Inhibition of Nonenzymatic Protein Glycation by Pomegranate and Other Fruit Juices

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ABSTRACT The nonenzymatic glycation of proteins and the formation of advanced glycation endproducts in diabetes leads to the crosslinking of proteins and disease complications. Our study sought to demonstrate the effect of commonly consumed juices (pomegranate, cranberry, black cherry, pineapple, apple, and Concord grape) on the fructose-mediated glycation of albumin. Albumin glycation decreased by 98% in the presence of 10 μ L of pomegranate juice/mL; other juices inhibited glycation by only 20%. Pomegranate juice produced the greatest inhibition on protein glycation when incubated at both the same phenolic concentration and the same antioxidant potential. Both punicalagin and ellagic acid significantly inhibited the glycation of albumin by $\sim$ 90% at 5 μ g/mL. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed that pomegranate, but not apple juice, protected albumin from modification. These results demonstrate that pomegranate juice and two of its major constituents are potent inhibitors of fructose-mediated protein glycation.

KEY WORDS: • advanced glycation end products • apple • black cherry • Concord grape • cranberry • ellagic acid • fluorescence • phenolics • pineapple • punicalagin

INTRODUCTION

PROTEIN GLYCATION, also known as the Maillard reaction, is a complex series of sequential and parallel steps that begin with the nonenzymatic binding of reducing sugar or sugar derivatives to the amine group of a protein.¹ Molecular rearrangements (Schiff base formation and Amadori rearrangements) lead to the formation of advanced glycation endproducts (AGEs) and ultimately the crosslinking of proteins. This is clearly observed in proteins that are not readily recycled in the body such as collagen and lens crystallins. AGE formation is accelerated in diabetes² and has been recognized to participate in the pathogenesis of retinopathy,³ nephropathy,⁴ neuropathy,⁵ and atherosclerosis.⁶

Long-term consumption of foods that are rich in polyphenols can ameliorate or provide prevention against certain disease states.^{7,8} One such fruit, the pomegranate, has been used for medicinal purposes since ancient times.⁹ The high phenolic content of the pomegranate is a natural defense against environmental stressors in the very arid regions of the world. The antioxidant potential of pomegranate juice has been reported to exceed that of red wine and tea and phenolically rich ready to drink (RTD) juices (Concord grape, blueberry, black cherry, acai, and cranberry).¹⁰ Po-

megranate juice contains significant amounts of hydrolyzable ellagitannins, gallotannins, ellagic acid, and various flavonoids.¹¹ Hydrolyzable tannins account for more than 90% of the antioxidant potential of pomegranate fruit; the major phytochemical contributor is punicalagin.¹² In recent years, studies have documented that punicalagin has significant anti-inflammatory, antiproliferative, and apoptotic properties.¹³ Punicalagin and other hydrolyzable tannins originate from the peel of pomegranate fruit and are found with ellagic acid in the juice.¹⁴

Numerous polyphenolic compounds have been shown to inhibit nonenzymatic glycation of proteins both *in vitro* and *in vivo*.^{15–19} In this study, we investigated the effect of pomegranate juice and other commonly consumed RTD juices on the fructose-mediated formation of AGEs.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA; essentially fatty acid free), D-(–) fructose, Chelex 100 (sodium form), Folin-Ciocalteu reagent, TPTZ (2, 4, 6-tri[2-pyridyl]-s-triazine, ellagic acid, and 2-mercaptoethanol were purchased from Sigma Chemical Company (St. Louis, MO, USA). Punicalagin was obtained from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, Sichuan, China). Organic apple, black cherry, Concord grape, organic cranberry, organic pineapple, and pomegranate juice, were purchased locally. Laemmli sample buffer, Coomassie blue, and Criterion precast gels (4–15% in

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Tris HCl, pH 8.6) were purchased from Bio-Rad (Hercules, CA, USA).

