IN VITRO ANTI-INFLAMMATORY POTENTIAL OF PHENLICS FROM DIGESTED AND ABSORBED GEORGIA-GROWN BLACKBERRIES

Progress Report
SRSFC Project # 2013-15
Research Proposal

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Objectives: To determine the level and anti-inflammatory activity of polyphenolic constituents (*i.e.*, anthocyanins) from blackberries before and after digestion as well as absorption.

Justification:
Owing to their perishability, blackberries (*Rubus* spp.) have been the subject of much horticulture research and many breeding programs aimed at producing a more robust berry. Production challenges such as harvesting by hand, fungus & disease prevention, and safe transportation create a thin profit margin on which the berries can be sold. Improving yield and shelf-life are important routes to increasing profitability and decreasing waste; yet, the industry would also be well-served by research that generated greater consumer demand. Research that raises public awareness for the health effects of blackberries can aid in creating such demand. While cranberries, pomegranates, and gogi berries have benefited from the nutraceutical/ functional food market boom and still do, other berries have not yet been adequately recognized for their health potential. By researching and documenting the physiological benefits associated with blackberry consumption progress can be made toward a qualified health claim for the anthocyanin-rich blackberries.

Research conducted in my lab has demonstrated the antioxidant and anti-inflammatory efficacy of polyphenolics from southeastern U.S. range blackberry cultivars (Srivastava *et al.* 2010. *Journal of Agricultural and Food Chemistry, 58,* 6102-6109; Hart *et al.* 2010. Blackberry polyphenolic inhibition of proinflammatory mediators released from murine RAW 264.7 macrophage cell lines. Institute of Food Technologists Annual Meeting + Food Expo, IFTSA Undergraduate Research 2nd Place Winner, July 18, Chicago, IL, oral & poster presentation; Bierwirth *et al.* 2012. The influence of *in vitro* digestion on the anti-inflammatory capacity of blackberry phenolics. 5th International Conference and Exhibition on Nutraceuticals & Functional Foods, International Society for Nutraceuticals and Functional Foods, Kailua-Kona, Hawaii, USA, December 2-6). The present research differs from previous research on blackberries and other fruits by more closely approximating the assimilation of phytochemicals during intestinal digestion and absorption. Much of the existing antioxidant research is done on food products that have not undergone digestion or absorption. The conclusions reached have not accounted for the potential breakdown of polyphenols in gastric juice nor the bioavailability of those antioxidants that survive. Human studies have their limitations as well. Because of a highly confounded food matrix and wide variability in gastrointestinal function, doses of the food-of-interest must be very high and trials very lengthy to achieve significant results. Even

then, the health effects are often measured by proxy, with subtle biomarkers of interest subject to the forces several competing physiological processes.

Contrary to previous trials, the proposed research is investigating the effects of digestion and absorption on antioxidant form, function, and availability in a controlled setting. Three southeastern U.S. range blackberry cultivars (*i.e.,* Navaho, Ouachita, and Natchez) from Paulk's Vineyard (Wray, GA) were harvested in June 2013 and frozen for analysis. Samples have undergone *in vitro* digestion with standardized enzymes, times, and temperatures. The glucuronidated, sulfonated, and methylated anthocyanin derivatives as well as other polyphenolic metabolites are being extracted from the resulting chyme and isolated. Caco-2 cell monolayers, a cultured intestinal cell system used for drug development and testing, will be treated on their apical surface with the digested, purified blackberry phenolics to assess bioavailability. The basal layer, containing the absorbed antioxidants, will then be used to treat RAW 264.7 murine macrophage cells and to determine the anti-inflammatory potential of the digested, absorbed anthocyanin derivatives. Preliminary research to date has been promising and indicates that sufficient quantities of phenolic antioxidants do indeed survive the digestive process and reduce inflammation in the cultured macrophage cells.

Methodologies

Collection of samples
Mature blackberries *(Rubus* spp.) of the Navaho, Ouchita, and Nachez cultivars were hand-picked from Jacob W. Paulk Farms, Inc. (Wray, GA) in June 2013. The blackberries were transported to the Department of Food Science & Technology, UGA, in Athens, GA, then sorted, cleaned, and frozen in polyethylene pouches. Samples were stored at -40 °C prior to analysis. Much of the sample remained frozen to be digested, while representative samples that were to be analyzed raw (undigested) were lyophilized using a FreeZone 2.5 L bench-top freeze dryer (Labconco Corp., Kansas City, MS).

