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Food Chemistry 101 (2007) 1052–1058
Food Chemistry
www.elsevier.com/locate/foodchem

Antioxidant assessment of an anthocyanin-enriched blackberry extract

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Received 12 December 2005; received in revised form 23 February 2006; accepted 23 February 2006

Abstract

Gel filtration of black berry (*Rubus fruticosus sp)* ethanolic extracts was employed to obtain an anthocyanin-enriched extract. The anthocyanin profile identified cyanidin-3-glucoside as the primary (e.g., 90% of total) anthocyanin present in blackberry. Gel filtration of crude extracts resulted in a 20-fold increase in total anthocyanin content, with no change in the proportion of cyanidin-3-glucoside. Antioxidant activities of both the crude and anthocyanin-enriched blackberry extracts were determined using cell-free (ORAC) and cell-based (INT-407 intracellular) antioxidant assays. Antioxidant activity, assessed by the ORAC assay, indicated a 7-fold increase in activity for the anthocyanin-enriched fraction. Similar results were obtained for the anthocyanin-enriched extract using the intracellular antioxidant assay with INT-407 cells. Our results indicate that the anthocyanin content, and more specifically the presence of cyanidin-3-glucoside, in blackberry, contributes a major part of the antioxidant ability to suppress both peroxyl radical-induced chemical and intracellular oxidation.

Keywords: Blackberry anthocyanidins; Enriched extract; ORAC; Intracellular oxidation

1. Introduction

Anthocyanins are pigments that give rise to the red to blue colours observed in many soft fruits and flowers (Markides, 1982). There is an increased interest in the use of anthocyanins in the functional food and nutraceutical industry with recent reports on the various potential health benefits (Zhang, Kou, Fugal, & McLaughlin, 2004), that include a reduced risk of coronary heart disease (Renaud & Lorgeril, 1992), visual improvement (Matsumoto, Nakamura, Tachibanaki, Kawamura, & Hirayama, 2003) and anti-carcinogenic (Bomser, Madhavi, Singletary, & Smith, 1996; Kamei et al., 1995), anti-mutagenic (Tate, Kuzmar, Smith, Wedge, & Larcom, 2003) and anti-inflammatory (Hu, Zawistowski, Ling, & Kitts, 2003; Wang & Mazza, 2002) effects. Many of these health benefits have been attributed to the antioxidant property of anthocyanins (Wu, Cao, & Prior, 2002).

Blackberries (*Rubus fruticosus sp.*) are of particular interest in this regard, due to the high anthocyanin and phenolic contents that contribute to its noted antioxidant capacity (Wang & Lin, 2000). In particular, cyanidin-3-glucoside, a common anthocyanin in many soft fruits, including blackberry, has also been reported to have the highest antioxidant capacity of 14 different anthocyanins tested (Wang, Cao, & Prior, 1997; Mazza & Miniati, 1993). Structure–activity relationship studies have revealed that the catechol structure at ring B with two adjacent hydroxl groups positioned 3′, 4′-sites and glycosylation are critical factors for antioxidant activity of anthocyanidins (Wang et al., 1997). Numerous procedures have been employed to concentrate the anthocyanin fraction from different plant sources. Gel filtration, a size exclusion chromatography method, has not been fully utilized, as an alternative method to solvent extraction, or solid phase extraction, to concentrate anthocyanins. For example, TSK-gel Toyopearl

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doi:10.1016/j.foodchem.2006.02.060

(HW-40F) was used to fractionate anthocyanin from black current on the basis of molecular weight (Slimestad & Solheim, 2002). Similar elution protocols have employed Bio-Gel P2 Columns for the separation of cyanidin-3-glucoside and peonidin-3-glucoside from black rice extract (Hu et al., 2003). The purpose of this study was to characterize and quantify the anthocyanin content in blackberry recovered by gel filtration and to assess the antioxidant activity by cells-free (ORAC) and cell-based assays, using a human intestinal cell line (INT-407).