Total phenolic content

The total phenolic content for all RTD juices was determined by the Folin-Ciocalteu method, as described,²⁰ utilizing gallic acid as a standard. Briefly, gallic acid standards (20 μ L) and each juice (20 μ L) were combined with distilled water (1580 μ L), Folin-Ciocalteu reagent (100 μ L), and 300 μ L of sodium carbonate (1.6 M). This mixture was allowed to incubate for 45 min, after which absorbance was read at 765 nm. The results are expressed as gallic acid equivalents (GAE)/mL.

Ferric-reducing antioxidant potential assay

The ferric-reducing antioxidant potential (FRAP) of all RTD juices was determined by a modified method described by Benzie and Strain,²¹ where iron (II) sulfate heptahydrate was the standard. Briefly, iron (II) sulfate heptahydrate standards (10 μ L) and each juice (10 μ L) were combined with distilled water (30 μ L) and 300 μ L of reagent (25 mL of 300 mM acetate buffer, pH 3.6, 25 mL of 10 mM TPTZ solution, and 2.5 mL of 20 mM ferric chloride solution). The mixture was allowed to incubate for 6 min after which each sample was combined with distilled water (340 μ L) and the absorbance read at 593 nm. The results are expressed as mmol FeSO₄ equivalents/L.

Fructose-mediated glycation of albumin

The glycation of BSA was determined fluorometrically by a method described by Farrar *et al.*²² Albumin (10 mg/mL) was incubated in the presence of D-(–) fructose (250 mM) and various concentrations of RTD juices, ellagic acid, and punicalagin in 200 mM potassium phosphate buffer containing 0.02% sodium azide (pH 7.4) at 37°C for 72 h. The potassium phosphate buffer was treated with Chelex 100 before use. Ellagic acid and punicalagin were dissolved in 99.5% H₂O/0.5% 1 N NaOH and 50% EtOH, respectively. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm using a Perkin-Elmer LS 55 Luminescence Spectrometer with slit widths set at 3 nm. All samples were corrected for the native fluorescence of albumin incubated in the presence of juices, ellagic acid, or punicalagin. Neither the juices nor the phenolics punicalagin or ellagic acid caused significant quenching of the fluorescence induced by protein glycation at the wavelength pair of 370 and 440 nm.

The fluorescence emission spectrum resulting from a 72-h incubation of albumin and fructose in the absence and presence of pomegranate juice was determined by setting the excitation wavelength at 370 nm. The emission spectra were scanned from 380 to 550 nm with the slit width set at 3 nm.

Modification of albumin by methylglyoxal

The glycation of BSA by methylglyoxal was performed using a method described by Lee *et al.*²³ BSA (100 μ M) was

incubated in the presence of methylglyoxal (1 mM) in Chelex 100 treated 0.1 M sodium phosphate, pH 7.0 at 37°C. After 96 h, the fluorescence was measured at a wavelength pair of 350/409 nm. All samples were corrected for the native fluorescence of albumin incubated with pomegranate juice.

Analysis of protein modifications

BSA (10 mg/mL) was incubated in the presence of D-(–) fructose (250 mM), in the presence and absence of pomegranate and apple juice (5 μ g GAE/mL) at 37°C for 14 days. In this experiment, 10 μ L of apple and pomegranate juice was added to the incubations every 3 days. Protein modifications induced by protein glycation were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).²⁴ Aliquots for each sample were diluted 1:1 with the Laemmli sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 20 mg/mL SDS, 0.01% (v/v) Bromophenol Blue, and 5% (v/v) 2-mercaptoethanol. Samples were boiled at 100°C for 5 min after dilution and then centrifuged. Centrifuged samples were loaded onto a 15% resolving polyacrylamide gel with a 4% stacking polyacrylamide gel and subjected to electrophoresis for 45 min. Gels were stained with Coomassie Blue for 1 h and destained with distilled water.

