RESEARCH ARTICLE

Bioaccessibility, bioavailability, and anti-inflammatory effects of anthocyanins from purple root vegetables using mono- and co-culture cell models

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Scope: Immune-inflammatory signaling and metabolic effects are the main pillars for bioactivity of anthocyanins derived from highly pigmented root vegetables. This study aims to assess the bioaccessibility and bioavailability of purple carrot and potato derived anthocyanins and the molecular mechanisms of their ability to ameliorate cellular inflammation in a mono- and co-culture cell models.

Methods and results: An in vitro gastrointestinal model was used and demonstrated bioaccessibility of 44.62 and 71.8% for anthocyanins of purple carrot and potato, respectively. These accessible anthocyanins significantly inhibited cellular inflammation in Caco-2 cells. Intact cyanidinglycoside or petunidinglycoside (respectively from carrots and potatoes) were transported across a transmembrane cell model and detected by LC-MS/MS. Computational docking and glucose uptake analyses suggested uptake of anthocyanins was mediated by hexose transporters. Subsequent experiment using an inflamed Caco-2 BBe1/THP-1 co-culture cell model showed these transported anthocyanins inhibited IL-8 and TNF-α secretion,and expression of pro-inflammatory cytokines by blocking NF-κB, and MAPK mediated inflammatory cellular signaling cascades, but with varying degrees due to structural features.

Conclusion: Anthocyanins from purple carrots and potatoes possess a promising anti-inflammatory effect in model gut system. They can be absorbed and act differently but are in general beneficial for inflammation-mediated diseases.

Keywords:
Anthocyanins / Bioaccessibility / Bioavailability / Inflammation / Purple root vegetables

1 Introduction

Profound inflammatory responses that arise from an impaired immune homeostasis can promote the development of chronic health conditions such as degenerative diseases and metabolic syndromes [1]. Even though the aetiology of chronic inflammation is not fully understood, environmental stimuli, dietary factors, genetic predisposition, and immune sensitivity are believed to be causative factors for ongoing innate immune responses and impairment of immune homeostasis [2]. The gut is an integral organ of the human digestive system and acts as an intermediate between external and internal environments in the body. This renders the gut as the most vulnerable organ exposed to harmful milieus generated from pathogens, toxins or autoimmune responses [3]. Prolonged exposure to harmful stimuli results in dysregulated immune functions in the gut, evokes the amplification of local and systemic inflammation to mediate tissue damage and an altered host–microbiota relationship. Collectively these lead to an increased risk of developing adverse

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Abbreviations: **GI**, gastrointestinal; **SPE**, solid phase extraction; **HBSS**, Hank's balanced salt solution; **FBS**, foetal bovine serum; **IL**, interleukin; **TNF**, tumor necrosis factor; **LPS**, lipopolysaccharide; **PBS**, phosphate buffer solution; **RT-PCR**, real time-polymer chain reaction; **WB**, Western blotting; **NF-κB**, nuclear factor-kappa B; **JNK**, c-Jun N-terminal kinase; **MAPK**, mitogen-activated protein kinase; **IECs**, intestinal epithelial cells; **ACE**, atomic contact energies

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metabolic symptoms and chronic disease such as diabetes, obesity, and inflammatory bowel diseases [4, 5]. Inducible inflammatory mediators such as cytokines are governed by key cellular signaling molecules such as nuclear factor (NF)-κB in response to harmful milieus that has arisen in inflamed intestinal mucosal systems [6]. Owing to direct interaction with the gut system, components of our daily diet pose both health risks and benefits and are essential epigenetic factors regulating human physiological homeostasis. An in-depth understanding of the implication of food bioactive constituents in regulating these effects is a prerequisite to developing effective dietary intervention for inflammatory disease prevention strategies.

Anthocyanins are natural pigments belonging to the general flavonoid group of polyphenols that are widely found in fruits and vegetables [7]. Anthocyanins engage in chemoprotective functions by attenuating inflammation and regulating cellular signaling transductions, making them effective immune modulatory agents against a variety of inflammatory diseases [8–10]. The effects of various dietary polyphenols on amelioration of intestinal inflammation have been extensively studied; however those of anthocyanins have only recently been investigated [11]. In addition, most of the current evidence regarding the bioavailability and anti-inflammatory actions of anthocyanins are related to ones derived from fruits such as berries and grapes [7, 12]. Highly pigmented root vegetables are also important dietary sources of anthocyanins, however, very scant information is available with regard to their chemical characteristics, bioavailability and molecular mechanisms of their antioxidant and anti-inflammatory effects. Potential mechanisms of anthocyanin absorption and metabolism have been manifested, but only limited scientific evidences exist to provide information on the bioaccessibility and bioavailability of anthocyanins of colored root vegetables in the gut, and on the underlying mechanisms that are involved in modifying cellular processes [13, 14]. This information is crucial for validating the health claims of anthocyanins as functional bioactives.

The present study aims to assess the bioaccessibility of purple carrot and potato derived anthocyanins using an in vitro gastrointestinal digestion model. We determined the permeability and metabolic fate and characterized intact composition of trans-epithelial anthocyanins by employing an intestinal epithelial Caco-2 cell trans-membrane model. Furthermore, the underlying mechanism of bioavailability of purple carrot and potato derived anthocyanins was assessed. The potential of anthocyanin-rich purple carrot and potato extracts to ameliorate intestinal inflammation was finally studied in both Caco-2 mono- and Caco-2 plus human macrophage THP-1 co-culture systems. Expression of various pro-inflammatory cytokines and activation of pro-inflammatory signaling events such as NF-κB and mitogen-activated protein kinase (MAPK) pathways were evaluated in order to validate the anti-inflammatory activity of purple carrot or potato derived anthocyanins toward their actions of maintaining mucosal homeostasis.

2 Materials and methods

2.1 Chemicals and reagents

All standard reference materials including cyanidin and petunidin were obtained from Sigma-Aldrich Co. (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 Laboratories Ltd. (Georgetown, ON, Canada) unless otherwise specified. Western blot analysis was carried out using the following antibodies: anti-p-SAPK/JNK (81E11), anti-SAPK/JNK (56G8), anti-p-IκBα (5A5), and anti-IκBα (L35A5) (Cell Signaling Technology, Inc., Danvers, MA).

2.2 In vitro digestion

A simulated gastrointestinal digestion was carried out as described in our previous study [15] with slight modifications as described below. Fresh purple carrots (Purple Haze) or potatoes (MacIntosh) were harvested from the Muck Research Station and Elora Research Station of the University of Guelph, Ontario, 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). Briefly, 200 mg of purple carrot or potato freeze-dried powder was separately mixed with 1.5 mL of saliva (collected from a volunteer after consuming 250 mL milk) and made up to 8 mL by adding HBSS. The mixtures were incubated for 15 min at 37°C in a water bath shaker at 200 rev/min, and then adjusted to pH 1.5 using 6 N HCl. Porcine pepsin was added to reach a final concentration of 1.3 mg/mL. The mixture was continuously incubated for 1.5 h in a water bath shaker at 200 rev/min, and then added KH_2PO_4 (2 mL, 0.5 M), pancreatin (final concentration: 0.175 mg/mL) and procine bile salt (final concentration: 1.1 mg/mL). The pH of this solution was then adjusted to 8 using 10 M NaOH and incubated at 37°C in a water bath shaker for another 2 h. The digestion process was terminated by adding phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 1 mM. Subsequently, the supernatant was decanted for further assessment of bioaccessibility after centrifuging at $1600 \times g$ for 10 min.

2.3 Extraction and purification

Phenolic extract of purple carrot or potato was prepared using acidified aqueous methanol as previously described [16]. Briefly, 40 g of powder obtained from ground freeze-dried samples was extracted with 1 L 70% methanol (v/v) overnight at room temperature, filtered through Whatman filter paper (pore size 11 μm, VWR), and the residue was re-extracted

twice. The combined filtrate was concentrated to 700 mL under reduced pressure at <50°C, and further purified using Strata™-X polymeric solid phase extraction [17] cartridges (2 g, Phenomenex, Torrance, CA, USA), to remove undesired polar components such as soluble proteins, amino acids or sugars. The eluent was first evaporated using Refrigerated CentriVap (Labconco, Kansas City, MO, USA) and then freeze-dried. The semi-purified anthocyanin-rich phenolic extracts were used for HPLC analysis and in cell culture studies.

