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Neuroprotective effect of blackberry (*Rubus* sp.) polyphenols is potentiated after simulated gastrointestinal digestion

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ABSTRACT

Blackberry ingestion has been demonstrated to attenuate brain degenerative processes in rodents with the benefits ascribed to the (poly)phenolic components. The aim of this work was to assess the efficacy of blackberry polyphenolics in a neurodegeneration cell model before and after simulated gastrointestinal digestion.

Digested blackberry metabolites protected neuroblastoma cells from H_2O_2-induced death at low, non-toxic levels that approach physiologically-relevant serum concentrations. However, the original extracts were not protective even at fivefold higher concentrations. This potentiation may reflect alterations in the polyphenolic composition caused by the digestion procedure, as detected by liquid-chromatography-mass spectrometric analysis. This protection was not caused by modulation of the intracellular antioxidant capacity or through alteration of glutathione levels, although the original extract influenced both of these parameters. This work reinforces the importance of evaluating digested metabolites in disease cell models and highlights the possible involvement of other mechanisms beyond antioxidant systems.

1. Introduction

Epidemiological studies have shown that dietary habits can influence the incidence of Alzheimer's and Parkinson's diseases (Dai, Borenstein, Wu, Jackson, & Larson, 2006; De Rijk et al., 1997; Engelhart et al., 2002; Gao et al., 2007). Foods and food ingredients, in particular, components chemically classified as antioxidants, have been reported to exert a beneficial effect in neurodegeneration (Mandel et al., 2008; Mandel, Amit, Weinreb, Reznichenko, & Youdim, 2008; Ramassamy, 2006; Vauzour, Vafeiadou, Rodriguez-Mateos, Rendeiro, & Spencer, 2008).

In the developed world, the population lifespan is increasing, with a concomitant increased incidence of many age-related diseases, such as cancer, cardiovascular troubles and neurodegeneration (Lau, Shukitt-Hale, & Joseph, 2006). The impact of this, at the financial and social level, is immense, with the health care costs in 2008 for Alzheimer's disease and other forms of dementia recently estimated at €160 billion for the EU27 and €177 billion for the whole of Europe (Wimo et al., 2011). Clearly it is paramount that preventative amelioration and/or, ideally, inhibition strategies are developed to retard or reverse neuronal and behavioural deficits that occur in ageing (Lau et al., 2006). Indeed, these foci are areas of intense research effort but the delivery of (pharma) products (Dumont & Beal, 2011; Williams, Sorribas, & Howes, 2011) and therapeutic strategies (Maiese, Chong, Hou, & Shang, 2009) have been limited. Furthermore the mechanisms involved in the behavioural deficits during ageing remain to be discerned (Thibault, Gant, & Landfield, 2007; Wiecki & Frank, 2011). Substantial evidence supports the hypothesis that oxidative stress plays a major role in neurodegenerative disease pathogenesis (Joseph, Cole, Head, & Ingram, 2009; Joseph, Denisova, Bielinski, Fisher, & Shukitt-Hale, 2000; Shadrina, Slominsky, & Limborska, 2010). Oxidative stress is generally caused by the excessive accumulation of reactive oxygen species (ROS) in cells and has been implicated in the development of many neurodegenerative diseases, including Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and Alzheimer's disease (Gandhi & Wood, 2005; Lin & Beal, 2006; Shadrina et al., 2010). In tissues from patients with neurodegenerative disorders, an increase in markers of ROS damage has been found (Andersen, 2004; Ramassamy, 2006). In the affected regions of brain, these markers are elevated levels of lipid

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(Singh, Nam, Arseneault, & Ramassamy, 2010), protein (Butterfield, Hardas, & Lange, 2010; Sesti, Liu, & Cai, 2010) and DNA (Gella & Durany, 2009; Sesti et al., 2010) oxidation products.

Behavioural studies in rodents have revealed an attenuation of brain ageing when strawberries, blueberries or blackberries are ingested (Bickford et al., 2000; Duffy et al., 2007; Shukitt-Hale, Cheng, & Joseph, 2009; Williams et al., 2008) and the authors propose that the benefits are due to the presence of (poly)phenolic compounds. These phytochemicals, and by association the foods, are accruing a significant evidence base for beneficial effects on human health and the reduction of risk of cardiovascular disease (Mulvihill & Huff, 2010; Pandey & Rizvi, 2009), cancer (Russo, 2007; Yang, Landau, Huang, & Newmark, 2001) and type II diabetes (Borriello, Cucciolla, Della Ragione, & Galletti, 2010). Driving these beneficial endpoints are the numerous pathways and protein kinases that have been reported as being targets for phenolic compounds, thereby demonstrating the broad spectrum of targets and strengthening their usefulness in addressing multifactorial diseases (Ramassamy, 2006).

In many of the *in vitro* studies focused on polyphenol-derived health benefits, the doses used are significantly higher than those to which humans are exposed through the diet or that could be found in the blood (Collins, 2005). On average, and depending on the polyphenol class, plasma bioavailabilities in healthy humans are in the range of 0.5–1.6 μM (Paganga & Rice-Evans, 1997). Furthermore, it was concluded, from an analysis of 97 bioavailability studies, that total polyphenol-derived metabolite concentration in plasma after an intake of 50 mg of aglycone equivalents ranges from 0 to 4 μM (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). However, most *in vitro* studies use polyphenol concentrations ranging from 10 to 100 μM (Virgili & Marino, 2008): around 2–25-fold difference. Additionally, most of the *in vitro* cell-based studies evaluate metabolites "as they are in food", ignoring the chemical alterations occurring during digestion, absorption and metabolism, with a consequential impact on bioavailability and bioefficacy. Moreover, many studies have evaluated the effect of single purified phenolic compounds, thereby losing possible synergic/cooperative or competitive activities between phenolic compounds (Virgili et al., 2008). Thus, in order to evaluate the potential role of fruit phytochemicals in the human body, we should take into account the physiochemical changes occurring in the gastrointestinal tract (Virgili et al., 2008).

The aim of this work is to compare the neuroprotective effect of non-digested blackberry extract against digested blackberry metabolites. This work encompasses a more physiological approach which takes into account: (i) the chemical changes occurring during digestion; (ii) the effects of phytochemical mixtures found in authentic foods; (iii) treatment of cell models with relevant *in vivo* concentrations of phytochemical metabolites.

