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Antioxidant Status in Humans after Consumption of Blackberry (*Rubus fruticosus* L.) Juices With and Without Defatted Milk

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The present study was designed to evaluate the possible effect of the consumption of blackberry juices (BJ) prepared with water (BJW) and defatted milk (BJM) on the plasma antioxidant capacity and the enzymatic and nonenzymatic antioxidants. A significant ($p < 0.05$) increase in the ascorbic acid content in the plasma was observed after intake of both BJs. However, no changes were observed in the plasma urate and α-tocopherol levels. An increase on the plasma antioxidant capacity, by ORAC assay, was observed only after consumption of BJW but not statistically significant. Plasma antioxidant capacity had a good positive correlation with ascorbic acid ($r = 0.93$) and a negative correlation with urate level ($r = -0.79$). No correlation was observed between antioxidant capacity and total cyanidin or total ellagic acid contents. Further, it was observed that plasma catalase increased following intake of BJ's. No change was observed on the plasma and erythrocyte CAT and glutathione peroxidase activities. A significant decrease ($p < 0.05$) in the urinary antioxidant capacity between 1 and 4 h after intake of both BJs was observed. A good correlation was observed between total antioxidant capacity and urate and total cyanidin levels. These results suggested association between anthocyanin levels and CAT and a good correlation between antioxidant capacity and ascorbic acid in the human plasma after intake of BJs. Follow-up studies investigating the antioxidant properties and health benefits are necessary to demonstrate the health benefits of polyphenols.

KEYWORDS: anthocyanins; ellagitannins; ellagic acid; blackberry; *Rubus fruticosus* L.; antioxidant capacity; antioxidant enzymes.

INTRODUCTION

Reactive oxygen species (ROS) have been implicated in the mediations of several pathological processes such as ischemia, inflammatory diseases, diabetes, and atherosclerosis. Mammalian cells have a complex detoxification system including the endogenous antioxidants, such as urate and tocopherol, and the antioxidant enzymes that act as scavengers of the ROS. The antioxidant enzymes, i.e., superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), play an important role in scavenging oxidants and preventing cell injury (*1*). Briefly, superoxide anion can be quickly converted to H_2O_2 by SOD, while CAT and GPx protect cells from the harmful effects of H_2O_2, converting it into water and oxygen (*1*).

Bioactive compounds from the diet could modulate both the activity and expression of these enzymes. Among them, flavonoids have been associated to protection against ROS formed during cellular metabolism (*2*). Anthocyanins and ellagitannins (ETs) are the major phenolic compounds present in blackberry (*Rubus fruticosus* L.) fruits. These two groups of phenolics have been studied mainly because of their potential health benefits reported in many in vitro and in vivo studies (*3*−*5*). Anthocyanins are well known as antioxidant, chemopreventive, and anti-inflammatory agents (*6*−*11*). Sanguiin H-6 and lambertianin C are considered the two major ETs of strawberries (*Fragaria* × *ananassa* Duch.), blackberries, and raspberries (*Rubus idaeus* L.), and they have been reported as responsible for the high antioxidant capacity of these berries (*12*). Mullen et al. (*4*) observed also that the antioxidant capacity of raspberry extracts was contributed mainly by ETs. According to Aaby et al. (*13*), ETs and anthocyanins from strawberries had the highest contribution to the antioxidant capacity, 19% and 13%, respectively, although ascorbic acid was the most overall contributor (24%). On the other hand, Tulipani et al. (*14*) showed that total phenolic and ascorbic acid makes an important contribution in total antioxidant capacity in strawberry not anthocyanins.

Despite several studies showing the high in vitro antioxidant capacity of the anthocyanins and ETs, it is not clear if this

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property could be observed in vivo since it would depend on the extent of absorption and metabolization of these compounds (*15*). Some studies have correlated the increase in the plasma antioxidant capacity to the presence of anthocyanins after consumption of anthocyanin-rich foods (*16, 17*). However, Lotito and Frei (*18*) associated the increase in the plasma antioxidant capacity, after apple consumption, to the increase on the urate formed as an effect of the fructose intake. Although anthocyanins can be quickly detected in plasma and rapidly excreted after intake, they have been associated with reduction of urinary infection (*19, 20*). In a previous study from our group, we observed that food matrix had an important effect on anthocyanin and ETs absorption. In the case of anthocyanins, the rate of absorption was retarded without alteration of the total amount absorbed. For ETs, a total inhibition of absorption after intake of blackberry juice prepared with defatted milk was observed. Therefore, the objective of this study was to verify if the consumption of two different blackberry juice preparations, with and without defatted milk, had any effect on the antioxidant status of human plasma and urine.

