Cytoprotective effects of anthocyanins and other phenolic fractions of Boysenberry and blackcurrant on dopamine and amyloid β-induced oxidative stress in transfected COS-7 cells†

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Abstract: There is growing interest both from consumers and researchers in the role that berries play in human health. In the experiments reported here, we assessed the ability of anthocyanins and phenolic fractions of Boysenberry and blackcurrant to ameliorate the deleterious effect of the amyloid β_{25-35} (100 μmol L^{-1}, 24 h) and dopamine (1 mmol L^{-1}, 4 h) on calcium buffering (recovery) of M1 muscarinic receptor-transfected COS-7 cells. Cell viability was also studied. Our results demonstrate that extracts of Boysenberry and blackcurrant showed significant protective effect and restored the calcium buffering ability of cells that had been subjected to oxidative stress induced by dopamine and the amyloid β_{25-35}. Blackcurrant polyphenolics showed slightly higher protective effect against dopamine, whereas Boysenberry polyphenolics had a higher effect against the amyloid β_{25-35}. In viability studies, all extracts showed significant protective effects against dopamine and amyloid β_{25-35}-induced cytotoxicity. Our results provide further evidence for the protective effects of berries against the neurotoxic effect of dopamine and amyloid β_{25-35} in brain cells.

Keywords: oxidative stress; calcium homeostasis; muscarinic receptors; dopamine; amyloid beta; berry extracts

INTRODUCTION

Polyphenolics have long been recognised as possessing many properties including antioxidant, anti-allergic, anti-inflammatory, anti-viral, anti-neurodegeneration, and anti-carcinogenic.[1–3] Despite the wide health-promoting benefits, considerable interest over the past decade has primarily been focused on their roles in reducing risk factors associated with cancer and heart disease.[4,5] Consequently, there still remains a huge gap in actual scientific information on their role in modulating brain functions, such as learning and memory, any decrements of which have very negative impacts on the quality of life. Polyphenolic compounds occur ubiquitously in foods of plant origin, with over 8000 different structures having been already identified and described.[3] As a result of our increasing understanding and awareness of the potential human health benefits of polyphenols, research in this area has recently intensified.[3–6]

Vulnerability to oxidative stress (OS) has been implicated in aging and other neurodegenerative diseases such as Alzheimer's disease (AD),[7–10] and is considered to be responsible for the decrements in such functional indices as cognitive[11,12] and motor behaviours.[13,14] Senescent humans as well as rodents show age-correlated impairments in performance and neurotransmitter plasticity in the brain. Research has shown that sensitivity to OS induces an increase in such parameters as DNA fragmentation,[15] astrocytic injury,[16] loss of glutathione,[17] excitotoxic injury,[18] bcl-2 activity in brain[15,19] and striatal muscarinic receptor (MAChR) sensitivity.[20] Joseph *et al*.[21] have described one factor determining vulnerability to OS which involves qualitative and quantitative differences in receptor subtypes in various neuronal populations. They exposed transfected COS-7 cells with one of five muscarinic receptors subtypes (M1–M5 AChR) to dopamine and examined the intracellular Ca^{2+} levels via fluorescent imaging analysis prior to and following 750 μmol L^{-1} oxotremorine (a non-hydrolysed muscarinic acetylcholine receptor agonist, Oxo). Results indicated that cells transfected with M1, M2 or M4 showed greater ability to clear excess Ca^{2+} (i.e. Ca^{2+} recovery) than those transfected with M3 or M5 subtypes when exposed to dopamine. The results of viability of cells also supported the