2. Materials and methods

2.1. Materials

Blackberries were supplied from a local farm in Abbotsford, BC, Canada, and were stored at −35 °C prior to extraction. Bio-Gel P2 was obtained from Bio-Rad Laboratories (Richmond, CA). Acetic acid, phosphoric acid (HPLC grade), methanol (HPLC grade) and hydrochloric acid were obtained from Fisher Scientific (Nepean, Ont.). Cyanidin-3-glucoside and cyanidin-3-chloride were purchased from Polyphenol AS (Sandnes, Norway). AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride) was obtained from Wako Chemicals USA (Richmond, VA). Trolox and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma–Aldrich Canada Ltd. (Oakville, Ont.). Minimum Essential Medium Eagle was purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Fetal bovine serum, penicillin and streptomycin were from obtained from Gibco (Grand Island, NY). The CellTiter-Glo® Luminescent Cell Viability Assay kits were from Promega Corporation (Madison, WI).

2.2. Extraction of blackberry anthocyanins

Four batches of 100 g of frozen blackberry were blended with 100 ml of 80% (v/v) ethanol using a Waring blender for 5 min. The slurry was transferred to an Erlenmeyer flask, and extracted overnight. The extract was filtered through a Buchner funnel using Whatman filter paper (No. 1) and rinsed twice with 25 ml of 80% ethanol. The filter cake was transferred to a new Erlenmeyer flask and was re-extracted with 150 ml of 80% ethanol using an orbital shaker at 400 rpm (Innova 4000, New Brunswick Scientific, NJ) for 1 h. The filter cake was extracted for the third time with 80% ethanol overnight and all filtrates were pooled together. Sample extractions were performed in quadruplet. The ethanol in the pooled filtrate was removed under vacuum at 35 °C. The residue was then freeze-dried and kept at 4 °C.

2.3. Enrichment of total anthocyanin content by gel filtration

Gel filtration chromatography was performed to concentrate the total anthocyanin contents of the crude extract as described by Hu et al. (2003). Blackberry crude extract was reconstituted with acidified water (acetic acid, pH 4.5, 300 mg/ml) and loaded onto a Bio-Gel P2 gel filtration column (2.5 cm × 14.5 cm). Acetic acid (pH 2.5) was used to elute the anthocyanin fractions (flow rate = 2 ml/min). The extract constituents were separated, based on molecular weight, with higher molecular weight compounds being eluted earlier. The eluted fraction was monitored at 520 nm and the red-coloured fraction was collected and pooled together over several loadings. This anthocyanin-rich fraction was lyophilized to a powder and stored at 4 °C.

2.4. Total anthocyanin content

Total anthocyanin content of the blackberry extract was measured using the pH differential method described by Wrolstad and Giusti (2001). A crude blackberry extract and the anthocyanin-enriched extract were separately dissolved in potassium chloride buffer (KCl, 0.025 M, pH 1.0) and sodium acetate ($CH_3CO_2Na \cdot 3H_2O$, 0.4 M, pH 4.5) with a pre-determined dilution factor. Sample measurement absorbancies were read at 510 and 700 nm against a blank cell containing distilled and deionized water (ddH_2O). The absorbance (A) of the diluted sample was then calculated as follows:

$$A = (A_{\lambda vis\text{-}max} - A_{700\ nm})_{pH\ 1.0} - (A_{\lambda vis\text{-}max} - A_{700\ nm})_{pH\ 4.5}$$

The monomeric anthocyanin pigment concentration in the original sample was calculated according the following formula:

$$\text{Anthocyanin content (mg/l)} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

where cyanidin-3-glucoside molecular weight (MW = 449.2) and the molar absorptivity ($\varepsilon = 26{,}900$) constants were used.