2. Materials and methods

2.1. Plant material and extract preparation

Blackberry (*Rubus* L. subgenus *Rubus* Watson) cv. Apache was grown in Fataca experimental field (Odemira, Portugal) and berries were harvested at full ripeness. Berries were harvested (yield approximately 500 g), frozen and then freeze-dried. Afterwards, fruits were ground without seed separation in an IKA M20 mill, to pass a 0.5 mm sieve, and stored at −80 °C prior to extraction. Fruit extracts were prepared as previously described (Tavares et al., 2010b). Briefly, to each 1 g of lyophilised powder, 12 ml of hydroethanolic solvent (50% (v/v) ethanol/water) were added and the mixture was shaken for 30 min at room temperature in the dark. The mixture was then centrifuged at 12,400*g* for 10 min at room temperature. The supernatant was filtered through filter paper and then through 0.2 μm cellulose acetate membrane filters. The resulting extracts were stored frozen at −80 °C.

2.2. In vitro digestion (IVD)

Phytochemical alterations during digestion were mimicked using the IVD model previously described by McDougall, Fyffe, Dobson, and Stewart (2005b). Briefly, the original extract (final volume 20 ml) was adjusted to pH 1.7 with 5 M HCl; then pepsin (Sigma Product number P6887) was added at 315 units ml^{-1} and incubated at 37 °C in a heated water bath for 2 h with shaking at 100 rpm. Aliquots (2 ml) of the post-gastric digestion were removed and frozen. The remainder was placed in a 250 ml glass beaker and 4.5 ml of 4 mg ml^{-1} pancreatin and 25 mg ml^{-1} bile salts mixture were added. A segment of cellulose dialysis tubing (molecular mass cut-off 12 kDa), containing sufficient 0.1 M $NaHCO_3$ to neutralise the sample's titratable acidity, was added and the beaker sealed with parafilm. The $NaHCO_3$ diffused out of the dialysis tubing and the pH reached neutrality within 45 min. After 2 h of incubation at 37 °C, the solution inside the dialysis tubing (fraction D1) and the solution outside the dialysis tubing (fraction D2) were collected. Small samples (2 ml) were immediately frozen. The digested fractions (D1 and D2) were acidified to 0.5% (v/v) by slow addition of 10% formic acid.

After centrifugation (2500*g*, 10 min, 5 °C), the soluble materials from fractions D1 and D2 were applied to C18 solid phase extraction columns (GIGA tubes, 1000 mg capacity, Phenomenex Ltd.), which had been pre-equilibrated in ultra pure water (UPW) containing 0.25% (v/v) formic acid (FA). After a wash with two volumes of FA/UPW, the bound material was eluted by the addition of 0.25% (v/v) FA in 25% (v/v) acetonitrile. This afforded complete separation of total phenolics from the bile salts present in samples (Coates et al., 2007). The fractions were then concentrated in a Speed-Vac to suitable phenol concentrations.

2.3. Chemical characterisation

2.3.1. Total phenolic quantification

Determination of total phenolic compounds was performed by the Folin–Ciocalteau method, adapted to a microplate reader (Tavares et al., 2010a). Gallic acid was used as the standard and the results were expressed as mg of gallic acid equivalents (mg GAE).

2.3.2. Peroxyl radical-scavenging capacity determination

Peroxyl radical-scavenging capacity was determined by the ORAC (Oxygen Radical Absorbance Capacity) method, as described by Tavares et al., (2010b). The final results were calculated using the differences in area under the fluorescence decay curves between the blank and the sample, and were expressed as μM trolox equivalents (μM TE).

2.3.3. Phenolic profile determination by LC-MS

Extracts and digested fractions were applied to a C-18 column (Synergi Hydro C18 column with polar end capping, 4.6 mm × 150 mm, Phenomonex Ltd.) and analysed by a LCQ-DECA system controlled by the XCALIBUR software (2.0, ThermoFinnigan), as reported by Tavares et al., (2010b). The LCQ-Deca system comprised a Surveyor autosampler, pump and photo diode array (PDA) detector and a Thermo Finnigan mass spectrometer iontrap.

2.4. Cell culture

Human neuroblastoma SK-N-MC cells were obtained from the European Collection of Cell Cultures (ECACC) and cultured in DMEM (Sigma) supplemented with 2 mM L-glutamine (Sigma),

10% (v/v) heat-inactivated foetal bovine serum (FBS, Gibco), 1% (v/v) non-essential amino acids (Sigma), and 1 mM sodium pyruvate (Sigma) containing 50 U ml^{-1} of penicillin and 50 μg ml^{-1} (w/v) OPA of streptomycin. The cells were maintained at 37 °C in 5% CO_2 and split at sub-confluence of 70–80%, using 0.05% trypsin/EDTA (Gibco).

2.5. Cytotoxicity profile

The original blackberry extracts and fraction D1 were concentrated under vacuum and dissolved in cell medium for the cytotoxicity tests (Fortalezas et al., 2010). The cell viability assay was performed in a 96-well plate cell and employed the neuroblastoma human cell line SK-N-MC to identify the non-toxic range of extract concentrations. Cells were seeded at 1.25×10^5 cells ml^{-1} and grown for 48 h prior to incubation with extracts. Toxicity tests involved 24 h incubation in the range 0–500 μg GAE ml^{-1} of medium. Cell viability was assessed using the CellTiter-Blue® Cell Viability Assay (Promega), according to the manufacturer's instructions. Non-viable cells rapidly lose their metabolic capacity and thus do not generate the fluorescent signal.

