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Metabolic Profiling of Phenolic Acids and Oxidative Stress Markers after Consumption of *Lonicera caerulea* L. Fruit

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ABSTRACT: This study investigated the effect of one-week consumption of 165 g/day fresh blue honeysuckle berries (208 mg/day anthocyanins) in 10 healthy volunteers. At the end of intervention, levels of benzoic (median 1782 vs 4156), protocatechuic (709 vs 2417), vanillic (2779 vs 4753), 3-hydroxycinnamic (143 vs 351), *p*-coumaric (182 vs 271), isoferulic (805 vs 1570), ferulic (1086 vs 2395), and hippuric (194833 vs 398711 μg/mg creatinine) acids by LC/MS were significantly increased in the urine. Clinical chemistry safety markers were not altered. Oxidative stress markers, erythrocyte glutathione peroxidase (0.73 vs 0.88 U/g Hb) and catalase (2.5 vs 2.8 μkat/g Hb) activities, and erythrocyte/plasma thiobarbituric acid reactive substance (522 vs 612/33 vs 38 μmol/g Hb/protein) levels were significantly increased, without change in plasma antioxidant status. Nonsignificant changes of advanced oxidation protein products and oxidized LDL were observed. The results provide a solid base for further study of metabolite excretion and antioxidant parameters after ingestion of anthocyanins.

KEYWORDS: *blue honeysuckle fruit, anthocyanins, human, oxidative stress markers, urine*

INTRODUCTION

There is epidemiological evidence that a diet rich in fruit and vegetable polyphenols may be protective against metabolic syndrome, a cluster of factors associated with increased risk of some chronic diseases.[1] Polyphenols are among the most abundant antioxidant compounds in our diet, and they could play a key role in the prevention of cardiovascular and neurodegenerative diseases and cancer.[2,3]

The average intake of polyphenols in humans is approximately 1 g daily. However, the distribution of polyphenols in body tissues is usually significantly lower due to rapid excretion or to metabolic changes. Partial absorption and deglycosylation occur in the digestive tract from the oral cavity to the small intestine. Phenolic substances that are not absorbed in the small bowel can be further metabolized by bacteria in the large intestine. Apart from deglycosylation, there are also reduction and hydrolysis reactions leading to the decomposition of polyphenols to phenolic acids and organic acids, e.g., hippuric acid. After absorption in the digestive tract, they are enzymatically metabolized in individual tissues. The levels of nonconjugated phenols in tissues following intake is generally very low, usually in nanomolar concentrations or less, but the end-products of polyphenol biotransformation can be found in urine at significantly higher levels.[2,3]

Supplementation with fruits mainly from the genus *Vaccinium* has a positive influence on human health, e.g., antioxidant, anti-inflammatory, antiatherogenic, antithrombotic, and also bacterial antiadherence.[2] The berries of *Lonicera caerulea* L. (blue honeysuckle) have a similar spectrum of phenolics[4,5] to other berries of the genus *Vaccinium*. The main spectrum of phenolics contained in blue honeysuckle fruit is shown in Table 1.[6] We recently reviewed the components of blue honeysuckle fruit, their biological activities, and uses in traditional medicine.[5,7] Blue honeysuckle extracts, containing high levels of phenolic compounds, were tested *in vitro* for

Table 1. Anthocyanins and Anthocyanidins in Fresh Blue Honeysuckle (*L. caerulea*) Fruit[a]

anthocyanin	*L. caerulea* (mg/100 g fw)
cyanidin-3-glucoside	47.1 ± 2.7
cyanidin-3,5-diglucoside	26.5 ± 1.8
peonidin-3,5-digalactoside	11.0 ± 0.6
peonidin-3-glucoside	10.3 ± 0.2
delphinidin-3-rutinoside	9.5 ± 0.8
pelargonidin-3-glucoside	7.2 ± 0.5
cyanidin-3-rutinoside	5.2 ± 0.6
petunidin-3-rutinoside	3.1 ± 0.6
cyanidin	2.9 ± 1.2
peonidin-3-rutinoside	2.0 ± 0.6
delphinidin-3-glucoside	0.6 ± 0.1
pelargonidin	0.3 ± 0.1
delphinidin	0.1 ± 0.05
malvidin-3-glucoside	0.1 ± 0.02
peonidin	0.1 ± 0.05
total	125.9

[a]Values are given as means ± SD.

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antimicrobial, antiadherence, and antioxidant effects and protective activity against UVB-caused injury of HaCaT keratinocytes, lipopolysaccharide-induced inflammation, and gingival fibroblast oxidative damage by lipopolysaccharide.[8−10] No pilot clinical trial with blue honeysuckle berries in humans has been conducted to date.

