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In Vitro and in Vivo Antioxidant and Anti-inflammatory Capacities of an Antioxidant-Rich Fruit and Berry Juice Blend. Results of a Pilot and Randomized, Double-Blinded, Placebo-Controlled, Crossover Study

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This study investigated the in vitro and in vivo antioxidant and anti-inflammatory properties of a juice blend (JB), MonaVie Active, containing a mixture of fruits and berries with known antioxidant activity, including açai, a palm fruit, as the predominant ingredient. The phytochemical antioxidants in the JB are primarily in the form of anthocyanins, predominantly cyanidin 3-rutoside, cyanidin 3-diglycoside, and cyanidin 3-glucoside. The cell-based antioxidant protection of erythrocytes (CAP-e) assay demonstrated that antioxidants in the JB penetrated and protected cells from oxidative damage ($p < 0.001$), whereas polymorphonuclear cells showed reduced formation of reactive oxygen species ($p < 0.003$) and reduced migration toward three different pro-inflammatory chemoattractants: fmlp ($p < 0.001$), leukotriene B4 ($p < 0.05$), and IL-8 ($p < 0.03$). A randomized, double-blinded, placebo-controlled, crossover trial with 12 healthy subjects examined the JB's antioxidant activity in vivo. Blood samples at baseline, 1 h, and 2 h following consumption of the JB or placebo were tested for antioxidant capacity using several antioxidant assays and the TBARS assay, a measure of lipid peroxidation. A *within subject* comparison showed an increase in serum antioxidants at 1 h ($p < 0.03$) and 2 h ($p < 0.015$), as well as inhibition of lipid peroxidation at 2 h ($p < 0.01$) postconsumption.

KEYWORDS: Antioxidant; anti-inflammatory; lipid peroxidation; cell-based antioxidant protection assay (CAP-e); oxygen radical absorbance capacity (ORAC) assay; açai, *Euterpe oleracea*; fruit juice; thiobarbituric acid reactive substances assay (TBARS)

INTRODUCTION

Reactive oxygen and nitrogen species play key roles in normal physiological processes, including cellular life/death processes, protection from pathogens, various cellular signaling pathways, and regulation of vascular tone (*1*). Oxidative stress is caused by an insufficient capacity of biological systems to neutralize excessive free radical production, which can contribute to human diseases and aging (*2*), including cardiovascular disease (*3*), neurodegenerative disease and age-related cognitive decline (*4*), obesity and insulin resistance (*5*), as well as immune system dysfunction (*6*). Oxidative stress also contributes to the accumulation of damaged macromolecules and organelles, including mitochondria (*4, 7*).

The antioxidant capacity of foods, juices, and teas has been linked to in vivo protection from oxidative stress in numerous studies. A recent study assessed the increase in plasma antioxidant capacity after the consumption of either an antioxidant-poor meal or the same antioxidant-poor meal with the addition of a known quantity of whole fruits added (*8*). This study

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showed that the consumption of an antioxidant-poor meal results in a decrease in plasma antioxidant capacity and that adding fruits to the same meal not only prevented this decrease but also led to an increase in antioxidant capacity. Furthermore, the study is important because it addresses fruits and juices consumed in normal amounts, in contrast to studies of highly enriched extracts or purified compounds.

There is a diverse array of methods currently employed for testing the antioxidant capacity of various foods and natural products. Novel research is currently directed toward possible therapies aimed at reversing the decline in mitochondrial ATP production and increased production of reactive oxygen species (ROS) (*3, 4*). For the chemical estimation of antioxidants in foods, the oxygen radical absorbance capacity (ORAC) test has been proposed as a preferred assay with possible relevance to human biology (*9*). The assay has been used for the assessment of total antioxidant capacity in human serum (*10*) and antioxidant uptake after the consumption of fruits and berries (*8*). We have developed a validated cell-based antioxidant protection assay using human erythrocytes (CAP-e), which we have used to document antioxidant capacity both in antioxidant-rich natural products and in serum obtained after the consumption of such products (*11, 12*).

The proprietary fruit and berry juice blend (JB) MonaVie Active contains the Amazonian palm fruit, açai (*Euterpe oleracea* Mart.) as the predominant ingredient (*13*), along with lesser amounts of the following fruits and berries in descending order of dominance: white grape (*Vitis* L.), Nashi pear (*Pyrus pyrifolia*), acerola (*Malpighia glabra*), aronia (*Aronia melanocarpa*), purple grape (*Vitis* L.), cranberry (*Vaccinium macrocarpon*), passion fruit (*Passiflora edulis*), apricot (*Prunus armeniaca*), prune (*Prunus* L.), kiwifruit (*Actinidia deliciosa*), blueberry (*Vaccinium* L.), wolfberry (*Lycium barbarum*), pomegranate (*Punica granatum*), lychee (*Litchi chinensis*), camu camu (*Myrciaria dubia*), pear (*Pyrus* L.), banana (*Musa acuminata*), and bilberry (*Vaccinium myrtillus*).

This study was undertaken to examine whether the antioxidants and anti-inflammatory compounds known to be present in the individual components of the unprocessed ingredients present in the JB are in a form able to enter into and protect human cells in vitro. Furthermore, the study also investigated the bioavailability of these compounds following ingestion of the JB and its effect on serum indicators of oxidative damage.