2.4 Cell culture

The human colorectal adenocarcinoma-derived intestinal epithelial cell (IEC) lines Caco-2 and THP-1 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and Sigma-Aldrich (Oakville, ON, Canada), respectively. Caco-2 cells were grown in 5 mL of Dulbecco's Modified Eagle Medium (DMEM) high glucose medium (Therm Fisher Scientific, Mississauga, ON, Canada) supplemented with 20% fetal bovine serum (FBS) (HyClone, Invitrogen Life Technologies Inc., Burlington, ON, Canada) and 50 U/mL penicillin-streptomycin (Invitrogen Life Technologies Inc., Burlington, ON, Canada). THP-1 cells were grown on RPMI 1640 (Invitrogen Life Technologies Inc., Burlington, ON, Canada) supplemented with 10% (v/v) FBS and 50 U/mL penicillin-streptomycin. Caco-2 cells between passages 15–45 or THP-1 cells between passages 3–20 were seeded at a density of 2×10^5 cells/well in 24- or 48-well plates (Corning Life Science, Tewksbury, MA, USA). Caco-2 cells were grown for 5–7 days with fresh media replacements every 2–3 days at 37°C in a humidity controlled 5% CO_2 cell culture incubator, and THP-1 cells were differentiated into mature macrophage-like state by stimulating with 50 ng/mL of Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 48 h before use in co-culture experiments.

Caco-2 BBe1 cell line obtained from ATCC was seeded between passages 50 to 60 onto polyester (PET) membrane permeable support inserts (6.5 mm, 0.4 μM pore size, Corning Inc., Corning, NY, USA) at a density of 3×10^4 cells/well, and grown in DMEM supplemented with 10% (v/v) FBS, 50 U/mL penicillin-streptomycin, 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; Thermo Fisher Scientific), 1 mM sodium pyruvate and non-essential amino acid (Thermo Fisher Scientific) and 0.01 μg/mL Human Transferrin (Sigma-Aldrich) at 37°C in a humidity controlled 5% CO_2 cell culture incubator. The culture medium was changed every second day until the cells became confluent monolayer. Only monolayers displaying trans-epithelial electrical resistance (TEER) values greater than 400 $\Omega \cdot cm^2$, generally on the post-seeding day 9th were used in the experiment. TEER values were measured when cells are equilibrated in HBSS for 30 min at 37°C using a Millicell-ERS Volt-Ohm Meter (Millipore, Bedford, MA, USA).

2.5 Co-culture

Co-culture was performed following a protocol [18] with slight modifications as described below. Caco-2 BBe1 cells were seeded onto PET membrane permeable support inserts and grown in a 24 well-plate for about 10 days. A 24 well-plate of THP-1 cells were stimulated for differentiation at 2 days before Caco-2 BBe1 cells were ready. After 48 h, THP-1 developed into macrophage-liked cells, which were attached at the bottom of cell plates. At day 10, the Transwell inserts on which Caco-2 BBe1 had been cultured were added into the 24-well plate preloaded with the differentiated THP-1 cells after removing the cell media. The insert which is referred to as the apical compartment was filled with Hank's buffered salt solution (HBSS), containing 10 mM HEPES, pH 6.4. Each well in the 24 well-plate is referred to as the basolateral compartment in which a double volume of HBSS containing 10 mM HEPES, pH 7.4 with 10% (v/v) FBS was added. Semi-purified phenolic extract of purple carrots or purple potatoes (200 μg/mL) was applied to the apical compartment and then continuously incubated for 6 h.

2.6 Inflammatory stimulation

Confluent cell monolayers were rinsed with HBSS and treated with different concentrations of semi-purified purple carrot or purple potato extracts dissolved in culture medium, at doses and times indicated. For the monoculture experiments, Caco-2 were stimulated with recombinant human TNF-α (2 ng/mL; Invitrogen) to induce inflammation. For the co-culture experiments, *Escherichia coli* O111:B4 lipopolysaccharide (LPS, 15 ng/mL; Sigma-Aldrich) was added into the basolateral compartment that contained the THP-1 cells to induce inflammation as previous described by others [18] with slight modification. Briefly, after 4 h incubation, co-cultured THP-1 cells were stimulated with LPS in the basolateral compartment for an additional 2-h incubation. Culture supernatants of the treated cells were then collected and stored at −80°C before for measuring IL-8 and TNF-α secretion using ELISA. Cells at the end of experiment 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) or Western blotting analyses. Protein concentration of cell lysate was determined by DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as standard.

2.7 Transport and absorption

Transport experiments were carried out by following a previously described procedure [19] with slight modifications. Briefly, after Caco-2 BBe1 cells were washed with HBSS, the semi-purified purple carrot or purple potato extract (200 μg/mL) dissolved in apical HBSS, pH 6.4, was applied to the insert and incubated with the Caco-2 BBe1, which

were attached at the bottom of the insert for a total of 6 h, and a double volume of HBSS containing 10mM HEPES, pH 7.4, was added in basolateral compartment. Transepithelial transport samples were assessed at every hour during the 6-h incubation in order to determine metabolic kinetics of the phytochemicals in the extracts. The solution collected from the basolateral compartment was applied to Strata™-X polymeric solid phase extraction [17] cartridges (30 mg, Phenomenex, Torrance, CA, USA) and evaporated to dryness for HPLC analysis. The cellular uptake of purple carrot or potato derived anthocyanins was measured following Faria's procedure with slight modification [19]. Specifically, after the removal of the apical HBSS from each individual insert, 500 μL of 70% (v/v) methanol was added into the insert to detach cells and extracted the cellular up-taken anthocyanins. The methanol mixtures containing Caco-2 BBe1 cells were subsequently transferred into a 1.5 mL tube and then homogenized for 1 min by a Qsonica Sonicator Q500 (Fisher Scientific) and centrifuged at 2000 × *g* for 5 min, in order to thoroughly extract the anthocyanins up-taken by the cells. The supernatant was collected and then cleaned up similarly to the procedures used for transepithelial samples before HPLC analysis. For quantification, all collected transepithelial transports and cellular uptakes were acidified with 2 N HCl, heated at 85°C for 1.5 h before injected into HPLC. Compounds were ionized in mass spectrometry (MS) using a heated electrospray ionization operating in positive mode.

2.8 HPLC and LC-MS/MS analysis of transported anthocyanins

The anthocyanins in the in vitro digesta of purple carrots or potatoes and those transported by intestinal epithelial cells were analyzed by an Agilent 1100 series HPLC system consisting of an auto-sampler, a degasser, a quaternary pump, and a diode array detector (DAD). Data were analyzed using Aglient 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 anthocyanidin compounds was performed after hydrolysis with external standard of cyanidin, using a linear curve generated between 0.125 and 50 μg/mL.Compound identities were determined by high resolution LC-DAD-MS and LC-MS/MS performed on a Thermo® Q-Exactive™ Orbitrap mass spectrometer coupled to an Agilent 1290 HPLC system. The formula generation tool in the Xcalibur Qualview software was used to determine the molecular formula of the major neutral losses (<10 mDa) and major product ion (<3 ppm) in MS/MS spectra which correspond to the molecular formula of the conjugates and the core anthocyanidin, respectively.

2.9 Cellular glucose uptake assay

For the glucose uptake assay, Caco-2 cells were seeded at 1×10^5 cells per well in a 96-well black/clear flat bottom plate (Corning, Costar) and then grown for overnight. Glucose uptake was measured using a glucose uptake cell-based assay kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, after washed with PBS, cells were thereafter treated with different concentrations (0.01–100 μg/mL) of purple carrot or potato semi-purified extract in 100 μL glucose-free DMEM containing 150 μg/mL 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose) (2-NBDG) and incubated for 1 h. The fluorescence intensity was measured using a fluorescence spectrophotometer PLX800 at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.10 Molecular modeling and computational docking

A 3D molecular model of full-length human *Homo sapiens* glucose transporter 2 (GLUT2), (#P11168) was retrieved from Protein Model Portal (PMP) [20]. The computational modeling was used to study the impact of anthocyanin on interacting with hexose transporters. The 3D model of human GLUT2 spanning amino acid sequences 7 through 489 was based on the crystal structures of human glucose transporter GLUT1 [21] with 54% sequence identity and the inward-facing structure of glucose transporter of *Staphylococcus epidermidis* [22, 23] with a sequence identity of 33%. The 3D structures of cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside and petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside were drawn and optimized using by ChemBioDraw software (Perkin Elmer; Waltham, Massachusetts, USA). *Homo sapiens* sodium/glucose cotransporter 1 (SC5A1) [accession number P13866 and 669 amino acids] was obtained from PMP and modeled based on sodium/galactose transporter from *Vibrio parahaemolyticus* (K294A MUTANT with an accession number 2XQ2 and 30% sequence identity with the template spanning amino acids 25–553) [24]. The docking of cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside or petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside with the GLUT2 and SGLT1transporter was refined by PATCHDOCK. Surfaces were treated facing an aqueous phase. 3D modeling of GLUT2/ SGLT1/cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside/petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside interactions were completed through the visual inspection of representations generated by PyMol [25] and Deep View [26].

