

CHARACTERIZATION OF THE ANTI-INFLAMMATORY PROPERTIES OF GEORGIA-GROWN BLACKBERRIES (*RUBUS* SPP.)

Progress Report

SRSFC Project #2009-13

Research Proposal

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Objectives

The objectives of this project are to investigate the anti-inflammatory and antioxidant properties of selected blackberry cultivars (Navaho, Ouachita, and Natchez) grown in Georgia using several *in vitro* models.

Justification

The blackberry (*Rubus* spp.), of the family Rosaceae, is a deciduous crop that grows best in temperate climates. In the southeastern U.S., blackberry production is on the rise due to consumer demand for this high-value crop and the recent release of varieties that can grow in the “deep South” and give good fruit size, yields, flavor/sweetness, and some resistance to the fungal disease double blossom/rosette. Although horticulture programs monitor certain traits (*e.g.*, disease resistance), bioactive constituents with antioxidant, anti-inflammatory, and health-promoting activities/potentials of newer cultivars have not been investigated. Therefore, this work presents a great opportunity to bridge the gap between the horticulturist, grower, and health-conscious consumer.

Collaborating with Jacob W. Paulk Farms, Inc. (Wray, GA), we have been analyzing some of the bioactives associated with the newer blackberry varieties grown at this industrial operation. In addition to eliciting the scientific information surrounding the health-promoting potential afforded by bioactives in blackberries, an aim of this collaboration is to provide marketing strategies for the company to better promote whole berries and the production of functional foods containing blackberries or their constituents.

Research is suggesting that very strong linkages exist between antioxidant balances in the mammalian system with mediators involved in inflammatory processes. This is significant as the production of proinflammatory cytokines and interruptions in signaling pathways may lead to the onset of a number of chronic disease states, including cancer. Based on a collaboration with researchers in the Nutraceutical Laboratory of UGA’s Pharmacy Department, we have examined the inhibitory effects of blackberry polyphenolics toward the formation of advanced glycation endproducts and the anti-inflammatory properties of blackberry polyphenolics by assessing the effects on edema and polymorphonuclear leukocyte infiltration after topical application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) using the mouse ear model. The main finding of the latter study is that topical application of a polyphenolic extract (PPE) from blackberries results in a significant ($p < 0.05$) inhibition of TPA-induced skin inflammation. This also suggests that the polyphenolics penetrate far enough into the epidermal layer of the ear tissue to effectively reduce the inflammatory response. The TPA model of ear inflammation is an important pharmaco-

logical screening test employed for studying topical acute and chronic inflammation. The dietary intervention of a PPE can ameliorate topical and systemic inflammation. However, biochemical and cell culture model tests, such as those described in this project, will further characterize the anti-inflammation activity of blackberry polyphenolics. Such findings can add to blackberry's other known benefits, and may enhance its market potential as a constituent in functional food/nutraceutical beverage formulations.

Methodologies

Collection of Samples

Mature blackberries (*Rubus* spp.) of the Navaho, Ouachita and Natchez cultivars were hand-picked at Jacob W. Paulk Farms, Inc. (Wray, GA) industrial operation in May 2009. The blackberries were transported to the Department of Food Science and Technology, UGA, in Athens, GA, then sorted, cleaned, and frozen in polyethylene pouches -40 °C. Representative samples from each cultivar were lyophilized using a FreeZone 2.5 L bench-top freeze dryer (Labconco Corp., Kansas City, MS) and transferred to polyethylene pouches.

Preparation of crude blackberry extracts (CBEs)

Freeze-dried blackberry samples (*i.e.*, containing fruit receptacles, skins and seeds) from each cultivar were ground in 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 0.1% [v/v] HCl) and blended using 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 extraction process was repeated 2× as described above. All filtrates were pooled and acetone was evaporated with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE) at 40 °C.

Preparation of polyphenolic extracts (PPEs)

Ten milliliters of each CBE (containing 13% solids) 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 the first 100 mL, the pH of the eluent was checked with pH paper test strips every 20 mL until a neutral pH was reached. 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 PPE was lyophilized to ensure all traces of moisture were removed and then stored in amber-glass bottles in a 4 °C refrigerator until further analyzed.

To date all PPEs have been prepared and antioxidant assays (*i.e.*, FRAP and TPC) conducted. Anti-inflammatory assays are in progress. To that end, all supplies with the exception of the RAW 264.7 cell lines have been ordered, and a temperature/humidity-controlled CO₂ incubator has been secured. The cell culture work will begin in early January 2010. Details for the anti-inflammatory activity measurements have been worked out and sandwich ELISA tests

employing a FLUOstar Omega microplate reader with existing ELISA kits in Dr. Pegg's lab have been used for practice, as the actual cytokine and COX screening kits are fairly expensive.

Antioxidant Assays

FRAP (Ferric Reducing Antioxidant Power) assay

The antioxidant capacity of each blackberry PPE preparation was determined using the FRAP assay. Briefly, the FRAP reagent was prepared fresh each day by adding 2.5 mL of a 10 mM TPTZ [2,4,6-tri(2-pyridyl)-s-triazine] solution in 40 mM HCl, 2.5 mL of 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, and 25 mL of acetate buffer (300 mM, pH 3.6). The FRAP reagent was kept warm at 37 °C in a 5-L Isotemp[®] Digital-Control Water Bath (Model 205, Fisher Scientific) until further use. Eighteen hundred microliters of the FRAP reagent and 180 μL of 37 °C deionized water were pipetted into borosilicate glass tubes, positioned in the 37 °C water bath. To respective test tubes, 60 μL of each sample solution [*i.e.*, 50 $\mu\text{g}/\text{mL}$ of 50% (v/v) ethanol] were added. The contents were mixed well by pumping the solution through the pipette tip twice. Using a micropipette, 250- μL aliquots were transferred to a prewarmed COSTAR[®] 96-well clear, non-sterile, non-treated microtiter assay plate and inserted into a FLUOstar Omega microplate reader (BMG LABTECH Inc., Durham, NC) set at 37 °C. Absorbance readings were recorded by bottom scanning every 20 s over 30 min at $\lambda = 595 \text{ nm}$. Aqueous solutions of known Fe^{2+} concentrations in the range of 0.1 to 2.5 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were employed to construct a calibration curve. All analyses were replicated a minimum of three times and FRAP values were expressed as mmol Fe^{2+} equivalents/100-mg fraction.