2.6. Neuroprotective effect against oxidative stress

To evaluate the neuroprotective effect of extracts, SK-N-MC neuroblastoma cells were incubated in the presence of H_2O_2. Cells were seeded at 7.4×10^4 cells ml^{-1} and, 24 h after seeding, growth medium was removed and wells were washed with PBS. Cells were pre-incubated with medium containing 0.5% (v/v) FBS supplemented with non-toxic concentrations of blackberry extracts. After 24 h of pre-incubation, cells were washed again with PBS and medium was replaced by medium containing 0.5% (v/v) FBS and H_2O_2 at a final concentration of 300 μM. After 24 h, medium was removed, and cells were washed with PBS, collected by trypsinisation and incubated with two fluorescent probes for 30 min at 37 °C. 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6(3)$, 20 nM, Invitrogen) was used to evaluate the mitochondrial transmembrane potential ($\Delta\Psi m$) and propidium iodide (PI, 1 μg ml^{-1}, Invitrogen) was used to determine cell viability, based on plasma membrane integrity (Queiroga et al., 2010). Cells were then analysed by flow cytometry. A flow cytometer (Partec), containing a blue solid state laser (488 nm) with FL1 green fluorescence channel for $DiOC_6(3)$ at 530 nm and a FL3 red fluorescence channel for PI detection at 650 nm, was used. The acquisition and analysis of the results were performed with FlowMax® (Partec) software.

2.7. Intracellular ROS production

To evaluate the ability of extracts to reduce ROS levels produced by cells, the conversion of 2′,7′-dichlorofluorescein diacetate (H_2DCFDA, Invitrogen) to fluorescent 2′,7′-dichlorofluorescein (DCF) was monitored (Wang & Joseph, 1999; Wolfe & Liu, 2007). SK-N-MC neuroblastoma cells were seeded in a 96-well plate at 1.25×10^5 cells ml^{-1}. Cells were grown for 24 h and then they were washed with PBS and then pre-incubated with extracts prepared in medium (0.5% (v/v) FBS) for 2 or 24 h. After pre-incubation, cells were washed with PBS and incubated with 25 μM H_2DCFDA in PBS for 30 min at 37 °C. Cells were washed and H_2O_2 (200 μM) in PBS, was added. Fluorescence was measured (λex: 485 nm, λem: 530 nm) using a FLx800 Fluorescence Microplate Reader (Biotek) for 1 h at 37 °C. ROS generation was calculated as an increase in fluorescent signal between control and H_2O_2-treated cells.

2.8. Glutathione (GSH) and glutathione disulphide (GSSG) quantification

To quantify GSH and GSSG, cold 10% (w/v) metaphosphoric acid was carefully added to samples or standards. After incubation (4 °C, 10 min) and centrifugation (16,000*g*, 20 min, 4 °C) supernatants were transferred into 1.5 ml propylene tubes (50 μl for determination of GSH and 200 μl for determination of GSSG).

Derivatisation was performed accordingly to Kand'ar, Zakova, Lotkova, Kucera, and Cervinkova (2007), adapted from Hissin and Hilf (1976). Briefly, for GSH analysis, 1 ml of 0.1% (w/v) EDTA in 0.1 M sodium hydrogen phosphate, pH 8.0, was added to 50 μl of supernatant. To a 20 μl portion of this mixture, 300 μl of 0.1% (w/v) EDTA in 0.1 M sodium hydrogen phosphate, and 20 μl of 0.1% (w/v) orthophthalaldehyde (OPA) in methanol, were added. Tubes were incubated at 25 °C for 15 min in the dark. The reaction mixture was then stored at 4 °C prior to analysis. For GSSG analysis, 200 μl of supernatant were incubated at 25 °C with 200 μl of 40 mM *N*-ethylmaleimide for 25 min in the dark. To this mixture, 750 μl of 0.1 M NaOH were added. A 20 μl portion was taken and mixed with 300 μl of 0.1 M NaOH and 20 μl of 0.1% (w/v) OPA. Tubes were incubated at 25 °C for 15 min in the dark and stored at 4 °C prior to analysis.

Chromatographic analysis was accomplished using isocratic elution on a C18 analytical column (Supelcosil™ABZ+Plus HPLC Column 15 cm × 4.6 mm, 3 μm (Supelco)) at 40 °C in an Acquity™ Ultra Performance LC system (Waters). The mobile phase consisted of 15% (v/v) methanol in 25 mM sodium hydrogen phosphate, pH 6.0. The flow rate was kept constant at 0.7 ml min^{-1}. The excitation and emission wavelengths were set at 350 and 420 nm, respectively. The amounts of GSH and GSSG were quantified from the corresponding peak areas, using Empower® Pro 2.0 software. The concentrations of GSH and GSSG in the samples were determined from standard curves with ranges 0–100 μM for GSH and 0–5 μM for GSSG. Values were normalised for total protein content, determined by the Lowry method (Bensadoun & Weinstein, 1976).

2.9. Statistical analysis

The results reported in this work are the averages of at least three independent experiments and are represented as the means ± SD. Differences amongst treatments were detected by analysis of variance with the Tukey HSD (honest significant difference) multiple comparison test ($\alpha = 0.05$) using SigmaStat 3.10 (Systat).

3. Results and discussion

3.1. Chemical characterisation

Blackberry fruits are well known to be a rich source of polyphenols and to exhibit high antioxidant capacity (Moyer, Hummer, Finn, Frei, & Wrolstad, 2002; Siriwoharn, Wrolstad, Finn, & Pereira, 2004). Furthermore, like many of the *Rubus* genus they have increasingly been attributed with a significant potential for human health (Dai, Patel, & Mumper, 2007; Lu, Li, Zhang, Stoner, & Huang, 2006; Wang & Stoner, 2008) and, in particular, blackberries have been reported to enhance short-term memory performance in animal models (Shukitt-Hale et al., 2009). After ingestion, blackberry phytochemicals undergo many modifications or even degradation by the processes of gastrointestinal (GI) digestion. Although transport and metabolic mechanisms cannot be effectively reproduced, *in vitro* studies can provide a simple predictive instrument to investigate the potential bioavailability under conditions mimicking the GI tract. In the present work, an IVD model was used to

Table 1
Total phenolic content (TP), antioxidant capacity (AC) and ratio antioxidant capacity per total phenolic content (AC/TP) of original extract of blackberry and IVD fractions.

