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URINARY EXCRETION OF ANTIOXIDANTS IN HEALTHY HUMANS FOLLOWING QUEEN GARNET PLUM JUICE INGESTION: A NEW PLUM VARIETY RICH IN ANTIOXIDANT COMPOUNDS

MICHAEL NETZEL[1,2,5], KENT FANNING[2,6], GABRIELE NETZEL[1], DIMITRIOS ZABARAS[3], GLORIA KARAGIANIS[2], TONY TRELOAR[2], DOUGAL RUSSELL[4] and ROGER STANLEY[2]

[1]CSIRO Food and Nutritional Sciences, Coopers Plains, Qld 4108, Australia
[2]Innovative Food Technologies, Agri-Science Queensland, Department of Employment, Economic Development and Innovation, Coopers Plains, Qld 4108, Australia
[3]CSIRO Food and Nutritional Sciences, North Ryde, 2113, NSW, Australia
[4]Horticulture and Forestry Sciences, Agri-Science Queensland, Department of Employment, Economic Development and Innovation, Applethorpe, Qld, 4380, Australia

[5]Corresponding author. TEL: 617-3214-2172; FAX: 617-3214-2062; EMAIL: michael.netzel@csiro.au;
[6]TEL: 617-3276-6011; FAX: 617-3216-6591; EMAIL: kent.fanning@deedi.qld.gov.au

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ABSTRACT

In recent years, there has been intense interest in the potential health benefits of dietary derived plant polyphenols and antioxidants. A new variety of *Prunus salicina*, Queen Garnet plum (QGP), was developed as a high anthocyanin, high antioxidant plum, in a Queensland Government breeding program. Following consumption of 400 mL QGP juice (QGPJ; 1,117 mg anthocyanins) by two healthy male subjects, QGP anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) were excreted mainly as methylated and glucuronidated metabolites in urine (0.5% of the ingested dose within 24 h). Furthermore, QGPJ intake resulted in a threefold increase in hippuric acid excretion (potential biomarker for total polyphenols intake and metabolite), an increased urinary antioxidant capacity and a decreased malondialdehyde excretion (biomarker for oxidative stress) within 24 h as compared with the polyphenol-/antioxidant-free control. Results from this pilot study suggest that metabolites, and not the native QGP anthocyanins/polyphenols, are most likely the bioactive compounds *in vivo*.

PRACTICAL APPLICATIONS

In recent years, there has been increasing consumer interest in antioxidant-rich foods, which have guided breeders of different crops to consider antioxidant compounds, such as anthocyanins and other polyphenols, as interesting targets in breeding programs. In this manuscript, we are presenting for the first time (1) data about the outstanding antioxidant and anthocyanin content of Queen Garnet plum (QGP), a new variety of *Prunus salicina*, which was developed in a Queensland Government breeding program, and (2) data about the absorption and metabolism of QGP anthocyanins/antioxidants in healthy humans as assessed by their urinary excretion. The results of this initial work indicate that QGP and/or derived products could represent a new and promising source of anthocyanins as natural ingredients and colorants for the food and nutraceutical industries.

INTRODUCTION

In recent years, there has been much interest in developing fruit and vegetable cultivars with increased levels of various phytochemicals such as polyphenols, for increased health benefits and/or as a source for food and nutraceutical ingredients (Botella-Pavia and Rodriguez-Concepcion 2006; Kopsell and Kopsell 2006).

The present study describes, for the first time, a new variety of the Japanese plum *Prunus salicina* Lindl., named Queen Garnet plum (QGP), which was developed within a Queensland Government breeding program. The strategy of this breeding program was to produce a plum with an outstanding antioxidant capacity and anthocyanin content. Anthocyanins, a polyphenol subclass, are one of the most abundant phenolic compounds in nature and are responsible for the red, purple and blue colors of many fruits and vegetables, including plums. Anthocyanins also provide the food industry with natural replacements for some synthetic food colorants (Downham and Collins 2000; Kumar and Sinha 2004).

The six anthocyanidins (aglycons) commonly found in plants are classified according to the number and position of hydroxyl and methoxyl groups on the flavan nucleus and are named pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Mazza and Kay 2008). *P. salicina* has been shown to contain significant levels of anthocyanins, predominantly cyanidin-3-glucoside and cyanidin-3-rutinoside (Mazza and Miniati 1993; USDA 2007). Numerous studies, mostly *in vitro*, and some animal experiments, have demonstrated a broad range of biological properties for anthocyanins, including antioxidant, anti-inflammatory, antimicrobial and anticarcinogenic activities (Mazza and Kay 2008). Their daily intake has been estimated at between 180 and 215 mg in the U.S.A. and 82 mg in Finland, which is considerably higher than the intake estimated for many other polyphenols (Mazza and Kay 2008). Anthocyanins must be bioavailable in some form to exert their biological effects. However, detailed information on the absorption and metabolism of anthocyanins from raw and processed food is still limited. Several studies have found that anthocyanins are absorbed as intact glycosides and appear in blood and urine, whereas some other studies have found anthocyanins to be present mainly as metabolites (for overview see Mazza and Kay (2008)). To date, no animal or human bioavailability study has been performed with QGP or QGP products.

In this study, we present for the first time data about the anthocyanin composition and antioxidant capacity of QGP (compared with several other commercial varieties) and QGP juice (QGPJ) as well as the absorption and metabolism of QGP anthocyanins in healthy humans as assessed by their urinary excretion. Furthermore, we evaluated the effect of QGPJ ingestion on the urinary antioxidant capacity and the concentration of malondialdehyde, a biomarker for oxidative stress (Korchazhkina *et al*. 2003). Hippuric acid, as a potential biomarker for total polyphenols intake (Dupont *et al*. 2002) as well as ascorbic acid and uric acid as important antioxidant compounds in biological fluids (Serafini *et al*. 1998; Ghiselli *et al*. 2000), were also quantified in the urine samples.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all chemicals were purchased from Merck (Kilsyth, Vic., Australia) or Sigma-Aldrich (Castle Hill, NSW, Australia), and were of analytical or high performance liquid chromatography (HPLC) grade. Deionized water was used throughout. Cyanidin-3-glucoside and cyanidin-3-rutinoside were purchased from ChromaDex (Irvine, CA).

