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Pomegranate Phenolics from the Peels, Arils, and Flowers Are Antiatherogenic: Studies *in Vivo* in Atherosclerotic Apolipoprotein E-Deficient (E^0) Mice and *in Vitro* in Cultured Macrophages and Lipoproteins

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We have analyzed *in vivo* and *in vitro* the antiatherogenic properties and mechanisms of action of all pomegranate fruit parts: peels (POMxl, POMxp), arils (POMa), seeds (POMo), and flowers (POMf), in comparison to whole fruit juice (PJ). Atherosclerotic E^o mice consumed POM extracts [200 μg of gallic acid equivalents (GAE)/mouse/day] for 3 months. Blood samples, peritoneal macrophages (MPM), and aortas were then collected. All POM extracts possess antioxidative properties *in vitro*. After consumption of PJ, POMxl, POMxp, POMa, or POMf by E^o mice, the atherosclerotic lesion area was significantly decreased by 44, 38, 39, 6, or 70%, respectively, as compared to placebo-treated group, while POMo had no effect. POMf consumption reduced serum lipids, and glucose levels by 18–25%. PJ, POMxl, POMxp, POMf, or POMa consumption resulted in a significant decrement, by 53, 42, 35, 27, or 13%, respectively, in MPM total peroxides content, and increased cellular paraoxonase 2 (PON2) activity, as compared to placebo-treated mice. The uptake rates of oxidized-LDL by E^o-MPM were significantly reduced by ~15% after consumption of PJ, POMxl, or POMxp. Similar results were obtained on using J774A.1 macrophage cell line. Finally, pomegranate phenolics (punicalagin, punicalin, gallic acid, and ellagic acid), as well as pomegranate unique complexed sugars, could mimic the antiatherogenic effects of pomegranate extracts. We conclude that attenuation of atherosclerosis development by some of the POM extracts and, in particular, POMf, could be related to the combined beneficial effects on serum lipids levels and on macrophage atherogenic properties.

KEYWORDS: Pomegranate; polyphenols; atherosclerosis; oxidative stress; macrophages

INTRODUCTION

Macrophage cholesterol accumulation and foam cell formation is the hallmark of early atherogenesis (*1*). Oxidative stress has been shown to contribute to the development and progression of atherosclerosis (*2*). Indeed, low-density lipoprotein (LDL) can undergo oxidative modification by arterial macrophages, followed by an enhanced cellular uptake, resulting in a significant increase in macrophage cholesterol and oxidized lipids content (*2*). Cholesterol accumulation in macrophages can also result from a decreased rate of high-density lipoprotein (HDL)-mediated cholesterol efflux from the cells (*1*). *In vivo*, macrophage cholesterol accumulation and foam cell formation can also result from abnormal patterns of serum lipids, i.e., increased levels of cholesterol and triacylglycerol, and/or enhanced serum oxidative stress.

In atherosclerotic patients, as well as in the apolipoprotein E-deficient (E^0) mice (which develop atherosclerotic lesions that resemble those present in humans), an increased oxidative stress was shown both in their serum lipoproteins and also in arterial (as well as peritoneal) macrophages (*3–6*). We have previously shown that the increased oxidative stress in macrophages affects

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their biological activities. "Oxidized macrophages" can oxidize LDL, and also take up oxidized LDL (Ox-LDL) at enhanced rates (*7*).

Because oxidative stress plays an important role in atherogenesis, its inhibition by nutritional antioxidants should retard the progression of the disease. The pomegranate fruit contains several very potent antioxidants. Pomegranate juice (PJ), prepared from squeezing whole fruit, contains several unique polyphenolics and sugars. Pomegranate soluble polyphenols contain hydrolyzable tannins such as the ellagitannin punicalagin, gallic and ellagic acids, as well as anthocyanins and catechins (*8*). Consumption of PJ by healthy subjects for as little as 2 weeks, significantly reduced the oxidation of both LDL and HDL, and increased HDL-associated paraoxonase 1 (PON1) activity (*9*). Studies in patients with carotid artery stenosis (CAS, suffering from a partial blockage in the arteries that supply blood to their brain) that consumed PJ for 3 years, clearly demonstrated reduced serum oxidative stress, increased serum PON1 activity, and most important, a reduction in atherosclerotic lesion size (*10*). Similarly, daily consumption of PJ improved stress-induced myocardial ischemia in patients with coronary artery disease (*11*). Furthermore, in diabetic patients, PJ consumption did not aggravate their diabetic condition and, in fact, resulted in a significant reduction in the oxidative stress in their serum, as well as in their monocyte-derived macrophages, and also in the uptake of Ox-LDL by their cells (*5*). Furthermore, in diabetic patients with hyperlipidemia, PJ consumption moderately lowered serum cholesterol levels (*12*). As shown in humans, in the E^0 mice that were supplemented with PJ, serum oxidative stress, macrophage-mediated oxidation of LDL and the progression of atherosclerotic lesions were all significantly inhibited (*9*). Administration of PJ to hypercholesterolemic mice at various stages of the disease significantly reduced the progression of atherosclerosis development (*13*). The above effects of PJ could be attributed to the pomegranate antioxidative properties (*14*), secondary to its effect on oxidation sensitive genes, such as macrophage paraoxonase 2 (*15*), and endothelial NO synthase (*16*). It may also be related to pomegranate phenolics role in inhibition of macrophage uptake of Ox-LDL and/or cholesterol biosynthesis attenuation (*17*).

PJ, which is prepared from whole squeezed fruit, contains constituents from both the outer peel and the arils. In the present study, we analyzed separately the antiatherogenic effects *in vivo* (in the E^0 mice) of the different parts of the pomegranate fruit, i.e., peels, arils, seeds, and flowers (the source of the pomegranate fruit), and compared them to the effects observed for the whole fruit juice (PJ). To get an insight into the mechanisms of action, we have also determined antiatherogenic activities of the fruit parts *in vitro*, using cultured macrophages and isolated plasma lipoproteins. Finally, PJ-purified phenolics and sugars were studied for their antiatherogenic properties in relation to their role in the effects of the pomegranate fruit parts.

MATERIALS AND METHODS

Preparation of POM Extracts. Throughout the study, we used California-grown and processed Wonderful variety pomegranate (POM) extracts supplied by POM Wonderful (Los Angeles, CA) (**Table 1**).

Pomegranate Juice (PJ). Pomegranate juice was prepared from the whole fruit that was cut to expose arils during the squeezing process. The juice was filtered, pasteurized, concentrated to 65 Brix and stored at −18 °C. Then, it was diluted 1:4 (v/v) to 16 Brix with water to obtain the single strength PJ which was used in the study.

Pomegranate Fruit Liquid Extract (POMxl). After expelling most of the juice from the pomegranate whole fruit, the remaining fruit (peels and membrans), which include aril residues, were collected and processed to remove the seeds before going through a screw press to produce a puree. The puree was enzymatically treated to break down the colloidal structure of the husks and to solubilize sugars, minerals, acids, and polyphenol compounds. The puree polyphenols were concentrated via membrane system and the resultant cloudy POMxl was filtered to produce a pomegranate polyphenol extract. The obtained extract was concentrated after passing through an evaporator and pasteurized. The final product has a 65–70 Brix concentration and was stored at 4 °C.

