Journal of Functional Foods 22 (2016) 363–375

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jff

Anthocyanin-rich phenolic extracts of purple root vegetables inhibit pro-inflammatory cytokines induced by H_2O_2 and enhance antioxidant enzyme activities in Caco-2 cells

Hua Zhang, Ronghua Liu, Rong Tsao *

Guelph Research and Development Centre, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, ON N1G 5C9, Canada

ARTICLE INFO

Article history:
Received 15 October 2015
Received in revised form 11 December 2015
Accepted 5 January 2016
Available online 12 February 2016

Keywords:
Purple carrot
Purple potato
Phenolics
Anthocyanins
Antioxidant activity
Anti-inflammatory activity

ABSTRACT

Highly pigmented root vegetables are ideal sources of phytochemical antioxidants particularly anthocyanins. The objective of this study was to characterise the phenolic composition in a purple variety of carrot and potato by examining the aglycone profiles and their antioxidative stress activity. Cyanidin and petunidin were the dominant phenolics among 11 identified aglycones in carrot and potato, respectively. The anthocyanin-rich phenolic extracts not only showed strong antioxidant activities by direct radical scavenging, but more importantly they significantly reduced H_2O_2-induced interleukins (IL)-1β, IL-6, IL-8 and tumour necrosis factor (TNF)-α in Caco-2 cells. In addition, the extracts were able to restore the intrinsic antioxidant defences as seen in significantly elevated antioxidant enzyme activities and glutathione concentration. These results suggest that purple varieties of carrots and potatoes rich in anthocyanins can ameliorate oxidative stress mediated intestinal inflammatory responses, leading to restoration of the impaired redox balance, ultimately improved health.

1. Introduction

In recent years, chronic non-communicable diseases such as cardiovascular disease, cancer and diabetes have become the leading causes of death around the world (Murray et al., 2012). Changing lifestyle due to improved socio-economic conditions, medical treatments, and advancement in science and technology has been attributed to the high prevalence of these diseases, particularly in developed countries. The most contributing factors include diet, physical activity, smoking and body mass index (Ford et al., 2009). Lack of intake of antioxidant-rich fruits and vegetables and increased exposure to environmental pollutants have led to imbalance between the

* *Corresponding author.* Guelph Research and Development Centre, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, Ontario, Canada, N1G 5C9. Tel. +1 226 217 8180; fax: +1 226 217 8183.
E-mail address: Rong.Cao@agr.gc.ca (R. Tsao).

Abbreviations: PC, purple carrot; PP, purple potato; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; GR, glutathione reductase; GPx, glutathione peroxidase; GSH, glutathione; GI, gastrointestinal; IBD, inflammatory bowel disease; GA, garlic acid; DPPH, 2,2-diphenyl-l-picrylhydrazyl; AAPH, 2,2′-azobis-(2-methylpropionamidine) dihydrochloride; SPC, solid phase extraction; TPC, total phenolic content; TAC, total anthocyanin content; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; TE, Trolox equivalent; AAE, L-ascorbic acid equivalent; HBSS, Hank's balanced salt solution; FBS, foetal bovine serum; CAA, cellular antioxidant activity; DCFH, 2′,7′-dichlorofluorescein diacetate; IL, interleukin; TNF, tumour necrosis factor; PBS, phosphate buffer solution; RT-PCR, real time-polymerase chain reaction

http://dx.doi.org/10.1016/j.jff.2016.01.004
1756-4646/

oxidative stress and antioxidants and the inability to restore redox homeostasis in the human body, which can further develop into chronic inflammation. Long term inflammation is the culprit of most chronic and degenerative diseases including cardiovascular disease (Nishiyama, Ikeda, Haramaki, Yoshida, & Imaizumi, 1998), renal diseases (Baud & Ardaillou, 1993), diabetes mellitus type 2 (Bondor et al., 2015), hypertension (Rubattu et al., 2015), chronic respiratory disease (Moylan & Reid, 2007), Alzheimer disease (Hritcu, Stefan, Brandsch, & Mihasan, 2015) as well as cancer (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). Excessive accumulation of reactive oxygen species (ROS) or depletion of intermediates with antioxidant capacity alters the redox balance and leads to oxidative stress. Exogenous ROS, generated by exposure to various stimuli such as pollutants, smoke, drugs, xenobiotics, ionizing radiation heavy metal ions, are all causative factors for oxidative stress (Chen, Muramoto, & Yamauchi, 1995). The physiological level of ROS is normally beneficial for the human body system, such as in fighting against pathogen invasion, depleting malignant cells or improving wound healing. Excess ROS, however, damage vital biological molecules, especially lipids, proteins and DNA (Kaneko et al., 2008). Living organisms have an intrinsic cellular redox enzyme system that contains enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR), however, this defensive mechanism is usually deficient in people suffering from chronic diseases (Pham-Huy, He, & Pham-Huy, 2008). Furthermore, oxidative stress induces overproduction of pro-inflammatory cytokines and augmentation of inflammatory responses caused by ROS which upregulates cellular inflammatory signalling transductions (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014). Eventually, the sustained inflammation can in turn contribute to developing an irreversible disequilibrium redox state in the body leading to permanent tissue damage.

As the primary digestion system, the gastrointestinal (GI) tract is prone to the exogenous oxidant attack due to direct encounter with various stimuli, including pollutants, smoking, drugs, xenobiotics, food toxins and heavy metal ions and intestinal microflora (Cross, Halliwell, & Allen, 1984). When the GI tract is under oxidative stress, the excessively produced ROS damages the intestinal mucosa and provokes immune response, leading to chronic inflammation. This eventually causes permanent mucosal injury and mediates development of inflammatory gut diseases such as inflammatory bowel diseases (IBD) (Bhattacharyya et al., 2014). IBD, e.g. both Crohn's disease and ulcerative colitis (UC), involves chronic inflammation along the GI tract resulting from relapsing activation of local innate immune response. The persistent ROS production and defective antioxidant enzyme activities have been observed and considered as contributors to the pathological mechanisms that lead to IBD (Colgan & Taylor, 2010). Intervention studies have shown that dietary antioxidants owing to their ROS scavenging capacity, are able to ameliorate inflammation and mucosal injury, and therefore can be used potentially to treat patients with complications of gut inflammation (Farzaei, Rahimi, & Abdollahi, 2015; Kruidenier & Verspaget, 1998). However, since IBD patients are experiencing conditions of mal-absorption of nutrients, it is difficult to ensure the right amount of antioxidants absorbed by these patients (Han, Burke, Baldassano, Rombeau, & Lichtenstein, 1999). In addition, inadequate intake or overdose of dietary antioxidants can have potential counteractive effects on disease conditions (Dryden, Deaciuc, Arteel, & McClain, 2005). Hence, finding a safe dietary source of antioxidants is critical for developing an effective dietary approach for IBD. In human, a beneficial daily intake of polyphenols, including flavonoids, has been estimated to be from 50 mg to 1 g for chronic disease prevention (Scalbert & Williamson, 2000). This indicates that polyphenols as a main source of natural antioxidants are quite safe to consume for people who suffer from gut inflammation. Dietary polyphenols have been found to be effective in treating gut inflammation in recent cell and animal studies (Romier, Schneider, Larondelle, & During, 2009). Polyphenols, including flavonoids and anthocyanins, are hydrophilic phytochemicals found widely in plant foods, especially highly pigmented fruits (Khanizadeh et al., 2008), vegetables (Li et al., 2011, 2012) and grains (Chen et al., 2015; Chung & Woo, 2001; Tang et al., 2015), and are strong antioxidants that may help reduce the development of chronic diseases.

Anthocyanins are a group of natural phytopigments belonging to the flavonoid group of polyphenols and are found in high amounts in fruits and vegetables (Nunez & Magnuson, 2013). Anthocyanins engage in functions such as alleviating oxidative stress processes, inhibiting inflammatory responses and regulating cellular signalling transductions, making them good protectants against a variety of degenerative diseases including cardiovascular disease and cancer (Butelli et al., 2008; Hou, Fujii, Terahara, & Yoshimoto, 2004). Dietary polyphenols in general have been studied extensively for their modulatory effects on intestinal inflammation (Romier et al., 2009), however, those of anthocyanins have only recently been investigated. A recent study indicates that anthocyanin supplementation improves the antioxidant status in humans and it prevents development of chronic diseases (Kuntz et al., 2014). Tsuda (2012) determined the therapeutic potential of dietary anthocyanins in ameliorating diabetes and obesity through reducing hyperglycaemia and inflammation. Moreover, intake of anthocyanin-rich bilberries was found to reduce the expression of two pro-inflammatory cytokines, tumour necrosis factor (TNF)-α and interferon (IFN)-γ, in a chemically induced colitis mouse model (Piberger et al., 2011), and to ameliorate the disease severity of UC patients (Biedermann et al., 2013). However, Janšáková et al. (2015) showed that the antioxidant status and disease conditions were not improved by intake of anthocyanin-rich diet in a chemically induced colitis mouse model. Such inconsistency between animal models means that it is necessary to first study the anti-inflammation mechanisms of anthocyanins at the cellular level. A direct immunomodulatory effect of anthocyanin-rich blueberry extract in human Caco-2 cells has been observed (Taverniti et al., 2014). However, the potential effects of anthocyanins on the oxidative stress mediated inflammatory responses have not been reported. Small berry fruits are the main dietary source of anthocyanins, hence, the majority of studies on the health benefits of anthocyanins are based on the administration of berries or their extracts (Tsuda, 2012). Nevertheless, these fruits are seasonal and costly, and not normally consumed daily in significant amounts. On the other hand, vegetables rich in anthocyanins, particularly root vegetables such as purple carrots and potatoes are advantageous compared to small berry fruits and

other leafy vegetables (Hu, Tsao, Liu, Alan Sullivan, & McDonald, 2012). Root vegetables rich in anthocyanins are inexpensive, not limited by season and easy to preserve, and they can be readily incorporated with grains and cereals to form a variety of food products (Hu et al., 2012). Insofar, only a few studies have been carried out to evaluate the phytochemical contents of purple carrots or potatoes and their potential health effects (Albishi, John, Al-Khalifa, & Shahidi, 2013; Han et al., 2006; Leja et al., 2013; Navarre, Pillai, Shakya, & Holden, 2011; Sun, Simon, & Tanumihardjo, 2009). The antioxidant and anti-inflammatory effects of phenolic compounds, including anthocyanins prepared from root vegetables, and the molecular mechanism of these compounds have not been fully studied.