The aim of this open, controlled dietary intervention study was to investigate the effects of a one-week consumption of fresh *L. caerulea* fruit on the level of simple phenolics and organic acids in urine, clinical chemistry safety markers in blood, selected oxidative stress markers in erythrocytes and plasma, total plasma antioxidant capacity, and levels of advanced oxidation protein products and oxidatively modified low-density lipoproteins in healthy human volunteers.

MATERIALS AND METHODS

Chemicals. The following standards were used. Benzoic acid, salicylic acid (2-hydroxybenzoic acid), 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), gentisic acid (2,5-dihydroxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), phenylacetic acid, 2-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid), 2-hydroxyphenylpropanoic acid, 3-hydroxyphenylpropanoic acid, phloretic acid (4-hydroxyphenylpropanoic acid), dihydrocaffeic acid (3,4-dihydroxyphenylpropanoic acid), dihydroferulic acid (4-hydroxy-3-methoxyphenylpropanoic acid), cinnamic acid, 3-hydroxycinnamic acid, *p*-coumaric acid (4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), isoferulic acid (3-hydroxy-4-methoxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid), hippuric acid, 2-hydroxyhippuric acid, chlorogenic acid, rosmarinic acid, ellagic acid, quercetin, and catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyanidin chloride and malvidine-3-glucoside were purchased from ChromaDex Inc. (Irvine, CA, USA). Methanol, acetonitrile, and *β*-glucuronidase/arylsulfatase (from *Helix pomatia*) were obtained from Merck (Darmstadt, Germany). Buffer components, acetic acid, trifluoroacetic acid, and H1-sulfatase were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solutions were prepared using reverse-osmosis deionized water (Ultrapur, Watrex, Prague, Czech Republic). Nitrogen, argon, and helium (99.999% for all) were obtained from Linde Gas (Prague, Czech Republic).

Fruit. *Lonicera caerulea* L. (subspecies *kamtschatica*, cultivar Blue Triumph) berries were harvested at full ripeness in the horticulture farm Chovanec in Lipnik nad Becvou (Czech Republic) from May 20 to June 5, 2010. Immediately after harvest, the fruit was frozen and stored at −20 °C.

Characterization of the Fruit. The fresh berry contained 82.7% water, 1.6% proteins, 1.5% lipids, 7.2% saccharides, 6.7% fiber, and 0.5% ash. Glutamic acid and arginine were the most abundant amino acids. Among others, aspartic acid, leucine, phenylalanine, and glycine predominated. The energetic value of *L. caerulea* fruit was calculated to be 1380 kJ/kg (330 kcal/kg). The fruit contained potassium, calcium, phosphorus, magnesium, and other minerals. The dominant vitamins were ascorbic acid, niacin, and tocopherols. The total content of anthocyanins in berries was 1.259 mg/g. The anthocyanin profile is shown in Table 1; the amount of other phenolics was 0.057 mg/g (quercetin 16.0 μg/g; rutin 7.4; chlorogenic acid 31.4; vanillic acid 2.6). The amounts of compounds besides those listed here were under the limit of detection. For other details see ref 5.

Subjects and Study Design. Ten healthy nonsmoking volunteers, employees of Palacky University (8 women and 2 men) between 25 and 39 years old with a body mass index of (mean ± SD) 22.9 ± 2.0, were recruited in August 2010. The study design was adapted from recent similar studies[11−14] and was approved by the Ethics Committee of the University Hospital and the Faculty of Medicine and Dentistry, Palacky University in Olomouc, Czech Republic. None reported any history of CVD, homeostatic disorders, or other diseases, and none took any medication or food supplements. Fasting baseline characteristics of the subjects are presented in Table 2.

Table 2. Fasting Baseline Characteristics of Subjects[a] on Day 0

	1st quartile	median	3rd quartile
BMI (kg/m^2)	21.8	23.0	24.3
systolic blood pressure (mmHg)	109.5	119.5	123.3
diastolic blood pressure (mmHg)	73.3	74.0	78.5
heartbeat (min^{-1})	67.5	71.5	74.0
TAC (mmol/L)	1.84	1.87	1.89
urea (mmol/L)	3.80	4.35	4.57
creatinine (μmol/L)	63.0	64.5	73.5
bilirubin (μmol/L)	7.50	10.5	12.0
ALT (μkat/L)	0.22	0.29	0.32
AST (μkat/L)	0.36	0.38	0.45
GGT (μkat/L)	0.19	0.22	0.27
CRP (mg/L)	0.65	0.90	2.90
cholesterol (mmol/L)	4.39	4.53	4.68
TAG (mmol/L)	0.74	0.84	1.08
HDL (mmol/L)	1.42	1.58	1.80
total/HDL cholesterol	2.37	2.84	3.30
LDL (mmol/L)	2.35	2.45	2.71
glucose (mmol/L)	4.25	4.65	4.77

[a]Age 25−39 years old, 2 male/8 female.