Table 1. POM Extracts Chemical Composition

extract/juice	pomegranate extract composition
POM (PJ)	total phenolics (GAE), 3600 μg/mL (0.36%), including mainly hydrolyzable tannins (ellagitannins), such as oligomers and punicalagin/punicalin, with a smaller amounts of ellagic acid and anthocyanins (delphinidin, cyaniding, pelargonidin) and their glycosides other components: sugars, 13.8%; organic acids, 0.6%; ash, 0.5%, including potassium, 0.21%; protein, 0.1% (16 Brix)
POMxl	total phenolics (GAE), 130 000 μg/mL (13%), including mainly hydrolyzable tannins (ellagitannins), such as oligomers and punicalagin/punicalin, with a smaller amounts of ellagic acid and anthocyanins (delphinidin, cyaniding, pelargonidin) and their glycosides other components: sugars, 52%; organic acid, 2.0%; ash, 2.0% (70 Brix)
POMxp	total phenolics (GAE), 930 μg/mg (93%), including mainly hydrolyzable tannins (ellagitannins), such as oligomers and punicalagin/punicalin, with a smaller amounts of ellagic acid and trace anthocyanins other components: sugars, 3.2%; organic acids, 1.9%; ash, 2.5%; protein, 5.0%; and moisture, 1.2%
POMa	total phenolics (GAE), 14 μg/mg (1.4%), which consist of 70.5% other hydrolyzable tannins (ellagitannins), including punicalagin/punicalin, 25% anthocyanins and 5% ellagic acid derivatives other components: carbohydrate, 79% (11% dietary fiber, 57% sugar); protein, 7%; ash, 2.5%; fat, 0.2%; and moisture, 10%
POMo	total phenolic: no phenolics are present. Fatty acids: POMo fatty acids include 64.3% 9,11,13-linolenic (punicic acid), 14.1% C18:3 conjugated linolenic acid isomers, 6.1% linoleic, 5.2% oleic, 2.6% palmitic, 2.1% stearic antioxidant mixture: TBHQ, tocopherol, and ascorbyl palmitate
POMf	total phenolics (GAE), 166 μg/mg GAE (16.6%), including mainly hydrolyzable tannins (ellagitannins), such as oligomers and punicalagin/punicalin, with a smaller amounts of ellagic acid and trace anthocyanins other components: 30.2% total dietary fiber (26.1% insoluble fiber), 7.7% sugars, 2.0% sugar alcohols, 22% other carbohydrate, 5.6% ash, including 2.2% potassium, 9.4% protein, 0.9% fat, and 5.7% moisture

Pomegranate Polyphenol Powder Extract (POMxp). POMxl, diluted with water, was passed through a resin column, which preferentially absorbs polyphenols. Polyphenols adsorbed on the resin were recovered using ethanol in water. The recovered polyphenol solution was dried to produce POMxp.

Pomegranate Arils Extract (POMa). POM Wonderful arils including the seeds are dried by tray lyophilization and grounded to produce a powder. Pomegranate arils powder (1 mg) was dissolved in 1 mL of water.

Pomegranate Ground Flowers Extract (POMf). POM Wonderful flowers ("persistent calyx") were ground to a powder form. The pomegranate ground flowers powder (10 mg) was dissolved in 1 mL of water.

Pomegranate Sugars (POMs). POM Wonderful POMs were prepared from POMxl after removing the polyphenolic components. The sugars and acids did not bound to the resin column, and were collected. The acids were neutralized using potassium hydroxide. The juice sugar fraction was filtered to remove the resin. The POMs solution was first concentrated to 65−70 Brix. In our study we used POMs diluted 1:10

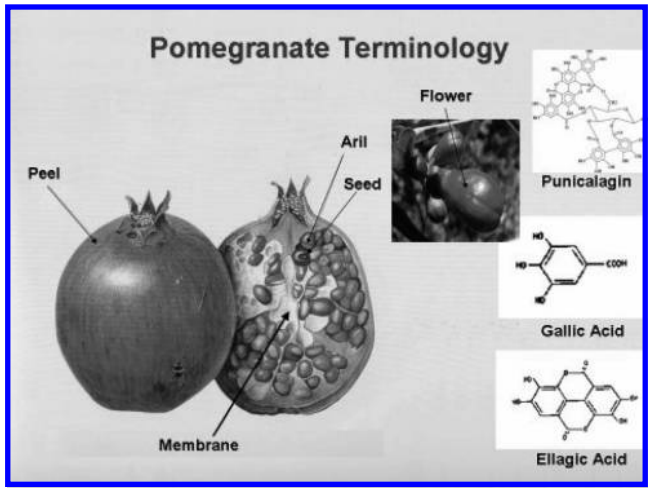

Figure 1. Schematic illustration of pomegranate fruit parts and their terminology and the chemical structure of punicalagin, ellagic acid, and gallic acid.

(v/v) with water. POMs polyphenol concentration was 89 ± 5 μg of gallic acid equivalent (GAE) per mL.

Pomegranate Seed Oil (POMo). POM Wonderful pomegranate oil was extracted from the pomegranate seeds by hexane followed by refining, bleaching, and deordorizing processing. The refined seed oil was stabilized with an antioxidant mixture of TBHQ, tocopherols, and ascorbyl palmitate.

Determination of Polyphenols. Total polyphenols were determined by the method of Singleton (*18*) using gallic acid as a standard.

Purification of Pomegranate Phenolics. PJ was partitioned with EtOAc and *n*-BuOH. The EtOAc extract was subjected to column chromatography on Amberlite XAD-16 (500 g, 6 × 35 cm) and eluted with water and MeOH. The MeOH fraction contained a mixture of tannins. This fraction was subjected to column chromatography on Sephadex LH-20 with 1:9 H_2O/MeOH (600 mL) and MeOH (500 mL) as eluents to yield pure ellagic acid. The *n*-BuOH extract was subjected to Amberlite XAD-16 column chromatography and eluted with water and MeOH following by further purification on Sephadex LH-20 CC (6 × 55 cm), and elution with 2:8 H_2O/MeOH (350 mL), and 1:9 (500 mL), MeOH (450 mL), and 1:1 MeOH/Me_2Co (600 mL), to finally yield nine fractions. Subfractions 1 + 2, 3, 4, 5 + 6, and 8 + 9 were further purified on Sephadex LH-20 column, to yield gallic acid, gallogyl dilactone, gallagic acid, punicalin, or punicalagin, respectively. The aqueous layers were lyophilized, and the residue were absorbed on XAD-16 and eluted with water and MeOH, consecutively. The MeOH eluate yielded the XAD−H_2O tannin mixture, which was further purified on Sephadex LH-20 column to give 13 fractions. Fraction 3 or 5 were further purified by Sephadex LH-20 column to obtain glucogallin or 2,3-hexahydroxydiphenyl-β-D-glucopyranose, respectively.