2.5. HPLC measurement of cyanidin-3-glucoside

Blackberry extracts were dissolved in a mixture of 50% acidified water (3% phosphoric acid, HPLC grade) and 50% methanol (HPLC grade). The reconstituted extracts were filtered through a 0.45 μM nylon membrane-filter. HPLC analyses was performed using an Agilent 1100 HPLC system (Agilent Technology 1100 series, Palo Alto, Ca), equipped with quaternary pumps, autosampler and a diode array detector. Anthocyanin separation were performed using a Zorbax RX-C18 column (5 μm, 4.6 mm × 250 mm) at 30 °C. Mobile phases constituted of 100% methanol (A) and 3% phosphoric acid (B) at a flow rate of 1 ml/min. The gradient condition started with 23% A, linearly increased to 24.3% A at 15 min, then to 50% A at 20 min and 55% A at 25 min. Standard anthocyanins, (i.e., cyanidin-3-glucoside) was used to identify the unknown peaks in samples. Peak identification of unknown compounds of interest in this study was performed by matching the retention time of unknown compounds to external standards. The ChemStation software (version A.10.02, Agilent Technology) was

used to analyze the chromatograms of blackberry extracts and to calculate the proportion of cyanidin-3-glucoside relative to the total anthocyanin content.

2.6. Antioxidant activity of blackberry extracts

The antioxidant capacities of both crude and anthocyanin-enriched blackberry extracts were determined using the oxygen radical absorption capacity assay (ORAC), as described by Kitts and Hu (2005). Briefly, blackberry samples, a range of trolox standards in phosphate buffer (50 mM, pH 7.0) and 60 nM fluorescein were applied to a 96-well plate (Nunc, Fluorescent microplate). Plates were incubated at 37 °C for 15 min. The peroxyl radical initiator, AAPH, was added to a final concentration of 12 mM and fluorescence (Ex = 485 nm, Em = 527 nm) was continuously taken for 60 min (Fluoroskan Ascent FL, Labsystems). Data transformation was performed according to Davalos, Gomez-Cordoves, and Bartolome (2004). The ORAC value was expressed as μmol trolox/g of sample (Kitts & Hu, 2005).

2.7. Intracellular oxidation assay

The effect of blackberry extracts on peroxyl radical-initiated intracellular oxidation was evaluated as described by Hu, Kwok, and Kitts (2005). Human intestinal INT-407 cells (ATCC CCL-6) were seeded into 96 well black plates. Final concentrations of crude blackberry extract (8 μg/ml–1 mg/ml) or anthocyanin-enriched extract (0.02–50 μg/ml) were added to culture media. The blackberry extracts were co-incubated with the 5 μM DCFH-DA probe for 3 h, followed by the addition of 1 mM AAPH to initiate intracellular oxidation. Fluorescence readings were taken from cells using a microplate reader (Fluoroskan Ascent FL, Labsystem) at 0 min (e.g., immediately upon addition of AAPH), and hourly for 7 h and at 24 h, respectively. The excitation wavelength was set at 485 nm and emission wavelength was set at 527 nm. A negative control was constructed to consist of cells exposed to only the DCFH-DA probe. The positive control consisted of cells cultured with the DCFH-DA probe and the peroxyl radical initiator (AAPH). Results were expressed according to the following formula:

$$\text{Fluorescence} = \frac{\text{Fluorescence } t_i}{\text{Fluorescence } t_0}$$

where Fluorescence t_i is fluorescence reading taken at time 1–7 h and 24 h, Fluorescence t_0 is initial fluorescence reading taken upon AAPH addition at 0 min.

