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The protective effect of blackberry anthocyanins against free radical-induced oxidative stress and cytotoxicity

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ABSTRACT

Anthocyanin represents a group of naturally occurring pigments present in many fruits and some vegetables. In addition to contributing to the hues of blue, purple, or deep red common to several plants, these pigments also have potent antioxidant activity. Blackberry in particular is a rich source of antioxidants, largely attributed to the high anthocyanin content associated primarily with the cyanidin-3-glucosides. Despite the *in vitro* chemical assays that have shown antioxidant activity, very few studies exist which demonstrate the biological implication of the potent antioxidant activity of blackberry anthocyanins, against free radical-induced cellular damage. The purpose of this study was to examine the protective effect of blackberry anthocyanins against free radical-induced intracellular oxidation and cytotoxicity in multiple cell lines, cultured *in vitro*. Blackberry crude and enriched extract were evaluated against AAPH initiated-intracellular oxidation and AAPH induced-cytotoxicity in one prostate (LNCaP) and two breast cancer cell lines (MCF-7 and MDA-MB-453). Blackberry anthocyanins effectively suppressed AAPH-initiated intracellular oxidation as monitored using DCFH-DA probe. The extracts also decreased AAPH-induced cytotoxicity as assessed using MTT and CellTiter-Glo assay. This study thus demonstrates the effectiveness of blackberry anthocyanins as a potent source of antioxidants that have the affinity to mitigate free radical induced-oxidative damage in different cell systems.

KEYWORDS: blackberry, anthocyanins, oxidative stress, cytotoxicity, antioxidant

INTRODUCTION

Reactive oxygen species (ROS) are highly unstable molecules that are continuously generated as byproducts of ordinary metabolism [1, 2]. Excessive generation of ROS will result in oxidative damage to biomolecules that compose cellular components, and thus initiate a process commonly referred to as oxidative stress [3, 4]. The oxidative damage to cellular proteins, membrane lipids or nucleic acids will eventually lead to impaired metabolism and result in adverse biochemical and physiological changes that play a key role in the progression of more than 50 diseases [5].

Dietary antioxidants, especially those present in edible plant sources, could be the link between soft fruit consumption patterns and protection against free radical associated diseases. Anthocyanins, the blue-red colored pigments found in soft fruit berries have been reported to exhibitpotent antioxidant activity [6]. Blackberry (*Rubus fruticosus*) in particular, is one of the richest sources of anthocyanins and has been considered to have excellent antioxidant capacity which can be attributed to the high anthocyanin content [7, 8]. Blackberry is also of particular interest, since its simplicity as an anthocyanin

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source lies with the major anthocyanin being identified as cyanidin-3-glucoside [8, 9]. Despite the fact that the antioxidant capacity of blackberry has been well demonstrated in various *in vitro* chemical systems, there is a paucity of information concerning modeling antioxidant capacity in cell culture systems which are relevant to *in vivo* biological systems.

This study aims to report on the *in vitro* chemical assessment of the antioxidant capacity of blackberry anthocyanins and furthermore demonstrate the effect of blackberry crude extract and a derived anthocyanin-enriched fraction, on free radical-initiated intracellular oxidation and inhibition of free radical-induced cytotoxicity in multiple cell lines.

MATERIALS AND METHODS

Materials

Blackberries were supplied from Sandhu Farm, Abbotsford, B.C., Canada. Biogel P2 was obtained from Bio-Rad Laboratories (Richmond, Ca). AAPH (2, 2'-azobis (2-amidinopropane) dihydrochloride) was obtained from Wako Chemicals USA (Richmond, VA). MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was from Sigma Co. (St. Louis, ML). Sodium dodecyl sulfate (SDS) was purchased from Fisher Scientific (Springfield, NJ). CellTiter-Glo® Luminescent Cell Viability Assay kit was obtained from Promega Corporation (Madison, WI). DCFH-DA (2',7'-dichlorofluorescin diacetate) was from Sigma-Aldrich Canada Ltd. (Oakville, ON). Minimum Essential Medium Eagle, RPMI and Leibovitz-15 were purchased from ATCC (Manassas, VA). Fetal bovine serum, penicillin and streptomycin were from Gibco (Grand Island, NY).

Extraction and enrichment of blackberry anthocyanins

Anthocyanins were extracted from one hundred grams of frozen blackberry that was blended with 100 mL of 80% ethanol using a Waring blender, for five minutes. The slurry was extracted overnight and this was followed by filtration through a Buchner funnel using Whatman number 1 paper. Extraction of anthocyanins was taken to completion by re-extracting the filter cake with 80% ethanol until a colorless filtrate was obtained. The different filtrates were then pooled together and the ethanol in the pooled filtrate was removed under reduced pressure at 35°C. The residue was then lyophilized to powder and kept at 4°C until ready for analysis.