Figure 1 demonstrates the pomegranate different pars terminology, as well as the chemical structures of punicalagin, gallic acid, and ellagic acid.

Statistics. Statistical analysis was performed using the one-way analysis of variation (ANOVA) test. Results are given as mean ± standard deviation (SD).

Other methods are available free of charge via the Internet as Supporting Information at htpp://pubs.acs.org.

RESULTS

***In Vivo* Studies in the Atherosclerotic E^0 Mice.** *a. Effects of Pomegranate Extracts Consumption by E^0 Mice on Atherosclerotic Lesion Size.* After consumption of PJ, POMxl, POMxp, or POMf by E^0 mice (200 μg of GAE/mouse/day, for 3 months), the atherosclerotic lesion area was significantly decreased by 44, 38, 39, or by as much as 70%, respectively, as compared to lesion area observed in the placebo-treated group (**Figure 2**).

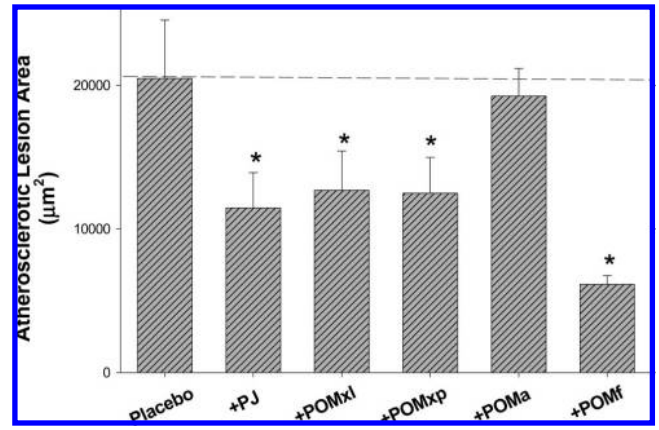

Figure 2. Effects of pomegranate extracts consumption by E^0 mice on their atherosclerotic lesion size. Apolipoprotein E-deficient mice (E^0 mice) consumed PJ, POMxl, POMxp, POMa, or POMf (200 μg of GAE/mouse/day) for 3 months. The placebo mice received only water. At the end of the study, the mice aortas were collected. The atherosclerotic lesion size was measured as described in the Materials and Methods. Results are given as mean ± SD of 10 mice in each group. (*) $p < 0.01$ versus placebo.

Table 2. Effects of PJ, POMxl, POMxp, POMa, or POMf Consumption by E^0 Mice on Serum Lipids and Glucose Concentrations[a]

treatment	total cholesterol (mg/dL)	triacylglycerol (mg/dL)	glucose (mg/dL)
placebo	574 ± 38	378 ± 43	122 ± 8
PJ	572 ± 28	380 ± 42	125 ± 10
POMxl	570 ± 25	381 ± 39	126 ± 9
POMxp	568 ± 34	385 ± 46	124 ± 3
POMa	614 ± 29	257 ± 28[b]	107 ± 6
POMf	471 ± 28[b]	284 ± 31[b]	99 ± 4[b]

[a] E^0 mice consumed 200 μg of GAE/mouse/day given as either pomegranate juice (PJ), pomegranate byproduct liquid (POMxl), pomegranate byproduct powder (POMxp), pomegranate arils (POMa), or pomegranate flowers (POMf). The placebo mice received only water. At the end of the study, blood samples were collected. Results are expressed as mean ± standard error of the mean (SEM) ($n = 10$). [b] $p < 0.01$ versus placebo ($n = 10$).

In contrast, POMa consumption, reduced the lesion size by only 6% (**Figure 2**), whereas POM seeds oil consumption had no effect (data not shown).

b. Effects of Pomegranate Extracts Consumption by E^0 Mice on Their Serum Lipids and Glucose Concentrations. PJ, POMxl, or POMxp consumption by the E^0 mice did not affect the serum levels of total cholesterol, triacylglycerol, and glucose (**Table 2**). POMa or POMf consumption, however, resulted in a significant reduction by 32 or 25%, respectively, in serum triacylglycerol concentration. POMf was the only one that decreased also serum glucose and cholesterol levels by 18–19%, as compared to the levels observed in the placebo group (**Table 2**).

c. Effect of Pomegranate Extracts Consumption by E^0 Mice on Serum Oxidative Stress. PJ, POMa, or POMf consumption by E^0 mice resulted in a significant decrement, by 29, 10, or 8%, respectively, in the basal levels of serum lipid peroxides, as compared to placebo-treated mice serum, whereas POMxl or POMxp consumption had no effect (**Figure 3A**). Serum paraoxonase 1 (PON1) is a HDL-associated lactonase, which can hydrolyze oxidized lipids, and which is inactivated under oxidative stress (*19, 20*). PJ or POMa consumption, significantly increased serum PON1 arylesterase activity by 43 or 22%, respectively, as compared to PON1 activity in the placebo-treated mice serum, whereas POMxl, POMxp, and POMf had no significant effect (**Figure 3B**).

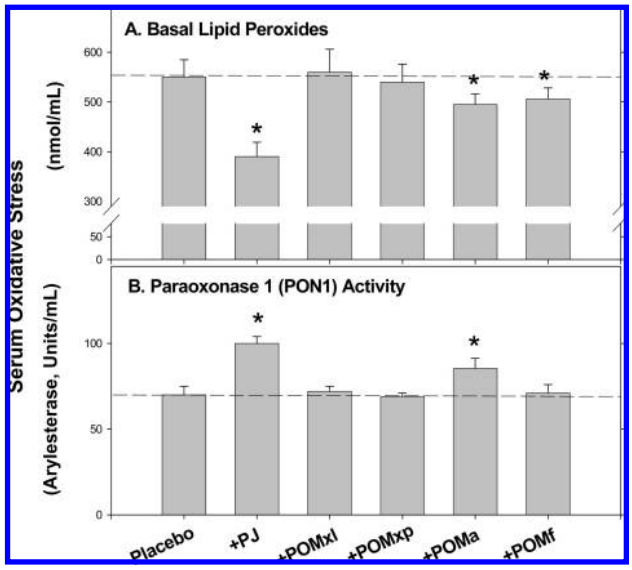

Figure 3. Effects of pomegranate extracts consumption by E^0 mice on serum oxidative stress. Apolipoprotein E-deficient mice (E^0 mice) consumed PJ, POMxl, POMxp, POMa, or POMf (200 μg of GAE/mouse/day) for 3 months. The placebo mice received only water. At the end of the study, the mice blood samples were collected and pooled. (**A**) Basal level of serum lipid peroxides was determined. (**B**) Serum paraoxonase 1 (PON1) arylesterase activity was measured using phenyl acetate as substrate. Results are given as mean ± SD ($n = 10$). (*) $p < 0.01$ versus placebo.