Antioxidant and Anthocyanin Screening

Fruit. In the 2003/2004 growing season, eight commercial varieties of Japanese plum (*P. salicina*) were compared with the QGP for anthocyanin content and antioxidant capacity. All plums were collected from Applethorpe Research Station (Queensland, Australia). Between 4 and 10 individuals of each sample were tested. The plums were frozen upon ripening and stored in ziplock bags at −20C prior to analysis.

Extraction. Each plum was diced separately, weighed and homogenized in a food processor for 1 min with an equal quantity of water. The crushed samples were centrifuged at 10,621 g for 15 min, to separate the fiber and skin, collectively referred to as the pulp, from the juice. The supernatant was removed by filtering through Whatman paper (GE Healthcare Australia, Rydalmere, NSW, Australia) and stored at −20C for later testing. Residual juice was pressed from the pulp manually and stored, and the remaining pulp was sealed in tubes with 50% ethanol solution (v/v) on a 1:3 (w/v) basis and placed on an automated shaker overnight. The juice and pulp extracts were stored at −20C prior to antioxidant and anthocyanin assay.

Antioxidant Capacity. Antioxidant capacity was measured using the 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) reduction assay as described by Re *et al*. (1999). The antioxidant levels in the pulp and juice extracts were measured against a standard curve of 0–0.7 mM ascorbic acid and expressed as ascorbic acid equivalents (AAE) per 100 g of fresh weight (FW). The stock solution was diluted with water to produce a working solution with an A_{734} between 0.98 and 1.02. For the assay, 50 μL of diluted sample extracts were added to 1,000 μL of ABTS working solution, mixed and left in the dark for 45 min, at room temperature, prior to reading the absorbance at 734 nm.

Anthocyanins. All plum extracts were prepared and analyzed for anthocyanin content as described below for QGPJ.

TABLE 1. ADMINISTERED DOSES OF ANTIOXIDANTS BY ANALYSIS

	QGPJ (400 mL)
Antioxidant capacity	
FRAP (mmol Fe^{2+})	32.6
ORAC (mmol TE)	15.6
Total phenolics (g GAE)	2.66
Anthocyanins (g)	
Cyanidin-3-glucoside	0.827
Cyanidin-3-rutinoside	0.290
Total anthocyanins	1.117

Note: Anthocyanins, calculated as cyanidin-3-glucoside equivalents. FRAP, ferric-reducing antioxidant power; ORAC, oxygen radical absorbance capacity; GAE, gallic acid equivalents; TE, Trolox equivalents.

Effect of Harvest Date on Anthocyanin Content. In 2008, the effect of harvest date on anthocyanin content was examined over a 5-week period. QGP were harvested weekly over a 35-day period and then stored at −20C until extraction and analysis were undertaken as described.

QGPJ Preparation. QGP which had been stored at −20C was thawed and manually halved and de-pitted. QGP was then placed into a bowl chopper for 30 s and then heated in a steam vat to 50C before enzyme treatment with a commercial pectinase (PectinexMash, 200 ppm, Novozymes, Sydney, NSW, Australia). The QGP puree was incubated at 50C for 1 h before being juiced in a Browns screw press (Brown, Winter Haven, FL). Collected QGPJ was then pasteurized (by heating to 80C and holding for 5 min) and stored at −10C prior to ingestion. Microbial analysis (plate count) was undertaken on QGPJ to ensure juice was fit for human consumption.

Pilot Study (Urine)

Study Design. Ethical permission was obtained from the CSIRO Human Experimentation Ethics Committee (code 08/22). Two healthy, nonsmoking male volunteers were recruited with ages of 26 and 41 years and a mean body mass index of 24.1 ($\pm$ 2.1 kg/m^2). Participants adhered to their usual diet, but abstained from food and beverages rich in polyphenols and ascorbic acid from 24 h prior to treatment. Alcohol and medication, including over the counter drugs, were refrained from during the course of the pilot study. Both subjects had the following experimental treatments by which each subject served as his own control: 400 mL of QGPJ (Table 1) or 400 mL of water as an antioxidant-free control beverage separated by a 1-week washout phase. At 8:00 a.m. after an overnight fast, volunteers took 400 mL of one of the two beverages, respectively, together with white bread rolls. During the experimental periods, only the consumption of water and of three further standardized meals (white bread rolls with cheese for lunch, afternoon snack and dinner) was allowed. Urine samples were collected quantitatively predose and in six intervals up to 24 h after dosing (0–2, 2–4, 4–6, 6–8, 8–10 and 10–24 h). Aliquots of 7 mL and 1 mL, respectively, were acidified and stabilized with concentrated formic acid (1:1.3) for anthocyanin analyses and with 5% metaphosphoric acid (10:1) for ascorbic acid and uric acid analysis. The sample material and aliquots were stored frozen at −80C until assayed.

Analysis of Anthocyanins in QGPJ. QGPJ was diluted 1:100 with HPLC mobile phase A (water/formic acid/acetonitrile, 87:10:3, v/v/v) and filtered through a 0.45-μm GHP Acrodisc Minispike filter (Pall Life Sciences, MI) before injection into the HPLC. The juice was analyzed in triplicate with the HPLC method and system described below.

Analysis of Anthocyanins, Anthocyanin Metabolites and Hippuric Acid in Urine. Urinary anthocyanins were extracted with a solid-phase extraction (SPE) cartridge (Sep-Pak C18, Waters Corporation, Milford, MA) according to Felgines *et al.* (2003) with slight modifications. Acidified urine samples were thawed and maintained for 60 min at room temperature before SPE extraction to obtain the maximal yield of the colored flavylium cations. The SPE cartridge was activated with 10 mL of methanol and equilibrated with 10 mL of 12 mM aqueous HCl before use. Subsequently, 9 mL of acidified urine was applied to the equilibrated cartridge. The cartridge was then washed with 10 mL of 12 mM aqueous HCl, and anthocyanins were eluted with 15 mL of 12 mM HCl in methanol. The methanolic extract was evaporated under nitrogen to a volume of 1 mL by use of a Rotavapor R-210 (Buchi, Flawil, Switzerland) at 35C. Aliquots of 1 μL (for identification of anthocyanins and anthocyanin metabolites) and of 50 μL (for quantification) were used for HPLC.