A concentrate of pure blackberry anthocyanins was also obtained using gel filtration chromatography according to methods previously described by Elisia *et al.* [10], with minor modifications. Briefly, blackberry crude extract was reconstituted with aqueous acetic acid (pH 2.5) and this was then loaded (300 mg/mL) onto a Biogel P-2 gel filtration column (2.5 cm x 14.5 cm). Acetic acid (pH 2.5) was used to elute the anthocyanin fractions (flow rate = 2 mL/min). The reddish-blue colored fraction was collected and pooled together over several loadings. The resulting anthocyanin-rich fraction was freeze-dried to a powder and stored at 4°C until ready for analysis.

Total anthocyanin content

The total anthocyanin content of both blackberry crude and enriched extracts were evaluated using the pH differential method as previously described by Wrolstad and Giusti [11]. The results were expressed as milligrams of cyanidin-3-glucoside equivalent per 100 g of frozen weight.

$ORAC_{FL}$ antioxidant activity

Antioxidant capacities of blackberry crude and enriched extracts were assessed using the Oxygen Radical Absorption Capacity assay ($ORAC_{FL}$), as described by Tijerina-Saenz *et al.* [12]. Blackberry samples and a standard antioxidant (Trolox) were dissolved in phosphate buffer (50 mM, pH 7.0), followed by 60 nM fluorescein in a 96-well plate. Plates were incubated at 37ºC for 15 min. AAPH, a peroxyl radical initiator, 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). The data transformation and interpretation was performed according to [13]. The $ORAC_{FL}$ value was expressed as µmol Trolox/g sample [12]. An $ORAC_{FL}$ antioxidant capacity value for the principal anthocyanins found in blackberry, namely cyanidin-3-glucoside (C3G), cyanidin-3-rutinoside (C3R) and malvidin-3-glucoside (M3G) was also determined.

Cell cultures

The human prostate cancer cell line (LNCaP), breast cancer cell lines (hormone sensitive MCF-7 and hormone insensitive MDA-MB-453) were obtained from ATCC (Manassas, VA) and were maintained in RPMI-1640, Minimum Essential Medium Eagle (MEME) and Leibovitz's L-15, respectively. All media were supplemented with 10% fetal bovine serum, penicillin (100 U) and streptomycin (100 μg/mL). Cells were incubated at 37°C in a 5% CO_2 humidified incubator except for MDA-MB-453 cells which was maintained at 37°C in 100% air incubator. Upon reaching confluence, cells were sub-cultured according to ATCC recommendation. Cell number was assessed with trypan blue dye exclusion using a haemocytometer.

Intracellular antioxidant assay

The effect of blackberry extracts on AAPH-initiated intracellular oxidation was evaluated using DCFH-DA intracellular probe. Individual cell lines were initially seeded at a concentration of 2.5 x 10^4 cells/well in a 96 well plate and left overnight in a 37ºC, 5% CO_2 incubator for attachment and attainment of growth. Final concentrations of blackberry crude extract (7.8 μg/mL - 1 mg/mL), or the anthocyanin-enriched extract (0.02 μg/mL - 50 μg/mL) were added to culture media used specifically for different cell lines. The blackberry extracts were co-incubated with the 5 μM DCFH-DA probe for three hours, 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 zero minutes immediately upon addition of AAPH and at 1 to 7 hours and 24 hours, respectively, after the initial addition of AAPH. 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. A positive control consisted of cells cultured with the DCFH-DA probe and the peroxyl radical initiator (AAPH). All results were expressed according to the following formula, where:

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

where
Fluorescence t_i is fluorescence reading taken at time 1 to 7 hours and 24 hours, Fluorescence t_0 represents the initial fluorescence reading taken upon AAPH addition at 0 minute.

Protection against free radical-induced cytotoxicity

Experiments designed to evaluate protection against free radical-induced cytotoxicity involved exposure of different cell lines to both the blackberry crude and anthocyanin-enriched extracts at final concentrations of 31 μg/mL - 1 mg/mL and 0.8 - 25 μg/mL respectively, for three hours. Different concentrations of AAPH (10 or 15 mM) were added to cells to induce cytotoxicity and the mixture was further incubated for 24 hours at 37ºC. Cell viability was measured using both MTT and CellTiter-Glo assay. The negative control consisted of cells cultured in media only, whereas a positive control consisted of cells treated with the AAPH without the blackberry extract. Cell viability of treated cells was expressed as a percent of viable cells present in the negative control.

For the MTT redox assay, the anthocyanin containing media was replaced with fresh media followed by the addition of 0.5 mg/mL MTT reagent. Microtiter plates were incubated for four hours at 37ºC in the dark, followed by the addition of SDS (10% in 0.01 HCl) to solubilize the formazan crystal formed. Absorbance readings were read at 570 nm using a microplate reader (Multiskan Spectrum, ThermoLabsystem, Chantilly, VA).

The CellTiter-Glo assay was performed according to the manufacturer instructions, with a slight modification. Briefly, the blackberry containing medium was replaced with fresh medium before the addition of CellTiter-Glo reagent to the cells. The plates were shaken for two minutes at 1200 rpm and an equal volume of CellTiter-Reagent was added. The plates were left at room temperature for 15 minutes before the luminescence was read using a luminometer (Fluoroskan Ascent FL, Labsystem, Helsinki, Finland).