Participants signed an informed consent, and they were aware of the study goals and instructed to abstain from polyphenol-rich food, especially color-pigment-containing fruit and coffee or tea at least 24 h before and during the experiment period. The volunteers consumed 165 g/day fresh berries (208 mg/day anthocyanins) for one week. Venous blood and midstream urine samples were taken on day 0 before the first intake and on day 7 two hours after the last intake of berries. Samples were stored at −80 °C until analysis. Blood pressure was measured after sample collection. Basic clinical chemistry markers (urea, creatinine, bilirubin, ALT, AST, GGT, CRP, total cholesterol, triacylglycerols, HDL-cholesterol, LDL-cholesterol, glucose) and advanced oxidation protein products (AOPP) were determined in serum samples at the Department of Clinical Biochemistry and Immunogenetics, University Hospital, Olomouc, using a Hitachi Modular Evo P analyzer (Hitachi, Japan). The concentrations of oxidized LDL were measured in blood plasma using the Mercodia Oxidized LDL ELISA test (Mercodia, Uppsala, Sweden). Standard urinanalysis was performed using the IQ200 Automated Urinalysis System (IRIS International, Inc., USA). Glutathione (GSH), glutathione reductase (GSR), glutathione transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase, and the level of lipoperoxidation products as thiobarbituric acid reactive substances (TBARS) were measured in erythrocytes; total plasma antioxidative capacity and content of SH-groups and TBARS in plasma were determined. For other details see ref 15.

Phenolics Determination in Plasma. *Analysis of Anthocyanins in Plasma.* Plasma was applied (0.5 mL) on a Strata SDB-L column for solid phase extraction (styrene−divinylbenzene copolymer, 500 mg of sorbent/3 mL reservoir, Phenomenex, CA, USA), preconditioned with methanol and 0.01% (v/v) aqueous HCl. The column was then washed with 3 mL of 0.01% HCl, and the anthocyanin dyes were eluted using 3 mL of 0.01% (v/v) HCl in methanol. The eluates were evaporated using a gentle stream of N_2 at 35 °C. The solid residues were dissolved in mobile phase A (0.12% trifluoroacetic acid, 5% acetonitrile) and analyzed by LC/MS using gradient elution as described in ref 16.

Analysis of Phenolic Acids in Plasma. The sample (1 mL) was applied on an SPE column (mixed sorbent RP/anex, Strata Screen A,

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Table 3. HPLC/MS Parameters of Standards Used for Determination of Compounds in Urine

compound	t_R (min)	λ_{max} (nm)	MS/MS^2 (m/z)
Benzoic Acid and Its Hydroxy Derivatives			
benzoic acid; BA	9.4	229/273	121
salicylic acid (2-hydroxybenzoic acid)	13.3	236/299	137/93
3-hydroxybenzoic acid	4.6	204/235/296	137/93
4-hydroxybenzoic acid; 4-HBA	3.4	193/255	137/93
2,3-dihydroxybenzoic acid	6.9	208/245/323	153, 167, 169
protocatechuic acid (3,4-dihydroxybenzoic acid); 3,4-DHBA	2.2	203/259/293	153/109
gentisic acid (2,5-dihydroxybenzoic acid)	6.6	209/246/322	153/109
gallic acid (3,4,5-trihydroxybenzoic acid)	1.6	202/213/277/312	169
vanilic acid (4-hydroxy-3-methoxybenzoic acid); 4-HMBA	3.8	203/216/260/291	167/152, 123
syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid)	3.9	215/274	197
Phenylacetic Acid and Its Hydroxy Derivatives			
phenylacetic acid; PA	9.8	205/257	135
2-hydroxyphenylacetic acid; 2-HPA	6.1	216/273	151/107
3-hydroxyphenylacetic acid; 3-HPA	4.5	218/274	151/107
4-hydroxyphenylacetic acid; 4-HPA	3.5	220/275	151/107
3,4-dihydroxyphenylacetic acid; 3,4-DHPA	2.4	195/282	167/123
homovanilic acid (4-hydroxy-3-methoxyphenylacetic acid); 4-HMPA	4.0	197/227/280	181/137
Phenylpropanoic Acid and Its Hydroxy Derivatives			
2-hydroxyphenylpropanoic acid	9.1	216/273	165/147, 121
3-hydroxyphenylpropanoic acid	5.6	206/251	165/165
phloretic acid (4-hydroxyphenylpropanoic acid)	5.7	220/277	165/121
dihydrocaffeic acid (3,4-dihydroxyphenylpropanoic acid); 3,4-DHPPA	3.3	195/279	181/137
dihydroferulic acid (4-hydroxy-3-methoxyphenylpropanoic acid); 4-HMPPA	6.4	197/227/280	195
Cinnamic Acid and Its Hydroxy Derivatives			
cinnamic acid	14.6	223/276	147
3-hydroxycinnamic acid; 3-HCA	8.8	232/277	163/119
p-coumaric acid (4-hydroxycinnamic acid); 4-HCA	6.9	225/309	163/119
caffeic acid (3,4-dihydroxycinnamic acid)	3.9	218/247/320	179
isoferulic acid (3-hydroxy-4-methoxycinnamic acid); 3-HMCA	8.4	240/297/322	193/178
ferulic acid (4-hydroxy-3-methoxycinnamic acid); 4-HMCA	7.8	237/321	193/149, 178, 134
sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid)	7.7	235/323	223
Other Phenolics			
hippuric acid; HA	4.2	192/227	178/134
2-hydroxyhippuric acid; 2-HHA	8.1	202/238/299	194
chlorogenic acid	3.9	215/249/322	353
rosmarinic acid	12.9	248/326	359
ellagic acid	8.9	251	301
quercetin	14.9	255/296/368	301
catechin	2.8	202/228/278	289
rutin	8.18	210/227/256/354	609

