JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Biosafety, Antioxidant Status, and Metabolites in Urine after Consumption of Dried Cranberry Juice in Healthy Women: A Pilot Double-Blind Placebo-Controlled Trial

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This study assessed the effect of an 8 week consumption of dried cranberry juice (DCJ) on 65 healthy young women. Basic biochemical and hematological parameters, antioxidant status, presence of metabolites in urine, and urine ex vivo antiadherence activity were determined throughout the trial. A 400 mg amount of DCJ/day had no influence on any parameter tested. A 1200 mg amount of DCJ/day resulted in a statistically significant decrease in serum levels of advanced oxidation protein products. This specific protective effect against oxidative damage of proteins is described here for the first time. Urine samples had an inhibitory effect on the adhesion of uropathogenic *Escherichia coli* strains, but no increase in urine acidity was noted. Hippuric acid, isomers of salicyluric and dihydroxybenzoic acids, and quercetin glucuronide were identified as the main metabolites. In conclusion, cranberry fruits are effective not only in the prevention of urinary tract infection but also for the prevention of oxidative stress.

KEYWORDS: ***Vaccinium macrocarpon*; human study; blood; biomarkers; AOPP; urine; metabolites; *Escherichia coli*; adherence**

INTRODUCTION

Cranberry (*Vaccinium macrocarpon*, Aiton, Ericaceae) fruits and juice have been used to prevent urinary tract infection (UTI) and to protect humans against oxidative stress (*1*). Cranberries contain mainly vitamin C, dietary fiber, glucose and fructose, flavonoids [flavonols, anthocyanins, and proanthocyanidins (condensed tannins)], and gallic, benzoic, citric, and oxalic acids. The medicinal effectiveness and safety of cranberry juice/pills have been critically evaluated recently (*2*, *3*). Cranberries seem to be the most effective in preventing the adhesion of *Escherichia coli* to uroepithelial cells, which is responsible for 85% of UTIs (*4*). Several human studies of cranberry for the treatment of UTIs have been reported (*5*−*7*), but their meta-analysis failed to show any therapeutic effect (*3*). The antiadherence activity was mostly assigned to A type proanthocyanidin trimers (*8*, *9*), whereas anthocyanic or proanthocyanic fractions exhibited no marked antibacterial activity (*10*). No evidence was found in the literature for antiadherent cranberry A type proanthocyanidin trimers in human urine (*11*−*13*). The ex vivo antiadherence activity was found in mice in a 30 day experiment (*14*) and in volunteers (n = 5 or 6, respectively) after a single dose of 42.5 g of dried cranberries (*15*) or 240−750 mL of cranberry juice (*9*, *16*). It has been suggested that fructose is responsible for the antiadherence activity of cranberries (*17*). However, fructose is contained in most fruits, but the effects on the urinary tract were found only in cranberries. The antiadherence activity of citrus juice was found on some *E. coli* strains but not on the strains equipped with P-fimbriae (*17*). An antiadherence effect of fruits with comparable contents of fructose as cranberries (grape and apple juices and raisins) has not been proven (*9*, *15*). Raz et al. (*18*) assume that, considering its low bioavail-

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10.1021/jf0636014 CCC: $37.00 © 2007 American Chemical Society
Published on Web 03/24/2007

ability, the activity of cranberry components is primarily localized in lower parts of the gastrointestinal tract, where they selectively influence potentially pathogenic bacteria.

Cranberry should be avoided by people with allergy/hypersensitivity to the *Vaccinium* species. A risk of kidney stones (*19*−*21*) and increased salicylate concentration in urine (*22*) has been described in the literature. Recently, Aston et al. (*23*) reviewed the literature on possible interactions between cranberry juice and warfarin in humans: Several cranberry components may inhibit the activity of cytochrome P450 (CYP) 2C9 (*24*, *25*), thus potentiating the anticoagulant effects of warfarin.

However, none of the human studies with cranberries, cranberry juice, concentrate, and/or pills evaluates the safety of long-term regular consumption. In this study, we assessed the effect of an 8 week consumption of 400 and 1200 mg/day of dried cranberry juice (DCJ) in young healthy women. Basic biochemical and hematological parameters, antioxidant status, the presence of metabolites in urine, and urine ex vivo antiadherence activity were determined in the volunteers throughout the trial. The contents of anthocyanins, phenolic acids, and sugars were identified and determined, and in vitro antimicrobial and antiadherence activities were tested in DCJ.

EXPERIMENTAL PROCEDURES

Material. DCJ (NutriCran90, Decas Botanical Synergies, United States), declared to contain 89% saccharides, 0.5% proteins, 7% ash, and 2.8 mg ascorbic acid/100 g, was used in the form of a food supplement for the clinical part. One gelatin capsule of the food supplement contained 200 mg of DCJ (equivalent to 5 g of fresh cranberries), 290 mg of soy oil, 15 mg of lecithin, and 10 mg of beeswax; gelatin capsules of placebo contained 443 mg of soy oil. Both capsules were kindly provided by Walmark a.s. (Třinec, Czech Republic).