Statistical analysis

Experiments were performed in triplicate and are expressed as mean $\pm$ SEM. Data were analyzed utilizing one-way analysis of variance and multiple comparisons were performed employing the Tukey's test. Statistical significance was set at $P < .05$.

RESULTS

The RTD fruit juices were initially tested for their phenolic content employing the Folin-Ciocalteu assay.²⁰ Pomegranate juice contained the highest concentration of total phenols (Table 1), nearly 4 mg GAE/mL, which is in agreement with previous findings.²⁵ Concord grape and black cherry juice contained the second and third highest concentration of total phenols. The juices were also analyzed for their antioxidant capacity as determined by the FRAP assay (Table 1). Pomegranate juice had the highest antioxidant capacity of all the

TABLE 1. PHENOLIC CONTENT AND FERRIC-REDUCING ANTIOXIDANT POTENTIAL VALUES OF FRUIT JUICES

Juice	Total phenolic content (mg gallic acid/mL)	FRAP values (mmol FeSO ₄ /L)
Apple	0.78 $\pm$ 0.01	2.7 $\pm$ 0.1
Black cherry	1.58 $\pm$ 0.01	10.5 $\pm$ 0.1
Concord grape	2.13 $\pm$ 0.01	16.5 $\pm$ 0.1
Cranberry	1.13 $\pm$ 0.01	8.7 $\pm$ 0.1
Pineapple	0.66 $\pm$ 0.02	3.5 $\pm$ 0.1
Pomegranate	3.96 $\pm$ 0.01	45.9 $\pm$ 0.1

Data represent mean $\pm$ SEM, $n = 3$.

FRAP, ferric-reducing antioxidant potential.

juices examined, approximately three times greater than the next highest juice. Similar to that observed with the content of phenolic compounds, Concord grape and black cherry juice possessed the second and third highest antioxidant capacity. These results are in agreement with Seeram *et al.*¹⁰ It is interesting to note that while pomegranate juice contained approximately five times the phenolic content of apple juice, the FRAP value of pomegranate juice was nearly 20 times greater compared with apple juice.

To examine the effect of the RTD juices on protein glycation, each juice (10 μ L/mL) was incubated with a solution containing BSA (10 mg/mL) and fructose (250 mM). The control incubation of albumin and fructose in the absence of any juice resulted in a significant increase in fluorescence (Fig. 1). When albumin and fructose were incubated in the presence of the juices (10 μ L of juice/mL), pomegranate juice produced the greatest inhibition; the fluorescence intensity observed in the presence of pomegranate juice was 2% of control. Black cherry, Concord grape, cranberry, and pineapple juice all resulted in a decrease in fluorescence of $\sim$ 20%. The least effective inhibitor of protein glycation was apple juice.

The emission spectrum of the albumin and fructose solution after the 72-h incubation in the absence of any juice is shown in Figure 2. With the excitation set at 370 nm, a broad fluorescence spectrum is observed having an emission maximum at 440 nm. When pomegranate juice was present at a concentration of 10 μ L/mL during the course of the 72-h incubation, the resulting emission spectrum resembles a plateau with no major emission maximum observed at 440 nm; the emission spectrum is quite similar to the spectrum observed when albumin is incubated with pomegranate juice (Fig. 2). These results demonstrate the near absence of fluorescent AGEs in the presence of pomegranate juice.

The superior inhibition observed with pomegranate juice on protein glycation when performed on the basis of volume

FIG. 1. Inhibition of albumin glycation by ready to drink (RTD) juices on the basis of volume. Albumin (10 mg/mL) and fructose (250 mM) were incubated with 10 μ L of RTD juices/mL for 72 h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean $\pm$ SEM for triplicate determinations. * P < .05 when compared with control; # P < .05 when compared with other RTD juices; POM, pomegranate.