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† Presented at the 2nd International Conference on Polyphenols and Health, UC Davis, USA, 4–7 October 2005
Contract/grant sponsor: HortResearch, New Zealand Boysenberry Council and Blackcurrants New Zealand Ltd; contract/grant number: KDF603-13
(*Received 13 June 2006; revised version received 7 February 2007; accepted 1 March 2007*)
Published online 25 June 2007; DOI: 10.1002/jsfa.2964

above phenomenon. Interestingly, the results from another study[22] indicated similar findings when the cells were exposed to amyloid $\beta(A\beta)_{25-35}$ and $A\beta_{1-40}$ showed similar effects on M1 and M3 AChR. In previous experiments from the same laboratory, results showed that α-phenyl-*n*-butylnitrone (PBN, a nitrone-trapping agent), Trolox (a vitamin E analogue) and several fruit extracts (blueberry, Boysenberry, cranberry, blackcurrant, plum and grape) had differential levels of recovery protection in comparisons with the non-supplemented controls.[21,23] PBN and Trolox prevented the cell death induced by dopamine (DA) pre-treatment, but they did not prevent the DA-induced decrements in recovery.[21] More recently, apple juice concentrate has been shown to prevent amyloid β-induced increase of reactive oxygen species, calcium influx and apoptosis.[24] It has been previously shown that the extracts used in this work from Boysenberry and blackcurrant have a protective effect against oxidative stress-induced DNA damage,[25] while having no effect on the growth or death of the cells at human physiological doses, both in plasma[26] and urine.[27] In the present work we investigated the effect of purified polyphenolic fractions of Boysenberry and blackcurrant to determine their protective effects at human physiologically relevant concentrations against insult by dopamine and/or the amyloid β_{25-35}.

MATERIALS AND METHODS

Extract preparation and HPLC analysis

Boysenberry (*Rubus loganbaccus* × *baileyanus* Britt 'Riwaka Choice') and blackcurrant (*Ribes nigrum* L. 'Ben Ard') fruit are predominately grown in New Zealand and were used in this project for extract preparation.[25] These were supplied by Berryfruit Export Ltd (Richmond, New Zealand) and Blackcurrants New Zealand Ltd (Christchurch, New Zealand). To extract non-anthocyanin polyphenols, portions of berries were first homogenised in a Waring blender with ethyl acetate and anhydrous sodium sulfate in a ratio of berries/ethyl acetate/sodium sulfate (1:2:1, w/v/w). The ethyl acetate extract was removed by filtration and the solid residue was further homogenised with methanol (2 × 2 volumes) to extract the anthocyanins. The methanol extract was separated from the solid residue by filtration and the methanol was evaporated by rotary evaporation (40 °C). The resulting aqueous anthocyanin extract was loaded onto an XAD-7 (Sigma, Sydney, Australia) column previously conditioned with 5% formic acid. The column was eluted with 5% formic acid to remove sugars and other water-soluble compounds not bound to the column. The anthocyanins were then eluted with 5% formic acid in methanol. This methanolic extract was concentrated and dried under vacuum to yield an anthocyanin-rich fraction of Boysenberry (ByAcy) and blackcurrant (BcAcy). The ethyl acetate extract containing the non-anthocyanin polyphenols was filtered twice and concentrated on a rotary evaporator (40 °C). Residual aqueous-soluble compounds were removed by washing the ethyl acetate extract with 0.1 mol L^{-1} HCl (2 × 1 volume) followed by drying with anhydrous sodium sulfate. It was then evaporated to dryness (40 °C). The residue was re-dissolved in 5% formic acid/methanol and washed three times with hexane (0.5 volume) to remove lipids and other fat-soluble compounds such as carotenoids. Finally the dark-brown methanol extract was evaporated to dryness and freeze dried to yield a polyphenolic-enriched fraction of Boysenberry (ByPhen) or blackcurrant (BcPhen). Anthocyanins and non-anthocyanin polyphenol concentrations in the extracts were determined by HPLC following solid-phase extraction (Fig. 1).

Cell culture and transfection

COS-7 cells (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified medium (D-MEM, GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO) and containing 100 U mL^{-1} penicillin (GIBCO) and 100 μg mL^{-1} streptomycin (GIBCO). Since they do not express MAChR, COS-7 cells were utilised for these determinations. Twenty-four hours prior to transfection, cells were harvested with trypsin, counted and plated on 100 mm^2 tissue culture plates at 5×10^6 cells per plate. Cells were transiently transfected with rat muscarinic receptor subtype 1 DNA, by the DEAE–dextran method.[28] After transfection cells were incubated for 2.5 h in growth medium containing 80 μmol L^{-1} chloroquine to minimise degradation of the DNA. Transfected cells were then maintained in growth medium for 48 h, harvested with trypsin, plated onto coverslips in 35 mm plates, and incubated overnight.