		Original extract	PG	D1	D2
Total phenolic (TP) content	mg GAE	16.7 ± 0.2	15.5 ± 0.4	0.9 ± 0.2	4.6 ± 0.2
	%	100.0	93.0	5.5	27.5
	Significance		*	***	***
Antioxidant capacity (AC)	μmol TE	544 ± 26.9	269 ± 71.1	91.7 ± 6.2	294 ± 23.5
	%	100.0	49.4	16.9	54.1
	Significance		**	***	**
AC/TP		32.6	17.3	102	64.0

Values are reported as means ± SD ($n = 3$) and percentage in comparison to the original extract. Statistical differences compared to the original extract are denoted as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. PG, post-gastric; D1, post-pancreatic (inside dialysis bag); D2, post-pancreatic (outside dialysis bag).

mimic the effect of GI digestion on blackberry phenolic extract. This model was first described by Miller, Schricker, Rasmussen, and Van Campen (1981) and then adopted to evaluate secondary metabolites (McDougall, Dobson, Smith, Blake, & Stewart, 2005a; McDougall, Fyffe, Dobson, & Stewart, 2007). IVD produces different digested fractions: post-gastric digest (PG) and two pancreatic digested fractions (D1 and D2). There was a large reduction in both total phenolic content (TP) and antioxidant capacity (AC) following digestion, in particular for the pancreatic digest fractions D1 and D2 (Table 1). Gastric digestion reduced the antioxidant capacity by 50.6%, although the total phenolic content was reduced by no more than 7%. The results for total phenolic content of PG digest are in accordance with other studies performed with pomegranate, red cabbage and chokeberry (Bermudez-Soto, Tomas-Barberan, & Garcia-Conesa, 2007; McDougall et al., 2007; Perez-Vicente, Gil-Izquierdo, & Garcia-Viguera, 2002). The TP content of D1 was reduced to 5.5% and the AC to 16.9% compared to the original blackberry extract. In D2, these values were 27.5% and 54.1%, respectively. Both TP and AC values are in the same range as those obtained for grapes by Tagliazucchi, Verzelloni, Bertolini, and Conte (2010) (55.5% for total phenolic compounds and 62.4% for antioxidant capacity). Interestingly, IVD increased the relative antioxidant capacity (expressed as a ratio of total phenolic content) of fraction D1, by threefold and by twofold, in fraction D2 (AC/TP in Table 1).

There were substantial alterations in phytochemical composition caused by IVD as assessed by LC-MS (Fig. 1; Table 2). The major anthocyanins, cyanidin-3-*O*-glucoside (CyGlc), cyanidin-3-*O*-xyloside (CyXyl) and cyanidin-3-*O*-dioxayl-glucoside (CyDAGlc), were greatly reduced by pancreatic digestion, with recoveries in

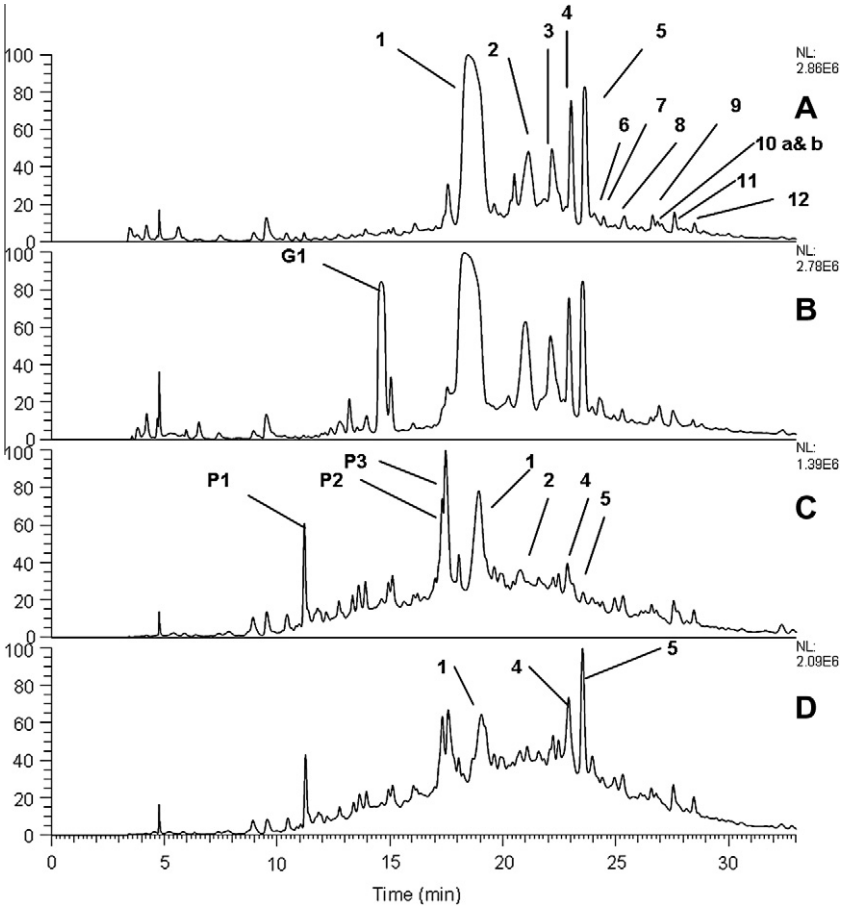

Fig. 1. Analysis of blackberry samples by liquid chromatography-mass spectrometry (LC-MS). (A) Original extract; (B) post-gastric; (C) D1 post-pancreatic; (D) D2 post-pancreatic. All graphs are representative traces recorded at 280 nm. The full scan deflection is shown in the upper right corner of each panel. Peaks are labelled as described in Table 2 with components appearing in the PG or the pancreatic digests, D1 and D2, labelled, e.g. G1 and P1, respectively.

Table 2
Identification of indicated phenolic components from blackberries.

