Ingestion of Japanese plums (*Prunus salicina* Lindl. cv. Crimson Globe) increases the urinary 6-sulfatoxymelatonin and total antioxidant capacity levels in young, middle-aged and elderly humans: Nutritional and functional characterization of their content

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Summary

Plums are considered a source of phytochemicals with beneficial health effects. The study was aimed at characterizing Japanese plums (*Prunus salicina* Lindl. cv. Crimson Globe) nutritionally and functionally and evaluating the antioxidant effect of a plum-enriched diet in young, middle-aged and elderly individuals. Participants consumed 195 g of plums twice a day for 5 days. First-void morning urines were collected before treatment (basal values), the immediate day after the last ingestion of plums (assay), and 1 day afterwards (post-assay); subsequently, urinary 6-sulfatoxymelatonin (aMT6-s) and total antioxidant capacity were measured. Nutritionally, plums were shown to contain low amounts of saccharides. From a functional point of view, serotonin, melatonin, phenolic compounds and anthocyanins were detected. Fruit consumption increased significantly the participants' urinary aMT6-s and total antioxidant capacity levels in relation to their corresponding basal and post-assay values. Plums may be taken as a source of antioxidants with a potential to counteract oxidation.

Keywords

antioxidant; anthocyanin; plum; *Prunus salicina*; serotonin; 6-sulfatoxymelatonin

Plums are considered a source of phytochemical compounds with beneficial effects on health [1–3]. Their consumption has traditionally been associated with an elevated dietary fibre supply [4]. However, an increasing number of reports have shown that phenolic compounds, anthocyanins, carotenoids and vitamin C are also present in plums [5–8]. These compounds, collectively known as antioxidants, are thought to be protective against a variety of diseases, as indicated by several epidemiological studies [9]. Detoxification of free radicals, which are implicated in a number of pathophysiological processes including aging, inflammation, ischemia-reperfusion injury, atherosclerosis or cancer, is believed to be one of the underlying mechanisms in the health protection exerted by antioxidants contained in fruits and vegetables [10].

Serotonin (5-hydroxytryptamine) and melatonin (*N*-acetyl-5-methoxytryptamine) are biogenic amines structurally related with other important compounds, including tryptophan, their precursor, and indole-3-acetic acid (IAA). These classically-considered animal indoles have been found to be potentially active constituents in plants [11, 12]. One of their functions may be the scavenging of free radicals, thereby protecting plants against oxidative stress and reducing the damage of macromolecules [12, 13]. Melatonin also possesses auxinic activity and seems to pro-

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mote vegetative growth in a manner analogous to IAA [14].

In recent years, research has been focused on the possible actions that newly identified plant bioactive compounds, i.e. melatonin and related indolic compounds serotonin and tryptophan, may exert on health. Particularly, it has recently been suggested that the presence of melatonin in edible plants may improve human health, by virtue of its biological activities and its good bioavailability [15]. This is of importance due to the numerous experimental data showing that melatonin may have utility in the treatment of several cardiovascular and neurodegenerative conditions [16, 17]. Moreover, endogenous melatonin production wanes with increasing age, leading some authors to speculate that its loss contributes to the aging process [18].

In previous reports, we showed that the consumption of cherries, either fresh or as a nutraceutical, had positive effects on nocturnal rest as well as elevating the levels of 6-sulfatoxymelatonin and antioxidants in the urine of middle-aged and elderly subjects [19, 20]. These actions were most probably attributed to the high contents of tryptophan, serotonin and melatonin [21, 22], among other phytochemicals [23] in cherries. Other workers obtained similar results with walnuts, which are also rich in melatonin [24]. Consumption of walnuts was found to provoke a threefold increase in circulating melatonin levels, also improving serum antioxidant capacity measured in Trolox equivalents [24]. Little is known, however, about Japanese plums. Hence, the aim of the present work was to characterize Japanese plums (*Prunus salicina* Lindl. cv. Crimson Globe) nutritionally and functionally. Another aim was to evaluate the possible antioxidant effect of diets enriched with this plant cultivar in young, middle-aged and elderly individuals.