2.11 Cytokine ELISAs

IL-8 or TNF-α concentration in cell supernatants was detected using a human IL-8 or TNF-α enzyme-linked

immunosorbent assay (ELISA) kits following the manufacturer's instructions (eBioscience, Inc., San Diego, CA, USA). Mouse anti-human IL-8 or TNF-α antibody were coated in 100 μL of PBS buffer in a 96-well plate (Corning, Costar) and incubated overnight at 4°C. The wells were washed three 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 or TNF-α standard were added into the wells and incubated for 2 h at room temperature. A 100 μL of secondary anti-human IL-8 or TNF-α 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 color development. This reaction was stopped by adding 50 μL 0.5N H_3PO_4. The absorbance was measured at 450 nm using EL340 UV/vis spectrophotometer (Bio-Tek). IL-8 concentrations were calculated from the IL-8 standard calibration curve.

2.12 RNA isolation and RT-PCR

Total RNA was extracted from cells using the PerfectPure RNA Cultured Cell Kit (5 Prime, Gaithersburg, MD, USA) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed into cDNA using a qScript™ cDNA Synthesis Kit (Quanta Biosciences, Inc., Gaithersburg, MD). RT-PCR was carried out using iQ SYBR Green Supermix (Quanta Biosciences) on Applied Biosystem 7500 Fast and 7500 RT-PCR system (Thermo Fisher Scientific) using the following conditions: 45 cycles of denaturization at 95°C for 15 s, annealing and extension at 60°C for 1 min using the primers listed in previous publication [16]. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [27] using GAPDH as the reference gene. Results were presented as fold expression change relative to the negative control representing untreated samples.

2.13 Western blot

Cells were washed twice with 1 mL of cold PBS and lysed in 100 μL ice-cold radio-immunoprecipitation assay (RIPA) buffer (Thermo Fisher) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher). Western blot analysis was carried out using the following procedure as previously described [28] with slight modifications. Total protein samples were resolved by SDS-PAGE (10%) and transferred onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked using 25 mL of 5% non-fat milk powder in Tris-buffered saline (TBS), and incubated with 10 mL primary antibody at a dilution of 1:2000 (v/v) overnight at 4°C. Detection was carried out using 10 mL of HRP-conjugated anti-mouse or anti-rabbit IgG (Promega, Madison, MI, USA) at the dilution rate of 1:10 000 (v/v) and ECL Western Blotting Detection Reagent (GE Healthcare, Mississauga, ON, Canada). Densitometry was performed using Image J software (Image Processing and Analysis in Java, National Institutes of Health, http://rsbweb.nih.gov/ij/).

2.14 Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) of at least triplicated measurements 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 or one-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 In vitro gastrointestinal digestion of anthocyanins from purple carrot or potato

In the present study, we first investigated the bioaccessibility of purple carrot or potato anthocyanin-rich extracts after simulated GI digestion, including gastric and intestinal phases, using in vitro digestion model [29]. As previously demonstrated, anthocyanins are the primary group of phytochemicals contained in our purple carrot and potato samples [16]. Our observation indicated a substantial impact of the digestion process on the availability of anthocyanin contents in purple carrots and potatoes. After simulated digestion, only 45 ± 1% of the originally applied cyanidin glycosides in purple carrot samples were detected, while 71.8 ± 0.3% of the petunidin glycosides from purple potato samples were detected. The significant reduction of anthocyanins observed in this study may be due to degradation under intestinal digestive condition such as mild alkaline environment [30, 31]. Glyco-conjugatged forms of anthocyanins such as cyanidin-3-glucoside occur more frequently in nature than aglycone forms [13, 14]. Woodward et al. have demonstrated that anthocyanidin glycosides are more stable compared with their aglycone derivatives during the simulated digestion [32]. Our results show that degradation of anthocyanins in purple carrot or potato during gastrointestinal digestion is relatively milder compared with findings in apples [30]. We validated that purple carrot or potato derived anthocyanins are able to reach the colonic epithelia after gastrointestinal digestion and absorption, suggesting they may directly influence anti-inflammatory action in intestinal epithelial system.

3.2 Anthocyanin-rich purple carrot or potato extracts reduce intestinal inflammation

Dysregulated mucosal immune system evokes the amplification of local and systemic inflammation resulting in

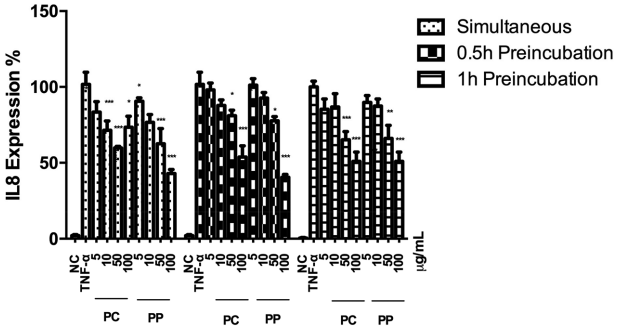

Figure 1. Effect of the anthocyanin-rich phenolic extracts of purple carrot and potato on TNF-α-induced interlukin (IL)-8 release from Caco-2 cells. Caco-2 cells were either pre-treated with 5 μg/mL, 10 μg/mL, 50 μg/mL and 100 μg/mL of the extract for 30 min or 1 h before stimulated with 2 ng/mL of TNF-α for another 4 h. In the treatment model, TNF-α was simultaneously added into cell culture with the above extracts. IL-8 expression was shown as presentation of released IL-8 from the positive control cells within each model. The negative control (NC) represents untreated cells. Values are presented as mean ± SEM of at least three independent experiments. Significant differences from cells treated with TNF-α alone were set at $p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

irreversible tissue damage by elevated production of inflammatory mediators [33]. This is the primary causative factor for chronic inflammation occurring in the GI tract and can significantly affect human health. Prolonged exposure to unhealthy dietary components such as saturated fats causes elevated local expression of TNF-α [3]. Over production of TNF-α further initiates and magnifies local inflammation and inhibits immune regulatory activity, eventually leading to tissue injury [34]. Because of this, TNF-α is a substantial and essential therapeutic target of intestinal inflammation. Caco-2 cells have been widely used as a model to study intestinal epithelial physiology and interactions between food substances and the gut [35].

Interleukin (IL) 8 is recognized as a primary histopathologic indicator of inflammation and IL-8 secretion from IECs results in recruitment of innate immune cells perpetuating progression of gut inflammation [36]. Similar to our previous study, a series of concentrations of semi-purified purple carrot or potato extract were simultaneously or preventively added to TNF-α-induced Caco-2 cells [16]. Results of the present study showed that IL-8 productions in cells pre-exposed to different concentrations of extracts prior to TNF-α stimulation and in cells simultaneously exposed to the extracts and TNF-α were all reduced in a dose-dependent manner (Fig. 1). In the simultaneous model, production of IL-8 was inhibited very significantly ($p < 0.01$) at nearly all concentrations except at the lowest concentration of 5 μg/mL at which only the purple potato extract showed significant inhibition ($P < 0.05$), suggesting components in this extract may be more effective. In the pre-incubation model, only cells treated with 100 μg/mL of extracts showed very significant inhibition of IL-8 ($p < 0.01$). Shorter incubation (30 min) was more effective than longer exposure, especially in cells pre-treated with purple potato extract (Fig. 1). Similar to other findings [37], the weakened activity of the latter might be due to degradation of the phenolics. These results suggest that the anthocyanin-rich phenolic extracts of the purple carrot and potato variety are potential anti-inflammatory agents and may act to prevent intestinal inflammation. In order to study the preventive effect on gut inflammation, a shorter pre-incubation (30 min) model was chosen in the follow-up studies.