MATERIAL AND METHODS

Chemicals. Cyanidin chloride was obtained from Extrasynthese (Genay, France). Ellagic acid, α-tocopherol, 2,2′-azobis (2-amidinopropano) hydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), BHT (butyl hydroxytoluene), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and fluorescein were purchased from Sigma Chemicals Co. (St. Louis, MO). Glutathione, glutathione reductase, and NADPH were obtained from Merck Chemical Co. (Darmstadt, Germany). All other reagents were analytical or HPLC grade.

Materials. Fully ripened blackberry (*Rubus fruticosus* L.) fruits were harvested (from December to January 2007) from the same local plantation (Minas Gerais state, Brazil). The fruit samples were stored at −18 °C until analyses. The anthocyanin content was 112 ± 2 mg of cyanidin aglycone equivalent/100 g of fresh weight (FW), with cyanidin 3-glucoside (cy 3-glu) (102 ± 2 mg/100 g FW) being the major anthocyanin present. The ETs content, expressed as total ellagic acid, was 146 ± 6 mg/100 g FW, with Sanguiin H-6 being the main ET.

Subjects and Study Design. Six healthy human subjects (6 females) with ages ranging from 24 to 40 years and body weight from 50 to 65 kg were recruited for the study. Subjects were asked to consume a "polyphenol-free" diet (no fruits, vegetables, juices, alcohol, tea, chocolate, or orange juice) 24 h before and during the study. The beverages were prepared with an amount of blackberry corresponding to 400 mg of cyanidin equivalent/50 kg of body weight, homogenized with 200 mL of water (BJW) or 200 mL of defatted milk (BJM). As per feeding, 200 mL of defatted milk (Mk) was provided too. In an open, single-center study under controlled conditions the volunteers ingested, after an overnight fasting, a single portion of either blackberry juice (BJ) prepared with water or juice prepared with defatted milk, both with identical total anthocyanin doses of 400 mg/50 kg body weight, or 200 mL of defatted milk, separated by a 4-week interval. Each person was used as her own control. During each experiment 250 mL of water was provided after 2 h juice intake. The order of the beverage ingestion was not randomized. Eight milliliters of venous blood was collected in EDTA tubes, and 4 mL was collected in anticoagulant free tubes from all volunteers at 0, 0.5, 1, 2, and 4 h postingestion of BJs and Mk. Urine was collected prior to consumption and over four time periods, 0−1, 1−2, 2−4, and 4−6 h, after BJs and Mk consumption. The total volume for each period was recorded. All samples were stored at −70 °C until analysis. The Ethics Committee of the Faculty of Pharmacy at University of Sao Paulo (Sao Paulo state, Brazil) approved the study design. The protocol was fully explained to all subjects, and informed consent was obtained prior to participation.

Preparation of Plasma and Urine Samples. Plasma was prepared according to the method described by Hassimotto et al. (*21*). The EDTA blood samples were centrifuged at 3000*g* for 10 min at 4 °C. An aliquot of plasma and urine was acidified with 5% trifluoracetic acid (TFA) (1:5, v/v). Serum was obtained after coat formation. The human erythrocyte was washed three times with saline and hemolized with distilled water (1:3, v/v). All samples were stored at −70 °C until analyses.

Anthocyanins and ETs. An aliquot of the acidic plasma (1.5 mL) or urine (10 mL) was diluted with 10 mM of oxalic acid and applied to Sep-Pak C18 cartridges (0.2 g of Supelclean LC-18, Supelco, Bellefonte, PA), which had been washed with methanol containing 5% TFA and equilibrated with 10 mM oxalic acid before use. Impurities were cleaned up with 10 mM oxalic acid. Anthocyanins, ETs, and free ellagic acid (EA) were eluted with methanol containing 5% TFA. The eluate was evaporated to dryness under reduced pressure at 40 °C, dissolved in methanol:acetic acid (99:5 v/v), and filtered through a 0.22 μm tetrafluoroethylene (PTFE) filter (Milipore Ltd., Bedford, MA) prior to quantification by high-performance liquid chromatography (HPLC). Total plasma anthocyanin was expressed as micomoles of cyanidin aglycone equivalent/milliliter of plasma. ETs were quantified as total EA as described below.

Total Ellagic Acid Content. After anthocyanins HPLC analysis the methanolic solution obtained above was dried under N_2 and hydrolyzed with 2 N trifluoracetic acid (TFA) at 120 °C for 1 h, as described by Pinto et al. (*22*). The solution was evaporated until dryness, resuspended with methanol, filtered through a 0.22 μm filter, and analyzed by HPLC. The results were expressed as micromoles of total free EA/milliliters of plasma.

