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Phenolic Antioxidants and Antiatherogenic Effects of Marula (*Sclerocarrya birrea* Subsp. *caffra*) Fruit Juice in Healthy Humans

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Antioxidant activity and composition of Israeli-grown marula (*Sclerocarrya birrea* subsp. *caffra*) fruit juice and health-promoting aspects of juice consumption on serum lipids and lipoproteins pattern in healthy volunteers were studied. Marula juice was found to contain high vitamin C and potassium levels and low sugar concentration (267 mg dL^{-1}, 328 mg dL^{-1}, and 7.3 g dL^{-1}, respectively). The juice contains a significant level of phenolics (56 mg of pyrogallol equiv dL^{-1}) and was found to be a potent antioxidant (382 mg of vitamin C equiv dL^{-1}). The antioxidant activity was resistant to pasteurization regimens and long-term freezing and slowly decreased during refrigeration, losing up to 14% of its capacity after 4 weeks. Three-week administration of the juice as a food supplement to healthy subjects significantly reduced their serum total cholesterol (by 8%), LDL-cholesterol concentration (by 17%), and triglyceride level (by 7%), increased their serum HDL-cholesterol level (by 10%), and attenuated serum oxidative stress. Upon a 4 week "washout" period, most of these parameters returned toward baseline values. Separation of the juice soluble phenolics by HPLC produced potent antioxidant fractions, tentatively containing hydrolyzable tannins, catechins, and hydroxycinnamic acid derivatives, which could be responsible for the observed protection against atherosclerosis risk factors following marula fruit juice consumption.

KEYWORDS: Marula; *Sclerocarrya birrea* subsp. *caffra*; antioxidants; radical scavenging; atherosclerosis; lipids; lipoproteins; triglycerides; cholesterol; phenolics

INTRODUCTION

The marula tree (*Sclerocarrya birrea* subsp. *caffra*) is native to sub-Saharan Africa. The tree bears edible aromatic and fleshy fruits that have been an important component in the local population diet since ancient times (*1*). The fruit is eaten fresh or processed into alcoholic and nonalcoholic beverages or jam. Local communities have also used marula fruit in ethnomedical and cultural practices (*2*−*4*). The tree was imported to Israel and successfully domesticated (*5, 6*). Several marula plantations exist now in the Israeli Negev and southern Arava Valley made up of selected plantlets of screened lines that were grafted in the Institute of Applied Sciences at Ben-Gurion University, Beer Sheva, Israel (*6, 7*). These lines differ in fruit ripening dates (August through January) and attributes—weight, juice content and quality, and seed size.

The ripened marula fruit has a pale yellow leather-like rind and succulent white pulp. The pulp is exceptionally rich with vitamin C (*8*−*11*) and exhibits high antioxidative capacity, as demonstrated by 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical formation inhibition (*12*), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing power, superoxide anion radical scavenging activity, and inhibition of phospholipid peroxidation (*13*). The presence of polyphenolic compounds in the fruit juice was proposed on the basis of polyphenol oxidase activity measurements (*12, 14*). Ndhlala et al. (*15*) reported on the high content of total polyphenolics, flavonoids, and condensed tannins in marula fruit pulp; however, the values should be taken with caution as no correction was made for the substantial interference of the endogenous vitamin C with the assays used in the study. The authors also reported on high-performance liquid chromatography (HPLC) analysis of methanolic extracts of the pulp and the detection of caffeic, ferulic, and *p*-coumaric acids.

In recent years, extensive evidence from epidemiological studies, animal research, clinical trials, and research in nutritional biochemistry indicated that fruit and vegetable consumption improves oxidative-stress related chronic and degenerative diseases (*16*−*21*). Extracts from certain fruits and vegetables

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may be beneficial against coronary heart disease, atherosclerosis, cancer, diabetes, and Parkinson's and Alzheimer's diseases. The beneficial effects were attributed to high contents of flavonoids, the polyphenolic compounds that, besides having high antioxidant and free radical scavenging activities, appear to regulate signaling pathways involved in cellular survival, growth, and differentiation (*22*). Thus, diets with a high content of such phenolic-rich antioxidants emerge as a promising approach to help strengthen the physiological antioxidant defense system and to improve chronic diseases. The marula fruit juice, with its high antioxidative capacity, is a potential candidate for this approach.

Atherosclerosis, the leading cause of morbidity and mortality among people with a western life style, develops as a result of various risk factors. Hypercholesterolemia is a major risk factor for atherosclerosis, and reduction in plasma low-density lipoproteins (LDL) cholesterol concentration by drug therapy reduces cardiovascular incidence (*23*). Oxidative modification of LDL is thought to play a key role in the pathogenesis of atherosclerosis. Oxidized LDL is a major contributor to the development of atherosclerotic lesion, because it stimulates macrophage cholesterol accumulation and foam cell formation (*24*−*28*). In contrast to the atherogenicity of LDL, serum high-density lipoprotein (HDL) levels are inversely related to the risk of atherosclerosis. HDL exerts an inhibitory effect on LDL oxidation, and this effect may be related to its associated enzyme paraoxonase 1 (PON1) (*29*−*31*).

Fruit and vegetable extracts of high antioxidative capacity and unique polyphenolic composition were reported to be beneficial in atherosclerosis prevention and attenuation (*18*−*21*). The current study explored antioxidant composition and activity aspects of Israeli-grown marula fruit juice and the antiatherogenic effects of the juice when administered to healthy subjects as a food supplement. The initial characterization of distinct fractions of marula fruit juice, containing phenolic compounds with prominent antioxidant activity, is also described.