Other TNF-α-induced pro-inflammatory cytokines, including IL-8, TNF-α, IL-6, and IL-1β, also play important roles in the progression of intestinal mucosal inflammation [38]. These biomarkers of inflammation were measured after Caco-2 cells were first pre-treated with low dose (50 μg/mL) or high dose (100 μg/mL) extracts for 30 min followed by incubation with 2 ng/mL exogenous TNF-α for 2 h. Expression of the above pro-inflammatory cytokines in treated cells was subsequently measured by RT-PCR. Significant suppression of IL-8, IL-6 and IL-1β expressions over the TNF-α control was observed in all treatments except for IL-6 at 50 μg/mL (Fig. 2). TNF-α expression was also significantly lower in cells pre-treated with low dose of purple carrot extract and high dose of purple potato extract (Fig. 2). The two extracts were able to restore the IL-6 and IL-1β expressions to a level observed in the untreated normal cells (negative control) at all doses with a couple of exceptions (Fig. 2). No significant dose dependence was found between the two doses (2), indicating the range of dosages used might be too narrow. These results suggest that the anthocyanin-rich extracts of purple root vegetables can attenuate inflammatory responses in the intestinal epithelial layer thus potentially improve intestinal homeostasis, and preserving normal intestinal physiological functions.

The underlying molecular basis for TNF-α-induced cellular pro-inflammatory cytokine production is to activate the NF-κB pathway and the MAPK pathway [28]. These two pathways are specific molecular targets for food bioactive constituents possessing chemopreventive actions [11]. The potential of food bioactives to suppress these pro-inflammatory signaling pathways affords new opportunities for developing alterative preventive and therapeutic strategies for chronic diseases through diet. A recent study has demonstrated that polyphenols are able to suppress the activation of TNF-α-induced cellular inflammatory pathways, including MAPK and NF-κB, which can be evaluated by expression of phosphorylated IκBα and c-Jun-N-terminal kinase (JNK) [39]. A significant suppression of TNF-α-induced phosphorylation of IκBα or JNK was observed in all anthocyanin-rich extract supplemented cells at both high and low doses when compared to cells treated with TNF-α alone (Fig. 3). Even though a significant dose-dependence was not identified in this study, a stronger suppression of TNF-α-induced activation of both inflammatory signaling pathways, especially NF-κB, was observed at all high doses. It is well-known that redox transcriptional regulator nuclear factor E2-related factor 2/antioxidant responsive element (Nrf2/ARE) are involved in inhibition of

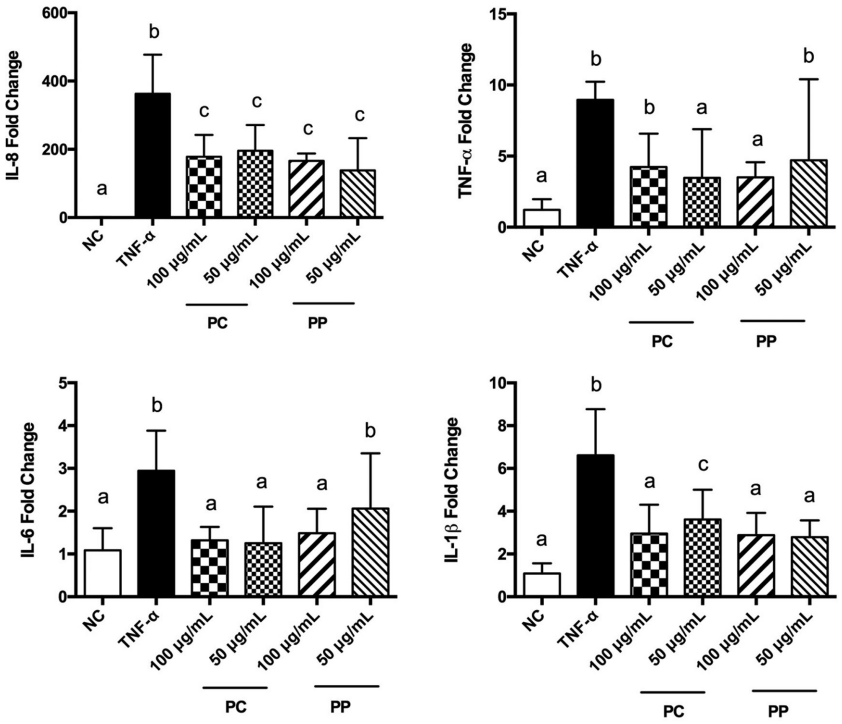

Figure 2. Effect of the anthocyanin-rich phenolic extracts of purple carrot and potato on TNF-α-induced pro-inflammatory cytokines expression in Caco-2 cells. Caco-2 cells were pre-treated with 50 μg/mL and 100 μg/mL extracts for 30 min, followed by incubation with 2 ng/mL of TNF-α for another 2 h. The 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 of at least three independent experiments. Values without a common letter are significantly different at $p < 0.05$.

NF-κB and MAPKs activation. Speciale and colleagues (2013) demonstrated the ability of cyanidin-3-*O*-glucoside to activate the Nrf2 pathway to counteract with the TNF-α-activated NF-κB and MAPK pathways in endothelial cells [17]. Several alternative molecular mechanisms have also been recently proposed for phenolic compounds in attenuating inflammatory responses [40]. A notable cross-talk between the aryl hydrocarbon receptor (Ahr) and Rel-like domain-containing protein A (RelA) was revealed, which ascertains the molecular basis of phenolic compounds interfering with the TNF-α-induced NF-κB pathway [41]. Although not statistically significant, a clear trend of dose effect was observed in the inhibition of phosphorylation of IκBα by the extracts (Fig. 3), suggesting NF-κB pathway was particularly targeted by the bioactives in treated IECs. Results of the present study also imply that multiple signaling molecules such as Nrf2 and RelA controlled pathways might be regulated by the bioactive constituents in purple carrot and potato extracts resulting in blocking TNF-α-induced inflammation. Together with our above data, it can be concluded that the anti-inflammatory activity of anthocyanin-rich purple carrot and potato extracts may be due to their ability to attenuating inflammatory signaling events, through suppressing the production of inflammatory mediators in the intestinal epithelia.

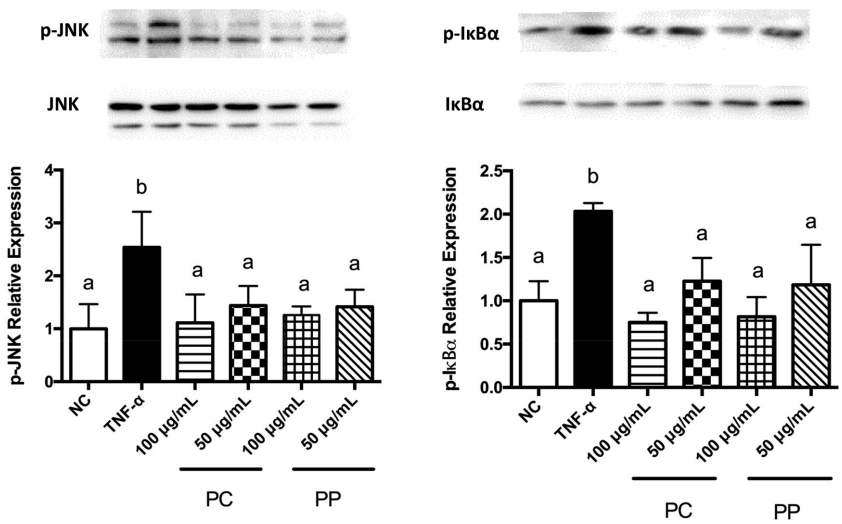

Figure 3. Suppressive effect of the anthocyanin-rich phenolic extracts of purple carrot and potato on TNF-α-stimulated phosphorylation of JNK and IκBα. Caco-2 cells were pre-treated for 30 min with 50 μg/mL and 100 μg/mL extracts and followed by stimulation with TNF-α for another 2 h, and then Western blot analysis of cells lysates was performed. The representative images and quantitative analysis of relative levels of phosphorylated JNK and IκBα are shown. Values are expressed as fold change relative to untreated cells, and presented as mean ± SEM of at least three individual replicates. Values without a common letter are significantly different at $p < 0.05$.