2.8. Cell culture

A human embryonic-derived intestinal cell line (INT-407) was maintained in Minimum Essential Medium Eagle (MEME), supplemented with 10% fetal bovine serum, penicillin (100 U) and streptomycin (100 μg/ml). Upon reaching confluency, intestinal cells were sub-cultured and maintained at 37 °C in a 5% CO_2 humidified incubator. INT-407 cells were seeded at 2.5×10^4 cells/well in a 96-well plate and left overnight to allow for attachment. Blackberry crude extract was dissolved in culture medium with serial dilutions made to achieve a concentration range of 0.01–1 mg/ml for final evaluation. The blackberry extracts were added to the cells and were incubated in 96 well plates for 24 h. The test control contained untreated cells and the appropriate cell culture medium without blackberry extracts. Each treatment was done in triplicate. At the end of the 24 h incubation, cells were recovered for cytotoxicity testing, using CellTiter-Glo assay. In a separate experiment, the effect of a blackberry anthocyanin-enriched extract (2–500 μg/ml) was assayed on cells cultured for 24 h.

2.9. CellTiter-Glo assay

After 24 h exposure to blackberry extracts, culture medium was replaced with fresh medium and cell viability was assessed using the CellTiter-Glo assay using a luminometer (Fluoroskan Ascent FL, Labsystem, Helsinki, Finland).

2.10. Statistics

Experiments were performed in triplicate and results were expressed as means ± standard deviation. One-way ANOVA was performed to detect significant difference in total anthocyanin contents between extractions of blackberry extract.

3. Results and discussion

3.1. Determination of total anthocyanin content of blackberry extracts

The total anthocyanin content of the blackberry crude extract, as determined by pH differential method, was 17.1 ± 0.9 mg/g of freeze-dried powder, which was equivalent to 176 ± 9.5 mg/100 g of blackberry (Table 1). These values were found to be within the range of the total anthocyanins reported for blackberry (83–326 mg of anthocyanins per 100 g) (Mazza & Miniati, 1993). The small batch-to-batch variation in total anthocyanin content measured from blackberries (Table 1) may be accounted for by differences in blackberry size. The anthocyanin-enriched extract obtained from gel filtration of the crude extract contained 371 ± 15.4 mg of total anthocyanin per gram of freeze dried powder (Table 1). This yield corresponded to an approximately 20 times greater concentration of total anthocyanins determined in the crude blackberry extract.

3.2. Cyanidin-3-glucoside content of blackberry anthocyanins

The blackberry crude extract contained a single major peak that eluted at 13.7 min, which corresponded to the

Table 1
Anthocyanin profile and associated antioxidant activity of blackberry extracts[a]

	Total anthocyanin content[c] (mg/g)	Cyanidin-3-glucoside[b] (% of total anthocyanin)	ORAC (μmol trolox equivalent/g)
Crude extract	17.1 ± 0.9	87.5	674 ± 52.4
Anthocyanin-enriched extract	371 ± 15.4	90.1	4885 ± 54.2

[a] Total anthocyanin content and ORAC value are expressed per gramme of freeze dried blackberry ($n = 8$).
[b] Cyanidin-3-glucoside as characterized using HPLC.
[c] Total anthocyanin contents as determined by pH differential method. Total anthocyanin content from four different batch samples of blackberry were 167 ± 5.3 (batch #1); 169 ± 3.0 (batch #2); 181 ± 2.6 (batch #3) and 188 ± 1.1 (batch #4) mg/100 g of blackberry.

cyanidin-3-glucoside standard. Cyanidin-3-glucoside in the crude blackberry represented 87.5% of total anthocyanins (Fig. 1). This result is in agreement with other reports indicating that cyanidin-3-glucoside is the primary anthocyanin in blackberry (Mazza & Miniati, 1993; Siriwoharn, Wrolstad, & Durst, 2005). In addition to cyanidin-3-glucoside, cyanidin-3-xyloside, cyanidin-3-(6″-malonyl) glucoside and cyanidin-3-dioxaloylglucoside have been identified in blackberry using HPLC–MS (Wu & Prior, 2005).

The anthocyanin-enriched extract showed a similar elution profile to the blackberry crude extract, with a single major peak representing cyanidin-3-glucoside that constituted 90.1% of the total anthocyanin content in the anthocyanin-enriched extract.

