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Pomegranate juice consumption increases GSH levels and reduces lipid and protein oxidation in human blood

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ABSTRACT

The aim of the present study was the assessment of the antioxidant effects of pomegranate juice (PJ) consumption in humans. Thus, 14 healthy volunteers consumed PJ daily for a period of 15 days and the changes of oxidative stress markers in their blood were assessed at four different time points, immediately before the experiment (T1), after 15 days of juice administration (T2), one (T3) and three weeks (T4) after the interruption of PJ administration. The markers studied were total antioxidant capacity (TAC), levels of malondialdehyde (MDA), and protein carbonyls (CARB) measured in plasma, as well as reduced glutathione (GSH), and catalase activity (CAT) measured in erythrocytes. The MDA was reduced by 24.4% at T3 and CARB were reduced by 19.6% and 17.7% at T2 and T3, respectively, supporting the evidence that PJ consumption enhances the antioxidant status in humans by decreasing lipid peroxidation and protein oxidation. Moreover, GSH levels were significantly increased (22.6%) at T2, indicating that PJ consumption improves the antioxidant mechanisms in erythrocytes by increasing GSH levels. Finally, it was shown that even a week after stopping PJ consumption some of its beneficial effects on antioxidant status still remained in the organism.

1. Introduction

Pomegranate (*Punica granatum* L.) constitutes one of the first recorded cultivated trees by humans. This tough, native plant of Himalayas (northern India), is currently cultivated in many regions including the Mediterranean basin countries, Iran, Afghanistan, India, China, Japan, Russia and some parts of the United States. The pomegranate fruit has been used in folk medicine from ancient times as antimicrobial (Gurib-Fakim, 2006) and as natural astringent for the treatment of diarrhea and harmful internal parasites (Das et al., 1999). Nowadays, the research interest on pomegranate fruit is increased as a consequence of reports establishing its benefits on human health (Faria and Calhau, 2011). In this respect, pomegranates have been studied as protective means of the cardiovascular system, the treatment of the acquired immune deficiency syndrome, in hormone replacement therapy, in oral hygiene (Faria and Calhau, 2011), in chemoprevention (Lansky et al., 2005), as microbicidal (Neurath et al., 2004) and as antihyperlipidemic (Fuhrman et al., 2005).

Pomegranate juice (PJ) is the greatest contributor for pomegranate ingestion which contains 85% water, 10% total sugars, 1.5% pectin, ascorbic acid, and polyphenols (Aviram et al., 2000). Several studies have reported a series of results for its clinical benefits, such as reduction of systolic blood pressure in hypertensive patients, decrease of common carotid artery intima-media thickness (IMT) (Aviram et al., 2004), attenuation of myocardial ischemia and the lipid profile improvement of diabetic patients

Abbreviations: BHT, 2,6-Di-tert-butyl-4-methylphenol; CARB, protein carbonyls; CAT, catalase activity; CV, coefficients of variation; DNPH, 2,4-dinitrophenylhydrazine; DPPH, 2,2′-diphenylpicrylhydrazyl; DTNB, 5,5′-dithiobis(2-nitro-benzoic acid); EDTA, ethylenediamine tetraacetic acid; GCL, glutamate cysteine ligase; GS, GSH synthetase; GSH, reduced glutathione; HDL, high density lipoprotein; HCl, hydrochloride; IMT, intima-media thickness; MDA, malondialdehyde; PJ, pomegranate juice; ROS, reactive oxygen species; SH, sulfhydryl group; TAC, total antioxidant capacity; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid; TPC, Total Phenolic Content.

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(Rosenblat et al., 2006). In addition, its chemopreventive, chemotherapeutic, antiatherosclerotic and anti-inflammatory (Aviram and Dornfeld, 2001; Kaplan et al., 2001; Rozenberg et al., 2006; Adams et al., 2006; Malik et al., 2005) have also been investigated. All the aforementioned beneficial effects of PJ on human health have been mainly attributed to its strong antioxidant properties (Rosenblat et al., 2006; Balasundram et al., 2006), since PJ is rich in free radical scavenging compounds such as anthocyanins (3-glucosides and 3.5-glucosides of delphinidin, cyanidin, and pelargonidin), ellagitannins (e.g. punicalagin, the most abundant polyphenol reaching levels of over 2 g/L juice), flavonoids (e.g. quercetin, kaempferol and luteolin glycosides) and polyphenolic acids (e.g. ellagic and gallic acid) (Gil et al., 2000; Seeram et al., 2005; Lansky, 2006). Moreover, the *in vitro* antioxidant activity of PJ has been determined as three folds higher in comparison to those of red wines and green teas and two to sixfold more potent from other natural beverages (Castilla et al., 2008).