HPLC Analysis. Identification and quantification of anthocyanins and EA were achieved using analytical reversed-phase HPLC in a Hewlett-Packard 1100 system with autosampler and quaternary pump coupled to a 1100 DAD (Hewlett-Packard, Palo Alto, CA). The column used was a Prodigy 5 μm ODS3 (250 mm × 4.6 mm i.d., Phenomenex Ltd.), and elution solvents were (A) water:tetrahydrofuran:trifluoroacetic acid (98:2:0.1) and (B) acetonitrile. The solvent gradient consisted of 8% B at the beginning, 10% at 5 min, 17% at 10 min, 25% at 15 min, 50% at 25 min, 90% at 30 min, 50% at 32 min, 8% at 35 min (run time, 35 min). Eluates were monitored at 270 and 525 nm. Flow rate was 1 mL/min, column temperature was 30 °C, and injection volume was 40 μL. Calibration was performed by injecting the standards three times at five different concentrations ($R^2 > 0.999$). Peak identification was performed by comparison of retention times and diode array spectral characteristics with the standards and library spectra. Cochromatography was used when necessary.

Determination of Antioxidants. Ascorbic acid (AA) content was determined according to Rizzolo et al. (*23*) with modification. Plasma was deproteinated with 5% metaphosphoric acid (0.3% w/v) and analyzed by HPLC in a Hewlett-Packard 1100 system with an autosampler and a quaternary pump coupled to a diode array detector. The column used was a μ-Bondapack (300 mm × 3.9 mm i.d., Waters, Milford, MA), and elution (flow rate of 1.5 mL/min) was performed in isocratic condition with 0.2 M sodium acetate/acetic acid buffer (pH 4.2), monitored at 262 nm. Total AA was estimated after reduction of dehydroascorbic acid (DHA) with 10 mM dithiothreitol. The results were expressed as micromoles/milliliter of plasma.

α-Tocopherol content was determined according to the method described by Teissier et al. (*24*). Plasma (0.1 mL) was precipitated using methanol/BHT (0.8 mL) and analyzed by HPLC in a Hewlett- Packard 1100 system with an autosampler and a quaternary pump coupled to a diode array detector. The column used was a Synergy (250 mm × 4.6 mm i.d., Phenomenex Ltd.), and elution (flow rate of 1.0 mL/min) was performed in isocratic condition with methanol, monitored at 292 nm. The concentration was determined using α-tocopherol as standard. The results were expressed as micromoles/milliliter of plasma.

Urate. The urate content in serum and urine samples was performed by colorimetric method using a kit (Labtest Uric Acid Kit, Labtest Diagnostica S/A). The results were expressed as micromoles/milliliter of serum or urine.

Enzymatic Activity. All enzymatic assays in plasma and erythrocyte were carried out at 37 °C using a Hewlett-Packard 8453 UV−vis DAD Spectrophotometer. Catalase (CAT) activity was determined according to Aebi (*25*). Briefly, catalase activity was expressed as the first-order kinetic constant of the rate of disappearance of 10 mM H_2O_2 as

measured by absorbance at 240 nm. Changes in absorbance were measured after 30 s from addition of H_2O_2 and during 4 min in 60 s intervals. Glutathione peroxidase (GPx) activity was determined according to the method described by Günzler and Flohé (*26*). GPx catalyzes the oxidation of glutathione by *tert*-butyl hydroperoxide. In the presence of glutathione reductase and NADPH the oxidized glutathione is converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$, which is reflected as a decrease in the absorbance at 340 nm. Changes in the absorbance were measured during 6 min in 60 s intervals. All enzymatic assays were expressed as Activity unit (UA-corresponding to 0.1 absorbance change) per minute per milligram of protein or milligram of Hemoglobin (Hb). Protein concentration in plasma was determined by the method of Lowry et al. (*27*). Hb was measured using Drabkins reagent (Sigma Diagnostics, Poole, U.K.).

Plasma and Urinary Antioxidant Capacity. Plasma and urinary antioxidant capacity was measured by two methods: DPPH free radical scavenging activity and ORAC (oxygen radical absorbance capacity). The antioxidant capacity for both methods was expressed as micromoles of Trolox equivalent/milliliter of plasma or urine.

DPPH Free Radical Scavenging Activity. The DPPH assay was performed according to the method described by Brand-Williams et al. (*28*) with modifications (*29*). A 0.1 mM solution of DPPH (1,1-diphenyl-2-picrylhydrazyl) in methanol was prepared. An aliquot of 40 μL of deproteinated sample was added to 250 μL of this solution. The decrease in absorbance was determined at 517 nm using a microplate spectrophotometer (Benchmark Plus, Biorad, Hercules, CA) when the reaction reached a plateau (after 20 min).

