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Research Article

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Inhibition of Non-Enzymatic Protein Glycation by Pomegranate (*Punica granatum*) Whole Fruit and its Components

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Abstract

The non-enzymatic glycation of proteins, an oxidative-dependent process, initiates the formation of advanced glycation endproducts (AGEs) which leads to protein crosslinking. This study investigates the effect of a phenolic whole pomegranate fruit extract and various parts (aril, peel, and membrane) of pomegranate fruit on the *in vitro* fructose-mediated glycation of albumin. Compared to apple, whole pomegranate fruit exhibited a much higher total phenolics content and antioxidant potential. Pomegranate fruit decreased glycation by 80% when incubated at a phenolic concentration of 2.5 μg gallic acid equivalents (GAE)/ml; apple, at this phenolic concentration, inhibited protein glycation by only 20%. Pomegranate membrane exhibited the highest total phenolics content and antioxidant potential compared to the pomegranate aril and peel. At 2.5 μg phenolics/ml, the membrane fraction decreased glycation by 85% compared to the aril (42%), and peel (75%). Pomegranate membrane also produced the greatest decrease in glycation when these fractions were incubated at the same antioxidant capacities. These results demonstrate that pomegranate fruit is a potent inhibitor of fructose-mediated albumin glycation when compared to whole apple. The inhibitory activity was concentrated in the pomegranate membrane and is attributed to the presence of punicalagin, which is not found in whole apple.

Keywords

Albumin glycation, Pomegranate, Aril, Membrane, Peel, Antioxidant capacity

Abbreviations

AGEs: Advanced Glycation Endproducts; BSA: Bovine Serum Albumin; GAE: Gallic Acid Equivalents; ROS: Reactive Oxygen Species; TPC: Total Phenolics Content

Introduction

Complications stemming from diabetes mellitus such as neuropathy [1], nephropathy [2], and retinopathy [3] are initiated by the accumulation of advanced glycation endproducts (AGEs) in various tissues. AGEs are formed from the non-enzymatic binding of a protein, nucleic acid, or lipid to a reducing carbohydrate [4]. Hyperglycemia is known to cause oxidative damage and an imbalance between reactive oxygen species (ROS) and antioxidant detoxification pathways. Diabetic individuals are more susceptible to oxidative processes due to an increased production of ROS [5], and a lower concentration of inherent

antioxidants (vitamins C and E) [6]. Many mechanisms are involved in hyperglycemia-mediated oxidative stress including the oxidation of glucose, protein glycation, and the formation of AGEs; free radicals and oxidation reactions are involved in the glucose-mediated modification of proteins [7].

Diets rich in fruits and vegetables reduce the risk of cancer and other chronic diseases [8]. Fruits and vegetables are also an important dietary source of polyphenols [9], and the typical human intake of phenolics is reported to be 0.5 g per day [8]. Diets rich in fruits and vegetables (5 or more servings a week) significantly reduced $HbA1_C$ levels in a cohort of over 5,000 adults [10]. In the United States, apples (33.1%) are the largest contributors of polyphenols to the American diet followed by oranges (14.0%), grapes (12.8%), and strawberries (9.8%) [11]. A wide variety of polyphenols have been shown to inhibit the formation of AGEs [12-14]; the degree of inhibition of flavonoids has been correlated with their antioxidative properties [15].

Pomegranate fruit is rich in polyphenols and found in the arid regions of the world and the health benefits of pomegranate stem from its antioxidative, anti-inflammatory, antiproliferative and antiangiogenic effects [16-18]. Previously we have shown that polyphenols found in commercially-available pomegranate juice are superior inhibitors of fructose-mediated protein glycation when compared to commonly consumed juices at the same phenolics content and antioxidant capacity [19]. This suggests that the major polyphenols contained within the pomegranate are unique inhibitors of the glycation process. Punicalagin is found in the peel and membrane of the fruit [20] and is a potent inhibitor of protein glycation [19]. During the juicing process, pomegranate is squeezed whole, leaching ellagitannins from the peel and membrane into the resultant juice. The current study examined the effect of extracts from whole pomegranate fruit and its various components (i.e., the peel, membrane, arils) on the glycation of bovine serum albumin (BSA) mediated by fructose.

Materials and Methods

Materials

Bovine serum albumin (essentially fatty acid free), D-(-) fructose, Chelex 100 (sodium form), Folin & Ciocalteu's phenol reagent, TPTZ (2,4,6-tri[2-pyridyl]-*S*-triazine), ferrous sulfate heptahydrate, anhydrous ferric chloride, Amberlite XAD-16 and punicalagin were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Pomegranates (POM Wonderful variety) and apples (Red Delicious) were purchased locally from a Publix Supermarket (Athens, GA, USA).

