Summary

The effect of a single oral dose of a beverage rich in polyphenols and ascorbic acid/vitamin C (study beverage, SB) on the antioxidant status (FRAP, TEAC and PCL), total phenolic content, levels of ascorbic acid, and uric acid as well as on the renal excretion of malondialdehyde (MDA, biomarker of oxidative stress), and hippuric acid (potential biomarker of polyphenol consumption), was investigated in the present study with six human subjects. The SB intake resulted in a statistically significant 9.5 fold and 8.9 fold increase in ten hour exposure to total phenolics and ascorbic acid in blood plasma, respectively, as compared to water (control). A ten-fold tendency to higher antioxidative exposure in plasma as assayed by FRAP, TEAC and PCL was observed without reaching statistical significance. Compared to the ingestion of water, SB consumption resulted in a significantly increased urinary excretion of total phenolics (+33 %), ascorbic acid (+330 %), total antioxidants estimated by the PCL (+43 %), and FRAP (+29 %) assay, as well as hippuric acid (+115 %) within 24 h. Furthermore, urinary MDA was significantly reduced (-26 % vs. control) following SB consumption. These results suggest that consumption of a beverage rich in bioactive compounds, like polyphenols and ascorbic acid, could reduce oxidative stress in vivo.

Keywords:
Study beverage; polyphenols; vitamin C; human plasma and urine; antioxidant activity; malondialdehyde; hippuric acid

Introduction

Many epidemiological studies have shown an inverse relation between fruit and vegetable intake and the risk of chronic diseases [1-3]. The reduction of disease risk has been attributed to antioxidants present in foods of plant origin, due to their ability to scavenge free radicals and thus reduce oxidative damage. Polyphenols are the most abundant antioxidants in our diets [4], and their consumption may contribute to prevent different kinds of cancer, stroke, CHD, neurodegenerative diseases or diabetes [5]. In addition to these non-nutritive antioxidant compounds, a diet rich in fruits and vegetables also contains antioxidant vitamins such as vitamin A, carotenoids, vitamin E, and especially vitamin C (ascorbic acid). Increasing the blood antioxidant status has been implicated as a possible preventative means to reduce the development of cardiovascular disease [6,7], diabetes [8], and cancer [9,10]. Durak et al. [11] have determined a correlation between increased ex vivo blood antioxidant status and risk of cardiovascular disease and cancer. They found atherosclerotic patients to have significantly lower blood antioxidant status as compared with healthy controls. Furthermore, an Australian study has determined that increasing the serum antioxidant status is associated with a decreased risk of breast cancer [12]. However, little is known from...
human experimental studies about the bioavailability, metabolism, and physiological effects of these bioactive compounds consumed as part of processed vegetables and fruits. Such studies serve as important links between nutritional epidemiological studies and the in vitro and animal studies [2]. Juices and juice-containing beverages are besides fruits and vegetables suitable food products in terms of ingestion of health protective compounds. These bioactive compounds may even be better absorbed from juices than from raw plant tissues, as it was demonstrated already for ascorbic acid [13].

The effect of a single oral dose of a beverage rich in polyphenols and ascorbic acid (study beverage, SB) on the antioxidant status, total phenolic content, levels of ascorbic acid, and uric acid as well as on the renal excretion of malondialdehyde (MDA, biomarker of oxidative stress), and hippuric acid (potential biomarker of polyphenol consumption), was investigated in the present study with six human subjects.

Material and Methods

Equipment
Antioxidant activity and total phenolics. Measurements were done in disposable cuvettes or microplates or reaction tubes using a spectrophotometer model Uviedec-610 (Jasco, Groß-Umstadt, Germany), a microplate reader model anthos ht2 (Anthos, Krefeld, Germany), and a Photochem® (Analytik Jena AG, Jena, Germany).