Peak No.	RT	PDA	*m/z*	MS^2	Putative ID
1	18.46	515, 280	+**449**, 287	287	Cyanidin-3-*O*-glucoside (Cho, Howard, Prior, & Clark, 2004); Mertz, Cheynier, Gunata, & Brat, 2007)
2	21.14	515, 208	+**419**, 287	287	Cyanidin-3-*O*-xyloside (Cho et al., 2004; Mertz et al., 2007)
3	22.18	520, 280	+**593**, 287	287	Cyanidin-3-*O*-dioxayl-glucoside (Cho et al., 2004)
4	23.03	240–300	**1401**, 1250, 934, 301	1869, 1567, **1250**, 1235, 934, 633	Lambertianin C (Gasperotti, Masuero, Vrhovsek, Guella, & Mattivi, 2010; Mertz et al., 2007; Mullen, Yokota, Lean, & Crozier, 2003)
5	23.63	240–300	**1869**, 935, 301	**1567**, 1265, 1235, 1103, 933, etc	Sanguiin H6 (Gasperotti et al., 2010; Hager, Howard, & Prior, 2008; Mertz et al., 2007; Mullen et al., 2003)
6	24.06	240–300	1103	Multiple	Unidentified ellagitannin
7	24.47	355	**433**, 301	301	Quercetin-xyloside (Wojdylo, Oszmianski, & Laskowski, 2008)
8	25.39	355	**433**, 301	301	Quercetin-xyloside (Wojdylo et al., 2008)
9	25.83	355	**609**, 301	301	Quercetin-rutinoside (Cho et al., 2004; Cho, Howard, Prior, & Clark, 2005; Mullen et al., 2003)
10a	26.64	355	**463**, 301	301	Quercetin-glucoside (Cho et al., 2004, 2005; Mertz et al., 2007; Mullen et al., 2003)
10b	26.86	355	**477**, 301	301	Quercetin-glucuronide (Cho et al., 2005; Mertz et al., 2007)
11	27.61	355	607, 505, 463, 301	545, 505, **463**, 301	Quercetin-HMG-glucoside (Cho et al., 2005)
12	28.50	365	447	315	Methyl ellagic acid pentoside (Mertz et al., 2007; Mullen et al., 2003)
G1	14.61	250–300, 270 max	None	–	Unknown
P1	11.21	220-300, 260 max	282, 150	150, 133	Unknown
P2	17.32	290	None	–	Unknown
P3	17.47	265	153	109	Dihydroxy benzoic acid

There was also evidence for cyanidin-3-*O*-rutinoside which co-eluted with the main anthocyanin peak. Assignments are supported by previous work. Peak numbers refer to Fig. 1. Compounds 1–12 were identified in the original extract; compound G1 was more apparent in PG and compounds P1–P3 were more apparent in the D1 and D2. + denote detection in the positive mode MS only. In bold are denoted the most abundant ions.

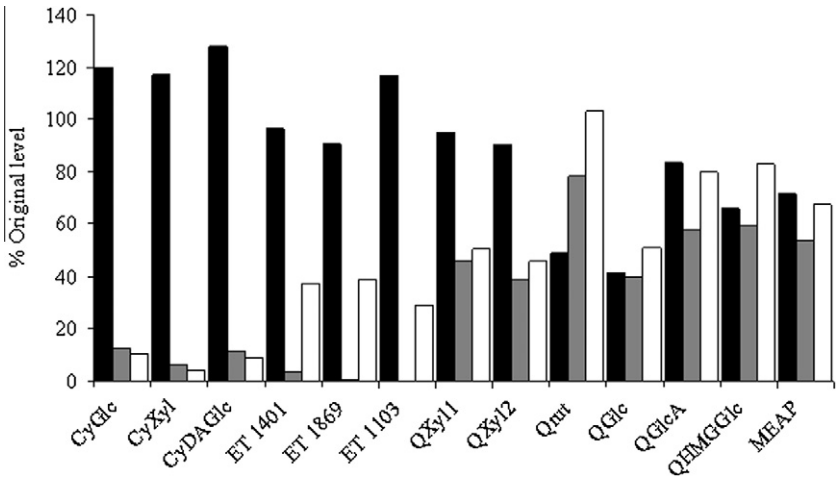

Fig. 2. Recovery of selected blackberry phenolics after *in vitro* digestion. The main metabolites identified in original blackberry extract were relatively quantified and values reported as percentage of recovery, based on the peak area of the mass spectrometer response for each *m/z*. ■, PG (post-gastric); ▒, D1 post-pancreatic (inside dialysis bag); □, D2 post-pancreatic (outside dialysis bag). Metabolites analysed: CyGlc, cyanidin-3-*O*-glucoside; CyXyl, cyanidin-3-*O*-xyloside; CyDAGlc, Cyanidin-3-*O*-dioxayl-glucoside; ET 1401, Lambertianin C; ET 1869, Sanguiin H6; ET 1103, unidentified ellagitannin; QXyl1, quercetin-xyloside 1; QXyl2, quercetin-xyloside 2; Qrut, quercetin-rutinoside; QGlc, quercetin-glucoside; QGlcA, quercetin-glucuronide; QHMGGlc, quercetin-HMG-glucoside; MEAD, methyl ellagic acid pentoside.

fractions D1 and D2 at around 10% of the original extract (Fig. 2). These low recoveries are concordant with the values reported after IVD of pomegranate juice (Perez-Vicente et al., 2002) and raspberry (McDougall et al., 2005a). Anthocyanins are generally stable in the acidic conditions of the stomach, but less stable at the higher pH of the small intestine (Gil-Izquierdo, Gil, & Ferreres, 2002; McDougall et al., 2005b; Perez-Vicente et al., 2002). Quercetin derivatives were more highly recovered after digestion with recoveries ranging from 40% to >80%. In some cases, the total recovery in fractions D1 plus D2 was greater than 100%, which may be due to interconversion of quercetin components during digestion or enhanced detection of quercetin derivatives due to reductions in other components. Increased relative recovery of flavonols was also noted after IVD of green tea (Okello, McDougall, Kumar, & Seal, 2010). The recovery of the major ellagitannin components was substantial in the PG fraction but much lower in the pancreatic digests: around 30–40% for fraction D2 and essentially zero for D1. Ellagitannins are more stable to pH changes than are anthocyanins but degrade to smaller components (Larrosa, Tomas-Barberan, & Espin, 2006).

It was also notable that new peaks were identified after IVD. For example, after gastric digestion, a major new peak was present (G1, Fig. 1B), but the MS properties did not permit identification.