Cell treatment

Both pure anthocyanin and other phenolic fractions[25] from Boysenberry (By) and blackcurrant (Bc) were used at a concentration of 100 μg mL^{-1} to 500 μg mL^{-1} in growth medium. The concentration ranges used in these experiments are within the human physiological range.[26,27] Anthocyanin metabolism in humans has yet to be adequately characterised partly due to the large variations in dosages used in clinical studies (high pharmacological dose versus low physiological dose). The dietary consumption of anthocyanins has been estimated at up to 200 mg day^{-1}.[29,30] The average urinary excretion is reported to be between 0.03 and 4% of the ingested dose.[31] Despite the increase of serum antioxidant level by 14% after ingestion of red wine,[32] the maximum plasma concentration are reported to be anywhere between 1.4 and 592 nmol L^{-1}.[31]

Cells were pre-treated for 45 min, then washed three times with growth media before the addition of dopamine (DA, Sigma, St Louis, MO, USA) or

DOI: 10.1002/jsfa

amyloid β_{25-35} (Aβ, Sigma) or with fresh growth media for the untreated controls.

For DA treatment, growth medium was removed and replaced with fresh growth medium containing 1 mmol L^{-1} DA. The cells were exposed to DA for 4 h, and DA solution was changed once each hour during the 4 h. For Aβ treatment, 100 μmol L^{-1} of amyloid β_{25-35} was used to preincubate the cells for 24 h. Following these incubations the cells were evaluated for either (i) alterations in Ca^{2+} flux or (ii) viability (see below). For determination of Ca^{2+} flux, cells were washed three times with growth medium, and loaded for 40 min with Fura 2 acetoxymethyl ester (Fura-2 AM, CalbiochemNovabiochem, La Jolla, CA, USA) in D-MEM containing 1% FBS. Fura-containing medium was removed, cells were incubated for 30 min in Kreb's–Ringer HEPES (KRH: 1.3 mmol L^{-1} $CaCl_2$; 131 mmol L^{-1} NaCl; 1.3 mmol L^{-1} $MgSO_4$; 5.0 mmol L^{-1} KCl; 0.4 mmol L^{-1} KH_2PO; 6.0 mmol L^{-1} glucose; 20 mmol L^{-1} HEPES; pH 7.4) buffer and tested immediately afterwards.

Ca^{2+} imaging

Ca^{2+} image analysis was performed as previously described.[23] Briefly, the transfected cells were treated under one of the above experimental conditions, washed with fresh media, and loaded with Fura-2 AM (2 μmol L^{-1}) in loading medium (99% DMEM 1% FBS) for 40 min at 37 °C with 5% CO_2 in air incubator (HERA Cell 150, Heraeus, Germany), followed by a 30 min incubation in Kreb's–Ringer HEPES buffer. A coverslip with treated COS-7 cells was inserted into a Leiden coverslip dish and 0.9 mL of KRH buffer added. This was placed into a Medical Systems Corp open perfusion micro-incubator with temperature control, which was mounted on the stage of an Olympus IMT-2 microscope and illuminated with a fluorescent light source. All tests were carried out at 37 °C. Simultaneous images of cells at λ_{ex} 340/380 nm and λ_{em} 510 nm were captured by using Simple PCI, a software package designed by Compix (Mars, PA, USA), to control a MAC 2000 filter/shutter controller (Ludl Electronic Products, Hawthorne, NY, USA). Pixel-by-pixel comparisons of the captured images were carried out and a ratio of Ca^{2+}-bound Fura (340 nm excitation wavelength) to unbound Fura (380 nm excitation wavelength) was generated for each pair of images. Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined using the method of Grynkiewicz *et al*.[33] The interval between capture of images ranged from 1.0 to 1.5 s. After approximately 45 s, the cells were depolarised by the addition of 750 μmol L^{-1} Oxo in the presence of 30 mmol L^{-1} KCl, and image capture continued for an additional 6 min.