MATERIALS AND METHODS

Plant Material and Processing Procedures. *Plant Material.* Ripe fruit of a variety of marula cultivars, differing in fruit properties and ripening dates, were used in the study. Fruit was collected from several sites in the Israeli Negev and southern Arava Valley on different harvest dates from August 2005 through January 2007.

Juice Extraction and Storage. Marula fruits were squeezed by a hydraulic press under 35 bar (Enorossi olive press, model 250). Juice was collected and stored frozen at −20 °C until further use.

Juice Pasteurization and Bottling. Extracts from eight preparation dates (August 30, 2005; September 2, 2005; October 17, 2005; August 16, 2006; August 30, 2006; November 5, 2006; November 20, 2006; and January 2, 2007) were thawed, combined, and thoroughly mixed on January 14, 2007. The homogenized juice was pasteurized at 72 °C for 30 s at the facilities of Yotvata Dairy Ltd. (Kibbutz Yotvata, Israel) using a locally constructed semi-industrial pasteurizer.

Aliquots of 200 mL were transferred under aseptic conditions to sterile 250 mL plastic screw-cap bottles and kept refrigerated throughout the preclinical study.

Juice Concentration. Three-fold concentration was achieved by placing juice-containing aluminum trays in a vacuum oven (Tuttnauer, oven dry sterilizer model 11-900), set at 57 °C and −760 mm mercury for 24 h.

Chemical Analysis. *Minerals.* Sodium and potassium were determined by a flame photometer (JENWAY, PFP7); calcium, magnesium, iron, zinc, and manganese were analyzed by an atomic absorption spectrometer (Perkin-Elmer, 3100); chloride ions were measured with a chloride analyzer (Sherwood, 926).

Total Soluble Solids (TSS). TSS (in percent) was measured with a refractometer (Atago, ATC-1E; Brix 0−32%).

pH and Titratable Acidity (TA). The pH was measured using a specialized food electrode (pH 211 microprocessor pH-meter and FC 200B food electrode, Hanna Instruments). TA (in mequiv of acid dL^{-1}) was measured colorimetrically by titration with 0.1 N NaOH using the pH indicator phenolphthalein.

Protein Content. Protein concentration was measured spectrophotometrically (Shimadzu, UV-1650PC) by the Coomassie Blue binding assay (*32*) using γ-globulin (Bio-Rad) as standard.

Total Soluble Phenolic Content. Marula juice was extracted (1:3) with 80% methanol supplemented with 2 mM NaF and centrifuged (10000 rpm for 10 min at 4 °C, Sorvall Instruments RC5C), and the supernatant was diluted 10−20-fold with double-distilled water (DDW). Concentration of total soluble phenolics was measured colorimetrically with Folin Ciocalteu 2 N phenol reagent (Sigma Chemical Co.) according to the method of Singleton and Rosssi (*33*). Aliquots of 100 μL were added to 900 μL of reaction solution consisting of 200 μL of freshly prepared 10-fold diluted Folin−Ciocalteu reagent, 100 μL of Na_2CO_3, and 600 μL of DDW. Pyrogallol (Sigma Chemical Co.) was used for the calibration curve (0−100 μg mL^{-1}). The absorbance at 765 nm was measured after 1 h of incubation, and the results were expressed in pyrogallol equivalents. It should be noted that the same assay was used when the phenolic content was measured in fractions collected during HPLC runs, with the exception that the fraction aliquots were added to the reaction solution without prior dilution. Also, in whole juice, the contribution of the juice's vitamin C to the measurement was subtracted to derive the phenolic concentration.

Sugar Analysis. Marula juice was extracted with 80% ethanol. The suspension was centrifuged as described above and the supernatant filtered; the clear filtrate was dried under a stream of nitrogen and dissolved in DDW. The individual sugars were separated by HPLC using a Merk Hitachi LaChrom system composed of pump L7100, column oven L7350 (set at 90 °C), and manual injector Rheodyne and equipped with a cation exchange column and precolumn (Merck, Polysphere CH CA) and a refractive index (RI) detector (Merck, LaChrom, L-7490). The mobile phase consisted of column-filtered water further distilled by a Corning Megapure System, MP-6A, and passed through a 0.20 μm nylon membrane. Highly pure sucrose, glucose, and fructose (HPLC grade, Sigma Chemical Co.) served as standards.

Vitamin C and Soluble Phenolic Compound Analysis. Marula juice was extracted (1:3) with 80% methanol supplemented with 2 mM NaF. The suspension was centrifuged as described above and the supernatant filtered through a 0.45 μm filter before injection. Samples of 20 μL were analyzed using the LaChrom Merck Hitachi HPLC system, consisting of pump L7100, column oven L7350 (set at 28 °C), mixer−degasser L-7614, and manual injector Rheodyne, coupled with multiwavelengh detector (Jasco MD-2010 Plus), interface (Jasco LC-Net II/ADC), and scientific software (EZChrom Elite Client/Server version 3.1.6 build 3.1.6.2433). An end-capped PurospherStar RP-18 column (250 × 4 mm LichroCART cartridge, 5 μm particle size) with an end-capped Lichrospher100 RP-18 guard column (4 × 4 mm LichroCART cartridge, 5 μm particle size) was used. Column ending was connected to a fraction collector (Pharmacia Fine Chemicals, FRAC-100). The mobile phases consisted of (A) phosphoric acid (0.1%), pH 2.4, and (B) methanol; the elution gradient was set to go from 0 to 100% methanol in 30 min. The flow rate was 0.6 mL min^{-1}. Vitamin C concentration was evaluated from the area under the corresponding chromatogram peaks using HPLC-grade vitamin C (Fluka, Buchs, Switzerland) for calibration. Methanol was of HPLC grade (LiChrosolv Merck); water was purified and filtered as described earlier; phosphoric acid (Frutarom) and NaF (Sigma) were of analytical grade. A phenolics standard library was constructed using catechin and chlorogenic, caffeic, and tannic acids from Sigma and 2-hydroxybenzoic (salicylic) acid, quercetin-3-β-glucoside, and ellagic acid from Fluka.