MATERIALS AND METHODS

Plant material

Plums *(Prunus salicina* Lindl. cv. Crimson Globe) were harvested from a local crop in Extremadura (Spain) and grown according to integrated production. Three hundred fruits were sorted and selected for uniform size (between 60 mm and 66 mm in diameter) and with absence of visual defects. On their arrival at the laboratory, they were rapidly pre-cooled by air-cooling until reaching 3 °C. Thirty fruits were analysed immediately to establish physical and chemical properties at day 0. Sixty fruits were frozen whole and stored at –80 °C for further analysis. For nutritional melatonin and serotonin studies, fifteen fruits were de-stoned and a homogenate was prepared with an Omni-Mixer (Omni International, Kennesaw, Georgia, USA). The homogenates were lyophilized and the samples were stored in vacuum dark containers. The remaining freshly harvested fruits were taken to volunteers.

Physicochemical results

The total soluble solids (TSS) of three homogenates obtained from fifteen fruits were determined by refractometry using an RE40 refractometer (Mettler Toledo, Coslada, Madrid, Spain). Values for pH and acidity were obtained using a DL50 Graphix automatic titrator (Mettler Toledo). 3 g homogenate were mixed with 50 ml deionized water and titrated with sodium hydroxide 0.1 $mol.l^{-1}$ to pH 8.1. The results were expressed as percentage of malic acid. Firmness was determined using a TA.XT2i texture analyser (Aname, Pozuelo, Madrid, Spain) with a cylindrical probe (8 mm diameter) in fifteen intact fruits. Force/deformation curves were recorded and the maximum force (N) was calculated. The colour of fifteen fruits was measured with a Minolta CM-3500d spectrophotometer (Aquateknica, Valencia, Spain) using a measurement area of 8 mm and 30 mm diameter for skin and pulp, respectively, to obtain the parameters L^* (lightness), C^* (chroma) and H^* (hue angle). C^* and H^* were calculated as follows:

$$C^* = (a^2 + b^2)^{1/2} \quad (1)$$

$$H^* = [\mathrm{tg}^{-1}\,(b/a)(180/\pi)] \quad (2)$$

Nutritional analysis

The moisture content was determined by the loss of weight of 3 g homogenate in a Selecta Vaciotem vacuum oven (JP Selecta, Barcelona, Spain) at 70 °C. Dietary fibre was measured using enzymatic digestion of 1 g freeze-dried homogenate. The protein content was determined by the Kjeldahl method, using a Gerhardt Kjeldatherm digestor and a Gerhardt Vapodest distillation system (Izasa, Seville, Spain). The ash content was measured by calcination of 1 g freeze-dried homogenate in a Selecta muffle furnace (Andaluza de Instrumentación, Mairena del Aljarafe, Seville, Spain) at 550 °C for 24 h. The total content of saccharides was calculated by difference and the energy value assessed in accordance with Livesey et al. [25]. The glucose, fructose and saccharose contents were measured by making up 1 g homogenate to 10 ml with deionized water, passing it through a 0.45 μm filter and injecting it into

an HPLC 1050 chromatograph (Agilent Technologies, Palo Alto, California, USA) using a Zorbax NH2 5 μm 4.6 × 250 mm column and a refractive index detector. Calibrations were carried out for each saccharide: D(+)-anhydrous glucose (Merck, Madrid, Spain), D(–)-fructose (Fluka, Tres Cantos, Madrid, Spain), saccharose (Sigma, Tres Cantos, Madrid, Spain).

Functional analysis

Phenolics were extracted from 5 g homogenate following the method described by LIMA et al. [26] and were determined using the Folin–Ciocalteu reagent in a UV-2450 PC spectrophotometer (Shimadzu Scientific Instruments, Columbia, Maryland, USA). Chlorogenic acid was used as a standard and the results were expressed as milligrams per kilogram of fresh weight.

Anthocyanins were extracted from 10 g following the method of GONZÁLEZ-GÓMEZ et al. [23]. The absorbance of this extract was measured at 520 nm in a UV-2450 PC spectrophotometer. Quantification was carried out using cyanidin 3-O-glucoside as a standard, and the results were expressed as milligrams per kilogram of fresh weight.