MATERIALS AND METHODS

Chemicals and Apparatus. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and fluorescein (sodium salt) (Fl) were obtained from Aldrich (Milwaukee, WI). Randomly methylated β-cyclodextrin (Trappsol) (Pharm grade) (RMCD) was obtained from Cyclodextrin Technologies Development Inc. (High Springs, FL). Folin−Ciocalteu's phenol reagent, sodium carbonate, sodium hydrogencarbonate ($NaHCO_3$), Sephadex LH-20, formic acid, gallic acid, and all other phenolic acids were purchased from Sigma (St. Louis, MO). Potassium phosphate dibasic (K_2HPO_4) and potassium phosphate monobasic (KH_2PO_4) were obtained from VWR (West Chester, PA). Standards of 3-*O*-β-glucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (six mixed anthocyanin standards, HPLC grade) were obtained from Polyphenols Laboratories (Sandnes, Norway). Methanol, acetonitrile, methylene chloride, and acetic acid (HPLC grade) were from Fisher (Fair Lawn, NJ). Phosphate-buffered saline, hydrogen peroxide, and RPMI-1640 cell culture medium were purchased from Sigma-Aldrich (St. Louis, MO). DCF-DA was from InVitrogen (Carlsbad, CA). Flow cytometry was performed using a FacsCalibur flow cytometer (Becton-Dickinson, San Jose, CA), and fluorescence reading of 96-well microtiter plates was performed using a TECAN SpectraFluor plate reader (TECAN US, Durham NC).

Study Overview. This study was conducted in three stages. Initial testing was performed to evaluate the phytochemical contents of the JB, as well as the antioxidant capacity of the JB in cell-based assays in vitro. The second stage was a pilot study with five healthy adults who were tested on a single study day, to identify the time course for antioxidant absorption and bioactivity of the JB in vivo. Blood samples were collected immediately prior to consumption of 120 mL (4 oz) of the JB and at 30, 60, and 120 min following consumption. The selection of these time points was based on previous studies using pomegranate juice (*14*). The third stage was a randomized, double-blinded, placebo-controlled, crossover study of 12 healthy adults ranging from 19 to 52 years of age, in which all 12 study participants were tested on both the placebo and JB on different days. Serum antioxidant and lipid peroxidation levels were compared at baseline and in response to the placebo or JB in a *within subjects* study design.

Polyphenols and Antioxidant Capacities of JB. *Sample Preparation.* Ten milliliters of the JB was centrifuged at 4000*g* for 10 min. Supernatant was harvested and extracted by hexane twice (5 mL × 2) to separate lipophilic components. The hexane layer was used to measure lipophilic ORAC, whereas the aqueous layer was used for phytochemical analysis, hydrophilic ORAC, and total phenolic measurements.

Phytochemical Analysis. One milliliter of aqueous solution was filtered using a 0.22 μm Teflon syringe filter (Cameo, MN) for qualitative and quantitative analysis of ACN analysis following the method reported previously (*15*). Five milliliters of aqueous solution was fractioned by Sephadex LH-20 for proanthocyanidin analysis following the published method for proanthocyanidin analysis and quantization (*16*). Phenolic acids analysis was carried out using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) coupled to the 4000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA) according to a recently published method (*17*).

ORAC Assay. The hydrophilic and lipophilic $ORAC_{FL}$ assays were carried out on a FLUOstar Galaxy plate reader (BMG Labtechnologies, Durham, NC), based on published procedures (*18, 19*). The results are expressed as micromoles of Trolox equivalents per milliliter of juice (μmol TE/mL).

Total Phenolic Assay. The aqueous layer was subjected to total phenolics measurement by Folin−Ciocalteu reagent according to the method of Wu et al. (*18*) The results are expressed as milligrams of gallic acid equivalents per milliliter of juice (mg GAE/mL).

Consumable Test Products: Preparation for in Vitro and in Vivo Testing. Five 750 mL bottles from the same batch of JB were received from the manufacturer. Because many antioxidants are degraded by exposure to light and air, a new bottle was opened on each clinical study day to ensure a consistent antioxidant concentration. The in vivo pilot study was performed to examine any possible effect of juice consumption. Therefore, a dose of 120 mL was chosen. This dose is at the high end of the manufacturer's recommended daily dose of 60−120 mL and has been mentioned in testimonials of consumers as supporting a generalized sense of well-being and relieving symptoms of an inflammatory nature.

For the in vitro cell-based studies, one bottle of JB was opened under sterile conditions and aliquoted into a series of vials. Care was taken to fill each vial to a maximum, to minimize exposure to oxygen and thus to prevent potential oxidation of the compounds during the storage period. The vials were stored at 4 °C and protected from light until required for in vitro testing. On each in vitro test day a vial of JB was prepared for addition to cell cultures by removal of solids and nonaqueous compounds by standard laboratory procedures, including centrifugation and filtration using a cellulose acetate syringe filter. Serial dilutions were then prepared in phosphate-buffered saline (PBS) and were used only on the day of preparation.

The in vitro antioxidant assays were performed using the sterile filtrate, whereas the clinical study evaluated ingestion of the whole juice. The filtration removes solids and lipid agglomerates, which removes the antioxidants responsible for the $ORAC_{lipophilic}$ value from the test product while retaining water-soluble compounds that are responsible for the $ORAC_{hydrophilic}$. Dry weight assessment of the amount of dissolved material to be added to cell cultures found that 100 mL of the JB contains 40% solids, which were removed by centrifugation

and filtration prior to addition to the cell cultures. The filtrate contained 100 g/L of dissolved material. Taking the 40% solids into account, unaltered JB contains 60 g/L of dissolved material. Thus, consumption of 120 mL of the whole juice is equivalent to 7.2 g of dissolved material.