Antioxidative Capacity Evaluation. Three methods were used to examine the antioxidant activity of marula fruit juice, including ferric reducing power, free radical scavenging capacity, and inhibition of LDL oxidation.

The ferric reducing power of marula fruit juice was measured by the colorimetric test originally developed to assess the ferric reducing antioxidant power (FRAP) of plasma (*34*). Clear methanolic extract was prepared as described earlier and diluted 10−20-fold with DDW.

Fifty microliters was added to 950 μL of freshly prepared FRAP working solution [50 mL of 300 mM acetate buffer + 5 mL of 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ) + 5 mL of 20 mM ferric chloride] in a 37 °C water bath. Absorbance at 593 nm was measured after 4 min. Vitamin C (Fluka) was used for the calibration curve (0−100 μg mL^{-1}), and the results were expressed in terms of vitamin C equivalents.

The free radical scavenging capacity of marula juice was analyzed by the DPPH assay. DPPH 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 nonradical form is produced. Aliquots from the analyzed juice were mixed with 1 mL of 0.1 mM DPPH in ethanol, and the change in optical density (OD) at 517 nm was continuously monitored (*35*).

LDL Oxidation. *LDL Preparation.* LDL was isolated from plasma derived from healthy normolipidemic volunteers, by discontinuous density gradient ultracentrifugation. The LDL was washed at $d = 1.063$ g mL^{-1} and dialyzed against 150 mM NaCl, 1 mM 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. Prior to oxidation, LDL was dialyzed against EDTA-free, phosphate-buffered saline (PBS) solution, pH 7.4, at 4 °C (*36*).

LDL Oxidation. LDL (100 μg of protein mL^{-1}) was incubated for 10 min at room temperature with the indicated concentrations of marula juice. Then, 5 μM $CuSO_4$ was added, and the tubes were incubated for 2 h at 37 °C. At the end of the incubation, the extent of LDL oxidation was determined by measuring the generated amounts of lipid peroxides and thiobarbituric acid reactive substances (TBARS) using spectrophotometric methods (*37*). The lipid peroxide test analyzes lipid peroxide formation by their capacity to convert iodide to iodine after incubation for 18 h at 25 °C, as measured spectrophotometrically at 365 nm (*38*).

Preclinical Study Protocol. Ten healthy volunteers, nonsmokers, and with no metabolic disorders, with plasma cholesterol levels of <200 mg dL^{-1} and under no drug treatment, were recruited from the Faculty of Medicine students and staff and included in the study. All subjects signed a consent form before entering the study. The study protocol was approved by the Rambam Helsinki Committee (No. 2452).

The participants consumed 200 mL (1 glass) of marula juice per day, with their main meal, for a period of 3 weeks. All subjects included in the study continued with their habitual lifestyle and were advised to refrain from polyphenol-rich food other than the marula juice during the entire period of the study.

Blood pressure was measured at time zero (before study entry), after 3 weeks, and at the end of the study (after 4 weeks of washout).

Blood samples (25 mL) were collected for analyses at time zero (baseline, before study entry), after 3 weeks of marula juice consumption, and 4 weeks after the end of juice consumption (washout).

Specific Methods. All serum samples were frozen at −80 °C until analyses of all of them at one time. Serum samples were analyzed in the Lipid Research Laboratory at the Technion Faculty of Medicine, Haifa, for the following:

Biochemical analyses in serum were performed by commercial diagnostic kits and instrumentation. Glucose was measured using an automated enzymatic UV hexokinase test (Olympus, Medtechnica, Israel); calcium and function parameters of the kidney [blood urea nitrogen (BUN), creatinine, and the electrolytes natrium and kalium], and liver [creatine kinase (CK), aspartate aminotransferase (AST), and total bilirubin] were measured with a commercial analyzer (Cobas Mira, Roche Diagnostics Inc., Mannheim, Germany).

Serum total cholesterol, HDL-cholesterol, and total triglycerides were measured using automated enzymatic color tests (Olympus, Medtechnica). The values were used to calculate the content of LDL-cholesterol.

Serum samples were also subjected to oxidative stress analyses: uric acid levels (as a possible antioxidant) in serum were measured with a commercial analyzer (Cobas Mira, Roche Diagnostics Inc.).

Table 1. Chemical and Antioxidant Aspects of Israeli-Grown Marula Fruit Juice

component	units	content[a]
water content	g dL^{-1}	85.1 ± 1.2
TSS	%	13.4 ± 0.9
DW	g dL^{-1}	14.9 ± 0.9
organic matter	g dL^{-1}	13.9 ± 0.6
ash	g dL^{-1}	1.01 ± 0.03
pH		3.7 ± 0.3
TA	mequiv dL^{-1}	19 ± 3
sucrose	g dL^{-1}	6.2 ± 0.8
glucose	g dL^{-1}	0.5 ± 0.3
fructose	g dL^{-1}	0.6 ± 0.4
protein	g dL^{-1}	0.32 ± 0.04
nutritional fiber[b]	g dL^{-1}	0.70 ± 0.12
vitamin C	mg dL^{-1}	267 ± 72
soluble phenolics	pyrogallol equiv, mg dL^{-1}	56 ± 13
ferric reducing power	vitamin C equiv, mg dL^{-1}	382 ± 118
K	mg dL^{-1}	328 ± 11
Na	mg dL^{-1}	10 ± 2
Ca	mg dL^{-1}	40 ± 6
Mg	mg dL^{-1}	44 ± 4
Fe	mg dL^{-1}	0.71 ± 0.08
Zn	mg dL^{-1}	0.19 ± 0.02
Mn	mg dL^{-1}	0.05 ± 0.01

[a] Mean ± SD of measurements in juice from eight harvests. [b] Analyzed by Bactochem laboratory, Ness Ziona, Israel.