Total antioxidant activity (TAA) was determined according to CANO et al. [27] on freshly prepared plum juice. The measurement was carried out in a UV-2401 PC spectrophotometer (Shimadzu Scientific Instruments). The results were expressed as Trolox equivalents in milligrams per kilogram of fresh weight.

The analysis of carotenoid pigments was performed applying the method proposed by MINGUEZ-MOSQUERA et al. [28] and modified by GARCÍA et al. [29]. Briefly, carotenoids were extracted with acetone, saponified overnight, and the obtained extract was injected in a HPLC 1200 chromatograph (Agilent Technologies). The separation was accomplished in a reversed phase C18 column (10 μm, 200 mm × 4.6 mm; Agilent Technologies). The initial mobile phase was acetone:water (75:25, v/v) for 5 min, raised to (95:5, v/v) over 10 min with a flow rate of 1 ml·min^{-1}. Chromatograms were recorded at 460 nm. Melatonin and serotonin were extracted from fruits and quantified according to the method of GONZÁLEZ-GÓMEZ et al. [22].

Subjects and experimental design

The study was carried out in young (20 ± 10 year-old, N = 6), middle-aged (45 ± 10 year-old, N = 6) and elderly (75 ± 10 year-old, N = 6) participants. It was approved by the Ethical Committee of the University of Extremadura (Badajoz, Spain), in accordance with the Declaration of Helsinki, the Council of Europe and the Universal Declaration of UNESCO on human rights, biomedicine, and human genome. Each volunteer was ascertained to be in good health by means of their medical history and a clinical examination including routine laboratory tests and screening. They consumed 390 g plums without seeds per day divided into two portions: 195 g as the lunch dessert and 195 g as the dinner dessert for 5 days. First-void morning urines were collected before treatment (basal values), the immediate day after the last ingestion of plums (assay) and 1 day afterwards (post-assay).

Determination of aMT6-s levels

For the quantification of urinary aMT6-s, a commercial ELISA kit (IBL, Hamburg, Germany) was used according to the manufacturer's instructions. In order to adjust for variation in the dilution of urine, aMT6-s concentrations were expressed as urinary aMT6-s/urine creatinine. Creatinine concentration was determined by means of the Jaffe test [30].

Urine total antioxidant capacity

The urine total antioxidant capacity was evaluated by means of a colorimetric assay kit (Cayman Chemical, Ann Arbor, Michigan, USA), according to the manufacturer's instructions. This assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS$^{\bullet+}$ by metmyoglobin. The capacity of the antioxidants in the sample to prevent ABTS oxidation was compared with that of Trolox, a water-soluble tocopherol analogue, and quantified as millimolar Trolox equivalents.

Statistical analysis

Friedman and Kruskal-Wallis non-parametric tests followed by Dunns' multiple comparison tests were used to analyse the results. Each value represents the mean ± standard deviation (SD) of the number of determinations carried out in duplicate. The degree of significance was set at $p < 0.05$. All analyses were performed using GraphPad Prism version 5.0, 2007 (GraphPad Software, San Diego, California, USA).

RESULTS AND DISCUSSION

The relationship between diet and health has led to intense research into bioactive compounds in foods. These extranutritional constituents include flavonoids, phytoestrogens, organosulfur

Tab. 1. Functional compounds of 'Crimson Globe' plums (*Prunus salicina* Lindl.)

Parameter	Mean ± *SD*
Biogenic amines	
Serotonin [ng·kg^{-1}]	213.63 ± 7.66^{a}
Melatonin [ng·kg^{-1}]	not quantifiable^{a}
Total phenolics [mg·kg^{-1}]	1744.97 ± 113.4^{b}
Total anthocyanins [mg·kg^{-1}]	168.20 ± 19.87^{b}
Total antioxidant activity [mg·kg^{-1}]	1510.21 ± 146.11^{b}
Total carotenoids [mg·kg^{-1}]	10.46 ± 0.2^{a}
β-carotene [mg·kg^{-1}]	1.90 ± 0.2^{a}
Lutein [mg·kg^{-1}]	0.69 ± 0.1^{a}
β-cryptoxanthin [mg·kg^{-1}]	not quantifiable^{a}

SD – standard deviation, a – mean of 3 independent replicates, b – mean of 4 independent replicates.

compounds, isothiocyanates, monoterpenes and plant sterols, among others. In the present study, phenols, anthocyanins and carotenoids were found in Crimson Globe Japanese plums (Tab. 1). Particularly, the phenol content was similar to that measured in other commercial plums [1, 8]. Total anthocyanins were lower in comparison to Black Diamond, a plum with the same characteristics as Crimson Globe in terms of skin and pulp, which are red in both cultivars [8]. In relation to carotenoids, the highest concentration was detected for β-carotene, while lutein and β-cryptoxanthin were present in lower amounts. Total antioxidant capacity was slightly lower as compared to other plum cultivars [8]. This is indicative of the cultivar effect.

