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ORIGINAL ARTICLE

Antioxidative activity and health benefits of anthocyanin-rich fruit juice in healthy volunteers

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ABSTRACT

Oxidative cell damage has been linked to the pathogenesis of numerous diseases such as atherosclerosis, type 2 diabetes, and cancer. The consumption of foods rich in polyphenols (e.g. anthocyanins) has been shown to exert preventive effects against such diseases. We investigated the biological effects of anthocyanin-rich fruit juice in a 9-week, placebo-controlled intervention study with 57 healthy male volunteers. The study design encompassed an initial 1 week of wash-out, followed by 8 weeks of intervention period with anthocyanin-rich fruit juice or placebo. The anthocyanin-rich fruit juice demonstrated DNA-protective and antioxidant effects; however, the placebo beverage, rich in vitamin C, showed similar effects based on the tested biomarkers. A significant reduction in background and total DNA strand breaks was observed in both groups within 24 h as well as after 8 weeks of intervention. Only anthocyanin-rich fruit juice consumption provided a significant reduction in body fat and an increase in fat-free mass. The activity of superoxide dismutase (SOD) was significantly elevated after consumption of anthocyanin-rich fruit juice. Both groups showed decreased levels of LDL and total cholesterol (TC) within the first week of the intervention. Similar results in both groups could be explained by the relatively high vitamin C contents of both beverages (>500 mg/L), which may have masked the effects of anthocyanins and other antioxidants in the studied juice. Taken together, anthocyanin-rich fruit juice as well as the placebo drink, both of which had high vitamin C content, can improve DNA integrity and might influence lipid metabolism in humans.

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Anthocyanin-rich fruit juice; antioxidant; blood lipids; comet assay; DNA strand breaks

Introduction

The increased formation of reactive oxygen species (ROS) has been shown to play a role in the pathogenesis of chronic diseases such as diabetes mellitus type 2, Parkinson's, liver diseases, and cardiovascular diseases [1]. ROS include free radicals like hydroxyl- and superoxide anion radicals, as well as neutral molecules like hydrogen peroxide and singlet oxygen. These harmful species can damage all cellular macromolecules, including proteins, lipids, and DNA and exert adverse effects on the organism [2]. However, antioxidants are able to reduce ROS formation by scavenging radicals, deactivating redox-active transition metals, and/or inducing specific signalling pathways to modulate cell defence [3].

It is well established that numerous polyphenols from fruits, including anthocyanins, mediate various biological effects [4]. Anthocyanins represent a class of plant pigment constituents that occur in many fruits of the daily diet, e.g. grapes, black currants, cherries and blueberries. They possess antioxidant, anti-inflammatory and DNA protective properties and are associated with health benefits [5–8]. Moreover, it has been reported that anthocyanin consumption can decrease total cholesterol (TC), LDL and triglyceride (TG) concentrations in humans [9,10]. Furthermore, numerous human intervention studies have demonstrated the DNA protective effects of anthocyanin-rich products [5,11,12]. Additionally, anthocyanins modulate the Nrf2-ARE signalling pathway to increase

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Nrf2 transcription as well as the activity of antioxidative enzymes SOD, CAT, and glutathione peroxidase (GPx) [6,9,12]. A previous intervention study that compared ileostomists and volunteers with a healthy gastrointestinal tract found that the consumption of bilberry extract modulates the Nrf2/ARE-signalling pathway with concomitant reduction of total DNA damage within 8 h [12–14]. This suggests that not only anthocyanins but also compounds containing flavylium cations, contribute to the beneficial effects of anthocyanin-rich juice [13–15]. However, we realised that it is important to clarify whether the observed short-term effects could be prolonged by continuous, long-term uptake of anthocyanin-rich products. Therefore, we first conducted a short-term human intervention study [16] that investigated red fruit products including bilberry juice, red grape skin extract and a juice produced mainly from red berries (red grapes, apples, blackberries, strawberries, cranberries, chokeberries, and acerola). The results showed that the juice produced from red berries had the most potent effects. Consequently, the study reported here addressed the chemopreventive properties of an anthocyanin-rich fruit juice similarly to the one tested before. The aim of this placebo-controlled intervention study of healthy volunteers was to determine whether the daily consumption of 750 mL of anthocyanin-rich fruit juice over an 8-week period could favourably alter biomarkers of oxidative stress response such as DNA damage, antioxidative enzymes (SOD, CAT), the inflammation marker interferon-γ-induced-protein 10 (IP10) and blood lipids (TG, TC, LDL, HDL). Additionally, on the first day of the study, we evaluated short-term effects by giving volunteers a bolus of the respective study beverage, with the goal to verify results from our previous research.

Table 1. Baseline characterisations of the subjects.

	All	Juice group	Placebo group
n	57	30	27
Age (years)	24 ± 3	23 ± 3	24 ± 3
High (cm)	180 ± 10	180 ± 5	180 ± 10
Weight (kg)	76 ± 8	77 ± 8	75 ± 8
BMI (kg/m^2)	23 ± 2	23 ± 2	23 ± 2

Data are means and SD.

Materials and methods

Chemicals

All chemicals used in the presented research were of analytical grade.