In addition to cyanidin-3-glucoside and its aglycone, several minor peaks were also detected but not identified herein, due to the fact that they represented less than 5% of the total anthocyanin content. Other anthocyanins that have been reported in smaller concentrations in blackberry include cyanidin-3-rutinoside, cyanidin-3-xyloside, malvidin-3-glucoside, cyanidin-3-glucoside acylated with malonic acid, and cyanidin-3-dioxalylglucoside (Dugo, Mondello, Err-

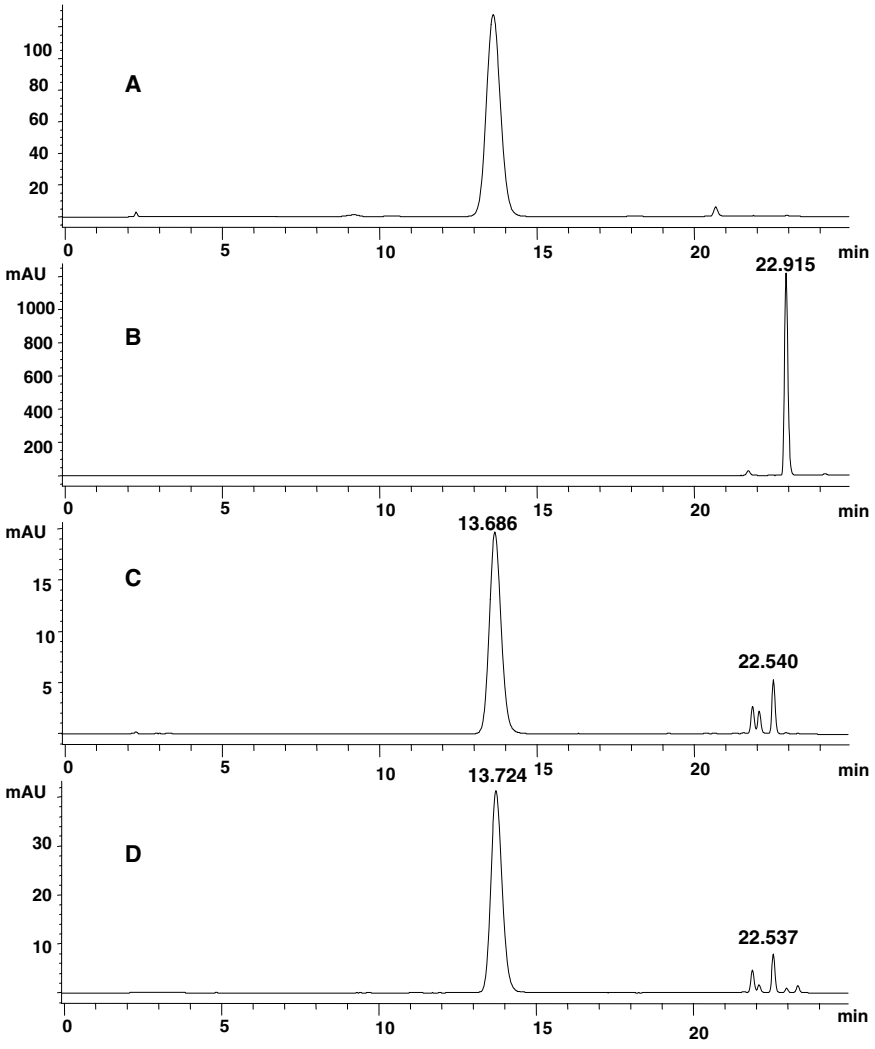

Fig. 1. HPLC profile (at 525 nm) of A = cyanidin-3-glucoside standard, B = cyanidin-3-chloride, C = anthocyanins from crude extract, D = anthocyanins from anthocyanin-enriched extract.

ante, Zappia, & Dugo, 2001; Fan-Chiang & Wrolstad, 2005; Markides, 1982; Stintzing, Stintzing, Carle, & Wrolstad, 2002).

