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Safety and Antioxidant Activity of a Pomegranate Ellagitannin-Enriched Polyphenol Dietary Supplement in Overweight Individuals with Increased Waist Size

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The consumption of pomegranate juice (PJ), a rich source of antioxidant polyphenols, has grown tremendously due to its reported health benefits. Pomegranate extracts, which incorporate the major antioxidants found in pomegranates, namely, ellagitannins, have been developed as botanical dietary supplements to provide an alternative convenient form for consuming the bioactive polyphenols found in PJ. Despite the commercial availability of pomegranate extract dietary supplements, there have been no studies evaluating their safety in human subjects. A pomegranate ellagitannin-enriched polyphenol extract (POMx) was prepared for dietary supplement use and evaluated in two pilot clinical studies. Study 1 was designed for safety assessment in 64 overweight individuals with increased waist size. The subjects consumed either one or two POMx capsules per day providing 710 mg (435 mg of gallic acid equivalents, GAEs) or 1420 mg (870 mg of GAEs) of extracts, respectively, and placebo (0 mg of GAEs). Safety laboratory determinations, including complete blood count (CBC), chemistry, and urinalysis, were made at each of three visits. Study 2 was designed for antioxidant activity assessment in 22 overweight subjects by administration of two POMx capsules per day providing 1000 mg (610 mg of GAEs) of extract versus baseline measurements. Measurement of antioxidant activity as evidenced by thiobarbituric acid reactive substances (TBARS) in plasma were measured before and after POMx supplementation. There was evidence of antioxidant activity through a significant reduction in TBARS linked with cardiovascular disease risk. There were no serious adverse events in any subject studied at either site. These studies demonstrate the safety of a pomegranate ellagitannin-enriched polyphenol dietary supplement in humans and provide evidence of antioxidant activity in humans.

KEYWORDS: Pomegranate extract; polyphenol; POMx; ellagitannins; safety; TBARS; human

INTRODUCTION

The pomegranate (*Punica granatum* L.) fruit has a long history of human consumption and has been associated with various health benefits dating back to ancient times (*1*). The potent in vitro antioxidant activity of pomegranate juice (PJ), by comparison to other high-antioxidant foods, has led to its utilization as a functional food by consumers for heart and prostate health. The high antioxidant activities of the phytochemicals found in the pomegranate have led to the development of dietary supplements that contain these biologically active polyphenols, namely, ellagitannins. Ellagitannins are the major polyphenols found in the pomegranate fruit and juice and account for >90% of the antioxidant activity of PJ (*2*). The overall antioxidant activity of PJ has been previously reported to exceed that of other red-purple fruits, red wine, and green tea (*3*).

A pomegranate ellagitannin-enriched polyphenol extract (POMx), enriched in ellagitannin content, has been prepared for dietary supplement use from partially juice-pressed whole fruit, arils, and seeds. The supplement contains predominantly monomeric and oligomeric ellagitannins as found in PJ but lacks the sugars and calories naturally found in the juice. This research was part of POMx's new dietary ingredient (NDI) safety

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submission, which to the best of our knowledge is currently the only pomegranate dietary supplement to be submitted to the U.S. Food and Drug Administration (FDA). The submission of safety data of dietary supplements is not required prior to marketing in the Untied States. The no-observed-adverse-effect-level (NOAEL) of POMx was considered to be 1500 mg/kg of body weight (unpublished data, POM Wonderful LLC., 2006). This study was undertaken at two different clinical sites in the United States to understand the human clinical and safety effects of POMx. Because excess abdominal fat has been associated with increased inflammation and oxidative stress, otherwise healthy overweight individuals with excess abdominal fat indicated by an increased waist circumference were recruited for this study to evaluate the safety and antioxidant activity of this dietary supplement.