Study design

Healthy male volunteers ($n = 62$, BMI = 19–25, age = 20 – 50) who fulfilled the inclusion criteria (healthy non-smokers, no practice of excessive sports, no intake of pharmaceutical drugs or food supplements during the study period) were recruited. After providing their informed written consent, the volunteers were subjected to a standard medical health check including a questionnaire, blood pressure measurements, and standard clinical blood biochemistry tests. The volunteers were then randomly divided into two groups (placebo group, $n = 31$ and juice group, $n = 31$). Five volunteers dropped out of the study for private reasons or illness. The remaining 57 volunteers ($n = 30$ in the juice group and $n = 27$ in the placebo group, see Table 1) completed the 9-week study.

This study was approved by the local ethics committee of Rhineland-Palatine, Mainz, Germany (no. 837.013.14 (9252-F)). The 9-week human intervention study (1 week wash out period and 8-week intervention period) had a prospective, randomised, placebo-controlled parallel design. After a 1-week wash-out period (no food rich in polyphenols), the volunteers consumed a 750 mL bolus of anthocyanin-rich fruit juice or placebo drink on the first day of the study. Over each of the following 55 days, the volunteers drank 750 mL of the beverage assigned to their group in three equal portions (Figure 1). Subjects were instructed to maintain their normal dietary and lifestyle habits during the study but to abstain from consuming foods rich in

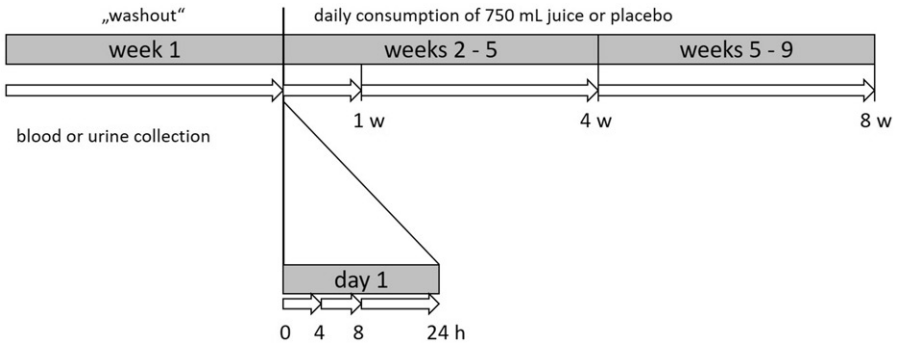

Figure 1. Design of the 9-week placebo-controlled intervention study. w: week; h: hour.

polyphenols (anthocyanin-rich food sources), dietary supplements or caffeine-containing products. Blood samples were collected from all subjects on an empty stomach immediately before (0 h), as well as 4, 8 and 24 h after bolus consumption (day 1, after 1 week wash out period). During the rest of the study, blood samples were collected from an empty subject after 1, 4 and 8 weeks. Each of the collected blood samples was analysed for biomarkers, namely, DNA strand breaks, SOD and CAT activity, blood lipids (TG, TC, LDL, HDL, and oxLDL), and IP10. For compliance within the study requirements, spot urine samples (50 mL) of all volunteers were collected after 1, 4, and 8 weeks of intervention time and their anthocyanin contents were quantitated. Prior to sampling, all volunteers completed a food record for the past week so that individual dietary habits and compliance to the instructions could be fully documented. Anthropometric measurements were taken on an empty bladder each time when venous blood samples were collected.

Sample preparation and analysis of study beverages

Both study beverages (anthocyanin-rich fruit juice and placebo drink) were provided by Eckes-Granini GmbH (Niederolm, Germany) in brown 750 mL bottles. The anthocyanin-rich fruit juice was produced from red grape juice, lingonberry juice from concentrate, apple, blueberry and strawberry puree, aronia juice from concentrate and acerola puree (100% fruit content). The placebo beverage included water, fructose, glucose, sucrose, citric acids, vitamin C and natural flavours. Brix values were measured using an Abbé-refractometer (Kruess GmbH, Hamburg, Germany), while vitamin C content was determined by potentiometric titration with dichlorophenol-indophenol [17]. Titratable acidity was measured by titration with sodium hydroxide solution and expressed as the amount of tartaric or citric acid in solution [18]. Total polyphenol contents were measured using the Folin-Ciocalteu reagent and expressed as the concentration of catechin in solution [19]. The total anthocyanin content of the fruit juice was determined chromatographically using HPLC-UV/VIS as previously reported [20]. Quantification was carried out as cyanidin-3-O-glucoside equivalents using peak areas detected at 540 nm and based on external calibration using the reference substance malvidin-3-glucoside. Characterisation of the anthocyanins was performed by comparing the retention times as performed before [20]. Antioxidative capacity was measured by comparing the results from a reaction with ABTS (diammonium-2,2′-azino-di-(3-ethylbenzothiazoline)-6-Sulfonate) with the reaction between the artificial vitamin E derivative "trolox" and ABTS (trolox Equivalent antioxidative Capacity TEAC) [21].

Anthropometric measurements

The body height and weight of volunteers were measured using a Seca delta 707 digital scale (Seca, Hamburg, Germany) and their BMI (kg/m^2) calculated. A bioelectrical impedance analyser 101 (BIA 101, SMT medical GmbH, Wuerzburg, Germany) was used to estimate body composition (total body water, TBW, fat mass, FM, and fat-free mass, FFM). The measurements were performed in the morning with fasted subjects, at the horizontal position and emptied bladder. Special skin electrodes were placed on the right hand and foot on dry skin according to the manufacturer's instructions.

