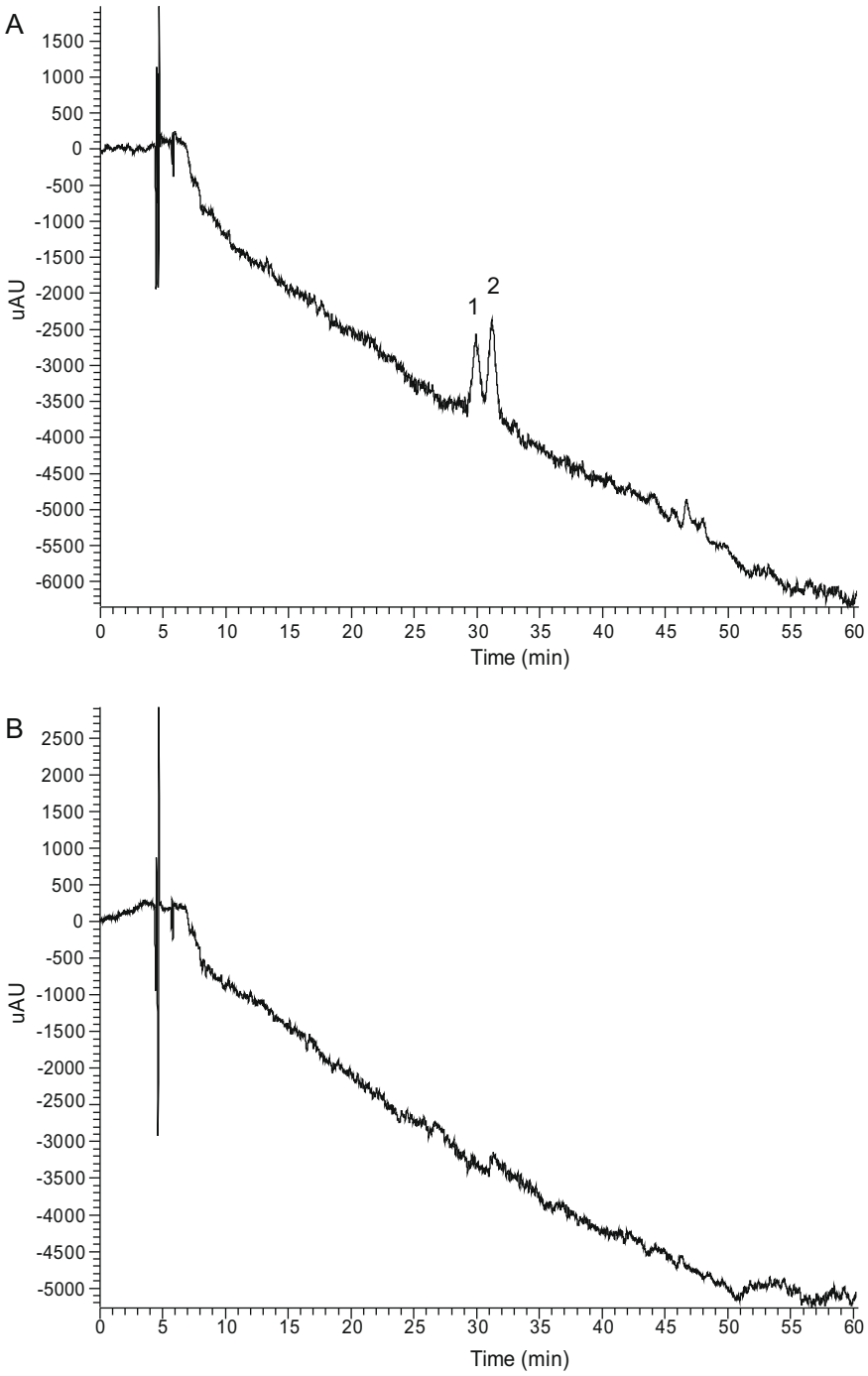

Fig. 3. Typical HPLC chromatogram (520 nm) of anthocyanins present in the basolateral compartment of Caco-2 cell monolayers following incubation with anthocyanin monomer fractions (A) and anthocyanin polymer fractions (B) for 2 h. Peak assignments: 1. cyanidin-3-glucoside and 2. cyanidin-3-rutinoside.

Table 2
Percent transport of anthocyanins from apical to basolateral side of Caco-2 cell monolayers following incubation for 2 h with monomeric and polymeric anthocyanin fractions from açai fruit.