Extraction of pomegranate and its components

Pomegranates are characterized as large berries covered by a leathery exocarp (peel). A white, spongy, and very bitter tissue connected to the peel extends into the interior of the fruit (membrane). As illustrated by Aviram and coworkers, the membrane provides a matrix for edible seeds surrounded by a sack of juice (arils) [21]. POM Wonderful pomegranates were cut into quarters (exposing the pericarp of the fruit), and placed in distilled water to facilitate the separation of the arils, membrane, and peel. The separated parts were blotted with a paper towel and then left to air dry in a fume hood for 6 h. Arils, membrane, peel, and whole fruit were macerated separately in a Super 5000 Vitamix (Cleveland, OH, USA) for approximately 1 min. Each resultant slurry was subjected to a hot water extraction at 95 °C at a material to solvent ratio of 1:5 (w/v) in a shaking water bath for 2 h. After this period, the samples were suction filtered. A column (40 cm height x 3 cm diameter) packed with Amberlite XAD-16 was washed with four column volumes of deionized water and eluent was discarded. The fruit slurries (25 ml) were loaded onto the column and the simple sugars and organic acids were removed with deionized water until the eluent reported a zero degrees Brix reading with a PAL-1 Atago pocket refractometer (Tokyo, Japan). Phenolic compounds were then eluted from the Amberlite XAD-16 using methanol. Methanol was removed from the extract using a Büchi Rotavapor R-210 (Büchi Corporation, New Castle, DE, USA) connected to a V-700 vacuum pump and V-850 vacuum controller. The aqueous residue was transferred to a crystallization dish, frozen and then lyophilized in a Labconco FreeZone 2.5 liter system (Kansas City, MO, USA). All sample extracts were reconstituted in 50% (v/v) ethanol at a concentration of 1 mg/ml before analysis.

Total phenolics content (TPC)

The TPC for all fruit extracts was determined by the Folin-Ciocalteu method [22] utilizing gallic acid as a standard. Briefly, gallic acid standards (20 µl) and each fruit extract (20 µl) were combined with distilled water (1580 µl), Folin and Ciocalteu's phenol reagent (100 µl), and 300 µl of 1.6 M sodium carbonate solution. Distilled water (20 µl) was used as a blank. The mixtures were incubated for 45 mins at room temperature, after which absorbance was read at 765 nm on a Beckman DU 600 series spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). The data are expressed as mg gallic acid equivalents (GAE)/ml.

Ferric reducing antioxidant potential (FRAP) assay

The ferric reducing antioxidant potential of all fruit extracts was determined [23] where iron (II) sulfate heptahydrate was the standard. Briefly iron (II) sulfate heptahydrate standards (10 µl) and each fruit extract (10 µl) were combined with distilled water (30 µl), and 300 µl of freshly prepared FRAP reagent (25 ml of 300 mM acetate buffer (pH 3.6), 2.5 ml of 10 mM TPTZ dissolved in 40 mM HCl and 2.5 ml of 20 mM ferric chloride solution) at 37 °C. Distilled water (10 µl) was used as a blank. The mixtures were allowed to incubate for 6 min after which each sample was combined with distilled water (340 µL). Absorbance was read at 593 nm on a Beckman DU 600 series spectrophotometer. The data are expressed as mM $FeSO_4$.

Modification of albumin by fructose

The glycation of bovine serum albumin was determined

[24]. Briefly, BSA (10 mg/ml) was incubated in the presence of D-(-) fructose (250 mM) and various concentrations of the fruit extracts in 200 mM potassium phosphate buffer (pH 7.4) containing 0.2 mg/ml sodium azide at 37 °C for 72 h in a laboratory incubator. Potassium phosphate buffer was treated with Chelex 100 prior to use. All samples were corrected for the fluorescence of albumin incubated with each fruit extract. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm using a PerkinElmer LS 55 luminescence spectrometer (PerkinElmer, Waltham, MA, USA) with slit widths set at 3 nm.

HPLC Characterization of Pomegranate Parts

Reverse phase HPLC was performed as previously described [25]. The detection wavelength was 260 nm. The injection volume was 20 µl. The extracts of the pomegranate components were standardized at 200 µg GAE/ml prior to analysis so that 4 µg of phenolics were analyzed by HPLC.