Nutrient intake

Based on the validated 7-day dietary records intake of nutrients/kcal was assessed (7-day dietary records) using the nutrition software package PRODI 5 Expert (Nutri-Science, Hausach, Germany).

Processing and storage of blood, plasma and urine samples

Venous blood was collected in EDTA tubes and centrifuged at $2000 \times g$ for 10 min at room temperature. The resulting plasma was used to determine CAT and SOD activity, as well as the concentrations of oxLDL and IP10. To assess the amount of DNA strand breaks, the blood sample in EDTA tubes was immediately prepared for the comet assay. For TG, TC, HDL and LDL measurements, the venous blood was collected in Li-heparin tubes and immediately sent to the Department of Laboratory Medicine (Westpfalzklinikum Kaiserslautern). For the stabilisation of anthocyanins in urine, the collected spot urine samples were adjusted to pH 2.5 with hydrochloric acid (1 M) and stored at $-80\,^{\circ}C$ prior to analysis.

Comet assay

Alkaline single cell gel electrophoresis was performed to determine background and total (after additional treatment with formamidopyrimidine-DNA glycosylase) DNA strand breaks following Collins et al. [22] with slight modifications, reported earlier [23] and was expressed as mean tail intensity (TI%) from two gels (tail intensity percentage; DNA in the comet tail relative to total DNA).

Measurement of plasma antioxidative and inflammatory biomarkers

SOD and CAT activities were determined by colourimetric methods using a Biotek Synergy 2 microplate reader (Biotek Instruments GmbH, Bad Friedrichshall, Germany) according to the manufacturer's instructions (Cayman Chemical, MI, USA). Plasma oxLDL (Mercodia AB, Uppsala, Sweden) and IP10 levels were measured with commercially available ELISA kits (R & D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Blood lipids

Routine blood parameters such as TC, TG, HDL, and LDL were measured at the Department of Laboratory Medicine (Westpfalzklinikum Kaiserslautern).

Compliance analysis

For compliance analysis, the anthocyanin content of spot urine samples was determined. Urine samples (6 mL) were applied to a solid-phase extraction cartridge (Strata C18, 500 mg, Phenomenex, Aschaffenburg, Germany) preconditioned with methanol (4 mL) and equilibrated with water/acetic acid (95/5, v/v, 4 mL). Elution was performed with water/acetic acid (95/5, v/v, 3 mL), eluates were concentrated (30 °C, without light exposure) using a vacuum concentrator (Eppendorf, Hamburg, Germany). The residue was resuspended in 300 µL of water/acetic acid/acetonitrile (92/5/3, v/v), after which 20 µL of the internal standard delphinidin-3,5-O-diglucoside (100 µg/mL) was added to 180 µL of the solution. An HPLC-ESI-MS/MS analysis was performed using an Agilent HPLC-system (Agilent, Santa Clara, CA, USA) coupled to an API 3200 MS (AB Sciex, Framingham, MA, USA). The HPLC parameters were as follows: flow 0.6 mL/min; Solvent A (water/formic acid/acetonitrile, 92/5/3, v/v); solvent B (acetonitrile/water/formic acid, 50/45/5, v/v), increasing from 2% B to 14% B within 40 min. MS/MS parameters are displayed in the Supplementary Table 1. Calibration curves ranged from 0.002–2 µg/mL with LOD was 0.007 µg/mL and LOQ was 0.025 µg/mL.

Statistical analysis

Results of tested parameters are reported as mean and SD. The Anderson–Darling test was used for the analysis of normal distribution. The statistical significance of differences in parameters between the study phases within each respective group were analysed with a one-sided paired *t*-test (differences normally distributed) or a one-sided paired Wilcoxon's signed rank test (differences without normal distribution). The statistical significance of differences in parameters between the anthocyanin-rich juice and placebo groups were analysed by either a two-sample *t*-test (normal distribution) or a Mann–Whitney *U*-test (non-normal distribution).

Results

In the presented placebo-controlled intervention study, we investigated the biological effects of anthocyanin-rich fruit juice (750 mL/day) in 57 healthy male volunteers. Furthermore, we studied both the short-term (within 24 h) and long-term (over 8 weeks) effects of anthocyanin-rich fruit juice consumption relative to a placebo.

Composition of the anthocyanin-rich fruit juice and placebo drink

Data for the compounds analysed in both study beverages are summarised in Table 2. The anthocyanin-rich fruit juice exhibited high antioxidant activity, corresponding to a TEAC value of 34 mmol/L, and had a high concentration of total phenols (3.6 g/L, expressed as catechin equivalents). Both beverages had identical Brix values (14.2° Brix) as well as glucose, fructose, sucrose and acid concentrations, but differed in their ascorbic acid contents; the anthocyanin-rich fruit juice contained 564 mg/L whereas the placebo drink contained 689 mg/L. In general, both beverages showed substantially higher ascorbic acid contents than other commercially-available fruit juices (<350 mg/L). The total anthocyanin content of red fruit juice was 274 mg/L. The anthocyanin detected were malvidin-3-glucoside (mal-3-glc, 33%), followed by cyanidin-3-galactoside (cy-3-gal, 14.3%), peonidin-3-glucoside (peo-3-glc, 11.6%), petunidin-3-glucoside (pet-3-glc, 10.3%), delphinidin-3-glucoside (del-3-glc, 7.7%), cyanidin-3-arabinoside (cy-3-ara, 6.8%), cyanidin-3-glucoside (cy-3-glc, 6.4%), delphinidin-3-arabinoside (del-3-ara, 3.8%), malvidin-3-galactoside (mal-3-gal, 2.5%),

Table 2. Composition of the anthocyanin-rich fruit juice and placebo drink.