d. Effect of Pomegranate Extracts Consumption by E^0 Mice on Oxidative Stress in Their Peritoneal Macrophages (MPM). PJ, POMxl, POMxp, or POMf consumption by the E^0 mice resulted in a significant decrement, by 53, 42, 35, or 27%, respectively, in cellular total peroxides levels (**Figure 4A**), as compared to MPM derived from the placebo-treated mice. POMa was the least potent extract with only 13% reduction in macrophage peroxides content (**Figure 4A**). Paraoxonase 2 (PON2) is a cellular esterase/lactonase, which is expressed in macrophages and acts as a cellular antioxidant (*21, 22*). Consumption of PJ, POMxl, or POMxp resulted in a significant increment in MPM PON2 lactonase activity by 102, 50, or 40%, respectively, as compared to PON2 activity in MPM from the placebo-treated mice (0.234 ± 0.010, 0.197 ± 0.013, or 0.183 ± 0.014 versus 0.131 ± 0.013 units/mg cell protein, respectively).

e. Effect of Pomegranate Extracts Consumption by E^0 Mice on Cholesterol Metabolism in Their Peritoneal Macrophages. Only PJ consumption significantly decreased by 27% the extent of native LDL uptake by MPM, as compared its uptake by the placebo-treated mice MPM, whereas, all of the other extracts had no significant effect (**Figure 4B**). The uptake rates of Ox-LDL by the mice MPM, however, were significantly reduced by 15, 10, or 10% after consumption of PJ, POMxl, or POMxp, respectively, whereas POMf had only a minor effect (6% reduction) and POMa had no effect (**Figure 4C**). Macrophage cholesterol accumulation can also result from reduced rates of HDL-induced macrophage cholesterol efflux (*1, 2*). PJ and to a lesser extent POMa consumption significantly stimulated HDL-mediated cholesterol efflux from the mice MPM by 39 or 27%, respectively (**Figure 4D**). In contrast, POMxl, POMxp, and POMf consumption had no significant effect.

Upon measuring cellular cholesterol biosynthesis rate, we observed in MPM from the mice that consumed POMf a significant inhibition, by 41%, as compared to MPM from the placebo-treated mice (9862 ± 100 versus 16715 ± 167 cpm/mg cell protein, respectively). Furthermore, the cellular cholesterol content in the mice MPM was significantly decreased after POMf consumption by 17%, as compared to MPM from placebo-treated mice (40 ± 2 versus 48 ± 2 μg/mg cell protein, respectively). All of the other pomegranate extracts had no effect on these parameters.

***In Vitro* Studies.** *a. Antioxidative Properties of Pomegranate Extracts.* Whereas PJ contained constituents from both the arils and the peels, POMxl and POMxp were obtained only from the peels and POMa only from the arils.

All extracts were potent antioxidants and the free radicals scavenging capacity of the various pomegranate extracts, analyzed on the basis of a similar total polyphenolic concentration (3 μg of GAE/mL), was in the following order: POMxl > POMxp > PJ > POMf > POMa. The absorbance measured at 517 nm decreased after 5 min of incubation with DPPH by 39, 34, 30, 26, and 22%, respectively (**Figure 5A**).

Next, we examined the effects of increasing concentrations of polyphenols (0–3 μg of GAE/mL) of the above pomegranate extracts on copper ion-induced LDL oxidation. All extracts, except for POMa, substantially inhibited the extent of LDL oxidation, with IC_{50} values (the concentration needed to obtain 50% inhibition of LDL oxidation) for PJ, POMxl, POMxp, POMa, and POMf of 0.34, 0.14, 0.23, 1.15, and 0.31 μg of GAE/mL, respectively (**Figure 5B**), indicating that POMxl was the most potent antioxidant against LDL oxidation, whereas POMa was the least potent one.

b. Effects of Pomegranate Extracts on J774 A.1 Macrophage Oxidative Stress. Incubation of J774 A.1 macrophage-like cell line with POMxl, POMxp, PJ, or POMf (10 μg of GAE/mL) for 20 h at 37 °C resulted in a significant decrement in cellular total peroxides content by 76, 56, 40, or 38%, respectively (**Figure 6A**), as compared to control cells. POMa was the least potent extract with only 19% reduction in cellular oxidative stress (**Figure 6A**).

c. Effects of Pomegranate Extracts on J774 A.1 Macrophage Cholesterol Metabolism. To explore possible mechanism for pomegranate extracts effects on macrophage cholesterol metabolism, J774A.1 macrophages were incubated without (control) or with 10 μg of GAE/mL of the pomegranate extracts for 20 h. The extent of native LDL uptake by the cells was significantly inhibited by 27, 40, 30, or 14% after macrophage incubation with PJ, POMxl, POMxp, or POMa, respectively, as compared to its uptake by control cells (**Figure 6B**). After incubation of J774A.1 macrophages with PJ, POMxl, POMxp, or POMa, a significant decrement in the extent of Ox-LDL uptake by the cells, by 42, 69, 67, or 27%, respectively, was noted (**Figure 6C**), as compared to its uptake by control cells. In contrast, but similar to the *in vivo* results (parts **B** and **C** of **Figure 4**), POMf did not significantly affect the extent of native LDL uptake (**Figure 6B**) or Ox-LDL uptake by the cells (**Figure 6C**).

Unlike the *in vivo* data, HDL-mediated cholesterol efflux from J774 A.1 macrophages was significantly stimulated by 52, 44, 43, or 52%, after cell incubation with PJ, POMxl, POMxp, or POMa, respectively, as compared to cholesterol efflux rate observed in control cells (**Figure 6D**). In contrast, POMf again, as shown *in vivo*, did not stimulate cholesterol efflux from the cells (**Figure 6D**).

Similarly to the *in vivo* results, in J774A.1 macrophages that were incubated with 10 μg of GAE/mL of POMf, cholesterol biosynthesis rate was decreased by 41%, as compared to control cells (9027 ± 90 versus 15300 ± 110 cpm/mg cell protein, respectively), and the cellular cholesterol mass decreased by 14% (32 ± 1 μg/mg cell protein in POMf-treated cells versus 37 ± 2 μg/mg cell protein in control cells).