3.3 Bioaccessibility and cellular uptake of anthocyanins from semi-purified purple carrot and potato extracts

The potential availability of anthocyanins after gastrointestinal digestion is important. The immune regulatory effects of anthocyanins derived from purple carrot or potato depend on their bioaccessibility, which must be demonstrated and confirmed that these bioactives can be delivered to the intestinal lamina propria where they can directly interact with immune cells to modulate inflammatory processes [42]. However, absorption and metabolism of anthocyanins derived from purple root vegetables are still not well understood. Caco-2 cells possess many physiological functions of the human intestinal epithelium in expressing intestinal enzymes and transporter proteins, thus are widely used as an in vitro model to study intestinal nutrient or drug permeability and uptake [43, 44]. Caco-2 BBe1 cell line was used in the present study to evaluate the absorption and metabolic fate of anthocyanins contained in the above mentioned extracts, and to determine the active compounds in each extract, thereby unravelling their bioavailability and bioefficacy.

Permeation of anthocyanins across the Caco-2 BBe1 monolayer was studied by applying 200 μg/mL of each extract to the apical compartment and then the absorbed or transported anthocyanins were assessed in collected cells or in the basolateral compartment at 1-h intervals from 1 to 6 h. Only slight drop in TEER values were observed in cells treated by the extracts at 1 h (4), but the values were restored quickly after, suggesting the change of TEER value may be caused by temperature drop. This also indicates that exposure to anthocyanin-rich extracts has limited effects on the integrity of the Caco-2 monolayer. The transport efficiency was then evaluated as the percentage of anthocyanins detected in the basolateral side of the cell monolayer of that originally added to the apical side. The permeating curve of cells indicates the metabolic kinetics of different treatments (Fig. 4). Elevated cellular uptake of anthocyanins in treated cells was observed as incubation time increased; however, the trend of uptake of the anthocyanins in the two extracts was different (Fig. 4). A dramatically increased uptake of anthocyanins was observed in cells exposed to purple carrot extract between 1 and 3 h of incubation, but the uptake started to drop thereafter until 6 h (Fig. 4A). The absorption curve however had an opposite trend. Concentrations of carrot anthocyanins in the cellular and basolateral sides were similar at the end of the experiment (Fig. 4A). Different absorption and uptake patterns were seen for the potato anthocyanins; both curves accumulatively and continuously increased throughout the experimental period (Fig. 4B). Two mechanisms have been proposed for the transport of anthocyanins in intestinal lumen,one by passive diffusion and the other via active transport mediated by hexose transporters such as sodium-dependent glucose cotransporter (SGLT)-1 or glucose transporters (GLUTs) distributed in luminal membrane of the intestinal epithelium [45]. Passive diffusion is the mechanism proposed particularly for the

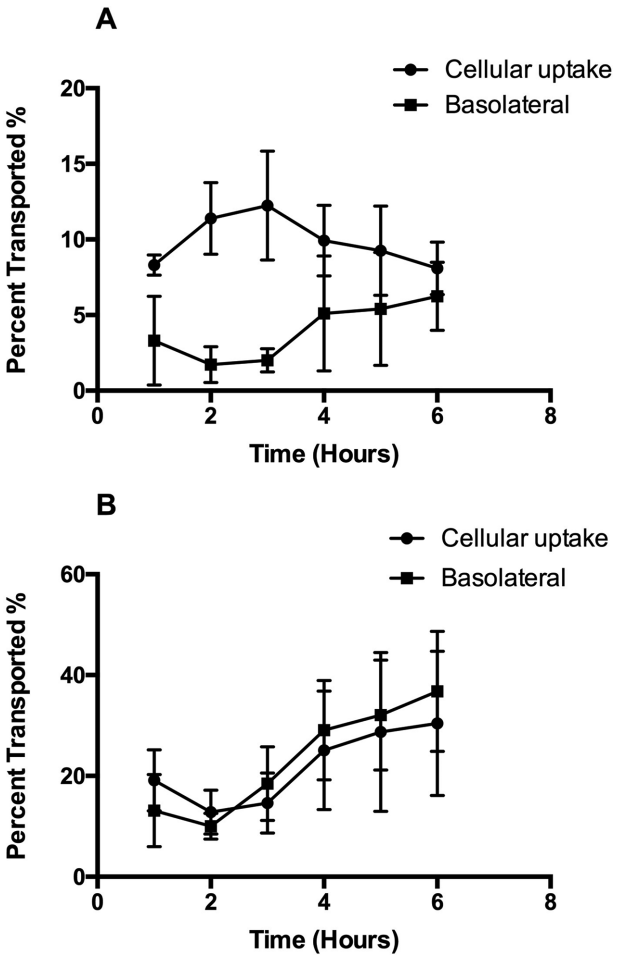

Figure 4. Transport of anthocyanins of purple carrot and potato across Caco-2 BBe1 monolayers. The permeability efficiency was assessed by HPLC analysis of the content of anthocyanins collected from cell lysate or basolateral compartment normalized by anthocyanins in apical compartment throughout the incubation of 6 h. Percent (%) cellular uptake of anthocyanins of purple carrot (A) and potato (B) in Caco-2 BBe1 cells are shown in filled circles; and trans-epithelial anthocyanins collected from basolateral compartment of Caco-2 BBe1 monolayers exposing to purple carrot (A) and potato (B) extracts are shown in squares, respectively. Data are mean ± SEM of at least three independent experiments.

aglycones (anthocyanidins) after anthocyanin glycosides were extracellularly hydrolysed by lactase phlorizin hydrolase [46]; whereas the hexose transporters are considered to be able to facilitate the transport of intact anthocyanidin glycosides (anthocyanins) into the enterocyte [13,14]. A fast uptake of purple carrot anthocyanin by Caco-2 cells observed in the present study suggests it has a higher affinity to SGLT-1 (Fig. 4A). However, accumulative uptake and continuous increase of anthocyanins of the purple potato extract by Caco-2 cells indicate their cellular absorption mainly depends on the active transport mechanism. In addition, due to the wide distribution of GLUT2 along the intestinal basolateral membrane, it

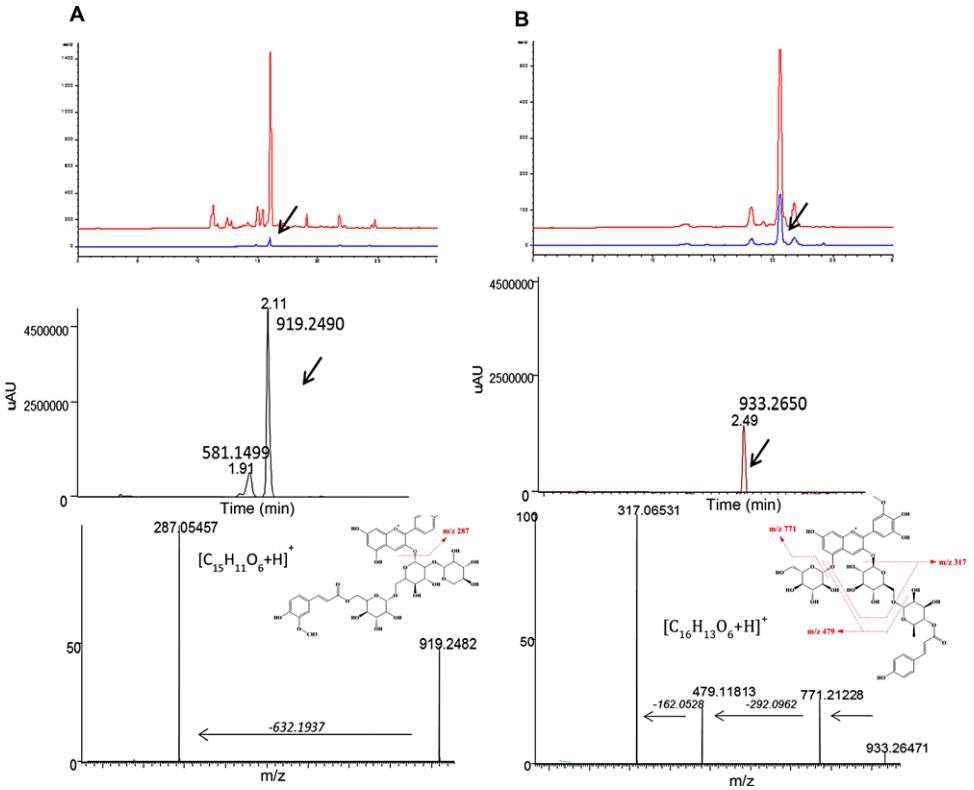

Figure 5. Trans-epithelial anthocyanins of purple carrot (Panel A, left) and potato (Panel B, right). Top: HPLC profiles (520 nm) of anthocyanins before and after in vitro transport across Caco-2 BBe1 monolayers. Purple carrot or potato extract (200 μg/mL) was directly applied into the apical compartment of the transwell system. Following incubation, solution from the basolateral compartment was collected and then analyzed by HPLC after purification. Middle: The peaks detected in transported samples collected from purple carrot or potato treated cells by LC-MS. Bottom: Identification of the major anthocyanin peaks by MS/MS and fragmentation pattern of identified anthocyanins.

is highly likely that GLUT2 is involved in the excretion of the intact anthocyanins into the lamina propria [47]. This is supported by the continued increase of transported anthocyanins in the basolateral side of the IECs (Fig. 4).