ORAC assay was performed according to Cao et al. (*30*). Briefly, in the final assay mixture (2 mL) 1.2 mL of fluorescein was used as a target of free radical damage, 0.6 mL of AAPH (4 mM) as a peroxyl radical generator, and Trolox as a control standard. The analyses were performed using a spectrophotometer with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Final results were calculated using the differences of the areas under the quenching curves of fluorescein between a blank and a sample.

Statistical Analysis. Data were expressed as means ± SD of six subjects. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($p < 0.05$) between the means between times after administration were analyzed by the Tukey test.

RESULTS AND DISCUSSION

Blackberry juices (BJs) in two different preparations, with water (BJW) and defatted milk (BJM), were administrated to subjects in order to evaluate the possible effects of food matrix on the plasma antioxidant capacity and enzymatic and nonenzymatic antioxidants. Defatted milk (Mk) was also provided to the subject in order to eliminate the milk compounds influence on the biochemical parameters evaluated.

Plasma Endogenous and Exogenous antioxidants. Figure 1 shows the content of the majors antioxidants, cyanidin (cy), total ellagic acid (EA), ascorbate (AA), α-tocopherol, and urate that could contribute to the plasma antioxidant capacity. A significant increase in the AA level between 0 to 4 h (**Figure 1D**) after intake of both BJs was observed, ranging from 0.04 ± 0.01 to 0.08 ± 0.02 μmol/mL for BJW, from 0.05 ± 0.01 to 0.08 ± 0.02 μmol/mL for BJM, and from 0.07 ± 0.01 to 0.07 ± 0.01 μmol/mL for Mk. The area under the curve ($AUC_{0\rightarrow 4\ h}$) were 0.11 ± 0.06 (BJW), 0.09 ± 0.03 (BJM), and 0.009 ± 0.028 μmol h mL^{-1} (Mk), and no significant difference ($p < 0.05$) was observed between AUC for BJW and BJM; however, a significant difference among both BJs and Mk was observed. The vitamin C content of the blackberry was 14 ± 1 mg/100 g FW, and around 60% was found as dehydroascorbic acid (DHA), indicating that even the oxidized form could be bioavailable. Jacob et al. (*31*) demonstrated that administration of DHA-rich fruits as cherries increased the plasma AA levels

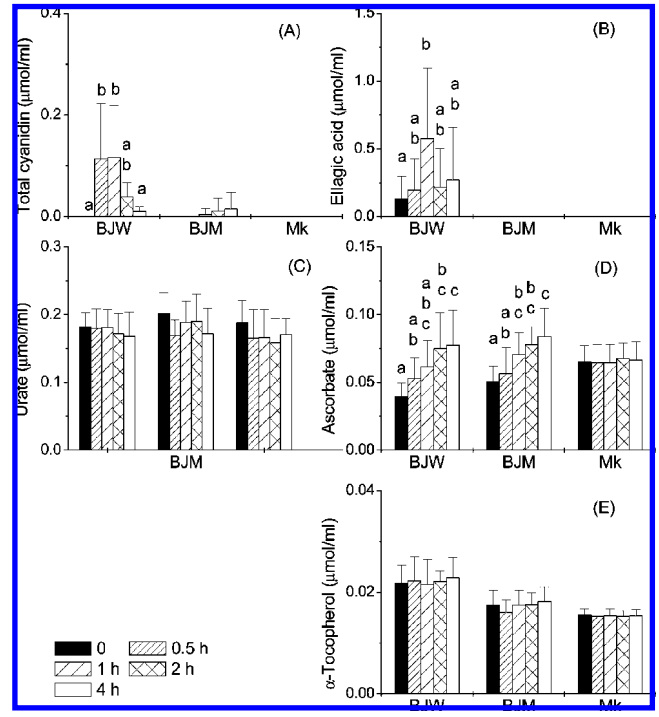

Figure 1. Plasma antioxidants levels at 0, 0.5, 1, 2, and 4 h after intake of blackberry juices prepared with water (BJW) and defatted milk (BJM) and only defatted milk (Mk). Cyanidin aglycone (A), total ellagic acid (B), urate (C), ascorbate (D), and α-tocopherol (E): mean ± SD ($n = 6$ subjects). Different letters mean significant differences ($p < 0.05$) among time in the same treatment. Letter absence means no significant difference.

in humans, suggesting that DHA is bioavailable. In spite of the lower in vitro antioxidant capacity of DHA than the reduced form, DHA is reported to be immediately reduced within erythrocytes to L-ascorbic acid by a glutathione-mediated pathway (*32, 33*), and therefore, its absorption at oxidized form has a physiological importance.