FIG. 2. Emission spectra of 10 mg/mL albumin incubated with fructose (250 mM) for 72 h (A), albumin incubated with fructose in the presence of pomegranate juice (5 μ g gallic acid equivalents [GAE]/mL) (B), and albumin incubated in the presence of pomegranate juice (C). The excitation wavelength was set at 370 nm.

was not unexpected due to the fact it contains the highest concentration of phenolic compounds (Table 1). The effect of RTD juices on protein glycation was studied when each juice was incubated at the same phenolic concentration (5 μ g GAE/mL). When pomegranate juice was incubated at this concentration, it still provided the greatest inhibition of protein glycation; the decrease in fluorescence intensity was $\sim$ 90% (Fig. 3). Pineapple yielded the next greatest decrease in fluorescence intensity (66%). The other juices produced lesser levels of inhibition. The resulting degree of inhibition among juices varied greatly and demonstrates that not all phenolic compounds inhibit glycation equally.

To further characterize the effect of pomegranate juice on protein glycation, an experiment was performed in which the different RTD juices were added at a single antioxidant capacity FRAP value (200 nmol FeSO₄ equivalents/mL, Fig. 4). Pomegranate juice still produced the greatest decrease in fluorescence intensity (98%); black cherry juice was the second best inhibitor with an $\sim$ 60% decrease in fluorescence intensity. These results demonstrate that the degree of inhibition of protein glycation among these juices can vary significantly even when they are present at the same antioxidant capacity.

Pomegranate juice, prepared by squeezing the whole fruit, is rich in hydrolyzable tannins, namely punicalagin, the major ellagitannin found in the commercial pomegranate fruit juices.¹⁴ The concentration-dependent inhibition of protein glycation by punicalagin is shown in Figure 5. Similar to that seen with pomegranate juice, punicalagin was an extremely effective inhibitor at 5 μ g/mL. At 2.5 μ g/mL, the punicalagin inhibition of protein glycation was found to be greater than 90%. No inhibition of protein glycation was observed at 1 μ g/mL. Ellagic acid is present in pomegranate juice and is also an intestinal microbial metabolite of ellagitannins (*i.e.*, punicalagin) after ingestion;²⁶ the data in

FIG. 3. Inhibition of albumin glycation by RTD juices on the basis of phenolic content. Albumin (10 mg/mL) and fructose (250 mM) were incubated with RTD juices (5 μ g GAE/mL) for 72 h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean $\pm$ SEM for triplicate determinations. * P < .05 when compared with control; # P < .05 when compared with other RTD juices.

Figure 6 demonstrate that ellagic acid inhibits protein glycation to the same extent as punicalagin.

Methylglyoxal is a reactive α -dicarbonyl, which is formed from the auto-oxidation of glucose. Reactive carbonyls have the ability to bind with amino groups of proteins to form AGEs directly. When methylglyoxal was incubated with BSA for 96 h, inhibition of fluorescence intensity was not observed by pomegranate juice at phenolic concentrations of 5 and 10 μ g GAE/mL (Fig. 7). These results suggest that pomegranate juice, at the concentrations employed in these studies, is not an effective inhibitor of glycation mediated by reactive α -dicarbonyls such as methylglyoxal.

The effect of pomegranate juice on fructose-mediated protein modification was analyzed by SDS-PAGE electro-

phoresis (Fig. 8). Albumin (10 mg/mL) incubated in the potassium phosphate buffer for 14 days (lane A) produced a distinct protein band at 65 kDa. Albumin (10 mg/mL) incubated in the presence of fructose for 14 days (lane B) resulted in a widening of the band. These results are similar to those of Yamagishi *et al.*²⁷ who observed a widening of the albumin band when incubated with glucose. Albumin incubated in the presence of fructose and apple juice (5 μ g GAE/mL; lane C) resulted in the same band widening as lane B. Interestingly, albumin incubated in the presence of fructose and 5 μ g GAE/mL pomegranate juice (lane D) resulted in a sharp 65 kDa band, similar to that of albumin incubated in the absence of fructose (lane A). Thus, pomegranate juice prevented the band widening seen when albumin is incubated with fructose.