Therefore, the present study aims to assess the capacity of anthocyanin-rich purple root vegetables to reduce oxidative stress in a model gut epithelial system using Caco-2 cells. We characterized the phenolic composition of a purple carrot and a purple potato, and then examined their antioxidant activities using chemical assays and measured various pro-inflammatory cytokines induced by hydrogen peroxide on ROS. The effects of these phenolics on antioxidant enzymes were also assessed. These effects were primarily studied in the preventive model described below.

2. Materials and methods

2.1. Chemicals and reagents

All standard reference materials including chlorogenic acid, kaempferol, ferulic acid, caffeic acid, myricetin, quercetin, and cyanidin, delphinidin, pelargonidin, peonidin, petunidin and malvidin were obtained from Sigma (St. Louis, MO, USA). The reagents such as gallic acid, Folin–Ciocalteu's phenol reagent, fluorescein, 2,2-diphenyl-1-picrylhydrazyl (DPPH), trolox and 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) were also purchased from Sigma (St. Louis, MO, USA). Sodium acetate, ferric chloride hexahydrate, sodium phosphate monobasic, sodium phosphate dibasic and HPLC-grade solvents, including methanol, formic acid and hydrochloric acid (HCl) were purchased from EMD Chemicals (Gibbstown, NJ, USA), VWR (Westchester, PA, USA) and Caledon Labs (Georgetown, ON, Canada) unless otherwise specified. All solvents were HPLC-grade from Caledon Labs.

2.2. Extraction and purification

Phenolic extracts of purple carrots or potatoes were prepared using acidified aqueous methanol extraction. Fresh purple carrots (Purple Haze) or potatoes (Machintosh) were harvested from the Muck Research Station and Elora Research Station of the University of Guelph (Elora, ON, Canada). Samples were first cut into $3 \times 3 \times 3$ cm^3 cubes, freeze-dried, and then ground into powder using a commercial grinder (Stanley Black & Decker Inc., New Britain, CT, USA). The dried powder (2 g) samples were accurately weighed and extracted with 30 mL 70% methanol containing 0.1% HCl (v/v) by shaking on a rotary shaker (Scientific Industries Inc., Bohemia, NY, USA) at $20 \times g$ for 2 h at room temperature followed by 15 min ultra-sonication (VWR, Mississauga, ON, Canada). The supernatant was collected after centrifuging at $4180 \times g$ for 15 min (Eppendorf centrifuge 5810R, Brinkman Instruments Inc., Westbury, NY, USA). The residue was re-extracted twice by 10 mL of the same solvent. The combined supernatant was made up to 40 mL, and used as crude extract for analyses of total phenolic content (TPC), total anthocyanin content (TAC) as well as for ORAC (oxygen radical absorbance capacity), FRAP (ferric reducing antioxidant power) and DPPH antioxidant assays.

Further purification of the crude extract was necessary for profiling and characterization of the phenolic compounds within these foods, and for measuring cell-based antioxidant activity. Briefly, 40 g of dried powder were extracted in 1 L 70% methanol (v/v) for overnight at room temperature, filtered through Whatman filter paper (pore size 11 μm, VWR), and the residue was re-extracted twice, each using 400 mL of the same solvent. The combined supernatant was concentrated to 700 mL under reduced pressure at 50 °C, and further purified using Strata™-X polymeric solid phase extraction (SPE) cartridges (2 g, Phenomenex, Torrance, CA, USA) to remove undesired polar components such as proteins or sugars. The SPE cartridge was first preconditioned with 20 mL of 100% methanol and equilibrated with 20 mL of water. Then 10 mL of concentrated extract were slowly loaded onto the cartridge and washed by 20 mL of water. The phenolic fraction was collected by eluting with 20 mL of 100% methanol. The eluent was first evaporated using Refrigerated CentriVap (Labcon Co., Kansas, MO, USA) and then freeze-dried. The purified phenolic extracts were used for HPLC analysis and in cell culture studies.

2.3. Total phenolic and anthocyanin contents

The total phenolic content (TPC) of purple carrot or potato extracts was determined using the Folin–Ciocalteu assay modified from Li et al. (2012). Briefly, 125 μL of 10x diluted Folin-Ciocalteu phenol reagent were added into each well of a 96-well plate preloaded with 25 μL of a sample or standard and mixed. After 10 min of reaction, 125 μL of 7.5% sodium carbonate (w/v) were added into each well and the assay mix was then incubated for another 60 min before absorbance measured at 765 nm by a UV/vis spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). TPC was expressed as mg of gallic acid (GA) equivalents/ g DW. The total anthocyanin content (TAC) was measured using a pH differential method (Giusti & Wrolstad, 2001). Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 or 4.5, respectively using the same spectrophotometer. TAC was expressed as mg cyanindin-3-glucoside equivalents/ g DW.

2.4. Antioxidant assays

ORAC assay was carried out in an automated spectrofluorometer PLX 800 (BioTek Instruments Inc., Winooski, CT, USA) with a dual scanning microplate reader according to the protocol described by Li et al. (2012). ORAC value was expressed as micromoles of Trolox equivalents per gram of DW sample (μmol TE/g DW) calculated based on Trolox standard calibration curve with linear range of 6.25–100 μM ($r^2 = 0.99$).

DPPH assay was also done according to the method described by Li et al. (2012). The DPPH radical scavenging activity

was expressed as μmol Trolox equivalents (TE) (μmol TE/g DW) calculated based on Trolox standard calibration curve with linear range of 62.5–1000 μM ($r^2 = 1$).

FRAP assay also followed the protocols of Li et al. (2012). The FRAP value was expressed as micromoles of L-ascorbic acid equivalent (AAE) per gram of DW (μmol AAE/g) and calculated on the basis of the AAE standard curve with linear range of 62.5–1000 μM ($r^2 = 1$).

2.5. *Identification and quantification of phenolics*

The SPE-purified extract of purple carrot or potato was dissolved in 70% methanol (v/v) with a final concentration of 2 mg/mL and hydrolysed with 6M HCl and heated at 85 °C for 2 h. The samples were subjected to HPLC analysis after filtered through a 0.2 μm PTFE membrane filter (VWR, Mississauga, ON, Canada). HPLC analysis was carried out on an Agilent 1100 series HPLC system consisting of an auto sampler, a degasser, a quaternary pump, and a diode array detector (DAD). Data were analysed using Agilent ChemStation software. Compounds were separated on a Phenomenex Luna phenyl-hexyl column (5 μm, 250 × 4.6 mm). A binary mobile phase consisting of 5% formic acid in water (v/v) (solvent A) and 95% methanol mixed with 5% acetonitrile (v/v) (solvent B) was used. The solvent gradient used was as follows: 0–40 min, 80% B; 40–42 min, 80–100% B; 42–44 min, 100% B; 44–44.5 min, 100–0% B. Peaks were monitored at 280 and 520 nm. Quantification of phenolic compounds was performed with external standards, using linear curves generated between 0.125 and 50 μg/mL.

2.6. *Cell culture and induced oxidative stress*

The Caco-2 human intestinal cell line (American Type Culture Collection, Rockville, MD, USA) was used in *in vitro* studies. The cells were grown in MEM medium (Gibco, Burlington, ON, Canada) with 20% foetal bovine serum (FBS; Hyclone Co., Logan, UT, USA) and 50 units/mL of penicillin-streptomycin (Gibco), and incubated at 37 °C in 5% CO_2. Cell medium was replaced every 3 days. Cell passages 20–50 were used in all monolayers for this experiment.

The oxidative stress in Caco-2 cells was induced by adding 1 mM H_2O_2 as described in Shi, Kovacs-Nolan, Jiang, Tsao, & Mine (2014). Caco-2 cells were cultured in 24 or 48-well culture plates (Corning Costar) at 2×10^5 cells/mL and grown for 5–7 days to reach 80–90% confluence. After washed twice with Hank's balanced salt solution (HBSS), cells were treated with various concentrations of purified extract of purple carrot or potato prepared in MEM with 5% foetal bovine serum (FBS). Cells were either pre-incubated with the extract for 30 min or 1 h before adding H_2O_2 or with the extract being added at the same time of H_2O_2. H_2O_2 was added to the well to a final concentration of 1 mM and incubated for 6 h. The supernatants of the negative control cells (untreated), positive control cells (treated with H_2O_2 alone) and treated cells were then collected and stored at –80 °C for measuring IL-8 release. The negative and positive control cells as well as cells pre-incubated with the extracts were washed twice with ice-cold phosphate buffer solution (PBS) pH 7.0 and then stored at –80 °C for future real time-polymerase chain reaction (RT-PCR) analyses. Protein concentration of cell lysate was determined by DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as standard.