Statistical analysis

All experiments were performed in triplicate and expressed as mean ± SEM. Data were analyzed utilizing a one-way analysis of variance (ANOVA) and multiple comparisons were performed employing Tukey's test. Statistical significance was set at $P < 0.05$.

Results and Discussion

The phenolics fractions of whole apples, whole pomegranates and the various components of pomegranates were freeze dried and resuspended in 50% ethanol at a concentration of 1 mg/ml. Whole pomegranate fruit had a higher content of total phenols than whole apple (Table 1), in agreement with a study by Martin and coworkers [26]. Whole apple and pomegranate fruit were also analyzed for their antioxidant capacity as determined by the FRAP assay (Table 1); pomegranate exhibited an antioxidant capacity that was approximately four times greater than that of apple.

Table 1: The total phenolics content and ferric reducing antioxidant potential (FRAP) values of extracts of apple, pomegranate and components of the pomegranate.

Fruit	Phenolics Content (mg GAE/ml)	FRAP units (mM $FeSO_4$)
Apple	0.39 ± 0.01	3.6 ± 0.1
Pomegranate	0.60 ± 0.01[a]	13.8 ± 0.1[a]
Pomegranate Arils	0.39 ± 0.01	5.0 ± 0.1
Pomegranate Peel	0.79 ± 0.02[b]	14.9 ± 0.1[b]
Pomegranate Membrane	0.81 ± 0.02[b]	16.0 ± 0.1[b]

Results represent mean ± SEM of triplicate determinations. [a]$P < 0.05$ when compared to apple; [b]$P < 0.05$ when compared to arils.

The total phenolics content and antioxidant capacity of the parts of pomegranate's pericarp were analyzed. Peel and membrane extracts exhibited total phenolics contents which are greater than the phenolics content of the aril extract (Table 1), which is in agreement with work by Tzulker and coworkers [27]. It is noteworthy to mention that the peel and membrane extracts exhibited an antioxidant capacity that was approximately three times greater than the aril extract (Table 1).

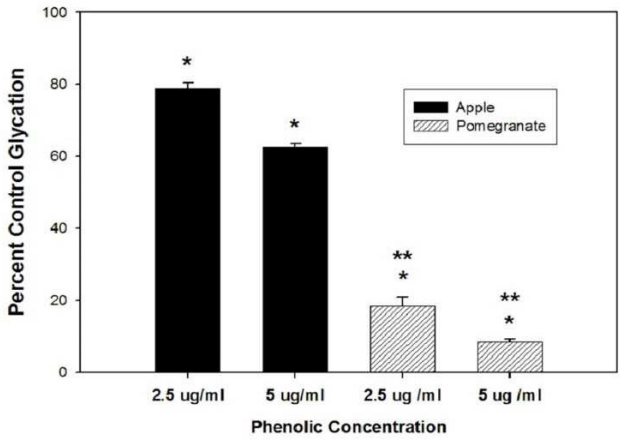

Figure 1: Inhibition of albumin glycation by whole pomegranate and apple at a concentration of 2.5 and 5 µg phenolics/ml. Results represent the mean ± SEM for triplicate determinations. *$P < 0.05$ when compared to control values; **$P < 0.05$ when compared to apple extract.

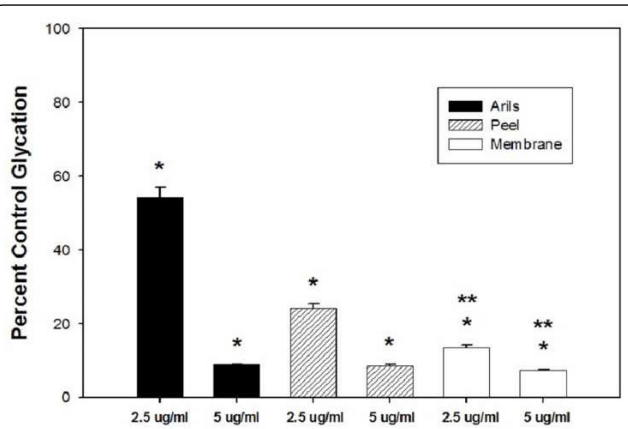

Figure 2: Inhibition of albumin glycation by components of the pomegranate pericarp (aril, peel, membrane) at a concentration of 2.5 and 5 µg phenolics/ml. Results represent the mean ± SEM for triplicate determinations. *$P < 0.05$ when compared to the control; **$P < 0.05$ when compared to aril and peel extracts.