The JB is a complex, robustly flavorful, dark purplish liquid, with a substantial amount of pulp, giving the juice a characteristic appearance that is very difficult to reproduce for placebo purposes. Therefore, we decided against developing a liquid placebo. An encapsulated placebo product was created, and study participants were purposefully left uncertain about whether they were being fed a new test product or a placebo. The placebo was prepared by mixing white potato flakes, which in contrast to some other types of potato flakes do not increase serum antioxidant levels (*20*), with a purplish food-coloring blend. The mix was redried, ground, and put into vegetable-based capsules. Two capsules containing 0.5 g each were consumed by the study participants.

Purification of Peripheral Blood Cell Subsets for in Vitro Testing. Peripheral venous blood for the in vitro work was obtained from healthy human volunteers between 19 and 52 years of age after informed consent was obtained and upon approval by the Sky Lakes Medical Center Institutional Review Board (FWA 2603). Blood samples were obtained using a sterile technique and processed immediately. The whole blood was layered onto a Histopaque 1119 density gradient. Following centrifugation, the polymorphonuclear (PMN) cell fraction and the erythrocyte fraction were harvested and washed. The PMN cells were used for testing of ROS formation and migration, and the erythrocytes were used for the CAP-e assay (see below).

Cell-Based Antioxidant Protection of Erythrocytes (CAP-e) Assay. The CAP-e assay (*11, 12*) utilizes human red blood cells as a model for antioxidant uptake and protection of a simplistic, noninflammatory cellular model that is intimately involved in redox processes in vivo. The CAP-e assay was used to evaluate the antioxidant capacity of JB in vitro and in vivo. For the in vitro assay, serial dilutions of JB were added to the assay. For testing antioxidant uptake in vivo, serum samples obtained before and after consumption of either the JB or placebo were added to the cells.

Purified red blood cells from a type O+ donor were washed extensively in physiological saline and stored at 4 °C. Red blood cells were distributed in triplicate in 96-well V-bottom microplates (NUNC, Roskilde, Denmark) and pretreated with either the JB or serum from the subjects receiving either the placebo or JB. Repeated washing ensured that any antioxidant compounds not absorbed by the cells were removed prior to analysis. The cells were stained with DCFDA—a probe that becomes fluorescent with free radical damage. One triplicate set of wells was left untreated as a control to record the background level of oxidation in the red blood cells. All other wells were treated with hydrogen peroxide, which penetrates the cells and triggers severe intracellular oxidative damage. The fluorescence intensity of the cells was analyzed by flow cytometry, and the subsequent data analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR).

Evaluation of Reactive Oxygen Species (ROS) Formation by PMN Cells. Evaluation of the inhibition by the JB of ROS production was done using freshly purified human PMN cells, as previously described (*21, 22*). In brief, PMN cells were incubated at 37 °C and 5% CO_2 for 90 min, either untreated or treated with serial dilutions of JB. The PMN cells were washed twice in PBS and resuspended in the precursor dye DCF-DA (Molecular Probes, Eugene, OR) and incubated for 1 h at 37 °C. All samples, with the exception of the negative controls, were then exposed to hydrogen peroxide for 45 min to induce ROS formation. Samples were washed twice in PBS to remove the peroxide, transferred to cold RPMI 1640, and stored on ice. The intensity of ROS-induced DCF-DA fluorescence was analyzed immediately by flow cytometry, as described above for the CAP-e assay. The mean fluorescence intensity (MFI) of PMN cells was compared between untreated, hydrogen peroxide-treated, and JB-pretreated cells. A reduction in MFI in samples pretreated with the JB prior to challenge with hydrogen peroxide would indicate that the JB mediated a reduction in ROS production.

PMN Migration in Vitro. The evaluation of random and directed PMN migration in vitro was performed using Millipore double-chamber migration 96-well culture plates; the top and bottom chambers are separated by a 3 μm porous filter. PMN cells were plated at 10^6/mL in the top chambers, with or without the addition of serial dilutions of JB. The bottom chambers contained either culture medium alone to assess random migration or one of the following three different proinflammatory chemoattractants: bacterial peptide f-Met-Leu-Phe (fmlp), leukotriene B4 (LTB4), or interleukin-8 (IL-8) to assess directed migration. All culture conditions were performed in triplicate. Plates were incubated for 18 h at 37 °C and 5% CO_2 to allow for PMN migration from the top into the bottom chambers. The following day the top chambers were removed, and the cells in the bottom chambers were transferred to V-bottom 96-well plates and stained with CyQuant (Molecular Probes). The fluorescence intensity, proportional to the numbers of cells that had migrated from the top to the bottom wells, was measured using a TECAN SpectraFluor plate reader.

In Vivo Testing of Antioxidant Uptake and Lipid Peroxidation. *General.* The study was approved by the Sky Lakes Medical Center Institutional Review Board (FWA 2603). Recruitment of study participants included an initial interview by a nurse to evaluate whether each volunteer met the inclusion/exclusion criteria. Volunteers who met these criteria were scheduled for a medical examination. Subjects who met the requirements of the medical examination were enrolled in the study. Exclusion criteria were as follows: under 18 or over 55 years of age; pregnancy; severe stress; asthma and/or allergies requiring daily medication; any known chronic illness; obesity, smoking; recreational drug use; impaired digestive function (including previous major gastrointestinal surgery); regular consumption of the JB or other products with a high concentration of known antioxidants. A total of 19 study subjects were interviewed. Two subjects were excluded due to obesity, and one was excluded on the basis of a high daily antioxidant intake. Two subjects were unable to participate due to their work schedule. Of the remaining 14, 7 participated in the pilot study and 12 participated in the clinical trial.

