Consumption of polyphenolic-rich beverages (mostly pomegranate and black currant juices) by healthy subjects for a short term increased serum antioxidant status, and the serum's ability to attenuate macrophage cholesterol accumulation

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The present study analyzed the antioxidative effects of various beverages, *in vitro*, and also the effect of short term consumption of beverages richest in polyphenols by healthy subjects on serum anti-atherogenic properties. Healthy subjects consumed 250 mL of the selected beverages for 2 h, or daily, for up to 1 week.

We hypothesized that differences in the anti-atherogenic properties of the studied beverages could be related, not only to the quantity of polyphenols, but also to their quality. Furthermore, we hypothesized that consumption of these juices by healthy subjects for just a short-term, will increase their serum anti-atherogenic properties, as was demonstrated previously in long-term consumption studies.

Of 35 beverages studied, both 100% Wonderful-variety pomegranate and 100% black currant juices were the most potent antioxidants *in vitro*, as they inhibited copper ion-induced LDL oxidation by up to 94% and AAPH-induced serum lipid peroxidation by up to 38%. Furthermore, they increased *in vitro* serum paraoxonase 1 (PON1) lactonase activity by up to 51%. Consumption of five selected polyphenol rich beverages by healthy subjects increased serum sulfhydryl group (SH) levels and serum PON1 activities after 2 h, and more so after 1 week of drinking these beverages. These effects were most pronounced after the consumption of 100% Wonderful-variety pomegranate and 100% black currant juices. In conclusion, polyphenolic-rich juices with impressive *in vitro* antioxidant properties, also demonstrate antioxidant effects *in vivo* when analyzed for short term consumption. In this respect, 100% Wonderful-variety pomegranate and 100% black currant juices were most the potent.

Introduction

Macrophage cholesterol accumulation and foam cell formation are hallmarks of early atherogenesis.[1] Oxidative stress contributes to the development and progression of atherosclerosis.[2] Cholesterol accumulation in macrophages can result from increased uptake of oxidized LDL and/or a decreased rate of HDL-mediated cholesterol efflux from the cells.[1,2]

Dietary polyphenols, such as those present in some beverages, were shown to be potent antioxidants[3] and cardioprotective agents.[4–7] Grape juice consumption by hypertensive individuals for 8 weeks increased serum antioxidant potential,[8] and exerts hypolipidemic and anti-inflammatory effects in both hemodialysis patients and in healthy subjects.[9] Red wine consumption by healthy subjects inhibited LDL oxidation[10] and decreased monocyte migration.[11] A more potent cardioprotector than grape juice or red wine was found to be 100% Wonderful-variety pomegranate juice (WPJ), as it protected atherosclerotic patients from further atherosclerosis development.[12,13] Consumption of WPJ by healthy subjects for at least 2 weeks, significantly reduced the oxidation of both LDL and HDL and increased HDL-associated paraoxonase 1 (PON1) activity.[14] Studies in patients with carotid artery stenosis (CAS) that consumed WPJ for 3 years, clearly demonstrated reduced serum oxidative stress, increased serum PON1 activity, and most importantly - a reduction in atherosclerotic lesion size.[12] Furthermore, in diabetic patients, WPJ consumption decreased serum and macrophage oxidative stress, decreased oxidized LDL uptake by their cells,[13] and increased PON1 association with HDL.[15] Similarly, WPJ *in vitro* was shown to increase PON1 binding to HDL,[16] and also to up-regulate PON1 expression in hepatocytes.[17] In all the above studies, the effects of beverages were noted after relatively long periods of consumption (weeks, months, and years). There are a limited number of studies which analyzed the acute effects of beverage consumption on serum oxidative stress and atherogenicity. Plasma antioxidant power was increased postprandially after red wine consumption by healthy subjects.[18] Black currant juice consumption for 1 week decreased serum lipid peroxidation and increased urinary excretion of quercetin.[19] Similarly, twelve hours after consumption of acai juice by healthy subjects, the plasma antioxidant capacity was significantly increased.[20] Finally, consumption of a mixture of berry juices, which include acai and palm fruit as the predominant ingredients, increased the serum antioxidant level, and inhibited lipid peroxidation, two hours post-consumption.[21]

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The aim of the present study was to compare the antioxidative effects of polyphenolic-rich beverages (and especially, those of 100% Wonderful-variety pomegranate juice, 100% black currant juice, 100% Concord grape juice, acai juice blend and red wine) *in vitro*, and also to study the effects of their short term consumption by healthy subjects (either 2 h, or daily intake for 1 week) on serum anti-atherogenic properties.

Experimental

In vitro studies

Table 1 demonstrates the list of the 35 beverages used in the study.

Polyphenols. Total polyphenols were determined spectrophotometrically by the method of Singleton, modified for small volumes.[22] Gallic acid served as a standard. Gallic acid stock solution was prepared in water at a concentration of 2 mmol/L. Volumes of 10, 20, 40 and 60 microlitres diluted in 1 mL solution (equivalent to 20, 40, 80 and 120 nmol respectively) were used for the standard curve.

Free radical scavenging capacity. The free radical-scavenging capacity of the various beverages was analyzed by the DPPH assay.[23] DPPH (1,1-diphenyl-2-picrylhydrazyl) is a radical generating substance that is widely used to monitor the free radical scavenging abilities (the ability of a compound to donate an electron) of various antioxidants. The DPPH radical has a deep violet color due to its impaired electron, and radical scavenging can be followed spectrophotometrically by the loss of absorbance at 517 nm, as the pale yellow non-radical form is produced. The beverages were diluted with water 1 : 10, v : v. Aliquots of 20 μL from the diluted beverages were mixed with 1 mL of 0.1 mmol DPPH/L in ethanol, and the change in optical density at 517 nm was monitored after 5 min.