The plasma levels of α-tocopherol and urate had no significant increase after 4 h of intake of both BJs and Mk (**Figure 1C** and **1E**). The average of plasma level of α-tocopherol was 0.022 ± 0.005 (BJW), 0.0173 ± 0.0008 (BJM), and 0.0154 ± 0.0001 μmol/mL plasma (Mk). These results are in accordance with Stahl et al. (*34*), who reported an average from 0.020 to 0.035 μmol/mL. The average of serum urate level was 0.177 ± 0.006 (BJW), 0.18 ± 0.01 (BJM), and 0.17 ± 0.01 (Mk) μmol/mL serum, which are in accordance with the serum normal level of the 0.09 to 0.36 μmol/mL (*34*). The plasma levels of bioactive compounds, such as anthocyanins (**Figure 1A**) and ETs (**Figure 1B**), were quite variable after intake of both BJs. These groups of phenolics were not detected before consumption of both BJs and Mk and even after Mk intake. After ingestion of BJW the anthocyanin average was 0.14 ± 0.09 μmol of cy equivalent/mL of plasma, at 30−60 min postintake, with maximum concentration (C_{max}) ranging from 0.021 ± 0.009 to 0.30 ± 0.08 μmol of cy equivalent/mL of plasma. However, after BJM intake no anthocyanin was detected in plasma samples from four volunteers until 4 h after ingestion. The total area under the curve ($AUC_{0\rightarrow 4\ h}$), calculated from the time-course curve of plasma, was 0.2 ± 0.1 μmol h mL^{-1} for BJW and 0.04 ± 0.05 μmol h mL^{-1} for BJM.

No ET present in the blackberry fruit was found intact in the plasma after consumption of BJW; however, after hydrolysis the amount of free EA increased, suggesting the presence of

Table 1. Antioxidant Capacity (mmol of Trolox equivalent/g of standard) of Purified Antioxidant Compounds Evaluated by DPPH and ORAC Assays

antioxidants	ORAC	DPPH
ellagic acid	0.0111 ± 0.0003	19 ± 2
urate	0.047 ± 0.003	1.6 ± 0.1
tocopherol	2.0 ± 0.3	7.9 ± 0.3
ascorbate	3.3 ± 0.1	7.4 ± 0.6
cyanidin 3-rutinoside	11.1 ± 0.4	3.8 ± 0.1
cyanidin 3-glucoside	12.9 ± 0.2	3.9 ± 0.5
cyanidin	15.2 ± 0.7	4.8 ± 0.5

some metabolized form of ET. Neither free EA nor ETs were detected in plasma after BJM and Mk intake, demonstrating that the defatted milk addition affects their bioavailability.

Plasma Antioxidant Capacity. Table 1 shows the antioxidant capacity of the purified antioxidant compounds. Among the antioxidants analyzed in the present study it was observed that the antioxidant that had high antioxidant capacity by one assay had low antioxidant capacity by the other one. It is known that both methods evaluate the antioxidant capacity of lipophilic and hydrophilic compounds; however, the nature of the solvent used in both methods can contribute to efficiency of the lipophilic compound in DPPH rather than ORAC assay. In ORAC assay cyanidin, cy-3-glucoside, and cy-3-rutinoside presented the highest antioxidant capacity than the most common antioxidants detected in plasma. In addition, among cyanidin derivatives the aglycone form had higher antioxidant capacity than the glycoside one in both methods. The higher value for cy aglycone is probably due to the presence of a *O*-catechol group in the B ring and 3-hydroxyl group, conferring higher stability to the radical formed (*35*). Since in the glycoside form the 3-hydroxyl group is blocked forming the glycosidic linkage with sugars, it explains the lower value of antioxidant capacity. Although urate has been considered the main plasma endogenous antioxidant (*18*), in the present study this compound had the lowest values for antioxidant capacity in both methods. Thus, evaluation of antioxidant capacity depends on the methods used and type of compounds analyzed.

Ellagic acid and α-tocopherol had the highest values for antioxidant capacity in DPPH radical scavenging assay and however the lowest values for ORAC assay. According to Priyadarsini et al. (*36*) EA has high antioxidant capacity, similar to vitamin C and vitamin E; however, its absorption and presence in plasma is unknown since this compound has low water solubility.

The antioxidant capacity for plasma samples was evaluated after intake of BJW, BJM, and Mk by ORAC and DPPH radical scavenging activity assays (**Figure 2A** and **2B**). No significant increase ($p < 0.05$) in the plasma antioxidant capacity after consumption of BJW, BJM, and Mk evaluated by both methods was observed. However, by the ORAC assay, when compared the initial time and 4 h after BJW consumption, an increase of 23% on the antioxidant capacity was observed. The same results were observed by Otaolaurruchi et al. (*37*) after 1 week of wine consumption.