HPLC-MS Analysis of DCJ Components. *Instrumentation.* Microliquid chromatograph CapLC (μLC/MS, Waters Corp., Milford, United States), coupled with an ion-trap mass spectrometer (LCQ, Finnigan, United States) or a hybrid mass spectrometer Q-TOF Premier (Waters Corp.) was used for all analyses. A microcolumn Gemini C-18 (150 mm × 300 μm, Phenomenex, United States) was used for chromatographic separation. The sample injection volume of 2 (partially filling 10 μL loop) or 1 μL (full loop injection mode using 1 μL loop), respectively, was used. Electrospray was the ionization technique used. Ionization parameters were optimized using standards of malvidin-3-glucoside and hippuric acid, respectively. Identification was based on the MS and MS/MS spectra collected over the chromatographic peaks and their comparison with spectra of standards. Calibration curves of 4-hydroxybenzoic acid, quercetin, malvidin-3-glucoside, and hippuric acid were used for quantification. Each compound also served for quantification of related compounds (3-hydroxybenzoic acid for the group of phenolic acids, quercetin, for flavonoids, malvidin-3-glucoside for anthocyanin dyes, and hippuric acid for conjugates of phenolic acids with glycine). Response factors were neglected. Calibration dependencies for 3-hydroxybenzoic acid, quercetin, and hippuric acid exhibited good linearity over the concentration range 1−200 mg/L (R^2 > 0.993 in all cases). The calibration dependence for malvidin-3-glucoside was sufficiently linear in the concentration range of 1−100 mg/L (R^2 = 0.997).

Anthocyanin Determination. The samples (10 mg of DCJ or 100 mg of freeze-dried urine) were dissolved in 1 mL of 0.01% HCl, v/v. The solutions (0.5 mL) were then applied on a conditioned solid-phase extraction (SPE) column (polymeric sorbent with reversed-phase separation mode, Strata SDB-L, Phenomenex, 500 mg/3 mL). The column was subsequently washed with 3 mL of 0.01% HCl, and the anthocyanins were eluted by 3 mL of acidified methanol (0.01% HCl in MeOH). The eluate was evaporated using a gentle stream of N_2 at 40 °C. The solid residue was dissolved in the respective mobile phase A and analyzed by μLC/MS using gradient elution: mobile phase A, 0.12% trifluoroacetic acid and 5% acetonitrile in water (v/v); mobile phase B, 0.12% trifluoroacetic acid in acetonitrile (v/v); gradient, 0−30 min, 10−90% B; 30−36 min, 90% B.

Determination of Phenolic Acids and Metabolites Using Preseparation. The samples (DCJ or freeze-dried urine) were dissolved in a 50 mM phosphate buffer, pH 7.0 (44 mg of the sample in 1.5 mL), and the solutions were filtered through a Teflon membrane microfilter (porosity, 0.45 μm). The sample was then applied on a conditioned SPE column (mixed sorbent RP/anex, Strata Screen A, Phenomenex, 200 mg/3 mL). The column was washed with 3 mL of deionized water and 3 mL of methanol and eluted with 3 mL of 1% HCl in methanol. The eluate was evaporated using an N_2 stream at 40 °C. The solid residue was dissolved in the respective mobile phase A and analyzed by μLC/MS using gradient elution: mobile phase A, 5.7 mM acetic acid and 5% acetonitrile in water (v/v); mobile phase B, acetonitrile; gradient, 0−5 min, 10% (v/v) B; 5−25 min, 10−90% (v/v) B; 25−40 min, 90% (v/v) B; 40−45 min, 90−10% (v/v) B; and 45−50 min, 10% (v/v) B.

Determination of Phenolic Acids and Metabolites without Preseparation. Freeze-dried urine samples were consequently analyzed directly after filtration, without prepurification using SPE. These analyses were designed in order to identify possible metabolites that were not selected by the SPE column used. The results of identified metabolites were consistent with those found using preseparation. The contamination of both chromatographic column and mass spectrometer was higher than using preseparation. The samples (10 mg of the freeze-dried urine) were dissolved in 1 mL of the respective mobile phase A, and solutions were filtered through a Teflon membrane microfilter (porosity 0.45 μm) and analyzed by gradient elution (described above).

Antiadherence Activity of DCJ and Urines. The antiadherence activity of DCJ and freeze-dried urine was tested on 12 pathogenic *E. coli* strains isolated from the urine of patients with UTIs. Strains that were able to agglutinate red blood cells (A_1, Rh+) were selected in order to confirm the presence of adherence factors. The selected strains were cultivated in tryptose broth (HiMedia, India) and then on an agar medium (Colonisation Medium, HiMedia) at 37 °C, in an aerobic atmosphere containing 10% CO_2 for 16 h. The testing was performed using red blood cells as described previously (*8*, *26*). Briefly, DCJ was dissolved in phosphate-buffered saline (PBS), and consecutive dilutions were prepared. Thirty microliters of each dilution was applied into wells of a U type 96 well microtiter plate (Gama, Czech Republic), and then, the bacterial suspensions (5×10^8 cells/mL in PBS, 10 μL) were added and incubated for 10 min at room temperature. Ten microliters of a red blood cell suspension (3% in PBS) was then added, and the plate was further incubated for 20 min. Inhibition of hemagglutination was detected microscopically in comparison with controls, containing only the respective bacterial strain and red blood cells.

Quantitative Biofilm Assay. Adhesion of the bacteria was then also tested using a microtiter polystyrene plate by a quantitative biofilm assay modified from Christensen et al. (*27*). Samples of DCJ (0, 0.037, 0.075, 0.15, 0.3, 0.6, 1.2, and 2.4 mg/mL), mannose (1%), and freeze-dried urine (to their initial concentration) were dissolved in BHI (brain heart infusion, Himedia) enriched with 0.25% glucose. These solutions (200 μL) were applied to the wells of sterile microtiter plates. The wells were then inoculated with 1 μL of bacterial suspension at a density corresponding to degree 1 of the McFarland scale. Thus, 10^5−10^6 colony-forming units (CFU)/mL were present in the wells. The plate was then incubated in an aerobic atmosphere at 37 °C overnight and then carefully washed with water. The biofilm formed was fixed by 200 μL of 99% methanol (15 min). After they were dried, the wells were stained with 160 μL of 1% crystal violet for 10 min and thoroughly washed with water. The fixed dye was then dissolved in 160 μL of 33% acetic acid. Color intensity, indicating the quantity of adhered bacteria, was determined spectrophotometrically at 570 nm (photometer MRX II, Dynex) (*28*, *29*).