Pilot Study. The pilot study involved seven study participants, of which two were excluded from analysis due to incomplete blood sampling. Thus, the analysis of the pilot study was based on five study participants from whom repeat blood draws were performed. Two methods for the evaluation of the antioxidant capacity of the serum samples were employed: (1) the ORAC assay and (2) the CAP-e assay. Samples from the initial pilot study of five subjects were shipped to Brunswick Laboratories (Norton, MA) for serum ORAC analysis. Samples were tested in serial dilutions and plotted onto a standard curve to acquire the data expressed as Trolox equivalents. An average was made based on the data from each serial dilution that was within the range of the standard curve. Because the ORAC testing did not result in a trend toward increased antioxidant activity, it was not used in the subsequent randomized controlled trial. Instead, the thiobarbituric acid reactive substances (TBARS) test was utilized for the reasons discussed below. Testing of the serum samples in the CAP-e assay showed an increase in serum antioxidants following JB consumption. Therefore, the CAP-e assay was utilized as well for the randomized controlled trial.

Randomized, Placebo-Controlled Crossover Clinical Trial. Twelve volunteers were scheduled for two study days at least one week apart. On each study day, the participants spent approximately 3 h at the clinic, including filling in initial daily intake questionnaires, baseline blood draws, consumption of the test product, and subsequent blood sample collection. The study participants were instructed to avoid vigorous exercise for a period of 24 h prior to arriving at the clinic. They were also instructed to eat a light meal and to avoid meat, fruits, or greens, as well as to abstain from consuming alcohol, coffee, or melatonin the night before. Subjects were scheduled to arrive at the clinic following an overnight fast and were instructed to consume no food, vitamins, or other nutritional supplements the morning of each study day. A daily intake questionnaire was used to track last meal, last snack, and amount of exercise within the past 24 h, to ensure that volunteers were in compliance. Sample collection was performed at the same time of the day (7:00−10:00 a.m.) to minimize the confounding effect circadian fluctuations may present on metabolic function. Upon arrival to the clinic, the study participants were allowed to consume water as needed. On each study day, volunteers were instructed to complete a questionnaire aimed at determining any exceptional stress-related circumstances

that might affect them, as well as to provide for the tracking of any adverse events or sickness during the past week. After completion of the questionnaire, a baseline blood sample was drawn. Immediately after collection of the baseline sample, 120 mL of the JB was provided for consumption. Blood samples were subsequently drawn at 1 and 2 h after ingestion of the test item. At each time point, 6 mL of blood was drawn into serum separator vials. The vials were placed at room temperature for 30 min to allow for complete coagulation. The vials were centrifuged at 400*g* for 10 min, and the serum was pipetted into multiple aliquots, which were frozen for later testing of antioxidant status. The serum antioxidant status was assessed using the CAP-e assay as described above. The extent of lipid peroxidation in the serum was determined using the TBARS assay. The TBARS assay is a well-established method for screening and monitoring lipid peroxidation (*23*). A commercially available kit for this assay was used according to the instruction manual from the manufacturer (Cayman Chemical Co., Ann Arbor, MI).

Statistical Analysis. For the in vitro antioxidant testing, a simple comparison of arithmetic means was performed using Student's *t* test, using Microsoft Excel (Microsoft, Redmond, WA). Probabilities (*p*) based on the statistics were computed as the tail area of the *t* distribution using the two-tailed *t* test, at a 5% level of significance. For the in vivo study of antioxidant uptake and the effect on lipid peroxidation, the normalized data for each person and each time point after placebo consumption were subtracted from the same data set after JB consumption. Statistical significance was performed using Student's *t* test. Statements regarding whether a given mean value differed from a reference mean value were evaluated by two-tailed independent as well as paired *t* tests and were supported if $p < 0.05$. To evaluate whether there was greater antioxidant uptake after JB consumption compared to placebo, a two-within factor and repeated measures analysis of variance (ANOVA) was used, where treatment (JB or placebo) was one within-group factor and time of blood draw was the other. The α levels were set at 0.05 with the two-tailed tests. Analysis was performed with SPSS version 12.5 (SPSS, Chicago, IL).

RESULTS

Polyphenols and Antioxidant Capacities of JB. Major polyphenol compounds including anthocyanins, proanthocyanidins, and phenolic acids, along with antioxidant capacities measured by ORAC and total phenolics, are presented in **Table 1**. Compared to freeze-dried açai (*13*), the polyphenol profile of the JB is different. However, certain key polyphenols, such as the anthocyanins cyanidin 3-glucoside and cyanidin 3-rutinoside, appeared as major anthocyanins in the JB as they are in the freeze-dried berry. The total antioxidant capacity of the JB measured by ORAC is 22.8 μmol TE/mL, which is higher than the range of ORAC values for most common juices (*18, 24*).

In Vitro Cell-Based Antioxidant Protection of Erythrocytes (CAP-e) Assay. The JB showed a clear dose-dependent antioxidant protection effect in the CAP-e assay, indicating that compounds in th JB are able to cross the plasma membrane of living cells and, subsequently, are able to provide significant protection from oxidative damage within the cells (**Figure 1**). The data were highly significant. At a concentration of 10 g/L, the *p* value was <0.001. Even at the lowest dose of JB (0.016 g/L) the antioxidant protection of the cells was statistically significant ($p < 0.045$).