LDL preparation. LDL was isolated from plasma taken from healthy normolipidemic volunteers, by discontinuous density gradient ultracentrifugation.[24] The LDL was washed at $d = 1.063$ g/mL, dialyzed against 150 mmol/L NaCl, 1 mmol/L Na_2EDTA (pH 7.4) at 4 °C. The LDL was then sterilized by filtration (0.45 μM), kept under nitrogen in the dark at 4 °C and used within 2 weeks. The LDL protein concentration was determined with the Folin Phenol reagent.[25] Prior to oxidation, LDL was dialyzed against EDTA-free, phosphate buffered saline (PBS) solution, pH 7.4, and at 4 °C.

Copper ion-induced oxidation. The beverages were diluted with water 1 : 100, v:v. LDL (100 μg of protein/mL) was then pre-incubated for 1 h at room temperature with 25 μL/mL of the samples of diluted beverages. Then, 5 μmol/L of $CuSO_4$ was

Table 1 List of beverages tested

Category	Brand	Product name
100% Pomegranate juice	POM Wonderful	100% Pomegranate Juice (Wonderful-variety)
	Knudsen	Just Pomegranate (variety unknown)
	Langers	100% Pomegranate Juice (variety unknown)
	Odwalla	Pomegranate Juice (variety unknown)
	Naked	Pomegranate (variety unknown)
100% Concord grape juice	Welch's	100% Concord Grape Juice
	Knudsen	Concord Grape
	Lakewood	Pure Concord Grape
100% Black cherry juice	Knudsen	Just Black Cherry
	Lakewood	Pure Black Cherry
100% Black currant juice and blends	Knudsen	Just Black Cherry
	Currant C	Black Currant Nectar
100% Blueberry juice	Knudsen	Just Blueberry
	Wyman's	100% Wild Blueberry
100% Yumberry	Frutzzo	100% Yumberry
Acai juice blends	Bossa Nova	Acai Juice- The Original
	Naked	Acai Machine
	Sambazon	Original Blend
"Superfruit" blends	Naked	Red Machine
		Green Machine
	Odwalla	Red Rhapsody
	Lakewood	Mangosteen/antioxidant fruit
	Born Dia	Acai Mangosteen
		Acai Blueberry
	Bossa Nova	Mangosteen Dragonfruit
	Goji Lania	Goji Cherry
		Goji Acai
		Goji Mangosteen
	Minute Maid	Pomegranate Blueberry
	Tropicana	Pomegranate Blueberry
Green tea	Nestea	Green Tea with natural flavor
	Lipton	Green Tea with citrus
Red wine	Beaulieu	BV Napa Cabernet Sauvignon
	Mondavi	Robert Mondavi Napa Merlot
	Cline	Zinfandel (California)

added and the tubes were incubated for 2 h at 37 °C. At the end of the incubation, the degree of LDL oxidation was determined by measuring the generated amount of thiobarbituric acid reactive substances (TBARS). The TBARS assay was performed at 532 nm, using malondialdehyde (MDA) for the standard curve.[26]

AAPH-induced serum lipid peroxidation. Serum samples from healthy subjects were diluted ×4 with phosphate buffered saline (PBS) and were pre-incubated with no addition (control), or with five polyphenol rich beverages (2 μL/mL of concentrated beverages) for 1 h at room temperature. Then 100 mmol/L of 2,2′-azobis, 2-amidinopropane hydrochloride (AAPH, Wako, Japan) was added to all samples and they were further incubated for 2 h at 37 °C.[27] The extent of lipid peroxidation was measured by the TBARS assay.[26]

In vivo studies

Subjects. Six healthy male subjects (aged 25–30 years) participated in the study. The subjects drank 250 mL of each beverage after an overnight fast. Blood was collected from the subjects 2 h after consumption. They continued daily beverage consumption for 1 week with the evening meal. After 1 week of beverage consumption, blood samples were collected. After a 1 week "washout period", this protocol was repeated with another beverage until each subject had consumed all 5 of the tested beverages. The order of beverages consumed was: first – acai juice blend (Naked), second – 100% Concord grape juice (Lakewood), third – 100% black currant juice (Knudsen), fourth – 100% Wonderful-variety pomegranate juice (POM Wonderful), and last – red wine (Merlot/Mondavi). The subjects served as their own control, as we compared all post-consumption data to the baseline values. All subjects were non-smokers. The study protocol was approved by the Helsinki Committee of the Rambam Medical Center, Israel Ministry of Health, application no. 3073.

Serum biochemical parameters. Serum sodium, potassium, glucose and lipids (total cholesterol, HDL cholesterol and triacylglycerol) concentrations were measured using automated enzymatic tests (Tayco Diagnostics- Agis Commercial Agencies, Israel).

Serum oxidative stress parameters

Basal serum oxidation status. The extent of lipid peroxidation was measured by the TBARS assay.[26]

Total thiols (SH groups) in serum. The assay procedure determines the amount of protein bound SH groups, as well as of glutathione.[28] An aliquot of 10 μL of the above serum samples was mixed with 200 μL of Tris-EDTA buffer, and the absorbance at 412 nm was measured. To these samples 8 μL of 10 mmol/L DTNB was added, and after 15 min of incubation at room temperature, the absorbance was measured again together with a DTNB blank. The total amounts of SH groups were then calculated.

Serum PON1 activities

Serum PON1 arylesterase activity. The assay was performed in a 96 well UV plate, in a total volume of 200 μL per well. The above serum samples were diluted 1 : 10 with "activity buffer" (1 mmol/L $CaCl_2$ in 50 mmol/L Tris HCl, pH 8.0) and then 5 μL were taken for a total reaction volume of 200 μL. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm for 3 min (every 15 s). The assay mixture included 1.0 mmol/L phenyl acetate in "activity buffer". One unit of arylesterase activity equals 1 μmol of phenyl acetate hydrolyzed/min/mL.[29]

Serum PON1 lactonase activity towards DHC. The assay was performed in 96 well UV plates, in a total volume of 200 μL per well. The above serum samples were diluted 1 : 10 with "activity buffer" (1 mM $CaCl_2$ in 50 mmol/L Tris HCl, pH 8.0), and 3 μL were then taken for the assay. Lactonase activity was measured using dihydrocoumarin (DHC) as the substrate. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm, for 10 min (every 15 s). The assay mixture included 1 mmol/L DHC in "activity buffer". Non-enzymatic hydrolysis of DHC was subtracted from the total rate of hydrolysis. One unit of lactonase activity equals 1 μmol of DHC hydrolyzed/min/mL.[29]

Macrophage cholesterol metabolism

J774A.1 macrophage cell line. The J774A.1 murine macrophage cells were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD). The cells were grown in DMEM containing 5% FCS.