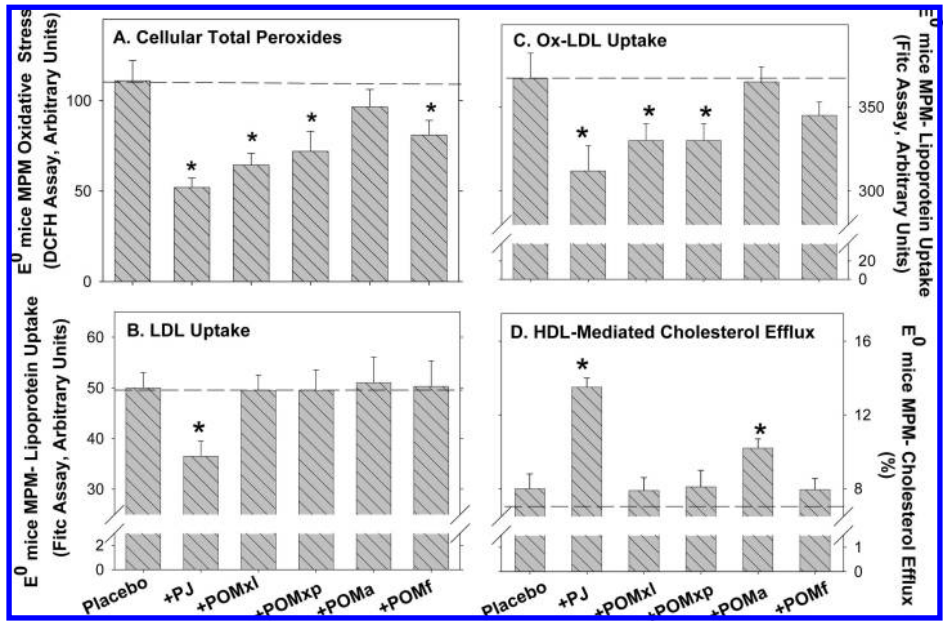

Figure 4. Effect of pomegranate extracts consumption by E^0 mice on their peritoneal macrophages oxidative stress and cholesterol metabolism. (**A**) Apolipoprotein E-deficient mice (E^0 mice) consumed PJ, POMxl, POMxp, POMa, or POMf (200 μg of GAE/mouse/day) for 3 months. The placebo mice received only water. At the end of the study, the mice peritoneal macrophages were harvested and pooled. (**A**) Amount of cellular total peroxides was measured by the DCFH assay. (**B** and **C**) Cells were then washed and the extent of fluoroisothiocyanate (FITC) labeled native LDL (**B**) or FITC-labeled oxidized LDL (Ox-LDL) uptake (**C**) by the macrophages were determined by flow cytometry. (**D**) Cells were washed and incubated with [^{3}H]-cholesterol for 1 h at 37 °C. After cell wash, they were further incubated for 3 h with HDL (100 μg of protein/mL), and the extent of HDL-mediated cholesterol efflux from the cells was determined. Results are given as mean ± SD of three different experiments. (*) $p < 0.01$ versus placebo.

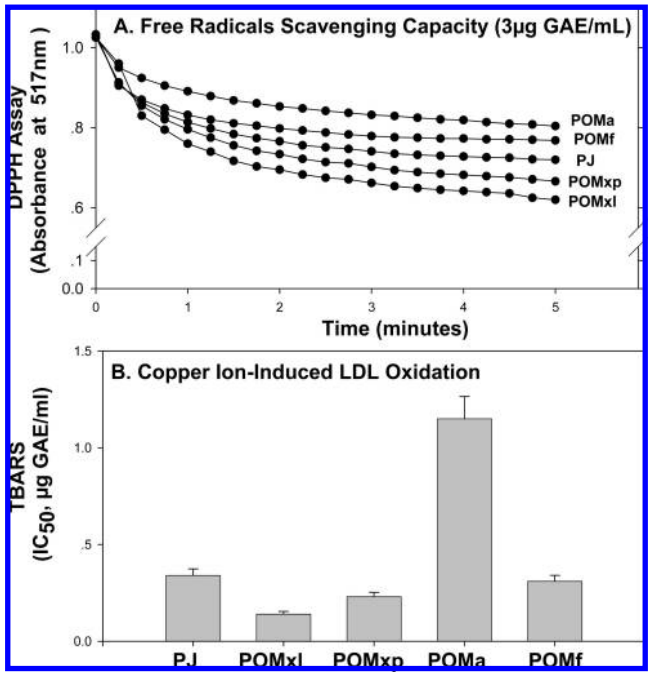

Figure 5. *In vitro* antioxidative properties of various pomegranate extracts. (**A**) Free-radical scavenging capacity of PJ, POMxl, POMxp, POMa, or POMf was analyzed by the DPPH assay, using 3 μg of GAE/mL. The absorbance at 517 was kinetically monitored for up to 5 min. (**B**) LDL (100 μg of protein/mL) was preincubated with increasing concentrations of polyphenols (0–3 μg of GAE/mL) of PJ, POMxl, POMxp, POMa, or POMf, and then 5 μmol/L $CuSO_4$ was added. The extent of LDL oxidation was determined after 2 h incubation at 37 °C. The IC_{50} values (the concentration needed to obtain 50% inhibition) are shown. Results are given as mean ± SD of three experiments.

d. Antiatherogenic Properties of Purified Pomegranate Phenolics. The potent antiatherogenic effects of pomegranate peel's extracts and also of PJ could be related to the pomegranate specific phenolics (**Table 1**). Thus, we have next isolated from PJ 8 purified phenolics: gallic acid, ellagic acid, punicalagin, punicalin, glucopyranose, gallagyl dilactone, glucogallin, or gallagic acid and analyzed directly their antiatherogenic properties *in vitro* using LDL and J774 A.1 macrophages.

The whole PJ (4 μg of total polyphenols/mL) reduced DPPH optical density, by up to 82% (**Figure 7**). Gallic acid, punicalagin, ellagic acid, punicalin, or glucopyranose, at a similar concentration were very potent free-radical scavengers, because they reduced DPPH optical density by 80, 62, 56, 51, or 50%, respectively (**Figure 7**). In contrast, gallagyl dilactone, glucogallin, or gallagic acid were much less potent free-radical scavengers, with only 25, 23, and 14% reduction in the optical density, respectively (**Figure 7**).

The addition of increasing PJ concentrations (0–0.5 μg/mL), substantially inhibited LDL oxidation by up to 97% (**Figure 8A**) with a maximal effect observed already at 0.1 μg of PJ polyphenols/mL. Gallic acid was again the most potent inhibitor of LDL oxidation among the studied pomegranate purified phenolics, followed by glucopyranose, punicalagin, punicalin, and ellagic acid (**Figure 8A**). The IC_{50} value for gallic acid versus the whole PJ was 0.03 versus 0.02 μg/mL, respectively. Punicalagin, punicalin, and glucopyranose were somewhat less potent with an IC_{50} of 0.16 μg/mL, and for ellagic acid, the IC_{50} was 0.2 μg/mL (**Figure 8A**). Upon adding increasing concentrations of gallagic acid, glucogallin, and gallagyl dilactone (0–2.5 μg/mL), LDL oxidation was completely inhibited in a dose-dependent manner. Although these phenolics were much less potent than the previous phenolics (**Figure 8A**), the most potent one among them was glucogallin (IC_{50} values of 0.4 μg/mL), followed by gallagyl dilactone (IC_{50} values of 0.7μg/mL) and gallagic acid (IC_{50} values of 1.8 μg/mL, **Figure 8B**).