To examine the effect of pomegranates and apples on protein glycation, pomegranate and apple extracts (2.5 and 5 µg phenolics/ml) were incubated with a solution containing BSA (10 mg/ml) and fructose (250 mM). When the BSA/fructose mixture was incubated in the presence of whole pomegranate extract (2.5 µg phenolics/ml), glycation was inhibited by approximately 80% (Figure 1); however, the same concentration of apple phenolic compounds resulted in only a 20% decrease in control glycation. Increasing the concentration of whole pomegranate extract to 5 µg phenolics/ml resulted in a further decrease in glycation to approximately 10% of control values. Apple phenolics, at 5 µg/ml, did not produce the same degree of inhibition observed with pomegranate phenolics at 2.5 µg/ml.

The effect of extracts from various parts of the pomegranate pericarp (arils, peel, membrane) on protein glycation was also examined. In this study (Figure 2), the phenolics content for each component was normalized either to 2.5 or 5 µg phenolics/ml in the incubation mixture. All three extracts from the various components significantly inhibited protein glycation at both concentrations; yet, the membrane extract produced the greatest decrease in glycation followed by the peel extract (Figure 2).

The effect of each pomegranate part extract, set at two different antioxidant capacities, on protein glycation was then examined (Figure 3). At an antioxidant capacity of 0.04 mM $FeSO_4$, the membrane extract demonstrated the greatest inhibition in glycation followed by the peel extract, with arils still showing significant inhibitory activity. At 0.05 mM $FeSO_4$ all extracts inhibited glycation by over 90%, with the membrane extract yielding the greatest inhibition among the components. In agreement with these findings, ellagitannins (the major phenolic group in pomegranate fruit and potent inhibitors of glycation) are mainly located in the peel and outer pericarp of pomegranate fruit [20], which includes the pomegranate membrane. The chromatograms of the phenolic compounds of the arils, peels and membranes are shown in figure 4. The content of punicalagin in the membranes was found to be 0.72 mg punicalagin/mg GAE; this was approximately 40% greater than that found in the peels. The amount of punicalagin found in the arils was approximately 20% of that found in the membranes. These results are in essential agreement with the work of Fischer and coworkers [28]. Membranes and peels were found to constitute 13% and 21% of the fresh weight of a whole pomegranate. Punicalagin content was found to be highest in the membrane (1.5% w/w) while peels contained 1.2% w/w.

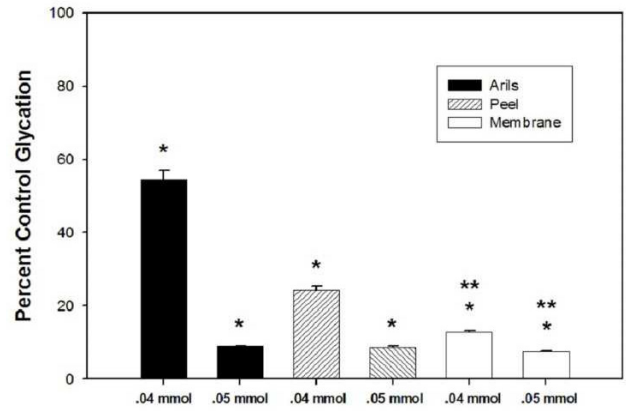

Figure 3: Inhibition of albumin by components of the pomegranate pericarp (aril, peel, membrane) at FRAP values of 0.04 and 0.05 mM $FeSO_4$. Results represent the mean ± SEM for triplicate determinations. *$P < 0.05$ when compared to control values; **$P < 0.05$ when compared to aril and peel extracts.

There are numerous studies centered on the antiglycation activity of natural products; however, there are few focused on the inhibition of this process by functional foods. The antioxidant and anti-inflammatory effects of pomegranate fruit