Serum-mediated changes in net cholesterol mass in macrophages. J774A.1 macrophages were incubated with 20 μL/mL of the above serum samples for 20 h at 37 °C. Then the cells were washed and their lipids extracted with hexane: isopropanol (3 : 2; v : v). The hexane phase was collected and dried under N_2. The amount of cellular cholesterol was determined using a kit (CHOL, Roche Diagnostics GMbH, Mannheim, Germany). After extraction of cellular lipids, the cells were dissolved in 0.1 mol/L NaOH for measurement of cellular protein by the Lowry assay.[25]

Serum-mediated cholesterol efflux from macrophages. J774A.1 macrophage cell line was incubated with [^{3}H]-labeled cholesterol (2 μCi/mL) for 1 h at 37 °C followed by cell wash in ice-cold PBS (×3) and further incubation in the absence or presence of 20 μL/mL of the above serum samples for 3 h at 37 °C. Cellular and medium [^{3}H]-labels were quantified[30] and serum-mediated cholesterol efflux was calculated as the ratio of [^{3}H]-label in the medium/([^{3}H]-label in the medium + [^{3}H]-label in cells).

Statistics. For comparison of the mean differences between paired groups we used the Wilcoxon rank test and a p-value <0.05 was considered significant, and p-values <0.01 were considered highly significant. Results are given as mean ± standard error of the mean (SEM).

Results

In vitro studies

a. Antioxidative properties of all beverages in a serum-free system. The highest content of total polyphenols was observed in 100% pomegranate and 100% black currant juices (Table 2). All

100% pomegranate juices studied are rich in polyphenols and, POM Wonderful is the richest in total polyphenol concentration. Unlike 100% black currant (Knudsen) juice however, Currant C brand was relatively low in polyphenols (Table 2). The polyphenol contents of one acai blend (Naked) and that of the red wines were also relatively high (Table 2), whereas very low polyphenol concentrations were observed in the various other juice blends, as well as in green tea, with the lowest polyphenol content observed in green tea (Nestea brand) (Table 2).

100% Pomegranate juice showed very potent free radical scavenging capacity, decreasing the optical density of the DPPH solution by as much as 62%–76% (Table 2). 100% Black currant juice decreased the optical density by 48%, while the red wines and 100% Concord grape juice decreased it by 23%–37% (Table 2). The Tropicana blend, 100% blueberry (Knudsen), 100% Concord grape (Welch's), 100% Yumberry (Frutzo) and green tea (Lipton) exhibited very similar, limited free radical scavenging capacity, as demonstrated by only 20%–26% reduction in the DPPH optical density (Table 2). Most beverages from the "Super fruit" blends category, and green tea (Nestea) were very limited in their free radical scavenging abilities (Table 2).

All beverages, when used at a concentration of 0.25 μL/mL, inhibited copper ion-induced LDL oxidation, but the extent of inhibition was very different. 100% Pomegranate and 100% black currant juices were the most potent antioxidants (Table 2). Red wines and 100% Concord grape juices were somewhat less potent than the 100% pomegranate and 100% black currant juices (Table 2). Red wine (Mondavi) was the best one among all the tested wine brands, with a reduction in LDL oxidation of as high as 37%. Acai juice blends and some of the superfruit blends were very weak inhibitors of LDL oxidation (reductions of only 3–11%) (Table 2). Green tea (Nestea) was the weakest inhibitor of LDL oxidation, as it decreased TBARS formation by only 2% (Table 2). Many of the superfruit blends reduced TBARS by only 1%. These results indicate that all pomegranate beverages except Langers and 100% black currant juice have superior total polyphenol concentration, free radical scavenging capacity and are the most potent inhibitors of LDL oxidation.

b. Antioxidative properties of the beverages richest in polyphenols in a serum

In vitro *system.* According to the results obtained in the *in vitro* studies (Table 2), we chose the five beverages containing the highest polyphenol concentrations *i.e.*, 100% Wonderful-variety pomegranate juice (POM Wonderful), 100% black currant juice (Knudsen), red wine (Mondavi), acai juice blend (Naked) and 100% Concord grape juice (Knudsen) for further *in vitro* study. All five beverages decreased the serum susceptibility to AAPH-induced lipid peroxidation (as compared to control serum, with no beverage addition), as measured by the TBARS assay (Fig. 1A). 100% Wonderful-variety pomegranate juice, 100% black currant juice and red wine were the most potent antioxidants in this respect, with reduction rates of serum lipid peroxidation of 38%, 38% or 31%, respectively (Fig. 1A). 100% Concord grape juice decreased serum oxidation by 23%, and acai juice blend was the least potent antioxidant juice in this respect, with only 8% inhibition of AAPH-induced serum lipid peroxidation (Fig. 1A).

Paraoxonase 1 (PON1) is associated in serum with HDL, and possesses anti-atherogenic properties.[31,32] Antioxidants have been shown to preserve PON1 activity.[33] Thus, we next analyzed the effects of these selected beverages on serum PON1 catalytic activities. PON1 lactonase activity (DHC hydrolysis) was significantly increased by 48%, 51% or by 41% on serum incubation with 100% Wonderful-variety pomegranate juice, 100% black currant juice or red wine, respectively, as compared to activity in non-treated (control) serum (Fig. 1B). Grape juice or acai juice were less potent, as they increased serum PON1 lactonase activity by 35% or 29%, respectively (Fig. 1B). A similar pattern was observed upon measuring serum PON1 arylesterase activity (phenyl acetate hydrolysis, data not shown).