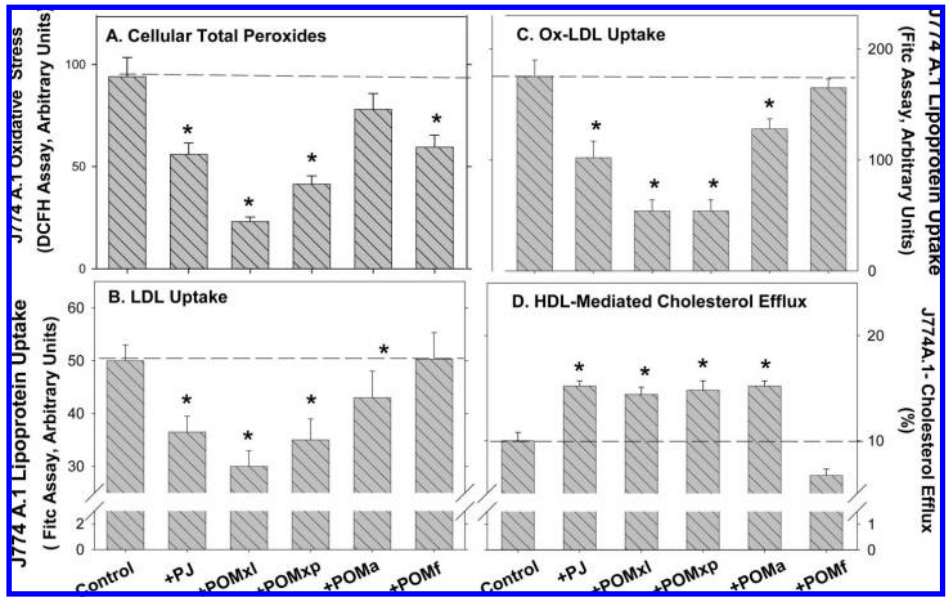

Figure 6. Effects of pomegranate extracts on J774 A.1 macrophage oxidative stress and cholesterol metabolism. (**A**) J774 A.1 macrophages were incubated for 20 h at 37 °C with no addition (control) or with pomegranate extracts (10 μg of GAE/mL). At the end of the incubation period, the cells were washed and the amount of total peroxides were determined by the DCFH assay. (**B** and **C**) Cells were then washed and the extent of fluoroisothiocyanate (FITC) labeled native LDL (**B**) or FITC-labeled oxidized LDL (Ox-LDL) uptake (**C**) by the macrophages were determined by flow cytometry. (**D**) Cells were washed and incubated with [^{3}H]-cholesterol for 1 h at 37 °C. After cell wash, they were further incubated for 3 h with HDL (100 μg of protein/mL), and the extent of HDL-mediated cholesterol efflux from the cells was determined. Results are given as mean ± SD of three different experiments. (*) $p < 0.01$ versus control.

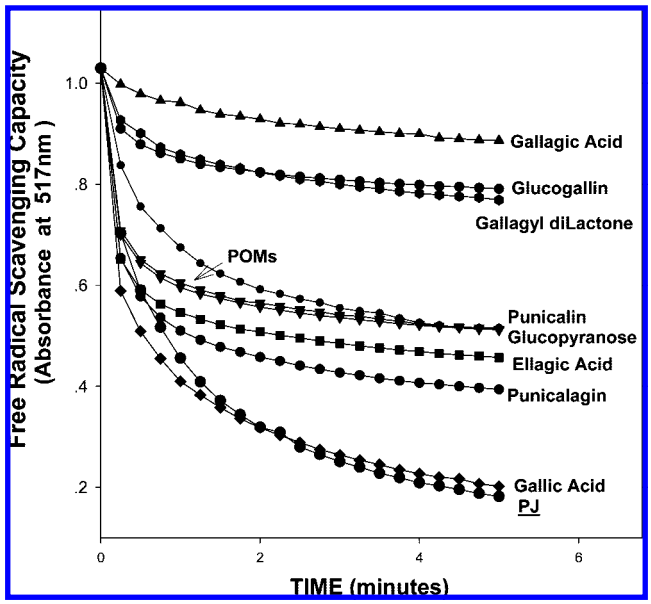

Figure 7. Free-radical scavenging capacity of pomegranate-purified phenolics and sugars. The free-radical scavenging capacity of PJ phenolics: gallagic acid, glucogallin, gallagyl dilactone, punicalin, glucopyranose, ellagic acid, punicalagin, gallic acid, PJ, or POM sugars was determined by the DPPH assay using (4 μg of total polyphenols/mL). A representative experiment, out of three similar studies, is shown.

Cellular oxidative stress, as measured by the DCFH assay, was significantly decreased upon incubation of J774 A.1 macrophages for 20 h with 30 μg/mL of PJ (total polyphenols) or of PJ purified phenolics (**Figure 9A**). Whole PJ, glucopyranos, punicalagin, punicalin, glucogallin, gallic acid, or ellagic acid reduced cellular oxidative stress by 48, 44, 37, 33, 33, 30, and 27%, respectively (**Figure 9A**). The less potent phenolics were gallagyl dilactone and gallagic acid with only 23 and 15% reduction in cellular oxidative stress (**Figure 9A**).

The extent of native LDL or Ox-LDL uptake by J774 A.1 macrophages was next measured, after cell incubation for 20 h with 30 μg/mL of PJ total phenolics or of purified phenolics. PJ inhibited the uptake of FITC-labeled native LDL by 15% (from 75 ± 5 to 64 ± 3, arbitrary units) whereas the purified phenolics inhibited its uptake by 30–60%, as compared to control cells (**Figure 9B**).

The extent of Ox-LDL uptake by the cells was significantly inhibited by 74–76%, after cell incubation with PJ, punicalagin, or punicalin (**Figure 9C**). Ellagic acid, glucopyranose, and gallic acid inhibited Ox-LDL cellular uptake by 64, 51, and 44%, respectively. In contrast, gallagic acid, gallagyl dilactone, and glucogallin reduced the extent of Ox-LDL uptake by only 25, 22, and 14%, respectively, as compared to the extent of its uptake by control cells (**Figure 9C**).

e. Antiatherogenic Properties of Pomegranate Sugars Fraction. Pomegranate sugars fraction (POMs, 4 μg of GAE/mL), decreased DPPH optical density by 50% (**Figure 7**), whereas glucose had no scavenging activity of free radicals (data not shown).

The addition of increasing POMs concentrations (0–20 μg of GAE/mL) to LDL also significantly inhibited copper ion-induced LDL oxidation in a POMs dose-dependent manner, by up to 96% (**Figure 8C**). The maximal effect was demonstrated already after adding 10 μg of GAE/mL, with an IC_{50} value of 4.5 μg of GAE/mL (**Figure 8C**). In contrast, glucose at a concentration of 20 μg/mL had no protective effect against LDL oxidation (The TBARS values were 36 ± 5 nmol/mg LDL protein in control LDL versus 34 ± 3 nmol/mg LDL protein in LDL incubated with glucose).

POMs (30 μg/mL) decreased J774A.1 macrophage oxidative stress by only 26% (**Figure 9A**) and the extent of native LDL (**Figure 9B**) or Ox-LDL (**Figure 9C**) uptake by the cells, by 40 or 25%, respectively, as compared to the extent of these lipoproteins uptake by control cells.