For the ferric reducing antioxidant power (FRAP) assay in serum, working FRAP reagent was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution. Aqueous solutions of $FeSO_4 \cdot 7H_2O$ at concentrations of 5, 10, 20, 30, 40, 50, and 100 μM were used for a standard calibration curve. FRAP reagent (freshly prepared) was warmed to 37 °C, and a reagent blank was read at 593 nm spectrophotometrically. Serum sample (30 μL) was mixed with 90 μL of water. Then, 900 μL of FRAP reagent was added and mixed quickly. Absorbance was read after 0.5 s and every 15 s during 4 min. The change in OD (OD_{593nm}) between the final and the initial values was calculated for each sample and was then related to Fe^{2+} concentrations in the standard curve (tested in parallel) (*34*).

For serum lipid peroxidation, serum was diluted 1:4 (v/v) with PBS and then incubated in the absence or presence of 100 mM 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH, free radical generator) for 2 h at 37 °C. Serum lipid peroxidation was determined by measuring the generated amounts of TBARS and lipid peroxides using spectrophotometric methods as described above.

Statistics. Statistical analysis was performed using Student's *t* test. Results are given as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Antioxidant Properties and Stability Aspects of Marula Fruit Juice. Nutritional and antioxidant parameters of Israeli-grown marula fruit juice were studied in fruits from different ripening dates and cultivation sites. The content of selected components and juice ferric reducing power in freshly extracted juice from eight harvests during the period from August 2005 to January 2007 are presented in **Table 1**.

The juice was found to contain high concentrations of vitamin C and phenolic compounds, comparable to levels previously reported for marula fruit pulp (*8−11, 13, 15*). The remarkably high ferric reducing capacity of the marula juice (22 ± 7 mM vitamin C equiv) was somewhat below that measured in pomegranate whole fruit juice and considerably greater than the levels in red wine and green tea (*39*).

The study employed Israeli-grown marula fruit from plantations established from selected plantlets of screened lines differing greatly in ripening dates and fruit properties (*5−7, 11*). The substantial standard deviations measured for the levels of

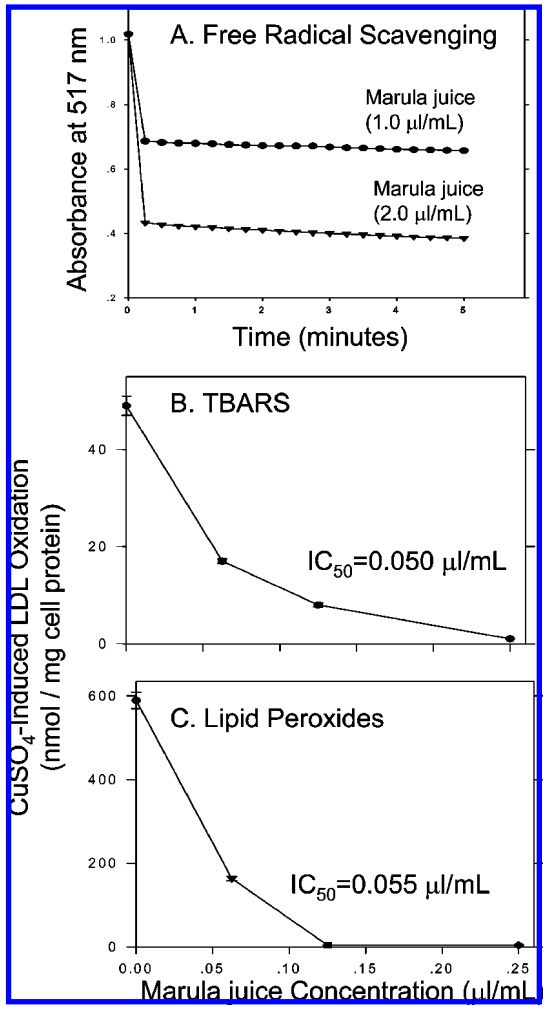

Figure 1. Free radical scavenging capacity (**A**) and LDL oxidation inhibition (**B** and **C**) by marula juice: concentration study.

vitamin C, soluble phenolics, and ferric reducing power reflect the large variations in marula fruit properties from different cultivars and harvest dates (*11*). The lowest values were measured in fruit harvested in August, whereas the highest values were measured in fruit harvested in January (approximately 200 and 600 mg of vitamin C equiv dL^{-1}, respectively).

Marula juice exhibited a remarkable capacity to scavenge free radicals (**Figure 1A**). One microliter per milliliter of marula juice induced a rapid decline in the OD of the DPPH solution (within 20 s), by 35%, and 2 μL mL^{-1} of marula juice resulted in a 62% decline in the OD of the DPPH solution.

The capacity of marula juice to inhibit LDL oxidation was next analyzed (**Figure 1B**,**C**). The juice inhibited copper ion-induced LDL oxidation with IC_{50} (the concentration needed to inhibit LDL oxidation by 50%) values of 0.050 and 0.055 μL mL^{-1} for the formation of TBARS (**Figure 1B**) and lipid peroxides (**Figure 1C**), respectively.