FIG. 4. Inhibition of albumin glycation by RTD juices on the basis of ferric reducing antioxidant potential values. Albumin (10 mg/mL) and fructose (250 mM) were incubated with RTD juices (200 nmol FeSO₄ equivalents/mL) for 72 h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean $\pm$ SEM for triplicate determinations. * P < .05 when compared with control; # P < .05 when compared with other RTD juices.

FIG. 5. Effect of punicalagin on albumin glycation. Albumin (10 mg/mL) and fructose (250 mM) were incubated with punicalagin for 72 h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean $\pm$ SEM for triplicate determinations. * P < .05 when compared with control.

DISCUSSION

Glucose or fructose can react with amino groups of proteins to initiate the Maillard reaction forming a glycosylamine, which degrades to a Schiff base. The Schiff base undergoes an Amadori rearrangement to form fructosamine,²⁸ after which the degradation of fructosamine forms stable products known as AGEs. However, α -oxoaldehydes (carbonyls) formed from degraded glucose, fructose, or Schiff bases can react with amino groups of proteins to form AGEs directly; therefore AGEs can be formed in the early or late stages of glycation. The mechanism of action of phe-

FIG. 6. Inhibition of albumin glycation by ellagic acid. Albumin (10 mg/mL) and fructose (250 mM) were incubated with ellagic acid for 72 h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean $\pm$ SEM for triplicate determinations. * P < .05 when compared with control.

FIG. 7. Effect of pomegranate juice on albumin glycation mediated by methylglyoxal. Albumin (100 μ M) was incubated with methylglyoxal (1 mM) and various concentrations of pomegranate juice for 96 h at 37°C. The fluorescence intensity was measured at 350/409 nm. Results represent the mean $\pm$ SEM for triplicate determinations.

nolic compounds on glycation is thought to be related to their antioxidant properties;²⁹ they prevent the oxidation of Amadori products and the subsequent formation of AGEs, sometimes referred to as glycooxidation. Some polyphenols may inhibit the later stages of glycation by preventing the binding of dicarbonyls to protein amino groups.³⁰ Verzelloni *et al.*³¹ suggest that phenolic compounds may inhibit the formation of Amadori products because of their specific binding to albumin. However, the effect of pomegranate

FIG. 8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of (A) 2 μ g bovine serum albumin (BSA), (B) 2 μ g BSA incubated with fructose (250 mM), (C) 2 μ g BSA incubated with fructose (250 mM) and apple juice, and (D) 2 μ g of BSA incubated with fructose (250 mM) and pomegranate juice. The mixtures were incubated for 14 days at 37°C.

TABLE 2. MAJOR PHENOLIC COMPOUNDS FOUND IN FRUIT JUICES

Juice	Phenolic compounds
Pomegranate	Ellagitannins, anthocyanins ¹⁴
Concord grape	Proanthocyanidins, anthocyanins ³²
Black cherry	Anthocyanins, flavan-3-ols ³³
Cranberry	Proanthocyanidins, anthocyanins ³⁴
Apple	Polyphenolic acids, proanthocyanidins ³⁵
Pineapple	<i>p</i> -Coumaric acid ³⁶

juice on glycation appears to be independent of its binding to albumin; a similar pattern of inhibition is also observed in proteins that do not extensively bind phenolics (gelatin and immunoglobulin G, data not shown). Pomegranate juice does not appear to trap dicarbonyl species for there was no effect on AGEs formed from α -oxoaldehydes (methylglyoxal; Fig. 7). This pattern of inhibiting glycation mediated by fructose, but not by methylglyoxal, has been observed with other phenolic compounds.¹⁸