Antibacterial Activity of DCJ. The antimicrobial activity of DCJ was tested using standard dilution micromethod (*2*) by determining the minimal inhibition concentration (MIC) of the sample, needed to inhibit bacterial growth. Testing was performed in microtiter plates, and the samples were diluted geometrically in Mueller−Hinton broth (Difco). Standard reference bacterial strains *Staphylococcus aureus* CCM 3953,

Enterococcus faecalis CCM 4224, *E. coli* CCM 3954, and *Pseudomonas aeruginosa* CCM 3955 (according to the Culture collection of the Masaryk University, Brno, Czech Republic) and strains isolated from patients from the Olomouc University Hospital, Czech Republic [*S. aureus*, methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococcus faecium* and *Clebsiella peumoniae* (producing wide broad β-lactamase AmpA)], were used. A standard quantity of bacteria (10^{5-6} CFU/mL) was inoculated in all wells. The MIC was determined following 24 h of incubation at 37 °C as the lowest sample concentration that visibly inhibited bacterial growth. The type of the effect (bacteriostatic or bactericide) was determined by inoculating the content of the wells without visible growth into blood agar.

Study Subjects. A total of 65 healthy women, students, and staff of Palacký University, Olomouc, aged 19−28 years (mean, 21.6 ± 1.6 years) with no known history of kidney calculi or antibiotic treatment participated in the study. The study was approved by the Ethics Committee of the University Hospital and the Faculty of Medicine and Dentistry, Palacký University in Olomouc, Czech Republic. All of the participants signed an informed consent, and they were aware of the study goals and instructed not to consume food rich in phenolics, especially anthocyanin-containing fruits, but also coffee and tea 24 h prior to each assessment. The volunteers were randomly divided into three groups: group I [$n = 23$, placebo (two capsules once/day), aged 21.7 ± 2.0 years, body mass index (BMI) 21.2 ± 2.1 kg/m^2], group II [$n = 20$, 400 mg of DCJ (two capsules once)/day, aged 21.4 ± 2.0 years, BMI 21.2 ± 1.5 kg/m^2], and group III [$n = 22$, 1200 mg of DCJ (two capsules three times)/day, aged 21.7 ± 2.0 years, BMI 20.5 ± 1.8 kg/m^2]. The volunteers consumed the capsules over 8 weeks with three blood and urine collections and blood pressure measurements at days 0, 28, and 56. An additional blood collection was performed 8 months following the end of the supplementation period (day 296) only in 10 volunteers from group III.

Clinical Biochemistry and Hematology. Basic biochemical and hematological parameters were determined in all samples—cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triacylglycerols, ALT, AST, GMT, urea, creatinine, uric acid, and advanced oxidation protein products (AOPP) levels in serum, a blood count including a differential leukocyte count in morning fasting blood, pH, and sediment examination of midstream urine. These were determined at the Department of Clinical Biochemistry of Šternberk Hospital (Czech Republic) using an ADVIA 1650 analyzer (Bayer, SRN). In group III, these examinations were expanded with 12 h urine collections, determination of total phenolic contents in urine using Folin−Ciocalteu phenolic reagent (*30*), and selected parameters for evaluation of oxidative stress [total antioxidant capacity and SH groups in plasma, lipid peroxidation products such as malondialdehyde (MDA) in plasma and erythrocytes, glutathione (GSH), superoxide dismutase (SOD), and glutathione peroxidase (GPX) in erythrocytes as described by Psotová et al. (*31*)]. The collected urine was stored at 4 °C up to the transmission to the trial organizers and then immediately evaporated to ca. 50 mL and freeze-dried.

Statistics. All of the results were evaluated using analysis of variance at $p < 0.05$.

RESULTS

Components of DCJ and Its in Vitro Biological Activity. DCJ contained 1−10% (m/m) phenolic acids, and benzoic, salicylic, ellagic, ferulic, caffeic, *p*-coumaric, sinapic, vanillic, and protocatechuic acids were identified. The total polyphenolic content was 3% (m/m). Quercetin [0.3% (m/m)] and anthocyanin dyes [0.44% (m/m); **Table 1**] were determined. Moreover, DCJ contained 42% (m/m) glucose, 26% (m/m) fructose, and 10% (m/m) MgO as a carrier. The antiadherence activity of DCJ was shown for concentrations ranging from 0.075 to 0.12 mg/mL; in the biofilm formation assay, significant reduction of adherence was observed at 0.037 mg/mL. Marked reduction was nevertheless observed in concentrations higher than 2.4 mg/mL (**Figure 1**). DCJ displayed a slight antimicrobial activity on tested bacterial strains (MIC 179−357 mg/mL). The highest activity was observed in the case of *P. aeruginosa* with MIC 179 mg/mL.

Table 1. Anthocyanins Determined in DCJ[a]

anthocyanin	% (m/m)	anthocyanin	% (m/m)
Pn-3,5-diGal	0.106	Cy-3-Ara	0.024
Cy-3-Gal	0.031	Pn-3-Gal	0.045
Cy-3-Glu	0.054	Pn-3-Glu	0.135
Pn-3-Ara	0.025	Dp	0.008
Cy-3-Pent	0.013	sum	0.44

[a] Pn, paeonidin; Cy, cyanidin; Dp, delphinidine; Gal, galactoside; Glu, glycoside; Ara, arabinoside; and Pent, nonidentified pentosid.

