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# Phytochemicals from berries and grapes inhibited the formation of advanced glycation end-products by scavenging reactive carbonyls

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ABSTRACT

Phenolic phytochemicals were extracted from blueberries, blackberries, strawberries, raspberries, cranberries, and Noble muscadine grapes. These extracts were purified to remove free sugars. Blueberry extract was separated into five fractions using a Sephadex LH-20 column. Berry extracts and fractions significantly inhibited AGEs generation in (bovine serum albumin) BSA-fructose, BSA-methylglyoxal, and arginine-methylglyoxal models, respectively. Their capacity to scavenge methylglyoxal suggested carbonyl scavenging as a major mechanism of protein glycation inhibition. Procyanidins were detected in all berry extracts and blueberry subfractions and were deduced to be one class of active compounds. (+)-Catechin, constituent unit of procyanidins, was used as a model compound to react with glyoxal and methylglyoxal. Five catechin-carbonyl adducts were detected and their structures were tentatively identified using HPLC-ESI-$MS^n$. Results in this study suggested that sugar-free phytochemicals extracted from berries were effective carbonyl scavengers and protein glycation inhibitors. These phytochemicals could be beneficial to prevent AGE-related chronic diseases.



## 1. Introduction

Reactive carbonyls are compounds that contain two or more carbonyl groups. The most common reactive carbonyls are dicarbonyls such as glyoxal and methylglyoxal. Reactive carbonyls react with proteins to generate advance glycation end products (AGEs). Accumulation of AGEs is a causing factor for diabetic complications, aging, and several types of cancers (Ahmed, 2005; Münch, Thome, Foley, Schinzel, & Riederer, 1997). A well-known AGE in human body is the glycated hemoglobin (HbA1c). Its level in blood is used to evaluate long term glucose control and risks for complications in diabetic patients (American Diabetes Association, 2009).

Reactive carbonyls are generated both in foods and the human body. Methylglyoxal and glyoxal are found in foods that contain large amounts of carbohydrates or in foods that are processed at high temperatures (Nemet, Varga-Defterdarovic, & Turk, 2006). Typical examples include baked meats, soy sauces, and carbonated soft drinks (Tan, Wang, Lo, Sang, & Ho, 2008). In the human body, methylglyoxal and glyoxal are generated from carbohydrate metabolism or auto-oxidation, as well as the oxidation of protein glycation products (Hollnagel & Kroh, 1998; O'Brien, Siraki, & Shangari, 2005).

Protein glycation, also known as the Maillard reaction when occurring *in vitro*, is a non-enzymatic reaction between reducing sugars and proteins. During such a reaction, reducing sugars, such as glucose, react with amino groups of proteins to produce Schiff bases. Schiff bases undergo Amadori rearrangement to produce relatively stable compounds called Amadori products. Under low pH or oxidative conditions, Schiff bases or Amadori products degrade to generate reactive carbonyls including methylglyoxal (Cho, Roman, Yeboah, & Konishi, 2007; O'Brien et al., 2005; Oya et al., 1999). Methylglyoxal is a major reactive carbonyl *in vivo*. Its concentration in the blood of diabetic patients was five folds of that in healthy individuals (Nagaraj et al., 2002). Methylglyoxal glycates proteins in a much faster rate than sugars, causing inter- and intramolecule cross-links of proteins.

Several types of phytochemicals have been suggested to be effective in scavenging carbonyls and inhibiting the formation of AGEs. For example, flavan-3-ols from green tea were found to scavenge toxic methylglyoxal under simulated physiological conditions (Lo et al., 2006). Procyanidins from cinnamon were shown to inhibit protein glycation (Peng et al., 2008). Pure resveratrol scavenged carbonyls by trapping them and form adducts (Lv et al., 2010). Berries and grapes are rich sources of phenolic nutraceuticals, such as catechins, resveratrol, and anthocyanins (Lo et al., 2006; Lv et al., 2010; Morata, Calderón, González, Gómez-Cordovés, & Suárez, 2007; Sang et al., 2007); however, it is not known whether phytochemicals in berries can scavenge carbonyls and inhibit the formation of AGEs. The objective of present study is to evaluate such effect.