EXPERIMENTAL METHODS

Pomegranate Polyphenol Extract Dietary Supplement (POMx). POMx is a proprietary standardized (to at least 90% pomegranate polyphenols) pomegranate fruit extract provided as a dietary supplement (POM Wonderful, LLC, Los Angeles, CA). It is produced from pomegranate (*Punica granatum* L.) fruits of the Wonderful variety grown in California by Paramount Farms (Lost Hills, CA). POMx is produced by a two-step process: (1) aqueous extraction of the fruit residue obtained after pressing for juice and (2) solid phase extraction of the liquid concentrate to produce a powder with a high content of polyphenols. Extraction is performed during fruit harvest using pressed pomegranate fruit skins and arils. In step 1, the remaining fruit matter, which includes pressed arils and rind obtained after the first press juice process, is milled and screened to remove the seeds. The deseeded fruit (mash) is then immersed in water and treated with pectinase, after which the mash solids are removed using a decanter. The resulting liquid is then ultrafiltered to remove insoluble solids and pasteurized. In step 2, the filtered aqueous extract is concentrated in an evaporator (at 130–150 °F) to produce an extract of concentrated pomegranate polyphenols. This concentrated liquid extract is then further purified using a food grade ion-exchange resin commonly used for "debittering". The liquid extract is diluted with water and adsorbed onto the resin column, and the polyphenols thus adsorbed are recovered using ethanol/water. The polyphenol eluate is vaccuum-dried at 140–150 °F for 8 h, yielding the powdered extract called POMx. The extract is stored at room temperature under dark anhydrous conditions until encapsulation for consumption. POMx has an ellagitannin profile similar to that of pomegranate juice (Jess Reed, University of Wisconsin–Madison, WI).

Folin–Ciocalteu Assay for Estimation of Phenolic Content. The Folin–Ciocalteu method (*4*) was used to quantify total phenolic compounds in POMx. Stock solutions of gallic acid (1 mg/mL) were used to generate four-point (0.005, 0.015, 0.03, and 0.05 mg) standard curves for the estimation of total polyphenols. Water (3 mL), Folin–Ciocalteu reagent (200 μL of stock solution as received from Sigma-Aldrich), and 100 μL of the powder analyte solution (1 mg/mL, dissolved in 50% aqueous MeOH) were added to a test tube. The test tube was vortex mixed and incubated at room temperature for 10 min. A 20% Na_2CO_3 solution (600 μL) was added to the tube, vortex mixed, and incubated at 40 °C for 20 min. The test tube was then cooled to room temperature in an ice bath. The absorbance of the standards and analytes was measured at a wavelength of 755 nm.

Method for Total Carbohydrate Determination. The POMx powder was analyzed for total carbohydrate content using the anthrone method after hydrolysis (*5*). The analyte (0.1 g) was dissolved in the least possible volume of water (0.1% trifluoroacetic acid, TFA; v/v) and loaded onto a reverse-phase C18 Cartridge (Waters Sep-Pak Vac 20 cm^3, 5 g), previously conditioned with methanol and equilibrated in water (0.1% TFA; v/v). The column was washed with 100 mL of water (0.1% TFA; v/v). The water eluate was dried by rotary evaporation (<30 °C) under vacuum. The remaining solid was dissolved in 5 mL of water, and the carbohydrate content was determined as glucose equivalents.

High Performance Liquid Chromatography (HPLC). The POMx powder was dissolved in the mobile phase (1 mg/mL) and injected (100 μL) onto a Waters Spherisorb ODS2, C-18 column (10 μm, 25 cm × 0.45 cm). The solvents for elution were water (0.1% TFA; solvent A) and methanol (solvent B). The elution profile was a series of step gradients: 100% A to 73% A over 30 min; 73% A to 45% A over the next 15 min; 45% A to 0% A over 5 min. The flow rate was maintained at 2 mL/min, and the elution was monitored by a Waters 996 diode array detector using Waters Millennium software for collecting and analyzing three-dimensional chromatograms at 280 nm.

Dry Matter and Ash. The hot weighing procedure (*6*) was used to determine the dry matter and ash contents of the POMx powder. Crucibles were hot weighed, and 0.5 g of the standard or powder analyte was added to the crucible. The crucible and analyte were heated at 100 °C in a forced-air oven for 12 h and were then placed in a muffle furnace and heated at 550 °C for 12 h to remove all organic matter. Crucibles were transferred back to the 100 °C forced-air oven and hot weighed to determine ash content.

Nitrogen. The POMx powder was assayed for nitrogen with a semimicro-Kjeldhal procedure as reported (*7*).

Human Study. *Recruitment.* A total of 88 subjects were recruited at a San Diego, CA (Accelovance Inc.), site and at a Denver, CO (University of Colorado Health Sciences Center), site; 86 subjects completed the study. At the clinical site in San Diego, 64 generally healthy male and female subjects between 35 and 65 years of age were recruited. At the clinical site in Denver, 24 adults ages 40–70 years were recruited, and 22 completed the 4-week study. At both sites, study participants were overweight and had excess abdominal fat as evidenced by a waist circumference of ≥35 in. for women and ≥40 in. for men. Subjects were counseled to eat a low-flavonoid diet for the duration of the study. Individuals with any change in smoking status or those taking any supplements or drugs that might interfere with absorption of polyphenols were excluded.