Ascorbic acid, uric acid, malondialdehyde and hippuric acid. These substances were analysed by HPLC. The system (Merck-Hitachi, Merck, Darmstadt, Germany) consisted of a L-6200 high precision pump, an AS-4000 autosampler, a L-4200 UV-Vis detector, a F-1050 fluorescence detector, and a Waters 990 photodiode array detector (Waters, Milford, USA).

Chemicals
Unless otherwise stated, all chemicals were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Taufkirchen, Germany), and were of analytical or HPLC grade. Deionised water was used throughout.

Instant (beverage) powder
The instant powder used for preparation of SB was obtained from Dr. B. Scheffler Nachf. GmbH & Co. KG (Bergisch Gladbach, Germany) and contained the following bioactive ingredients: concentrated juices (a mixture of blueberry, elderberry, and black currant juice), extracts of rosehip and hibiscus, extracts of raspberry and blackberry leaves, black carrot powder, and ascorbic acid.

Study design
Ethical permission was obtained from the Ethics Committee of the Friedrich-Schiller-University Jena, Faculty of Medicine (code 1550-05/09), and each subject gave his written informed consent prior to participating in the study. Six healthy, non-smoking volunteers (five women and one man) were recruited with ages ranging from 22 to 26 years and a mean body mass index of 22.8 (± 3.7 kg/m²). Before entering the study, subjects underwent a screening evaluation including medical history and physical examinations. Participants adhered to their usual diet, but had to abstain from food and beverages rich in polyphenols and ascorbic acid from 24 h prior to treatment. They were instructed to refrain from alcohol and medication, including over the counter drugs, throughout the study. Each subject ingested 180 mg ascorbic acid/day for a period of two weeks prior to the treatments, in order to saturate body stores of the vitamin. Twenty-four hours prior to the first treatment, the supplementation with ascorbic acid was stopped to allow for plasma level stabilization. The study followed an open-label, randomised, two-way (two treatments, two periods) cross-over design. The subjects were randomly allocated to one of two possible treatment sequences of equal size. In each of the two study periods, 400 mL of SB (containing 780 mg of total phenolics, 97.6 mg of ascorbic acid, and a total antioxidant capacity of 6.64 mmol [TEAC assay], 3.53 mmol [PCL assay], and 10.6 mmol [FRAP assay] per 400 mL, respectively) or 400 mL of tap water (as an antioxidant-free control beverage) were administered (referred to as treatments). The periods were separated by a two-week wash-out 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. The SB was prepared immediately prior to drinking by dissolving the instant powder (68 g) in 400 mL water. During the experimental periods, only the consumption of water and of two further standardised meals (white bread rolls with cheese for lunch and dinner) was allowed. Venous blood samples were drawn predose (t = 0 h) as well as 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 10 h after administration. Each blood sample (18 mL) was collected in a EDTA coated tube. The blood samples were centrifuged (10 min, 4500 rpm, 8 °C) within 10 min after collection. The resulting plasma was aliquoted and stored frozen at -80 °C until analysed. In addition, the volunteers collected the complete urine during 24 h in dark bottles. During the collection period urine was stored at 4 °C, and no preservatives were added. Aliquots (10 mL) were stored frozen at -80 °C. For analysis of ascorbic acid, an aliquot of plasma (0.6 mL) and urine (1 mL) was stabilised with an equal volume of 10 % (w/v) meta-phosphoric acid and stored until analysis as described above.
Analytical procedure

Antioxidant activity. The antioxidant activity (SB, plasma and urine) was determined using three different assays, the Trolox Equivalent Antioxidant Capacity (TEAC), the Photochemiluminescence (PCL), and the Ferric Reducing Antioxidant Power (FRAP).

The TEAC assay [14,15] is based on the reduction of the ABTS (2,2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)) radical cation by antioxidants. The ABTS radical cation was prepared by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture has to remain for 12-24 h until the reaction is complete and the absorbance is stable. For the photometric assay, 1 mL of the ABTS solution and 100 µL antioxidant solution (sample) were mixed for 45 sec and measured immediately after 1 min at 734 nm. The antioxidant activity of the samples was calculated by determining the decrease in absorbance at different concentrations.