2.7. *Cellular antioxidant activity (CAA)*

The antioxidant activity at the cellular level was evaluated by the method described by (Li, Deng, Liu, Loewen, & Tsao, 2014) with slight modifications. Caco-2 cells were grown in a 96-well black/clear flat bottom plate (Corning, Costar) until reaching monolayer. Cells were treated with 200 μL of purple carrot or potato purified extract at different concentrations (5, 10, 50 and 100 μg/mL) and 100 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA) at the same time, and then incubated for 30 min at 37 °C. After washing twice with PBS, cell media were removed and replaced with 100 μL of 50 μM H_2O_2. The fluorescence intensity was measured using a fluorescence spectrophotometer PLX800 at an excitation wavelength of 485 nm and an emission wavelength of 528 nm for 1 h. The results were calculated according to the following equation:

$$\text{Eq. CAA unit (\%)} = \left(100 - \left(\int \text{SA} - \int \text{BA}\right) \Big/ \int \text{CA}\right) \times 100$$

where ∫SA is the integrated area under the sample fluorescence versus time curve, ∫BA and ∫CA are the integrated area from the blank and control curves, respectively.

2.8. *Biomarker assays*

2.8.1. *Interleukin-8 immunoassay*

IL-8 concentration in cell supernatants was determined by using a human IL-8 enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instructions (eBioscience, Inc., San Diego, CA, USA). Mouse anti-human IL-8 antibodies were coated in 100 μL of PBS buffer in a 96-well plate (Corning, Costar) incubated overnight at 4 °C. The wells were washed 3 times with 300 μL wash buffer and then blocked with 200 μL blocking buffer for 1 h. The plate was washed 3 times between each of following steps. The 100 μL sample and IL-8 standard were added into the wells and incubated for 2 h at room temperature. A 100 μL of secondary anti-human IL-8 antibody was added into the well for an additional 1 h incubation, followed by 30 min incubation with 100 μL avidin-horseradish peroxidase conjugate (Av-HRP). A 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma Aldrich) for colour development. This reaction was stopped by adding 50 μL 0.17 M H_3PO_4. The absorbance was measured at 450 nm using EL340 UV/vis spectrophotometer (BioTek). The concentration (pg/mL) of IL-8 was extrapolated from the standard calibration curve and expressed as IL-8 percent relative to positive control.

2.8.2. *RNA isolation and real-time RT-PCR*

Pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α were analysed by RT-PCR. After 6 h incubation with H_2O_2, cells were rinsed twice with 1 mL/well of PBS at each time and then lysed by lysis buffer. The total RNA was purified and extracted from cell lysate using the Perfect Pure RNA Cultured Cell Kit (5 Prime, Gaithersburg, MD, USA) according to the manufacturer's instructions. The quantity of the RNA was determined by measuring the A260 and A280 (NanoDrop® ND-1000; Thermo

Scientific, Wilmington, DE, USA). One microgram of total RNA was reverse transcribed into cDNA using a qScript™ cDNA Synthesis Kit (Quanta Biosciences, Inc., Gaithersburg, MD, USA). RT-PCR was carried out using iQ SYBR Green Supermix (Quanta Biosciences) on Applied Biosystems 7500 Fast and 7500 RT-PCR system (Thermo Scientific, Burlington, ON, Canada) using the following conditions: 45 cycles of denaturisation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min using the primers listed in supplemental Table S1. The gene expression levels were calculated relative to the expression of the gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Results were presented as fold expression change relative to the negative control representing untreated samples.

2.9. Determination of intracellular antioxidant enzyme activity

Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were measured using colorimetric Assay Kits (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, Caco-2 cells in a 24-well plate were pre-incubated with or without 50 and 100 μg/mL extracts for 30 min, and then H_2O_2 was directly added into the wells to reach a final concentration of 1 mM and incubated for 6 h. At the end of this period, the cells were washed twice with PBS and then collected into 1.5 mL Eppendorf. And then, the lysate was sonicated in sample buffer provided in the kits. The sample buffer prepared for SOD assay contained 20 mM pH 7.2 HEPES, 1 mM ethylene glycol tetraacetic acid (EGTA), 210 mM mannitol and 70 mM sucrose. The sonicated mixture was centrifuged at 10,000 × *g* for 15 min at 4 °C, and the supernatant collected. The activity of each enzyme was calculated in milliunits per mg of protein (mU/mg protein). The results were reported as percent of the negative control.

2.10. Determination of total intracellular glutathione

The total glutathione (GSH) was quantified using a flourometric enzyme immunoassay (Cayman Chemical) according to the manufacturer's instruction with slightly modifications. At the end of incubation, cells were washed twice with PBS, added 1 mL of cell-based lysis buffer into each well, and incubated for 15 min on an orbital shaker at room temperature. The cell lysate supernatants were centrifuged at 10,000 × *g* for 10 min at room temperature. An aliquot of the lysate (90 μL) of each sample was loaded into the corresponding wells of the black plate and mixed with 10 μL substrate solution. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Concentration of GSH was calculated against to a standard curve, and reported as % of the negative control.

2.11. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) of at least three independent experiments unless otherwise specified. Statistical analyses were carried out using GraphPad software (San Diego, CA, USA). The statistical significance of the data was determined using two-way ANOVA followed by Tukey's multiple-comparison test with a $P < 0.05$ taken as value of significance.

3. Results and discussion

3.1. Total phenolic and anthocyanin contents and antioxidant activities

TPC and TAC of the crude extracts of the purple carrot and potato are shown in Table 1. TPC of purple carrots was higher than that of potato, and the difference in TAC was even greater. While these are consistent with our previous observation (Li et al., 2012), they were different from other reported data as these phytochemicals are often significantly affected by genetic and environmental factors (Navarre et al., 2011; Reyes, Miller, & Cisneros-Zevallos, 2004; Xu, Li, Lu, Beta, & Hydamaka, 2009). According to the TPC and TAC results, anthocyanins were 59% and 26% of the total phenolic content, in purple carrot and potato, respectively, suggesting they are major phenolic compounds in both vegetables (Table 1).

The higher TPC and TAC in carrot also led to stronger antioxidant activities as measured in ORAC, DPPH and FRAP assays. This suggests that phenolics are contributors to the antioxidant activity of these two vegetables, and anthocyanins play a major role (Table 1). The results also point to the varied mode of antioxidant actions of these phenolics, as the three assays are based on different mechanisms (Dai & Mumper, 2010).

Table 1 – Total phenolic and anthocyanin contents, individual phenolic and anthocyanidin concentrations (mg/100 g DW) as well as antioxidant activities of the methanolic extracts of purple carrot and potato (n = 3)[a].

Peak #	Phenolics	Purple carrot	Purple potato
1	Chlorogenic acid	8.62 ± 0.17	1.84 ± 0.10
2	Ferulic acid	5.44 ± 0.47	1.27 ± 0.02
2′	Ferulic acid derivative[g]	21.23 ± 0.69	ND[b]
3	o-coumaric	4.52 ± 0.04	2.31 ± 0.08
4	Myricetin	10.24 ± 0.06	6.01 ± 0.11
5	Quercetin	ND[b]	3.88 ± 0.15
6	Kaempherol	4.58 ± 0.02	ND[b]
7	Pelargonidin	10.24 ± 0.08	2.88 ± 0.01
8	Cyanidin	161.4 ± 6.94	ND[b]
9	Petunidin	ND[b]	15.27 ± 1.72
10	Peonidin	ND[b]	ND[b]
11	Malvidin	ND[b]	ND[b]
	TPC[c]	394.33 ± 4.67	151.33 ± 0.33
	TAC[d]	232.15 ± 14.05	39 ± 2.65
	ORAC[e]	143.24 ± 3.70	97.26 ± 2.24
	FRAP[f]	75.01 ± 4.05	42.68 ± 0.66
	DPPH[e]	201.97 ± 2.44	47.56 ± 4.39

[a] Values are mean ± SEM, n = 3. Refer to Fig. 1 for peak numbers.
[b] ND: not detectable (<1 mg/100 g DW).
[c] TPC: total phenolic content (mg GA/100 g DW).
[d] TAC: total anthocyanin content (mg Cyanidin-3-glucoside/100 g DW).
[e] ORAC and DPPH: oxygen radical absorption capacity assay and 2,2-diphenyl-1-1 picrylhydrazyl assay (μmol TE/g DW).
[f] FRAP: ferric reducing antioxidant power assay (μmol AAE/g DW).
[g] By UV spectral data see (Supplemental Fig. S2).