The high ferric reducing ability, free radical scavenging activity, and capacity to efficiently inhibit LDL oxidation of the marula fruit juice most likely originated from the juice soluble phenolic compounds and vitamin C. The exceptionally high antioxidative capacity suggests that marula fruit juice has a potential health value. The relatively low sugar and high potassium content of marula juice (**Table 1**) further add to the juice health benefits.

Before putting the health-promoting value of marula fruit juice tothe in vivo test in humans, it was necessary to address juice tolerance to customary processing conducts. Marula juice was subjected to a variety of simulated pasteurization processes (**Table 2**), as well as to long-term storage at −18 °C (**Table 3**). Juice properties associated with organoleptic traits (TSS, TA, and pH, **Table 2**) and antioxidative capacity (ferric reducing ability, **Tables 2** and **3**) exhibited high stability under the employed conditions. Thus, pasteurized and long-term frozen-stored juice carry antioxidant benefits similar to those exhibited by the freshly extracted juice, thereby, further enhancing its value as a food product.

Table 2. Stability of Marula Juice Chemical Parameters under Simulated Pasteurization Conditions[a]

treatment	TSS (%)	pH	TA (mequiv dL^{-1})	ferric reducing ability (vitamin C equiv, mg dL^{-1})
control	12.5 ± 0.1	3.5 ± 0.1	16 ± 3	342 ± 21
30 s at 72 °C	12.5 ± 0.1	3.4 ± 0.1	16 ± 2	341 ± 16
30 min at 63 °C	12.4 ± 0.1	3.5 ± 0.1	16 ± 3	347 ± 19
60 min at 55 °C	12.5 ± 0.1	3.5 ± 0.1	16 ± 4	369 ± 11

[a] Values are the mean ± SD of four replicates.

Table 3. Reducing Ability of Marula Fruit Juice Prior to and Following Long-Term Frozen Storage at −18°C[a]

	ferric reducing ability (vitamin C equiv, mg dL^{-1})	
storage duration (months)	fresh juice	after storage
6	412 ± 21	402 ± 15
18	387 ± 28	393 ± 27

[a] Values are the mean ± SD of three replicates.

Table 4. Marula Juice Quality from Pasteurization Date Throughout the Consumption Phase of the Preclinical Study[a]

treatment	refrigeration duration (weeks)	TSS (%)	pH	TA (mequiv dL^{-1})	Fe^{3+} reducing capacity (vitamin C equiv, mg dL^{-1})
fresh		12.6 ± 0.2	3.4 ± 0.0	21 ± 1	306 ± 32
pasteurized		12.8 ± 0.0	3.4 ± 0.1	21 ± 0	315 ± 23
pasteurized	1	12.9 ± 0.1	3.6 ± 0.1	21 ± 0	311 ± 12
pasteurized	2	12.9 ± 0.1	3.6 ± 0.0	21 ± 0	282 ± 27
pasteurized	3	12.9 ± 0.0	3.7 ± 0.0	21 ± 0	240 ± 33
pasteurized	4	12.8 ± 0.0	3.6 ± 0.0	21 ± 0	268 ± 37

[a] Values are the mean ± SD of measurements on three bottles.

Dietary Supplementation of Marula Juice to Healthy Subjects: Effect on Plasma Lipid and Lipoprotein Pattern, on Serum Oxidative Stress, and on Serum Atherogenicity. Fruit and vegetable extracts of high antioxidative capacity and unique polyphenolic composition were reported to be beneficial in atherosclerosis prevention and attenuation (*18*−*21*). The antioxidant qualities of the marula fruit juice indicate that it may have favorable effects on major risk factors of atherosclerosis. A study was conducted to examine in detail possible antiatherogenic effects of marula juice administered as a food supplement to healthy subjects.

The preclinical study consisted of two phases: 3 weeks of juice consumption and a 4 week "washout" period. Juice parameters relating to organoleptic (TSS, pH, TA) and antioxidant (ferric-reducing capacity) qualities were monitored weekly throughout the consumption phase of the study (**Table 4**). The results indicate that juice characteristics were significantly preserved during pasteurization and throughout the consumption period. A slow decline in antioxidative capacity was measured after the first 2 weeks of refrigeration; however, loss of activity was only 14% by the end of juice consumption phase. The slow decrease in antioxidant activity is likely to reflect the slow