In the PCL assay [15,16], the photochemical generation of free radicals is combined with the sensitive detection by using chemiluminescence. This reaction is induced by optical excitation of a photosensitiser which results in the generation of superoxide radicals O$_2^{-}$-•. The free radicals are visualised with a chemiluminescent detection reagent. Luminol works as photosensitiser as well as oxygen radical detection reagent. This reaction takes place in the Photocem®. The antioxidant potential of the samples was evaluated by measuring the lag phase, and the results were standardised using Trolox, a water soluble analogue of tocopherol.

The FRAP assay is a simple and frequently used method to assess the total reducing capacity of samples and was adopted with minor modifications [15,17]. Thirty microlitres of water and 10 µL antioxidant solution (SB, plasma or urine) were mixed with 200 µL FRAP reagent consisting of ferric chloride and 2,4,6-tripyridyl-s-triazine (TPZ). The absorbance was measured after 8 min at 595 nm. The reducing capacity was calculated using the absorbance difference between sample and blank and a further parallel Fe(II) standard solution.

Total phenolics. The total phenolic content in plasma, urine, and SB was measured using the Folin-Ciocalteu method [18], with modifications for plasma according to Serafini et al. [19].

Pharmaceutical Analysis. The total phenolic compounds were determined after a procedure of hydrolyses (with HCl and NaOH), precipitation (with meta-phosphoric acid), and extraction (with aceton/water). The samples (supernatants) were assayed for total phenolics with the Folin-Ciocalteu reagent. Results were expressed as mg gallic acid equivalents (GAE) per litre plasma.

SB and urine: diluted SB and urine samples were directly assayed with gallic acid serving as standard. Results were expressed as total phenolics (GAE) per litre SB and as total phenolic excretion per 24 h, respectively.

Malondialdehyde (MDA). Urinary MDA concentrations were determined by isocratic RP-HPLC according to Volpi and Tarugi [20]. Urine was mixed with TBA (2-thiobarbituric acid) and BHT (2,6-di-tert.-butyl-4-methylphenol) and incubated at 95 °C for 45 min. After cooling at room temperature, the samples were centrifuged, and the supernatants were used for HPLC analysis. The separation of the MDA-TBA complex was performed using a 125x4.6 mm LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany) with a mobile phase composed of 35 % methanol and 65 % 50 mM sodium phosphate buffer, pH 7.0. The complex was eluted at a flow rate of 1.0 mL/min and monitored by fluorescence detection (ex.: 515 nm; em.: 553 nm).

Ascorbic acid and uric acid. Ascorbic acid (SB, plasma and urine) and uric acid (important endogenous antioxidant which contributes substantially to the in vivo antioxidant activity; plasma and urine) were analysed using validated HPLC methods with UV-Vis and photodiode array detection, respectively [21, 22]. Briefly, plasma and urine (stabilised with meta-phosphoric acid) were centrifuged at 14 000 rpm for 5 min, and the supernatants were directly injected onto a Prontosil Eurobond RP-18 column (Bischoff, Leonberg, Germany). The samples were eluted under isocratic conditions with water/sulphuric acid (pH 2.2), and detection was carried out at 245 nm. The diluted SB was centrifuged (14 000 rpm for 5 min), and analysed for ascorbic acid under the same conditions as described above.

Hippuric acid. The urinary excretion of hippuric acid was analysed according to the HPLC method reported by Kubota et al. [23]. Urine was centrifuged at 14 000 rpm for 5 min, diluted with deionised water (1:200, v/v), and then directly injected onto a Prontosil Eurobond RP-18 column. The samples were eluted under isocratic conditions with water/acetonitrile/acetic acid (78:20:2, v/v/v; pH 2.2), and photodiode array detection was carried out at 235 nm.

All analyses/determinations were done in duplicate.