200 mg of sorbent/3 mL reservoir, Phenomenex, Torrance, CA, USA), preconditioned with methanol and phosphate buffer. The column was washed with 3 mL of deionized water and eluted with 3 mL of methanol and then with 3 mL of 1% HCl in methanol. The eluates (methanol and 1% HCl in methanol) were mixed and evaporated using a N_2 stream at 35 °C. The solid residue was dissolved in mobile phase A (10 mM acetic acid and 5% (v/v) acetonitrile in water), and the solution was analyzed by LC/MS as described previously.[16]

Phenolics Determination in Urine. *Analysis of Free Phenolics in Urine.* The samples of urine (160 μL) were mixed with 240 μL of 1% acetic acid in methanol (v/v) and centrifuged for 3 min (14000*g*, at laboratory temperature) using a Minispin Plus (Eppendorf, Hamburg, Germany), and supernatants (10 μL) were analyzed by HPLC/MS.

Analysis of Total Phenolics in Urine. The samples of urine (160 μL) were mixed with 40 μL of 1 M acetate buffer (pH 5.0), 4 μL of β-glucuronidase/arylsulfatase (40/20 U/mL as standard solution from Merck, K41682514), and 4 μL of H1-sulfatase from Sigma-Aldrich, S9626 (1000 U/mL, dissolved in 0.2 M acetate buffer, pH 5.0). After the incubation period (30 min, 37 °C) the samples were mixed with 200 μL of 1% acetic acid in methanol (v/v) and then centrifuged (14000*g*) for 3 min at room temperature. The supernatants (10 μL) were analyzed by HPLC/MS.

HPLC/MS of Phenolic Acids. The HPLC chromatographic system used was a Dionex UltiMate 3000 (Dionex Corp., Sunnyvale, CA, USA) equipped with a degasser (SRD-3400, 4 Degasser CH), a binary pump (HPG-3400SD), an autosampler (WPS-3000 TSL Analytical), a column compartment (TCC-3000RS), and a diode array detector (DAD-3000, 190–400 nm). A Gemini C18 110A chromatographic column (150 mm × 2.0 mm, 5 μm) with C_{18} guard column (4 mm × 2 mm, 5 μm) from Phenomenex (USA) was used. The injection volume was 10 μL, and the mobile phase consisted of acetonitrile (solvent B)/10 mM acetic acid in a 5% (v) acetonitrile aqueous solution, linear gradient elution (%, *v*): 0–3 min (10% B), 3–20 min (10–40% B), 20–20.1 min (40–90% B), 20.1–22 min (90% B), 22–22.1 min (90–10% B), 22.1–28 min (10% B). The mobile phase flow rate was 0.4 mL/min, the temperature of the autosampler was 10 °C, and the column oven was set at 25 °C. The LCQ Fleet quadrupole ion-trap MS instrument (Thermo Scientific, Waltham, MA, USA) operating in a negative ESI mode was used for the analysis. The ESI-MS parameters selected were as follows: spray voltage 4.75