3.2. *Characterization of phenolics in purple carrot and potato extracts*

The phenolic profiles of the two vegetables were obtained from a semi-purified extract using SPE. Characterization and identification of the individual phenolics including anthocyanins of the purple carrot and potato extracts were carried out based on similar HPLC-DAD methods previously used in our laboratory, according to congruent retention times and UV/Vis spectral data (Li et al., 2011; Wu et al., 2012). Natural phenolic compounds in fruits and vegetables often exist in glycosidic forms, and polyphenols particularly anthocyanins can be further acylated by aliphatic or aromatic acids which are less bioavailable than the non-acylated anthocyanins (Charron, Clevidence, Britz, & Novotny, 2007). Predominant peaks of phenolic compounds were identified by matching the retention times and UV/Vis spectra with those of the respective standards (Supplemental Fig. S1). Due to limited availability of standard reference materials, phenolics, particularly anthocyanins with complex glycosylation patterns were acid hydrolysed, and identification and quantification were done based on respective aglycones and individual standard curves. Fig. 1 shows the aglycone profiles of SPE semi-purified extracts of purple carrot and potato. The chlorogenic (peak 1) and ferulic acids (peak 2), o-coumaric acid (peak 3), and myricetin (peak 4) were found in both purple potato and carrot extracts, but a cinnamic acid derivative (peak 2′) and kaempferol (peak 6) were only found in purple carrot samples and quercetin (peak 5) was only found in purple potato samples. Peak 2′ was tentatively identified as one type of cinnamic acids only by similarity in UV spectrum (Supplemental Fig. S2), and further studies need to be carried out to validate this tentative identification. The anthocyanidin profiles of purple carrots or potatoes are relatively simpler than their phenolic profiles. Each vegetable sample showed only one predominant anthocyanidin in the extracts. The primary anthocyanidin found in the hydrolysed purple carrot and potato extract was cyanidin (peak 8) and petunidin (peak 9), respectively (Table 1). Pelargonidin (peak 7), peonidin (peak 10) and malvidin (peak 11) were also identified in purple carrot and potato extracts as very minor components (Fig. 1). Our results are consistent with previous findings, i.e. the majority of the anthocyanins are cyanidin glycosides in purple carrots and petunidin glycosides in purple potatoes (Li et al., 2012). The predominant anthocyanidins account for the largest proportion of phenolic contents at 59% and 26% in purple carrots and potatoes, respectively, based on TAC and TPC, and 77% and 54%, respectively, based on quantitative data derived from individual peaks from the chromatogram (Fig. 1). This suggests that anthocyanins based on the percentages may be the main hydrophilic antioxidant contributors and that this may especially be the case for purple carrot.

3.3. *Cellular antioxidant activity in H_2O_2-stimulated Caco-2 cells*

The antioxidant activities measured by chemical assays generally lack the physiological relevance. Cell-based antioxidant assay (CAA) is widely used to assess the activity, uptake, metabolism and distribution of antioxidants at a cellular level (Wolfe & Liu, 2007). In this study, Caco-2 cells were first treated with different concentrations (at final concentrations of 5, 10, 50 and 100 μg/mL cell culture medium) of purple carrot or potato extract for 30 min, and then exposed to H_2O_2 to induce cellular responses. These results showed that lower doses of both extracts had strong CAA values, but the activity was not dose-dependent, and there was no significant difference between the two extracts (Fig. 2). Higher dosages may trigger or induce the protective machinery through other potential mechanisms such as enhanced antioxidant enzyme activities, which was substantiated and further discussed in the following studies.

3.4. *Effects on pro-inflammatory cytokine production*

Living organisms have the ability to maintain the redox balance. Even though over production of ROS can be harmful, moderate level of prooxidant molecules are beneficial for health because they may activate mechanisms in modulating cell signalling

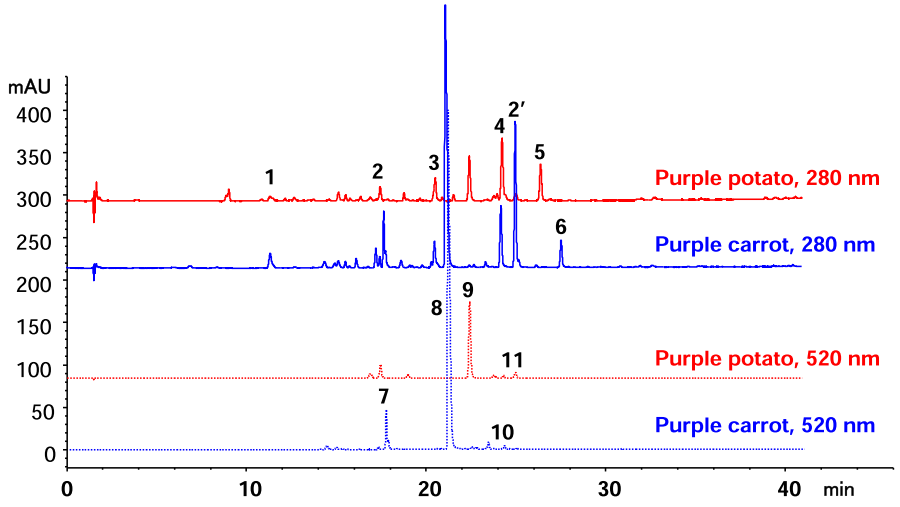

Fig. 1 – HPLC chromatograms of the anthocyanin-rich phenolic extracts of purple carrots and potatoes after acid hydrolysis. Top 2 chromatograms. HPLC profiles of phenolic compounds at 280 nm. Bottom 2 chromatograms. HPLC profiles of anthocyanidins at 520 nm.

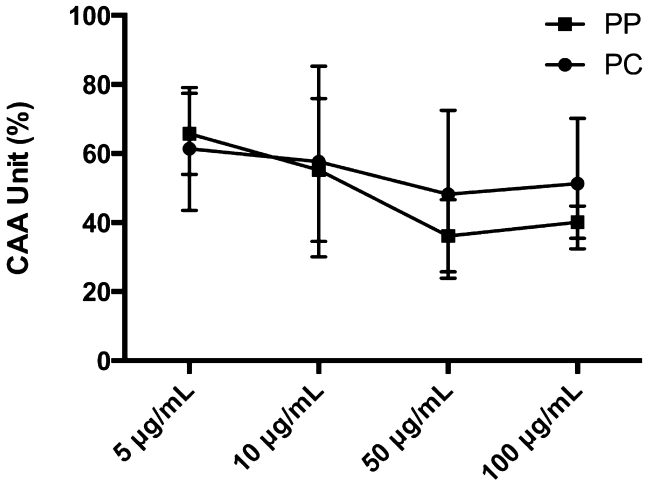

Fig. 2 – Cellular antioxidant activities (CAA) of the anthocyanin-rich phenolic extracts of purple carrot and potato. Caco-2 cells were treated with 5 µg/mL, 10 µg/mL, 50 µg/mL and 100 µg/mL of purple carrot or potato (PP) extracts for 30 min. Value are expressed as CAA Unit (%) and presented as mean ± SEM, n = 4.

transcriptions and/or eliminating pathogens (Martindale & Holbrook, 2002). In addition to the free radical scavenging capacity, effects of these phenolic compounds on the entire cellular redox balance, i.e. on the cytotoxic reactions and induction of inflammatory responses (Fernández-Iglesias et al., 2014), especially on cytokines of the inflammatory signalling pathways (Valko et al., 2007), must also be investigated.

One of such biomarkers is interleukin 8 (IL-8), which is known to be secreted abundantly from the intestinal epithelial cells into the mucosa layer to recruit neutrophils that mediate the activation of innate immune responses (Kucharzik et al., 2005). In Caco-2 cells, IL-8 can be drastically increased when stimulated by H_2O_2, making it a key indicator of oxidative stress (Chohan, Naughton, Jones, & Opara, 2012; Katayama & Mine, 2007; Katayama, Xu, Fan, & Mine, 2006; Shi et al., 2014). A series of concentrations of purple carrot or potato extract was supplemented to Caco-2 cell media in two models, i.e. pre-incubation without H_2O_2, and simultaneous incubation with H_2O_2, representing a preventive and a simultaneous model in the present study, respectively. IL-8 production in cells treated with both extracts was inhibited by an increasing concentration of extracts as compared to the control with H_2O_2 alone within each group (Fig. 3). Significant inhibition of IL-8 production was detected in Caco-2 cells treated with both 50 and 100 µg/mL levels of either purple carrot or potato extract ($P < 0.05$), except for purple potato extract in the simultaneous model at 50 µg/mL (Fig. 3). This result suggests the preventive model is sufficiently effective in managing oxidative stress related inflammation, even at lower concentrations and shorter pre-exposure to these compounds. The longer pre-incubation of 1 h also showed significant inhibition of H_2O_2-induced IL-8, however, due possibly to degradation of the phenolic compounds or formation of less effective metabolites (Xiao & HÖgger, 2015), the effect was weakened compared to 30 min pre-incubation (Fig. 3). A 24 h pre-treatment with phenolic-rich beverages has been reported by others to be unsuccessful in improving the redox status in H_2O_2-induced Caco-2 cells (Cilla, Laparra, Alegria, Barbera, & Farre, 2008), suggesting 24 h might be too long according to our results.

ROS acting as regulatory molecules are known to be involved in stimulation of the pro-inflammatory signalling transduction (Martindale & Holbrook, 2002). Cellular antioxidant status can also be improved by supplementation of phenolics or anthocyanins, resulting in the down-regulation of other pro-inflammatory mediators such as IL-6, TNF-α, IL-1β and IL-8, (Kuntz et al., 2014; Shih, Yeh, & Yen, 2007). In the

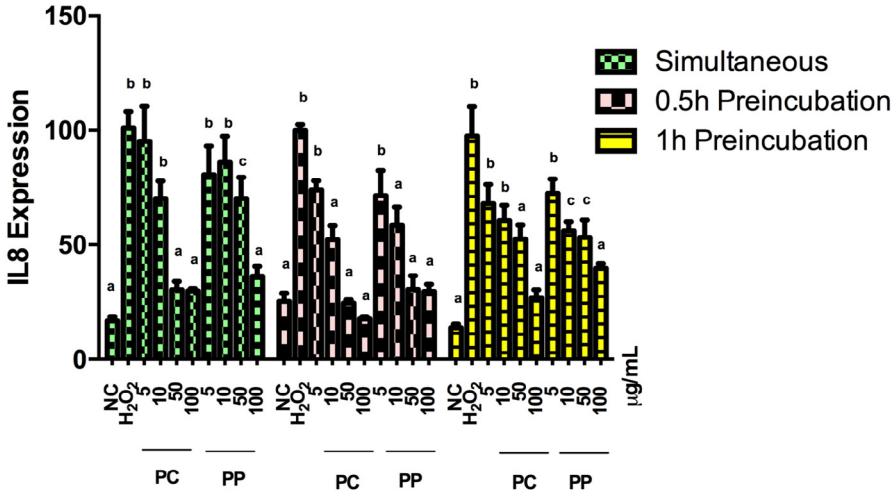

Fig. 3 – Effect of the anthocyanin-rich phenolic extracts of purple carrot and potato on H_2O_2-induced interleukin (IL)-8 released from Caco-2 cells. In the preventive model, Caco-2 cells were either pre-treated with 5 µg/mL, 10 µg/mL, 50 µg/mL and 100 µg/mL of the extract for 30 min or 1 h before stimulated with 1 mM of H_2O_2 for another 6 h. In the treatment model, 1 mM of H_2O_2 were simultaneously added into cell culture when Caco-2 cells were treated with the above extracts. The negative control (NC) represents untreated cells. Values are presented as mean ± SEM, n = 4. Values without a common letter are significantly different at $P < 0.05$.