Only 6 ± 1% of the total purple carrot anthocyanins were transported (Fig. 4A), whereas 36 ± 6% of those in the purple potato extract were transported to the basolateral side of the monolayer (Fig. 4B), suggesting anthocyanins of the purple potato have higher transport efficiency compared to those in the carrots. The anthocyanin compositions of both apical and basolateral collections were analyzed by LC-DAD-MS/MS. Each extract had one main anthocyanin peak as detected at 520 nm by DAD. MS/MS showed that in both apical and basolateral collections, the main peak in purple carrot extract had a neutral molecular mass of 918.25 and 932.27 Da in purple potato extract (Fig. 5). Based on the molecular formulas derived from the accurate mass measurement, the neutral mass losses in MS/MS and data reported in the literature, the main anthocyanins in purple carrot and purple potato are likely cyanidin-3-*O*-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside and petunidin-3-*O*-*p*-coumaroylrutinoside-5-*O*-glucoside, respectively (Fig. 5). These two main anthocyanins have been identified in various highly pigmented cultivars of carrots or potatoes [48–50]. To the best of our knowledge, this is the first report on the detection and identification of highly complex anthocyanins being transported across the monolayer of IECs. A previous study on the absorption of blueberry anthocyanins showed that a simpler anthocyanin petunidin 3-*O*-β-glucoside was more readily transported to the basolateral side of the Caco-2 monolayer compared with cyanidin 3-*O*-β-glucoside [12], suggesting the additional methoxy group on the B-ring of petunidin might play a role in the enhanced bioavailability. This and the present findings suggest that structural differences including the aglycone backbone and the glycosylation pattern may significantly affect the absorption or transport of native anthocyanins of plant foods.

3.4 Molecular mechanism of intestinal cellular uptake of anthocyanins from semi-purified purple carrot and potato extracts

In order to substantiate the impact of conformational features of anthocyanins on regulation of molecule's sensing and binding to the transporter proteins, we used computational modeling to assess SGLT-1 (belonging to SC5A1 gene family) and GLUT2 binding with purple carrot and potato derived anthocyanins identified in the previous section. The published structure of human SC5A1 modeled based on sodium/galactose transporter and human glucose transporter GLUT1 (4pypA) and glucose transporter of *Staphylococcus epidermidis* (4ldsA) were used as templates to model the 3D structure of SGLT-1 and GLUT2 respecitively. The proposed structure of GLUT2 spanned across amino acids 7 to 489 corresponding to 92% coverage of GLUT2's full-length

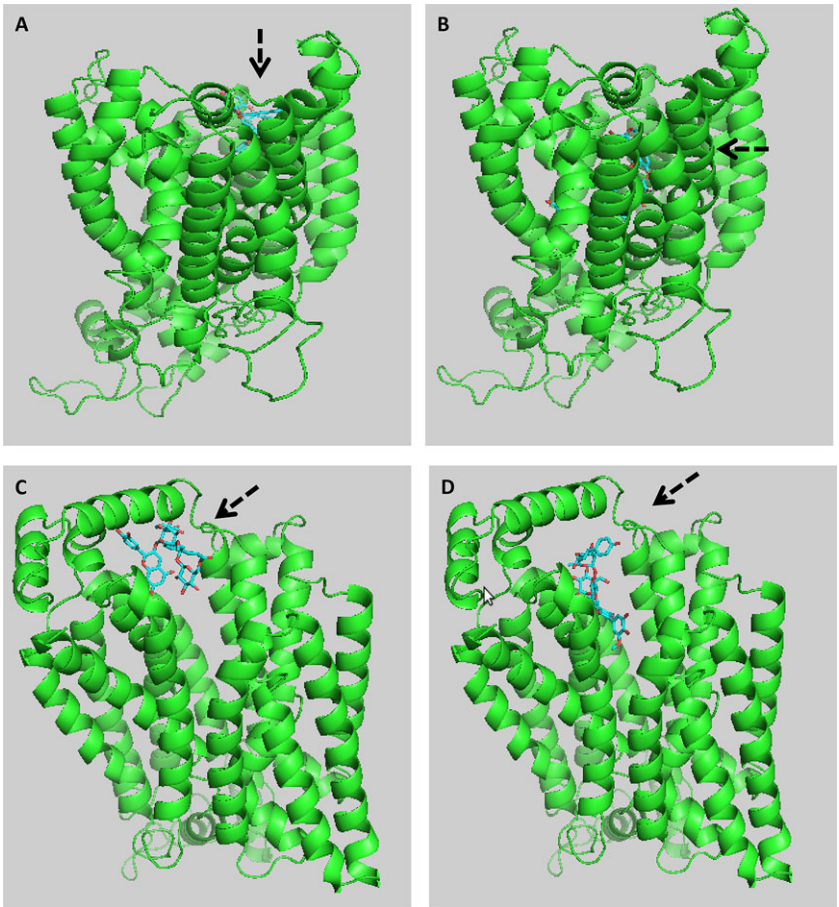

Figure 6. Molecular docking showing that both cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactosideand petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside can possibly be transported by GLUT2 and SGLT1. Larger interface-areas with lower desolvation energies were calculated for cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside interactions with SGLT1 (A) and GLUT2 (C), respectively in comparison to petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside interactions with SGLT1 (B) and GLUT2 (D). Furthermore, cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside has unique binding/interaction positions within each transporters that differ from the binding positions of petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside.

sequence (524 amino acids in total). Our docking calculations suggested that both anthocyanin molecules derived from purple carrot and potato spatially complemented human SGLT-1 with the selected top geometric shape complementarity score to be 9254 for petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside and 8436 for cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside, respectively; while GLUT2 had similar geometric scores (8128 for cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside and 8042 for petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside). The binding conformations of the above two anthocyanin molecules in the pocket shaped SGLT-1 and GLUT2 docking site are shown in Fig. 6. Despite the above similar geometric scores, clear differences in the calculated atomic contact energies (ACE), the free energy required to rearrange water molecules within the protein's interior in order for the protein-substrate interactions to proceed, and interface areas were observed between the two compounds. The complementarity area between SGLT1 and petunidin-3-O-*p*-coumaroylrutinoside-5-*O*-glucoside was 1144.20 with ACE close to -373 while it was 1194 for cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside with a desolvation energy of ACE = -632.01. Besides, cyanidin-3-*O*-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside endured a larger interface area (1022.4) with lower ACE (-365.70) to spatially bind with GLUT2 in comparison to petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside (area = 905.8, ACE = -183.54). The docking of cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside or petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside with GLUT2 showed similar tendencies but a slightly higher surface complementarity between SGLT1 and the above two compounds in comparison with that between GLUT2 and the two anthocyanins. Our results showed cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside has stronger binding affinity for both glucose transporters compared with petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside.