Analysis of anthocyanins and anthocyanin metabolites was carried out by HPLC-photodiode array detector (PDA) according to the method of Kammerer *et al.* (2004) with slight modifications. The HPLC system consisted of a 600E multisolvent delivery system (pump), in-line degasser, 2,996 PDA, 717 plus auto-injector and 600 system controller (Waters Corporation) equipped with an Aqua Luna C18(2) (250 × 4.6 mm) reversed phase column with a particle size of 5 μm (Phenomenex, Lane Cove, NSW, Australia) protected by a Phenomenex 4.0 × 3.0 mm C18 ODS guard column. The column was operated at a temperature of 25C. The mobile phase consisted of water/formic acid/acetonitrile (87:10:3, v/v/v; eluent A) and of acetonitrile/water/formic acid (50:40:10, v/v/v; eluent B). The gradient program was as follows: 10% B to 25% B (10 min), 25% B to 31% B (5 min), 31% B to 40% B (5 min), 40% B to 50% B (10 min), 50% B to

100% B (10 min), 100% B (5 min. isocratic), 100% to 10% B (1 min), and 10% B for 5 min before injecting the next sample. The injection volume was 50 μL and samples were filtered through a 0.45-μm GHP Acrodisc Minispike filter prior to injection. Detection was carried out at 520 nm at a flow rate of 0.8 mL/min. Anthocyanins and individual anthocyanin metabolites were quantified by comparison of their peak areas with an external calibration curve obtained using known concentrations of cyanidin-3-glucoside (Chandra *et al.* 2001; Kay *et al.* 2004; Netzel *et al.* 2007a). Results were expressed as micrograms cyanidin-3-glucoside equivalents per 24 h (μg cyanidin-3-glucoside equivalents/24 h).

Hippuric acid was analyzed and quantified according to the HPLC method reported by Kubota *et al.* (1988) with slight modifications. Untreated urine was diluted with deionized water (1:200, v/v), filtered through a 0.45-μm GHP Acrodisc Minispike filter and then directly injected onto an Aqua Luna C18(2) column. The samples were eluted under isocratic conditions with water/acetonitrile/acetic acid (78:20:2, v/v/v; pH 2.2), and monitored at 235 nm. Urinary hippuric acid concentrations were calculated from an external hippuric acid calibration curve and expressed as milligrams hippuric acid per 24 h (mg/24 h). The HPLC system used, including the analytical column and guard column, was the same as described for the analysis of anthocyanins and anthocyanin metabolites. Sample aliquots for liquid chromatography-mass spectrometry (LC-MS) analysis were prepared using SPE as described for anthocyanins and anthocyanin metabolites.

LC-PDA-MS analysis was carried out on a Quantum triple stage quadrupole mass spectrometer (ThermoFinnigan, Rydalmere, NSW, Australia) equipped with a quaternary solvent delivery system, a column oven, a PDA and an autosampler. An aliquot (20 μL) of a methanolic solution (250 μg/mL) was chromatographed on a Luna C18(2) column (150 × 2.1 mm, 5 μm particle size; Phenomenex), which was maintained at 25C. The mobile phase consisted of 1% formic acid in water (A) and 1% formic acid in acetonitrile (B) at the rate of 0.3 mL/min (no splitting). A linear gradient was used (10% B to 100% B over 40 min). Ions were generated using an electrospray source in the positive mode under conditions set following optimization using solutions of cyanidin-3-glucoside. MS experiments in the full scan (parent and product specific) and the selected reaction monitoring mode were carried out to confirm the identity of components.

Total Phenolic Content. The total phenolic content in QGPJ and urine was determined using the Folin–Ciocalteu assay (Singleton and Rossi 1965; Schlesier *et al.* 2002) and was performed in a Biochrom Ultraspec 3,100 spectrophotometer (Biochrom, Cambridge, U.K.). Juice and urine samples were diluted with deionized water (1:100 and 1:20, respectively), and were directly assayed at 750 nm with gallic acid serving as a standard. Total phenolic content was expressed as grams gallic acid equivalents (GAE) per 400 mL QGPJ (administered dose; g GAE/400 mL) or milligrams GAE per 24 h (mg GAE/24 h), respectively.

Ferric-Reducing Antioxidant Power (FRAP) Assay. The FRAP assay is a simple and frequently used method to assess the total reducing capacity of samples and was adopted with minor modifications (Benzie and Strain 1996). Thirty microliters of deionized water and 10 μL sample solution (QGPJ or urine) were mixed with 200 μL FRAP reagent consisting of ferric chloride and 2,4,6-tripyridyl-*s*-triazine in acetate buffer. The absorbance was measured after 8 min at 595 nm with a microplate reader (VICTOR3 2030 multilabel counter, Perkin Elmer, Melbourne, VIC, Australia). The reducing capacity was calculated using the absorbance difference between sample and blank and a further parallel Fe(II) standard solution. Results were expressed as mmol Fe^{2+} equivalents per 400 mL QGPJ (mmol Fe^{2+}/400 mL) or mmol Fe^{2+} equivalents per 24 h (mmol Fe^{2+}/24 h), respectively.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC assay for hydrophilic antioxidant compounds was carried out according to Huang *et al.* (2002) with modifications. Samples were assayed on a microplate reader (VICTOR3 2030 multilabel counter, Perkin Elmer) at an excitation wavelength of 490 nm and an emission wavelength of 515 nm. QGPJ and urine samples were diluted (1:1,000 and 1:200, respectively) with 75 mM phosphate buffer (pH 7.0). The assay was carried out in black 96-well flat bottom plates (Thermo Fisher Scientific, Lidcombe, NSW, Australia) and each well had a final volume of 245 μL. The following reactants were added: 200 μL fluorescein working solution (92 μM kept at 37C), 20 μL Trolox standard (6.25–100 μM), blank (phosphate buffer) or diluted sample. Reaction was started by addition of 25 μL 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH; 79 mM) and plates were placed immediately in the fluorescence plate reader. Plates were read every 2 min for 90 min. The AUC (area under the curve, fluorescence intensity versus time) was calculated by using the trapezoidal rule in Excel (Microsoft, Redmond, WA) and results were expressed as millimols Trolox Equivalents per 400 mL QGPJ (mmol TE/400 mL) or millimols Trolox Equivalents per 24 h (mmol TE/24 h), respectively.