present study, cells were treated at 50 μg/mL and 100 μg/mL for 30 min and then these pro-inflammatory cytokines, as expressed at the transcriptional level, were measured. The results showed that both purple carrot and potato extracts had approximately 50% of reduction in the expression of the aforementioned cytokines except for IL-6, compared with cells treated with H_2O_2 alone (Fig. 4). No significant effects on IL-6 at 50 μg/mL of purple carrot and potato extract or IL-1 β at 100 μg/mL of purple carrot extract were identified, respectively. Other than those, all inhibition was significant ($P < 0.05$) (Fig. 4). Similar to the CAA results (Fig. 2), no significant differences in cytokine production were found between the two concentrations of each extract (Fig. 4), indicating either the range of dosages used in this study was too narrow or the dosage at 50 μg/mL saturated the antioxidant stress activity at the cellular level. These results suggest that the phenolic antioxidants especially anthocyanins may play a vital role in reducing oxidative stress via both radical scavenging and inhibition of pro-inflammatory cytokines. Both mechanisms are important for gut health, and possibly for other diseases.

3.5. *Effects on cellular antioxidant enzyme activities*

The intrinsic antioxidant defence system also depends on various antioxidant enzymes including CAT, SOD, GR and GPx as well as the endogenous antioxidant GSH. SOD plays a key role in converting superoxide to hydrogen peroxide, which is then decomposed by CAT or GPx into water (Finkel & Holbrook, 2000). The protective effects against the oxidative stress were further evaluated by measuring activities of these antioxidant

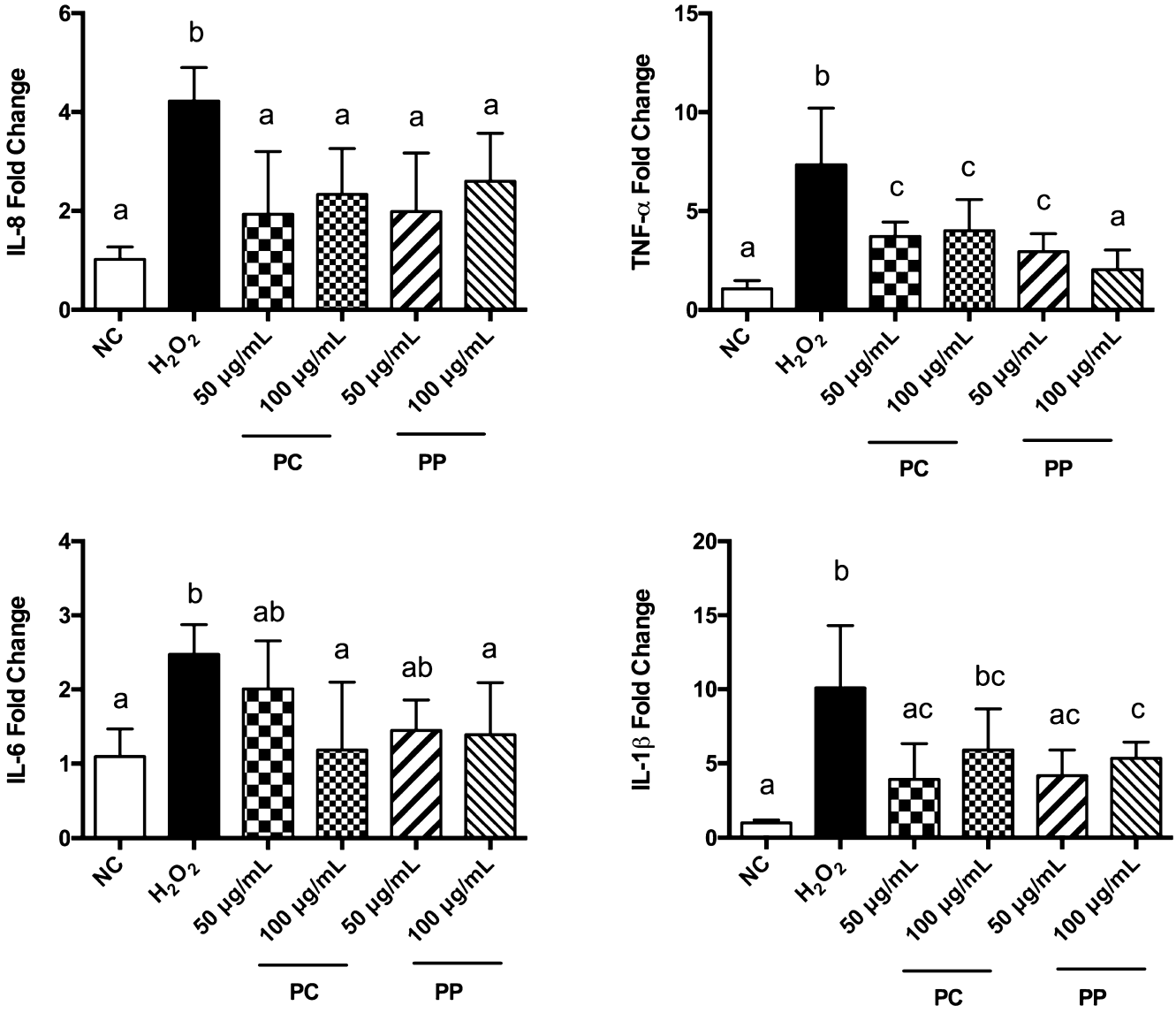

Fig. 4 – Effect of the anthocyanin-rich phenolic extracts of purple carrot and potato induced on H_2O_2-induced pro-inflammatory cytokines expression in Caco-2 cells. Caco-2 cells were pre-treated with 50 μg/mL and 100 μg/mL extracts for 30 min, followed by incubation with 1 mM of H_2O_2 for 6 h. The relative mRNA expression was measured by RT-PCR. The negative control (NC) represents untreated cells. Values are expressed as fold change relative to untreated cells, and presented as mean ± SEM, n = 6. Values without a common letter are significantly different at $P < 0.05$.

enzymes in Caco-2 cells pre-treated with 50 or 100 μg/mL of anthocyanin-rich extracts for 30 min prior to additional exposure to 1 mM H_2O_2 for 6 h. Pre-exposure to the phenolics from purple carrot or potato significantly diminished the effect by H_2O_2, i.e. the anthocyanin-rich phenolic extracts of the two vegetables were able to preserve the GR, SOD, CAT and GPx activities (Fig. 5). Similar results have been reported for bioactive peptides and other compounds (Guo, Zhang, Jiang, Miao, & Mu, 2014; O'Sullivan et al., 2013; Shi et al., 2014). As shown in Fig. 5, the SOD activity was enhanced by 61.8% and 68.6% by purple carrot extract, and 68.7% and 79.6% by purple potato extract at 50 μg/mL and 100 μg/mL, respectively. As the primary intrinsic antioxidant enzyme, SOD functions to protect cells from harmful damage through breaking down superoxide ($O_2^{\bullet-}$) radicals (Dryden et al., 2005). However, the protective effect on SOD was not observed in Caco-2 cells pre-treated with phenolics-rich fruit beverages with H_2O_2 for 24 h as reported by Cilla et al. (2008). This again suggests that long term exposure to ROS may render the phenolics ineffective. Similarly, compared to the cells treated with H_2O_2 alone, GR activity was 47.1% and 48.9% higher when pre-treated with 50 μg/mL and 100 μg/mL purple carrot extract, and 53.2% and 43.1% higher with 50 μg/mL and 100 μg/mL purple potato extract, respectively (Fig. 5). CAT activity was also significantly improved by the two extracts except for 50 μg/mL purple potato extract. GPx activity was increased by 69.7% and 93.1% for carrot and 61.2% and 75.5% for potato extract at 50 μg/mL and 100 μg/mL, respectively (Fig. 5). Elevated activities were observed for GPx and CAT at higher extract concentration (Fig. 5).