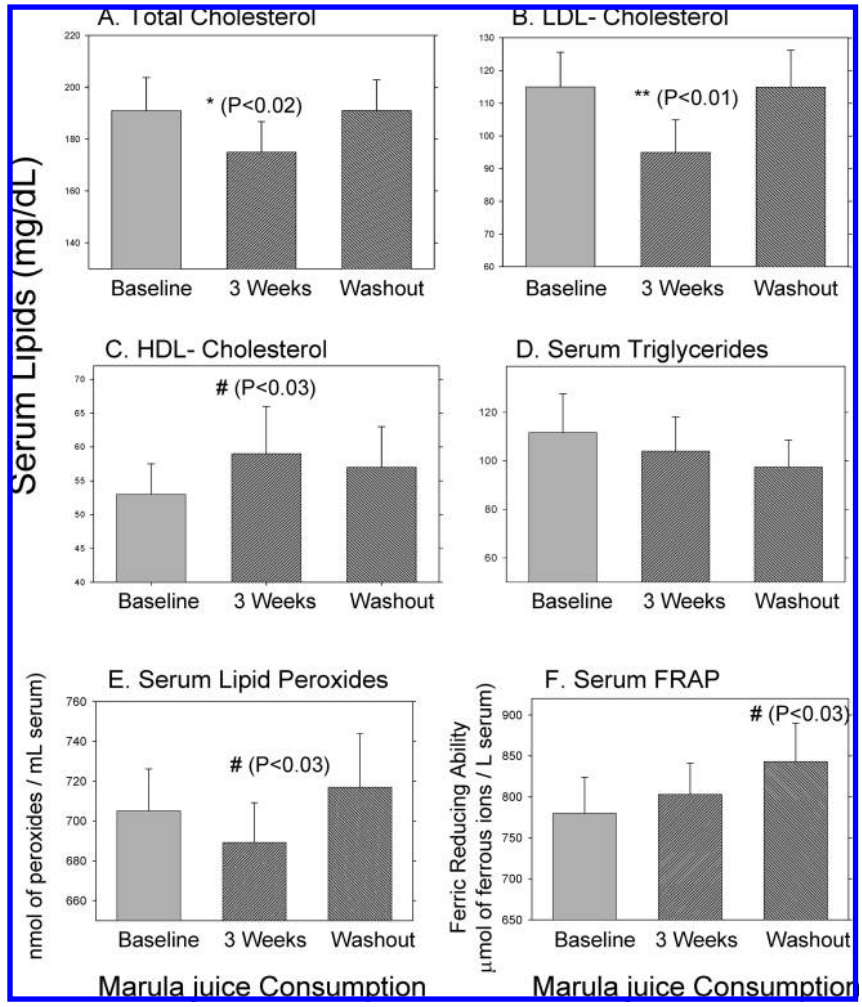

Figure 2. Effect of marula juice consumption on serum total cholesterol (**A**), LDL-cholesterol (**B**), HDL-cholesterol (**C**), and triglyceride (**D**) concentrations and on serum oxidative stress (**E** and **F**).

Table 5. Effect of Marula Juice Consumption on Blood Pressure, Serum Glucose and Calcium Levels, Kidney Function (BUN, Creatinine, and the Electrolytes Natrium and Kalium), and Liver Function (CK, AST, and Total Bilirubin)[a]

	study progress		
physiological parameter	baseline	3 weeks	washout
diastolic blood pressure	80 ± 11	78 ± 12	81 ± 12
systolic blood pressure	126 ± 12	130 ± 15	129 ± 16
serum glucose	88 ± 10	89 ± 8	73 ± 21
serum calcium	9.3 ± 0.4	9.8 ± 0.6	8.7 ± 0.7
BUN	14 ± 4	14 ± 4	13 ± 4
creatinine	0.83 ± 0.09	0.93 ± 0.12	0.91 ± 0.13
blood natrium	143 ± 1	141 ± 2	140 ± 1
blood kalium	4.4 ± 0.1	4.4 ± 0.3	4.2 ± 0.2
CK	142 ± 87	135 ± 60	112 ± 34
AST	22.8 ± 5.8	19.2 ± 3.1	22.3 ± 6.0
total bilirubin	0.5 ± 0.1	0.6 ± 0.3	0.5 ± 0.2

[a] Measurements were taken before study entry (baseline), after 3 weeks (3 weeks), and after 4 weeks of washout (washout). Values are means ± SD of measurements taken from 10 participants.

oxidation process in the juice as no attempt was made to remove oxygen during the pasteurization and bottling steps. Moreover, the screw caps were not absolutely airtight.

Participants' blood pressure, serum glucose and calcium, kidney function (BUN, creatinine, and the electrolytes natrium and kalium), and liver function (CK, AST, and total bilirubin) were measured at time zero (before study entry), after 3 weeks of juice consumption, and at the end of the study (after 4 weeks of washout). The results are summarized in **Table 5**, indicating that marula juice consumption had no significant effect on any of the above measures. Thus, juice consumption posed no adverse effects on participant's health indicators.

The effect of marula juice consumption on serum cholesterol concentrations is presented in **Figure 2A**−**C**. Drinking marula juice for 3 weeks significantly ($p < 0.02$) reduced the concentrations of total cholesterol (**Figure 2A**) in serum by 8%. This reduction could be related to a significant ($p < 0.01$) reduction in LDL-cholesterol (**Figure 2B**) levels, by 17%. However, these reductions did not persist after the washout period, as the levels of total cholesterol, as well as those of LDL-cholesterol, returned to baseline levels after 4 weeks of washout period, during which the subjects did not consume marula juice. The serum level of HDL-cholesterol (**Figure 2C**) increased significantly ($p < 0.03$), by 10%, after consumption of marula juice, and this increase persisted also after the washout period, although at a lower extent (by 7%). **Figure 2D** presents the effect of marula juice consumption on serum triglyceride concentrations. Serum triglyceride levels decreased by 7% after consumption of marula juice, and this effect persisted after the 4 week washout period. The reduction in serum triglycerides may explain the increment in serum HDL levels. Hydrolysis of triglyceride-rich lipoproteins is accompanied by removal of surface lipoprotein materials such as unesterified cholesterol and phospholipids. These surface lipoprotein lipids can then be

attached to the HDL and increase serum HDL concentration, as indeed observed in the study.

The effect of marula juice consumption on serum oxidative stress is summarized in **Figure 2E**,**F**. Serum samples were subjected to AAPH-induced oxidation. Lipid peroxide formation (**Figure 2E**) was significantly ($p < 0.03$) decreased in serum samples derived after consumption of marula juice for 3 weeks. However, this effect was not sustained after the washout period. The "antioxidant power", measured by the FRAP assay (**Figure 2F**), increased in serum samples derived after consumption of marula juice and, surprisingly, even further increased, reaching a significant elevation of 8% after the washout period.