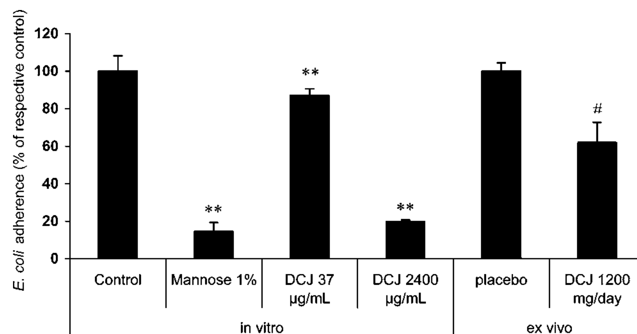

Figure 1. Effect of mannose, DCJ (in vitro), and urine of volunteers taking placebo and DCJ (1200 mg/day, ex vivo) on adherence of uropathogenic *E. coli*. Results are expressed as means ± SE. The effect of the urine is expressed per mmol of creatinine. The adherence in urines from the placebo group was considered to be 100%. **$p < 0.01$ vs control and $^{\#}p < 0.05$ vs placebo.

Biosafety of DCJ. Fifty-seven out of a total of 65 volunteers finished the study. Two women withdrew from group I [respiratory illness (*1*) and personal reasons (*1*)], and six withdrew from group III [respiratory illness (*2*), excessive urination (*1*), stomach acidity (*1*), and personal reasons (*1*)].

Statistically evaluated results are presented in **Tables 2−4**. A significant decrease in HDL cholesterol was observed in group I (placebo) on days 28 and 56 as compared to day 0. Moreover, serum AST and creatinine levels dropped on day 56 (**Table 2**). In group II (400 mg of DCJ /day), HDL cholesterol was lower on day 56 and AST was lower on days 28 and 56. The AST and hemoglobin levels on day 28 in group II were significantly lower than in group I (**Table 3**). In group III (1200 mg of DCJ /day), a significant but clinically nonrelevant increase in diastolic blood pressure was noted on day 0 as compared to groups I and II. This event did not occur in subsequent examinations. As compared to group I, ALT levels were lower on days 0 and 28, but AST levels were higher than in group II on days 28 and 56. A drop in the serum creatinine level was observed on day 28 as compared to days 0 and 56 and also with group II (**Table 4**). However, all of these observed changes were within the normal physiological range and thus not due to any adverse effect of the food supplement.

Antioxidant Status. The AOPP level was measured in subjects in all three groups. While in group II, this parameter of late oxidative stress was significantly increased on days 28 and 56, in group III, a very strong significant AOPP drop was observed even after 4 weeks of intervention. The values measured were much lower than those usually observed in the Czech population (Stejskal, personal communication); in some volunteers, the measured value was probably lower than 5.0 μM (detection limit of the method used; **Table 4**). Because of this unexpected result, we decided to perform additional analyses

Table 2. Parameters of Clinical Biochemistry and Hematology in Group I (Placebo)[a]

parameter	day 0	day 28	day 56
n	23	23	23
systolic blood pressure	110 ± 10	110 ± 11	114 ± 10
diastolic blood pressure	71 ± 10	73 ± 9	75 ± 8
cholesterol (mM)	4.8 ± 0.7	4.7 ± 0.7	4.8 ± 0.8
HDL cholesterol (mM)	2.01 ± 0.34	**1.77 ± 0.26**[b]	**1.71 ± 0.34**[b]
LDL cholesterol (mM)	2.64 ± 0.50	2.54 ± 0.53	2.45 ± 0.54
triacylglycerols (mM)	1.11 ± 0.38	1.03 ± 0.65	1.06 ± 0.59
ALT (μkat/L)	0.33 ± 0.14	0.34 ± 0.19	0.30 ± 0.18
AST (μkat/L)	0.37 ± 0.07	0.33 ± 0.09	**0.24 ± 0.11**[b,c]
GMT (μkat/L)	0.25 ± 0.12	0.30 ± 0.20	0.24 ± 0.10
urea (mM)	2.97 ± 0.71	3.21 ± 0.73	3.24 ± 0.51
creatinine (μM)	84.7 ± 5.5	81.0 ± 5.2	**79.0 ± 4.6**[b,c]
uric acid (μM)	273.8 ± 57.1	247.8 ± 40.0	249.1 ± 50.6
AOPP (μM)	55.6 ± 14.5	65.5 ± 39.9	51.2 ± 27.3
urine pH	6.18 ± 0.52	5.91 ± 0.69	5.98 ± 0.53
	blood count		
hemoglobin (g/L)	134.1 ± 9.2	137.7 ± 8.7	133.3 ± 8.0
erythrocytes (10^{12}/L)	4.63 ± 0.21	4.48 ± 0.23	4.54 ± 0.26
leukocytes (10^9/L)	7.6 ± 1.5	7.5 ± 1.6	7.7 ± 2.1
thrombocytes (10^9/L)	276 ± 42	285 ± 55	277 ± 61

[a] Data are expressed as means ± SD. [b] $p < 0.05$ vs day 0. [c] $p < 0.05$ vs day 28.