***In vivo* studies**

All the beverages used contain high concentrations of naturally occurring sugar (which can possibly increase serum glucose and triacylglycerol concentrations, as well as enhance serum oxidative stress). Nevertheless, they also possess potent *in vitro* antioxidative properties. Thus, we questioned the *in vivo* effects of the beverages on healthy subjects. In the present study, we analyzed the effects of the five selected polyphenol-rich beverages when consumed for a short period of time (2 h after consumption, or a daily intake for 1 week), on serum glucose and lipid profiles, on serum oxidative stress, on serum PON1 activities and on serum ability to affect cholesterol accumulation in macrophages.

a. The effects of consumption by healthy subjects of the polyphenol-rich beverages on serum biochemical parameters. None of the beverages tested (acai juice blend, 100% Concord grape juice, 100% black currant juice, 100% Wonderful-variety pomegranate juice or red wine – consumed for 2 h or for 1 week) significantly affected serum lipids (total cholesterol, HDL-cholesterol or triacylglycerol) concentration, compared to baseline (before beverage consumption, Table 3). Similarly, these beverages had no significant effects on serum glucose, sodium or potassium concentrations (Table 3).

b. The effects of beverage consumption by healthy subjects on serum oxidative stress

Measurement of TBARS levels in the serum samples (basal oxidative status) before and after 2 h or 1 week of beverage consumption, revealed that all of the five beverages used, did not significantly impact serum basal oxidative stress, which is already low in healthy subjects (Table 4). However, consumption of the five selected beverages increased serum antioxidant status, as measured by the serum concentrations of sulfhydryl (-SH) groups (Fig. 2). Two hours after consumption of 100% Wonderful-variety pomegranate juice, the concentration of serum SH groups significantly increased by 6% (Fig. 2A). After 1 week of consumption, 100% black currant juice or 100% Wonderful-variety pomegranate juice, significantly increased the concentration of serum SH groups by 11% or 8%, respectively (Fig. 2B). In contrast, consumption of all other beverages for 2 h or for one week had no statistically significant effect on the concentration of serum SH groups (Fig. 2B).

Table 2 Total polyphenol concentration and antioxidative properties of various beverages tested

Category	Product brand	Product name	Total polyphenols (mg GAE/mL)	Free radical scavenging capacity (% O.D. reduction of control[a])	$CuSO_4$-induced LDL oxidation (TBARS, % reduction of control[a])
100% Pomegranate juice	POM Wonderful	100% Pomegranate juice (Wonderful-variety)	4.80 + 0.30	76 ± 4	93 ± 9
	Knudsen	Just Pomegranate (variety unknown)	4.70 + 0.10	75 ± 2	93 ± 9
	Langers	100% Pomegranate Juice (variety unknown)	3.70 + 0.20	62 ± 3	57 ± 6
	Odwalla	Pomegranate juice (variety unknown)	4.20 + 0.20	71 ± 4	84 ± 7
	Naked	Pomegranate (variety unknown)	4.30 + 0.20	70 ± 4	92 ± 8
100% Concord grape juice	Welch's	100% Concord Grape	2.70 ± 0.20	23 ± 2	26 ± 3
	Knudsen	Concord Grape	3.60 + 0.20	32 ± 3	11 ± 2
	Lakewood	Pure Concord Grape	3.30 + 0.20	32 ± 3	15 ± 2
100% Black cherry juice	Knudsen	Just Black Cherry	2.40 + 0.10	18 ± 2	14 ± 1
	Lakewood	Pure Black Cherry	1.90 + 0.10	15 ± 1	5 ± 1
100% Black currant juice and blends	Knudsen	Just Black Currant	6.80 + 0.10	48 ± 4	94 ± 5
	Currant C	Black Currant Nectar	3.40 + 0.10	31 ± 3	11 ± 2
100% Blueberry juice	Knudsen	Just Blueberry	2.80 + 0.20	23 ± 2	8 ± 2
	Wyman's	100% Blueberry	2.60 + 0.20	15 ± 1	5 ± 1
100% Yumberry	Frutzzo	100% Yumberry	1.90 + 0.10	23 ± 2	15 ± 2
Acai juice blends	Bossa Nova	Acai Juice- The Original	1.60 + 0.18	11 ± 1	5 ± 1
	Naked	Acai Machine	4.40 + 0.40	18 ± 2	5 ± 1
	Sambazon	Original Blend	1.60 + 0.14	7 ± 1	3 ± 1
"Superfruit" blends	Naked	Red Machine	2.00 + 0.10	11 ± 2	10 ± 2
		Green machine	1.20 + 0.14	4 ± 1	4 ± 1
	Odwalla	Red Rhapsody	1.90 + 0.10	11 ± 1	5 ± 1
	Lakewood	Mangosteen/antioxidant fruit	1.10 + 0.10	10 ± 1	3 ± 1
	Born Dia	Acai Mangosteen	1.80 + 0.10	10 ± 1	1 ± 1
		Acai Blueberry	1.70 + 0.10	10 ± 1	2 ± 1
	Bossa Nova	Mangosteen Dragonfruit	1.00 + 0.06	5 ± 1	1 ± 1
	Goji Lania	Goji Cherry	1.20 + 0.16	8 ± 1	1 ± 1
		Goji Acai	0.80 + 0.03	5 ± 1	3 ± 1
		Goji Mangosteen	0.60 + 0.06	8 ± 2	1 ± 1
	Minute Maid	Pomegranate Blueberry	1.25 + 0.01	8 ± 2	8 ± 2
	Tropicana	Pomegranate Blueberry	1.50 + 0.06	26 ± 3	11 ± 2
Green tea	Nestea	Green Tea with natural flavor	0.20 + 0.02	2 ± 1	2 ± 1
	Lipton	Green Tea with citrus	0.90 + 0.02	20 ± 2	8 ± 2
Red wine	Beaulieu	BV Cabernet Sauvignon	3.70 + 0.30	29 ± 3	21 ± 3
	Mondavi	Robert Mondavi Napa Merlot	4.70 + 0.40	37 ± 4	37 ± 4
	Cline	Zinfandel (California)	3.30 + 0.20	27 ± 3	16 ± 2

[a] Control represents samples with no beverage added.