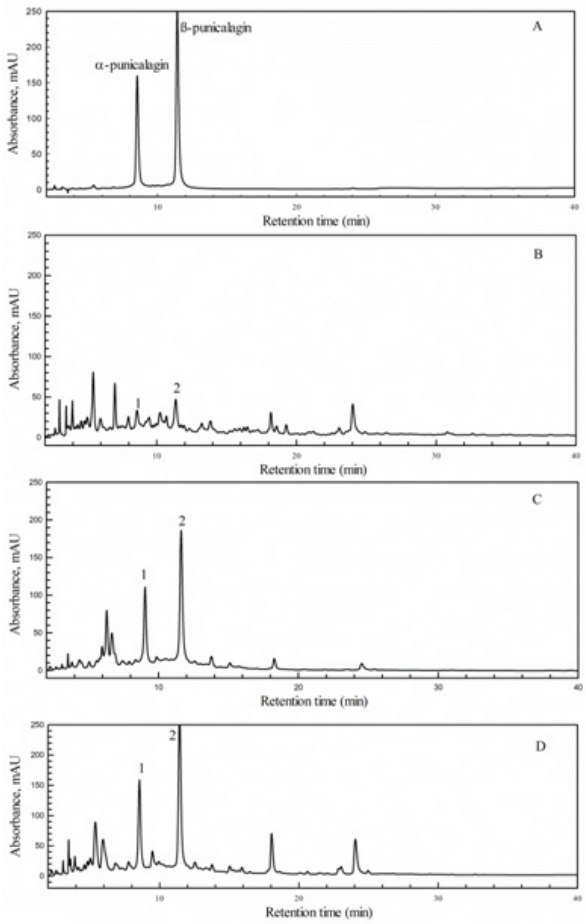

Figure 4: Reverse-phase HPLC chromatographs of punicalagin standard **(A)**, aril phenolics **(B)**, peel phenolics **(C)** and membrane phenolics **(D)**. 20 µl of the phenolic extract (200 µg phenolics/ml) from the different pomegranate components were analyzed.

are well documented, but its effect on protein glycation has only been recently investigated. Previously, we explored the effect of pomegranate juice and other commonly consumed juices (black cherry, Concord grape, pineapple, apple, and cranberry) on the formation of AGEs [19]. Pomegranate juice was the best inhibitor of the juices examined. Based on these findings, we explored the effect of the pomegranate fruit and the various parts of its pericarp on the inhibition of protein glycation. The extract of whole pomegranates produced a greater inhibition of protein glycation when compared to a whole apple extract (Figure 1). These results agree with our previous research [19]: apple juice was much less effective in inhibiting protein glycation when compared to pomegranate juice. This is not the first report on the effect of the apple on protein glycation. It has been previously reported that dehydrated apple extracts were effective inhibitors of protein glycation [29]; procyanidin B2 was found to be the most effective phenolic inhibitor, followed by (+)-catechin and (-)-epicatechin. In contrast, pomegranates and other berries (such as strawberries, blackberries, and raspberries) are phenolically rich in ellagitannins, ellagic acid (hydrolytic product of ellagitannins), and its derivatives [30]. The major ellagitannin in pomegranates is punicalagin [20]. The content of punicalagin, based on phenolic concentration, was found to be highest in the membranes of the pomegranate

(Figure 4). In a previous study [19], punicalagin was documented to inhibit protein glycation by over 80% at a concentration of 2.5 µg GAE/ml, in agreement with the results observed with the data presented in figure 2. Therefore, the differences observed in the inhibition of glycation by the components of the pomegranate can be attributed to their relative punicalagin content. The peels of many different fruits contain a high concentration of antioxidants, which provide protection against environmental stressors [31, 32]. In a similar matter, 10 µM of garcinol (purified product from *Garcina indica* fruit rind) inhibited glycation by 50% [33].

Hyperglycemia has been associated with the overproduction of the superoxide anion radical from the mitochondrial electron transport chain [34]. These free radicals damage biomolecules (lipids, proteins, DNA), that can lead to many chronic diseases and diabetic complications. Flavonoids with a strong scavenging activity (antioxidant capacity) tend to have a greater inhibitory effect on AGE formation than weaker scavenging phenolics [35]. However, depicted in figure 3, different pomegranate parts inhibit protein glycation to varying degrees even at the same antioxidant capacity. This would suggest that the antioxidant capacity is not the sole factor in determining inhibition of AGE formation.

Pomegranate has been extolled for many years due to its medicinal properties. The inhibitory properties of pomegranate fruit on protein glycation were first observed using a water-soluble extract of pomegranate rind [36]. The importance of the role of ellagic acid in inhibiting protein glycation was revealed in two separate studies [37, 38]. Several ellagitannins isolated from pomegranates were all shown to be more potent inhibitors of AGE formation than aminoguanidine [39]. The scavenging of carbonyl species by pomegranate phenolics has provided a possible mechanism for their activity [40] and inhibition of glycation by pomegranate phenolics has been studied in an *in vivo* model [41].

Conclusion

At the present time, there are no medications approved by the U. S. Food and Drug Administration to arrest the glycation of proteins and the associated complications observed in the diabetic state. To slow the glycation process, current therapy relies on lowering the plasma glucose concentration by a variety of pharmacological agents. The inhibitory effects of antioxidant, phenolic compounds, such as the ellagitannins found in the pomegranate, on protein glycation offers an alternate strategy to possibly delay the progression of diabetic complications.

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