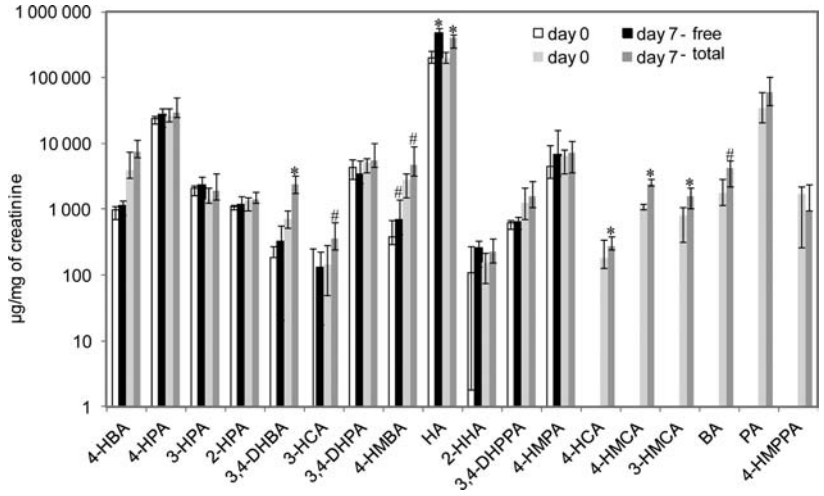

Figure 1. Free and total phenolic compounds in urine of volunteers (n = 10) before (day 0) and after (day 7) one-week consumption of *L. caerulea* berries. The analytes were identified on the basis of MS^2 fragmentation patterns, and data are expressed as medians (25th−75th percentile). *Median was significantly different from that on day 0 ($p < 0.05$). #Median was significantly different from that on day 0 ($p < 0.1$)

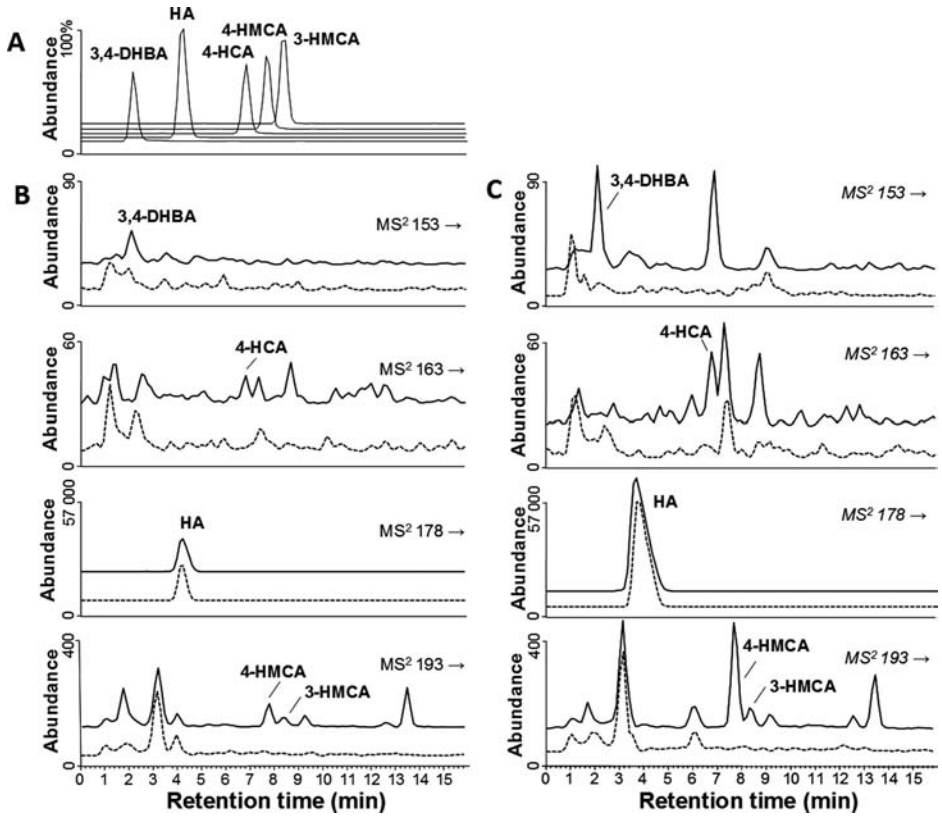

Figure 2. (A) LC/MS chromatograms of selected phenolic acids and hippuric acid standards. Representative chromatographic records of investigated compounds in urine (B) before and (C) after intervention. The chromatograms of native samples (dashed lines) and samples after enzymatic cleavage (solid lines) are shown. MS (for A in %) and MS^2 (for B and C) responses are presented as TIC. The quantitative data for the compounds (marked *) can be found in Table 4. For other details on preparation of urine samples, see Materials and Methods.

kV, capillary temperature 210 °C, and capillary voltage −1 V. Nitrogen was used as sheath, auxiliary, and sweep gas, and helium was used as the collision gas. The sheath, auxiliary, and sweep gas flow rates were 60, 5, and 2 (as arbitrary units). The MS^2 fragments were isolated from the following parent ions: 137, 151, 153, 163, 165, 167, 178, 181, 193 m/z; normalized collision energy was 25%. The analytical parameters for qualitative analysis of studied compounds (36 in total) are presented in Table 3. The quantification of the compounds was carried out according to the calibration curve in the range 7.5 ng to 10 μg per injection.

Data Analysis. The data were analyzed using the nonparametric Exact Wilcoxon test and reported as quantiles. Significant results are graphically presented with box plots and graphs of empirical cumulative distribution functions.