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## 2. Materials and methods

### 2.1. Chemicals and materials

Southern high-bush Blueberries, strawberries, cranberries, raspberries, blackberries, and Noble muscadine grapes were purchased from local supermarkets or obtained from local farms in central Florida. Only fully ripe fruits were used for experiments. AAPH (2,2′-azotis(2-amidinopropane)) was a product of Wako Chemicals Inc. (Bellwood, RI). Sephadex LH-20, (+)-catechin, and Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (St. Louis, MO). Amberlite XAD-7 resin was a product of Rohm and Haas Co. (Philadelphia, PA). Methylglyoxal (40% solution), glyoxal (40% solution), aminoguanidine, HPLC grade methanol, and other chemicals were purchased from Fisher Scientific Co. (Pittsburg, PA).

### 2.2. Phytochemical extraction and purification

Frozen berries or muscadine grapes were thawed under room temperature. Fruits (200 g) were blended with methanol (200 ml with 1% formic acid) in a kitchen blender. The mixture was sonicated in a water-bath sonicater (FS30, Fisher Scientific) for 10 min, then kept in room temperature for 20 min and filtered through a Whatman No.4 filter paper. The extraction was repeated once on the remaining solid and the methanol extracts were combined. Extracts were dried in a SpeedVac concentrator (Thermo scientific ISS110, Waltham, MA) under ambient temperature.

Dried berry crude extract was re-suspended in 20 ml distilled water containing 1% formic acid and loaded onto a glass column (2.8×55 cm, 500 ml) packed with Amberlite XAD-7 resin. After sample loading, elution was halted for 10 min to facilitate the adsorption of phytochemicals on resin beads. Resin column was eluted with 600 ml of acidified water (containing 1% formic acid) to remove sugars. Phytochemicals absorbed on resin were recovered with 250 ml of 80% methanol (with 1% formic acid). Eluents from the resin column were dried on a SpeedVac concentrator. About 0.84, 0.73, 1.045, 0.94, 0.50 and 1.32 g of dry extract were obtained from blueberries, strawberries, cranberries, blackberries, raspberries, and Noble muscadine grapes, respectively.

About 0.8 g of dry blueberry extract obtained after resin absorption was homogenized in 10 mL water and loaded into a column (2.8×55 cm, 500 ml) packed with Sephadex LH-20. Five fractions were collected by eluting the column with 500 ml water, 700 ml of 20% methanol, 700 ml of 50% methanol, 700 ml of 70% methanol, and 500 ml of 80% acetone. All eluting solvents were acidified with 1% formic acid to stabilize anthocyanins. Solvents in all the fractions were evaporated on a SpeedVac concentrator and the remaining solids were weighed. About 0.27, 0.13, 0.08, 0.17 and 0.10 g of dry extract were obtained for fraction I to fraction V, respectively.

### 2.3. Chemical analyses of extracts and fractions

The total phenolic contents of berry and extracts were determined with Folin-Ciocalteu assay using gallic acid as a standard (Singleton & Rossi, 1965). Total antioxidant capacity was measured with oxygen radical absorbance capacity (ORAC) assay using trolox as a standard (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). Total anthocyanin content was determined with a pH differential assay using molar absorptivity of cyanidin 3-glucoside of 26,900 (Giusti & Wrolstad, 2001). Total procyanidin content was analyzed with 4-dimethylaminocinnamaldehyde (DMAC) colorimetric method using (+)-catechin as a standard (Prior et al., 2010). An analysis of free sugars was conducted using a Hitachi HPLC system with a refractive index detector and a Shodex SP0810 column (300 mm×8 mm, Shodex, Colorado springs, CO) with a SP-G guard column (2 mm×4 mm). An isocratic solvent delivery of water was run at 1.0 mL/min. Purified berry phytochemicals extracts were dissolved in distilled water to a concentration of 15 mg/ml. Sample injection volume was 5 μL. Sucrose, glucose, fructose, lactose and maltodextrin were used as standards.