Table 3. Parameters of Clinical Biochemistry and Hematology in Group II (400 mg of DCJ/Day)[a]

parameter	day 0	day 28	day 56
n	20	19	18
systolic blood pressure	114 ± 9	114 ± 13	116 ± 10
diastolic blood pressure	76 ± 5	78 ± 9	77 ± 7
cholesterol (mM)	4.7 ± 0.7	4.7 ± 0.6	5.0 ± 0.8
HDL cholesterol (mM)	1.91 ± 0.30	1.75 ± 0.22	**1.69 ± 0.23**[b]
LDL cholesterol (mM)	2.50 ± 0.51	2.39 ± 0.51	2.30 ± 0.46
triacylglycerols (mM)	1.36 ± 0.45	1.22 ± 0.29	1.34 ± 0.58
ALT (μkat/L)	0.28 ± 0.09	0.30 ± 0.08	0.30 ± 0.08
AST (μkat/L)	0.36 ± 0.08	**0.25 ± 0.07**[b,c]	**0.25 ± 0.07**[b]
GMT (μkat/L)	0.22 ± 0.09	0.21 ± 0.08	0.23 ± 0.07
urea (mM)	3.11 ± 0.91	3.37 ± 0.95	3.19 ± 1.14
creatinine (μM)	**77.3 ± 15.3**[c]	**75.2 ± 6.9**[c]	81.4 ± 6.0
uric acid (μM)	257.9 ± 52.4	249.9 ± 62.7	242.3 ± 60.9
AOPP (μM)	42.9 ± 10.1	**57.5 ± 19.8**[b]	**61.8 ± 21.3**[b]
urine pH	6.13 ± 0.88	5.71 ± 0.51	5.97 ± 0.70
	blood count		
hemoglobin (g/L)	129.5 ± 10.4	**127.6 ± 11.0**[c]	129.2 ± 12.0
erythrocytes (10^{12}/L)	4.62 ± 0.30	4.58 ± 0.26	4.59 ± 0.28
leukocytes (10^9/L)	7.86 ± 1.83	7.89 ± 1.52	7.97 ± 1.25
thrombocytes (10^9/L)	303 ± 92	296 ± 91	296 ± 97

[a] Data are expressed as means ± SD. [b] $p < 0.05$ vs day 0. [c] $p < 0.05$ vs group I.

for evaluation of oxidative stress in group III. A drop of total plasma SH group level on day 56 and an increase in lipid peroxidation products (MDA) in erythrocytes on days 28 and 56 were measured. Neither plasma antioxidant capacity, plasma lipid peroxidation product level, nor SOD, GSH, and GPX levels in erythrocytes were affected. Eight months after the end of the intervention period, the AOPP level was measured in 10 volunteers from group III. The AOPP level at day 296 (27.64 ± 6.54 mM) was significantly higher than on days 28 and 56 but did not reach the level on day 0 (**Figure 2**).

Identification and Determination of Metabolites in Urine. No change in urine pH was observed throughout the study in volunteers from all groups. The total phenolic content in urine from group III, expressed per mol of creatinine, was significantly higher at day 28 but then dropped back ($p > 0.05$; **Table 4**). Organic acids and phenolic compounds were analyzed in

Table 4. Parameters of Clinical Biochemistry and Hematology in Group III (1200 of DCJ mg/Day)[a]

parameter	day 0	day 28	day 56
n	22	20	16
systolic blood pressure	**119 ± 11**[d]	115 ± 11	116 ± 10
diastolic blood pressure	80 ± 8	75 ± 9	78 ± 6
cholesterol (mM)	4.6 ± 0.8	4.7 ± 0.9	4.7 ± 1.0
HDL cholesterol (mM)	**1.79 ± 0.23**[d]	1.78 ± 0.27	1.72 ± 0.21
LDL cholesterol (mM)	2.69 ± 0.67	2.67 ± 0.72	2.60 ± 0.74
triacylglycerols (mM)	1.26 ± 0.43	1.16 ± 0.62	1.23 ± 0.57
ALT (μkat/L)	**0.25 ± 0.07**[d]	**0.25 ± 0.06**[d]	0.25 ± 0.07
AST (μkat/L)	0.40 ± 0.05	**0.39 ± 0.06**[e]	**0.43 ± 0.07**[d,e]
GMT (μkat/L)	0.26 ± 0.11	0.27 ± 0.10	0.25 ± 0.10
urea (mM)	3.41 ± 0.52	3.11 ± 0.59	3.35 ± 0.54
creatinine (μM)	79.4 ± 5.0	**73.8 ± 4.3**[b,d]	**82.6 ± 6.1**[c]
uric acid (μM)	265.8 ± 46.5	**289.7 ± 61.0**[d]	270.5 ± 51.6
AOPP (μM)	47.4 ± 25.0	**9.53 ± 16.8**[b,d,e]	**11.4 ± 12.1**[b,d,e]
antioxidant capacity[f]	6.90 ± 1.11	7.32 ± 1.38	6.87 ± 1.25
SH groups[g]	5.99 ± 1.07	5.87 ± 0.79	4.93 ± 0.57[b,c]
SOD-ery (U/g proteins)	2.59 ± 0.51	2.73 ± 0.42	2.68 ± 0.33
MDA-ery[g]	0.46 ± 0.10	**0.57 ± 0.13**[b]	**0.59 ± 0.13**[b]
GSH-ery[g]	116.3 ± 24.5	119.6 ± 23.4	124.3 ± 24.1
GPX-ery (μU/g proteins)	10.60 ± 2.28	11.06 ± 2.25	11.74 ± 3.01
MDA[g]	0.16 ± 0.041	0.16 ± 0.031	0.15 ± 0.036
urine pH	5.79 ± 0.49	5.89 ± 0.54	5.75 ± 0.58
total phenols urine (mg/mol creatinine)	37.7 ± 14.9	**54.2 ± 20.5**[b]	**37.8 ± 12.7**[c]
	blood count		
hemoglobin (g/L)	132.3 ± 10.63	132.0 ± 6.94	131.1 ± 8.96
erythrocytes (10^{12}/L)	4.54 ± 0.35	4.53 ± 0.27	4.47 ± 0.37
leukocytes (10^9/L)	7.31 ± 1.75	7.36 ± 1.54	7.41 ± 1.84
thrombocytes (10^9/L)	276 ± 52	301 ± 54	277 ± 54

[a] Data are expressed as means ± SD. [b] $p < 0.05$ vs day 0. [c] $p < 0.05$ vs day 28. [d] $p < 0.05$ vs group I. [e] $p < 0.05$ vs group II. [f] μA/mL of plasma. [g] μmol/g of protein.