Perturbation of ROS elimination mechanisms occurs as a result of oxidative stress. The GR activity was also significantly increased by pre-treatment with anthocyanin-rich phenolic extracts, suggesting a role for anthocyanins in the regeneration of GSH. CAT and GPx are antioxidant enzymes in the secondary steps of the antioxidant pathway and are more important than SOD (Dryden et al., 2005). Exposure to high H_2O_2 triggers the intrinsic defence system in Caco-2 cells which acts to scavenge the excess exogenous H_2O_2 by CAT and GPx (Finkel & Holbrook, 2000), and often in stimulated cells these two enzymes are upregulated to prevent H_2O_2 mediated oxidative damage (Pendergrass, Rafferty, & Davis, 2011). The reduction of antioxidant enzyme activities of H_2O_2-treated Caco-2 cells may be caused by oxidative-stress mediated cell death after a long period of exposure. Results of the present study suggest that the anthocyanin-rich phenolic extracts of the purple vegetables not only have direct antioxidant activities as shown in the chemical based assays, but more importantly, they may stimulate the expression of antioxidant enzymes such as CAT and GPx or directly stimulate enzyme activity via an allosteric action to further strengthen the antioxidant defence (Fig. 5). Similar results were found in other studies (Alía et al., 2006; Shi et al., 2014). In addition, even though CAT can reduce oxidative stress through decomposition of low concentrations of exogenous or endogenous H_2O_2 (Masaki, Okano, & Sakurai, 1998), it is incapable of reducing a concentration as high as 1 mM H_2O_2 which is beyond normal physiological level to a harmless level. The excess H_2O_2 can in turn result in inhibiting CAT activity. The direct antioxidant activities observed in

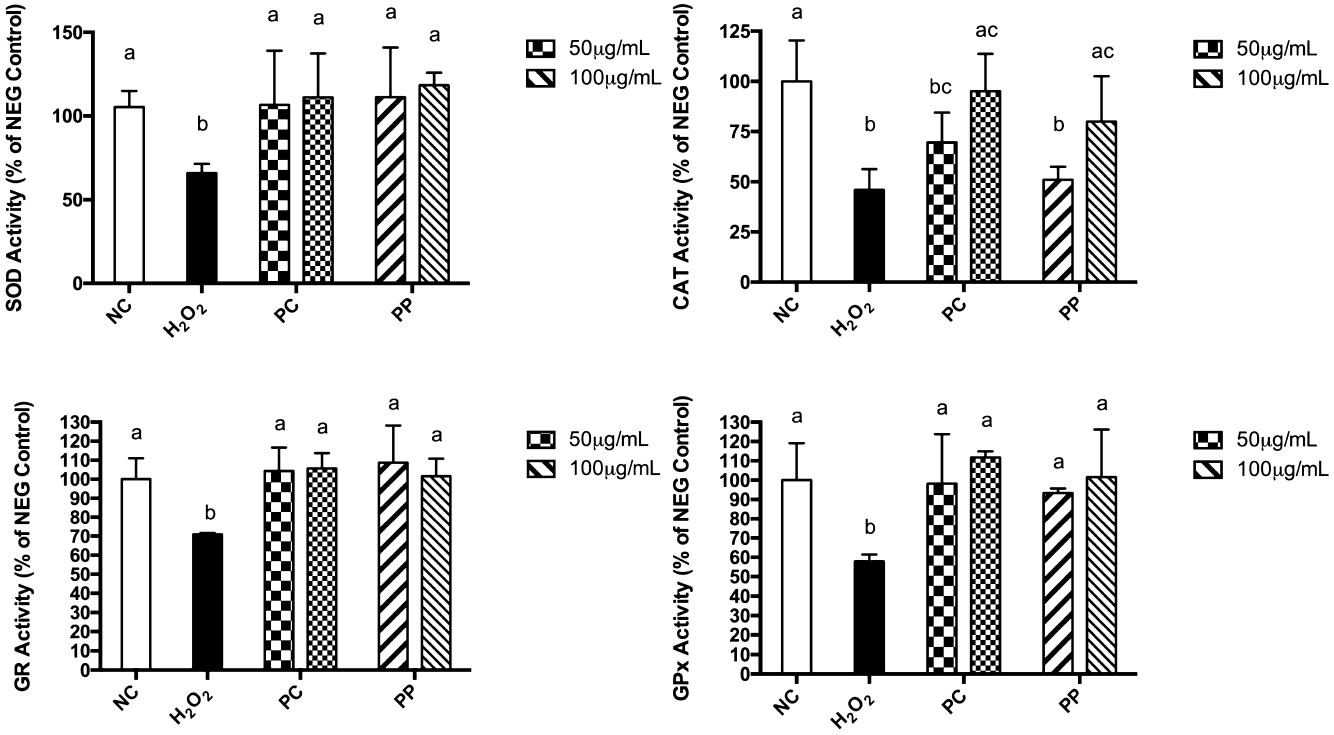

Fig. 5 – Effect of the anthocyanin-rich phenolic extracts purple carrot and potato on antioxidant enzyme activities in H_2O_2-stimulated Caco-2 cells. Caco-2 cells were pre-treated with 50 μg/mL and 100 μg/mL extracts for 30 min, followed by incubation with 1 mM of H_2O_2 for 6 h. The negative control (NC) represents untreated cells. Values are presented as mean ± SEM, n = 4. Values without a common letter are significantly different at $P < 0.05$.

ORAC, FRAP and DPPH assays might have contributed to the reduction of ROS such as excess H_2O_2, rendering the oxidative stress to a normal biological range so that CAT can be upregulated and its activity enhanced.

Glutathione (GSH) plays an essential role in the intrinsic defence mechanism for detoxification and antioxidation in nearly all cell types (Wu, Fang, Yang, Lupton, & Turner, 2004). GPx is an important antioxidant enzyme of this pathway, and plays a critical role in protecting living organisms from oxidative damage. GPx catalyses GSH to decompose H_2O_2 into water and to form GSSG (oxidized GSH) which is reduced back to GSH by GR (Wu et al., 2004). GSH, as an endogenous antioxidant, is widely involved in cellular reactions to detoxify electrophilic xenobiotics and scavenge the reactive intermediates and is thus an important indicator of intrinsic cellular antioxidant responses. The anthocyanin rich phenolic extracts of the two highly pigmented vegetables both significantly alleviated the reduced cellular GSH induced by H_2O_2 (Fig. 6). Supplementation of anthocyanins or fruit juices containing anthocyanins was found to increase GSH synthesis in reported cell and animal models or human studies (Diamanti et al., 2014; Juadjur et al., 2015; Riso et al., 2013; Spormann et al., 2008; You, Kim, Lim, & Lee, 2010; Zhu, Jia, Wang, Zhang, & Xia, 2012). However, such an effect has not been reported for anthocyanins-rich vegetables or extracts. The present study, therefore, could be the first to demonstrate how anthocyanin-rich phenolics of vegetables positively affect GSH production as part of the cellular antioxidant mechanism. The overall effect of these compounds on the activity of antioxidant enzymes and production of GSH demonstrated that consumption of food containing these may help to improve the redox-balancing mechanism which is compromised by oxidative stress.

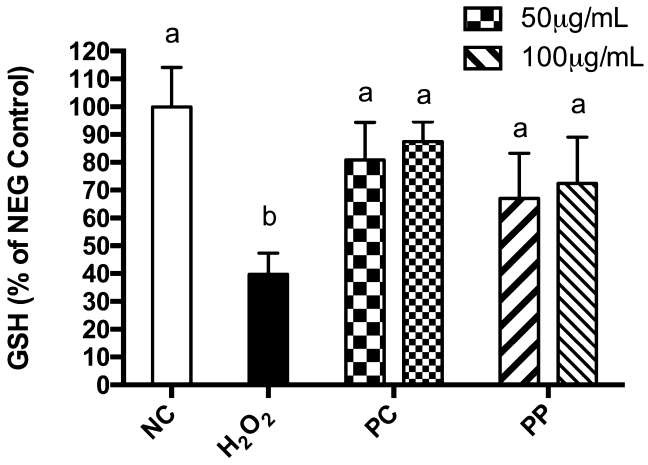

Fig. 6 – Effect of the anthocyanin-rich phenolic extracts of purple carrot and potato extracts on GSH production in H_2O_2-stimulated Caco-2 cells. Caco-2 cells were pre-treated with 50 μg/mL and 100 μg/mL extracts for 30 min, followed by incubation with 1 mM of H_2O_2 for 6 h. The negative control (NC) represents untreated cells. Values are presented as mean ± SEM, n = 4. Values without a common letter are significantly different at $P < 0.05$.

4. Conclusion

While most studies focus on anthocyanin rich fruits, the present study chose to investigate two most commonly consumed staple root vegetables rich in anthocyanins. The purple varieties of carrot and potato contained various phenolic compounds and anthocyanins that are considered as the main contributors to the antioxidant activities found in ORAC, FRAP and DPPH assays. These antioxidant activities might be important in direct scavenging of the ROS, however, the effect of these compounds on the activity of antioxidant enzymes and the production of the endogenous antioxidant GSH as found in the Caco-2 model in the present study strongly suggests that phenolics, particularly the anthocyanins, can act to aid the cellular antioxidant defence mechanisms beyond the direct radical scavenging activity. The enhanced activity of SOD, CAT, GPx and GR, and increased production of GSH in H_2O_2-stressed Caco-2 cells suggest that phenolic compounds of these two highly pigmented root vegetables, especially the anthocyanins thereof, can contribute to improved cellular redox state in the epithelial cell layer of the gut, therefore potentially prevent oxidative stress related chronic gastrointestinal disease. The potential benefits of the two purple root vegetables on gut health were further supported by the inhibitory effect of anthocyanin-rich phenolic content on H_2O_2-induced pro-inflammatory cytokines IL-8, IL-6, IL-1β and TNF-α. While future *in vitro* and *in vivo* studies on the bioaccessibility, bioavailability and on the effect of metabolites of the phenolics in the two vegetables must be done, findings of the present study do provide useful fundamental information for the development of anthocyanin-rich varieties of carrots and potatoes, and functional foods or beverages based on these two vegetables.

Acknowledgment

We thank Professors A. Sullivan and M.R. McDonald and Ms. Vanessa Currie of the Department of Plant Agriculture, University of Guelph for their kind donation of the purple carrot and potato samples. This project was funded by Agriculture and Agri-Food Canada (J-000237.001.02).

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2016.01.004.

REFERENCES

Albishi, T., John, J. A., Al-Khalifa, A. S., & Shahidi, F. (2013). Phenolic content and antioxidant activities of selected potato varieties and their processing by-products. *Journal of Functional Foods*, 5, 590–600.