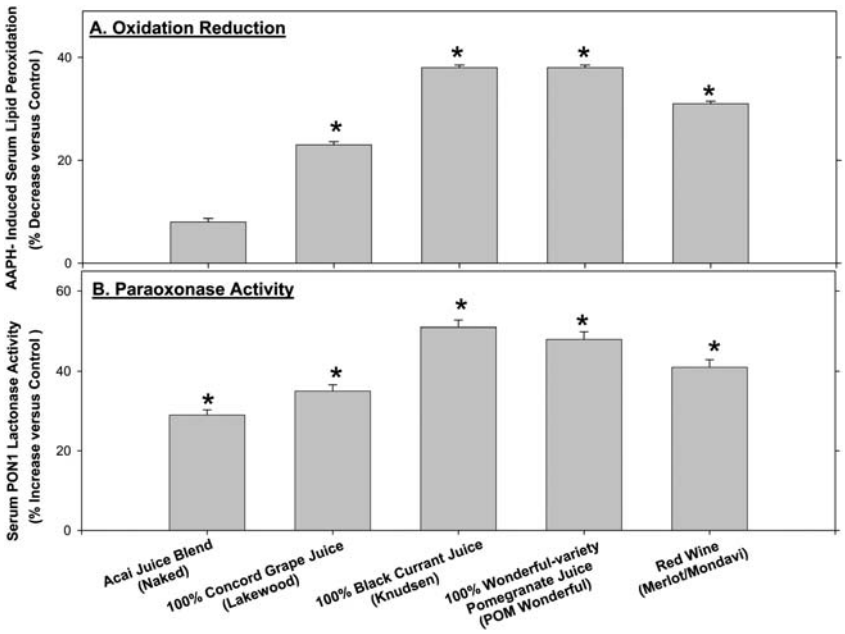

Fig. 1 The effect of polyphenol-rich beverages on *in vitro* AAPH-induced serum lipid peroxidation, and on serum PON1 lactonase activity. The serum from healthy subjects was pre-incubated for 1 h with no addition (control), or with 2 μL of the concentrated beverages [POM Wonderful, black currant (Knudsen), red wine (Mondavi), grape (Lakewood), acai (Naked)]. (A) The extent of AAPH-induced serum lipid peroxidation was determined by the TBARS assay. (B) Serum PON1 lactonase activity. Results are expressed as mean ± SEM of three different experiments. *p <0.01 *vs.* control.

c. The effects of consumption of beverages by healthy subjects on serum paraoxonase 1 (PON1) activities

PON1 has been shown to be inactivated under oxidative stress.[33] Since nutritional antioxidants increase its expression and activities,[17,33] we next analyzed the effects of the selected beverages (2 h or 1 week consumption) on serum PON1 catalytic activities (Fig. 3). Two hours after consumption of the selected beverages, serum PON1 lactonase activity was not significantly affected (Fig. 3A). After 1 week of consumption however, 100% black currant juice significantly increased serum PON1 lactonase activities by 20%, and 100% Wonderful-variety pomegranate juice increased it by 5% (Fig. 3B). The other beverages had no significant impact on PON1 lactonase activity after one week of consumption (Fig. 3B). Similar results were also found for serum PON1 arylesterase activity (data not shown).

d. The effects of beverage consumption by healthy subjects on serum-induced changes in J774A.1 macrophage cholesterol content

Serum lipoproteins can be taken up by macrophages, leading to cellular accumulation of cholesterol.[2,34] Under oxidative stress, lipoproteins undergo oxidation and the oxidized lipoproteins can be taken up by macrophages at an enhanced rate, further contributing to cellular cholesterol accumulation.[2] As 100% Wonderful-variety pomegranate and 100% black currant juices were the most potent antioxidants among all the beverages studied, we further analyzed them for their effects on serum-induced macrophage cholesterol accumulation. Serum samples collected 2 h after juice consumption did not significantly affect cellular cholesterol content, but consumption of these juices for 1 week resulted in a significant decrement (8%) in macrophage cholesterol content compared to baseline (Fig. 4A).

Finally, we questioned whether the above serum-induced decrement in macrophage cholesterol content could be associated with increased serum-mediated cholesterol efflux from cell cultured macrophages. As shown in Fig. 4B, consumption of 100% Wonderful-variety pomegranate and 100% black currant juices for 2 h or for 1 week, had no significant effect on the extent of the human serum-induced cholesterol efflux rate from the cells, compared to serum obtained at 0 time (before juice consumption).

Discussion

In the present study we have demonstrated, for the first time, that 100% Wonderful-variety pomegranate juice or 100% black currant juice, and to a lesser extent 100% Concord grape juice, or red wine, but not acai juice blend, consumed by healthy subjects in the short term (2 h, or daily consumption for 1 week) improved serum anti-atherogenic properties, *i.e.* increment in serum anti-oxidative status (SH groups, PON1 activity), and decrement in serum-induced macrophage cholesterol accumulation.