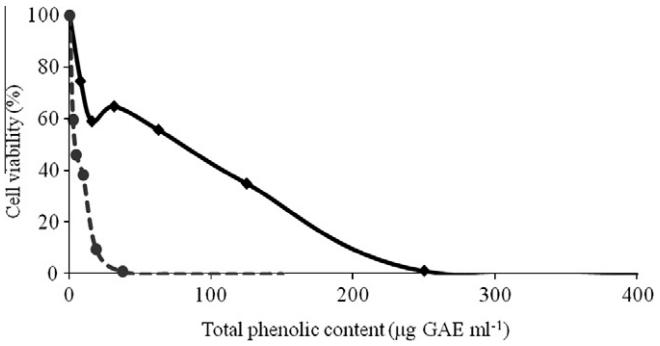

Fig. 3. Cytotoxicity profile obtained for original extract (solid line) and D1 (dashed line). Cell viability was determined by CellTiter-Blue® Cell Viability Assay (Promega) after cells being incubated with phenolic compounds for 24 h.

After pancreatic digestion, a number of new peaks appeared in fractions D1 and D2 (Fig. 1C and D, peaks P1–P3). Peak P3 gave PDA and MS properties consistent with assignment as dihydroxybenzoic acid, perhaps derived from the breakdown of cyanidin anthocyanins at pH > 7 (McDougall et al., 2005b, 2007).

As mentioned above, fraction D1 had approximately threefold higher antioxidant capacity than had the original extract when expressed as a ratio of phenolic content. This increased antioxidant capacity may result from its greatly altered phenolic composition (lower levels of anthocyanins and ellagitannins but relatively enhanced levels of quercetin derivatives and evidence of accumulation of breakdown products) (Fig. 2) and made it the obvious choice for further studies on neuroprotective effects.

3.2. Cytotoxicity profile determination

Prior to the assessment of the neuroprotective potential, cytotoxicity assays were performed, using the assessment of cell metabolism of SK-N-MC neuroblastoma cells. Fraction D1 was more toxic than was the original extract (Fig. 3), presumably as a result of digestion-induced chemical modifications. Non-toxic concentrations of the original extract and fraction D1 were selected for further assays. Interestingly, the non-toxic range of fraction D1 (maximum 1 μg GAE ml^{-1}, corresponding to 6 μM) was similar to that reported for dietary polyphenolic-derived metabolites in plasma at 0–4 μM (Manach et al., 2005), which is physiologically relevant with respect to polyphenolic-load. The maximum non-toxic concentration for the original extract was higher, 5 μg GAE ml^{-1} (equivalent to 30 μM). It should be noted that these concentrations of total polyphenols do not refer to specific chemical moieties; rather therefore values for the total mix of polyphenols obtained from fruits or their simulated digestion.

To evaluate if the effect of the polyphenols was due to interactions with extracellular receptors or to uptake, we took advantage of the intrinsic autofluorescence properties of polyphenolic compounds (Colin et al., 2008). Cells incubated for 1 h with increasing

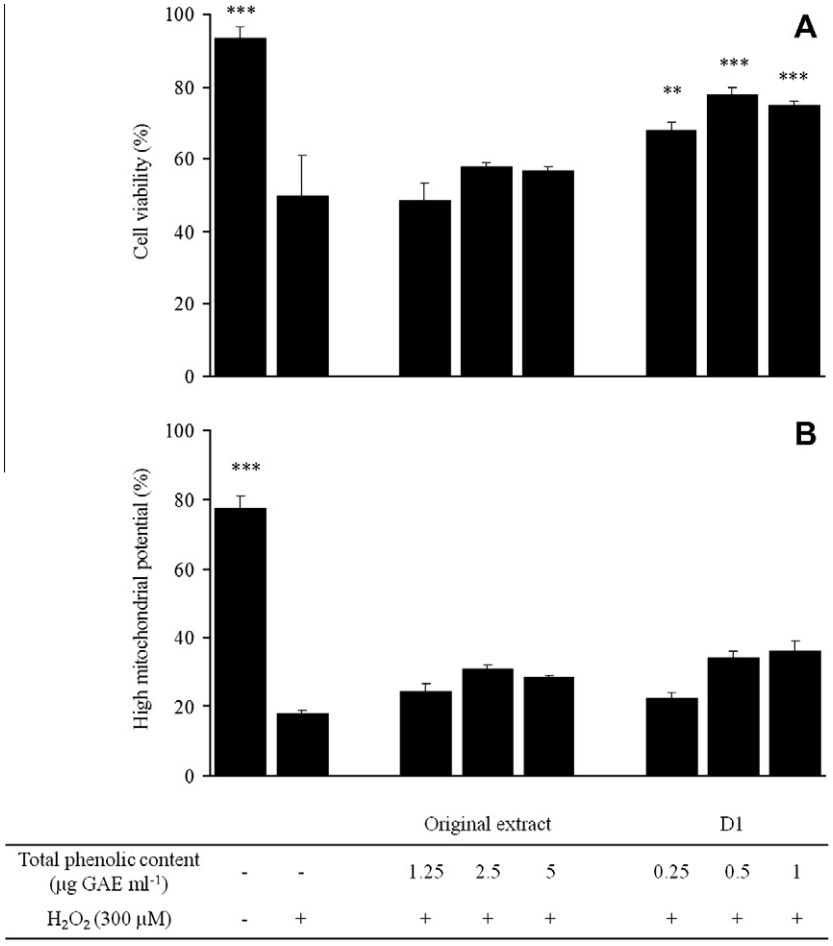

Fig. 4. Cell viability and mitochondrial transmembrane potential ($\Delta\Psi m$) assessed by flow cytometry. (A) Cell viability, expressed as percentage of cells containing intact membrane, using PI as fluorochrome. (B) Percentage of cells presenting high mitochondrial transmembrane potential, using $DiOC_6(3)$ as probe. Neuroblastoma cells were pre-incubated with blackberry original extract or D1 for 24 h and then injured by 300 μM H_2O_2 for 24 h. Statistical differences between treatments are denoted as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All values are means ± SD, $n = 3$.