RESULTS AND DISCUSSION

The clinical biochemistry and hematology data including liver and kidney function, blood glucose, and lipids of the subjects were within the normal range (Table 2). No side effects of

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Table 4. Changes in Urinary Levels of Phenolic, Benzoic, and Hippuric Acids (μg/mg of Creatinine) after Intervention[a]

compound		free acid		total acid	
		day 0	day 7	day 0	day 7
benzoic acid	BA	0/0/0	0/0/0	1169/1782/2870	2207/4156/5597[c]
protocatechuic acid	3,4-DHBA	0/184.4/273.2	20.6/323.2/571.5	523.1/708.8/945.3	1762/2417/3189[b]
vanilic acid	4-HMBA	291.9/373.1/683.8	414.6/701.8/1416.3[c]	1512/2779/3527	3233/4753/8933[c]
3-hydroxycinnamic acid	3-HCA	0/0/250.8	17.9/129.7/223.1	50.3/142.8/286.8	239.9/351.4/618.2[c]
p-coumaric acid	4-HCA	0/0/0	0/0/22.5	128.9/181.8/342.9	241.5/270.5/380.6[b]
ferulic acid	4-HMCA	0/0/0	0/0/0	1010/1086/1221	2256/2395/2905[b]
isoferulic acid	3-HMCA	0/0/0	0/0/246.2	317.8/805.0/1067	1020/1570/2112[b]
hippuric acid	HA	171/199/257 ($\times 10^3$)	379/477/567 ($\times 10^3$)[b]	167/195/244 ($\times 10^3$)	290/399/455 ($\times 10^3$)[b]

[a]Data are expressed as 1st quartile/median/3rd quartile. [b]Median was significantly different from that on day 0 ($p < 0.05$). [c]Median was significantly different from that on day 0 ($p < 0.1$).

intervention were reported by the volunteers after one-week-long intake of blue honeysuckle fruit. Routine clinical laboratory profiles of volunteers remained unchanged.

After evaluation of basic clinical data, we performed HPLC/MS analysis of phenolic compounds and their metabolites in the plasma and urine on days 0 and 7. Identification of phenolic compounds was carried out with a series of 36 standards, mainly phenolic acids, hippuric acid, and selected polyphenols (Table 3). The concentration of all analyzed phenolic compounds in plasma was under the quantification threshold before and after intervention. Phenolic substances in the urine were analyzed directly without enzymatic cleavage (free phenolics) and then after further incubation with a mixture of deconjugation enzymes, β-glucuronidase, and sulfatase (resulting in total phenolic content). The enzyme cleavage was used because most of the polyphenolics in the organism are subjected to glucuronidation and sulfatation.[17] Benzoic acid and its hydroxy derivatives (4-HBA, 3,4-DHBA, 4-HMBA), all analyzed phenylacetic acid derivatives (see Table 3), two hydroxy derivatives of phenylpropanoic acid (3,4-DHPPA, 4-HMPPA), and hydroxy derivatives of cinnamic acid (3-HCA, 4-HCA, 3-HMCA, 4-HMCA), as well as HA and 2-HHA were found in the urine of volunteers (Figure 1). Exemplary HPLC chromatograms are shown in Figure 2. A statistically significant increase in free acid fraction between the two time points was found only for HA ($p = 0.0098$) and 4-HMBA ($p = 0.064$). A significant increase in content after enzymatic cleavage was noted for 3,4-DHBA ($p = 0.0039$), 4-HCA (($p = 0.013$), 3-HCA ($p = 0.064$), HA ($p = 0.027$), 3-HMCA ($p = 0.0098$), 4-HMCA ($p = 0.0019$), and BA ($p = 0.064$) (Table 4). The most abundant metabolite was HA, whose baseline level in the free fraction was as high as 199 238 μg/mg of creatinine and increased to 476 937 μg/mg at the end of the study. The concentration of other phenolic compounds in urine was at least 40 times lower (Figure 1 and Table 4).

The increased content of simple phenolics and hippuric acid in urine (Table 4) is related to the metabolic transformation of dietary parent flavonoids. We hypothesize that most of the phenolic acids in urine, whose level was elevated on day 7, are colonic metabolites of flavonoids.[18] Of the parent flavonoids, mainly anthocyanins are probably subjected to microbial cleavage, as anthocyanins predominate in fresh blue honeysuckle fruit, ca. 80% of all phenolics.[5,19] With respect to the extensive biotransformation of flavonoids in the colon, it is uncertain that the biological effect observable after ingestion of the fruit is associated with the parent anthocyanins and/or their cleavage products (Table 4). This is supported by the fact that parent anthocyanins are extremely poorly adsorbed, as has been shown in detail for cyanidin-3-glucoside (main anthocyanidin in blue honeysuckle fruit; see Table 1) using radiolabeling on a mice model.[20] It is also important to note that the metabolic end-products found in the urine of volunteers were not found by the LC/MS method in ingested fruits with the exception of vanilic (2.6 μg/mL) and chlorogenic acid (31.4 μg/mL). In general, simple phenolic compounds as phenolic acids are minor components (<0.1%, w) of the blue honeysuckle fruit.[5]