### 2.4. Antiglycation assay in BSA-fructose model

This model evaluates all stages of protein glycation. Fructose (1.5 M, 1 ml) was mixed with berry extracts obtain from Amberlite resin column (0.15 mg/ml, 1 ml) or catechin (0.15 mg/ml, 1 ml) in sodium phosphate buffer (50 mM, pH 7.4, with 0.02% sodium azide) in capped test tubes and kept at 37 °C for 2 h. BSA (30 mg/ml, 1 ml) was added in each test tube and the mixtures were incubated at 37 °C for six days. Phosphate buffer (1 ml) with no berry extract was used as a blank control. Aminoguanidine (10 mM, final concentration) was used as a positive control. Fluorescent AGEs were monitored on a microplate reader (Spectra XMS Gemini, Molecular Device, Sunnyvale, CA) using 340 and 420 nm as the excitation and emission wavelengths. Experiments were conducted in triplicates. Percentage of the AGE inhibition was calculated by the following equation:

$$\text{Percentage inhibition} = \left(1 - \frac{\text{Fluorescent intensity with inhibitor}}{\text{Fluorescent intensity without inhibitor}}\right) \times 100\%$$

### 2.5. Antiglycation assay in BSA-methylglyoxal model

This model evaluates the middle stage of protein glycation. Methylglyoxal (60 mM, 1 ml) was mixed with berry extracts (1.5 mg/ml, 1 ml), catechin (1.5 mg/ml, 1 ml) in sodium phosphate buffer (50 mM, pH 7.4, with 0.02% sodium azide) and kept at 37 °C for 2 h. BSA (30 mg/ml, 1 ml) was added to each test tube and incubated at 37 °C for six days. Phosphate buffer (1 ml) with no berry extract was used as a blank control. Aminoguanidine (10 mM, final concentration) was used as a positive control. Fluorescent AGEs were monitored on a microplate reader using 340 and 380 nm as the excitation and emission wavelengths. Experiments were conducted in triplicates. The percentage of the AGE inhibition was calculated using the same equation in BSA-fructose model.

### 2.6. Antiglycation assay in arginine–methylglyoxal model

This model evaluates a major and specific source of AGE production. Methylglyoxal (60 mM, 1 ml) was mixed berry extracts (1.5 mg/ml, 1 ml) or catechin (1.5 mg/ml, 1 ml) in sodium phosphate buffer (50 mM, pH 7.4, with 0.02% sodium azide) and kept at 37 °C for 2 h. Arginine (60 mM, 1 ml) was added to each test tube and incubated at 37 °C for six days. Phosphate buffer (1 ml) with no berry extract was used as a blank control. Aminoguanidine (10 mM, final concentration) was used as a positive control. Fluorescent AGEs were monitored on a microplate reader using 340 and 380 nm as the excitation and emission wavelengths. Experiments were conducted in triplicates. Percentage of the AGE inhibition was calculated using the same equation as in the BSA-fructose model.

### 2.7. Methylglyoxal scavenging

Methylglyoxal scavenging was tested using a published method with minor modifications (Peng et al., 2008). Methylglyoxal and o-phenylenediamine were dissolved in phosphate buffer (50 mM, pH 7.4) to a concentration of 10 mM and 50 mM, respectively. Berry and grape extracts were dispersed in phosphate buffer to 2.5 mg/ml. Aminoguanidine (10 mM) was used as a positive control. Methylglyoxal solution (0.125 ml) was mixed with 0.125 ml of phosphate buffer (negative control) or berry extracts. The tubes were incubated at 37 °C. O-phenylenediamine (0.25 ml) was added into each tube at