Statistics

All treatments were performed in triplicate. Results were expressed as mean ± standard deviation. A T-test was used to determine significant ($P < 0.05$) differences between control and treatment means for both MTT and CellTiter-Glo assays.

RESULTS

Total anthocyanin content

Blackberry crude extract contained 17.1 ± 0.9 mg of total anthocyanins per gram of the freeze-dried powder. Gel filtration of the blackberry crude extract produced an anthocyanin enriched extract which total anthocyanin content was 371.1 ± 15.4 mg/g of freeze dried powder.

Antioxidant activity

The $ORAC_{FL}$ value for crude blackberry extract was 673.8 ± 52.4 µmole Trolox/g of freeze dried blackberry. The $ORAC_{FL}$ value for the anthocyanin-enriched extract was 4884.7 ± 52.4 µmole Trolox/g. The antioxidant capacity of pure cyanidin-3-glucoside (C3G), cyanidin-3-rutinoside (C3R), malvidin-3-glucoside (M3G) standards is presented in Table 1.

Intracellular oxidation

The effect of both the crude and anthocyanin-enriched extracts to reduce the extent of AAPH-generated free radical in three different cell lines is shown in Figures 1-3. Exposure of different cell lines to AAPH alone resulted in a time-dependent increase in free radical-induced fluorescence. The presence of the DCFH-DA probe alone produced minimum change in fluorescence. Both the blackberry crude extract and the anthocyanin-enriched extract consistently suppressed ($P < 0.05$) fluorescence development in a time-dependent concentration response for all cell lines tested, over a wide concentration range of anthocyanin (Figures 1-3).

Table 1. $ORAC_{FL}$ values for pure anthocyanin standards known to be present in blackberry.

	$ORAC_{FL}$ (µmol TE/ mg Std)[1]
Cyanidin 3-glucoside	6.4±0.4[a]
Cyanidin 3-rutinoside	6.1±0.36[a]
Malvidin 3-glucoside	4.5±0.60[b]

[1]Different superscript letters indicate significant difference ($P < 0.05$) in treatment means between anthocyanin standards. Values are mean ± standard deviation (n =6).

The IC_{50} values obtained from the blackberry crude extract from different cell lines measured at 24 hour treatment ranged from 25.8 µg/mL to 56.1 µg/mL (Figure 4, Table 2). This corresponded to an IC_{50} range of 2.6 µg/mL to 6.1 µg/mL for the blackberry anthocyanin-enriched extract (Figure 4, Table 2).

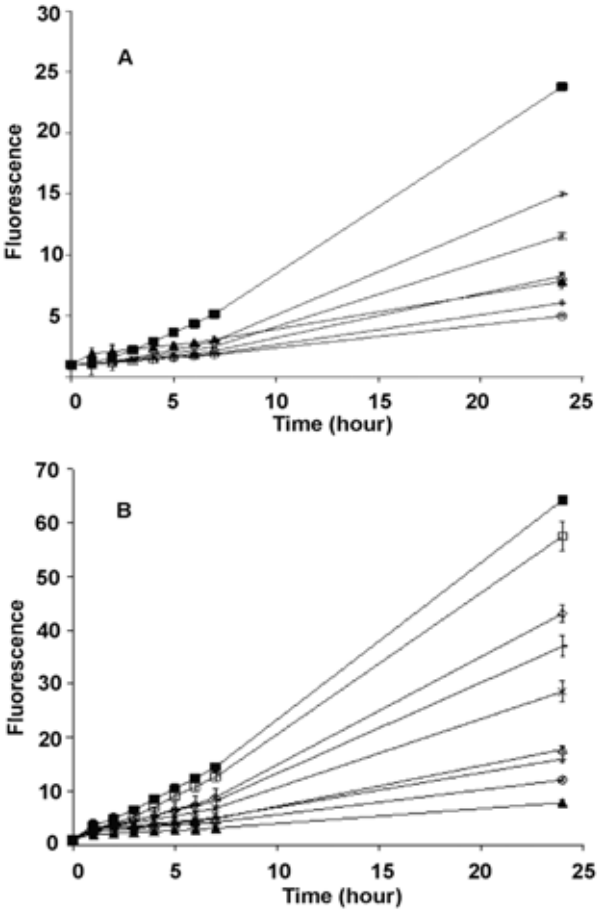

Figure 1. The suppression of intracellular oxidation in LNCaP cells cultured with A. anthocyanin-enriched extract and B. blackberry crude extract. Figure labels corresponding to the top panel (A) are represented by anthocyanin-enriched extract concentrations of: 50 µg/ml (○), 25 µg/ml (+), 12.5 µg/ml (△), 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 (△), 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 mean ± standard deviation (n = 3).