Analysis of Ascorbic Acid and Uric Acid. The microfiltered and diluted QGPJ and urine samples (1:25 with 5% metaphosphoric acid) were directly injected onto a Phenomenex Aqua Luna C18(2) column and eluted under isocratic conditions with water acidified with sulfuric acid to pH 2.2

(Ross 1994; Vazquez Oderiz *et al*. 1994). Detection was carried out at 245 nm at a flow rate of 0.8 mL/min. Ascorbic acid (in QGPJ and urine) and uric acid (in urine) were identified by comparing their retention times and characteristic UV-vis spectra with those of synthetic L-ascorbic acid and uric acid, respectively. The concentrations in the samples were directly calculated from external L-ascorbic acid and uric acid calibration curves and were expressed as mg ascorbic acid per 400 mL QGPJ (mg/400 mL) or mg ascorbic acid per 24 h (mg/24 h), respectively, as well as mg uric acid per 24 h (mg/24 h). The HPLC system used, including the analytical column and guard column, was the same as described for the analysis of anthocyanins and anthocyanin metabolites.

Analysis of Malondialdehyde (MDA). MDA concentrations in urine were determined by isocratic reversed phase (RP)-HPLC according to Volpi and Tarugi (1998). Urine was mixed with TBA (2-thiobarbituric acid) and butylated hydroxytoluene and incubated at 95C for 45 min. After cooling on ice, the samples were centrifuged (20,817 g for 10 min), and the supernatants were used for HPLC analysis. The separation of the MDA-TBA complex was performed using a Prontosil Eurobond C18 (125 × 4 mm) reversed phase column with a particle size of 5 μm (Bischoff, Leonberg, Germany) equipped with a LiChrospher 100 RP-18 (4 × 4 mm, 5 μm) guard column (Merck, Kilsyth, Vic., Australia) and a mobile phase composed of 35% methanol and 65% 50 mM disodium phosphate buffer, pH 7.0. The complex was eluted using a flow rate of 0.8 mL/min and monitored by a Waters 2,475 multi-wavelength fluorescence detector (ex. 515 nm; em. 553 nm). Urinary MDA concentrations were calculated from an external MDA calibration curve and expressed as micromoles per 24 h (μmol/24 h). The HPLC system used was the same as described for the analysis of anthocyanins and anthocyanin metabolites.

Data Analysis

All analysis and determinations were performed in duplicate unless otherwise stated. Values are given as mean +/− standard deviation (SD) or as mean and range. The differences in antioxidant capacity and anthocyanin content, for QGP and commercial varieties, were evaluated using one-way analysis of variance and the Tukey HSD procedure using JMP software version 7 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Queen Garnet Plum Anthocyanins and Antioxidants

The anthocyanins present in QGP and QGPJ were identified as cyanidin-3-glucoside (cya-3-glucoside) and cyanidin-3-rutinoside (cya-3-rutinoside) based on retention time (standards), UV-vis spectra and mass spectra. HPLC-ESI-MS data are presented in Fig. 1. Figure 2 shows that QGP had seven times greater anthocyanin content and antioxidant activity than the best commercial performing variety, Satsuma ($P < 0.05$). Similar standout results against commercial varieties were seen in the preceding seasons up until 2008 (data not shown). Anthocyanin content of QGP was seen to increase by 55% over a five week harvest period, from 175 mg cya-3-glucoside equivalents/100 g FW to 272 mg cya-3-glucoside equivalents/100 g FW. An increase in anthocyanin content with extended harvest date has been reported for both *P. salicina* (Diaz-Mula *et al*. 2008) and *P. domestica* (Usenik *et al*. 2009). Thus, QGP should be harvested as late as possible, considering fruit quality parameters, to ensure the highest anthocyanin content. The anthocyanin content in QGP compares favorably with that cited for the anthocyanin-rich berry fruits. Using data from a recent review article by Szajdek and Borowska (2008), QGP had more anthocyanins than strawberry (20–39 mg/100 g FW), raspberry (19–65 mg/100 g FW), cranberry (20–66 mg/100 g FW) and blackberry (135–152 mg/100 g FW) and values in the range of those quoted for blackcurrant (128–411 mg/100 g FW), blueberry (63–331 mg/100 g FW) and bilberry (215–300 mg/100 g FW).

Pilot Study (Urine)

Table 2 shows that there was increased excreted amounts of total antioxidants in the volunteers' urine 24 h after consumption of QGPJ as compared to water, as estimated by the FRAP (+21%), ORAC (+28%) and total phenolics (+16%) assays. Similar results were found in previous studies after consumption of polyphenol-rich beverages and/or food. Price *et al*. (2008) reported a significant increase in urinary total phenolics and antioxidant potential (assayed by FRAP) in 18 healthy subjects after ingestion of wheat bran. Studies by Roura *et al*. (2006, 2007) have shown significant increase in urinary total antioxidant capacity (assayed by Trolox Equivalent Antioxidant Capacity) and increased excretion of total polyphenols following the intake of a polyphenol-rich cocoa shake. Duthie *et al*. (1998) determined total phenolics in urine 4 h after the ingestion of red wine, 12-year-old malt whisky (matured in oak wood casks), or newly distilled whisky which contained a negligible amount of phenolics. They observed significantly more total phenolics in the volunteers' urine who had consumed the red wine and the 12-year-old whisky compared with the volunteers who consumed the virtually phenolic-free newly made spirit. Cao *et al*. (1998) could demonstrate that the total antioxidant activity of urine (eight elderly women) determined as ORAC increased significantly for strawberries, spinach and vitamin C, respectively, during the 24h period following ingestion. It should be noted that Tepel *et al*. (2002) reported in a review