	Anthocyanin-rich fruit juice	Placebo drink
Brix [°]	14.2	14.2
Acids (g/L)	6.2	6.4
Glucose (g/L)	65.2	65.2
Fructose (g/L)	70.5	70.5
Sucrose (g/L)	3.8	3.8
Polyphenols (Folin) (g/L)	3.6	0
TEAC (mmoL/L)	34	n.a.
Vitamin C (mg/L)	564	689
Total anthocyanins[a] (mg/L)	274.5	ND

n.a.: not analysed; ND: not detectable

[a]mal-3-glc (33.0%), cy-3-gal (14.3%), peo-3-glc (11.6%), pet-3-glc (10.3%), del-3-glc (7.7%), cy-3-ara (6.8%), cy-3-glc (6.4%), del-3-ara (3.8%), mal-3-gal (2.5%), pet-3-gal (2.0%), del-3-gal (1.6%).

petunidin-3-galactoside (pet-3-gal, 2%) and delphinidin-3-galactoside, (del-3-gal, 1.6%).

Urine analysis for compliance control

The monitoring of anthocyanins in the urine of volunteers from both groups after the 1-week wash-out period showed levels below the LOQ. During the intervention phase, no anthocyanins were detected in urine samples from the placebo group, while urine samples from the juice group demonstrated detectable anthocyanin concentrations (data not shown). In this way, the detection of anthocyanins in spot urine samples using HPLC-ESI-MS/MS seems to be a practicable method for compliance control.

Nutrient intake

The daily nutrient and energy intakes of volunteers were calculated based on 7-day food records, which were completed at the end of the 1 week wash-out period as well as after four and 8 weeks of intervention (Table 3). After 4 weeks of intervention, the consumption of the equicaloric study beverages had significantly increased volunteers' energy and carbohydrate intakes and decreased fat and protein intakes in comparison to the wash-out period. These effects were also observed during the next 4 weeks of the study. However, the two study drinks did not differ noticeably in any of the measured nutrient intake parameters.

Body weight and composition

The body weight and composition changes during the study are presented in Table 4. The body weight of volunteers consuming the anthocyanin-rich fruit juice increased after 1 week of intervention, after which it remained stable throughout the rest of the study period. Figure 2(A,B) demonstrates how the intervention and wash-out periods compare in terms of changes in the body fat and fat-free mass of volunteers. Surprisingly, volunteers who consumed the anthocyanin-rich fruit juice experienced a significant increase in fat-free mass ($p < 0.01$) over the entire study period. Likewise, the body fat of volunteers in the juice group changed significantly ($p < 0.05$). Here the significant decrease of this parameter was observed after one and 4-week intervention with anthocyanin-rich fruit juice. Eight weeks ingestion of anthocyanin-rich fruit juice led to a slight decrease in body fat mass, but this effect did not reach statistical significance. The consumption of placebo drink showed the same effect in the first week of intervention, whereas further consumption of placebo drinks showed no modulation of these parameters during the following weeks.

DNA strand breaks

Modulation of DNA strand breaks was assessed in volunteers from both groups after 24 h (short-term) and after the complete 8-week intervention period (long-term). In comparison to the wash-out period, volunteers showed a significant reduction in background and total DNA strand breaks within 24 h after consuming a bolus of 750 mL of anthocyanin-rich fruit juice or placebo drink (Figure 3(A,B)). Interestingly, the significant reduction in DNA strand breaks was already clearly observable 4 h after the consumption of each study beverage. Consequently, after 8 and 24 h only an incremental decrease in DNA strand breaks was detectable. Moreover, these effects ($p < 0.001$) were observed in both groups during the entire intervention (see Figure 3(A,B)). Contrary to the distinct effects observed after the consumption of red fruit juice and placebo drinks, the two groups did not significantly differ in terms of DNA strand breaks at any point of the study period.

Table 3. Average daily energy and nutrient intakes of volunteers, based on 7-day food records completed in the last week of the wash-out period, four and 8 weeks of intervention prior to blood and urine sampling (wo: wash out, w: weeks).

	After wo	4 w	8 w
Juice group			
Energy intake (kcal)	2384.8 ± 454.3	2507.2 ± 485.3	2526.0 ± 571.6*
Carbohydrates (g)	264.0 ± 59.2	343.4 ± 70.8***	338.1 ± 75.5***
Fat (g)	96.3 ± 24.9	78.0 ± 23.6***	80.5 ± 27.0***
Protein (g)	102.9 ± 26.7	88.7 ± 21.1	98.0 ± 32.6***
Placebo group			
Energy intake (kcal)	2199.2 ± 450.4	2408.5 ± 477.8*	2380.0 ± 499.9*
Carbohydrates (g)	237.7 ± 48	330.7 ± 61.3***	324.0 ± 65.1***
Fat (g)	90.4 ± 25	78.5 ± 22.4**	77.4 ± 22.7*
Protein (g)	97.1 ± 19.0	82.4 ± 18**	83.8 ± 20.0**

Data are means and SD; Significant differences in comparison to the wash-out period: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

Table 4. Body weight and composition of volunteers after the wash-out period and after 1, 4 and 8 weeks of intervention.