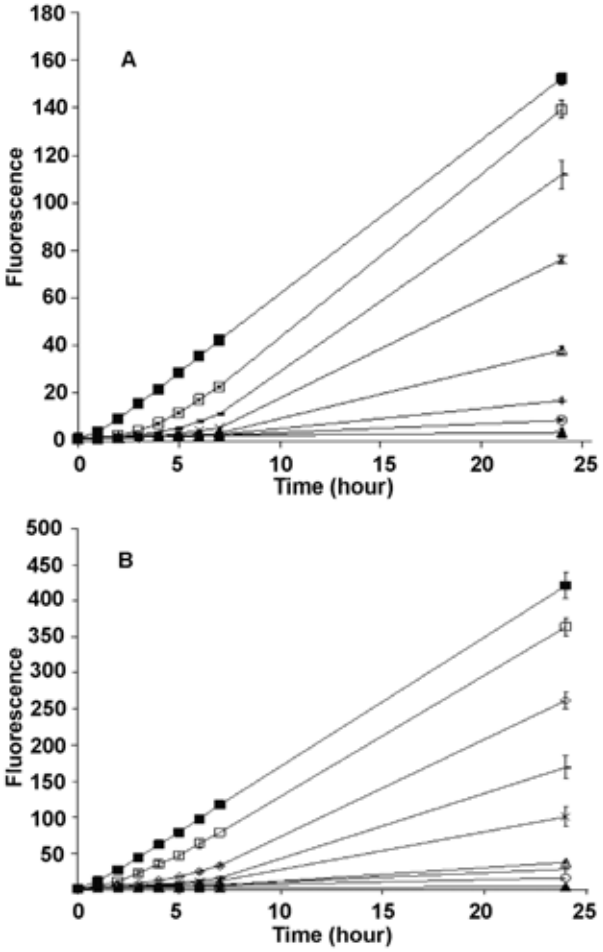

Figure 2. The suppression of intracellular oxidation in MCF-7 cells cultured with A. anthocyanin-enriched extract and B. blackberry crude extract. Figure labels corresponding to the top panel (A) are represented by anthocyanin-enriched extract concentrations of: 50 µg/ml (○), 25 µg/ml (+), 12.5 µg/ml (△), 6.2 µg/ml (×), 3.1 µg/ml (—), 1.5 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 (△), 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 mean ± standard deviation (n = 3).

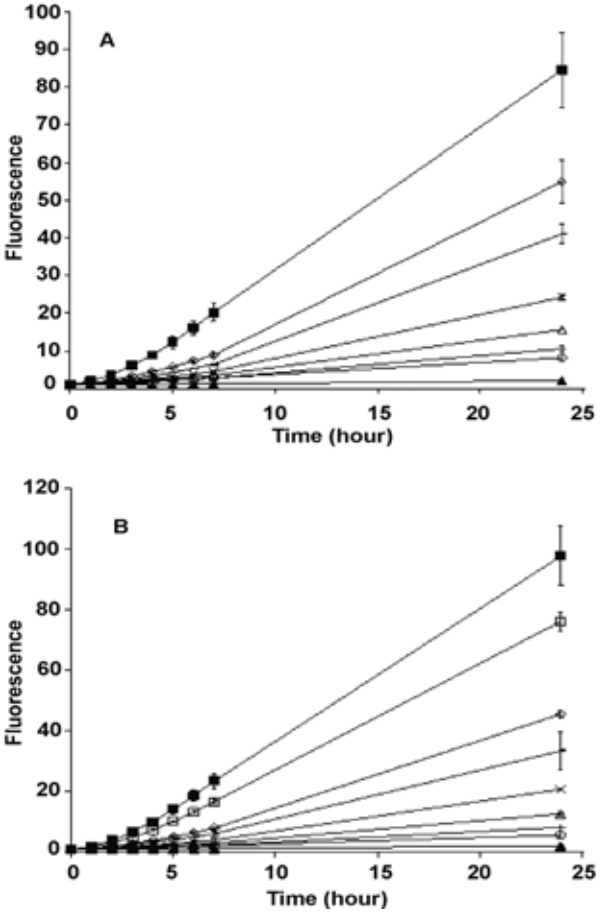

Figure 3. The suppression of intracellular oxidation in MDA-MB-453 cells cultured with A. anthocyanin-enriched extract and B. blackberry crude extract. Figure labels corresponding to the top panel (A) are represented by anthocyanin-enriched extract concentrations of: 50 µg/ml (○), 25 µg/ml (+), 12.5 µg/ml (△), 6.2 µg/ml (×), 3.1 µg/ml (—), 1.5 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 (△), 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 mean ± standard deviation (n = 3).

Protective effect against AAPH-induced cytotoxicity

The protective effect of both blackberry crude extract and anthocyanin-enriched extract against free radical-induced cytotoxicity is summarized in Tables 3-4 and Figure 5. AAPH effectively induced ($P < 0.05$) cytotoxicity in all cell lines tested in a concentration dependent manner as determined using both MTT and CellTiter-Glo assays.