Inhibition of Reactive Oxygen Species (ROS) Formation by Polymorphonuclear (PMN) Cells. PMN cells are highly reactive immune cells capable of producing ROS immediately upon stimuli. ROS production can be inhibited by antioxidants available to enter live cells and can also be further inhibited by anti-inflammatory compounds in a complex product. The pretreatment of PMN cells by the JB resulted in inhibition of th formation of ROS by the PMN cells (**Figure 2**). Untreated PMN cells (no JB, no H_2O_2) served as a baseline, and PMN cells treated with H_2O_2 in the absence of JB served to show maximum ROS production. PMN cells treated with the JB produced fewer ROS than cells treated with H_2O_2 in the absence of JB. The inhibition of ROS formation was dose-dependent, and at the highest dose of JB tested (1 g/L), the inhibition was highly significant ($p < 0.003$).

Effects on PMN Migration toward Inflammatory Stimuli. JB treatment of PMN cells showed differential effects under different culture conditions. The PMN response to JB treatment resulted in nonlinear, complex dose-responses for random

Table 1. Phytochemical Analysis of the Juice Blend

		μg/mL of juice
proanthocyanidins	total	0.472005
	1-mer	0.0118
	2-mer	0.001255
	3-mer	0.0108
	4-mer	0.00565
	polymer	0.4425
anthocyanins	cyanidin 3-diglycoside	57.3
	cyanidin 3-sambubioside	10.2
	cyanidin 3-glucoside	26.5
	cyanidin 3-arabinoside	13.3
	cyanidin 3-rutinoside	69.9
phenolic acids	total	1.50
	protocatechuic acid (3,4-OH BA)	0.58
	caffeic acid (3,4-OH CA)	0.20
	3-(4-hydroxyphenyl)propionic acid (4-OH PPA)	0.41
	p-coumaric acid (3-OH CA)	0.31

		mg GAE[a]/mL of juice
total phenolics		1.48

		μmol TE[b]/mL of juice
ORAC	H-ORAC	22.2
	L-ORAC	0.61

[a] GAE, gallic acid equivalents. [b] TE, Trolox equivalents.

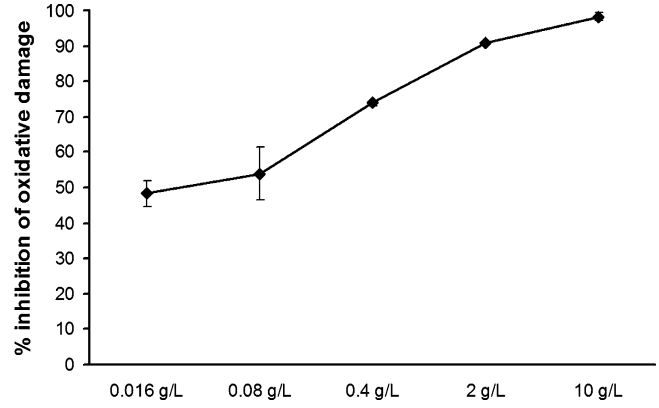

Figure 1. Antioxidant capacity of the JB evaluated in vitro, using the CAP-e assay. Red blood cells were used as a cellular model for evaluating the ability of antioxidants to cross the plasma membrane into living cells. Red blood cells were exposed to serial dilutions of the JB and subsequently exposed to an oxidative challenge. The graph shows the percent inhibition of oxidative damage. The treatment of cells with the JB resulted in statistically significant protection from oxidative damage over a broad range of dilutions, from the highest concentration of 10 g/L in cell culture ($p < 0.001$) to the lowest concentration tested ($p < 0.045$).

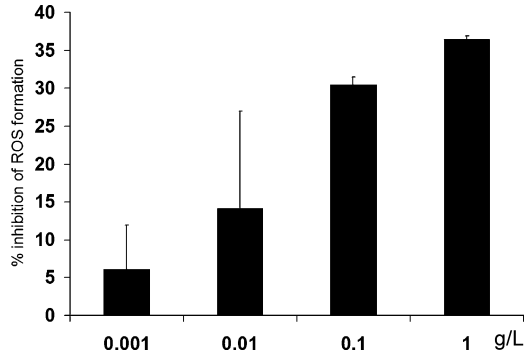

Figure 2. Capacity of the JB to reduce formation of ROS by PMN cells tested in vitro. PMN cells were exposed to serial dilutions of the JB and then challenged by H_2O_2 to produce ROS. Untreated cells (no JB, no H_2O_2) served as negative controls. The graph shows the percent inhibition of ROS formation as calculated means ± standard deviations of triplicate samples, compared to the negative and positive controls. A clear dose-dependent inhibition of ROS formation was caused by the JB. The effect was statistically significant with $p < 0.003$ at the highest dose of JB. All tests were performed in triplicate and repeated three times using cells from different donors.

migration (**Figure 3a**) as well as migration toward the bacterial peptide fmlp (**Figure 3b**). At the dose of 0.1 g/L, the JB significantly enhanced PMN random migration ($p < 0.05$). At the dose of 1 g/L, the PMN migration toward fmlp was significantly inhibited ($p < 0.001$). In contrast, the JB treatment of PMN cells showed a less complex, more linear, dose-response when PMN cells migrated toward two pro-inflammatory compounds made during inflammation, leukotriene B4 (LTB4, **Figure 3c**) and interleukin-8 (IL-8, **Figure 3d**). At the highest JB dose tested (1 g/L), significant inhibition of PMN migration was observed toward LTB4 ($p < 0.05$) and IL-8 ($p < 0.03$).