Anthocyanin fraction	% Anthocyanin transport			
	Cyanidin-3-glucoside		Cyanidin-3-rutinoside	
	50 μg/ml[a]	500 μg/ml	50 μg/ml	500 μg/ml
100% anthocyanin monomers	4.94 ± 0.35 a[b]	4.89 ± 0.31 a	4.51 ± 0.33 a	4.43 ± 0.31 a
75% anthocyanin monomers, 25% anthocyanin polymers	3.84 ± 0.26 b	3.80 ± 0.25 b	3.79 ± 0.27 b	3.51 ± 0.24 b
50% anthocyanin monomers, 50% anthocyanin polymers	3.04 ± 0.20 c	2.92 ± 0.23 c	3.11 ± 0.22 c	2.86 ± 0.19 c
100% anthocyanin polymers	–	0.49 ± 0.09 d	–	0.54 ± 0.10 d

[a] Anthocyanin concentrations initially loaded into the apical side of Caco-2 cell monolayers.
[b] Values with different letters within the same column are significantly different (LSD test, $p < 0.05$).

3.2. Cell proliferation

The antiproliferative activities of anthocyanin monomer, polymer, and mixed fractions were evaluated in a cell culture model using HT-29 colon carcinoma cells. The number of cells was related to HT-29 cell proliferation and declines in cell numbers were considered reflective of the cytotoxic effects of anthocyanin extracts. Both monomeric and polymeric anthocyanin fractions and their mixtures decreased ($p < 0.01$) total cell numbers in a concentration-dependent manner (Fig. 2). Monomeric anthocyanin fractions (5–20 μg/ml) were more effective in reducing cell proliferation when compared to similar concentrations of mixtures containing polymeric fractions. Likewise, anthocyanin concentrations at which cell proliferation was inhibited by 50% (IC_{50}) were lower ($p < 0.05$) for monomeric fractions (IC_{50} = 12.1 μg/ml) than for polymeric fractions (IC_{50} = 14.4 μg/ml). Mixtures containing both monomeric and polymeric fractions had intermediate IC_{50} values, varying from 12.2 to 13.6 μg/ml for mixtures with 75:25 and 50:50 monomeric-to-polymeric anthocyanin ratios, respectively.

Differences in the inhibitory effects of monomeric and polymeric anthocyanin extracts may be due to variations in their anthocyanin and/or non-anthocyanin composition, since the availability of functional groups with an ability to access target sites within the cells was likely higher for monomeric anthocyanins. The chemical structure of anthocyanins was also found to influence the chemopreventive and antiproliferative activities of anthocyanin-rich extracts in similar cell models with non-acylated, monoglycosylated anthocyanins having a higher inhibitory effect on HT-29 cell growth when compared to their acylated counterparts (Jing et al., 2008; Zhao, Giusti, Malik, Moyer, & Magnuson, 2004). Inhibitory concentrations (IC_{50}) for purple corn extracts, rich in cyanidin-3-glucoside, were estimated at ~14 μg/ml (Jing et al., 2008), which was in agreement with the findings of these trials.

Antiproliferative activity of monomeric anthocyanin glycosides on cancer cells has been attributed to their effects on growth stimulatory signals across the cell membrane, including inhibition of the epidermal growth factor receptor (Meiers et al., 2001) and to their ability to interfere with various stages of the cell cycle by effects on regulator proteins such as $p21^{WAF1}$, an inhibitor of cell proliferation (Malik et al., 2003; Wang & Stoner, 2008; Wu, Koponen, Mykkanen, & Torronen, 2007). Additional mechanisms, including induction of apoptosis in malignant cells are also likely to be involved in the antiproliferative action of anthocyanin monomers (Chen et al., 2005; Fimognari, Lenzi, & Hrelia, 2008; Hou, 2003; Neto, Amoroso, & Liberty, 2008; Seeram et al., 2006; Srivastava, Akoh, Fischer, & Krewer, 2007). Similar mechanisms might be responsible for the inhibitory effects of anthocyanin polymers on colon cancer cell growth, yet this is the first report on the antiproliferative activity of polymeric anthocyanin fractions on cancer cells *in vitro*.

3.3. Transepithelial transport model

Caco-2 cell monolayers were used as *in vitro* models to assess intestinal absorption of monomeric and polymeric anthocyanin fractions from açai fruit. Unidirectional anthocyanin transport was assessed from the apical to basolateral side of differentiated cell monolayers. Transport efficiencies were expressed as the percentage of anthocyanin concentrations initially loaded into the apical side detected on the basolateral side of cell monolayers following incubation for 0.5, 1.0, 1.5, and 2.0 h. Analytical HPLC chromatograms of anthocyanins present in the basolateral side of cell monolayers following incubation with monomeric and polymeric anthocyanin fractions for 2 h are presented in Fig. 3. Cyanidin-3-glucoside and cyanidin-3-rutinoside present in all açai fruit anthocyanin fractions were transported from apical to basolateral sides of cell monolayers, while additional polymeric anthocyanin fraction components were not transported following incubation for up to 2 h (Fig. 3).