Pre-incubation of blackberry crude extract decreased AAPH-induced cytotoxicity to various extents in all cell lines tested (Tables 3-4). Using the MTT assay to assess cell viability, maximum protection ($P < 0.05$) to LNCaP (e.g. 43.2%) was found to

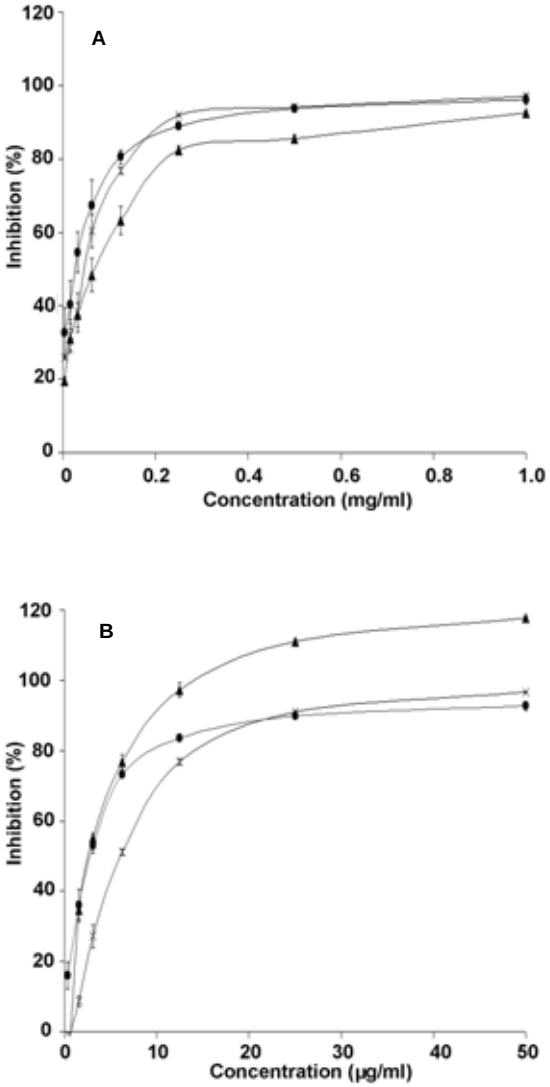

Figure 4. Percent inhibition of intracellular oxidation by blackberry crude extract (A) and anthocyanin-enriched extract (B) in all cell lines at 24 hour treatment. Figure labels corresponding to both panels are represented by LNCaP (▲), MCF7 (×), and MDA-MB-453 (●). Values represent mean ± standard deviation (n = 3).

occur at the minimum blackberry crude extract concentration tested (30 µg/mL) against 10 mM AAPH, with no further protection observed at higher concentrations. Exposure of MCF-7 cells to 10 mM AAPH on the other hand, produced a relatively lower apparent cytotoxicity (e.g. 20%); the extent of which was not altered by pre-incubation with blackberry crude extract at the concentration range tested (Table 3). Nevertheless, a significant protection ($P < 0.05$) of MCF-7 cells against 15 mM AAPH was afforded by the blackberry crude extract that began at the minimum concentration (30 µg/mL) tested and reached a maximum (e.g. 15.9 %) protection at the highest concentration tested (0.13 mg/mL) (Table 3). A similar protective effect trend was observed for MDA-MB-453 cells that were challenged with either 10 or 15 mM AAPH. The loss of cell viability associated with AAPH challenge was significantly ($P < 0.05$) ameliorated by blackberry crude extract at the minimum concentration used (30 µg/mL) and was maximal at the highest concentration examined (0.13 mg/mL; Table 3).

Despite the significant protective effect observed for blackberry crude extract when assessed using MTT-redox assay, evaluation of cell viability using the CellTiter-Glo assay, demonstrated that the blackberry crude extract at the concentrations tested was ineffective at reducing AAPH-induced cytotoxicity in all cell lines (Table 4).

The anthocyanin-enriched extract, at a concentration that was 20 times lower than the blackberry crude extract, was found to have a variable but significant ($P < 0.05$) protective effect against cytotoxicity induced by 15 mM AAPH. For LNCaP cells, the minimal concentration (0.8 µg/mL) of the anthocyanin rich extract gave a significant ($P < 0.05$) protective effect (e.g. 9.4%) against 15 mM AAPH,

Table 2. Inhibition (IC_{50}) of AAPH-induced intracellular oxidation by blackberry extracts in multiple cell lines[1].

	IC_{50} (µg/ml)		
	LNCaP	**MCF-7**	**MDA-MB-453**
Anthocyanin enriched extract	2.6 ± 0.1^{a}	6.1 ± 0.3^{b}	2.8 ± 0.2^{a}
Blackberry crude extract	56.1 ± 4.7^{b}	49.7 ± 8.5^{b}	25.8 ± 1.0^{a}

[1]Different superscript letters indicate significant difference ($P < 0.05$) in treatment means between cell lines of the same treatment. Values are mean ± standard deviation (n = 3).

Table 3. Protective effect of blackberry crude extract against AAPH-induced cytotoxicity for various cell lines as evaluated by MTT assay[1].