	After wo	1 w	4 w	8 w
Juice group				
Body weight [kg]	76.6 ± 7.8	77.0 ± 7.7**	76.9 ± 7.8	77.1 ± 7.8
FFM [kg]	63.1 ± 7.2	63.9 ± 7.3***	63.8 ± 7.5**	63.8 ± 7.1*
FM [kg]	13.5 ± 3.0	13.0 ± 4.0*	13.0 ± 3.7*	13.3 ± 3.5
TBW [L]	46.2 ± 5.3	46.8 ± 5.3	46.7 ± 5.5	46.7 ± 5.2
Placebo group				
Body weight [kg]	74.9 ± 7.3	74.8 ± 7.4	74.8 ± 7.8	74.6 ± 7.8
FFM [kg]	61.2 ± 4.4	62.0 ± 4.5*	61.2 ± 4.4	61.2 ± 4.5
FM [kg]	13.6 ± 4.8	12.8 ± 4.9*	13.6 ± 4.9	13.4 ± 4.6
TBW [L]	44.8 ± 3.2	45.5 ± 3.4	44.8 ± 3.2	44.8 ± 3.3

FFM: fat free mass; FM: fat mass; TBW: total body water; wo: wash out; h: hour; w: week

Data are means and SD; Significant differences in comparison to the wash-out period: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

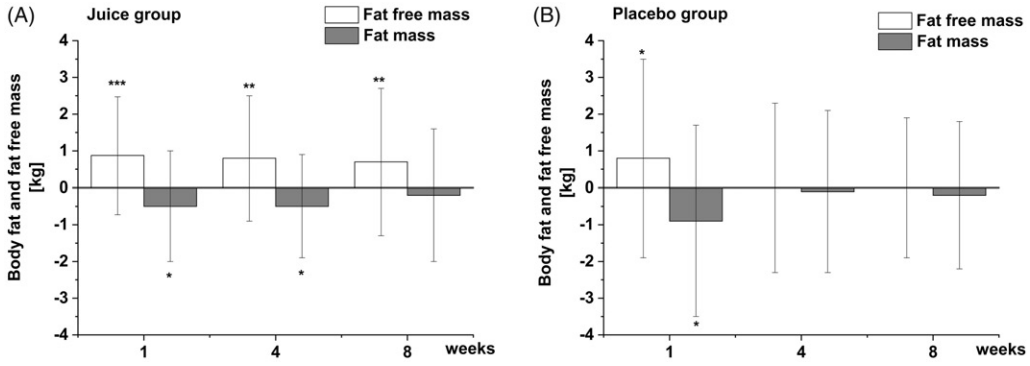

Figure 2. Changes in the body fat and fat free mass of volunteers in both groups during the intervention in comparison to the wash-out period: (A) juice group ($n = 30$); (B) placebo group ($n = 27$). Data are presented as mean values and SD of differences. Significant differences in body composition relative to the wash-out period: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

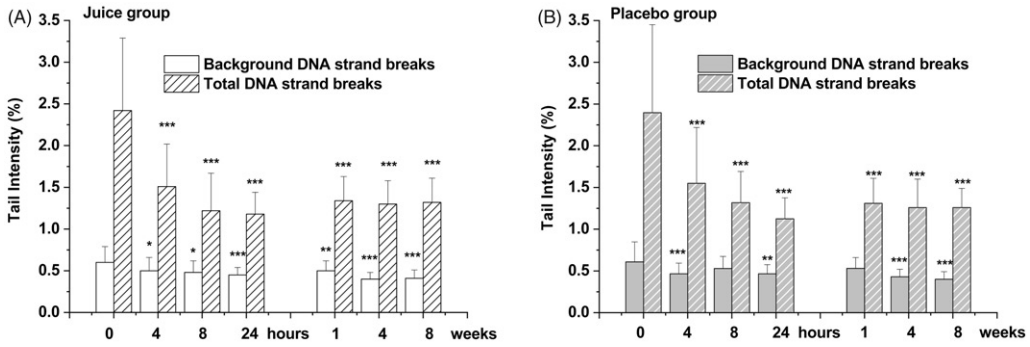

Figure 3. Background and total DNA strand breaks in (A) juice group ($n = 30$) and (B) in placebo group ($n = 27$) during the study. Data are expressed as tail intensity in % (TI%) with mean values and SD; significant differences in comparison to the wash-out: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

Table 5. The effects of study beverages on blood plasma parameters throughout the study period.