Berry polyphenols are associated with a broad spectrum of biological activities *in vivo* (see Introduction). In fact, the majority of these activities are ascribed to their antioxidant and/or prooxidant properties.[21] For this reason, we focused on comparison of oxidative status in volunteers before and after the fruit consumption. The oxidative status of the volunteers was evaluated using selected markers in both blood plasma and red blood cells. Neither total antioxidative capacity (TAC) of plasma nor plasma total concentration of SH groups was influenced by the intervention. Glutathione level, glutathione *S*-transferase, glutathione reductase, and superoxide dismutase activities in erythrocytes remained unchanged as well. A significant increase was found for glutathione peroxidase, catalase in erythrocytes, and TBARS levels in both erythrocytes and plasma (Figure 3) and an insignificant decrease in AOPP ($p = 0.084$) and oxidized LDL ($p = 0.164$) levels in plasma (Table 5). When the latter two parameters were evaluated for single volunteers, we observed a decrease for the clear majority of subjects. The results could indicate slight antioxidant effects in the plasma of volunteers after the intervention (Figure 4).

In fact, only slight changes in level of selected oxidative stress markers on day 7 were found in plasma as opposed to changes in erythrocytes. This finding is in agreement with nonsignificant changes in plasma TAC on days 0 and 7. The results suggest that the berries had no effect on antioxidant status. However, the polyphenol supplementation during the intervention significantly affected erythrocyte functions involved in oxidative stress metabolism. The changes in antioxidant parameters of erythrocytes could be connected with the disruptive (prooxidant) effects of blue honeysuckle polyphenols and their metabolites. The prooxidant action could be involved by formation of reactive transition metal complexes of polyphenolics in erythrocytes.[21−23] The participation of erythrocyte peroxidases in the formation of prooxidant phenoxyl radicals derived from the polyphenols contained in the diet also cannot be excluded.[24] Similar prooxidant effects were observed after application of high-dose plant polyphenolics (in the form of *Ginkgo biloba* L. leaf extract) on isolated erythrocytes.[25] There is an evidence that the prooxidant effects of polyphenols in erythrocytes can be observed not only under *in vitro* conditions

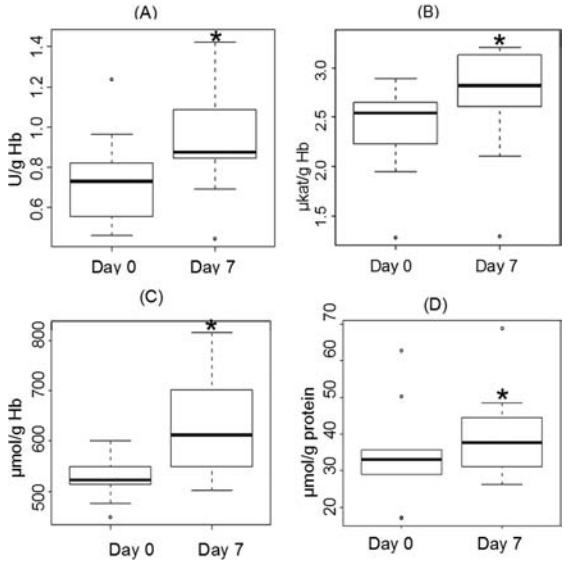

Figure 3. Effect of *L. caerulea* berry consumption on (A) glutathione peroxidase, (B) catalase, (C) TBARS in erythrocytes, and (D) TBARS in blood plasma of volunteers before (day 0) and after (day 7) the intervention. The box-and-whiskers graphs show the median as the middle line. The box extends from the 25th to the 75th percentile, and the whiskers extend from the lowest value to the highest; ∘, outside values. *The median was significantly different from that on day 0 ($p < 0.05$).