Cell lines	Concentrations of crude extract (mg/mL)				
	AAPH	**0**	**0.03**	**0.06**	**0.13**
LNCaP	**0 mM**	100	100.36	100.57	98.38
	10 mM	45.77	88.99*	76.54*	74.16*
	15 mM	12.45	17.68*	19.91*	19.51*
MCF-7	**0 mM**	100	101.28	96.22*	92.44*
	10 mM	80.02	75.49	80.79	81.56
	15 mM	21.60	26.42*	35.09*	37.50*
MDA-MB-453	**0 mM**	100	98.42	108.38	114.94*
	10 mM	73.28	80.49*	84.10*	92.32*
	15 mM	42.18	50.76*	60.59*	60.84*

Note: Cell viability was assessed upon 3 h pre-incubation of various cells to different concentrations of anthocyanin containing extract followed by 24 h exposure to AAPH peroxyl radical generator.
[1]Data are expressed as mean values of percent cell viability, n = 3.
*indicates significant difference ($P < 0.05$) between percent viability value of cells pre-treated with anthocyanin containing extract and cells treated with AAPH only (i.e. 0 mg/ml of the anthocyanin containing extract).

Table 4. Protective effect of blackberry crude extract against AAPH-induced cytotoxicity for various cell lines as evaluated by CellTiter-Glo[1].

Cell lines	Concentrations of crude extract (mg/mL)				
	AAPH	**0**	**0.03**	**0.06**	**0.13**
LNCaP	**0 mM**	100	88.63*	89.90*	88.77*
	10 mM	74.46	72.72	63.02*	57.14*
	15 mM	39.75	41.57	38.51	35.26
MCF-7	**0 mM**	100	99.21	98.60	99.49
	10 mM	89.32	89.24	89.71	85.03
	15 mM	81.71	79.78	81.72	79.68
MDA-MB-453	**0 mM**	100	101.21	101.04	96.85
	10 mM	54.40	61.30	61.02	60.23
	15 mM	21.20	35.48	32.97	32.67

Note: Cell viability was assessed upon 3 h pre-incubation of various cells to different concentrations of anthocyanin containing extract followed by 24 h exposure to AAPH peroxyl radical generator.
[1]Data are expressed as mean values of percent cell viability, n = 3.
*indicates significant difference ($P < 0.05$) between percent viability value of cells pre-treated with anthocyanin containing extract and cells treated with AAPH only (i.e. 0 mg/ml of the anthocyanin containing extract).

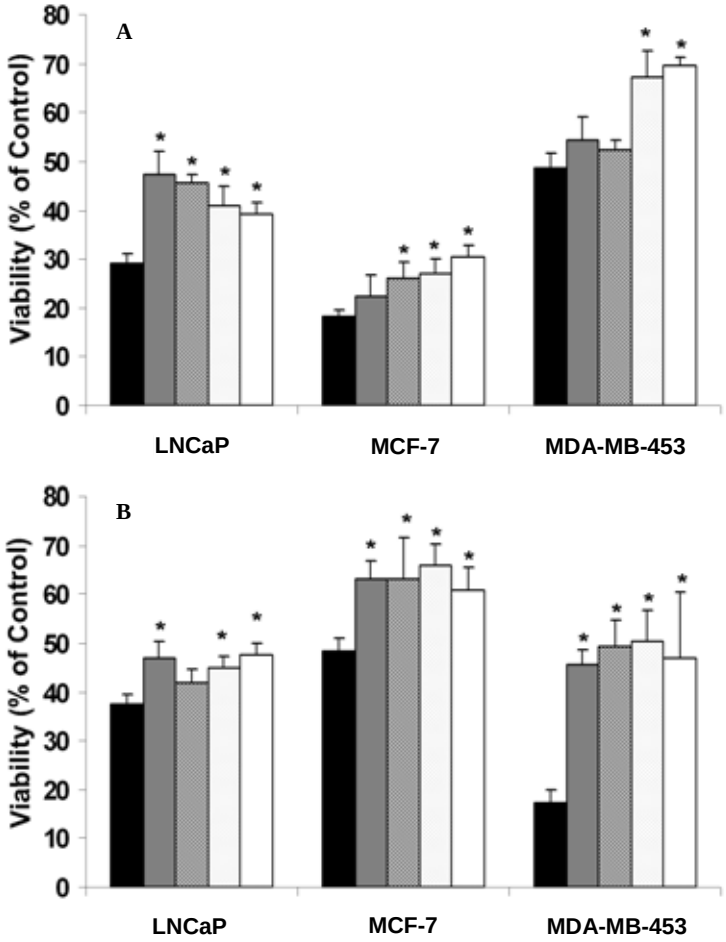

Figure 5. Protective effect of anthocyanin-enriched extract against AAPH-induced cytotoxicity for various cell lines as evaluated by CellTiter-Glo (A) and MTT (B) assay. Different bars indicate viability of cells pre-incubated with 0 µg/ml (■), 0.8 µg/ml (▒), 3.1 µg/mlv (▨), 12.5 µg/ml (⬚) and 25 µg/ml (□) for three hours prior to exposure to 15 mM AAPH.
*indicates significant difference ($P < 0.05$) between percent viability value of cells pre-treated with anthocyanin containing extract and cells treated with AAPH only (i.e. 0 mg/ml of the enriched extract).

as determined by the CellTiter-Glo assay (Figure 5) with no further additional protective effect at higher concentrations of anthocyanin enriched extract. The maximum extent by which the anthocyanin-enriched extract protected LNCaP cells was shown to be greater when evaluated using MTT assay (18%).