Exclusion. Exclusions included potential confounding factors such as those who were being treated for chronic diseases related to oxidative stress such as diabetes or hypertension. To enrich the study population with subjects who were more likely to have elevated levels of oxidative markers, subjects were required to have an above-normal body mass index (BMI) of 25–32 and to have central obesity determined by waist measurement (males, >40 in.; females, >35 in.).

Study Protocol. At the San Diego site, subjects consumed either one or two POMx capsules per day providing 710 and 1420 mg of extract containing 435 and 870 mg of gallic acid equivalents (GAEs), respectively. To maintain blinding, subjects in the 710 mg arm received one bottle of placebo and one bottle of POMx capsules. Subjects in the 1420 mg arm received two bottles of POMx capsules. In addition, 7 of the 64 subjects

Table 1. Electrolytes and Renal Function Laboratory Results in Subjects at the San Diego Site

		POMx 710 mg			POMx 1420 mg			placebo		
test	units	baseline	end of study	*p* value	baseline	end of study	*p* value	baseline	end of study	*p* value
BUN	mg/dL	14.1 ± 2.3	13.6 ± 2.5	0.133	13.7 ± 2.5	13.7 ± 2.8	0.842	12.7 ± 3.6	11.6 ± 2.3	0.423
creatinine	mg/dL	0.8 ± 0.1	0.9 ± 0.1	0.168	0.8 ± 0.1	0.8 ± 0.1	0.139	0.8 ± 0.08	0.8 ± 0.08	0.105
potassium	mequiv/L	4.5 ± 0.4	4.3 ± 0.3	0.252	4.4 ± 0.2	4.3 ± 0.2	0.358	4.2 ± 0.3	4.2 ± 0.2	0.782
sodium	mequiv/L	140.7 ± 1.7	138.9 ± 1.7	0.0013	140.2 ± 1.8	138.7 ± 1.4	0.006	140.2 ± 1.2	140.1 ± 1.3	0.842
chloride	mequiv/L	104.6 ± 2.1	103.6 ± 2.1	0.191	104.1 ± 1.1	103.7 ± 1.2	0.227	105.8 ± 1.6	104.4 ± 1.2	0.035
bicarbonate	mequiv/L	22.8 ± 2.0	23.9 ± 1.5	0.075	22.9 ± 1.8	24.1 ± 1.9	0.0009	22.7 ± 2.2	24.9 ± 1.7	0.015
calcium	mg/dL	9.3 ± 0.3	9.4 ± 0.3	0.71	9.3 ± 0.3	9.3 ± 0.3	0.64	9.2 ± 0.3	9.4 ± 0.3	0.115
uric acid	mg/dL	5.2 ± 1.4	5.0 ± 1.2	0.111	4.4 ± 1.0	4.2 ± 1.0	0.479	4.5 ± 1.1	4.6 ± 1.0	0.706

Table 2. Liver Function Laboratory Results in Subjects at the San Diego Site

		POMx710 mg			POMx1420 mg			placebo		
test	units	baseline	end of study	*p* value	baseline	end of study	*p* value	baseline	end of study	*p* value
alk phos	U/L	67.6 ± 16.5	67.7 ± 17.1	0.919	70.7 ± 11.9	72.5 ± 13.3	0.392	69.0 ± 13.8	69.3 ± 14.1	0.902
AST/GOT	U/L	19.0 ± 5.6	21.4 ± 8.4	0.343	17.0 ± 3.6	24.2 ± 13.7	0.284	19.3 ± 3.6	22.6 ± 6.3	0.088
ALT/GPT	U/L	20.8 ± 8.6	23.9 ± 12.5	0.459	17.9 ± 6.7	26.1 ± 17.1	0.258	18.2 ± 4.4	22.0 ± 6.9	0.251
total bilirubin	mg/dL	0.6 ± 0.2	0.6 ± 02	0.692	0.7 ± 0.2	0.6 ± 0.2	0.064	0.6 ± 0.2	0.5 ± 0.1	0.548
total protein	g/dL	7.4 ± 0.4	7.3 ± 0.3	0.4427	7.3 ± 0.4	7.4 ± 0.3	0.175	7.2 ± 0.3	7.3 ± 0.3	0.394
albumin	g/dL	4.4 ± 0.2	4.4 ± 0.2	0.784	4.4 ± 0.2	4.4 ± 0.1	0.197	4.4 ± 0.1	4.4 ± 0.1	0.594
direct bilirubin	mg/dL	0.1 ± 0.02	0.1 ± 0.02	1.0	0.1 ± 0.03	0.1 ± 0.02	0.489	0.09 ± 0.02	0.1 ± 0.02	0.347