The reduction in serum oxidative stress by marula juice consumption could be the result of the reduction in serum lipids concentrations (less substrate available for oxidation), as well as the effect of the marula juice potent antioxidants.

The results of the preclinical study suggest that marula juice is a hypocholesterolemic nutrient which favorably affects blood lipids, as shown by the reduction in serum LDL, the increment in serum HDL, and the attenuation of serum oxidative stress.

Analysis of Soluble Phenolic Compounds in Marula Fruit Juice. Two families of antioxidants are likely to generate the high antioxidative capacity of the marula juice: soluble phenolic compounds and vitamin C. In previous studies (*18*−*21*), the significant antiatherogenic effects of licorice, red wine, and pomegranate juice extracts were all attributed to their specific polyphenolic composition. It is possible that potent phenolic antioxidants present in marula juice exert the unique in vivo effect observed in the preclinical study. It is, thus, of interest to further study the composition and nature of the soluble phenolic compounds in the marula fruit juice.

Methanolic extracts of marula juice were analyzed by HPLC as described under Materials and Methods. A typical chromatogram at 270 and 15 nm window width is presented in **Figure 3A**. Undistinguishable chromatograms were obtained with extracts of pasteurized and concentrated juice. The major components coming through within the initial 7.5 min of the run had an absorption maximum at 243 nm. The chromatogram at 243 nm (presented as an insert in **Figure 3A**) reveals two main peaks matching vitamin C with a similarity factor of ≥0.999.

Figure 3B focuses on the chromatogram peaks eluting after vitamin C. The figure portrays a two-dimensional optical density map presentation of the HPLC run between 8 and 25 min and between 230 and 375 nm wavelength range. The peaks are clearly discerned by retention times and absorption spectra.

To detect and quantify phenolic groups and antioxidant activity of the above separated phenolic compounds within the chromatogram peaks, a methanolic extract of concentrated juice (prepared as described under Materials and Methods) was applied to the column; eluted fractions were collected during the HPLC run as described under Materials and Methods and then analyzed. The procedure was repeated five times with very similar results. A representative experiment is shown in **Figure 4**. The HPLC chromatogram of the concentrated juice extract at 270 nm (with a 15 nm window width) is presented in **Figure 4A**. Fractions were collected at a rate of one per minute (0.6 mL each) and analyzed for phenolic concentration (**Figure 4B**). The fraction number in the figure denotes the column runtime coinciding with the conclusion of the collection period; that is, fraction 10 refers to the HPLC runtime from just after 9 until 10 min, and so on. The highest phenolic concentrations were associated with compounds eluted in fractions 13, 14, and 17−21.

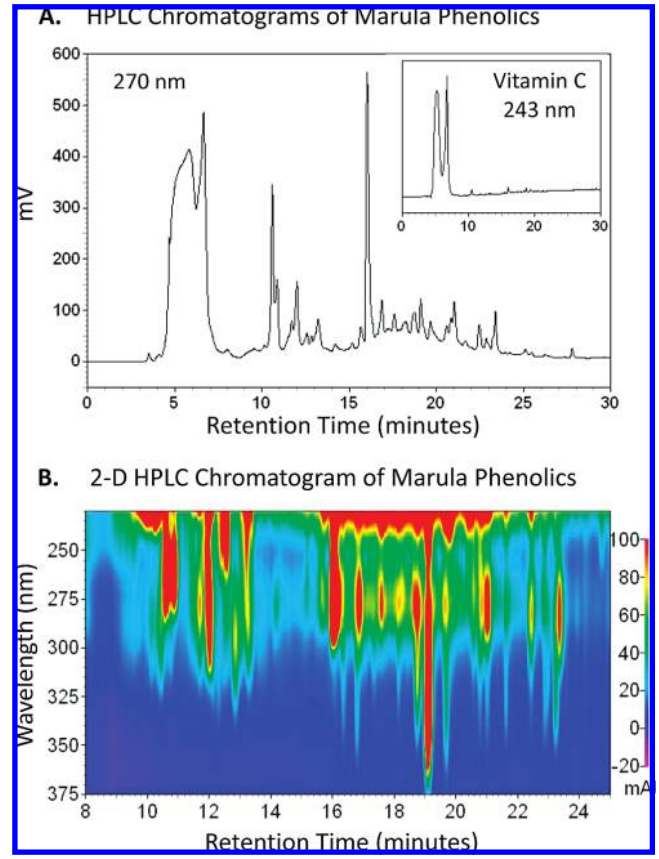

Figure 3. HPLC chromatograms of marula fruit juice methanolic extract at 270 nm (**A**) and 243 nm (insert in **A**) and in a two-dimensional optical density map presentation (**B**). Window width in (**A**) was 15 nm. Wavelength range in (**B**) is from 230 (top) to 375 nm (bottom), and runtime is from 8 to 25 min; a color scale ranging from blue (low) to red (high) is used to display concentrations in terms of optical density.

The free radical scavenging capacity of the isolated fractions was next measured (**Figure 4C**). The entire volume of each fraction was added to the DPPH solution, and the absorbance at 517 nm was monitored for a total of 5.5 min. The reaction kinetics of the absorbance loss consisted of two distinct phases, that is, fast and slow processes, similarly to the reaction course exhibited by whole marula juice (**Figure 1**). The values corresponding to the rapid drop during the initial 0.5 min and the subsequent slower decrease during the next 5 min were monitored. The results are displayed in **Figure 4C** as stacked columns; the full height of the column presents the total absorbance loss during the entire reaction period.