Response baseline, peak response (% increase), and 80% recovery

Response was determined by examining whether a cell showed increases in $[Ca^{2+}]_i$ to Oxo by >30% over baseline.[20,22] Only those cells (>80 % of total cells) that showed this magnitude of response were considered for further analysis.

Baseline levels were determined by averaging the $[Ca^{2+}]_i$ seen before Oxo-induced calcium flux (Fig. 2A).

Peak values (Fig. 2B) were the highest $[Ca^{2+}]_i$ following Oxo stimulation. Percent increase was determined by the formula: (peak − baseline)/peak ×100.

Recovery was determined by assessing the time (within 300 s) for the Ca^{2+} levels to return to 20% of the increase following Oxo stimulation in the cells that responded (Fig. 2C).

Viability

Viability of the cells at 24 h following a 4 h and 24 h exposure to 1 mmol L^{-1} DA and 100 μmol L^{-1} Aβ was determined using the Live/Dead Eukolight Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA) without detaching the cells from the 35 mm plates. Cells were stained for 30 min in D-PBS with 1 μmol L^{-1} calcein AM and 2 μmol L^{-1} ethidium homodimer (EthD-1) following the instructions in the kit. Between 200 and 1000 cells per well were differentially counted using the Olympus microscope with a DAPI/FITC/Texas Red filter block (Chroma Technology Corp., VT, USA), and the results were recorded as % viable cells. Live cells, stained green by the calcein, were counted versus the dead cells, stained red by EthD-1.

Statistical data analysis

Baseline and % increase of Ca^{2+}, as well as viability, were analysed by analysis of variance using Systat (SPSS, Inc., Chicago, IL, USA) and Tukey's Honest Significant Difference (HSD) multiple comparisons at the 5% level. Approximately 100 cells were run per condition from three separate experiments. Cells that were counted as responders exhibited a $\geq$ 30% increase in depolarisation following stimulation by Oxo. Of the responders, cells that were able to reduce $[Ca^{2+}]_i$ within 300 s were regarded as recovered and the % recovery for each condition was calculated.

RESULTS

Identification and characterisation of compounds

The Boysenberry (cv. Riwaka Choice) and blackcurrant (cv. Ben Ard) extracts were analysed by reversed-phase high performance chromatography (RP-HPLC). HPLC analysis confirmed the presence of the four major anthocyanins, cyanidin glucoside, cyanidin rutinoside, cyanidin sophoroside and cyanidin glucorutinoside in the Boysenberry anthocyanin extract (Fig. 2C). The Ben Ard blackcurrant anthocyanin extract showed the presence of cyanidin glucoside, cyanidin rutinoside, delphinidin glucoside and delphinidin rutinoside (Fig. 2A). Minor components