In recent years, the indole melatonin has been identified as another plant bioactive compound [12]. This may have major implications for animal and human health taking into account the potent antioxidant activity of the compound in a variety of disorders and diseases where an exacerbated production of free radicals has been reported [31]. Here, melatonin was detected in Japanese plums but it could not be quantified. However, serotonin as the precursor of melatonin in the tryptophan metabolic pathway, was detected and quantified, reaching a concentration of 213.6 ± 7.7 ng·kg^{-1}.

The physicochemical and nutritional parameters that characterize Crimson Globe Japanese plums are shown in Tab. 2. The saccharide content of the cultivar was low, the acidity high, firmness was medium, and skin and pulp red when the fruits were harvested. The ratio TSS/acidity was lower than that described by other authors in plum cultivars of early production [8, 32]. The mean values of humidity, fibre, protein and ash were in the range for plums. On the other hand, the content of saccharides and therefore energy content, was lower as compared to other plum cultivars [8].

In order to study nutritional properties and the contents of bioactive compounds in Japanese plums cv. Crimson Globe, diets enriched with these fruits were administered to young, middle-aged, and elderly volunteers in an attempt to elucidate whether the consumption of these plums may exert beneficial actions on health. Fig. 1

Tab. 2. Physicochemical properties and nutritional composition of 'Crimson Globe' plums (*Prunus salicina* Lindl.)

Physicochemical properties	
Parameter	Mean ± *SD*
pH	3.04 ± 0.02^{a}
Acidity [%]	1.6 ± 0.1^{a}
Total soluble solids (TSS) [°Brix]	10.60 ± 0.1^{a}
TSS/acidity	6.80 ± 0.3^{a}
Flesh firmness [N]	14.80 ± 2.2^{b}
Skin colour	
*L** (lightness)	26.69 ± 1.2^{b}
*C** (chroma)	10.26 ± 2.8^{b}
*H** (hue angle)	18.19 ± 2.5^{b}
Flesh colour	
*L** (lightness)	38.4 ± 4.0^{b}
*C** (chroma)	37.3 ± 1.8^{b}
*H** (hue angle)	35.7 ± 3.0^{b}

Nutritional composition	
Parameter	Mean ± *SD*
Moisture [%]	88.3 ± 0.5^{c}
Fibre [%]	1.5 ± 0.0^{c}
Protein [%]	0.7 ± 0.0^{c}
Ash [%]	0.7 ± 0.1^{c}
Saccharides [%]	8.9 ± 0.5^{c}
Glucose [%]	5.1 ± 0.3^{d}
Fructose [%]	3.2 ± 0.0^{d}
Saccharose [%]	0.6 ± 0.4^{d}
Energy [kJ·kg^{-1}]	1683.26 ± 75.35^{c}

Acidity is expressed as percentage of malic acid. *C** and *H** were calculated according to eq. 1 and eq. 2.
SD – standard deviation, a – mean of 6 independent replicates, b – mean of 15 independent replicates, c – mean of 4 independent replicates, d – mean of 3 independent replicates.