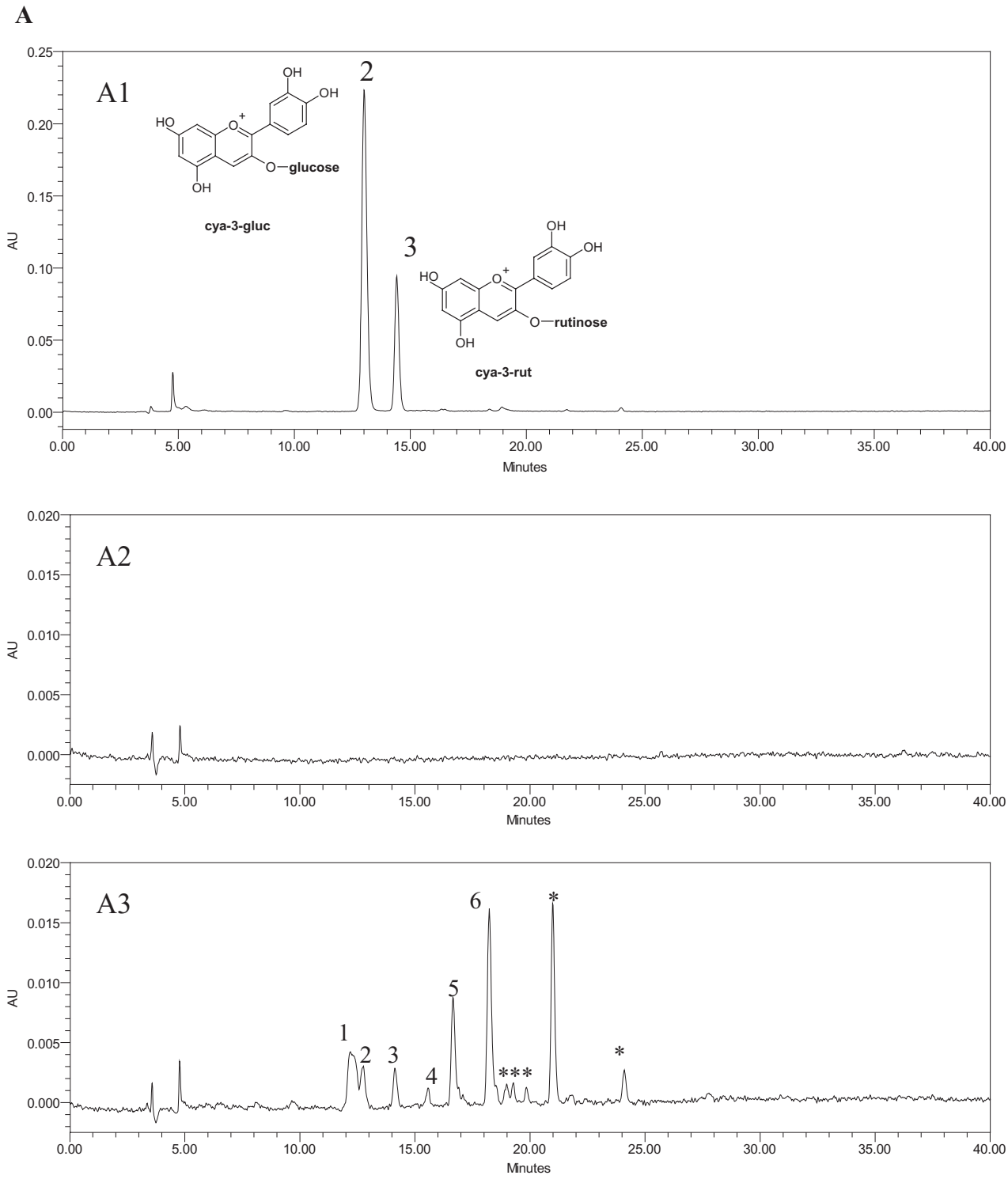

FIG. 1. HPLC CHROMATOGRAMS AND FRAGMENTATION PATTERNS
(A) Anthocyanin profile of the administered QGPJ (A1), and of human urine collected before (A2) and after (A3) the ingestion of 400 mL QGPJ (collection interval 2–4 h); detection was performed at 520 nm. Peak assignment: (1) cyanidin monoglucuronide, (2) cyanidin-3-glucoside, (3) cyanidin-3-rutinoside, (4) peonidin monoglucuronide, (5) peonidin-3-glucoside, (6) peonidin-3-rutinoside and (*) not yet identified metabolites. (B) HPLC-ESI-MS precursor scans for product m/z 287 (A–C) and m/z 301 (D–F) showing QGP anthocyanins and metabolites. Peak numbers in each scan refer to peaks in Fig. 1 (A).