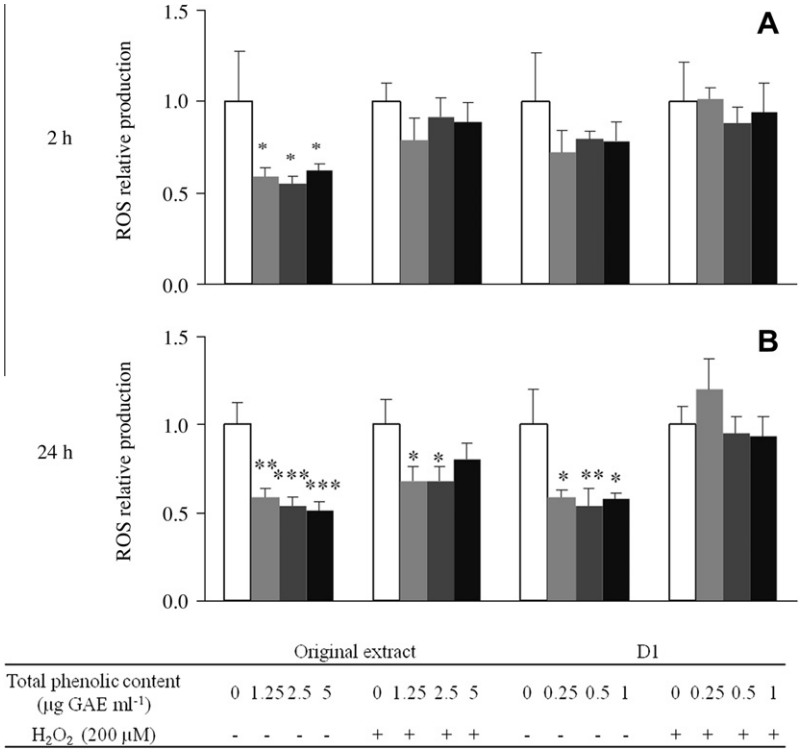

Fig. 5. Relative intracellular ROS production by SK-N-MC neuroblastoma cells in presence/absence of oxidative stress. Oxidative stress (200 μM H_2O_2) was applied for 1 h. Cells were pre-incubated with original blackberry extract or fraction D1 for (A) 2 h; (B) 24 h. ROS were detected by fluorimetry, using DCF as probe. Statistical differences between treatments are denoted as $^{*}p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$. All values are means ± SD, $n \geqslant 3$.

concentrations of blackberry extract showed a concentration-dependent autofluorescent signal within the cytoplasm. This suggests cellular uptake of, at least, some of the polyphenols.

3.3. Evaluation of neuroprotective effect

Concentrations of blackberry extract and D1 fraction, which were non-toxic as assessed by mitochondrial metabolism, were tested in an H_2O_2-stress neurodegeneration cell model. Production of H_2O_2 is related to age-related diseases and more particularly neurodegeneration (Cavazzoni, Barogi, Baracca, Parenti Castelli, & Lenaz, 1999; Tabner, Turnbull, Fullwood, German, & Allsop, 2005). Neuroblastoma cells were treated with 300 μM H_2O_2 for 24 h, which reduced cell viability to around 50% (based on cells with an intact cell membrane, negative for PI in flow cytometry assay; Fig. 4A).

Only fraction D1 was able to significantly protect neuroblastoma cells from H_2O_2 injury (Fig. 4A) even though the original extract was applied at fivefold higher concentration. The lowest concentration of D1 (0.25 μg GAE ml^{-1}, equivalent to 1.5 μM) was able to exert a protective effect and this concentration is in the same range as the serum bioavailability reported for individual polyphenols (maximum 4 μM; (Manach et al., 2005) which reinforces the biological significance of this neuronal protection. Furthermore, greater protection was achieved when higher metabolite concentrations were used (0.5 and 1 μg GAE ml^{-1}) and pre-incubation with D1 (0.5 μg GAE ml^{-1}) resulted in an increase in viability to 78% (Fig. 4A). However, in this neurodegeneration cell model, neither the original extract nor fraction D1 could prevent the dissipation of the mitochondrial transmembrane potential ($\Delta\Psi m$; Fig. 4B). This dissipation reflects the earlier stage of H_2O_2-induced cytotoxicity associated with its diffusion into the mitochondrial matrix and with subsequent loss of integrity, ability to generate ATP and finally cell death (Mronga, Stahnke, Goldbaum, & Richter-Landsberg, 2004; Perry, Norman, Barbieri, Brown, & Gelbard, 2011). Therefore, although not able to completely protect neuroblastoma cells, digested metabolites in fraction D1 were able to modulate molecular mechanisms of survivability in response to the imposed oxidative stress.

3.4. Intracellular ROS production determination

The same concentrations were also tested for intracellular antioxidant capacity (Fig. 5). Two different pre-incubation times were tested, at 2 and 24 h (Fig. 5A and B, respectively), to cover different timescale events. We presumed that the 2 h pre-incubation would evaluate the direct scavenging events caused by the phenolic compounds. As it turned out, as mentioned above (Section 3.2), we verified that 1 h should be enough for compounds presented in blackberry extract to be taken up by cells. We presumed that 24 h of pre-incubation with polyphenol-derived metabolites could influence ROS levels via indirect effects on endogenous antioxidant systems. At both time points and in the absence of an imposed oxidative stress, both original and D1 extracts significantly reduced the basal ROS production. This suggests that the original and D1 extracts could alter the oxidative environment of cells. H_2O_2 was used to promote an oxidative stress in cells and doubled ROS production compared with the control (Fig. 6) without promoting cell death. With a 2 h pre-incubation, neither the original extract nor D1 reduced intracellular ROS caused by H_2O_2 stress. With a 24 h pre-incubation, followed by H_2O_2 stress, the original extract but not the D1 reduced intracellular ROS production. Comparison of these results with that derived from the neuroprotection assay suggests that the neuroprotection exhibited by fraction D1 was not mediated by ROS-scavenging. Indeed, similar results were obtained by Cilla, Laparra, Alegria, Barbera, and Farre (2008), who reported that intracellular ROS production was not diminished by pre-incubating cells with an *in vitro* digested fruit beverage.