	After wo	4 h	8 h	24 h	1 w	4 w	8 w
Juice group							
SOD (U/mL)	3.06 ± 1.40	3.39 ± 1.50**	2.81 ± 1.58*	3.00 ± 1.53	3.15 ± 1.36	3.19 ± 1.53	3.33 ± 1.60*
CAT (nmol/min/mL)	35.7 ± 13.7	34.1 ± 13.8	30.3 ± 10.1*	24.7 ± 10.0***	33.5 ± 12.6	29.6 ± 7.3	29.9 ± 9.2
TC (mg/dL)	159.4 ± 27.4	155.8 ± 27.8***	153.8 ± 28.5***	148.0 ± 25.8**	154.1 ± 29.2	155.9 ± 24.3	166.9 ± 29.8
LDL (mg/dL)	88.8 ± 25.3	85.8 ± 25.4***	81.3 ± 28.9***	82.2 ± 23.6*	86.6 ± 26.0	102.2 ± 26.6	101.1 ± 27.8
HDL (mg/dL)	53.2 ± 10.7	53.3 ± 10.2	52.1 ± 10.4***	51.3 ± 9.2***	51.5 ± 10.5*	54.2 ± 10.1	53.3 ± 11.9
TG (mg/dL)	82.9 ± 43.0	80.2 ± 39.4	125.8 ± 70.7***	72.0 ± 32.0	80.1 ± 30.4	80.2 ± 36.5	85.6 ± 37.3
oxLDL (U/L)	39.8 ± 11.1	n.a	n.a	n.a	38.9 ± 11.5	41.4 ± 11.1	40.8 ± 10.6
IP 10 (pg/mL)	89.6 ± 48.9	n.a	n.a	n.a	96.3 ± 68.3	100.7 ± 74.3	86.4 ± 53.6
Placebo group							
SOD (U/mL)	2.69 ± 1.23	2.93 ± 1.40*	2.55 ± 1.63	2.65 ± 1.09	3.04 ± 1.33	2.91 ± 1.68	2.78 ± 0.80
CAT (nmol/min/mL)	37.83 ± 14.68	36.74 ± 12.25	34.32 ± 13.11	25.78 ± 10.4***	37.81 ± 11.10	32.32 ± 10.80	34.64 ± 10.21
TC (mg/dL)	163.2 ± 28.5	155.8 ± 28.3***	156.9 ± 26.8***	157.8 ± 27.5**	153.6 ± 23.6**	161.6 ± 27.6	175.2 ± 32.7***
LDL (mg/dL)	92.2 ± 26.0	87.8 ± 25.8***	88.5 ± 23.8***	90.0 ± 24.8*	87.0 ± 22.0*	110.5 ± 30.0***	111.8 ± 33.5***
HDL (mg/dL)	53.0 ± 12.3	51.8 ± 11.5**	51.5 ± 11.5***	50.4 ± 11.3***	49.2 ± 9.7**	52.1 ± 10.4	52.9 ± 10.6
TG (mg/dL)	80.1 ± 32.3	92.5 ± 50.7*	117.4 ± 84.9**	89.3 ± 37.1	99.9 ± 59.8*	90.3 ± 37.0	91.2 ± 48.4
oxLDL (U/L)	41.8 ± 11.9	n.a	n.a	n.a	38.9 ± 9.9	42.3 ± 11.5	42.6 ± 13.5
IP 10 (pg/mL)	71.9 ± 26.3	n.a	n.a	n.a	85.9 ± 36.7	90.6 ± 58.9	86.5 ± 33.5

SOD: super oxide dismutase, CAT: catalase, TC: total cholesterol, LDL: low-density lipoprotein, HDL: high-density lipoprotein, TG: triglycerides, ox LDL: oxidised low density lipoprotein, IP 10: interferon-γ-induced-protein 10; wo: wash out; h: hour; w: week
Data are means and SD. significant differences in comparison to the wash-out period: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

The effects of study beverages on blood plasma parameters

Table 5 summarises the results from analyses of blood lipids, IP10, CAT, and SOD in volunteers' plasma samples. Concerning antioxidative parameters, SOD activity significantly increased 4 h after the consumption of both beverages ($p < 0.01$ juice group, $p < 0.05$ placebo group) relative to the wash-out period. However, SOD activity had decreased at both the 8 and 24 h time points. In comparison to the placebo drink, the

consumption of anthocyanin-rich fruit juice led to a significant increase in SOD activity ($p < 0.05$) at the end of the intervention period. CAT activity decreased in both group during the whole study period.

Both groups of volunteers showed similar changes in plasma lipids over the course of the study (Table 5). The TC levels of all volunteers significantly decreased after 24 h, 1 week and 4 weeks relative to the wash-out period. By the end of the study period, the TC levels had increased from the earlier values and were higher than the values after wash-out period. In both groups, the LDL cholesterol levels reduced within 24 h of 750 mL bolus intake and after 1 week of intervention. The continuous consumption of both beverages over 4 and 8 weeks increased LDL levels so that they were slightly higher than the values after the wash-out period. In both groups, HDL cholesterol levels decreased within 1 week of intervention, after which the levels increased to reflect values similarly to after the wash-out period. In comparison to the wash-out period, the TG levels of volunteers from placebo group increased throughout the whole study period. Likewise, a distinct increase in TG levels was detected 8 h after volunteers consumed the red fruit juice bolus. However, TG levels decreased 24 h after the consumption of red fruit juice and remained almost unchanged throughout the course of the study. The oxLDL and inflammatory marker IP10 levels did not show significant changes in both groups at any point in the study.