Alía, M., Mateos, R., Ramos, S., Lecumberri, E., Bravo, L., & Goya, L. (2006). Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2). *European Journal of Nutrition*, 45(1), 19–28.

Baud, L., & Ardaillou, R. (1993). Involvement of reactive oxygen species in kidney damage. *British Medical Bulletin*, *49*(3), 621–629.

Bhattacharyya, A., Chattopadhyay, R., Mitra, S., & Crowe, S. E. (2014). Oxidative stress: An essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiological Reviews*, *94*(2), 329–354. doi:10.1152/physrev.00040.2012.

Biedermann, L., Mwinyi, J., Scharl, M., Frei, P., Zeitz, J., Kullak-Ublick, G. A., Vavricka, S. R., Fried, M., Weber, A., Humpf, H. U., Peschke, S., Jetter, A., Krammer, G., & Rogler, G. (2013). Bilberry ingestion improves disease activity in mild to moderate ulcerative colitis – An open pilot study. *Journal of Crohn's and Colitis*, *7*(4), 271–279.

Bondor, C. I., Potra, A. R., Moldovan, D., Rusu, C. C., Ciorba Pop, M., Muresan, A., Vladutiu, D. S., & Kacso, I. M. (2015). Relationship of adiponectin to markers of oxidative stress in type 2 diabetic patients: Influence of incipient diabetes-associated kidney disease. *International Urology and Nephrology*, *47*(7), 1173–1180.

Butelli, E., Titta, L., Giorgio, M., Mock, H. P., Matros, A., Peterek, S., Schijlen, E. G., Hall, R. D., Bovy, A. G., Lou, J., & Martin, C. (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nature Biotechnology*, *26*(11), 1301–1308.

Charron, C. S., Clevidence, B. A., Britz, S. J., & Novotny, J. A. (2007). Effect of dose size on bioavailability of acylated and nonacylated anthocyanins from red cabbage (Brassica oleracea L. Var. capitata). *Journal of Agricultural and Food Chemistry*, *55*(13), 5354–5362.

Chen, H. M., Muramoto, K., & Yamauchi, F. (1995). Structural analysis of antioxidative peptides from soybean beta-conglycinin. *Journal of Agricultural and Food Chemistry*, *43*, 574–578.

Chen, P. X., Bozzo, G. G., Freixas-Coutin, J. A., Marcone, M. F., Pauls, P. K., Tang, Y., Zhang, B., Liu, R., & Tsao, R. (2015). Free and conjugated phenolic compounds and their antioxidant activities in regular and non-darkening cranberry bean (*Phaseolus vulgaris* L.) seed coats. *Journal of Functional Foods*, *18*, 1047–1056.

Chohan, M., Naughton, D. P., Jones, L., & Opara, E. I. (2012). An investigation of the relationship between the anti-inflammatory activity, polyphenolic content, and antioxidant activities of cooked and in vitro digested culinary herbs. *Oxidative Medicine and Cellular Longevity*, *2012*, 627843.

Chung, H. S., & Woo, W. S. (2001). A quinolone alkaloid with antioxidant activity from the aleurone layer of anthocyanin-pigmented rice. *Journal of Natural Products*, *64*(12), 1579–1580.

Cilla, A., Laparra, J. M., Alegria, A., Barbera, R., & Farre, R. (2008). Antioxidant effect derived from bioaccessible fractions of fruit beverages against H2O2-induced oxidative stress in Caco-2 cells. *Food Chemistry*, *106*(3), 1180–1187.

Colgan, S. P., & Taylor, C. T. (2010). Hypoxia: An alarm signal during intestinal inflammation. *Nature Reviews Gastroenterology & Hepatology*, *7*(5), 281–287.

Cross, C., Halliwell, B., & Allen, A. (1984). Antioxidant protection: A function of tracheobronchial and gastrointestinal mucus. *The Lancet*, *323*(8390), 1328–1330.

Dai, J., & Mumper, R. J. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules*, *15*(10), 7313–7352.

Diamanti, J., Mezzetti, B., Giampieri, F., Alvarez-Suarez, J. M., Quiles, J. L., Gonzalez-Alonso, A., Ramirez-Tortosa, M. C., Granados-Principal, S., Gozáles-Paramás, A. M., Santos-Buelga, C., & Battino, M. (2014). Doxorubicin-induced oxidative stress in rats is efficiently counteracted by dietary anthocyanin differently enriched strawberry (Fragaria × ananassa Duch.) *Journal of Agricultural and Food Chemistry*, *62*(18), 3935–3943.

Dryden, G. W., Jr., Deaciuc, I., Arteel, G., & McClain, C. J. (2005). Clinical implications of oxidative stress and antioxidant therapy. *Current Gastroenterology Reports*, *7*(4), 308–316.

Farzaei, H. M., Rahimi, R., & Abdollahi, M. (2015). The role of dietary polyphenols in the management of inflammatory bowel disease. *Current Pharmaceutical Biotechnology*, *16*(3), 196–210.

Fernández-Iglesias, A., Pajuelo, D., Quesada, H., Díaz, S., Bladé, C., Arola, L., Salvadó, M. J., & Mulero, M. (2014). Grape seed proanthocyanidin extract improves the hepatic glutathione metabolism in obese Zucker rats. *Molecular Nutrition and Food Research*, *58*(4), 727–737.

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, *408*(6809), 239–247.

Ford, E. S., Bergmann, M. M., Kroger, J., Schienkiewitz, A., Weikert, C., & Boeing, H. (2009). Healthy living is the best revenge: Findings from the European prospective investigation into cancer and nutrition – Potsdam study. *Archives of Internal Medicine*, *169*(15), 1355.

Giusti, M. M., & Wrolstad, R. E. (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. *Current Protocols in Food Analytical Chemistry*, doi:10.1002/0471142913.faf0102s00.

Guo, Y., Zhang, T., Jiang, B., Miao, M., & Mu, W. (2014). The effects of an antioxidative pentapeptide derived from chickpea protein hydrolysates on oxidative stress in Caco-2 and HT-29 cell lines. *Journal of Functional Foods*, *7*, 719–726.

Han, K. H., Sekikawa, M., Shimada, K., Hashimoto, M., Hashimoto, N., Noda, T., Tanaka, H., & Fukushima, M. (2006). Anthocyanin-rich purple potato flake extract has antioxidant capacity and improves antioxidant potential in rats. *British Journal of Nutrition*, *96*(6), 1125–1133.

Han, P. D., Burke, A., Baldassano, R. N., Rombeau, J. L., & Lichtenstein, G. R. (1999). Nutrition and inflammatory bowel disease. *Gastroenterology Clinics of North America*, *28*(2), 423–443.

Hou, D. X., Fujii, M., Terahara, N., & Yoshimoto, M. (2004). Molecular mechanisms behind the chemopreventive effects of anthocyanidins. *Journal of Biomedicine and Biotechnology*, *2004*(5), 321–325.

Hritcu, L., Stefan, M., Brandsch, R., & Mihasan, M. (2015). Enhanced behavioral response by decreasing brain oxidative stress to 6-hydroxy-l-nicotine in Alzheimer's disease rat model. *Neuroscience Letters*, *591*, 41–47.

Hu, C., Tsao, R., Liu, R., Alan Sullivan, J., & McDonald, M. R. (2012). Influence of cultivar and year on phytochemical and antioxidant activity of potato (Solanum tuberosum L.) in Ontario. *Canadian Journal of Plant Science*, *92*(3), 485–493.

Janšáková, K., Bábíčková, J., Filová, B., Lengyelová, E., Havrlentová, M., Kraic, J., Celec, P., & Tóthová, Ľ. (2015). Anthocyanin-rich diet in chemically induced colitis in mice. *Folia Biologica (Folia Biol-Prague)*, *61*, 104–109.

Juadjur, A., Mohn, C., Schantz, M., Baum, M., Winterhalter, P., & Richling, E. (2015). Fractionation of an anthocyanin-rich bilberry extract and in vitro antioxidative activity testing. *Food Chemistry*, *167*, 418–424. doi:10.1016/j.foodchem.2014.07.004.

Kaneko, K., Akuta, T., Sawa, T., Kim, H. W., Fujii, S., Okamoto, T., Nakayama, H., Ohigashi, H., Murakami, A., & Akaike, T. (2008). Mutagenicity of 8-nitroguanosine, a product of nitrative nucleoside modification by reactive nitrogen oxides, in mammalian cells. *Cancer Letters*, *262*(2), 239–247.

Katayama, S., & Mine, Y. (2007). Antioxidative activity of amino acids on tissue oxidative stress in human intestinal epithelial cell model. *Journal of Agricultural and Food Chemistry*, *17*, 8458–8464.

Katayama, S., Xu, X., Fan, M. Z., & Mine, Y. (2006). Antioxidative stress activity of oligophosphopeptides derived from hen egg yolk phosvitin in Caco-2 cells. *Journal of Agricultural and Food Chemistry*, 54(3), 773–778.

Khanizadeh, S., Tsao, R., Rekika, D., Yang, R., Charles, M. T., & Rupasinghe, H. V. (2008). Polyphenol composition and total antioxidant capacity of selected apple genotypes for processing. *Journal of Food Composition and Analysis*, 21(5), 396–401.

Kruidenier, L., & Verspaget, H. W. (1998). Antioxidants and mucosa protectives: Realistic therapeutic options in inflammatory bowel disease? *Mediators of Inflammation*, 7(3), 157–162.