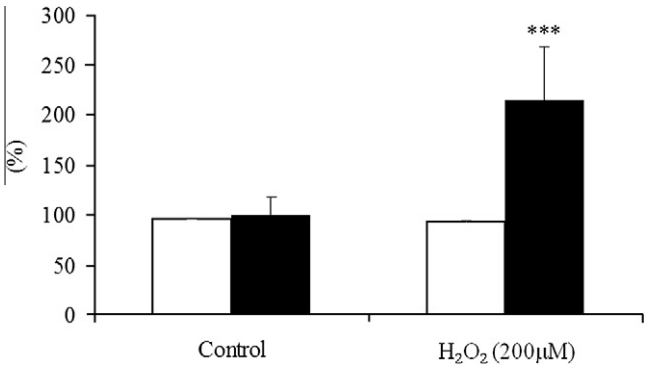

Fig. 6. Characterisation of stress conditions used to determine intracellular ROS production. □, Cell viability; ■, ROS production. Values are reported as percentages relative to the control condition. Cells were incubated in presence/absence of an oxidative stress (200 μM H_2O_2 for 1 h), cell viability determined by FACS with PI as probe and ROS production, determined by fluorimetry with DCF as probe. Statistical differences between treatments are denoted as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All values are means ± SD, $n = 3$.

Conversely, the original extract was able to promote ROS-scavenging, although not enough to protect cells from death. Protection from ROS over a 24 h time scale may be indicative of priming of the endogenous antioxidant systems via events such as nuclear receptor modulation, gene expression and enzyme activity regulation, subcellular signalling pathway modulation and involvement in mechanisms of DNA protection/repair, amongst others (Joseph et al., 2003; Seeram, 2008; Shukitt-Hale, Lau, & Joseph, 2008). Phenolic compounds present in the original blackberry extract could be modulating some endogenous antioxidant defences and consequently reducing the intracellular ROS, as suggested for grape-derived polyphenols (Rodrigo, Miranda, & Vergara, 2011; Vieira de Almeida et al., 2008). Indeed, recent work by Xiao (2010) showed that fruit polyphenols stimulated the expression and production of mammalian detoxification/antioxidant enzymes via the Nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor. Compounds responsible for decreasing ROS levels may have been removed during digestion, since the same response was not observed with D1. These results suggest that neuronal protection, caused by D1, is produced by mechanisms other than directly modulating ROS levels.

3.5. GSH and GSSG quantification

GSH is the major antioxidant within cells and is involved in maintaining a tight control of redox status (Ballatori et al., 2009). To verify the alterations induced by metabolite pre-treatment and post application of H_2O_2 stress, the redox pair GSH/GSSG was quantified. GSH and total thiols (GSH + 2 × GSSG) are presented in Fig. 7. Cells subjected to H_2O_2 (300 μM, 24 h) exhibited a reduction in the level of total thiols as a result of GSH depletion. Conversely, cells subjected to a 24 h pre-treatment with blackberry extract, at 1.25 and 2.5 μg GAE ml^{-1}, showed an increase in GSH and total thiols. Pre-treatment with D1 did not prevent the H_2O_2-dependent depletion of GSH levels. These changes in GSH levels are in accordance with ROS levels observed after the 24 h pre-treatment, followed by oxidative stress (Fig. 6). The augmentation in GSH, following pre-treatment with blackberry extract and induction of oxidative stress, could contribute to the reduction in ROS levels detected. Although D1 did not prevent GSH depletion, it promoted cell protection, unlike the original blackberry extract. Again, these results reinforce the differences noted between cell model studies performed with metabolites obtained from food digestion rather than the direct food components.

4. Conclusions

The potential of blackberry to contribute to dietary strategies to prevent or retard neurodegeneration was evaluated. The present work clearly compares and distinguishes the neuroprotective effect of non-digested blackberry extract (original extract) and digested blackberry metabolites (D1 fraction) at concentrations approaching physiological levels.

The original undigested blackberry extract, although exhibiting a significant antioxidant capacity *in vitro*, was not able to protect neurons in a neurodegeneration cell model, but enhanced GSH levels and reduced ROS production. However, enhancing intracellular antioxidant capacity *per se* was not enough to effectively protect neurons.

Conversely, digested polyphenol metabolites were able to maintain cell membrane integrity, protecting neurons from death. Interestingly, this protection was not related to enhanced intracellular antioxidant capacity, since the D1 fraction did not directly reduce ROS production or indirectly influence ROS-scavenging. In addition, there were no alterations in GSH redox status after D1 pre-incubation. This highlights the involvement of mechanisms

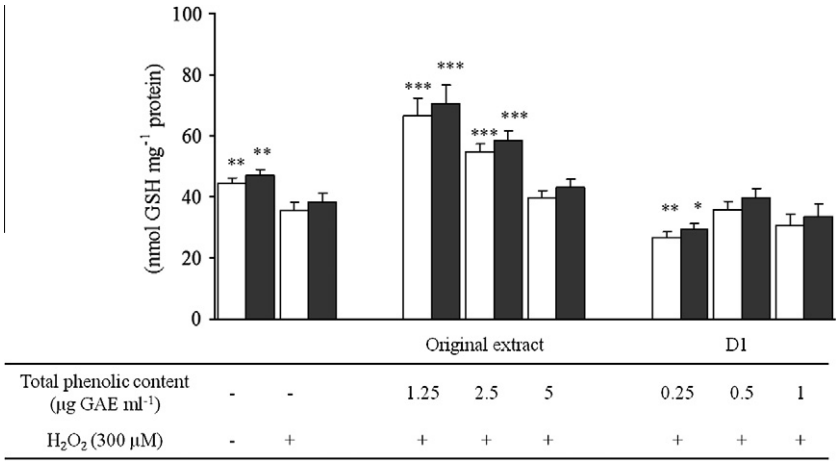

Fig. 7. Quantification of GSH and total thiols. □, GSH and ■, total thiol levels in SK-N-MC neuroblastoma cells pre-treated with original blackberry extract and fraction D1. After pre-treatment for 24 h, cells were subjected to 300 μM H_2O_2 for 24 h. Cells were harvested and analysed for their content in GSH and GSSG by UPLC. Total thiol content was calculated as GSH + 2 × GSSG and values are expressed in nmol GSH mg^{-1} protein. Statistical differences between treatments are denoted as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All values are means ± SD, $n \geqslant 3$.

other than antioxidant systems and the complexity of how, what may be thought of as a simple food, fruit, can interact at the fundamental level with our cells. Overall, this work illustrates the importance of evaluating the effect of digested metabolites in disease cell models.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.10.025.

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