Among the 35 beverages analyzed *in vitro*, 100% Wonderful-variety pomegranate juice and 100% black currant juice were the most potent antioxidants (free radical scavenging capacity, and inhibition of copper ion-induced LDL oxidation). 100% Black currant juice, which contains the highest polyphenol concentration among all the beverages studied, inhibited LDL oxidation to the same degree as 100% pomegranate juice, but its free radical scavenging capacity was lower than that of 100% pomegranate juice, suggesting that pomegranate polyphenols are more potent antioxidants than black currant juice polyphenols. However, we

Table 3 Effect of beverages consumption by healthy subjects on serum biochemical parameters. Cho – total cholesterol, TG – triacylglycerol, HDL-C – HDL cholesterol

Serum biochemical parameter		Acai juice blend (Naked)	100% Concord grape juice (Lakewood)	100% Black currant juice (Knudsen)	100% Wonderful-variety pomegranate juice (POM Wonderful)	Red wine (Merlot/Mondavi)
Cho (mg/dL)	0	152 ± 7	159 ± 7	158 ± 6	154 ± 7	159 ± 4
	2 h	157 ± 8	157 ± 8	162 ± 7	157 ± 9	159 ± 5
	1 w	157 ± 7	157 ± 7	159 ± 7	154 ± 3	164 ± 3
TG (mg/dL)	0	71 ± 14	89 ± 17	80 ± 11	92 ± 17	86 ± 18
	2 h	67 ± 13	81 ± 12	74 ± 11	78 ± 15	89 ± 14
	1 w	71 ± 15	84 ± 10	76 ± 11	84 ± 16	84 ± 17
HDL-C (mg/dL)	0	53 ± 4	51 ± 5	54 ± 3	49 ± 4	51 ± 4
	2 h	53 ± 4	52 ± 4	55 ± 3	51 ± 4	52 ± 3
	1 w	52 ± 4	51 ± 4	53 ± 4	50 ± 3	55 ± 4
Glucose (mg/dL)	0	80 ± 2	78 ± 4	87 ± 4	86 ± 4	85 ± 3
	2 h	77 ± 2	76 ± 3	78 ± 2	75 ± 4	77 ± 2
	1 w	81 ± 2	81 ± 3	83 ± 4	85 ± 2	84 ± 3
Sodium/nmol/L	0	145.0 ± 0.5	145.0 ± 1.0	144.0 ± 1.2	146.0 ± 0.6	144.0 ± 0.7
	2 h	144.0 ± 0.4	144.0 ± 1.6	144.0 ± 1.1	145.0 ± 0.8	144.0 ± 0.7
	1 w	145.0 ± 1.2	144.0 ± 0.9	145.0 ± 1.0	145.0 ± 0.5	144.0 ± 1.4
Potassium/nmol/L	0	4.50 ± 0.10	4.80 ± 0.24	4.30 ± 0.09	4.40 ± 0.10	4.50 ± 0.11
	2 h	4.60 ± 0.10	4.60 ± 0.13	4.60 ± 0.02	4.60 ± 0.08	4.60 ± 0.14
	1 w	4.50 ± 0.07	4.50 ± 0.12	4.60 ± 0.12	4.40 ± 0.07	4.40 ± 0.04

cannot exclude the effects of other potent antioxidants in these beverages. Red wine and 100% Concord grape juice were both less potent than 100% pomegranate juice and 100% black currant juice, while acai juice blend was the weakest antioxidant among those studied *in vivo*. A previous *in vitro* comparison of the antioxidant potency of commonly consumed polyphenol-rich beverages in the United States, demonstrated the following order of antioxidant potency: 100% Wonderful-variety pomegranate juice > red wine > 100% Concord grape juice > 100% blueberry juice > 100% black cherry juice, acai juice blend, 100% cranberry juice > 100% orange juice, iced tea beverages, and 100% apple juice.[3] This pattern is similar to that shown in the present study, where additional beverages and brands were tested, including 100% black currant juice. In comparison to Knudsen Black Currant juice, Currant C (another black currant juice blend) contains only one-half of the total polyphenol concentration, and demonstrated much lower anti-oxidative properties than the Knudsen 100% black currant juice.

We chose for the *in vivo* study, the five juices which contained the highest polyphenol concentration: 100% black currant (Knudsen), 100% Wonderful-variety pomegranate (POM Wonderful), red wine (Merlot/Mondavi), acai juice blend (Naked) and 100% Concord grape (Knudsen). The present study is the first to compare the acute effects of the above beverages on serum anti-atherogenic properties. We chose 1 week of beverage consumption since we have previously observed a significant reduction in serum oxidative stress after two weeks of 100% Wonderful-variety pomegranate juice or red wine consumption by healthy subjects.[14,10] Furthermore, in the current study we evaluated the acute effects 2 h after beverage consumption. Polyphenols in the above beverages were probably absorbed to some extent, as the levels of urinary quercetin,[19] or anthocyanins,[20,35] or those of plasma catechin,[18] were all shown to be significantly increased dose- and time-dependently after acai juice blend, 100% black currant juice or red wine consumption.

The bioavailability of the pomegranate active components and metabolites has been demonstrated previously, with ellagic acid detected in the blood and dimethylellagic acid glucuronide (DMEAG), as well as urolithin A and B, found in the urine of most subjects after consumption of 100% pomegranate juice.[36–39]

An important issue in the current study is that consumption of the beverages (which contain sugars) by healthy subjects for 2 h or for 1 week, did not increase serum glucose levels and did not affect serum lipids or electrolytes (potassium and sodium).

Serum PON1 activities (arylesterase and lactonase) were analyzed as an additional marker for oxidative stress. *In vitro* all the five beverages used significantly increased serum PON1 activities. The mechanism responsible for the PON1 activity increase could be related to the binding of the beverages' polyphenols to PON1. Such binding may change the enzyme conformation, thus affecting PON1 active site interactions with its substrates. Furthermore, certain polyphenols increase PON1 binding to the HDL, thus stabilizing PON1, as was shown previously upon incubation of serum *in vitro* with pomegranate juice or its most potent unique polyphenol punicalagin.[15,16] Furthermore, 100% Wonderful-variety pomegranate juice consumption over longer periods of time (weeks, months, or years) has been shown to increase PON1 binding to the HDL particles, and as a result, stabilize PON1 and enhance its catalytic

Table 4 Effect of polyphenol-rich beverages' consumption by healthy subjects on basal serum oxidation status