Pilot Study in Vivo. During an initial exploratory phase prior to the full study, the total antioxidant status in serum was assayed using both the ORAC and CAP-e assays. Whereas the CAP-e assay showed an increase in the antioxidant capacity in all five of the subjects, the ORAC test did not ($p < 0.2$, at 2 h postconsumption). On the basis of these findings the ORAC assay was not used in the full study and instead the TBARS test was employed.

In Vivo Protection from Oxidative Damage—Serum Antioxidant Status. The randomized, placebo-controlled trial involving 12 people in a within-subject design was performed as outlined in **Figure 4**. The description of the study population is shown in **Table 2**. Consumption of the JB resulted in an increase in the serum antioxidant capacity within 2 h of consumption in 11 of 12 study participants. The antioxidant capacity was tested using the CAP-e assay for serum samples obtained at baseline and at 1 and 2 h postconsumption (**Figure 5**). The increase in serum antioxidant capacity was statistically significant both at 1 h ($p < 0.027$) and at 2 h ($p < 0.015$) postconsumption. When a paired t test was performed on the normalized data from each person's response to placebo versus JB, the significance at 2 h postconsumption was even stronger ($p < 0.01$).

In Vivo Protection from Oxidative Damage—Serum Lipid Peroxidation Status. Consumption of the JB resulted in a decrease in serum lipid peroxidation within 2 h of consumption in 10 of the 12 study participants (**Figure 6**). The level of lipid peroxidation in the sera was evaluated using the TBARS test. The paired analysis, that is, the individual changes for each study participant consuming either JB or placebo, showed that the decrease in lipid peroxidation had not reached statistical

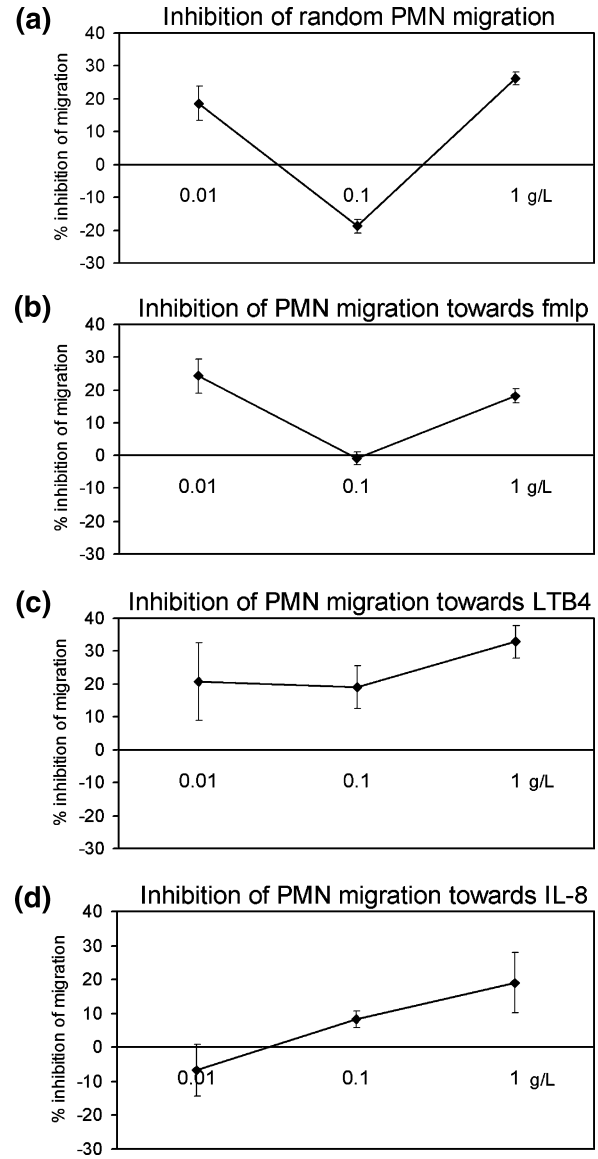

Figure 3. Treatment of PMN cells with the JB in vitro altered PMN migration under four different test conditions: random migration (**a**); and directed migration toward three chemoattractants bacterial peptide fmlp (**b**), LTB4 (**c**), and IL-8 (**d**). The graphs show percent inhibition of PMN migration as the mean ± standard deviations. At a dose of 1 g/L JB, random PMN migration was inhibited ($p < 0.05$), whereas at 0.1 g/L JB, random PMN migration was increased ($p < 0.05$). At the dose of 1 g/L JB, the PMN migration toward fmlp was significantly inhibited ($p < 0.001$). In contrast, pro-inflammatory, directed chemotaxis was inhibited for both inflammatory markers LTB4 ($p < 0.05$) and IL-8 ($p < 0.03$). All test conditions, serial dilutions, and positive and negative controls were performed in triplicates. The testing was repeated three times with cells from different donors.

significance at 1 h postconsumption ($p < 0.15$), but did achieve statistical significance at 2 h ($p < 0.01$). Analysis of the variance (ANOVA) of the repeated measures showed statistical significance for the treatment effect ($p < 0.02$).

The antioxidant uptake was correlated with the decrease in lipid peroxidation in vivo. A 45% correlation was observed between the increased antioxidant capacity in the serum and the reduction in lipid peroxidation at 2 h postconsumption (**Figure 7**).