Table 5. Erythrocyte and Plasma Markers of Oxidative Status[a]

erythrocyte	day 0	day 7
GSH (μmol/g Hb)	67.3/93.7/133.8	91.3/111.5/126.0
GST (μkat/g Hb)	2.07/2.4/2.78	1.68/1.97/2.42
GSR (μkat/g Hb)	0.85/1.15/1.7	0.87/1.12/1.56
GPx (U/g Hb)	0.57/0.73/0.81	0.85/0.88/1.08[b]
SOD (U/g Hb)	84.0/86.2/91.9	83.6/86.1/91.8
Catalase (μkat/g Hb)	2.2/2.5/2.6	2.6/2.8/3.1[b]
TBARS (μmol/g Hb)	513.8/522.1/544.4	550.1/611.8/697.8[b]
plasma	day 0	day 7
TAC (mmol/L)	1.8/1.9/1.9	1.9/1.9/1.9
SH groups (μmol/g protein)	1.8/2.2/2.6	1.9/2.1/2.5
TBARS (μmol/g protein)	29.4/33.1/35.4	32.4/37.7/43.6[b]
AOPP (μmol/L)	137.1/152.2/179.6	83.3/98.65/153.6[c]
oxLDL (U/L)	28.3/31.6/38.2	22.2/26.3/33.9

[a]Data are expressed as 1st quartile/median/3rd quartile. [b]Median was significantly different from that on day 0 ($p < 0.05$). [c]Median was significantly different from that on day 0 ($p < 0.1$).

but also at the whole organism level. The above-described prooxidant effects in erythrocytes are probably strictly connected to use of high doses of polyphenolic compounds. Interactions of polyphenolics with erythrocytes are the subject of our ongoing research.[26]

In conclusion, there are a large number of reports on the effect of a polyphenol-rich diet under *ex vivo* conditions or using animal models. In the present paper, we described the effect of blue honeysuckle (*L. caerulea* L.) fruit consumption on healthy human volunteers. The results show that intake of the fruit leads to the production of specific metabolic end-products (hippuric acid and phenolic acids) in the urine of volunteers as identified by LC/MS. In addition, oxidative stress metabolism has been modulated in erythrocytes after the berry consumption. This study provides a solid base for further more complex study on (a) formation and urinary excretion of metabolites of berry polyphenols and (b) antioxidant−prooxidant (dual) effects after ingestion of an anthocyanin-rich diet in humans.

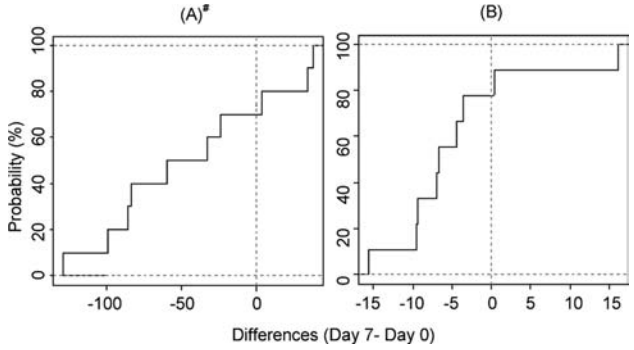

Figure 4. Effect of *L. caerulea* berry consumption on advanced oxidation products of proteins (A) and oxidized LDL (B) and levels in plasma of individual volunteers. The values are expressed as difference values based on day 7 and day 0; each step corresponds to one single volunteer. #Median was significantly different from that on day 0 ($p = 0.084$).

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Notes

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ABBREVIATIONS

Analyzed substances: BA, benzoic acid; 4-HBA, 4-hydroxybenzoic acid; 3,4-DHBA, 3,4-dihydroxybenzoic acid (protocatechuic acid); 4-HMBA, 4-hydroxy-3-methoxybenzoic acid (vanilic acid); PA, phenylacetic acid; 2-HPA, 2-hydroxyphenylacetic acid; 3-HPA, 3-hydroxyphenylacetic acid; 4-HPA, 4-hydroxyphenylacetic acid; 3,4-DHPA, 3,4-dihydroxyphenylacetic acid; 4-HMPA, 4-hydroxy-3-methoxyphenylacetic acid (homovanilic acid); 3,4-DHPPA, 3,4-dihydroxyphenylpropanoic acid (dihydrocaffeic acid); 4-HMPPA, 4-hydroxy-3-methoxyphenylpropanoic acid (dihydroferulic acid); 3-HCA, 3-hydroxycinnamic acid; 4-HCA, 4-hydroxycinnamic acid (*p*-coumaric); 3-HMCA, 3-hydroxy-4-methoxycinnamic acid (isoferulic acid); 4-HMC, 4-hydroxy-3-methoxycinnamic acid (ferulic acid); HA, hippuric acid; 2-HHA, 2-hydroxyhippuric acid; *Physical and biochemical parameters*: fw, fresh weight; BMI, body mass index; TAC, total antioxidant capacity; ALT, alanine transaminase; AST, aspartate transaminase; GGT, gamma-glutamyl transferase; CRP, C-reactive protein; TAG, triacylglycerols; HDL, high-density lipoproteins; LDL, low-density lipoproteins; GSH, glutathione; GST, glutathione *S*-transferase; GSR, glutathione reductase; GPx, glutathione peroxidase; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; AOPP, advanced oxidation protein products; oxLDL, oxidized low-density lipoproteins

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