Discussion

The aim of presented 9-week randomised, placebo-controlled intervention study was to investigate the effects of anthocyanin-rich fruit juice on biomarkers of oxidative stress response, such as DNA strand breaks, antioxidative enzyme activity, inflammation marker, and blood lipids. Additionally, body composition and energy/nutrient intake were monitored. In this intervention study, volunteers consumed a 750 mL bolus of anthocyanin-rich fruit juice or placebo beverage on day one of the study (after a 7-day wash-out period), after which they consumed 750 mL of the respective beverage in three equal portions per day for the remainder of the study period. The anthocyanin-rich fruit juice was a mixture of different red fruit juices/concentrates (red grape, lingonberry, aronia) and purees (apple, blueberry, strawberry, acerola), and contained relatively high amounts of polyphenols and total anthocyanins, as well as significant levels of vitamin C (564 mg/L). The high vitamin C concentration in the study juice was primarily attributed to the presence of acerola puree since acerola has one of the highest vitamin C contents among fruits. In a previous study, researchers prepared juice from acerola pulp and powder showed vitamin C concentrations as high as 10 g/L [24]. Most commercially-available red fruit juices have naturally occurring amounts of vitamin C less than 350 mg/L. The placebo drink was prepared by adding fructose, glucose, sucrose, citric acids, vitamin C, and natural flavours into water to obtain almost the same composition as the fruit juice under study. Unfortunately, because of excessive addition of vitamin C, the placebo drink contained more vitamin C (689 mg/L) than the red fruit juice (564 mg/L). Thus, both study beverages included substantially higher concentrations of vitamin C than other commercially-available red fruit juices. Volunteers in the anthocyanin-rich fruit juice group consumed 423 mg vitamin C and 205.5 mg anthocyanins daily while volunteers in the placebo group consumed 516 mg vitamin C daily.

The spot urine analysis verified that no volunteers had consumed anthocyanins during the wash-out period. Moreover, no anthocyanins were detected from volunteers in the placebo group during the intervention period, while anthocyanins could be detected from all samples from the group consuming anthocyanin-rich fruit juice. The obtained results clearly show that all volunteers had adhered to the study protocol. The intake of both study beverages led to an increase in energy and nutrient intake and a decrease in fat and protein intake during the whole study period. However, the body weight of volunteers consuming anthocyanin-rich fruit juice increased during the first week, after which it remained unchanged for the remainder of the intervention. Anthropometric measurements revealed that the consumption of anthocyanin-rich fruit juice has a distinct impact on body composition. The volunteers consuming anthocyanin-rich fruit juice experienced a significant increase in fat-free mass throughout the study period in comparison to the wash out. The body fat of volunteers from juice group was also significantly modulated. A significant reduction of this parameter was observed after one and 4-week intervention. Furthermore, the consumption of fruit juice rich in anthocyanins and vitamin C seems to influence lipid metabolism. Previous research has shown that body fat mass in humans can be influenced by the intake of flavons from citrus [25–28] or chlorogenic acids from coffee [29,30]. In an animal study, Prior et al. observed a reduction in body fat in mice fed isolated anthocyanins in drinking water (0.2 mg/mL) for 72 days, while this effect was not as pronounced, but still observable, in mice consuming blueberry juice (2.8 mL per mouse) [31]. Cañete da Costa et al. [32] reported a significant

reduction in the body mass index (BMI) and total body weight of 35 elderly women after the intake of juice prepared from red grapes (total phenolics 53.6 mg/mL). Other data highlight that certain polyphenols, such as citrus flavons, can inhibit phosphodiesterase and induce lipolytic effects in adipocytes [26].