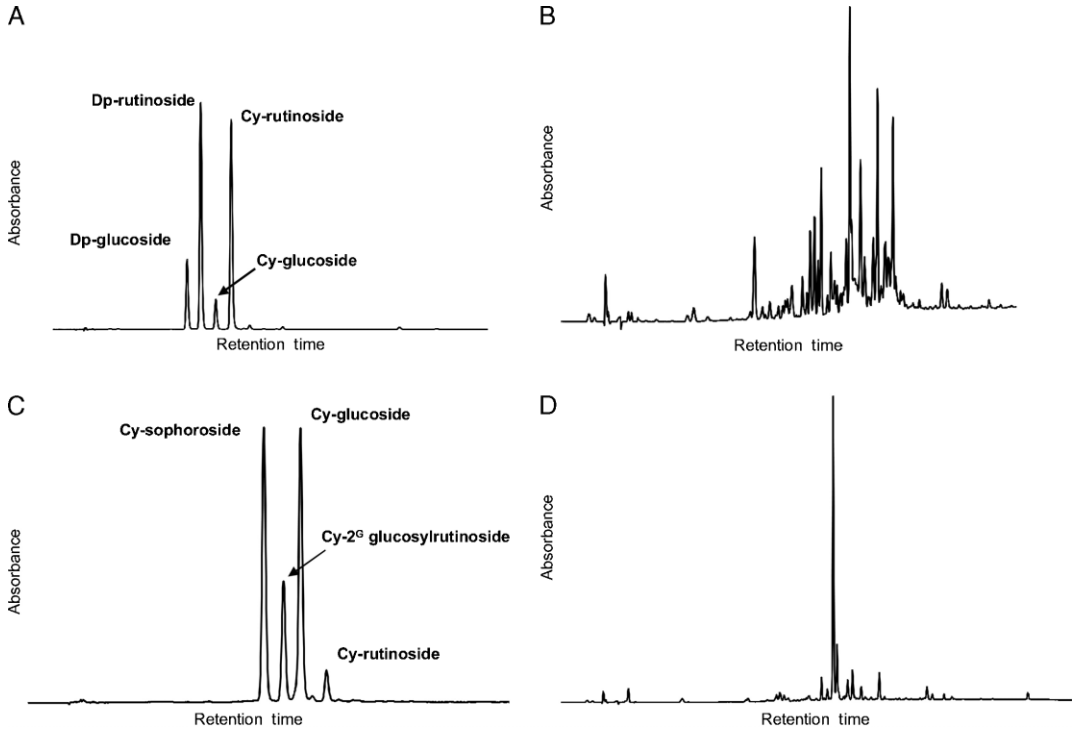

Figure 1. HPLC of extracts. (A) Blackcurrant anthocyanins; (B) blackcurrant polyphenolics; (C) Boysenberry anthocyanins; and (D) Boysenberry polyphenolics.

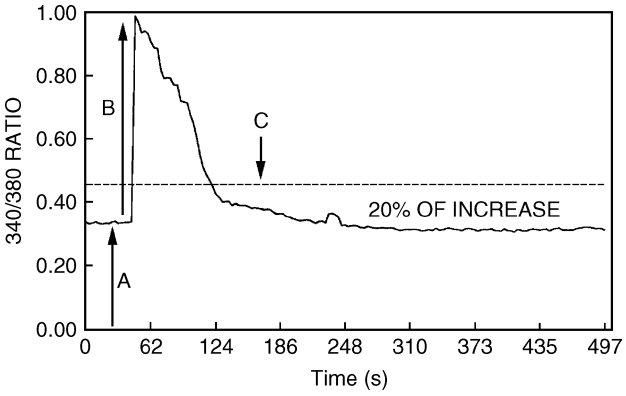

Figure 2. Typical image analysis response ratios for control cells transfected with MAChR receptor (See Materials and Methods). Three parameters were selected in order to analyse $[Ca^{2+}]_i$ levels. Level A, baseline: the initial intracellular Ca^{2+} level before depolarising the cell with 30 mmol L^{-1} KCl. Level B, the % increase (peak response): the increase in intracellular Ca^{2+} compared to the baseline, following oxo stimulation. Level C, recovery: the ability of the cell to remove Ca^{2+} extracellularly within 20% of the baseline level[21].

were also found in both extracts and confirmed by liquid chromatography–mass spectrometry (LC–MS). These were derivatives of anthocyanins and probably produced during the extraction and purification procedure. The chromatograms of both Ben Ard blackcurrant and Riwaka Choice Boysenberry phenolic extracts demonstrated a very complex mixture of phenolic compounds (Fig. 2B and D). We have also measured the total anthocyanin and phenolic concentrations of the four extracts. The values are in mg g^{-1}. The total anthocyanin in ByAcy and BcAcy are 261 and 451, respectively, whereas the total polyphenols in ByPhen and BcPhen are 241 and 474, respectively.