Beverage	TBARS/nmol/mL before beverage consumption	TBARS/nmol/mL 2 h after beverage consumption	TBARS/nmol/mL after 1 week of beverage consumption
Acai juice blend (Naked)	1.50 ± 0.16	1.60 ± 0.18	1.60 ± 0.07
100% Concord grape juice (Lakewood)	1.60 ± 0.07	1.70 ± 0.11	1.50 ± 0.09
100% Black currant juice (Knudsen)	1.60 ± 0.09	1.70 ± 0.11	1.60 ± 0.12
100% Wonderful-variety pomegranate juice (POM Wonderful)	1.50 ± 0.18	1.50 ± 0.12	1.50 ± 0.08
Red wine (Merlot/Mondavi)	1.60 ± 0.07	1.60 ± 0.08	1.60 ± 0.11

and biological activities.[12,13,15,16] We evaluated also serum sulfhydryl (SH) group concentrations, as they have been shown to be inversely related to oxidative stress, and positively correlated with PON1 activity.[40] Although all five selected polyphenol-rich juices inhibited AAPH-induced serum lipid peroxidation *in vitro*, *in vivo* their consumption for a short period of time (2 h or 1 week) did not significantly affect AAPH-induced serum lipid peroxidation. Serum SH group concentration and PON1 activity however, were modestly, but significantly increased following beverage consumption, with 100% Wonderful-variety pomegranate and 100% black currant juices being the most potent beverages after 1 week of consumption. Nevertheless, this protection by SH groups and PON1 may be insufficient to protect the serum from free radical-induced oxidative stress. PON1 was shown to protect serum from oxidative stress, by its ability to hydrolyze specific oxidized lipids in lipoproteins (such as specific oxidized phospholipids, lipid peroxides, cholesteryl linoleate hydroperoxides).[41–43] Longer periods of beverage consumption (up to months) may be needed to achieve more impressive effects on serum oxidative stress in healthy subjects. Among the *in vivo* studied beverages, acai juice blend was the least potent, even though it has a similar concentration of polyphenols. This suggests that acai juice blend polyphenols are weak antioxidants, in comparison to the polyphenols in 100% pomegranate juice, 100% black currant juice or 100% Concord grape juice (and also red wine). Similar to the present study, plasma antioxidant capacity increased twelve hours after consumption of acai juice blend,[20] or 2 h post-consumption of a mixture of berries, by healthy subjects.[21] In a recent study, 100% black currant juice consumption for 1 week, substantially decreased serum lipid peroxidation,[19] but this impressive effect is likely related to the high volume of juice consumed (1500 mL).

As 100% Wonderful-variety pomegranate juice and 100% black currant juice were the most potent antioxidants in the

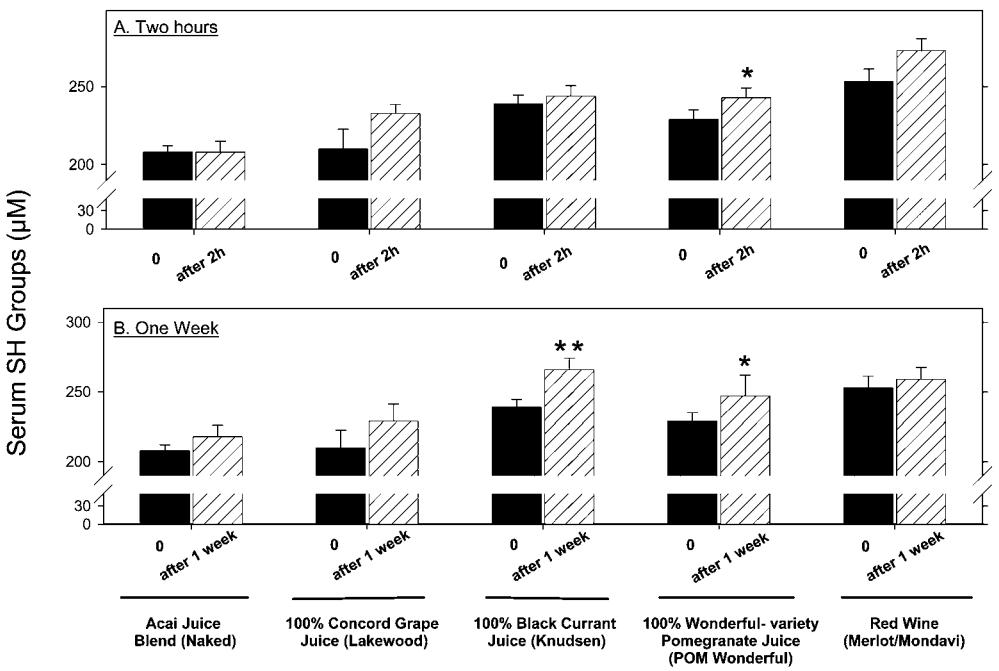

Fig. 2 The effect of polyphenol rich beverages consumed by healthy subjects on the concentration of their serum SH groups. Six healthy subjects consumed POM Wonderful, black currant (Knudsen), red wine (Mondavi), grape (Lakewood), and acai (Naked). Blood samples were collected before and after 2 h or 1 week of beverage consumption. Serum SH group concentrations were determined as described under the methods section. (A) 2 h after consumption of beverages. (B) 1 week after of consumption of beverages. Results are expressed as mean ± SEM. *p <0.05 *vs.* 0 time, **p <0.01 *vs.* 0 time.