In the present study, the consumption of both beverages markedly decreased background and total DNA strand breaks in the peripheral blood cells of volunteers. During the whole study period %TI values of FPG treated samples, indicating total DNA damage, were two to three-fold higher compared to the respective values of background DNA strand breaks (without FPG treatment). This finding substantiates that oxidative DNA damage largely accounts for the total DNA damage measured, in line with earlier findings by our and another group, respectively [5,11,33]. A marked decrease in DNA strand breaks was already observed 4 h after bolus intake, and the effect was prolonged over the entire 55-day intervention. This is in line with our previous findings, as the consumption of coffee rich in chlorogenic acids every 2 h reduced DNA strand breaks (background and oxidative ones) within 8 h [34]. Additionally, comparable results were observed in our previous investigations of fruit juices prepared from different red berries, namely, DNA strand breaks decreased within 8 h of the consumption of a 700 mL bolus of red fruit juice (unpublished data) [16]. Weisel et al. observed long-term protective effects on DNA strand breaks in healthy volunteers after a 4-week intervention of 700 mL/day red fruit juice [5]. In another study total (FPG-sensitive sites) and H_2O_2-induced DNA strand breaks decreased significantly after volunteers had consumed a drink prepared from wild blueberries for 6 weeks, while no effect was observed in the placebo group [35]. In the present study, both the placebo drink and anthocyanin-rich fruit juice showed the same effects on DNA strand breaks, with these effects probably the result of high vitamin C contents in both study beverages. In a human intervention study, Brennan et al. [36] found supplementation with vitamin C (2×500 mg/d) for 42 days to significantly decrease H_2O_2-induced DNA damage in peripheral blood cells. Furthermore, another human intervention study reported that the consumption of golden kiwifruits, which are rich in vitamin C, significantly decreases H_2O_2-induced DNA damage in lymphocytes. Additionally, the consumption of kiwifruit over a 4-week period decreased FPG-sensitive sites in lymphocyte DNA [37]. We hypothesise that the high amounts of vitamin C in both beverages used in this study masked the protective effects of anthocyanins. In a previous *in vitro* study, we demonstrated that a bilberry extract protects DNA against menadione-induced oxidative damage in Caco-2 cells [8]. Other studies with human subjects have also shown that anthocyanins, or foods rich in anthocyanins, protect DNA from oxidative damage [12,38]. Future studies should concentrate on using anthocyanin-rich juice with lower vitamin C content (i.e. similarly to other commercially-available fruit juices) to investigate whether anthocyanins and other polyphenols such as chlorogenic acids provide substantial DNA protective effects. However, it is still not clear whether the ingredients of the juice and/or vitamin C directly scavenge ROS or whether they confer an indirect protective effect by upregulating the expression of Nrf2/ARE-dependent antioxidative genes, as was also mentioned by Kropat et al. in a study of a bilberry pomace extract [12]. Their experiments demonstrated that the consumption of bilberry extract markedly reduces DNA strand breaks only 2 h after intervention, which was concomitant with significantly elevated transcription of the Nrf2-dependent gene NQO1 and diminished transcription of Nrf2. The observed impacts of Nrf2 and Nrf2-dependent gene transcription suggests a role for Nrf2 in the antioxidative defence system, especially when considering the long-term perspective and the associated chemoprevention. We also measured the activities of antioxidative enzymes related to the Nrf2/ARE pathway. The activity of SOD in the group consuming anthocyanin-rich fruit juice was significantly elevated ($p < 0.05$) within 8 weeks of intervention, whereas in the placebo group SOD activity was slightly diminished. These findings are partly in line with data presented by Kuntz et al. [6], who found that the intake of an anthocyanin-rich juice or smoothie increases plasma SOD and CAT activities, but does not influence SOD activity in erythrocytes after 14 days. However, in the present study, we observed a reduction in CAT activity in both groups. Rangel-Huerta et al. [28] reported that the consumption of orange juice with normal or high polyphenol content decreased CAT activity over a 12-week period, which is in line with our observations. Additionally, Broncel et al. reported a significant increase in SOD and GPx activities and a decrease in CAT activity in metabolic syndrome patients after the intake of aronia extract (3×100 mg/d) for 2 months [9]. Due to their scavenging activity, anthocyanins can reduce superoxide anions and consequently, hydrogen peroxide which is generated during normal cell metabolism. These effects result in the suppression of CAT biosynthesis. Hydrogen peroxide is also deactivated by GPx. In this line, elevated GPx activity can decrease the concentration of the CAT substrate and thus, inhibit

CAT activity [9]. We did not measure the GPx activity in volunteers' plasma samples in this study. Therefore, we cannot fully explain the reasons underlying the decline in CAT activity observed here. On the other hands, a study performed with 20 healthy young females showed no difference in CAT, GPx, SOD, lipid oxidation, and oxidative DNA damage in women which consumed 750 mL of cranberry juice/day for 2 weeks in comparison to the control group [39].

Our plasma lipid analyses show a significant decrease in total and LDL cholesterol within 24 h as well as after 1 week of intervention in the case of both beverages. Although these parameters increased towards the end of the study, they were still below the reference values (TC, <192 mg/dL; LDL, <135 mg/dL). Comparable effects were observed in a study that investigated the health benefits of frozen, stored bilberries. Consumption led to a significant decrease in total and LDL cholesterol in the blood of women, whereas in men, LDL increased significantly [10]. Broncel et al. reported a significant decrease in total and LDL cholesterol in metabolic syndrome patients who had consumed aronia extract for 2 months [9]. However, neither of these parameters showed noticeable changes when healthy volunteers consumed cranberry juice containing significant levels of vitamin C and total polyphenols [39]. In the present study, measured levels of oxLDL did not change after the consumption of anthocyanin-rich fruit juice or placebo drink. This is in agreement with findings from other studies in which healthy volunteers consumed polyphenol-rich fruit juice [38] or aronia extract [40]. In contrast, other studies have reported reduced LDL oxidation in volunteers after the consumption of foods rich in vitamin C or polyphenols [41,42]. The type of intervention, study subjects, product consumed and/or other differences in study conditions may be responsible for these conflicting results. A previous *in vitro* study showed that this marker was significantly reduced in HT29 colonic cells incubated with anthocyanins and bilberry extract [7]. Nevertheless, we did not detect any change in IP10 levels in our volunteers during the 2-month intervention. Another human intervention study, performed by Karlsen et al. with 120 healthy volunteers, found that the consumption of anthocyanins isolated from bilberries and black currants decreases plasma concentrations of several NF-kB-regulated proinflammatory chemokines and immunoregulatory cytokines [43]. However, the IP10 levels did not change significantly over the course of their study.

Taken together, anthocyanin-rich red fruit juice as well as the placebo drink both rich in vitamin C, demonstrate potential to improve DNA integrity and may influence lipid metabolism in human subjects. To better observe the effects of anthocyanins and other polyphenols in humans, future research should employ a juice that is rich in anthocyanins but has a noticeably lower vitamin C content than the red fruit juice used this study.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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