The fractions differed in both the total free radical scavenging activity and the relative contributions of the two kinetic components. These reflect both different concentrations and natures of the phenolic compounds in the various fractions. Phenolic hydroxyls were shown to exhibit different antioxidant activities in different phenolic compounds (*39*), which is to be expected considering the polarity and steric effects of adjacent groups in the molecule on the hydroxyl reactivity. The highest levels of free radical scavenging activity were associated with compounds contained in fractions 13, 14, and 18−20.

Notably, some inconsistency was observed in the habitual positive correlation between phenolic content and radical scavenging activity. Fractions 13, 14, and 17−21 were found to contain especially high concentrations of phenolic hydroxyls, whereas the most profound radical scavenging capacity was displayed by fractions 13, 14, and 18−20, indicating that the phenolics in fractions 17 and 21 had a lower specific activity.

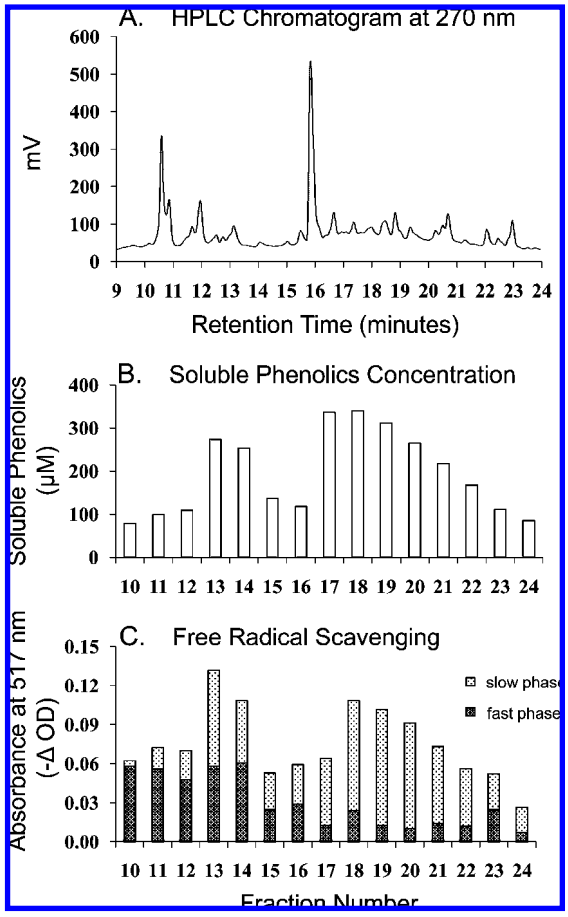

Figure 4. HPLC chromatogram at 270 nm of marula juice methanolic extract (**A**) and phenolic hydroxyls' concentration (**B**) and free radical scavenging activity (**C**) in HPLC fractions of the extract. Fractions were collected one per minute; fraction number denotes the column running time coinciding with the conclusion of the collection period. Free radical scavenging activity is expressed as the loss of OD at 517 nm during the initial 0.5 min (fast phase) and during an additional 5 min (slow phase).

By reference to the standard library, the major phenolic compounds in these fractions are, tentatively, derivatives of hydrolyzable tannins (fractions 13, 14, and 19), catechins (fractions 17, 18, and 21), and hydroxycinnamic acid (fraction 20). Further resolution of the above fractions to approximate a single chromatogram peak per fraction will facilitate the purification and identification of particularly potent compounds.

The present study explored health value aspects of Israeli-grown marula fruits. The juice extracted from these fruits exhibited an exceptionally high antioxidative capacity with a demonstrated high tolerance to customary pasteurization and storage conducts. In addition, the juice was relatively low in sugar and high in potassium contents. Taken together, these properties make the marula fruit juice a potential health-promoting food product. Major contributors to the highly stable antioxidant activity are likely to be the phenolic compounds, the presence of which in the juice was established. Other plant extracts (*18*−*21*) containing polyphenolics with high antioxidant activities were found to be beneficial in reducing the risk of atherosclerosis. When administered to healthy human subjects as a food supplement, marula fruit juice was found to induce distinct favorable effects on serum lipids: reduction in total cholesterol, LDL-cholesterol, and triglycerides concentrations and increased HDL-cholesterol level. Also, an attenuation of serum oxidative stress was established. Some of the effects persisted, at least partially, after a 4 week washout period. Marula fruit juice can be, thus, considered to be a hypolipidemic antioxidant nutrient. Natural food products of such health value are of prime relevance to the general population and are, therefore, the subject of an increasingly exhaustive pursuit.

Pertinent candidates to exert the health-promoting effects are the potent polyphenolic antioxidants of the juice that may act on their own, or, more likely, in synergy with other elements. Distinct fractions containing especially active phenolic compounds were obtained. Tentatively, hydrolyzable tannins, catechins, and hydroxycinnamic acid derivatives constitute the phenolics component in these fractions. Further resolution of these fractions will set the basis for the purification and identification of the compounds that are implicated in the antiatherogenic effects of marula fruit juice. Once established, their deployment can be extended to formulations other than whole juice, thus making the marula fruit health benefits more accessible to consumers everywhere.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane hydrochloride); ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; DDW, double-distilled water; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; HDL, high-density lipoproteins; HPLC, high-performance liquid chromatography; LDL, low-density lipoproteins; OD, optical density; PBS, phosphate-buffered saline; PON1, paraoxonase 1; SD, standard deviation; TA, titratable acidity; TBARS, thiobarbituric acid reactive substances; TSS, total soluble solids; TPTZ, 2,4,6-tripyridyl-*s*-triazine.

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