When the antioxidant capacity values in both assays were compared, ORAC had higher values than the DPPH method. Since plasma deproteinization is necessary in the DPPH method, the differences found could be due to the plasma protein contribution in antioxidant capacity when ORAC was used.

A significant increase in the total cy, total EA, and AA contents that could contribute to the increase on the plasma antioxidant capacity observed by the ORAC assay was observed (**Figure 1A**, **1B**, and **1D**). However, after BJW intake no

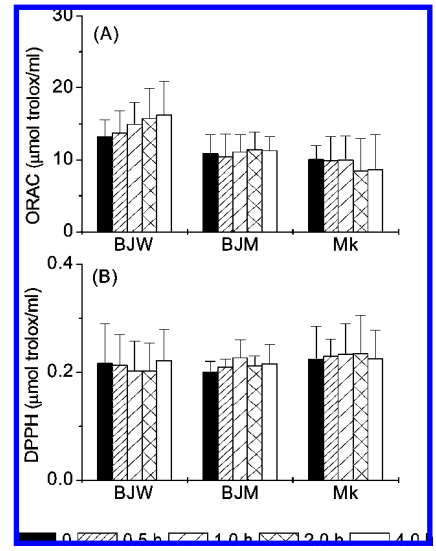

Figure 2. Plasma antioxidant capacity at 0, 0.5, 1, 2, and 4 h after intake of blackberry juices prepared with water (BJW) and defatted milk (BJM) and only defatted milk (Mk) evaluated by ORAC (A) and DPPH (B) assays: mean ± SD ($n = 6$ subjects). Letter absence means no significant difference ($p < 0.05$).

correlation was observed between antioxidant capacity and total EA ($r = 0.27$), α-tocopherol ($r = 0.30$), and total cy ($r = -0.09$). On the other hand, a good correlation was found between antioxidant capacity and AA levels ($r = 0.93$) and a negative correlation with urate levels ($r = -0.79$). Thus, the increase on the plasma antioxidant capacity after BJW consumption could be attributed mainly to the increase in the AA levels and not to other antioxidants considered in this study, such as anthocyanins and ET, present in high amounts in blackberry fruits.

Ascorbate and urate are considered important endogenous antioxidants; however, the contribution from the bioactive compounds should be taken into account, although the influence of food-rich anthocyanins consumption in the plasma antioxidant capacity is ubiquitous. Many authors have reported an increase on the plasma antioxidant capacity after consumption of red wine dealcoholized (*38*), lyophilized blueberries (*17*), and red grape juice (*39*). Hassimotto et al. (*21*) also reported a significant increase on the plasma antioxidant capacity after administration of blackberries to rats, correlating this effect to the plasma anthocyanin levels, despite the low absorption of these compounds. However, Lotito and Frei (*18*) attributed the increase on the plasma antioxidant capacity after consumption of apples to the presence of fructose and its well-known metabolic effect on urate levels and not only to the flavonoids or AA. Fructose has been described to increase the urate levels due to the rapid fructokinase-mediated metabolism to fructose 1-phosphate that leads to the AMP degradation to urate (*40*). Thus, these authors suggested that the fructose present in apples lead to the change in the serum urate contents and, consequently, the increase on the antioxidant capacity. However, Jacob et al. (*31*) reported an increase on the plasma antioxidant capacity and decrease on the urate levels after consumption of cherries, correlating with the bioactive compounds absorbed.

In the present study, no change in the urate levels was observed after consumption of BJs or Mk and the fructose amount ingested (20 g of fructose/50 kg of body weight) was lower than that reported by Lotito and Frei (*18*).

Urinary Antioxidant Capacity. Figure 3 shows the urinary antioxidant capacity evaluated by DPPH and ORAC assays after

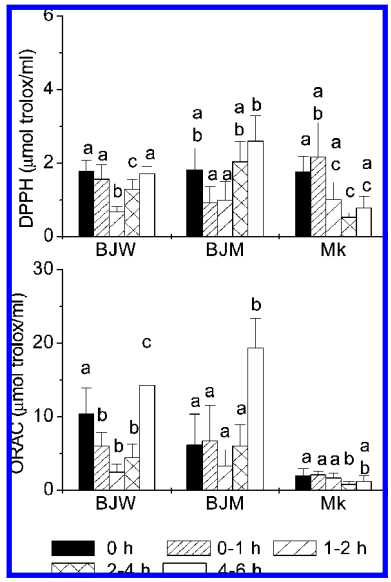

Figure 3. Urinary antioxidant capacity after intake of blackberry juice with water (BJW) and defatted milk (BJM) and only defatted milk (Mk) evaluated by DPPH (A) and ORAC (B) assays. Analyses were performed prior to and at 0−1, 1−2, 2−4, and 4−6 h after intake: mean ± SD ($n = 6$ subjects). Different letters mean a significant difference ($p < 0.05$) among time in the same treatment.