Pharmacokinetics and statistical analysis. Analogous to the evaluation of the effect versus time curve of a drug (pharmacodynamics), the individual time courses of antioxidative capacity in plasma were analysed according to non-compartmental pharmacodynamic
methods. The rationale is that plasma antioxidative capacity (operationally measured as concentrations) and their changes during the observed time span can be viewed as an effect or response upon ingestion of food/beverages containing antioxidative components. Any positive change in this response over time is indicative of the biological activity of antioxidative substances. Therefore, the integrated concentration versus time or Area Under the Curve (AUC), that is above (i.e. towards increasing Y values) the baseline concentration, was chosen as the main response variable. The baseline was defined as the last non-missing observation prior to the first dose of the study beverage, i.e. at t=0 h. The AUC was calculated using the linear trapezoidal rule, which summarizes partial trapezoids made of assayed concentrations and scheduled blood sampling times. The plasma response variables (AUC above baseline), and urinary response variables (amount excreted within 24 hours) were subjected to an analysis of variance (ANOVA). The ANOVA was performed on untransformed data and included treatment, period, sequence and subjects nested within sequence as factors. Effects were considered as statistically significant if the probability (p value) associated with F statistics was < 0.05. All testing was purely exploratory. All pharmacodynamic and statistical calculations were done using the WinNonlin® software, version 5.0 (Pharsight Co, Mountain View, CA, USA).

**Results and Discussion**

The values of AUC above baseline in plasma of the six subjects as measured by different tests are summarized by treatment in Tab. 1. Urinary excretion during 24 hours is summarised in Tab. 2. The intake of the study beverage rich in polyphenols and ascorbic acid resulted in a statistically significant 9.5 fold and 8.9 fold increase in 10 h exposure to total phenolics and ascorbic acid in blood plasma, respectively, as compared to control (Tab. 1 and Fig. 1). A tendency to higher antioxidative exposure in plasma as assayed by FRAP, TEAC and PCL was observed without reaching statistical significance (Tab. 1 and Fig. 2). Uric acid in plasma was not affected by the ingestion of SB or water (data not shown). This result is in accordance with a previous work of Serafini et al. [19]. They found no changes in plasma uric acid, vitamin E, thiol groups, ascorbic acid, and five carotenoids after the ingestion of water, alcohol-free red wine or alcohol-free white wine. The observed non-significant increase of plasma TEAC, PCL, and FRAP after SB consumption is mainly caused by the low power of the ANOVA to detect significant changes, i.e. the small number of subjects combined with the large variability. Enhanced ability of plasma to resist oxidative challenges has been observed following consumption of

<table>
<thead>
<tr>
<th>Parameter/Substances</th>
<th>Control</th>
<th>SB</th>
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<tbody>
<tr>
<td>TEAC (µmol*h/L)</td>
<td>10.2 ± 20.2</td>
<td>1431 ± 1162</td>
</tr>
<tr>
<td>PCL (µmol*h/L)</td>
<td>0.63 ± 1.07</td>
<td>121 ± 117</td>
</tr>
<tr>
<td>FRAP (µmol*h/L)</td>
<td>44.6 ± 103</td>
<td>228 ± 373</td>
</tr>
<tr>
<td>Total phenolics (mg*h/L)</td>
<td>1.01 ± 1.46</td>
<td>9.59 ± 7.13*</td>
</tr>
<tr>
<td>Ascorbic acid (mg*h/L)</td>
<td>1.80 ± 1.96</td>
<td>15.9 ± 12.6*</td>
</tr>
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</table>

**Tab. 1: Plasma AUC** of FRAP, PCL, TEAC, total phenolics, and ascorbic acid following ingestion of a single oral dose of 400 mL SB or 400 mL water (control) to six healthy subjects, respectively.

* Area Under the response Curve that is above baseline (the concentration at dose time t = 0 h); data represent mean ± SD. * p < 0.05, SB versus Control (ANOVA).

Total phenolics are expressed as gallic acid equivalents (GAE), FRAP values are expressed as µmol Fe++, TEAC and PCL values are expressed as µmol Trolox equivalents, respectively.