Transport efficiencies for anthocyanin glycosides present in monomeric and polymeric anthocyanin fractions and their mixtures are summarised in Table 2. The percentage of transported monomeric anthocyanin-glycosides following 2 h incubation ranged from 0.5% to 4.9% in each anthocyanin fraction tested, and was higher for monomeric fractions (4.4–4.9%). Transport efficiency was lower for monomeric anthocyanins when 25–50% polymeric anthocyanins were contained in the mixtures (2.9–3.8%), while anthocyanins in polymeric fractions had the lowest transport efficiencies, at 0.5 ± 0.1% (Table 2). Similar results were also observed following incubation for 0.5, 1.0, and 1.5 h (Fig. 4). Decreased monomeric anthocyanin transport (16.0–40.3%) in anthocyanin fraction mixtures containing polymeric fractions (25–50%) indicated anthocyanin polymers may interfere with monomeric anthocyanin transport mechanisms. Mechanisms for absorption and transport of monomeric anthocyanin glycosides through cell monolayers are not yet clear, and both active and passive transport mechanisms have been proposed (Brown, Khodr, Hider, & Rice-Evans, 1998; Cao et al., 2001; Gee et al., 2000; Hollman & Katan, 1998; Hollman et al., 1999; Manach, Williamson, Morand, Scalbert, & Remesy, 2005; Mulleder, Murkovic, & Pfannhauser, 2002; Williamson, Day, Plumb, & Couteau, 2000). Results from this study suggest the presence of anthocyanin polymers may interfere with either active or passive transport mechanisms

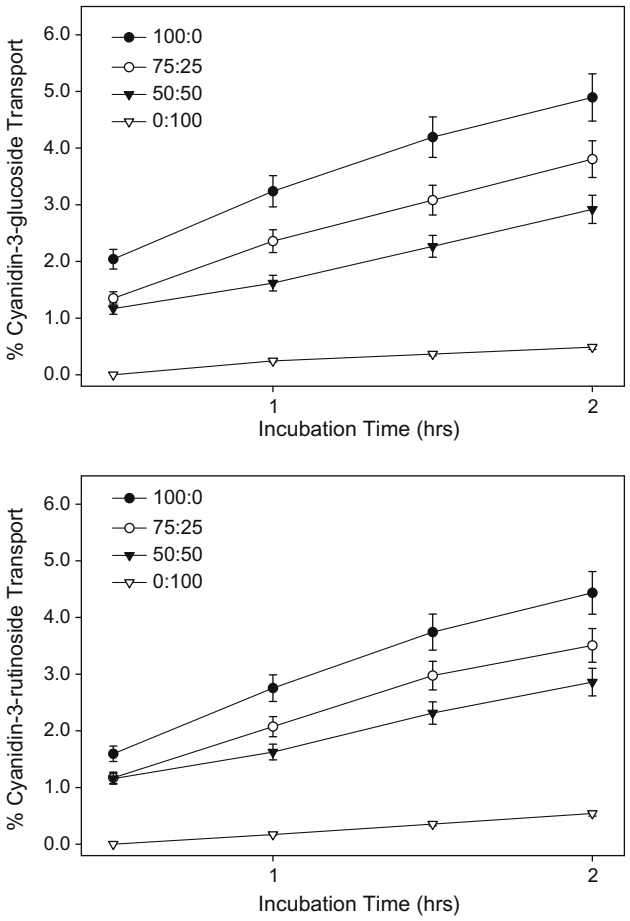

Fig. 4. Percent transport of cyanidin-3-glucoside and cyanidin-3-rutinoside from apical to basolateral side of Caco-2 cell monolayers following incubation with açai fruit anthocyanin mixtures of varying monomeric and polymeric anthocyanin ratios. Legends denote monomeric to polymeric anthocyanin ratios.

Table 3
Average anthocyanin transport rates (μg/l h) from the apical to the basolateral side of Caco-2 cell monolayers, following incubation with monomeric and polymeric anthocyanin fractions from açai fruit.