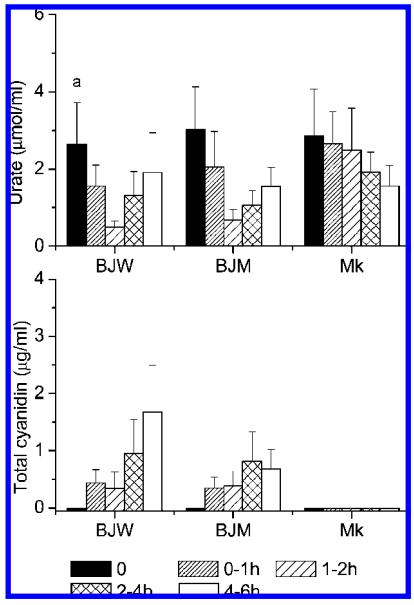

Figure 4. Urinary urate and total cyanidin levels before and at 0−1, 1−2, 2−4, and 4−6 h after intake of blackberry juice prepared with water (BJW) and defatted milk (BJM) and only defatted milk (Mk): mean ± SD ($n = 6$ subjects). Different letters mean a significant difference ($p < 0.05$) among time in the same treatment. Letter absence means no significant difference.

consumption of BJW, BJM, and Mk. A significant decrease ($p < 0.05$) in the antioxidant capacity, in both methods, between 1 and 4 h after the intake of BJs was observed followed by the increase after 4 h intake. The increase on the antioxidant capacity was coincident with the liberation of fasting. In this case, the subjects were allowed to eat but maintaining the restriction of the flavonoid-rich foods. Consumption of the foods from diet after 4 h intake increased the urate excretion (**Figure 4A**). The maximum level of anthocyanin excretion was observed from 2 to 4 h after intake of both BJs (**Figure 4B**). A good correlation was found between antioxidant capacity and total anthocyanin

Table 2. Correlation Coefficient (r) between Urinary Antioxidant Capacity (ORAC and DPPH assays), Urate, and Total Cyanidin (cy) Levels after Consumption of Blackberry Juices Prepared with Water (BJW) and Defatted Milk (BJM)

	BJW		BJM	
	ORAC	DPPH	ORAC	DPPH
urate	0.77	0.99	0.68	0.004
total cy	0.89	0.67	0.37	0.83

excreted and urate levels (**Table 2**). In spite of anthocyanins being quickly excreted after intake, a previous study demonstrated an increase in urine antioxidant capacity after consumption of fruit preparations (*41*), although another study reported a lack of antioxidant effect after consumption of açai preparation (*42*).

Plasma and Erythrocyte CAT and GPx Activities. Figure 5 shows the plasma CAT activity after intake of BJW, BJM, and Mk. A significant increase ($p < 0.05$) in the CAT activity between 30 min and 2 h after consumption of BJs, BJW (five subjects from six), and BJM (three subjects from six) was observed, coincident with the increase on the plasma anthocyanin levels. The highest increase (8 and 12 times) was observed for volunteers V3 and V5, respectively. The same subjects had an increase about three and seven times when compared to the basal level in the plasma CAT activity, respectively, after intake of BJM. However, no change ($p < 0.05$) in the plasma CAT activity was observed after consumption of Mk (**Figure 5**).

Many flavonoids have been shown to modulate CAT activity by binding to the heme group or a protein region of CAT structure contributing to enhancement of activity. Doronicheva et al. (*43*) reported that flavanols from green tea partially suppressed CAT activity, and flavone and flavonols could exert a positive effect on the CAT activity. In addition, oral administration of naringin to rats submitted to a severe oxidative stress leads to a significant increase on the myocardium SOD and CAT activities (*44*). However, Doronicheva et al. (*43*) demonstrated that dadzein (isoflavone) and naringenin (flavanone) had no effect on the CAT activity. Pedraza-Chaverrí et al. (*45*) reported that supplementation to rats of a garlic extract decreased significantly the liver and kidney CAT activity and expression. This decrease on the CAT expression could be associated with the direct antioxidant effect of garlic phytochemicals during the post-transcriptional events (*46*).