received only placebo throughout the trial to assess the incidence of adverse effects in a group receiving only placebo. At the Denver site, subjects consumed two POMx capsules per day providing 1000 mg of extract containing 610 mg of GAEs. Following a screening visit, subjects received dietary instruction as to avoidance of foods with strong antioxidant properties for the duration of the trial. At the San Diego site, safety laboratory determinations were made at each of three visits. At visits subsequent to the screening visit, subjects were queried for any adverse events since the previous visit and any changes in concomitant medications. Vital signs (height, weight, blood pressure, and pulse rate) were taken at each visit. Safety laboratories include complete blood count (CBC), chemistry, and urinalysis. At the Denver site, the primary measure of antioxidant activity as evidenced by thiobarbituric acid reactive substances (TBARS) in plasma was taken before and after POMx supplementation.

Antioxidant Assay: Plasma TBARS Assay. Plasma was assayed for TBARS by the reported method (*8*) using 1,1,3,3-tetramethoxypropane as a standard. The TBARS test measures lipid peroxidation products, including precursors that will continue to break down to yield malondialdehyde. Briefly, plasma samples were stabilized upon collection and separation from packed cells by the addition of 1 μM butylated hydroxytoluene to prevent any further lipid peroxidation. They were assayed on the day of collection, or, if necessary frozen at −70 °C for a few days. The TBARS assay was carried out at only the Denver site.

Statistical Analysis. Mean value ± SD was calculated for all outcome variables. A paired *t* test was performed to determine whether the difference between baseline and end-of-study was significant for the control, 710 and 1420 mg of POMx interventions separately. All statistical analyses were performed using PRISM statistical analysis software package version 4 (GraphPad Software, San Diego, CA). For the subjects at the Denver study site, observed baseline and 30-day mean values were compared using paired *t* tests. Linear regression models were used to obtain least-squares adjusted means to adjust for observed change in weight (adjusted means are estimated at zero weight change).

RESULTS

Chemical Composition of the Pomegranate Ellagitannin-Enriched Polyphenol Extract (POMx). POMx is a proprietary pomegranate polyphenol extract (POM Wonderful LLC) that appears as a red-brown free-flowing powder with a bulk density between 0.8 and 0.9 g/cm^3. POMx has a polyphenol content of 61% GAEs (approximately 90% pomegranate polyphenols; personal communication, Jess Reed, University of Wisconsin–Madison, WI). The POMx polyphenols measured by HPLC at 280 nm consist of (1) oligomers composed of 2–10 repeating units of gallic acid, ellagic acid, and glucose in different combinations (77%); (2) ellagitannins as punicalagins and punicalins (19%); (3) free ellagic acid (4%); and (4) anthocyanins (0%). The powdered POMx extract also contains 2.2% ash, 2.9% sugars, 1.9% organic acids (as citric acid equivalents), 0.7% nitrogen, and 3.3% moisture.

Safety Study (San Diego Site). At the San Diego site, there was a formal assessment of adverse reactions and a comprehensive series of blood tests for toxicity by comparison to the control group receiving placebo (see **Tables 1**–**3**). No subject discontinued the study due to an adverse event. There were no serious adverse events reported, but there were 11 minor adverse events (e.g., upper respiratory infections) reported by 9 of the 64 randomized subjects at the San Diego site. None of these minor adverse effects were deemed to be related to the supplement. These events were distributed between the two treatment groups. Two of 27 (7.4%) subjects in the low-dose group reported minor adverse effects, as did 5 of 28 (17.9%) high-dose subjects. There were no qualitative or quantitative differences between treatment groups or by comparison to placebo. There were no apparent treatment-related changes of clinical significance, and no laboratory results were outside the normal range in any of the chemistry, hematology, or urinalysis laboratory testing in the subjects at the San Diego site (see **Table 1**–**3**).

Antioxidant Activity Study (Denver Site). At the Denver site, 22 of 24 subjects (18 females, 4 males) completed the treatment. The study results at the Denver site are presented separately because a significant increase in body weight (1.30 ± 1.95 lb, $p = 0.005$) was observed during the study. This

Table 3. Glucose and Triglycerides Laboratory Results in Subjects at the San Diego Site