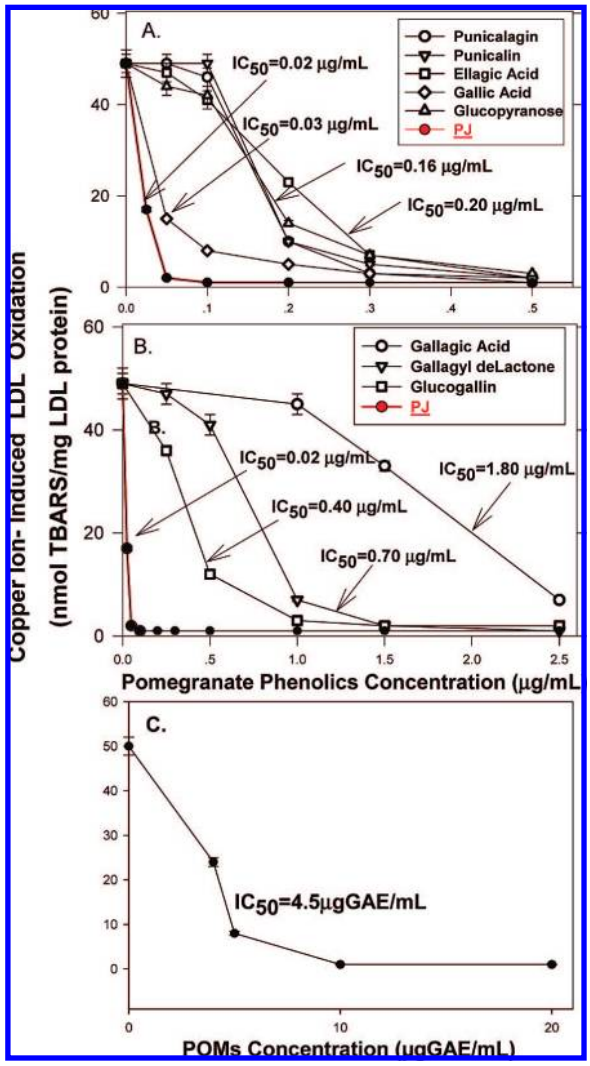

Figure 8. Effects of pomegranate phenolics and sugars on copper ion-induced LDL oxidation. (**A**). LDL (100 μg of protein/mL) was preincubated with increasing concentrations (0–0.5 GAE/mL) of punicalagin, punicalin, ellagic acid, gallic acid, glucopyranose, or PJ. (**B**) LDL (100 μg of protein/mL) was preincubated with increasing concentrations (0–2.5 GAE/mL) of gallagic acid, gallagyl dilactone, glucogallin, or PJ. (**C**) LDL (100 μg of protein/mL) was preincubated with increasing concentrations (0–20 GAE/mL) of POM sugars. Copper ions (5 μmol/L) were added to all LDL samples (**A**−**C**). The extent of LDL oxidation was determined after 2 h of incubation at 37 °C by the TBARS assay. The IC_{50} values (the concentration needed to obtain 50% inhibition) are also given. Results are given as mean ± SD of three different experiments.

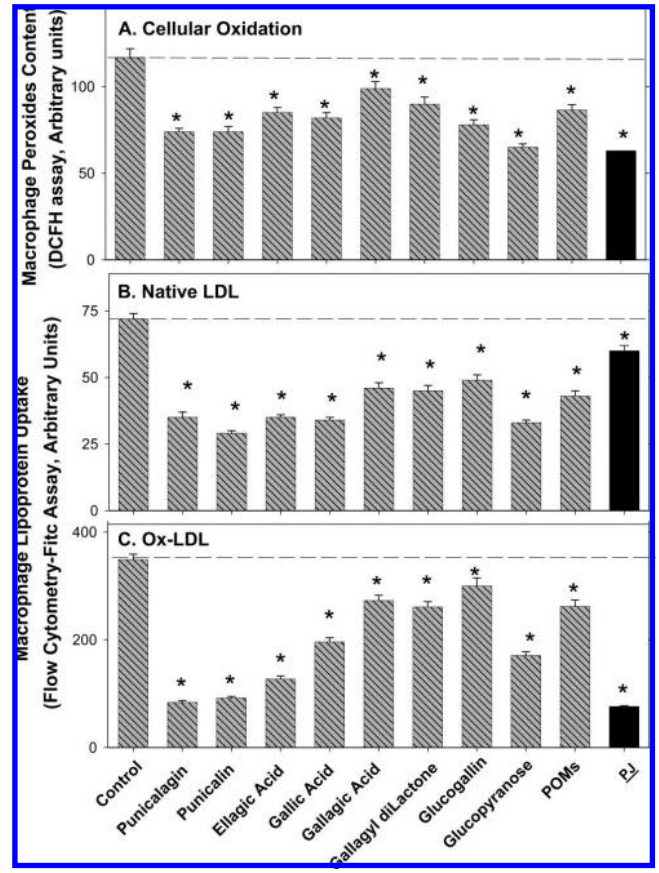

Figure 9. Effects of pomegranate purified phenolics and sugars on J774A.1 macrophages oxidative stress, and on native LDL or oxidized LDL (Ox-LDL) cellular uptake. J774 A.1 macrophages were incubated for 20 h at 37 °C with 30 μg of PJ total polyphenols, with POM sugars (POMs), or with PJ purified phenolics: punicalin, punicalagin, ellagic acid, gallic acid, gallagyl acid, gallagyl dilactone, glucogallin, and glucopyranose. (**A**) After cell washing, the amount of total peroxides was measured by the DCFH assay. The extent of FITC-labeled native LDL uptake (**B**) or that of oxidized LDL (Ox-LDL) uptake (**C**) were determined by flow cytometry. Results are given as mean ± SD of three different experiments. (∗) $p < 0.01$ versus control.

DISCUSSION

In the present study, we have clearly demonstrated, the *in vivo* antiatherosclerotic properties of pomegranate peel extracts (POMxl and POMxp), indicating their major contribution to the beneficial effects previously observed by whole fruit juice (PJ), mainly on macrophage oxidative stress and on the extent of Ox-LDL uptake by the cells. These effects of PJ could be attributed to the hydrolyzed tannins (including oligomeric ellagitannins and other phenolics, such as punicalagin, punicalin, ellagic acid, and gallic acid), as well as to the PJ unique complexed sugars, which form complexes with the pomegranate phenolics. A most potent antiatherosclerotic capacity was shown for the pomegranate flowers extract (POMf), which could be related to the decrement in serum cholesterol level together with a significant inhibition in macrophage uptake of Ox-LDL and in cellular cholesterol biosynthesis rate, which may be related to the synergistic effects of hydrolyzed tannins and other components such as dietary fiber and other carbohydrates.

In vitro, all POM extracts demonstrated antioxidative properties (free-radical scavenging capacity and inhibition of copper ion-induced LDL oxidation). All POM extracts contain polyphenols, but because a similar polyphenolic concentration was used, the differences in their antioxidative capacity could be attributed to different types of polyphenols present in the various extracts. The POM primarily peel extracts (POMxl, POMxp) were the most powerful antioxidants, whereas POM arils were much less potent, indicating that the peel polyphenols are the major contributors to PJ antioxidative capacity as was indeed recently shown (*23, 24*). POM flowers extract was previously shown to contain polyphenols with potent antioxidant properties, which were able to scavenge reactive oxygen/nitrogen species (ROS/RNS, *25*). Pomegranate flower extract also inhibited hydroxyl radical induced oxidation of lipids and proteins (*25*). The E^0 mice consumed 200 μg of GAE/mouse/day. This concentration was chosen on the basis of our previous studies (*9, 13*). Although we are not sure that each mouse in the treated group consumed similar polyphenol concentration, in most of our experiments,

we used pooled serum or pooled peritoneal macrophages. Using similar concentration of total polyphenols, the pomegranate extracts from the different parts did show different bioactivity, which could be attributed to the differences in the type of polyphenolic compounds.