3.3. Antioxidant capacity of blackberry extracts

The antioxidant capacity of the crude blackberry extract, as measured by ORAC was 674.2 ± 52.4 μmol trolox/g of blackberry. This compared to an ORAC value of 4885 ± 54.2 μmol trolox/g of the anthocyanin-enriched extract (Table 1). Other workers have reported ORAC values ranging from 33.3 to 78.8 μmol trolox equivalents/g fruit (Moyer, Hummer, Finn, Frei, & Wrolstad, 2002). It is of interest that, although the concentration of total anthocyanins in the anthocyanin-enriched extract was approximately 20 times greater than the crude extract, the antioxidant capacity (ORAC value) of the anthocyanin-enriched extract was increased relatively less (e.g., 7.3 times) than that measured in the original crude extract. This can be possibly be explained by the presence of other phenolic compounds, which have antioxidant capacity that results in either an additive or synergistic antioxidant activity with the anthocyanins present in the crude extract. Some phenolic compounds, such as quercetin glycosides, catechin and epicathechin, as well as ellagic acid derivatives, have also been reported in blackberry, the extent of relative composition being influenced by genotypes as well as cultivar and maturity stages (Cho, Howard, Prior, & Clark, 2005; Siriwoharn, Wrolstad, Finn, & Pereira, 2004; Siriwoharn et al., 2005). In our study, we suppose that these particular flavonols and phenolic acids did not co-elute with the blackberry anthocyanins using gel filtration; therefore, the contribution of other antioxidant phytochemicals, originating from blackberry, were minimal in the enriched sample. Notwithstanding this, it is noteworthy that a synergistic effect of anthocyanin mixture was observed in both blackberry and elder berry extracts compared to individual anthocyanins recovered from both berries (Stintzing, Stintzing, Carele, Frei, & Wrolstad, 2002).