Anthocyanin fraction	Anthocyanin transport rate (μg/l h)			
	Cyanidin-3-glucoside		Cyanidin-3-rutinoside	
	50 μg/ml[a]	500 μg/ml	50 μg/ml	500 μg/ml
100% anthocyanin monomers	19.6 ± 1.5 a[b]	19.0 ± 1.5 a	18.9 ± 1.4 a	19.0 ± 1.4 a
75% anthocyanin monomers, 25% anthocyanin polymers	15.7 ± 1.1 b	16.2 ± 1.3 b	16.0 ± 1.2 b	15.8 ± 1.2 b
50% anthocyanin monomers, 50% anthocyanin polymers	12.6 ± 1.0 c	11.8 ± 0.9 c	12.5 ± 1.1 c	11.6 ± 1.0 c
100% anthocyanin polymers	Not detected	3.18 ± 0.3 d	Not detected	3.62 ± 0.3 d

[a] Anthocyanin concentrations initially loaded into the apical side of Caco-2 cell monolayers.
[b] Values with different letters within the same column are significantly different (LSD test, $p < 0.05$).

involved in the absorption of anthocyanin glycosides. In order to determine a dose response on transport efficiency, 50 or 500 μg/ml of monomeric and polymeric anthocyanin fractions and two combination ratios were loaded into the apical compartment of the cell monolayers. No differences were found for the relative transport rates of cyanidin-3-glucoside or cyanidin-3-rutinoside for these fractions, suggesting intestinal transport efficiency of anthocyanin glycosides was not dose-dependent (Table 2). Moreover, both target anthocyanins were equally ($p < 0.05$) transported from the apical to basolateral side of the cell monolayers, indicating that the glycosidic moiety (glucose to rutinose) had no influence on absorption of these cyanidin-based anthocyanins (Table 2).

Average transport rates (μg/l h) of anthocyanin-glycosides following incubation with monomeric and polymeric anthocyanin fractions and their mixtures (50 or 500 μg/ml) were calculated based on the relative amount of anthocyanins transported from apical to basolateral side of cell monolayers over 0.5, 1.0, 1.5, and 2.0 h (Fig. 4). Anthocyanin transport rates varied with anthocyanin composition, and ranged from 3.2 to 19.6 μg/l h (Table 3). Transport rates were higher for anthocyanin glycosides present in monomeric fractions (18.9–19.6 μg/l h), and decreased to 11.6–16.2 μg/l h as the relative proportion of polymeric fractions increased from 25% to 50%. Transport rates were lowest (3.2–3.6 μg/l h) in the polymeric anthocyanin fraction. Similar to previous observations on anthocyanin transport efficiencies, variations in anthocyanin concentration levels loaded into the apical compartments of cell monolayers (50 or 500 μg/l) did not influence transport rates of cyanidin-3-glucoside or cyanidin-3-rutinoside and no differences were found between transport rates for cyanidin-3-glucoside and cyanidin-3-rutinoside in any of the fractions.

A strong correlation ($r = 0.98$) was found between anthocyanin transport efficiencies (%) and anthocyanin transport rates (μg/l h), suggesting that factors responsible for increased anthocyanin transport likely influenced the rate of anthocyanin transport in these models. Thus, higher transport efficiencies may be associated with transport mechanisms targeting monomeric anthocyanin glycosides, potentially inhibited or disrupted by the presence of polymeric anthocyanin fractions. Polymeric anthocyanin fractions may decrease transport of monomeric anthocyanin glycosides due to the presence of polar hydroxyl group ends, which have been associated with increased hydrogen-bond formation at the surface of the cell membrane, resulting in lower transport efficiencies (Ollila, Halling, Vuorela, Vuorela, & Slotte, 2002; Saija et al., 1995; Van Dijk, Driessen, & Recourt, 2000). Although mechanisms for anthocyanin transport are still in debate and may be influenced by numerous factors including molecular weight or substituent moieties, this is the first report to show that polymeric anthocyanins are prevented from absorption *in vitro* and that polymeric anthocyanins exert an inhibitory response to the absorption of monomeric anthocyanin glycosides.

4. Conclusion

The influence of anthocyanin polymerisation reactions on the chemical composition, antioxidant properties, antiproliferative activity, and *in vitro* absorption of anthocyanins from açai fruit were evaluated. Monomeric anthocyanin fractions were characterised by the predominant presence of cyanidin-3-glucoside and cyanidin-3-rutinoside, while several anthocyanin adducts were found in polymeric fractions. Both fractions inhibited HT-29 colon cancer cell proliferation in a concentration-dependent manner by up to 95.2%. *In vitro* absorption trials using Caco-2 intestinal cell monolayers demonstrated that cyanidin-3-glucoside and cyanidin-3-rutinoside were similarly transported from the apical to the basolateral side of the cell monolayers, while no anthocyanin adducts were transported following incubation for up to 2 h. The presence of polymeric anthocyanins also decreased transport of monomeric anthocyanin glycosides in a dose-dependent manner by up to 40.3%. Results from this study suggest the presence of anthocyanin polymers may significantly influence anthocyanin absorption properties in açai fruit products.

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