Kucharzik, T., Hudson Iii, J. T., Lügering, A., Abbas, J. A., Bettini, M., Lake, J. G., Evans, M. E., Ziegler, T. R., Merlin, D., Madara, J. L., & Williams, I. R. (2005). Acute induction of human IL-8 production by intestinal epithelium triggers neutrophil infiltration without mucosal injury. *Gut*, 54(11), 1565–1572.

Kuntz, S., Kunz, C., Herrmann, J., Borsch, C. H., Abel, G., Fröhling, B., Dietrich, H., & Rudloff, S. (2014). Anthocyanins from fruit juices improve the antioxidant status of healthy young female volunteers without affecting anti-inflammatory parameters: Results from the randomised, double-blind, placebo-controlled, cross-over ANTHONIA (ANTHOcyanins in Nutrition Investigation Alliance) study. *British Journal of Nutrition*, 112(06), 925–936.

Leja, M., Kamińska, I., Kramer, M., Maksylewicz-Kaul, A., Kammerer, D., Carle, R., & Baranski, R. (2013). The content of phenolic compounds and radical scavenging activity varies with carrot origin and root color. *Plant Foods for Human Nutrition (Dordrecht, Netherlands)*, 68(2), 163–170.

Li, H., Deng, Z., Liu, R., Loewen, S., & Tsao, R. (2014). Bioaccessibility, in vitro antioxidant activities and in vivo anti-inflammatory activities of a purple tomato (Solanum lycopersicum L. *Food Chemistry*, 159, 353–360.

Li, H., Deng, Z., Liu, R., Young, J. C., Zhu, H., Loewen, S., & Tsao, R. (2011). Characterization of phytochemicals and antioxidant activities of a purple tomato (Solanum lycopersicum L.). *Journal of Agricultural and Food Chemistry*, 59(21), 11803–11811.

Li, H., Deng, Z., Zhu, H., Hu, C., Liu, R., Young, J. C., & Tsao, R. (2012). Highly pigmented vegetables: Anthocyanin compositions and their role in antioxidant activities. *Food Research International*, 46(1), 250–259.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods (San Diego, Calif.)*, 25(4), 402–408.

Martindale, J. L., & Holbrook, N. J. (2002). Cellular response to oxidative stress: Signaling for suicide and survival. *Journal of Cellular Physiology*, 192(1), 1–15.

Masaki, H., Okano, Y., & Sakurai, H. (1998). Differential role of catalase and glutathione peroxidase in cultured human fibroblasts under exposure of H2O2 or ultraviolet B light. *Archives of Dermatological Research*, 290(3), 113–118.

Moylan, J. S., & Reid, M. B. (2007). Oxidative stress, chronic disease, and muscle wasting. *Muscle and Nerve*, 35, 411–429.

Murray, C. J. L., Vos, T., Lozano, R., Naghavi, M., Flaxman, A. D., Michaud, C., & Lopez, A. D. (2012). Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: A systematic analysis for the Global Burden of Disease Study 2010. *The Lancet*, 380(9859), 2197–2223.

Navarre, D. A., Pillai, S. S., Shakya, R., & Holden, M. J. (2011). HPLC profiling of phenolics in diverse potato genotypes. *Food Chemistry*, 127(1), 34–41.

Nishiyama, Y., Ikeda, H., Haramaki, N., Yoshida, N., & Imaizumi, T. (1998). Oxidative stress is related to exercise intolerance in patients with heart failure. *American Heart Journal*, 135(1), 115–120.

Nunez, M. F., & Magnuson, B. A. (2013). Anthocyanins in health and disease prevention. In T. C. Wallace & M. M. Giusti (Eds.), Anthocyanins in health and disease. Florida: Boca Raton, CRC Press. pp. 1.

O'Sullivan, A. M., O'Callaghan, Y. C., O'Grady, M. N., Hayes, M., Kerry, J. P., & O'Brien, N. M. (2013). The effect of solvents on the antioxidant activity in Caco-2 cells of Irish brown seaweed extracts prepared using accelerated solvent extraction (ASE®). *Journal of Functional Foods*, 5(2), 940–948.

Pendergrass, K. D., Rafferty, K., & Davis, M. E. (2011). H2O2 acutely upregulates glutathione peroxidase and catalase mRNA levels in cardiac progenitor cells. *The FASEB Journal*, 25(1_MeetingAbstracts), 826.823.

Pham-Huy, L. A., He, H., & Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science*, 4(2), 89–96.

Piberger, H., Oehme, A., Hofmann, C., Dreiseitel, A., Sand, P. G., Obermeier, F., Schoelmerich, J., Schreier, P., Krammer, G., & Rogler, G. (2011). Bilberries and their anthocyanins ameliorate experimental colitis. *Molecular Nutrition & Food Research*, 55(11), 1724–1729.

Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Biology and Medicine*, 49(11), 1603–1616.

Reyes, L. F., Miller, J. C., Jr., & Cisneros-Zevallos, L. (2004). Environmental conditions influence the content and yield of anthocyanins and total phenolics in purple- and red-flesh potatoes during tuber development. *American Journal of Potato Research*, 81(3), 187–193.

Riso, P., Klimis-Zacas, D., Del Bo, C., Martini, D., Campolo, J., Vendrame, S., Møller, P., Loft, S., De Maria, R., & Porrini, M. (2013). Effect of a wild blueberry (Vaccinium angustifolium) drink intervention on markers of oxidative stress, inflammation and endothelial function in humans with cardiovascular risk factors. *European Journal of Nutrition*, 52(3), 949–961.

Romier, B., Schneider, Y.-J., Larondelle, Y., & During, A. (2009). Dietary polyphenols can modulate the intestinal inflammatory response. *Nutrition Reviews*, 67(7), 363–378.

Rubattu, S., Pagliaro, B., Pierelli, G., Santolamazza, C., Di Castro, S., Mennuni, S., & Volpe, M. (2015). Pathogenesis of target organ damage in hypertension: Role of mitochondrial oxidative stress. *International Journal of Molecular Sciences*, 16(1), 823–839.

Scalbert, A., & Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *The Journal of Nutrition*, 130(8), 2073S–2085S.

Shi, Y., Kovacs-Nolan, J., Jiang, B., Tsao, R., & Mine, Y. (2014). Antioxidant activity of enzymatic hydrolysates from eggshell membrane proteins and its protective capacity in human intestinal epithelial Caco-2 cells. *Journal of Functional Foods*, 10, 35–45.

Shih, P.-H., Yeh, C.-T., & Yen, G.-C. (2007). Anthocyanins induce the activation of Phase II enzymes through the antioxidant response element pathway against oxidative stress-induced apoptosis. *Journal of Agricultural and Food Chemistry*, 55(23), 9427–9435.

Spormann, T. M., Albert, F. W., Rath, T., Dietrich, H., Will, F., Stockis, J. P., Eisenbrand, G., & Janzowski, C. (2008). Anthocyanin/polyphenolic-rich fruit juice reduces oxidative cell damage in an intervention study with patients on hemodialysis. *Cancer Epidemiology, Biomarkers and Prevention*, 17(12), 3372–3380.

Sun, T., Simon, P. W., & Tanumihardjo, S. A. (2009). Antioxidant phytochemicals and antioxidant capacity of biofortified carrots (Daucus carota L.) of various colors. *Journal of Agricultural and Food Chemistry*, 57(10), 4142–4147.

Tang, Y., Li, X., Zhang, B., Chen, P. X., Liu, R., & Tsao, R. (2015). Characterisation of phenolics, betanins and antioxidant activities in seeds of three Chenopodium quinoa Willd. genotypes. *Food Chemistry*, *166*, 380–388.

Taverniti, V., Fracassetti, D., Del Bo', C., Lanti, C., Minuzzo, M., Klimis-Zacas, D., Riso, P., & Guglielmetti, S. (2014). Immunomodulatory effect of a wild blueberry anthocyanin-rich extract in human Caco-2 intestinal cells. *Journal of Agricultural and Food Chemistry*, *62*(33), 8346–8351.

Tsuda, T. (2012). Dietary anthocyanin-rich plants: Biochemical basis and recent progress in health benefits studies. *Molecular Nutrition and Food Research*, *56*(1), 159–170.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology*, *39*(1), 44–84.

Wolfe, K. L., & Liu, R. H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry*, *55*(22), 8896–8907.

Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R., & Turner, N. D. (2004). Glutathione metabolism and its implications for health. *Journal of Nutrition*, *134*(3), 489–492.

Wu, T., Yan, J., Liu, R., Marcone, M. F., Aisa, H. A., & Tsao, R. (2012). Optimization of microwave-assisted extraction of phenolics from potato and its downstream waste using orthogonal array design. *Food Chemistry*, *133*(4), 1292–1298.

Xiao, J., & HÖgger, P. (2015). Stability of dietary polyphenols under the cell culture conditions: Avoiding erroneous conclusions. *Journal of Agricultural and Food Chemistry*, *63*(5), 1547–1557.

Xu, X., Li, W., Lu, Z., Beta, T., & Hydamaka, A. W. (2009). Phenolic content, composition, antioxidant activity, and their changes during domestic cooking of potatoes. *Journal of Agricultural and Food Chemistry*, *57*(21), 10231–10236.

You, J., Kim, J., Lim, J., & Lee, E. (2010). Anthocyanin stimulates in vitro development of cloned pig embryos by increasing the intracellular glutathione level and inhibiting reactive oxygen species. *Theriogenology*, *74*(5), 777–785.

Zhu, W., Jia, Q., Wang, Y., Zhang, Y., & Xia, M. (2012). The anthocyanin cyanidin-3-O-β-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: Involvement of a cAMP-PKA-dependent signaling pathway. *Free Radical Biology and Medicine*, *52*(2), 314–327.