For MCF-7 cells, pre-incubation with anthocyanin-enriched extract over a range of 1.56 µg/mL - 25 µg/mL produced a significant ($P < 0.05$) concentration-dependent recovery in cell viability (Figure 5) as evaluated with the MTT assay. The anthocyanin enriched extract, however began to elicit a protective effect at minimum concentration of 0.8 µg/mL when assessed by the CellTiter-Glo assay.

For MDA-MB-453 cells, the minimum concentration of anthocyanin-enriched extract found to protect against 15 mM AAPH (28.3%) was 0.8 µg/mL (28.3%; $P < 0.05$), when assessed using the CellTiter-Glo assay ($P < 0.05$; Figure 5). Pre-incubation of breast cells with a higher concentration of the extract also produced an increased degree of protection against AAPH-induced cytotoxicity, with a maximum protective effect obtained at 12.5 µg/mL (33%; $P < 0.05$). On the other hand, maximum protection, assessed by the MTT assay, was obtained when MDA-MB-453 cells were pre-incubated with 25 µg/mL of the anthocyanin-enriched extract (21%; $P < 0.05$).

DISCUSSION

The blackberry crude and anthocyanin-enriched extracts both suppressed generation of chemical and intracellular peroxyl free radicals-induced by AAPH in both the *in vitro* $ORAC_{FL}$ assay and, in culture with the three different cell lines used in this study. The affinity of blackberry anthocyanins to suppress intracellular free radicals induced by AAPH was tested using DCFHDA probe to monitor oxidative status. In this particular assay, a non-fluorescent probe (DCFH-DA) is uptaken into cell and subsequently hydrolysed by the intracellular enzyme esterase to form another non-fluorescent substrate (dichlorofluorescin, DCFH). Peroxyl radicals generated from AAPH oxidize the DCFH to a fluorescent product (dichlorofluorescein, DCF). The presence of antioxidants within the cell quenches the free radical, and thus in turn reduces the fluorescence intensity thereby indicating modulation of intracellular oxidation.

A concentration dependent suppression of intracellular oxidation over a 24 hour period by crude blackberry extracts was attributed in part to the antioxidant activity of blackberry anthocyanins which quench AAPH-generated free radicals.

While both blackberry crude and anthocyanin-enriched extracts effectively suppressed intracellular oxidation, there was greater potency for the anthocyanin-enriched extract to inhibit free radical generation. This result can be attributed to the greater antioxidant activity that would have resulted due to the increased concentration of anthocyanins present in the enriched extract; a final concentration that reached approximately 20 times more anthocyanin than that present in the blackberry crude extract. We have demonstrated herein that the antioxidant activity of anthocyanin as observed in chemical $ORAC_{FL}$-based assay, translate well to a protection against free radical-induced oxidative stress in different cell systems.

To further provide evidence that the anthocyanins were indeed the ones contributing to the antioxidant activity of the anthocyanin enriched extract, the antioxidant activity of selected pure anthocyanin standards was determined. Ninety percent of the anthocyanin in the enriched extract was identified to be cyanidin-3-glucoside, which exhibited ORAC value of 6.4 µmole TE/µM standard or 14.2 µmole/mg of C3G [10]. Based on the finding that the anthocyanin-enriched extract contains 371.1 mg of anthocyanin/g of freeze dried extract, the calculated ORAC value of the enriched extract is thus 4742 µmole TE/g of anthocyanin-enriched extract, which is comparable to the experimental ORAC value obtained in this study of 4884.7 µmole TE/g. Other anthocyanins present in blackberry include C3R and M3G which also yield $ORAC_{Fl}$ values; however, the contribution to the total antioxidant capacity from these particular anthocyanins is relatively minor due to the low concentrations in blackberry [14]. We therefore confirm that the antioxidant activity of the anthocyanins-enriched extract is largely contributed by cyanidin-3-glucoside.

The difference in ORAC value can be attributed to the subtle structure-activity relationships between specific anthocyanins and $ORAC_{FL}$ measurements [15]. Cyanidin has two hydroxyl groups at positions 3’ and 4’ on the B ring, and had a higher ORAC value compared to malvidin, which contains an *O*-methyl group on 4’ position on the B-ring. The sugar moiety on the other hand has no effect on the ORAC antioxidant capacity of same aglycone, as evidenced for cyanidin 3-glucoside and cyanidin-3-rutinoside. These results are supported by previous findings of Wang *et al.* [16] who also used the ORAC assay to make comparisons on relative antioxidant potency of a number of different anthocyanins.