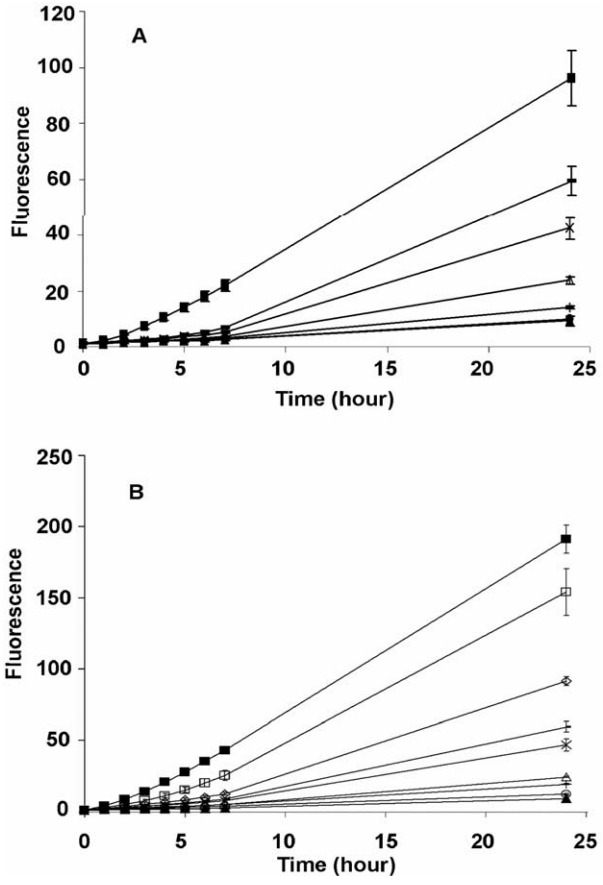

Fig. 2. The suppression of intracellular oxidation in INT-407 cells cultured with A. anthocyanin-enriched extract and B. blackberry crude extract. Figure labels corresponding to the top panel (A) are represented by anthocyanin-enriched extract concentrations of: 50 μg/ml (○), 25 μg/ml (+), 12.5 μg/ml (Δ), 6.2 μg/ml (×), 3.1 μg/ml (−), positive control or 0 μg/ml (■) and negative control (▲). The bottom panel (B) labels are represented by blackberry crude extract concentrations of: 1 mg/ml (○), 0.5 mg/ml (+), 0.25 mg/ml (Δ), 0.13 mg/ml (×), 62 μg/ml (−), 31 μg/ml (◆), 7.8 μg/ml (□), positive control or 0 μg/ml (■) and negative control (▲).Values represent means ± standard deviation ($n = 3$).

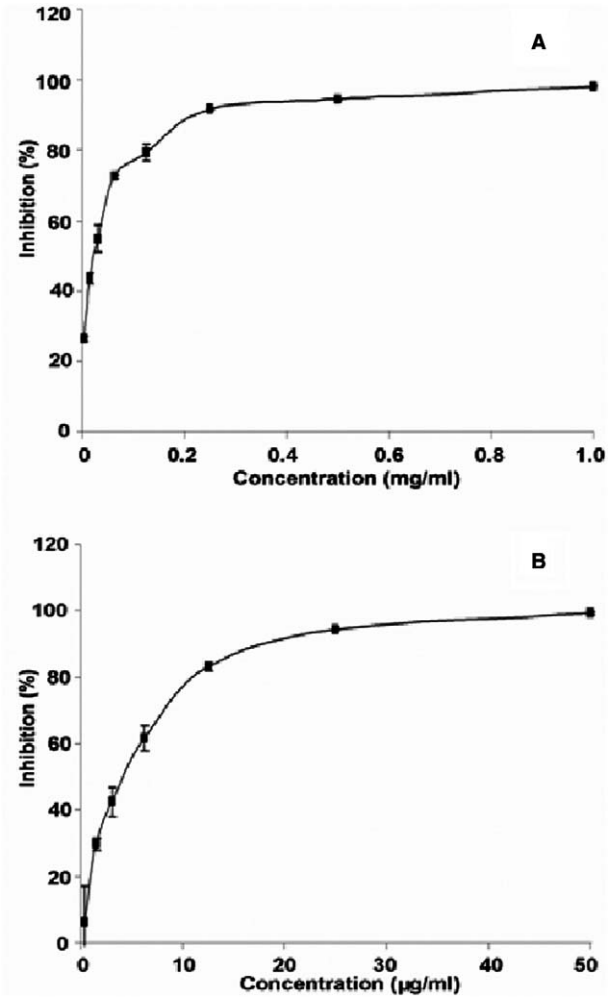

Fig. 3. Percent inhibition of intracellular oxidation by blackberry crude extract (A) and anthocyanin-enriched extract (B) in INT-407 at 24 h treatment. Values represent means ± standard deviation ($n = 3$).

3.4. Intracellular oxidation

The ability of both the crude and anthocyanin-enriched blackberry extracts to reduce the extent of AAPH-generated free radicals in INT-407 cell is shown in Fig. 2. This particular antioxidant assay is based on the cellular uptake of a non-fluorescent probe (DCFH-DA), which is subsequently hydrolyzed by intracellular esterase to form dichlorofluorescin, DCFH. The non-fluorescent substrate is oxidized by the peroxyl radicals generated from AAPH, producing a fluorescent product (dicholorofluorescein, DCF). The antioxidants within the cell act to quench the free radical and reduce fluorescence intensity, thus indicating modulation of intracellular oxidation.