Digestion of blackberry
Representative samples of 4.5-g frozen berries were thawed, crushed, and digested in triplicate at 37 °C in a shaking water bath according to an established *in vitro* digestion protocol. (Versantoort *et. al.*, 2005. *Food and Chemical Toxicology, 43*, 31-40). The resulting digest was lyophilized.

Preparation of crude blackberry extracts (CBEs)
Lyophilized berry and digested samples were ground using a commercial coffee mill (KitchenAid, St Joseph, MI). Fifteen grams of blackberry powder were mixed with 150 mL of 70% (*v/v*) acidified acetone (containing 1% [*v/v*] HCl) and blended with a PT-3100 Polytron™ homogenizer (Brinkmann Instruments, Westbury, NY) at 15,000 rpm for 10 min. The slurry was then filtered by gravity through fluted P8-filter paper (Fisher Scientific). The extract process was performed in triplicate and all filtrates were pooled and acetone evaporated using a Büchi Rotavapor R-210 with a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE) at 40 °C.

Preparation of polyphenol extracts (PPEs)
Ten milliliters of each CBE were applied to the top of a chromatographic column (30 mm *i.d.* × 340 mm *e.l.*, Kontes, Vineland, NJ) packed with Amberlite XAD-16 [(bead size: 20-60 mesh), Sigma-Aldrich] and washed with ~300 mL of deionized water to remove sugars and organic acids. After 300 mL had transversed through the column, the eluent was tested for its sugar content with a pocket refractometer PAL-1 (Tokyo, Japan). Water washing continued until the eluent reached a 0% Brix reading. The PPE was then eluted from the column with anhydrous methanol (~300 mL) as the mobile phase. Methanol was evaporated using the Rotavapor at 40 °C. The PPEs were lyophilized and stored in amber-glass bottles in a -12 °C freezer until further analyzed.

Fractionation of the PPEs
For each sample, 200 mg of lyophilized PPE were dissolved in 10 mL of 95% (*v/v*) ethanol, sonicated to facilitate dissolution, and then applied to a chromatographic column (30 mm *i.d.* × 360 mm *e.l.*, Kontes, Vineland, NJ) packed with Sephadex LH-20 [(bead size: 25-100 µm), Sigma-Aldrich]. Fractions were eluted with 95% (*v/v*) ethanol at a flow rate of ~0.6 mL/min. 9-mL Fractions were collected in 13 × 100-mm borosilicate glass culture tubes with a fraction collector (Model SC-100, Beckman Coulter Inc., Fullerton, CA). Using UV-Vis absorbance readings at 280, 360, and 520 nm from an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Inc., Wilmington, DE) as a guide, eluent was pooled into two major fractions (*i.e.*, FXN-I and FXN-II). In total, 500 mL of ethanol (95% [*v/v*]) was employed. Then, the eluting system was changed over to 50% (*v/v*) acetone, and ~300 mL were required to elute FXN-III, comprising mostly high-molecular-weight phenolics (*i.e.*, hydrolyzable and condensed tannins) from the Sephadex LH-20 column. Organic solvents were evaporated from the collected fractions using the Büchi Rotavapor at 40 °C. Fractions were then lyophilized with the bench-top freeze dryer to ensure all traces of moisture were removed, and then stored in amber-glass bottles in the refrigerator's freezer.

Total phenolics content (TPC) determination
The TPC was determined colorimetrically for blackberry crude extracts and fractions using the Folin-Ciocalteu assay. Briefly, 0.5 mL of a methanolic solution of PPE was pipetted into a test tube followed by the addition of 8.0 mL of deionized water, 0.5 mL of 2 N Folin-Ciocalteu's phenol reagent, and 1.0 mL of a saturated Na_2CO_3 solution. The contents were vortexed for 15 s. After an incubation period of 60 min at room temperature to allow optimal color development, absorbance readings were taken at λ = 750 nm with the Agilent spectrophotometer. Quantification was based on a standard curve generated with gallic acid. The results were expressed as mg gallic acid equivalents (GAE) per 100-g fruit (f.w.) or,100-mg fraction (d.w.). TPC results were used to standardize cell treatment doses; however, FRAP analyses will also be conducted at each research stage as a secondary comparison measure.