The extent to which a concentration of blackberry extract inhibits free radical generation was however shown to be cell-line specific. The anthocyanin-enriched extract exhibited a suppression of peroxyl radicals in cells that followed the order of LNCaP > MDA-MB-453 > MCF-7. This response was different to the blackberry crude extract which ranked MDA-MB-453 > MCF-7 > LNCaP for inhibiting intracellular free radical generation.

This relative difference between anthocyanin-induced protections against intracellular oxidation reflects the degree to which the blackberry crude or the anthocyanin-enriched extracts were taken up by individual cells to neutralize oxidative stress. For example, the fact that blackberry anthocyanins suppressed intracellular oxidation indicates that blackberry anthocyanin was incorporated into

cells to an extent that was required to reduce the presence of AAPH-initiated free radicals. This interpretation of our finding is supported by Youdim *et al.* [17], who reported incorporation of elderberry anthocyanins to cultured endothelial cells, and related this effect to an increased resistance against H_2O_2 and AAPH-induced oxidative stress [17]. A similar protective effect of anthocyanins against H_2O_2 induced-oxidative stress has been reported in our laboratory using anthocyanin containing Saskatoon berry [18] and pigmented black rice in cultured RAW264.7 cells [19].

The antioxidant activity of blackberry anthocyanins was further evaluated for protection against free radical-induced cell death. While lower concentration (1 mM) of AAPH was used to induce oxidative stress in cells, exposure of cells to higher concentration of AAPH (>10 mM) produced concentration-dependent decreases in cell viability, as measured using both MTT and CellTiter-Glo assays. Upon exposure to heat, AAPH dissociates and interacts with oxygen to form peroxyl radicals, which in turn initiates lipid peroxidation through a cascade of reactions [20, 21]. Lipid peroxidation is a major factor for inducing oxidative stress that results in cellular damage [22, 23]. Products of membrane lipid peroxidation include damaged membrane phospholipids, proteins and cellular DNA [22, 24]. Accumulation of oxidative damages ultimately leads to cellular dysfunction and cell death [25].

The blackberry crude and anthocyanin-enriched extracts used in the present study effectively reduced AAPH-induced cytotoxicity. The degree of protection obtained, however, was found to be dependent on several factors, such as the concentration of AAPH used to induce cytotoxicity (e.g. the exposure level to the oxidizing agent), the concentration of blackberry anthocyanin (e.g. the level of natural antioxidant agent) used to pre-incubate different cells, the cell type (susceptibility to oxidative stress due to level and activity of endogenous antioxidant defense mechanisms), and the cell viability assay used to measure cytotoxicity (the predictor endpoint measure for cell death).

The level of protection afforded by blackberry crude extract to all cells against low concentration of AAPH (5 mM) was relatively small and quite variable (data not shown). However, by increasing the AAPH exposure of cells to 10 and 15 mM, we were able to produce a markedly higher decline in cell viability which enabled us to determine the relative degree of protection afforded by the crude blackberry extract at different concentrations. Improved sophistication of the intracellular assay enable us to show that supplementation of cells with blackberry anthocyanins undoubtedly provided a beneficial effect against AAPH-induced oxidative stress, particularly when exposure to free radicals was generated at a relatively high level.

It should be noted that the protective effect of blackberry crude extracts against peroxyl radical-induced cytotoxicity was evident when evaluated using the MTT assay, but was shown ineffective in the CellTiter-Glo assay. This discrepancy can be explained in part by the potential of the MTT assay to overestimate cell viability, which was attributed to interference of antioxidant compounds with redox character by which the MTT assay is based on [26, 27]. The use of the CellTiter-Glo assay to estimate cell viability is based on changes in cellular ATP levels, therefore serving as a confirmation to the finding based on MTT assay.

The relatively greater efficacy of the anthocyanin-enriched extract to protect against AAPH-induced cytotoxicity compared to the blackberry crude extract was observed in all cell lines tested. Anthocyanin-enriched extract of blackberry at a concentration that was 20 times higher than blackberry crude extract was effective at inhibiting AAPH-mediated cytotoxicity as demonstrated using both the MTT and CellTiter-Glo assays, respectively. Our result extends the finding that that the antioxidant activity of the anthocyanin concentrate recovered from blackberry was not only effective in mitigating free radical-induced oxidative stress, but was also sufficiently potent to prevent oxidative damage leading to cell death.

CONCLUSION

Anthocyanins present in blackberry fruit were demonstrated to have potent $ORAC_{FL}$ activity that was related to an efficacy to suppress AAPH-initiated intracellular oxidation, and moreover lead to protection in various cell lines against free

radical-induced cytotoxicity. The protective effect of blackberry anthocyanins against peroxyl radical-induced cytotoxicity was dependent on the cell line. The anthocyanin-enriched extract was also shown to be consistently more effective than the crude extract at suppressing the intracellular oxidation as well as peroxyl radical-induced cytotoxicity; a response that was simply attributed to the greater concentration of anthocyanins in the blackberry extract.

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