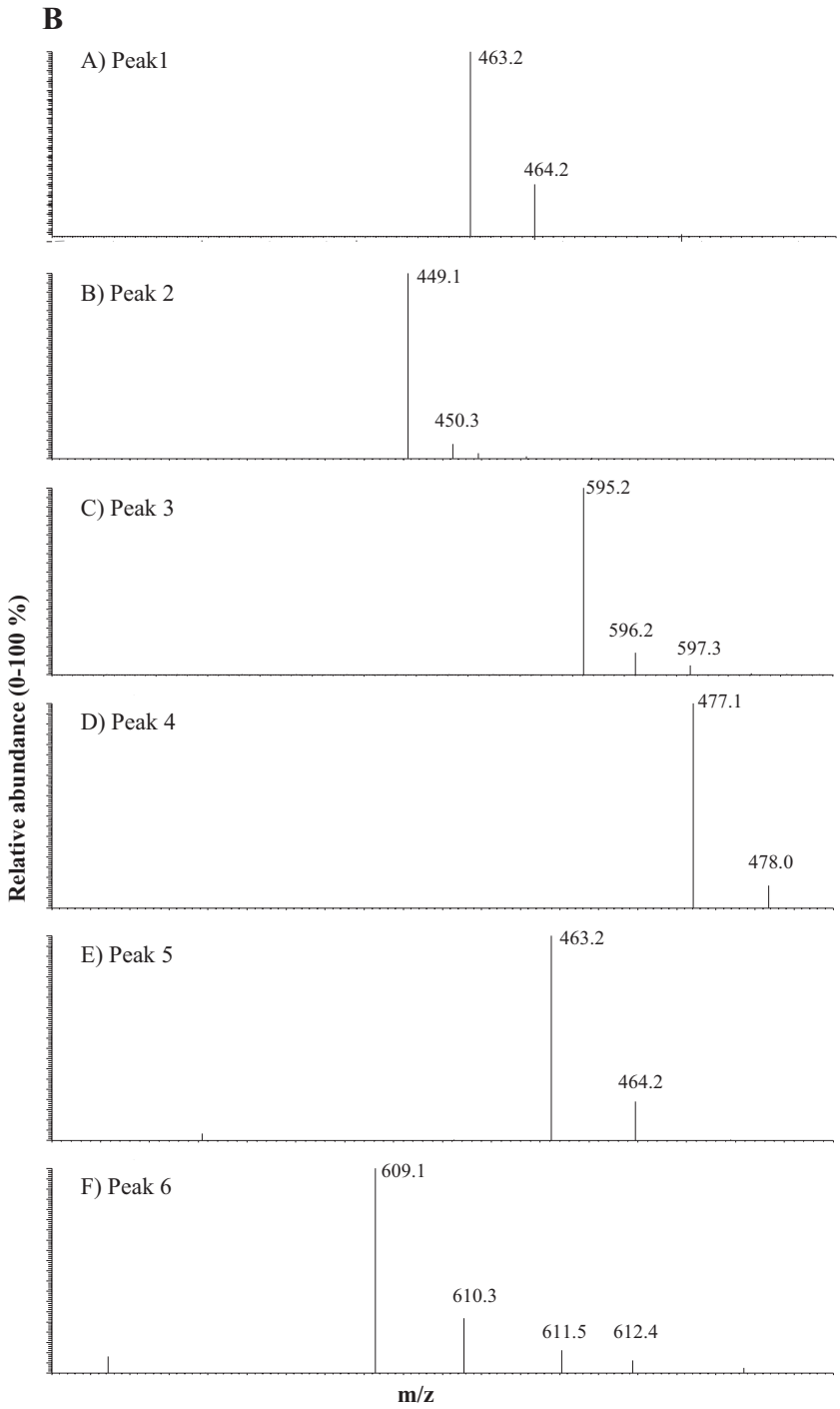

FIG. 1. CONTINUED

about antioxidative therapies and vascular diseases that antioxidants can prevent the acute decrease of renal function caused by ischemia, contrast media or drugs. Therefore, the enhancement of urinary concentration in antioxidants may have physiological relevance regarding the protection of functional renal tissue.

The consumption of QGPJ resulted in the appearance of both native QGP anthocyanins and at least four identified anthocyanin metabolites in the volunteers' urine (Table 3 and Fig. 1). Urinary excretion of each anthocyanin and identified anthocyanin metabolite, respectively, was calculated relative to cya-3-glucoside and expressed as cya-3-glucoside

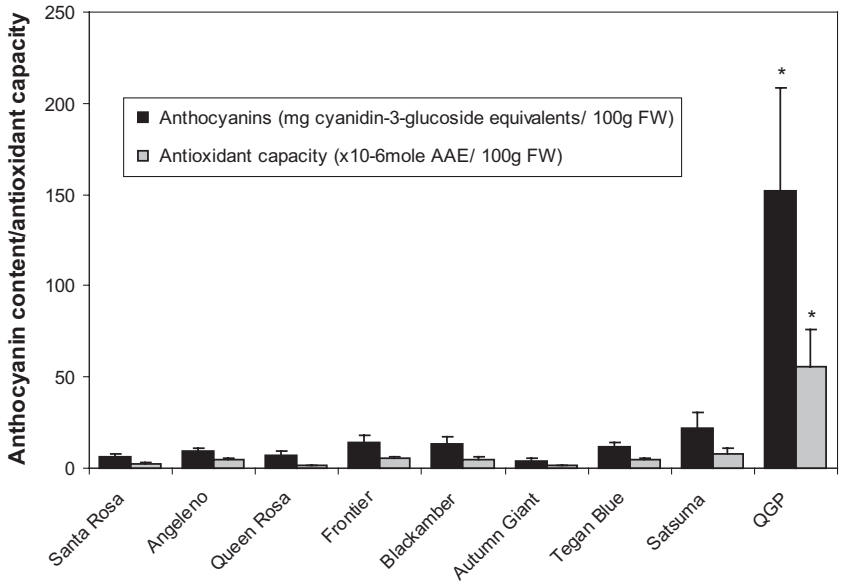

FIG. 2. COMPARISON OF ANTHOCYANIN CONTENT AND ANTIOXIDANT CAPACITY IN PLUM VARIETIES; VALUES ARE MEANS ± SD, $N = 4–10$ (*$P < 0.05$, QGP VERSUS SATSUMA)

equivalents/24 h. Cya monoglucuronide and peonidin-3-rutinoside (peo-3-rutinoside) were the primary anthocyanin metabolites identified in the urine samples accounting for ~20% and ~42% of the identified anthocyanins and metabolites, respectively. Intact cya-3-glucoside and cya-3-rutinoside accounted for ~17%, whereas the sum of the four identified metabolites (cya monoglucuronide, peo monoglucuronide, peo-3-glucoside and peo-3-rutinoside) accounted for ~83% of the total anthocyanin excretion. These results, methylated and glucuronidated derivatives of cyanidin as the main urinary metabolites, are in agreement with several animal and human studies carried out with purified cyanidin compounds or fruit extracts rich in cyanidins (Vitaglione *et al.* 2007; Lehtonen *et al.* 2009). Furthermore, Felgines *et al.* (2003) demonstrated that with six healthy volunteers after consumption of 200 g of strawberries (with pelargonidin-3-glucoside as the main anthocyanin) more than 96% of the excreted anthocyanins were related to pelargonidin metabolites (predominantly glucuronides). In the present study, the urinary recovery of intact anthocyanins and metabolites was 5,587.2 (range: 4,122.7–7,051.6) μg corresponding to 0.50 (range: 0.37–0.63) % of the administered anthocyanin dose. These data are within the range as reported in the literature for urinary excretion rates of anthocyanins and metabolites after ingestion of anthocyanin-rich food (reported range: 0.004–5%; Manach *et al.* 2005; Vitaglione *et al.* 2007). The maximal excretion of cya-3-glucoside and cya-3-rutinoside was observed during the first 2 h (~52% of total anthocyanin excretion) whereas the metabolites' excretion was maximal between 2 and 4 h (~41% of total metabolite excretion) after QGPJ consumption (Fig. 3). It is apparent that the urinary excretion of peo-3-rutinoside continued until the end of the experimental treatment (24 h post-ingestion). A similar excretion pattern was observed by Felgines *et al.* (2003) after the consumption of strawberries rich in pelargonidin-3-glucoside by six healthy volunteers: the maximal excretion of intact pelargonidin-3-glucoside was observed during the first 2 h, whereas the maximal excretion of pelargonidin metabolites was measured between 2 and 4 h after the strawberry breakfast. The rapid peak excretion of the native cyanidin gly-