In the present study, the increase observed on the plasma anthocyanin levels, after consumption of both BJs, could be

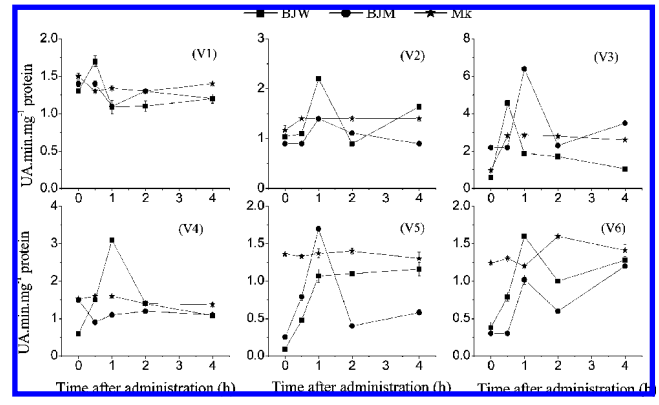

Figure 5. Plasma CAT activity (UA min mg^{-1} protein) before and after intake of blackberry juice prepared with water (BJW) and defatted milk (BJM) and only defatted milk (Mk). Values expressed by subjects (V1−V6).

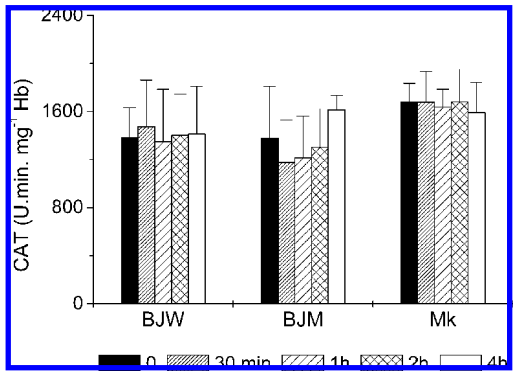

Figure 6. Erythrocyte CAT activity (CAT) at 0, 0.5, 1, 2, and 4 h after intake of blackberry juice prepared with water (BJW) and defatted milk (BJM) and only defatted milk (Mk): mean ± SD ($n = 6$ subjects). Letter absence means no significant differences ($p < 0.05$) among time in the same treatment.

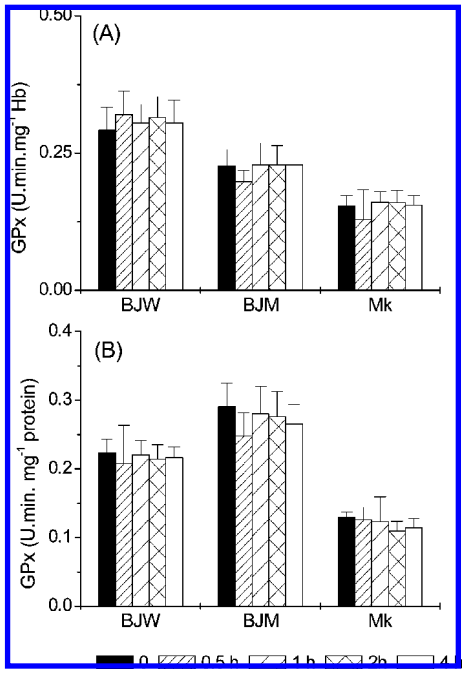

Figure 7. Erythrocyte (A) and plasma (B) GPx activity at 0, 0.5, 1, 2, and 4 h after intake of blackberry juice prepared with water (BJW) and defatted milk (BJM) and only defatted milk (Mk): mean ± SD ($n = 6$ subjects). Letter absence means no significant differences ($p < 0.05$) among time in the same treatment.

correlated with the increase in the plasma CAT activity since modulation of expression is improbable due to the acute experiment.

No significant difference ($p < 0.05$) was observed on the erythrocyte CAT activity for all subjects after intake of both BJs and Mk (**Figure 6**). Also, there were no significant differences on the plasma and erythrocyte GPx activities after intake of both BJs and Mk (**Figure 7**). The basal levels for plasma GPx activity were 0.22 ± 0.02 (BJW), 0.29 ± 0.03 (BJM), and 0.129 ± 0.008 (Mk) UA min mg^{-1} protein. The basal levels for erythrocyte GPx activity were 0.29 ± 0.04 (BJW), 0.23 ± 0.03 (BJM), and 0.15 ± 0.02 (Mk) UA min mg^{-1} Hb.

In conclusion, blackberry juices consumption led to an increase in plasma and urine antioxidant capacities, which could indicate the in vivo antioxidant potential. In plasma, this effect was more associated with the increase in the ascorbate level and not with polyphenols present in blackberry fruits. However, in the urine the antioxidant capacity was associated with the anthocyanins and urate levels. Therefore, an increase in the plasma CAT activity coincident with the plasma anthocyanins levels was observed. Further studies are necessary to find out which active polyphenols or other compounds are able to mediate the observed physiological effects and assess the optimal doses before recommending their regular consumption.

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