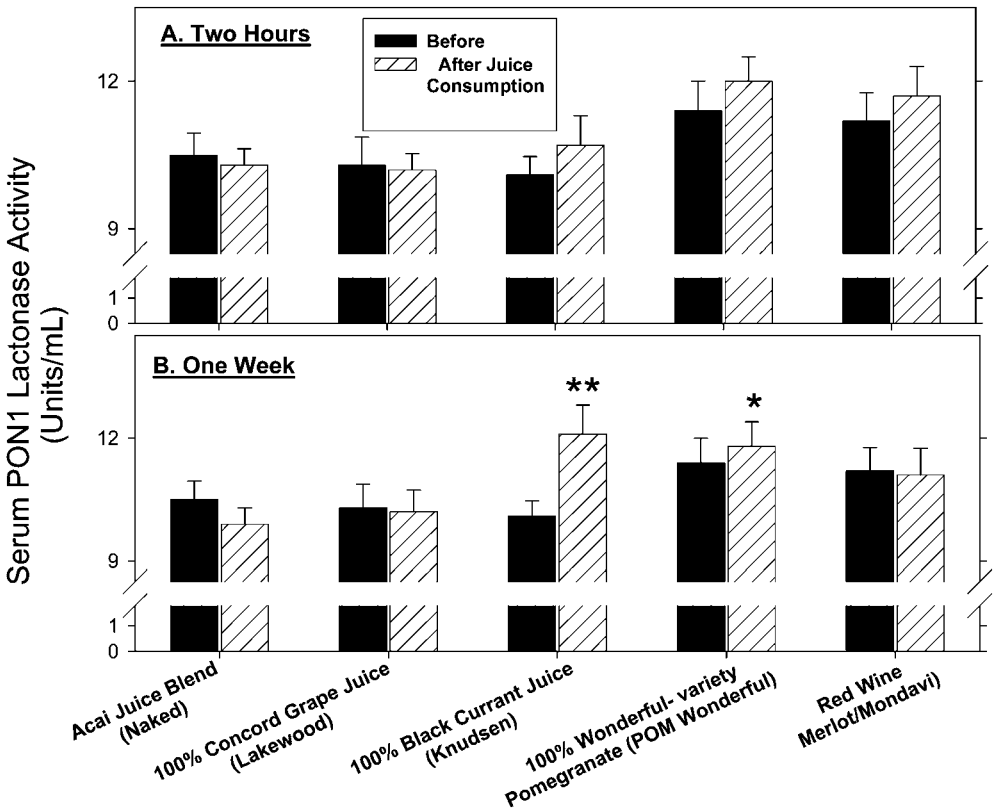

Fig. 3 The effects of consumption of polyphenol-rich beverages by healthy subjects on their serum paraoxonase 1 (PON1) catalytic activity. Six healthy subjects consumed POM Wonderful, black currant (Knudsen), red wine (Mondavi), grape (Lakewood), and acai (Naked). Blood samples were collected before and after 2 h or 1 week of beverage consumption. Serum PON1 lactonase activity was determined. (A) 2 h after beverage consumption. (B) 1 week after beverage consumption. Results are expressed as mean ± SEM. *p <0.05 *vs.* 0 time, **p <0.01 *vs.* 0 time.

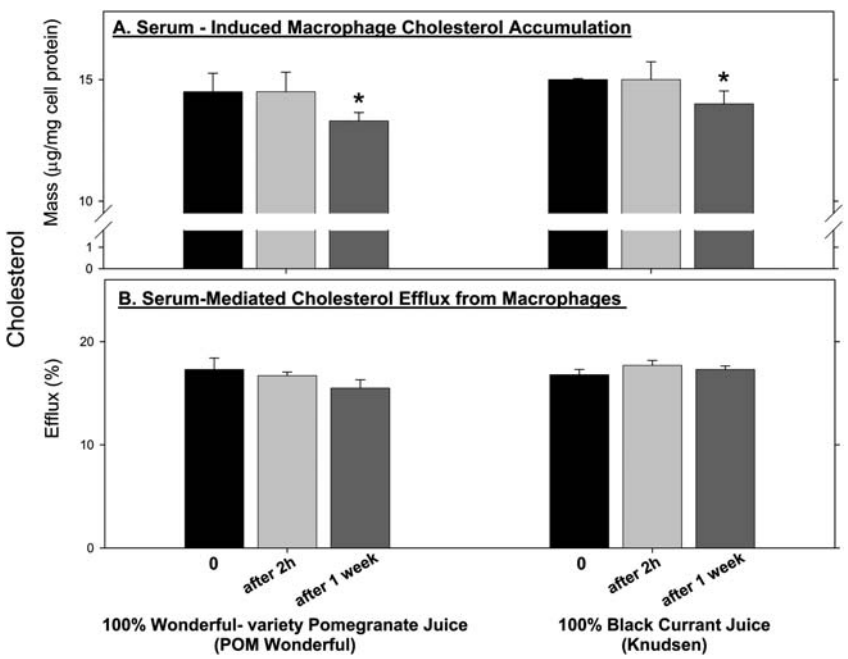

Fig. 4 The effect of pomegranate juice and black currant juice consumption by healthy subjects on serum-induced cholesterol accumulation in J774A.1 macrophages. J774A.1 macrophages were incubated with 20 µL/mL of the above serum samples for 20 h at 37 °C. (A) The cellular total cholesterol content was then determined in the cells' lipid extract. (B) The extent of serum-mediated cholesterol efflux from the cells was determined as described in the Methods section. Results are expressed as mean ± SEM. *p <0.05 *vs.* 0 time.

in vitro and in the *in vivo* studies, we chose them to assess an important feature of atherogenesis, *i.e.*, macrophage cholesterol accumulation and foam cell formation, the hallmark of early atherogenesis.[1,2] Consumption of 100% Wonderful-variety pomegranate juice or 100% black currant juice for 1 week, modestly, but significantly, decreased serum-induced cholesterol accumulation in macrophages. The reduction in macrophage cholesterol mass, however, was not the result of increased cholesterol efflux from the cells. In fact, it is probably the result of inhibition of cholesterol-rich lipoprotein uptake by the cells, mediated by serum associated polyphenols and/or polyphenol metabolites.[44,45]

In conclusion, we showed in the present study that 100% Wonderful-variety pomegranate juice and 100% black currant juice have potent antioxidant properties, both *in vitro* and *in vivo*.

These findings were observed even after short periods of daily juice consumption (two hours, or for one week), though the antioxidant and anti-atherogenic effects were modest, in comparison to previous studies conducted over longer periods of juice consumption.[12–15]

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