TABLE 2. URINARY EXCRETION OF ANTIOXIDANTS ASSAYED BY TOTAL PHENOLICS, FRAP, AND ORAC, MALONDIALDEHYDE (MDA), ASCORBIC ACID, URIC ACID AND HIPPURIC ACID FOLLOWING INGESTION OF A SINGLE ORAL DOSE OF 400 mL QGPJ OR 400 mL WATER (CONTROL) IN TWO HEALTHY MALE SUBJECTS

	Control	QGPJ
Antioxidant capacity:		
FRAP (mmol Fe^{2+}/24 h)	7.35 (7.01–7.71)	8.94 (8.51–9.37)
ORAC (mmol TE/24 h)	6.85 (6.45–7.26)	8.88 (8.44–9.33)
Total phenolics (mg GAE/24 h)	521.3 (512.6–529.9)	605.2 (589.5–620.8)
MDA (μmol/24 h)	2.65 (2.43–2.86)	2.21 (2.07–2.35)
Ascorbic acid (mg/24 h)	11.1 (6.7–15.6)	10.6 (4.6–16.5)
Uric acid (mg/24 h)	447.2 (411.2–483.3)	444.5 (410.2–478.8)
Hippuric acid (mg/24 h)	315.5 (265.2–365.8)	951.8 (836.4–1,067.2)

FRAP, ferric-reducing antioxidant power; ORAC, oxygen radical absorbance capacity; GAE, gallic acid equivalents; TE, Trolox equivalents; data are means and range ($n = 2$).

TABLE 3. URINARY EXCRETION OF ANTHOCYANINS AND ANTHOCYANIN METABOLITES FOLLOWING INGESTION OF A SINGLE ORAL DOSE OF 400 mL QGPJ CONTAINING 1.117 g TOTAL ANTHOCYANINS IN TWO HEALTHY MALE SUBJECTS

Anthocyanin compounds*	m/z	Concentration† (μg cyanidin-3-glucoside equivalents/24 h)
(1) Cyanidin monoglucuronide	463/287	1,108.5 (819.4–1,397.8)
(2) Cyanidin-3-glucoside	449/287	575.6 (491.9–659.4)
(3) Cyanidin-3-rutinoside	595/287	361.9 (340.6–383.4)
(4) Peonidin monoglucuronide	477/301	288.3 (101.3–475.2)
(5) Peonidin-3-glucoside	463/301	891.7 (675.5–1,107.8)
(6) Peonidin-3-rutinoside	609/301	2,361.0 (1,693.9–3,028.1)
Total amount		5,587.2 (4,122.7–7,051.6) = 0.50 (0.37–0.63) % of the ingested amount

* Numbers refer to Fig. 1.

† Quantified by HPLC-PDA; data are means and range (n = 2).

m/z, mass-to-charge ratio.

cosides within 2 h after QGPJ consumption appears to confirm the important role of gastric absorption. After absorption from the stomach into the bloodstream, the native QGP anthocyanins are glucuronidated and methylated through the activities of uridine diphosphate (UDP)-glucuronosyltransferase and catechol-O-methyltransferase, respectively, mostly in the liver and kidney which are the major sites of glucuronidation and methylation *in vivo* (Vitaglione *et al.* 2007). Although polyphenol metabolites sometimes have lower antioxidant activity than parent compounds, conjugated metabolites can contribute significantly to the antioxidant activity *in vivo* (Harada *et al.* 1999; Moon *et al.* 2001; Natsume *et al.* 2004).

At baseline and in the control treatment, all urine samples did not contain any native anthocyanins or methylated/glucuronidated anthocyanin forms.

In this study, the mean urinary excretion of hippuric acid (m/z 180/105 by positive ESI-LC/MS) increased by 636 mg/24 h after consumption of 400 mL of QGPJ (~threefold increase versus control; Table 2). Hippuric acid is considered as a metabolite that results from microbial degradation of polyphenols in the colon followed by hepatic conjugation with glycine (Dupont *et al.* 2002). Hippuric acid may be a consistent biomarker of intake of total polyphenols having been reported as an urinary indicator of polyphenol consumption from tea (Clifford *et al.* 2000). Clifford *et al.* (2000) described a massive increase in urinary hippuric acid excretion after black tea consumption in humans. Urinary hippuric acid increased by 1.50 mmol/24 h after the consumption of eight mugs of black tea per day. The urinary excretion of hippuric acid of 20 volunteers studied by Olthof *et al.* (2003) increased by 1.90 mmol/24 h after consumption of 4 g of black tea solids per day. Recently, Mulder *et al.* (2005) reported that black tea and green tea consumption had comparable effects on urinary hippuric acid excretion: the daily dose of polyphenols in the black tea solids was 9.08 mmol GAE, and the corresponding increase in hippurate excretion in urine was 1.86 mmol/24 h (20%). For green tea, the corresponding values were 13.3 mmol GAE consumed per day, resulting in an increase in urinary hippurate excretion of 2.33 mmol/24 h (18%). A similar effect on urinary hippuric acid excretion was found in the present study: a single oral dose of 400 mL of QGPJ (15.6 mmol GAE) resulted in an additional excretion of 3.55 mmol hippuric acid/24 h (23%). It has been noted that not only tea drinking but also the ingestion of red wine polyphenols (Gonthier *et al.* 2003), chlorogenic acid (main cinnamic acid in coffee) (Olthof *et al.* 2003), cider (Dupont *et al.* 2002) and dried cranberry juice