shows that the intake of 195 g plums twice a day induced an increase ($p < 0.05$) in urinary aMT6-s levels in all three groups of volunteers (Fig. 1A, 1B, and 1C) with respect to their corresponding basal and post-assay values. There was also an increase ($p < 0.05$) in the urine antioxidant capacity in all the groups with respect to their basal and post-assay values (Fig. 2). The middle-aged and elderly participants presented the greatest increase (Fig. 2B and 2C). This is of importance since numerous investigators, on the basis of their experimental results, have concluded that free radical mutilation of essential molecules is related to deteriorative changes in cells and the organism. These may be associated with aging and also with the development of a variety of age-related diseases [33, 34]. Thus, consuming Japanese plums cv. Crimson Globe may have a protective effect against oxidation. Phenols, anthocyanins or carotenoids, measured in the cultivar in the present research, may be responsible for the results obtained for the total antioxidant capacity. Other bioactive compounds, including phytosterols, phytic acid or even melatonin cannot, however, be precluded. In relation to this, it has been repeatedly shown that indirect evidence for an increase in blood melatonin levels is a significant rise in levels of urinary aMT6-s (the major urinary metabolite of melatonin) [19, 20, 35]. As stated before, urinary aMT6-s levels increased significantly after plum consumption. It is known that increased levels of circulating melatonin, directly by exogenous administration or indirectly by introducing in the diet vegetables rich in this compound, enhance the individual's antioxidant status [19, 20, 24, 36].

Although melatonin was not quantifiable in the plums evaluated here, the presence of serotonin and the detection of melatonin indicates that tryptophan, the precursor of both indoleamines, was also present in the cultivar. Since orally ingested tryptophan increases the levels of serotonin in brain during the day, and the levels of circulating melatonin during the immediately following night [37–39], this amino acid may presumably be responsible for both the values of serotonin observed in the fruits and the rise in the aMT6-s levels measured in the three groups of volunteers. Moreover, tryptophan contained in plums may stimulate melatonin release from the gastrointestinal tract [40]. In fact, oral application of tryptophan in rats causes a rapid elevation of circulating melatonin, the gastrointestinal tract being the major source of this rise [41].

In sum, the intake of 195 g plums twice a day increased both urinary aMT6-s and total antioxidant capacity levels in young, middle-aged and

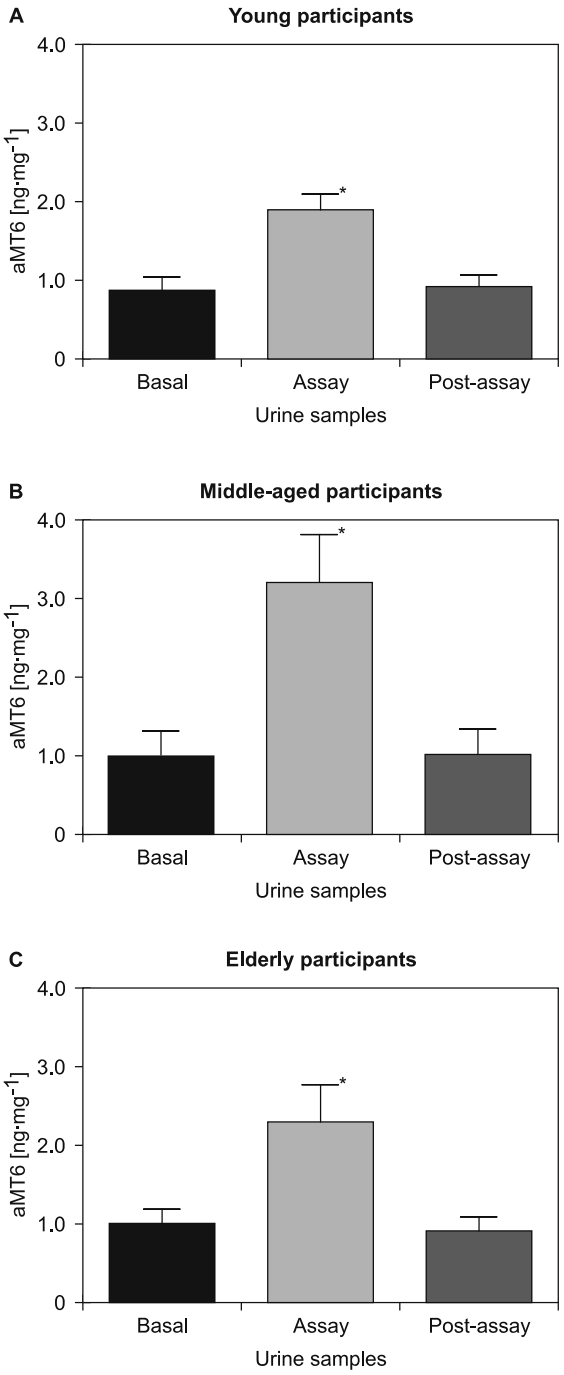

Fig. 1. Effect of the intake of plums on urinary aMT6-s levels.

aMT6-s levels were measured in basal (urine samples obtained before the intake of plums), assay (urine samples taken after 5 days of intake of 195 g plums twice a day) and post-assay (urine samples taken 1 day afterwards) conditions in young (A), middle-aged (B) and elderly (C) participants.