**Fig. 1: The effect of drinking 400 mL of SB or water on the total phenolic and ascorbic acid concentration in human plasma. Data as mean ± SD (n = 6). Total phenolics are expressed as gallic acid equivalents (GAE).**
Fig. 2: The effect of drinking 400 mL of SB or water on the antioxidant potential of human plasma as determined by the changes in TEAC, PCL, and FRAP values. Data as mean ± SD (n = 6). FRAP values are expressed as µmol Fe²⁺, TEAC and PCL values are expressed as µmol Trolox equivalents, respectively.

greater in vitro antioxidant activity than vitamin C or E. The effect of feeding eight elderly women strawberries, spinach, red wine, or vitamin C was determined by three methods (ORAC, TEAC, and FRAP) that measure the antioxidant activity in serum [25]. A single meal including strawberries, spinach, or red wine increased serum antioxidant activity to an extent equivalent or greater than a large dose of vitamin C (1250 mg). These increases were attributed to the antioxidant properties of polyphenols, which were effectively absorbed from a single serving.

The redox potential of polyphenols (and their metabolites), that reach the plasma, enables them to scavenge damaging radicals, but endogenous plasma antioxidants, especially vitamin C, are required for disposal of the resultant phenoxyl radicals [29]. Therefore, when attempting to ascribe nutritional antioxidant properties to the phenolic compounds in the plant-based foods, the contribution of vitamin C to antioxidant activity should not be overlooked.

Significantly higher excreted amounts of total phenolics (+33 %), ascorbic acid (+330 %), total antioxidants estimated by the PCL (+43 %), and FRAP (+29 %) assay, as well as hippuric acid (+115 %) were found in the volunteers’ 24 h urine after consumption of SB as compared to water (Tab. 2).

A tendency to higher amount excreted (+19 %) as measured by TEAC was observed following SB compared to control without reaching statistical significance. The increased urinary antioxidant activity was found to be paralleled by an increased excretion of alcohol-free red wine [19], red wine and whisky [24, 25, 26], green and black tea [27], as well as after eight kinds of juices produced from apples, orange, grape, peach, plum, kiwi, melon, and watermelon [28]. Some polyphenols have been shown to have many times

Tab. 2: Summary of urinary excretion data (for footnote) of antioxidant activity, total phenolics, MDA, ascorbic acid, uric acid, and hippuric acid following ingestion of a single oral dose of 400 mL SB or 400 mL water (control) to six healthy subjects, respectively.

<table>
<thead>
<tr>
<th>Parameter/Substances</th>
<th>Control</th>
<th>SB</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEAC (mmol/24 h)</td>
<td>4.21 ± 0.56</td>
<td>5.00 ± 1.00</td>
</tr>
<tr>
<td>PCL (mmol/24 h)</td>
<td>1.71 ± 0.14</td>
<td>2.44 ± 0.51</td>
</tr>
<tr>
<td>FRAP (mmol/24 h)</td>
<td>6.15 ± 0.73</td>
<td>7.91 ± 0.71*</td>
</tr>
<tr>
<td>Total phenolics b</td>
<td>259 ± 56.4</td>
<td>345 ± 22.5*</td>
</tr>
<tr>
<td>MDA (µmol/24 h)</td>
<td>1.65 ± 0.57</td>
<td>1.22 ± 0.37</td>
</tr>
<tr>
<td>Ascorbic acid (mg/24 h)</td>
<td>11.1 ± 3.1</td>
<td>47.8 ± 24.3*</td>
</tr>
<tr>
<td>Uric acid (mg/24 h)</td>
<td>306 ± 108</td>
<td>305 ± 144</td>
</tr>
<tr>
<td>Hippuric acid (mg/24 h)</td>
<td>149 ± 46.7</td>
<td>321 ± 88.7*</td>
</tr>
</tbody>
</table>

* Data represent mean ± SD.

b Corrected for ascorbic acid and uric acid.

p < 0.05, SB versus Control (ANOVA).