It must be noted however, that most of these studies concern the evaluation of the *in vitro* antioxidant properties of PJ and there are only scarce reports on their effects in humans. Thus, we were intrigued to investigate herein the *in vivo* antioxidant potency of PJ through the assessment of their total antioxidant capacity (TAC), MDA levels (a biomarker of lipid peroxidation) and the levels of protein carbonyls (CARB), which constitute a biomarker of protein oxidation in human plasma. Finally, the levels of glutathione (GSH) and catalase activity (CAT) were also evaluated in human erythrocytes.

2. Materials and methods

2.1. Reagents and standards

All solvents used for the qualitative and quantitative determination of polyphenols were purchased from J.T. Baker (Griesheim, Germany) as analytical (polyphenol extraction) or HPLC (chromatographic analyses) grades. The Folin–Ciocalteu reagent was purchased from Fluka (Steinheim, Germany). All remaining chemicals were of analytical grade and obtained from Sigma–Aldrich. For the chromatographic analyses HPLC-grade water was prepared using a Milli-Q system, while all HPLC-solvents were filtered prior to use through cellulose acetate membranes of 0.45 μm pore size.

2.2. Assessment of polyphenolic content of pomegranate juice

The pomegranate juice used for the experiments was kindly provided by VITOM Christodoulou Bros SA, Greece and derived from the same production batch. Before the consumption experiments, the bioactive polyphenolic content of the pomegranate juice was determined. For this purpose the contained polyphenols were extracted.

2.2.1. Pomegranate juice extraction

The pomegranate juice was lyophilized and 50 g of the resulting solid were weighed in Erlenmeyer flasks and extracted four times with 50 mL of MeOH/H_2O/HCl 1.0 N (90:9.5:0.5 v.v.) in a sonication bath for 10 min. After filtration the solvents were evaporated under vacuum in the absence of light at temperature below 35 °C to avoid polyphenols degradation. The extract was reconstituted with 30 mL of methanol–H_2O (50:50) and centrifuged for 10 min (70,000 rpm). The supernatants were combined and re-extracted with Petroleum Ether (3 × 30 mL). The methanol–H_2O layer was evaporated to dryness under vacuum at 35 °C and the residue was dissolved in 30 mL of brine and extracted four times with ethyl acetate. The combined organic layers were dried over $MgSO_4$ and evaporated under vacuum. The HPLC samples were prepared by re-dissolving this solid in MeOH (1 mg/mL) and membrane filtration (0.45 μm).

2.2.2. HPLC analysis

The HPLC analysis was carried out using a Hewlett Packard HP1100 system equipped with: quaternary pump, auto-sampler, degasser and diode array detector (DAD). Chromatographic data were acquired and processed using the Chemstation software. Polyphenols were separated on a Kromasil C18 column (250 mm × 4.6 mm, particle size 5 μm) connected with a guard column of the same material (8 × 4 mm).

The HPLC method used is a modified version of the method developed by Tsao and Yang (2003). More specifically, the analysis was carried out at 30 °C (maintained by a column thermostat) using samples of 20 μL, which were directly injected by means of a Rheodyne injection valve (model 7725I). The gradient eluted consisted of solvent A (obtained by the addition of 3% v/v acetic acid in 2 mM sodium acetate aqueous solution) and solvent B (acetonitrile, CH_3CN). Run time was set at 70 min with a constant flow rate at 1.0 mL/min in accordance with the following gradient time table: at zero time, 95% A and 5% B; after 45 min, the pumps were adjusted to 85% A and 15% B; at 60 min, 65% A and 35% B; at 65 min, 50% A and 50% B; and finally at 70 min, 100% B. This routine was followed by a 30 min equilibration period with the zero time mixture prior to injection of the next sample. The column effluent was monitored at 280, 320, and 360 nm simultaneously. Peaks were identified by comparing their retention time and UV–vis spectra against the corresponding commercial polyphenols (obtained from Sigma–Aldrich) which were used as reference standards. Data were quantified using the corresponding curves of the reference compounds as standards. All standards were dissolved in methanol.