Total phenolics content (TPC) assay

The TPC was determined by the classical Folin-Ciocalteu assay. Briefly, 0.5 mL of a methanolic solution (5 $\mu\text{g}/\text{mL}$) of each 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 for optimal color development of the samples, absorbance readings were taken at $\lambda = 750 \text{ nm}$ with an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Inc., Wilmington, DE). Quantification was based on a standard curve generated with gallic acid. The TPCs were determined from the standard curve, and results were expressed as mg gallic acid equivalents/100-mg PPE.

Anti-inflammatory Assays

Anti-inflammatory activities of the PPEs will be tested using a cell culture model of RAW 264.7 mouse cell lines (ATCC[®] TIB-71[™]) purchased from ATCC: The Global Bioresource Center[™]. Cells will be cultured in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin. The cells will be cultured at 37 °C in a humidified 5% (v/v) CO_2 incubator. Subcultures will be prepared by scraping and adding appropriate aliquots of the suspension into new culture vessels (subcultivated at a 1:4 ratio).

The anti-inflammatory assays to be performed include the following:

Measurement of Nitric Oxide/Nitrite

A nitrate/nitrite assay kit has been purchased from Cayman Chemicals (Ann Arbor, MI). The kit measures the total nitrate/nitrite concentration in a simple two-step process. Nitrate is first converted to nitrite using nitrate reductase. The second step is the addition of the Griess reagent which converts nitrite to a deep red azo compound. Absorbance readings of the azo dye at $\lambda = 540$ nm accurately determine the nitrite concentration. The assay will be repeated without the introduction of nitrate reductase. The difference in absorbance between the two readings represents the quantity of nitrate present. Cellular nitrate/nitrite production is quantitated by subtracting the level of nitrate/nitrite present in the media (*i.e.*, in the absence of cells) from the total nitrate/nitrite present during cell growth. The impact of the PPEs on nitrate/nitrite production during cell growth will be assessed.

A second nitrate/nitrite assay kit utilizing lactate dehydrogenase to oxidize excess NADPH has been purchased and will allow for the measurement of nitric oxide synthase (NOS) activity *in vitro*. NOS is a key enzyme that mediates the inflammatory process. Nitrate and nitrite are the stable end products for the reaction of NO with molecular O₂. The kit is designed to measure the total accumulation of nitrate and nitrite in tissue culture media and also that derived from NO production by NOS.

Determination of Cytokines (TNF- α & IL-1 β)

Tumor necrosis factor- α (TNF- α) is produced in the early stages of inflammation and is known to regulate the generation of the proinflammatory cytokine, interleukin-1 β (IL-1 β). The polyphenolics of berries are believed to be potent inhibitors of TNF- α and thereby have a significant effect in retarding inflammatory disease. The cytokines, TNF- α and IL-1 β , will be measured by a solid-phase sandwich ELISA (enzyme-linked immunosorbent assay) using kits already purchased from Invitrogen Corporation (Carlsbad, CA). The RAW 264.7 mouse cell line will be preincubated with various concentrations of the PPEs for 24 h, after which the cells will be pelleted by centrifugation and the culture supernates collected. The supernates will be assayed for IL-1 β and TNF- α according to the instructions provided with the kits. A standard curve will be prepared for each cytokine. The FLUOstar Omega microplate reader will be used to measure the absorbance of the colorimetric reaction in the ELISA assays for both the samples and controls (both positive and negative) at the specified wavelengths of each kit.

Cyclooxygenase (COX) Activity

A COX inhibitor screening assay kit will be purchased from Cayman Chemicals (Ann Arbor, MI) to assess the inhibitory effects of the PPEs from the blackberry cultivars against COX-1 and COX-2 activities. COX-2 is a key enzyme that mediates the inflammatory process. The assay is based on the competition between prostaglandins (PGs) and PG-acetylcholinesterase conjugate for a limited amount of PG antiserum. The product of the enzymatic reaction has a distinct yellow color that absorbs strongly at $\lambda = 412$ nm. The intensity of the color is proportional to the amount of PG tracer bound to

the well, which is inversely proportional to the amount of free PG present in the well during the incubation. The FLUOstar Omega microplate reader will be used to measure the absorbance.

Results

As determined by the FRAP assay, results indicate that the prepared PPEs from the Navaho, Ouachita, and Natchez blackberry cultivars have marked antioxidant activity. Furthermore, the results show a strong linear correlation ($r > 0.9$) with the TPCs determined from each preparation.

As has already been stated, anti-inflammatory studies using the cell culture model are slated for early January 2010.

Conclusions

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

Impact Statements

Clearly, funding from the SRSFC has been instrumental in allowing for these anti-inflammatory studies on select blackberry varieties grown in Georgia. When the research is complete, it is anticipated that the findings can assist the industry in marketing campaigns to better promote the health attributes associated with blackberries.