When the juices were normalized on the basis of phenolic content (5 μ g GAE/mL of mixture; Fig. 3) and antioxidant capacity (200 nmol FeSO₄/mL; Fig. 4), pomegranate juice was found to be the most effective in inhibiting glycation. The antiglycative activity of pomegranate juice was confirmed by SDS-PAGE electrophoresis showing that pomegranate, but not apple, juice protected albumin from protein modification. These findings suggest that pomegranate juice contains phenolic compounds that are more potent inhibitors of protein glycation than phenolic compounds found in other juices. The major polyphenols present in the juices examined are listed in Table 2.^{14,32–36} Pomegranate, unlike the other fruit juices, contains significant amounts of ellagitannins. Different phenols can inhibit protein glycation to varying degrees.^{16,18} Matsuda *et al.*²⁹ examined the relationship between the flavonoid structure, the inhibition of protein glycation, and radical scavenging properties of 62 flavonoids. Flavonoids with a strong 2,2-Diphenyl-1-picrylhydrazyl radical scavenging tended to be, with a few exceptions, robust inhibitors of AGE formation. When the juices were examined at the same antioxidative capacity (Fig. 4), pomegranate juice produced the greatest inhibition on glycation. These results agree with those of Kim and Kim³⁷ who found that compounds, which scavenge free radicals to the same extent, could have different effects on glycation.

Comparing the major polyphenolic groups listed in Table 2, the unique phenolic profile of pomegranate juice can be attributed to ellagic acid and hydrolyzable tannins.³⁸ In this study, we tested the effect of punicalagin and ellagic acid on the inhibition of albumin glycation; both were found to inhibit protein glycation at 2.5 μ g/mL (corresponding to 8 μ M for ellagic acid). It should be noted that condensed tannins have been reported to hydrolyze and form ellagic acid in neutral buffers, similar to that found in the gastrointestinal tract;²⁶ therefore, the effect of punicalagin on protein glycation may well be due to ellagic acid. Ellagic acid has previously been showed to inhibit glycation³⁹ and the intestinal microbial metabolites of ellagic acid, urolithins A and B also possess antiglycative activity.³¹ Unfortunately,

these studies did not compare the relative potency of these compounds to other major phenolics. Recently, the pomegranate seed extract has been shown to inhibit protein glycation at doses much higher than 5 μ g GAE/mL.⁴⁰ Muthenna *et al.*³⁹ have recently provided evidence that the mechanism of action of ellagic acid is independent of antioxidant potential, metal chelation, and post-Amadori inhibition. Rather, they propose, using much higher concentrations of ellagic acid than employed in these studies, that the flavonoid inhibits glycation by dicarbonyl trapping and preventing the formation of CML [N^ε-(carboxymethyl) lysine], an AGE. Inhibition of glycation was observed with different proteins and using different glycation agents (fructose, ribose, and methylglyoxal).

Currently, there are no U.S. Food and Drug Administration–approved medications that inhibit both the glycation of proteins and the progression of diabetic complications. For this reason, phenolic compounds offer an interesting approach to slow the rate of glycation. Nagasawa *et al.*⁴¹ illustrated this concept in diabetic rats fed G-rutin. After 1 month, G-rutin-treated rats had a much lower content of glycation products in serum and kidney without lowering blood glucose levels. Therefore, phenolic compounds can suppress the extent of glycation of tissue proteins independent of blood glucose concentrations.

Consumption of pomegranates results in the absorption of ellagic acid and urolithins from the gastrointestinal tract, resulting with their appearance in plasma and increasing plasma antioxidant capacity.⁴² Pomegranate juice consumption has been reported to inhibit the serum angiotensin converting enzyme activity⁴³ and slowed the rise in prostate-specific antigen concentration in men.⁴⁴ There have been two small human studies that found that the plasma concentration of hemoglobin A1c is not altered by pomegranate juice consumption.^{45,46} Unfortunately, these studies were not designed to examine the efficacy of pomegranate juice on lowering hemoglobin A1c levels. The pomegranate juice employed in this study is commercially available, and therefore, proper studies can be performed to ascertain whether the antiglycative properties of pomegranate juice are also observed in diabetic patients.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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