Ca^{2+} homeostasis in COS-7 cells

No treatment

The results demonstrated that in the absence of pretreatment with polyphenols there were significant effects of both DA and Aβ on recovery of the M1-transfected cells (e.g. control vs. DA- or Aβ-treated cells with no extract pre-treatment) (Fig. 1A and B, respectively). However, subsequent analysis revealed that there were no differences in calcium levels prior to Oxo stimulation between DA and Aβ treatment and control cells (data not shown).

Pretreatment

A range of concentrations of By and Bc fractions were used in a pilot study (data not included) and one concentration was chosen as a working standard. Both ByAcy and BcAcy were used at 250 µg mL^{-1}, whereas ByPhen and BcPhen were used at the 100 µg mL^{-1} concentration. Pretreatment of COS-7 cells with each of the fractions followed by exposure to DA affected Ca^{2+} recovery (Fig. 3A). A similar type of Ca^{2+} recovery profile was found after Aβ treatment (Fig. 3B). Overall, the results revealed that both anthocyanins (ByAcy and BcAcy) and phenolics (ByPhen and BcPhen) were effective in antagonising the effects of the oxidative insults in the treated cells (Fig. 3).

Viability

The possible protective effects of anthocyanins and other phenolic extracts of Boysenberry and

DOI: 10.1002/jsfa

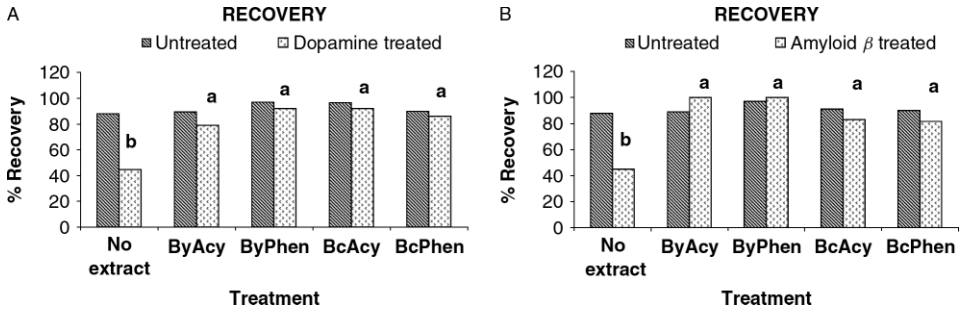

Figure 3. Percent Ca^{2+} recovery of M1-transfected control COS-7 cells exposed to dopamine (A) or amyloid β_{25-35} (B) after pre-treatment with Boysenberry or blackcurrant polyphenolics or left untreated (no extract). a = cells pre-treated with Boysenberry or blackcurrant and dopamine or amyloid β_{25-35} significantly different from untreated (no extract) cells exposed to dopamine or amyloid β_{25-35}. b = no extract control significantly different after dopamine or amyloid β_{25-35} exposure ($P < 0.05$).

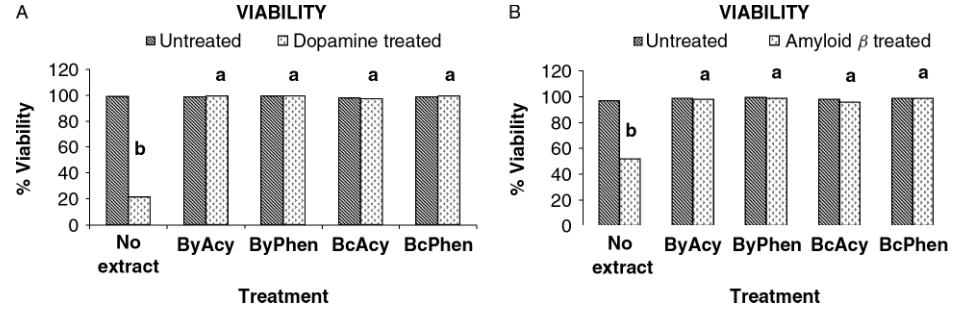

Figure 4. Percent viability of M1-transfected control COS-7 cells exposed to dopamine (A) or amyloid β_{25-35} (B) after pre-treatment with Boysenberry or blackcurrant polyphenolics or left untreated (no extract). a = cells pre-treated with Boysenberry or blackcurrant and dopamine or amyloid β_{25-35} significantly different from untreated (no extract) cells exposed to dopamine or amyloid β_{25-35}. b = no extract control significantly different after dopamine or amyloid β_{25-35} exposure ($P < 0.05$).