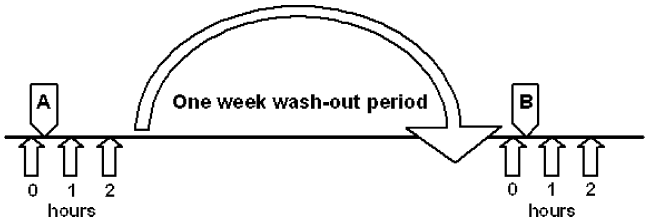

Figure 4. Schematic diagram of the randomized, placebo-controlled crossover study design for the in vivo study. Each of the 12 volunteers participated on two different clinic days, one week apart to allow for wash-out of active constituents. On each clinic day, a baseline blood draw was performed (0 h), and a consumable "A" or "B" was ingested. Two more blood samples were drawn at 1 and 2 h postconsumption. Volunteers were randomized to receive either placebo or JB on the first versus second clinic day (see also **Table 2**).

Table 2. Description of the Study Population

	males	females	total study population
average age (years)	25.5 ± 11.1	42.3 ± 13.8	33.9 ± 5.5
age (range)	19−48	20−52	19−52
height (cm)	177.8 ± 14.5	164.3 ± 7.3	171.0 ± 13.0
weight (kg)	79.5 ± 4.8	72.0 ± 13.5	75.7 ± 10.3
body mass index	25.6 ± 6.2	26.8 ± 5.2	26.3 ± 5.5
randomization: JB then PL	3	3	6
randomization: PL then JB	3	3	6

DISCUSSION

The study reported here was performed to evaluate whether the antioxidants present in the antioxidant-rich fruit and berry juice blend (JB) are capable of protecting living cells from oxidative damage in vitro, as well as to evaluate their bioavailability and biological effect in vivo. Using the CAP-e assay (*11*) to evaluate the activity of antioxidants in a noninflammatory cell-based system, it was determined that a significant amount of the antioxidants found in the JB are available to living cells, are capable of penetrating the plasma membrane of the cells, and able to protect the cells from intracellular oxidative damage in vitro.

The JB showed anti-inflammatory effects in several in vitro assays using the inflammatory PMN cell. JB treatment of PMN cells showed both a reduction in ROS formation and altered migratory behavior. In particular, the effect on PMN migration indicated altered cellular behavior beyond a simple contribution of antioxidants. PMN migration involves both random and directed migration behavior. Random migration reflects PMN cell scavenging for invaders as part of normal immune surveillance. In contrast, directed migration toward a chemoattractant involves migration toward and into sites of inflammation. It is biologically relevant to distinguish the effects of natural products on our innate defense mechanisms, such as random migration and migration in response to bacterial invaders, as opposed to inflammatory mediators made by the host. Therefore, it is of

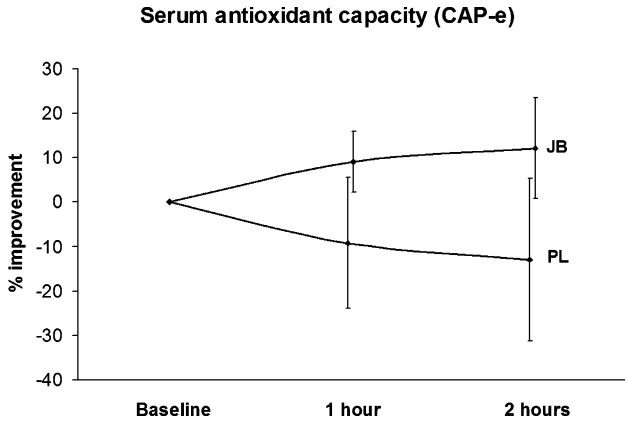

Figure 5. Consumption of JB resulted in an increase in antioxidants available to living cells. Serum samples obtained at baseline and 1 and 2 h after consumption of either JB or placebo (PL) were tested in the CAP-e assay. Data from each person per test day were normalized to examine changes in antioxidant capacity for each person over time after consumption of either JB or PL. The average and standard deviations for each group of data are shown for JB and PL. The difference in serum antioxidant capacity between JB and PL was statistically significant both at 1 h ($p < 0.03$) and 2 h ($p < 0.015$) postconsumption.

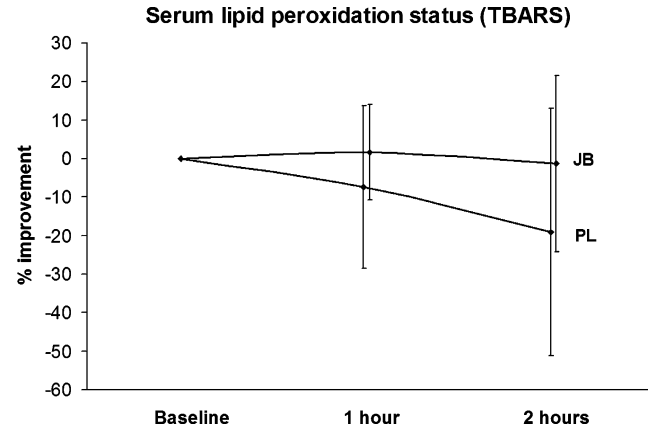

Figure 6. Consumption of JB resulted in a decrease in lipid peroxidation in vivo. Serum samples obtained at baseline and 1 and 2 h after consumption of either the JB or placebo (PL) were tested using the TBARS assay. Data from each person per test day were normalized to examine changes in antioxidant capacity for each person over time after consumption of either JB or PL. The average and standard deviations for each group of data are shown. The difference between JB and PL did not reach statistical significance at 1 h ($p < 0.11$) or 2 h ($p < 0.22$). However, when paired analysis was performed, by which each person's response to JB was compared to the same person's response to PL, a significant reduction of lipid peroxidation in the serum was observed at 2 h postconsumption ($p < 0.01$).