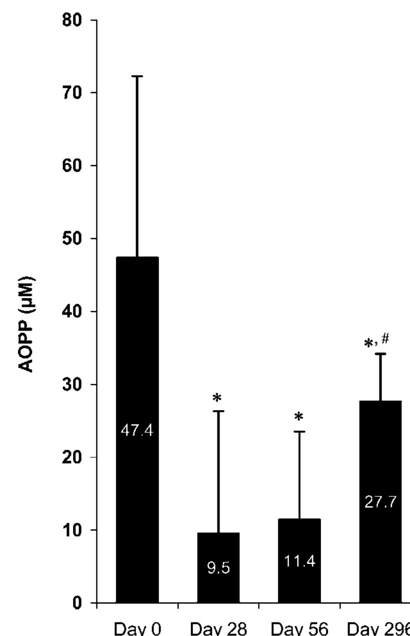

Figure 2. Serum AOPPs in volunteers taking DCJ (1200 mg/day). Day 296 corresponds to 8 months after the end of the intervention period. Data are expressed as means ± SD. *$p < 0.05$ vs day 0 and #$p < 0.05$ vs days 28 and 56.

collected urine from group III. Besides citric acid, hippuric and salicyluric acids, quercetin glucuronide, apigenin, and isomers of dihydroxybenzoic acids were found. Of these, the hippuric acid content dominated (7.01−42.82 mg/mmol of creatinine) followed by the isomers of the hydroxybenzoic acid−glycine

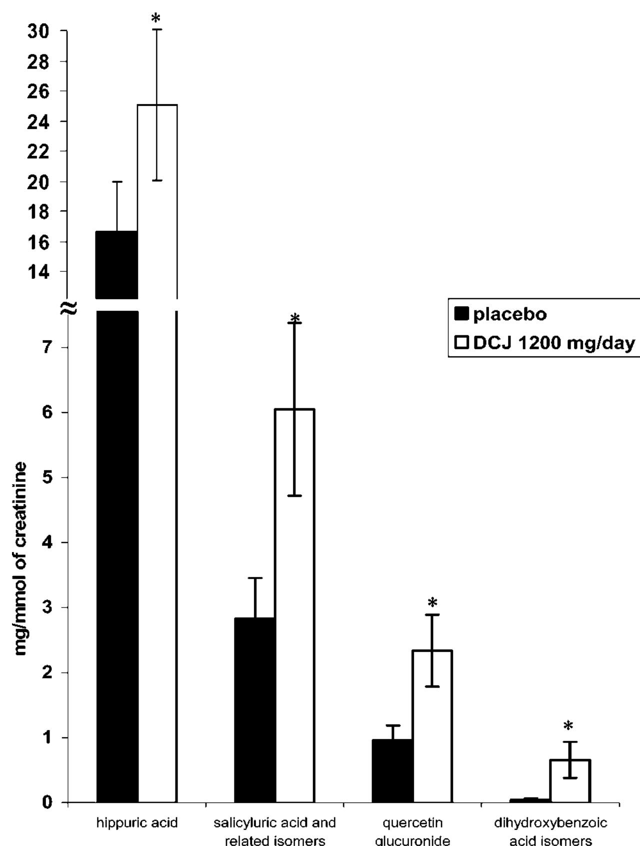

Figure 3. Content of cranberry metabolites (μLC/MS, see the Experimental Procedures) in urines from volunteers taking 1200 mg/day placebo or DCJ. Results are expressed as means ± SE. *$p < 0.05$ vs placebo.

conjugate (salicyluric acid, 2.00−12.53 mg/mmol of creatinine), glucuronide of quercetin (0.96−5.25 mg/mmol of creatinine), and isomers of dihydroxybenzoic acids (0−0.76 mg/mmol of creatinine; **Figure 3**). The level of anthocyanins in analyzed urine was very low. The sum of identified dyes (cyanidin, related arabinoside, galactoside and glucoside, delphinidin, and related arabinosides) was below the limit of quantification and did not exceed approximately 0.7 mg/mmol creatinine. No proanthocyanidin oligomers were detected using both positive and negative ionization modes. In comparison with group I (placebo), significantly higher levels of hippuric, salicyluric, and dihydroxybenzoic acids and quercetin glucuronide were found (**Figure 3**).

Reconstructed chromatograms and related collision spectra in **Figure 4** show a typical analysis of the dominant metabolites as well as unmetabolized dihydroxybenzoic acids in urine. As mentioned above, the content of hippuric acid was relatively high and a single peak dominated in the reconstructed chromatogram at *m/z* 178. The loss of CO_2 resulting in the formation of the ion *m/z* 134 can be clearly seen in the related collision spectrum collected over the chromatographic peak. Consequent fragmentation led to a peak *m/z* 77 corresponding to the deprotonated benzene.