		POMx 710 mg			POMx 1420 mg			placebo		
test	units	baseline	end of study	*p* value	baseline	end of study	*p* value	baseline	end of study	*p* value
glucose	mg/dL	95.3 ± 10.4	94.8 ± 12.0	0.9558	89.2 ± 7.6	90.3 ± 5.6	0.217	97.8 ± 9.0	96.3 ± 10.6	0.482
cholesterol	mg/dL	215.3 ± 28.0	209.5 ± 27.5	0.216	205.4 ± 28.4	210.9 ± 212.1	0.343	212.1 ± 29.2	207.1 ± 21.9	0.540
triglyceride	mg/dL	209.2 ± 155.1	204.0 ± 128.0	0.548	153.0 ± 80.9	127.9 ± 58.8	0.366	153.0 ± 42.7	148.2 ± 71.2	0.849
HDL	mg/dL	53.93 ± 10.0	52.6 ± 10.9	0.732	59.8 ± 11.3	59.7 ± 8.9	0.776	47.7 ± 6.2	48.7 ± 6.7	0.524
LDL	mg/dL	148.4 ± 29.6	139.8 ± 29.9	0.033	137.9 ± 26.2	144.5 ± 32.1	0.306	152.8 ± 37.5	150.8 ± 30.0	0.302

Table 4. Results of Study at the Denver Site Demonstrating a Significant Decrease in TBARS Despite an Increase in Body Weight

variable	baseline mean ± SD	30-day mean ± SD	change mean ± SD (95% CI)	*p* value[a]
weight (lb)	201.77 ± 55.27	203.07 ± 55.52	1.30 ± 1.95 (0.44, 2.17)	0.005
BMI (kg/m^2)	33.36 ± 8.52	33.57 ± 8.51	0.21 ± 0.35 (0.05, 0.36)	0.010
TBARS (μM)	1.02 ± 0.20	0.89 ± 0.21	−0.13 ± 0.23 (−0.23, −0.03)	0.044
AST (U/L)	25.45 ± 7.17	22.82 ± 5.93	−2.64 ± 5.36 (−5.01, −0.26)	0.172
ALT (U/L)	26.27 ± 14.17	23.18 ± 12.32	−3.09 ± 6.94 (−6.17, −0.01)	0.329
BUN (mg/dL)	12.86 ± 3.91	11.68 ± 3.09	−1.18 ± 2.77 (−2.41, 0.05)	0.090
creatinine (mg/dL)	0.86 ± 0.13	0.88 ± 0.16	0.02 ± 0.11 (−0.03, 0.06)	0.217

[a] Observed baseline and 30-day mean values were compared using paired *t* tests. Linear regression models were used to obtain least-squares adjusted means to adjust for observed change in weight (adjusted means are estimated at zero weight change).

weight gain is likely attributable to the timing of the study, which was carried out during the late fall when a number of holidays occurred. TBARS levels showed a significant decrease between baseline and 4 weeks ($-0.13 \pm 0.23\ \mu$M, $p = 0.011$). After adjustment for the change in weight, the decrease in TBARS was still significant ($p = 0.044$). There were no statistically significant changes in glucose, BUN, creatinine, lipids, insulin, c-peptide, paraoxonase-1, or electrolytes or liver enzymes (AST or ALT) after adjustment for changes in weight.

DISCUSSION

This study demonstrates in preliminary fashion that a pomegranate ellagitannin-enriched polyphenol (POMx) dietary supplement is safe when ingested by healthy human subjects in amounts up to 1420 mg/day providing a total of 870 mg of GAEs/day for 28 days. No adverse events related to the dietary supplement consumption or changes in hematology, serum chemistry, or urinalyses were observed. Furthermore, the pomegranate extract has been carefully characterized with regard to its chemical profile (Jess Reed, University of Wisconsin–Madison).

Preliminary evidence of a reduction in TBARS was seen in the subjects who were studied at the Denver site. Further studies are underway to document the effects of this supplement in subjects with type 2 diabetes, known to have a more marked increase in oxidant stress. TBARS are an important biomarker of oxidative stress, measuring harmful products of lipid (fat) oxidation found in the blood. Lower levels of TBARS are seen in healthy and younger individuals. As people age, and in certain diseases such as coronary heart disease, the amount of TBARS circulating in the blood increases, indicating elevated oxidative stress levels. Serum levels of TBARS are strongly predictable of cardiovascular events in people with stable coronary artery disease, independent of traditional risk factors and inflammatory markers (*9*).

No allergic reactions were observed in any of the 86 subjects who consumed the dietary supplement at the two clinical study sites. It is noteworthy that Greenblatt et al. (*10*) recently demonstrated that 100% PJ does not impair clearance of oral or intravenous midazolam used as a probe for cytochrome P450-3A activity, implying a lack of juice–drug interaction as has been well documented (*10*).

Therefore, these pilot studies demonstrate both the safety and efficacy of POMx, a pomegranate ellagitannin-enriched polyphenol dietary supplement, in humans. However, further studies need to be done to confirm the antioxidant properties of pomegranate ellagitannins administered as a dietary supplement.

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