Total phenolics are expressed as gallic acid equivalents (GAE), FRAP values are expressed as mmol Fe²⁺, TEAC and PCL values are expressed as mmol Trolox equivalents, respectively.
phenolic compounds and ascorbic acid. The low excretion of phenolic substances (11 % of ingested total phenolics) within 24 h is accompanied by a high excretion of ascorbic acid (38 % of ingested ascorbic acid dose). The recoveries found for total phenolics and ascorbic acid are in the same order of magnitude as found by other authors after ingestion of a mixed fruit juice [30] or seabuckthorn juice [13], respectively. Uric acid excretion was not affected by the ingestion of SB. Similar results, with respect to the antioxidant activity, were found in a previous study conducted by Cao et al. [25]. The total antioxidant activity of urine (eight elderly women) determined as ORAC increased significantly for strawberries, spinach, and vitamin C, respectively, during the 24 h period following these treatments. Tepel et al. [31] reported that therapies with different antioxidants prevent the acute decrease of renal function caused by ischaemia, contrast media or drugs. Therefore, the enhancement of urinary concentration in antioxidants may have physiological relevance.

Furthermore, urinary MDA was significantly reduced from 1.65 ± 0.57 to 1.22 ± 0.37 µmol/24 h (-26 % vs. control) following SB consumption. The fall in MDA excretion rate suggests that the renal generation of MDA and/or the systemic production of MDA was reduced after SB ingestion. MDA, one of the major secondary oxidation products of peroxidised polyunsaturated fatty acids, has been found elevated in various diseases purportedly related to free radical damage [32, 33]. The decrease in urinary MDA following SB ingestion suggests that the antioxidant compounds of the beverage have a reducing effect on oxidant stress in vivo which could be of physiological relevance.

In this study, the mean urinary excretion of hippuric acid increased by 172 mg/24 h after consumption of 400 mL of the study beverage (+115 % vs. control). Hippuric acid is a metabolite that results from microbial degradation of polyphenols in the colon followed by hepatic conjugation with glycine, as well as from the metabolism of endogenous catecholamine neurotransmitter [34]. A consistent biomarker of intake of total polyphenols may be hippuric acid, which is reported as an urinary indicator of polyphenol consumption from tea [35, 36]. Clifford et al. [35] described first the massive increase in urinary hippuric acid excretion after black tea consumption among humans. Among the nine subjects they studied, 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 by the twenty volunteers studied by Olthoff et al. [37] increased by 1.90 mmol/24 h, on average, after consumption of 4 g of black tea solids per day. Recently, Mulder et al. [36] 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 gallic acid equivalents (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 SB (4.59 mmol GAE) resulted in an additional excretion of 0.97 mmol/24 h urinary hippuric acid (21 %).

The health benefits of dietary polyphenols have often been attributed to their antioxidant activities, but the microbial metabolites of dietary polyphenols have sometimes lower antioxidant activities than do their parent compounds [38]. The lower antioxidant activity, however, may be offset by greater bioavailability for these smaller molecules. In addition, dietary polyphenols may have significant effects on the colonic flora [39] and thus confer a type of prebiotic effect. In this respect, it has been noted that not only tea drinking but also wine [40], cider [41], and coffee [37] consumption can result in increases in urinary hippuric acid excretion, indicating that polyphenols from different dietary sources may have similar effects on the colonic flora. Therefore, these and other microbial metabolites, and not the native polyphenols, may be responsible for at least some of the health effects attributed to the consumption of foods/beverages rich in polyphenols.

In conclusion, it could be demonstrated that after consumption of a beverage rich in bioactive compounds valuable ingredients like polyphenols and ascorbic acid are bioavailable for humans and are active as antioxidants in vivo. Considering that the results of the current study were obtained after a single oral administration of the study beverage, the long-term effects need to be investigated to identify the biological relevance of the observed short-term effects.

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**References**