Four peaks exhibiting analogous fragmentation were found in the reconstructed chromatogram at *m/z* 194 (considered isomers of salicyluric acid). The cleavage of CO_2 ($\Delta m/z$ 44 = 194 − 150) and the formation of phenoxide dominated in the collision spectra of all four peaks. Because the standards of isomers of "salicyluric acid" were not available, the identity of the last eluted peak (retention time 11.82 min) was not sufficiently elucidated. However, considering higher polarity of hydroxylated conjugate with respect to hippuric acid, we suggest that this peak did not correspond to the discussed conjugates and it was not taken into account in the quantification.

An intensive peak of quercetin glucuronide was found at *m/z* 477. The loss of dehydrated glucuronic acid could be found in the appropriate collision spectra. The second minor peak exhibited similar fragmentation and was ascribed to a quercetin glucuronide isomer. Three peaks could be found in the reconstructed chromatogram for *m/z* 153. A similar fragmentation pattern was observed in the related collision spectra of each peak. The cleavage of CO_2 or H_2O (losses of 44 and 18), respectively, were dominant processes. The fragment of *m/z* 135 consequently lost CO_2 and a fragment of *m/z* 109 lost H_2O. The fragmentation spectra were compared with the fragmentation of standards of protocatechuic and gentisic acids and a very good agreement was found. These acids are reported as the sum of dihydroxybenzoic acid isomers in **Figures 3** and **4**.

Antiadherence Activity of Urines. The effect of the urine on *E. coli* adhesion was tested using the biofilm assay. Uropathogenic *E. coli* bacteria formed a biofilm with abundant population only in milieu containing urine from group I (placebo; see **Figure 1**). Samples from group III lowered *E. coli* adhesion in comparison with group I ($p < 0.05$).

DISCUSSION

In vitro experiments with the cranberry juice and/or some its components display antioxidant, chemoprotective, and antiadherence activities (*18*). The comparison of clinical biochemistry and hematology data between volunteers consuming placebo vs 400 or 1200 mg/day of DCJ for 56 days demonstrated no significant effect of the food supplement on the markers evaluated (**Tables 2−4**). The history of kidney calculi was an exclusion criterion for participation in the study; therefore, evaluation of this risk factor was not included here. In group III, the dose of 1200 mg/day of DCJ (an estimated daily intake 35 mg of total phenols, 7.8 mg of anthocyanins, and 14.4 mg of proanthocyanidins) led to a drop in AOPP to values not usually found in our population after the 8 week intervention period (**Table 4** and **Figure 1**). Eight months after the end of the study, the serum AOPP level was increased again, although not to the initial value (**Figure 2**). This effect of plant antioxidants on AOPP level, apart from ascorbic acid, has not been described to date (*32*). Although ascorbic acid was also contained in DCJ, its daily intake in group III was only 0.034 mg, which is negligible as compared to its intake from a normal diet. No relationship between AOPP and other parameters of oxidative damage was found (**Table 4**). AOPP are generated by the reaction of free radicals with proteins. They activate proinflammatory cytokines, which initiate the oxidative burst of neutrophiles, monocytes, and T-lymphocytes (*33*). Patients with ischemic heart disease and uremic syndrome have higher AOPP levels. In both groups of patients, AOPP levels were significantly higher than in healthy volunteers (*34*, *35*). Our study demonstrated that DCJ may have not only antiadherence but also antiatherogenic effects. Ruel et al. (*36*) described a lowering of lipid peroxidation products after drinking cranberry juice [men, $n = 21$, 38 ± 8 years, 7 mL/kg body mass (BM), 2 weeks]. Juice components were not analyzed. Recently, the antioxidant status and biomarkers related to heart disease and cancer were evaluated in healthy female volunteers consuming 750 mL of 27% cranberry juice/day for 2 weeks. AOPP were not evaluated in this study, and no significant differences in plasma antioxidant potential, GPX, catalase, SOD and MDA, urine 8-oxo-deoxyguanosine, or DNA damage in lymphocytes were found (*37*). This is in accordance with our results,

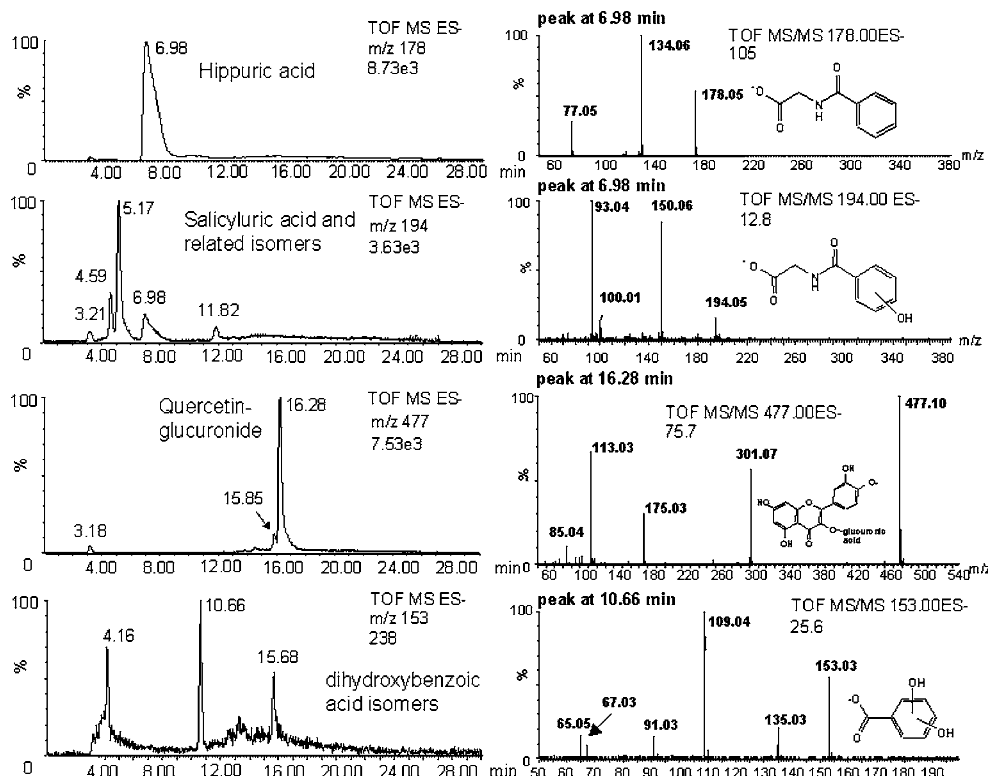

Figure 4. Typical chromatograms (μLC/MS, see the Experimental Procedures) of cranberry metabolites in the urine of volunteers taking 1200 mg/day DCJ.