In contrast to the *in vitro* results, *in vivo*, in the atherosclerotic E^0 mice, no effect on serum oxidative stress was noted after consumption of POMxl, POMxp, or POMf extracts. PJ and POMa consumption, however, slightly decreased serum oxidative stress, and this could have resulted from an increase in serum paraoxonase 1 (PON1) activity. Because in E^0 mice increased oxidative stress was shown in their serum (*3*), it could be that the concentration of POM extracts used (200 μg of GAE/mouse/day) was not sufficient to reduce the high serum oxidative stress.

Among the studied extracts, only POMf consumption decreased the levels off serum lipids (and glucose) in the mice, and similar effects were observed upon administration of pomegranate flower extract to diabetic rats (*26*). This phenomenon could be related to PPAR-α activation (*27*), to improved sensitivity of the insulin receptor (*27*), or to inhibition of intestinal α-glucosidase activity (*28*).

In contrast to the results obtained in the mice serum, consumption of the POM extracts significantly decreased oxidative stress in the mice macrophages. Pomegranate peel extracts (POMxl and POMxp) were most potent, just like PJ, whereas POM arils only slightly affected macrophage oxidative stress, both *in vivo* (in MPM) and *in vitro* (in J774A.1 macrophage cell line). These results indicate that polyphenolic constituents in the peels, and not in the arils, affect macrophage oxidative stress. Similarly, it was recently shown, using U937 pro-monocytes and HUVEC endothelial cells, that aqueous rinds extract protected the cells from oxidative stress-induced cytotoxic effects, even more than PJ, while the arils juice was much less potent (*29*). As arils juice, in contrast to PJ and POM rind extracts, contains small amount of ellagic acid and punicalagin (**Table 1**), this may explain the above results. We have previously shown that consumption of pomegranate byproduct (prepared from the whole pomegranate fruit, which is left after the juice preparation) by E^0 mice, resulted in a significant decrease in the MPM lipid peroxides content (*30*). Similarly, pomegranate peel extract administration to rats reduced oxidative stress in the liver (*31*). The decrement in macrophage oxidative stress by POM extracts could have resulted from a direct effect of their polyphenols or sugars (as shown using PJ purified phenolics or POMs) or secondary to their effects on oxidation sensitive genes (*16*), including the cellular antioxidant paraoxonase 2 (PON2, *15, 30*). Another possible mechanism, which may account for the attenuation of atherosclerosis development in E^0 mice after consumption of POM extracts, is decreased cholesterol influx by macrophages, measured as a reduction in the extent of Ox-LDL uptake by the cells. Both POMxl or POMxp, as well as PJ, decreased the extent of Ox-LDL uptake *in vivo* (by the MPM) and also *in vitro* (by J774A.1 macrophages), while POMa was less potent in this respect, indicating once again that the unique polyphenols in the peel could be responsible for the observed antiatherosclerotic effects of pomegranate extracts. Similarly to the current results, we have previously shown that PJ *in vitro* (*17*) or *in vivo* (*9, 13*), as well as pomegranate byproduct (*30*), significantly decreased the extent of Ox-LDL uptake by macrophages.

Recently, it was shown that the antioxidative action of punicalagin, a major hydrolyzable tannin polyphenolic antioxidant, is the result of its free radicals scavenging capacity, as well as its action as a metal chelator (*32*). Punicalagin, punicalin, and ellagic and gallic acids decreased macrophage oxidative stress and native LDL and Ox-LDL uptake by the cells. PJ, however, was more potent than the purified phenolics, in all studied antioxidant and antiatherogenic properties, indicating that more than one constituent in PJ contributes to the beneficial effects of pomegranate extracts. Indeed, it was shown that the antioxidant activities of punicalagin, ellagic acid, and total pomegranate tannin extract are enhanced when present in combination with other polyphenols, as exists in pomegranate juice (*33*). The results of the present study illustrate that phytochemicals in general, and pomegranate phenolics in particular, varied in various fruit parts, thus resulting in variations in the bioactivity of the fruit parts.

The present study demonstrated that POM sugars also possess antioxidative properties, and this may be due to the presence of complexes between pomegranate polyphenols and sugars. Similarly, a free-radical scavenging capacity of a polysaccharide fraction isolated from the pomegranate rind was recently demonstrated (*34*). We have recently shown that sugars separated from pomegranate juice, but not from grape juice, decreased macrophage oxidative stress, as well as the extent of Ox-LDL uptake by macrophages (*35*). This could be explained by the complexes formed between glucose and pomegranate phenolics, which possess antiatherogenic properties, as opposed to the free glucose present in grape juice that possesses proatherogenic characteristics (*35*).

Several different antiatherosclerotic mechanisms are involved in the beneficial activities of the various pomegranate extracts. These mechanisms include effects on concentration of serum lipids (POMf), or composition, effect on serum lipids peroxidation (PJ), effect on macrophage lipids peroxidation (POMxl, POMxp, PJ, and POMf), and effect on macrophage uptake of oxidized LDL (PJ, POMxl, POMxp, and POMf).

We have shown that not only the various pomegranate extracts act via several antiatherogenic mechanisms, but also the pomegranate phenolics and mainly oligomers, punicalagin, punicalin, and gallic and ellagic acids, are major contributors to the above beneficial effects of pomegranate juice.

ABBREVIATIONS USED

PJ, pomegranate juice; POMxl, pomegranate peel liquid extract; POMxp, pomegranate peel powder extract; POMa, pomegranate arils extract; POMf, pomegranate ground flowers extract; POMs, pomegranate sugars; POMo, pomegranate seeds oil; GAE, gallic acid equivalent; E^0 mice, apolipoprotein E-deficient mice; MPM, mouse peritoneal macrophages; Ox-LDL, oxidized LDL; TBARS, thiobarbituric acid reactive substances; PON1, paraoxonase 1; PON2, paraoxonase 2; DPPH, diphenyl picryl hydrazyl; AAPH, azobisamidinopropane hydrochloride; FITC, fluoroisothiocyanate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

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Nina Volkova performed all of the *in vitro* studies and took part in the mice studies together with Mira Rosenblat. Raymond Coleman performed the atherosclerotic lesion analysis. Muntha Kesava Reddy and Daneel Ferreira purified the polyphenols from PJ. Mark Dreher examined the pomegranate fruit parts composition. Prof. Michael Aviram initiated the study, was the supervisor of the whole project, and together with Mira Rosenblat wrote the manuscript.

Supporting Information Available: Materials and Methods to be published online only. This material is available free of charge via the Internet at htpp://pubs.acs.org.

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