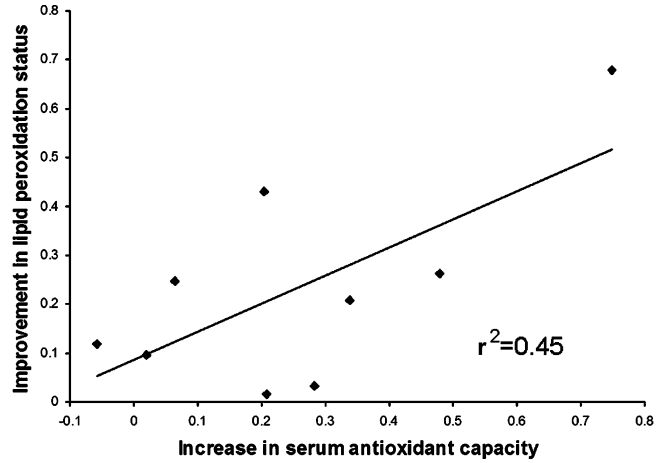

Figure 7. Comparison of the postconsumption reduction in lipid peroxidation and the increase in antioxidants capable of crossing the plasma membrane of living cells. The individual difference between a subject's levels of lipid peroxidation after consumption of JB versus placebo was plotted against the same individual's differences in serum antioxidant status. A linear trend line showed a 45% correlation coefficient between the improved serum antioxidant levels as measured by the CAP-e assay and the improvement in lipid peroxidation status as measured by the TBARS assay on serum samples collected 2 h postconsumption.

interest that JB treatment of freshly purified human PMN cells in vitro affected random and fmlp-directed migratory behavior differently from PMN migration toward the inflammatory mediators LTB4 and IL-8. We speculate that the anti-inflammatory properties of JB in vivo may allow normal immune surveillance while at the same time reducing inflammatory conditions.

On the basis of the preliminary in vitro data, the in vivo investigation of JB was performed. Consumption of the JB resulted in an overall increase in the antioxidant capacity of serum collected from the study subjects when compared to the placebo group, as measured by the CAP-e assay, but the trend was not as clear for the serum ORAC test. Because the CAP-e assay is cell-based, it is possible that some antioxidants from JB were able to accumulate and be retained in the cells, thus providing a more sensitive testing system, in contrast to the chemical ORAC assay. Therefore, the CAP-e assay was chosen for the subsequent randomized placebo-controlled crossover study.

The increase in serum antioxidant status upon consumption of JB was more clearly observed when the data from consumption of the JB were compared with the same study subject's data from placebo consumption in a paired statistical analysis. There was no evidence of a placebo effect in this study.

In only a few of the study subjects did the antioxidant capacity remain consistent with baseline values after consumption of the placebo. A slight increase in oxidative damage was observed in approximately 50% of the participants (i.e., a gradual decrease in antioxidant capacity) over the 2 h following consumption of the placebo. ROS are produced as a consequence of normal aerobic metabolism. We speculate that the observed increase in the potential for inducing oxidative damage in some of the study participants receiving the placebo may reflect a natural decline in their antioxidant status due to the overnight fast and increased depletion of food-derived antioxidants prior to the morning blood draws. Thus, by performing paired analysis, the decrease in antioxidant capacity as a result of fasting was taken into account when the same participant's response to JB consumption was analyzed.

Consumption of the JB resulted in an increase in serum antioxidant compounds that are able to enter living cells and protect them from oxidative damage in 11 of 12 study subjects, as evaluated by applying the serum samples to the CAP-e assay.

A decrease in lipid peroxidation in the sera of 11 of the 12 participants was observed 2 h after consumption of the JB, as demonstrated by comparing the normalized differences between the results of the TBARS data on sera collected 2 h following consumption of either the JB or placebo. This suggests that a rapid reduction in lipid peroxidation may occur in vivo within a 2 h period after consumption of the JB.

In conclusion, ingestion of the JB demonstrated a substantial antioxidant capacity for protecting cells from oxidative damage in vitro in this study. Furthermore, JB consumption led to a rapid increase in serum antioxidants, as measured by the cell-based assay for protection from oxidative damage. JB consumption also resulted in a statistically significant decrease in serum lipid peroxidation within 2 h of consumption. This effect is likely due to increased serum antioxidant capacity.

Antioxidant consumption, along with anti-inflammatory treatment, is being critically evaluated as a potential strategy for reversal of disease progression (*25*). It has been suggested that the apparent failure of multiple larger clinical trials to document reversal of disease processes may be linked to the choice of antioxidants. In particular, the frequent use of vitamins C and E in such studies may be due to availability and low cost, but may not have been the best choice, as both vitamins have pro-oxidant capacity as well (*26*). In contrast, studies on polyphenols may be much more promising, and may be more relevant, as these types of antioxidants are the most abundant in a health-conscious diet (*27*−*29*). Given the high content of certain specific polyphenols in the JB, the increased antioxidant protection in vivo after consumption of the JB, and the anti-inflammatory capacity in vitro, further research is warranted to evaluate whether JB consumption may provide reversal of risk markers in subjects with conditions such as arthritis, obesity, chronic viral diseases, cardiovascular disease, and compromised cognitive function as well as other conditions associated with chronic inflammation.

ABBREVIATIONS USED

CAP-e, cell-based antioxidant protection of erythrocytes; fmlp, bacterial peptide f-Met-Leu-Phe; GA, gallic acid; IL-8, interleukin-8; LTB4, leukotriene B4; ORAC, oxygen radical absorbance capacity assay; PMN, polymorphonuclear cells; RBC, red blood cell; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances assay; TE, Trolox equivalents.

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