Results are expressed as fold-change over the basal level (experimental/basal). Each value represents the mean ± standard deviation of 10 determinations carried out in duplicate.

* – $p < 0.05$ with respect to basal and post-assay values.

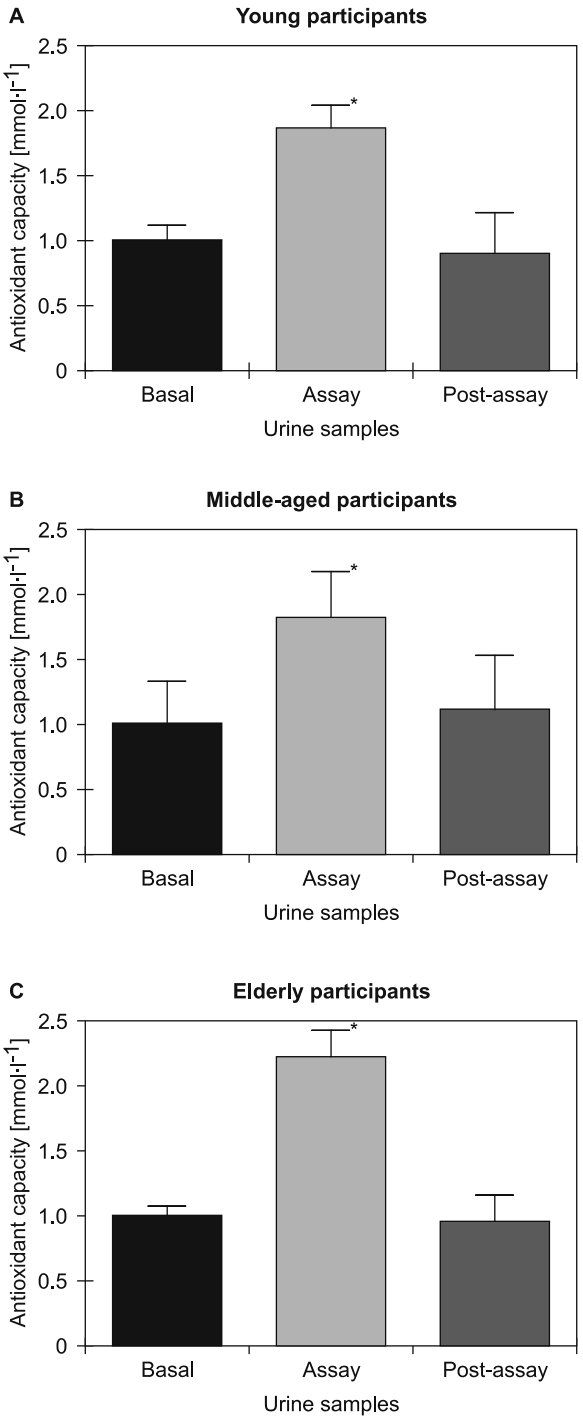

Fig. 2. Effect of the intake of plums on urinary antioxidant capacity.

Urinary antioxidant capacity was measured in basal (urine samples obtained before the intake of plums), assay (urine samples taken after 5 days of intake of 195 g plums twice a day) and post-assay (urine samples taken 1 day afterwards) conditions in young (A), middle-aged (B) and elderly (C) participants.

Results are expressed as fold-change over the basal level (experimental/basal). Each value represents the mean ± standard deviation of 10 determinations carried out in duplicate.

* – $p < 0.05$ with respect to basal and post-assay values.

elderly volunteers. This may be due to the content of bioactive compounds and due to nutritional properties. Japanese plums cv. Crimson Globe may be taken as a source of antioxidants with a potential to counteract oxidation.

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