This could possibly explain the higher efficacy of cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside in inhibiting glucose transport. In this assay, the less the intracellular glucose level, the lower fluorescent intensity is detected. So to achieve the same level of fluorescent inhibition during glucose transport, close to 100-fold higher concentration of petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside was needed (7). Our in vitro study indicated that both purple carrot and potato derived anthocyanins can interfere with the intestinal glucose uptake in a competitive manner due to steric hindrance, which has been reported by

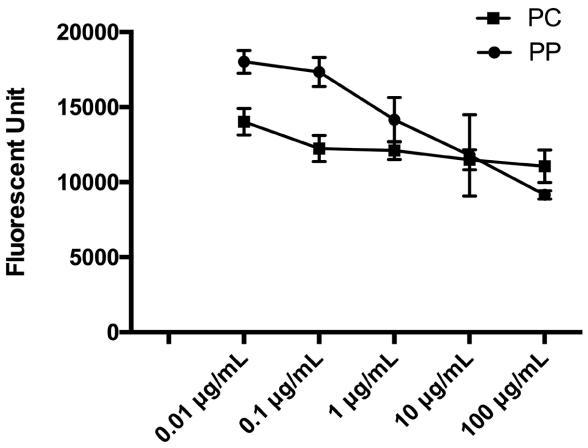

Figure 7. Effects of transported anthocyanins on glucose uptake in Caco-2 cells. Cells were treated with a series of concentrations of semi-purified anthocyanin-rich purple carrot (PC) or potato (PP) extract for 1 h. Data are presented as mean ± SEM of at least three independent experiments.

others [51]. A low concentration of cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside could already inhibit glucose uptake in Caco-2 cells due to its saturated binding to both SGLT-1 and GLUT2 as shown in Fig. 7. In the intestinal cells, both SGLT-1 and GLUT2 are distributed along apical side of brush border, while GLUT2 is the dominate isoform distributed on the basolateral side of the enterocytes [52]. Our observations suggest cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside has a strong steric hindrance potential to both glucose transporters, thereby particularly affecting its intestinal uptake and efflux. This finding agrees with a significant lower transport efficiency of purple carrot derived anthocyanin observed in our previous section.

The computational docking analysis demonstrated that both purple carrot and potato derived anthocyanins can directly bind with docking pocket of SGLT-1 and GLUT2. Our findings support that anthocyanins derived from purple carrots and potatoes can inhibit glucose uptake across the brush border membrane of enterocytes by competitively interacting with glucose transporters. The steric hindrance by the anthocyanins suggests that conformational features of these anthocyanins play a key role in affecting their sensing and transport efficiency in the case of active transport. The uptake and active transport of anthocyanins largely depend on the presence of a hexose moiety, especially a glucose residue. Inhibitors of hexose transporter GLUT2 were found to lower the bioavailability and transport of anthocyanins and to help stabilize the glycosides in Caco-2 cells [13]. The results observed in the present study suggest that a hexose transporter-mediated active transport mechanism is involved in the intact transport of these two anthocyanin compounds in IECs. Nevertheless, the modulatory effects of these transported anthocyanin molecules on immune cells distributed in the lamina propria are unknown, therefore further experiments were conducted to investigate the immunomodulatory effects of the transported anthocyanins in inflamed cells.

3.5 Transported anthocyanins modulate inflammatory responses of macrophages

Exposure to dietary antigen, pathogens and environmental stimulus has significant impact on intestinal epithelial barrier integrity and physiological functionalities, leading to increased intestinal permeability. As a result, commensal bacteria in the gut permeate into the mucosal layer to mount production of inflammatory mediators as they encounter with the innate immune cells. Persistent activation of innate immune responses leads to suppressed immune regulatory responses and onset of profound inflammation that ultimately causes damage to the intestinal barrier [53]. To assess the modulatory effects of transported anthocyanins on innate immune cells such as macrophages, a co-culture in vitro system consisting of intestinal epithelial Caco-BBe1(apical side) and macrophage THP-1 cells (basolateral side) was used in the present study to mimic the human intestinal mucosal system. Caco-BBe1 cells are a useful model for the study of intestinal transport of dietary bioactive constituents. Lipopolysaccharide (LPS) from pathogenic bacteria is a known endotoxin used to stimulate THP-1 macrophages, a human monocytic cell line, to mimic the in vivo inflammatory milieu of the intestine [18,54]. In the present study, LPS was directly applied into the basolateral side in order to prevent inhomogeneous transport of LPS by IECs and to ensure only TLR-4-mediated pro-inflammatory pathways will occur in macrophages upon LPS challenge. Intestinal macrophages are known as a prominent regulator in governing both innate and adaptive immune responses against pathogenic stimulation, due to the continuous exposure of the intestinal surface to enormous amounts of antigens. However, intestinal macrophages are dysfunctional under profound mucosal inflammation that can lead to inappropriate responses against even harmless commensal bacteria [55]. The effect of trans-epithelial anthocyanins of this study therefore may inhibit the expression of inflammatory cytokines from LPS-induced THP-1 macrophages at the protein and gene levels. This was indeed observed in the present study. Upon LPS stimulation, both TNF-α and IL-8 productions in THP-1 were drastically augmented when compared with untreated cells (negative control), but secretion of both LPS-induced cytokines was significantly suppressed by the two major transported anthocyanins in the THP-1 cells of the co-culture system ($p < 0.05$) (Fig. 8). Transported anthocyanins from the purple potato extract had a significantly stronger inhibition on both biomarkers as compared to those of the purple carrot extract ($p < 0.05$) (Fig. 8). Further studies showed that although distinctly decreased gene expression was found for all inflammatory cytokines TNF-α, IL-8, IL-1β, and IL-6, only TNF-α and IL-8 expressions were significantly suppressed by transported anthocyanins in both extract-treated groups (Fig. 9). The reduced IL-1β and IL-6

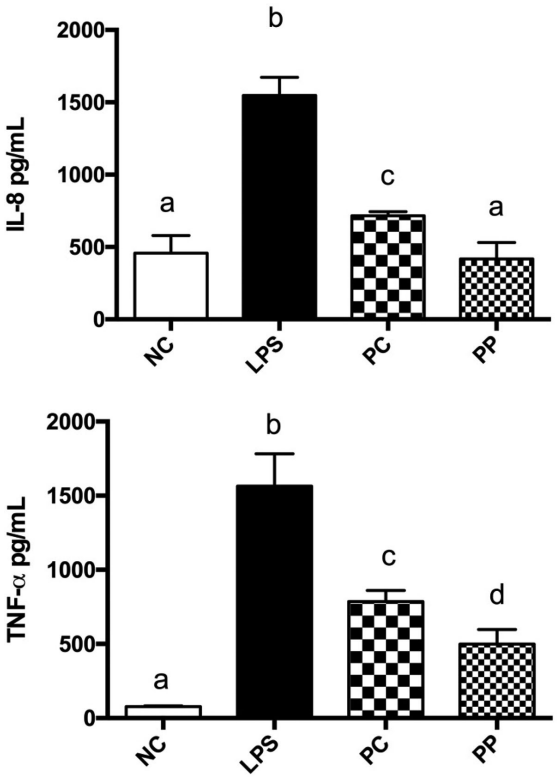

Figure 8. Anti-inflammatory activity of transported anthocyanins derived from purple carrot or potato in a co-culture system consisted of Caco-2 BBe1 and THP-1 cells. The anthocyanin-rich purple carrot or potato extracts were added into apical compartment and incubated with Caco-2 BBe1 for 4 h followed by LPS (15 ng/mL) added into basolateral compartment to stimulate THP-1 cells for another 2 h. Secretion of IL-8 and TNF-α were measured in basolateral supernatant by ELISA. Data are mean ± SEM of at least three independent experiments. Values without a common letter are significantly different at $p < 0.05$.

expression in LPS-induced THP-1 cells may be due to the higher variability (Fig. 9). The augmentation of pro-inflammatory cytokines produced by macrophage plays a key role in the aggravation of inflammation, which may result in the initiation and magnification of local and systemic inflammation that ultimately leads to irreversible tissue damage. The present findings suggest that anthocyanins from purple carrots or potatoes, when successfully absorbed and transported, can suppress LPS triggered inflammatory responses thereby acquiring immune regulatory ability to restore mucosal homeostasis.