2.3. Assessment of the Total Phenolic Content (TPC)

The TPC of the extracts was determined in accordance with a modified version of the Folin–Ciocalteu method (Singleton et al., 1999). In particular, a 100 μL of the solid sample of the juice was added to a 10 mL flask containing 6 mL of deionized water. One milliliter of Folin–Ciocalteu reagent was added to the mixture, and the flask was stoppered and allowed to stand at room temperature for 3 min. A 1.5 mL portion of 20% Na_2CO_3 was added and the solution was diluted to the desire volume (10 mL) with deionized water. Absorbance was measured at 725 nm versus a blank after 2 h at room temperature. The results are expressed as gallic acid equivalents using the standard curve (absorbance versus concentration) prepared from authentic gallic acid.

2.4. Determination of total flavonoids

The total flavonoid content of juice was determined using a modified colorimetric method developed by Jia et al. (1999). In particular, 1 mL of solid sample was added into a 10 mL flask containing 4 mL of deionized water. A 0.3 mL portion of 5% $NaNO_2$ was added to this mixture and allowed to stand for 5 min at room temperature. Then, 0.3 mL of 10% $AlCl_36H_2O$ was added, the mixture was allowed to stand for 1 min at room temperature and 2 mL of 1 M NaOH was added. The solution was diluted to 10 mL with the addition of deionized water and the absorbance of the solution versus a blank at 510 nm was measured immediately. The results are expressed as catechin equivalents using a standard curve (absorbance versus concentration) prepared from authentic catechin samples.

2.5. Participants

Fourteen volunteers (men: 8, women: 6; age, 33.5 ± 3.2 yr; height, 172 ± 2.2 cm; weight, 73.3 ± 4.5 kg; body fat, 22.5 ± 2.7%; body mass index, 25.4 ± 1.3 kg/m^2) participated in the present study. All were nonsmokers, were not receiving anti-inflammatory medication or nutritional supplements and did not perform any special physical effort either before or after juice administration. A written informed consent to participate in the study was provided to all subjects involved in the study after they were informed of all risks, discomforts and benefits. The procedures were in accordance with the Helsinki declaration of 1975 and an approval was received by the human subjects committee of the University of Thessaly.

The subjects visited the laboratory for the first time for a screening of their anthropometric parameters and completed a health and activity questionnaire. Each participant reported to the laboratory in the morning after an overnight fast and abstinence from alcohol and caffeine for 24 h. Body mass was measured to the nearest 0.5 kg (Beam Balance 710, Seca, UK) with the subjects lightly dressed and barefoot. Standing height was measured to the nearest 0.5 cm (Stadiometer 208, Seca), while the percentage of their body fat was calculated from seven skinfold measures (an average of two measurements of each site) using a Harpenden caliper (John Bull, UK), in accordance to published guidelines (American College of Sports Medicine, 2000). Their body mass index was calculated as the ratio of body weight (kg)/height (m^2).

2.6. Diet and activity before the experiment

The subjects were instructed to follow their usual eating habits during the days prior to the experiment. They were also asked to record their diet for 3 days before the blood collections on a dietary record sheet. The subjects received a copy of their dietary record sheets and were asked to exactly follow the same food intake patterns (as recorded in their dietary record sheets) before all blood collections.

2.7. Study design

The subjects visited the laboratory (08:00–09:00 in the morning) the first day for anthropometry measurements. Each subject received 30 packs of 250 ml of PJ. The PJ was kindly provided by VITOM BrO Christodoulou Co Ltd, Greece. Blood samples were obtained prior to the experiment (T1). Then, for the next 15 days the subjects consumed 0.5 L (2 packs of 250 mL a day) of pomegranate juice. After 15 days