Exposure of INT-407 cells to AAPH (positive control) resulted in a time-dependent increase in AAPH-induced fluorescence (Fig. 2). On the other hand, the addition of the DCFH-DA probe alone to the cells produced no change in fluorescence (Fig. 2). Both the blackberry crude extract and the anthocyanin-enriched extract consistently suppressed ($p < 0.05$) fluorescence development in a time-dependent response, over a wide concentration range of anthocyanin (Fig. 2A–B). To our knowledge, this is the first time that blackberry extracts have been shown to effectively suppress the generation of intracellular peroxyl free radicals-induced by AAPH. A concentration-dependent suppression of intracellular oxidation overd 24-h period by blackberry extracts can be attributed in part to the antioxidant activity of blackberry anthocyanins, most notably cyanidin-3-glucoside, which quenched AAPH-generated free radicals during the initial stage of intracellular oxidation. This was clearly demonstrated by the time-dependent delay in free radical generation up to 7 h, which occurred concomitantly with an increase in blackberry extracts concentrations.

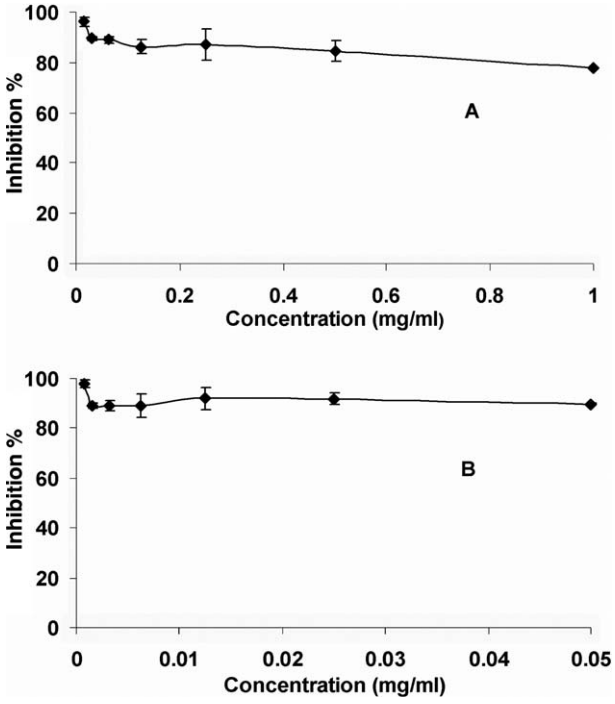

Fig. 4. Cytotoxicity of blackberry crude extract (A) and anthocyanin-enriched extract (B) on cultured intestinal cell line (INT-407).

The anthocyanin-enriched extract displayed a greater ($p < 0.05$) ability to suppress free radical generation than did crude blackberry extract (Fig. 2). For example, the concentration of crude blackberry extract (26.8 ± 1.5 μg/ml) required to inhibit 50% of the fluorescence was approximately fold greater than the concentration of the anthocyanin-enriched extract (4.1 ± 0.4 μg/ml) needed to produce the same inhibition (Fig. 3). This result is attributed to the approximately 20 times greater amount of anthocyanin that was present in the anthocyanin-enriched extract and agrees with the ORAC findings. Thus, antioxidant activity of anthocyanins, in both assays, indicates the ability of blackberry pigment to significantly scavenge peroxyl radical. The intracellular assay results also suggest that the antioxidant potential of anthocyanins against peroxyl radical is not limited to extracellular oxidation reactions alone.

3.5. Cytotoxicity of blackberry extracts

It is important to note that the suppression of intracellular oxidation by both the crude blackberry extract and the anthocyanin-enriched extract occurred at concentrations that were not cytotoxic to the INT-407 cells (Fig. 4A and B). This implies that the reduction in fluorescence in INT-407, observed in the presence of blackberry extracts, was due to the antioxidant property, and not to a decrease in cell viability.

4. Conclusion

The use of Bio-Gel P2 allowed the recovery of a principal anthocyanin, i.e., cyanidin-3-glucoside, resulting in an enriched blackberry anthocyanin extract that was concentrated more than 20 times compared to the crude extract. The anthocyanin cyanidin-3-glucoside showed marked antioxidant efficacy in both cell-free and cell culture-based antioxidant activity models.

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