which showed parameters of oxidative damage for group III within normal physiological values (**Table 4**). The daily intake of cranberry anthocyanins in the study of Duthie et al. (*37*) was comparable with our dose of 400 mg/day of DCJ, while the intake of proanthocyanidins corresponds more to 1200 mg/day of DCJ. Interestingly, the total phenolic content in the juice used by Duthie et al. (*37*) was much higher than that in our DCJ (405 vs 4.5 times the content of anthocyanins). However, the total intake of identified cranberry components in our study group III (1200 mg/day of DCJ) was 2.5 higher for proanthocyanidins and 10 times higher for anthocyanins.

The ex vivo antiadherence activity of human urine has been to date studied only after a single dose of cranberry products. Thus, the antiadherence activity on uropathogenic *E. coli* was found in the urine of six volunteers after drinking of 240 mL of cranberry juice (*9*) as well as in three out of five women after the consumption of 42.5 g of dried cranberries (*15*). Dose-dependent antiadherence activity was recently noted in 20 healthy volunteers after a dose of 750 mL of total drink combining cranberry juice with a placebo drink in a double-blind placebo-controlled crossover trial (*16*).

A significantly higher level of hippuric acid, conjugates of some other phenolic acids, and quercetin glucuronide was found in urine from group III as compared to group I. Salicyluric acid excretion in volunteers consuming cranberry juice (250 mL/day for 2 weeks) has been reported (*22*). Zhang and Zuo (*13*) found benzoic, *o*-hydroxybenzoic, *p*-hydroxybenzoic, 2,3- and 2,4-dihydroxybenzoic, and sinapic acids in the plasma and urine of one volunteer after consumption of a single dose of cranberry juice (1800 mL).

On the other hand, no significant increase in the concentration of anthocyanins or proanthocyanins in the urine of group III was found. A very low content of these compounds, reported as active factors in inhibition of *E. coli* adhesion, was found in the urine. Various berry (but nor cranberry) anthocyanin glycosides have been found to be absorbed and excreted into urine unmetabolized by both humans and rats. However, only 0.1% of the amount ingested was excreted into the urine (*12*). Ohnishi et al. recently showed that cranberry anthocyanins are excreted in a total amount corresponding to 5% of the dose consumed within 24 h after consumption with a maximum excretion between 3 and 6 h (*38*). Only one reference to a possible presence of proanthocyanidin trimers and oligomers in urine was found in the litterature (*11*). The authors describe administration of ^{14}C-labeled hawthorn proanthocyanidins (not indicating the type) to mice finding both trimers and oligomers in the urine. However, the authors measured only urine radioactivity without identification of the compounds responsible for it.

The present study demonstrated that after 8 weeks of consumption of DCJ, human urine had an inhibitory effect on the adhesion of bacteria causing acute and/or chronic UTI (**Figure 1**), but we failed to identify which cranberry metabolites were responsible for this effect. The in vitro antiadherence activity of single metabolites is the subject of our ongoing research.

In conclusion, DCJ in a daily dose of 400 mg of DCJ over 8 weeks in healthy women did not influence any of the parameters tested. In volunteers consuming 1200 mg/day of DCJ, a statistically significant decrease in the serum level of AOPP was observed. To date, this specific protective effect against oxidative damage of proteins has not been described in any antioxidant-containing food supplement. The urine of these volunteers had an inhibitory effect on the adhesion of uropathogenic *E. coli* strains, but no increase in urine acidity was found. Cranberry fruits, either as a functional food or a food supplement, are effective not only in the prevention of UTI, but they may also be beneficial in the prevention of oxidative stress in risk groups of a population, for example, people with metabolic syndrome and patients on dialysis.

ABBREVIATIONS USED

ALT, alanine aminotransferase, AOPP, advanced oxidation protein products; AST, aspartate aminotransferase; BHI, brain heart infusion; BM, body mass; BMI, body mass index; CFU, colony-forming units; DCJ, dried cranberry juice; GMT, D-glutamyl transferase, GPX, glutathione peroxidase; GSH, glutathione; HDL, high-density lipoproteins; LDL, low-density lipoproteins; MDA, malondialdehyde; MIC, minimal inhibition concentration; μLC/MS, microliquid chromatograph(y) coupled with mass spectrometr(y); PBS, phosphate-buffered saline; SOD, superoxide dismutase; SPE, solid phase extraction; UTI, urinary tract infection.

ACKNOWLEDGMENT

We declare that the results were interpreted independently and that they do not express any trade interest of Walmark, a.s., which provided the DCJ, DCJ-containing food supplement, and placebo.

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Received for review December 12, 2006. Revised manuscript received February 20, 2007. Accepted February 23, 2007. Financial support from the Czech Ministry of Education, Youth and Sport (MSM 6198959216) and Ministry of Trade and Commerce (FT-TA3/024) is greatly acknowledged.

JF0636014