RAW 264.7 cell treatment with PPEs
Murine RAW 264.7 cells (American Type Cell Culture, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium with additional heat-inactivated fetal bovine serum, 100 µg/mL penicillin/streptomycin, and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). After reaching confluence in Corning (Corning, NY) culture flasks, the cells were transferred to a Multiwell™ 24-well culture-treated plate and incubated at 37 °C with 5% (*v/v*) CO_2 until once

again confluent. Wells were treated with 1 μg/mL *Escherichia coli* O111:B4 lipopolysaccharide (Sigma-Aldrich Chemical Co., St. Louis, MO) in media to initiate inflammation via the NF-κB pathway. After 1-h incubation, cells were treated with 12.5, 25, and 50 μg/mL concentrations of digested or undigested samples in quadruplicate.

Griess assay to assess nitric oxide (NO) production

The media of each well was removed, centrifuged, and measured for NO production, as nitrite, using a Griess assay kit (Invitrogen, Carlsbad, CA) and a FLUOstar Omega microplate reader (BMG LABTECH Inc., Cary, NC).

MTT assay for cell viability

The MTT assay used 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT reagent) to determine the adverse effects of the treatment condition. After cells were emptied, 0.5 mL of the dissolved MTT reagent was added to each well, to be absorbed by only the living cells. After 1-h incubation at 37 °C, the wells were emptied and washed with 1-mL phosphate buffered saline (PBS). Cells were then lysed by adding 0.5 mL of dimethyl sulfoxide (DMSO) and brought up to a 1-mL volume with additional DMSO. Absorbance was measured at 540 nm to determine average cell viability within each treatment condition.

Reversed-phase HPLC

An Agilent 1200 Series HPLC system consisting of a quaternary pump with degasser, autosampler, thermostatted column compartment, UV-Vis diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies) was used for the chromatography. A reversed-phase Luna $C_{18}(2)$ column (4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA) was utilized. A gradient elution consisting of mobile phase A (H_2O:CH_3CN:CH_3COOH; 93:5:2, *v/v/v*) and mobile phase B (H_2O:CH_3CN:CH_3COOH; 58:40:2, *v/v/v*) from 0% to 100% B over a 50-min period at a flow rate of 1 mL/min was employed. Before subsequent injections, the system was re-equilibrated for 10 min using 100% A giving a total run time of 60 min. The injection volume of crude extracts and each fraction (0.5 mg/mL in methanol) was 20 μL. Detection wavelengths employed were 260 nm (ellagic acid and ellagic acid derivatives), 280 nm (phenolic acids, catechin, epicatechin), 320 nm (phenolic acids), 360 nm (flavonols), and 520 nm (anthocyanins).

To date, PPEs have been prepared from digested and undigested Navaho cultivar blackberries, and samples have been tested with RAW 264.7 cells to determine cell viability and NO production of each. Additionally, fractionated PPEs have been prepared for undigested Navaho and Ouchita cultivars. Preliminary RP-HPLC analysis is ongoing to access the polyphenolic composition of these samples. Caco-2 cell culture work and ELISA analyses will begin in January 2014.

Results

Preliminary results show that while digested samples retain much of their *ex vivo* antioxidant capacity (as measured by TPC), when standardized for that TPC, these samples do not achieve the same dose-dependent anti-inflammatory efficacy as their undigested counterparts. Undigested samples clearly show a dose-dependent decrease in NO concentration in media, as

compared to control samples. Digested samples, however, show no significant difference even at the highest TPC concentrations. The MTT assay results indicate that there is no significant different in cell viability between any of the treatment conditions and the control. These preliminary results do not account for the impact of *in vitro* absorption; however, further research is aimed at repeating these trials with HPLC analysis at each step to determine the influence of each phase of digestion on the chemical composition of the polyphenols.

Conclusions

It is still too early to draw conclusions from this project.

Impact Statement

Funding from the SRSFC has been pivotal in facilitating the UGA research team in the further understanding of blackberry antioxidants and their health effects.