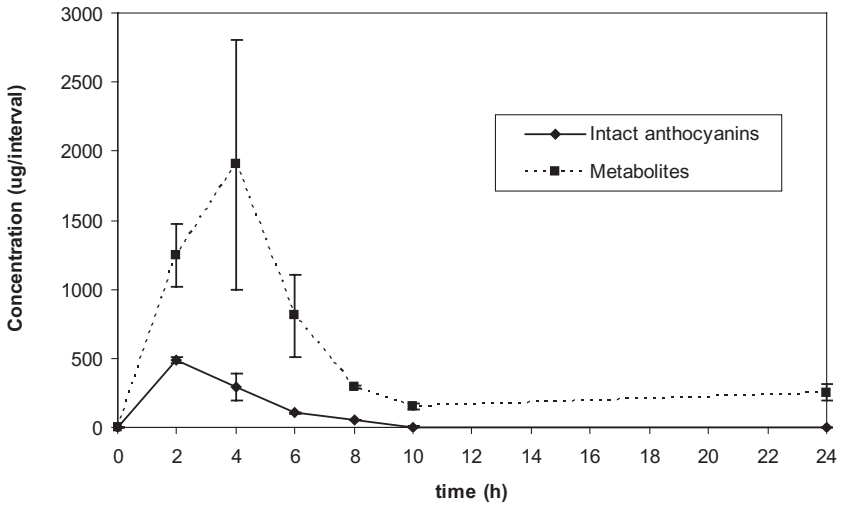

FIG. 3. URINARY EXCRETION PROFILES OF INTACT QGP ANTHOCYANINS (CYANIDIN-3-GLUCOSIDE AND CYANIDIN-3-RUTINOSIDE) AND OF ITS METABOLITES (SUM OF CYANIDIN MONOGLUCURONIDE, PEONIDIN MONOGLUCURONIDE, PEONIDIN-3-GLUCOSIDE, AND PEONIDIN-3-RUTINOSIDE); DATA ARE MEANS AND RANGE (N = 2)

(Valentova *et al*. 2007) as well as the consumption of blueberries and cherries (Toromanovic *et al*. 2008) can result in significant increases in urinary hippuric acid excretion, indicating that polyphenols from different dietary sources may have similar effects on the colonic flora.

Urinary MDA was reduced from 2.65 to 2.21 μmol/24 h (−17% versus control) following QGPJ consumption (Table 2) and values are in the same range as reported by Piche *et al*. (1988) for human adults (2.05 μmol/24 h). Urinary MDA has been widely used in animal models and in humans as a noninvasive biomarker of lipid peroxidation induced by oxidative stress (Korchazhkina *et al*. 2003). The fall in MDA excretion rate suggests that the renal generation of MDA and/or the systemic production of MDA were reduced after QGPJ ingestion. MDA has been found elevated in various diseases purportedly related to free radical damage (Suttnar *et al*. 1997). Recently, Gorelik *et al*. (2008) demonstrated significantly decreased excretion rates of MDA 6 h after the consumption of turkey cutlets with red wine compared with a control (in a randomized crossover study with 10 healthy subjects). Based on their results, they suggested that red wine polyphenols exert a beneficial effect by a novel function: inhibition of absorption of MDA due to the formation of an adduct between red wine polyphenols and aldehydes in the gastrointestinal tract. In another double-blind, randomized, crossover study with 12 healthy men, Weinbrenner *et al*. (2004) demonstrated a significant decrease in urinary MDA (24 h urine) following a 4-day intervention with olive oils containing different contents of phenolic compounds. The observed decrease in urinary MDA following QGPJ ingestion in the present study suggests that the antioxidant compounds of the plum, either in their intact form or as metabolites, may have a reducing effect on oxidative stress *in vivo* and could therefore provide protective benefits for human health.

Uric acid and ascorbic acid excretion (Table 2) were unaffected by the ingestion of QGPJ and were within the concentration range as it is reported for healthy humans (200–2,000 mg/24 h for uric acid (Fanguy and Henry 2002) and ~11 mg/24 h for ascorbic acid (Netzel *et al*. 2007b). Both compounds are important antioxidants in biological fluids like blood and urine and contribute substantially to the *in vivo* antioxidant capacity (Serafini *et al*. 1998; Ghiselli *et al*. 2000). Therefore, uric acid and ascorbic acid were determined to exclude the possibility that the increase in antioxidant capacity observed after the ingestion of QGPJ was not directly related to the polyphenols and metabolites excreted in urine. Lotito and Frei (2006) concluded that a significant increase of plasma antioxidant capacity measured after the ingestion of flavonoid-rich foods is not caused by the flavonoids themselves, but is probably the consequence of increased uric acid concentrations. Our results showed clearly that the consumption of QGPJ did not increase the levels of uric acid or ascorbic acid in the 24 h urine samples in either subject. Therefore, the observed increase in urinary antioxidant capacity cannot be attributed to an increase of these antioxidant compounds. Roura *et al*. (2007) found similar results regarding urinary uric acid excretion and total antioxidant capacity: the intake of a polyphenol-rich cocoa shake did not increase the uric acid levels in urine of 21 healthy volunteers. They concluded that the observed increase in urinary total antioxidant capacity after consumption of the cocoa shake was not due to an increase in uric acid, but most likely caused by the excreted cocoa flavonoid metabolites.

It should be noted that the increased urinary antioxidant capacity assayed by total phenolics, FRAP and ORAC, decreased MDA excretion as well as unaffected ascorbic acid and uric acid concentrations after QGPJ ingestion was consistent in both subjects.

CONCLUSION

This is the first published report of the anthocyanin and antioxidant content of QGP together with preliminary absorption and metabolism data. QGP showed significantly higher anthocyanin concentrations and antioxidant capacity compared to other plum cultivars tested. Based on its outstanding anthocyanin content, QGP, QGPJ and other derived products represent a new and promising source of anthocyanins as natural colorants for the food and nutraceutical industries. Results from the pilot study with two healthy male subjects, increased urinary antioxidant capacity and decreased MDA excretion, suggest that QGP polyphenols might be active as antioxidants *in vivo*. Furthermore, the urinary excretion of QGP anthocyanins, mainly as glucuronidated and methylated metabolites as well as the threefold increase of hippuric acid within 24 h after QGPJ consumption, indicates a high biotransformation of the ingested QGP polyphenols. Thus, these metabolites and not the native QGP polyphenols/anthocyanins are most likely the bioactive compounds *in vivo*. Therefore, additional human studies with QGP and/or derived products including blood plasma and urine as well as a larger number of subjects are warranted to identify the complete spectra of *in vivo* metabolites and to investigate their interaction with chronic disease processes by using appropriate cell based assays and animal models.

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