blackcurrant against DA- and Aβ-induced cell death were assessed. The cells were pre-treated with the same concentration of fraction used in the imaging experiments. Cell survival was dramatically reduced by DA and Aβ treatment (approximately 78% and 45% of untreated controls, Fig. 4A and B). Results demonstrated that all fractions gave very significant levels of protection in cell viability (Fig. 4A and B). An average cell death of 3% was observed in the absence of the OS, over the duration of the experiments.

DISCUSSION

Oxidative stress is considered a risk factor in the incidence and progression of cognitive decline in various neurodegenerative processes, such as Alzheimer's and Parkinson's diseases.[7,8] This work provides further evidence for the protective effects of berries against the cytotoxic or neurotoxic effect of dopamine and amyloid β_{25-35} in brain cells. Recently, food-derived antioxidants, such as polyphenols, have received growing attention, because of their chemopreventive potential against oxidative damage.

The loss of calcium homeostasis is an important factor in neuronal ageing and alterations in learning and memory.[34] Decline in calcium recovery can lead to increased intracellular Ca^{2+} which, in turn, can produce additional free-radical activity and further cell degeneration. Both DA and Aβ have been shown to significantly disrupt calcium (Ca^{2+}) regulation in M-1 transfected COS-7 cells.[20,21] Heo and Lee.[35] have shown that Aβ_{25-35} could trigger elevation of $[Ca^{2+}]_i$ levels in 90% of the cells. As MAChRs are intimately involved in various aspects of both neuronal (AβPP procession[36]) and vascular functioning,[37] the disruption of calcium homeostasis is particularly important in ageing and AD.[34] In a recent study with APP/PS1 transgenic mice (with the genetic mutation that promotes the production of Aβ), 8 months blueberry supplementation experiment showed performance similar to that of non-transgenic mice and significantly better than that of non-supplement-treated transgenic mice.[38] Moreover, the OS sensitive MAChRs[20] are predominantly located in the striatum,[39] as well as memory control areas.[40] Long-term exposure to DA, which generates OS and subsequent Ca^{2+} dysregulation, may also induce decreases in MAChR concentrations in ageing.[41] The results from the present study have demonstrated that polyphenolics from Boysenberry and blackcurrant have shown a degree of protection against the deleterious effects of DA and Aβ. There was a trend to suggest that blackcurrant polyphenolics showed a higher protective effect against DA, whereas Boysenberry polyphenolics have a stronger effect against Aβ.

In the present study, both DA and Aβ_{25-35} decreased the cell viability (approximately 78% and 45% of control, respectively), and their cytotoxic effects were attenuated in the presence of berry

polyphenols. It has been demonstrated previously that Aβ_{25-35} decreased neuronal cell viability (approximately 72%), and cocoa extracts, epicatechin and catechin were able to reduce these cytotoxic effects.[35] In addition, the synergistic cytoprotective effect of two phenolics was demonstrated. The research of Joseph and Fisher,[22] Joseph *et al*.[42] and Andres-Lacueva[43] strongly suggests that dietary supplementation with fruit or vegetable extracts might decrease the enhanced vulnerability to oxidative stress and inflammation leading to improving motor and cognitive behaviour.

The current findings provide evidence to suggest that the deleterious effect of compounds such as DA or Aβ on neurons can be reduced by polyphenolic fractions of Boysenberry and blackcurrant. Our findings add to the body of evidence implying that berries are edible superstars that may protect against oxidative stress-induced disorders, including ageing.[44]

ACKNOWLEDGEMENTS

This research was supported by HortResearch internal research project (KDF603-13), the New Zealand Boysenberry Council and Blackcurrants New Zealand Ltd. Thanks to Harry Martin and Margot Skinner for critically reading the manuscript.

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