The intestinal mucosal inflammatory responses are commonly actuated by Toll-like receptor (TLR-4) expressed by innate immune cells recognizing LPS, which leads to Toll-IL-1R signaling pathway activation, including both NF-κB and MAPK pathways to augment expressions of the above studied inflammatory cytokines. To confirm this, the anti-inflammatory activity of transported anthocyanins in the co-culture system was examined by expressing phosphorylated JNK or IkBα, as indicators of MAPK and NF-κB activation, respectively. As observed, both p-JNK and p-IkBα expressions were significantly suppressed in co-cultured THP-1cells in response to LPS stimulation ($p < 0.05$), particularly by transported anthocyanins from purple potatoes which restored the level to that of the untreated normal cells (Fig. 10). Reduction in p-JNK and p-IkBα expression was observed but not significant in co-culture cells treated by purple carrot extract (Fig. 10). These results suggest that transported anthocyanins derived from purple root vegetables are able to abrogate the TLR mediated inflammatory signaling cascades in mucosal innate immune cells, and those derived from purple potatoes are stronger anti-inflammatory agents compared with those from purple carrots. Even though our previous study indicated that anthocyanin contents were higher in purple carrots than in purple potatoes [16], the present results suggest that composition, i.e., type of anthocyanins

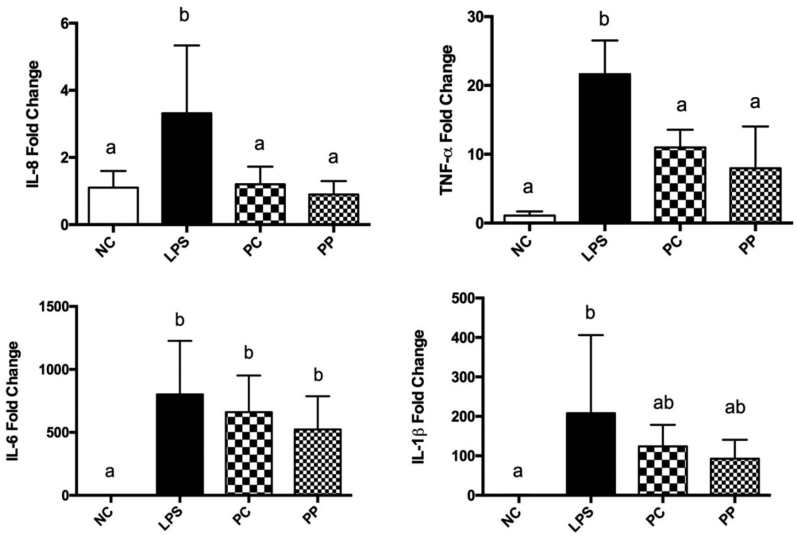

Figure 9. Effects of transported purple carrot or potato derived anthocyanins on the expression of pro-inflammatory cytokines in THP-1 cells. The anthocyanin-rich purple carrot or potato extracts were added into apical compartment and incubated with Caco-2 BBe1 for 4 h followed by LPS (15 ng/mL) added into basolateral compartment to stimulate THP-1 cells for another 2 h. Expression of pro-inflammatory cytokines, including TNF-α, IL-8, IL-1β, and IL-6, were measured in THP-1 cells collected from basolateral compartment. Data are presented as mean ± SEM of at least three independent experiments. Values without a common letter are significantly different at $p < 0.05$.

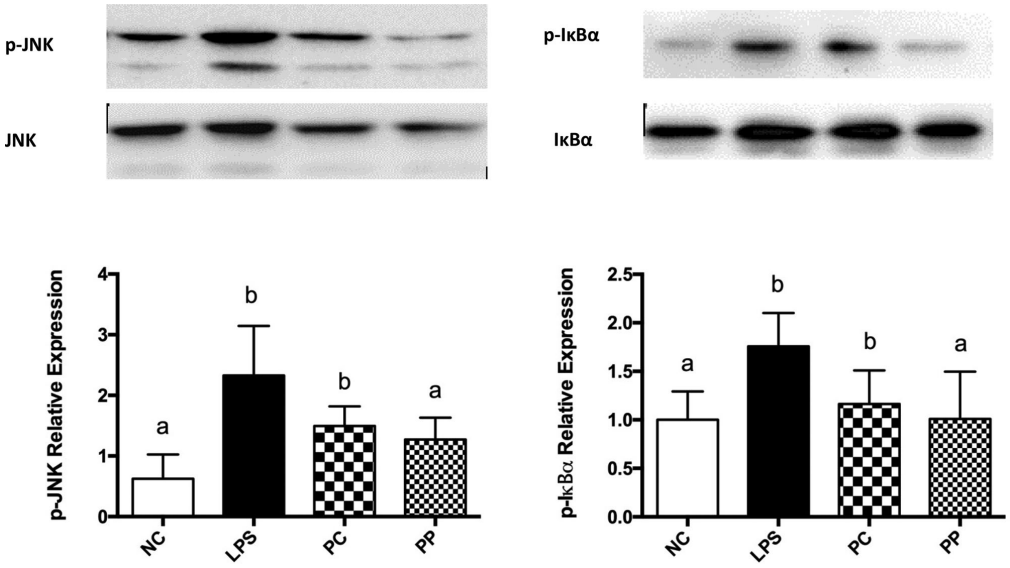

Figure 10. Modulatory effect of anthocyanins on LPS-induced phosphorylation of JNK and IκBα in THP-1 cells. The anthocyanin-rich purple carrot or potato extracts were added into apical compartment and incubated with Caco-2 BBe1 for 4 h followed by LPS (15 ng/mL) added into basolateral compartment to stimulate THP-1 cells for another 2 h, and then Western blot analysis of cells lysates was performed for THP-1 cells collected from the basolateral compartment. The representative images and quantitative analysis of relative levels of phosphorylated JNK and IκBα are shown. Values are expressed as fold change relative to untreated cells, and presented as mean ± SEM of at least three individual experiments. Values without a common letter are significantly different at $p < 0.05$.

rather than the amount may more significantly affect the efficiency of the transport across the epithelial layer of the intestine, and conformational features of these pigment molecules may have significant impact on the bioavailability and anti-inflammatory effects.

4 Concluding remarks

Increasing evidence has shown that anthocyanins and their metabolites circulated in the body system possess strong health-enhancing physiological activity arising from their anti-inflammatory effects [40]. Although the levels of anthocyanins in the circulating blood are often believed to be too low to engage in direct health promoting actions such as antioxidant activities, recent studies have demonstrated that low concentrations of these compounds do have significant regulatory effects on cellular biomarkers by modulating cellular signaling transductions [10]. In the present study, we demonstrated the bioaccessibility and bioavailability of anthocyanins from two purple root vegetables using in vitro models. Anthocyanins derived from these purple root vegetables were relatively resistant to digestion in the gastrointestinal tract, therefore potentially gaining access to the intestinal epithelium to initiate anti-inflammatory actions. The transported anthocyanins in the basolateral side of the Caco-2 monolayer were found to be in their original glycoside forms as was confirmed by LC-MS/MS. Both passive and active transports were likely involved, but between the two main anthocyanins, i.e. cyanidin-3-O-(2″-xylosyl-6″-(6‴-feruloyl-glucosyl)-galactoside from purple carrot and petunidin-3-O-*p*-coumaroylrutinoside-5-O-glucoside from purple potato, the latter was found to be more readily taken up by the cells in the in vitro transmembrane system, possibly *via* hexose transporters SGLT1 and GLUT2, which may preferably bind to a cyanidin glycoside than a petunidin glycoside as shown in the in silico docking experiment. This was seen in higher transport efficiency of purple potato derived anthocyanin (37 ± 6%) than purple carrot derived anthocyanins (6 ± 1%), ultimately leading to a difference in their bioavailability. Interactions between dietary anthocyanins and hexose transporters and the effect on the delivery of hexoses such as glucose have been receiving considerable attention in recent years as a mechanism for managing blood glucose by anthocyanins. The present study further showed that the two transported anthocyanins, especially the petunidin glycoside from purple potato, owing to its stronger transport efficiency, suppressed the secretion of pro-inflammatory cytokines IL-6, IL-8, IL-1β, and TNF-α *via* blocking NF-κB and MAPK involved inflammatory cellular signaling cascades, in inflamed THP-1 cells of a co-culture system. These findings strongly suggest that dietary anthocyanins in the two purple root vegetables, particularly the purple potatoes may help restore mucosal homeostasis in inflamed gut. Information obtained on the bioaccessibility, bioavailability, and anti-inflammatory effects are essential for further exploration and development of purple carrot and potato based functional foods or beverages for risk reduction of inflammation related chronic diseases.

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The authors have declared no conflict of interest.

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