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Constituents and Antimicrobial Properties of Blue Honeysuckle: A Novel Source for Phenolic Antioxidants

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The fruit of *Lonicera caerulea* L. (blue honeysuckle; Caprifoliaceae) and its phenolic fraction were analyzed for nutrients and micronutrients. The phenolic fraction was prepared from berries percolated with 0.1% H_3PO_4 and SPE using Sepabeads SP207. The sugar and lipid content was analyzed by HPLC and GC-MS. The total content of anthocyanins was determined using the pH differential absorbance method and aliphatic acids by capillary electrophoresis. μLC-MS/MS was used for determination of cyanidin-3-glucoside (the predominant anthocyanin), 3,5-diglucoside, and 3-rutinoside, paeonidin-3-glucoside, 3,5-diglucoside, and 3-rutinoside, delphinidin-3-glucoside and 3-rutinoside, pelargonidin-3-glucoside, 3,5-diglucoside, and 3-rutinoside, quercetin, its 3-glucoside, and 3-rutinoside, epicatechin, protocatechuic, gentisic, ellagic, ferulic, caffeic, chlorogenic, and coumaric acids. The phenolic fraction displayed Folin–Ciocalteu reagent reducing (335 $\pm$ 15 μg of gallic acid equivalent/mg) and DPPH and superoxide scavenging activity (IC_{50} 12.1 $\pm$ 0.1 and 115.5 $\pm$ 6.4 μg/mL) and inhibited rat liver microsome peroxidation (IC_{50} 160 $\pm$ 20 μg/mL). The freeze-dried fruit and its phenolic fraction reduced the biofilm formation and adhesion to the artificial surface of *Candida parapsilosis, Staphylococcus epidermidis*, *Escherichia coli*, *Enterococcus faecalis*, and *Streptococcus mutans*.

KEYWORDS: *Lonicera caerulea* var. *kamtschatica*; nutrition analyses; lipids; sugars; organic acids; anthocyanins; antimicrobial effect

INTRODUCTION

Currently there are a large number of studies on the beneficial effects of anthocyanins and proanthocyanidins in the prevention of a plethora of chronic diseases. These compounds provide the pigmentation of fruits and prove beneficial to human health (*1*−*3*). Their biological activities include protection against cancer (*4*) and ischemic heart disease (*5*). They also have antimicrobial (*6*) and anti-inflammatory (*7*) activities.

Lonicera caerulea L. (blue honeysuckle, honeyberry, edible honeysuckle, sweet berry honeysuckle; Caprifoliaceae) is a nutritionally valuable native northern temperate zone bush which can be successfully cultivated outside its original habitat. Its fruits, resembling in flavor that of bilberries, are widely harvested in Russia, China, and Japan but practically unknown as edible berries in Europe and North America. The health benefits of consuming these berries have long been acknowledged in Russia, and recent research has supported some of the folkloric claims of therapeutic uses for hypertension, gastrointestinal disorders, and bacterial infections. *L. caerulea* berries and their juice have been found to contain ascorbic acid

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and polyphenols as major components (*8*). Other studies have focused on the relationship between saccharide and protein content in *L. caerulea* shoot apices and the plant's frost resistance (*9, 10*). However, to the best of our knowledge, the complete chemical composition of *L. caerulea* berries has not been published to date.

In this study we analyzed the component profile and phenolic fraction of *L. caerulea* var. *kamtschatica* fruits harvested in Central Moravia (Czech Republic, i.e., Central Europe, out of its natural habitat). The radical scavenging activity of the phenolic fraction was also evaluated. The lyophilized, ground *L. caerulea* berries and fraction of phenolics were tested for antimicrobial and antiadherence activities.

MATERIALS AND METHODS

Plant Material. *L. caerulea* berries were harvested in Lipník nad Bečvou (Central Moravia; 2007). The fruits were frozen and stored at −20 °C and used for nutrition analyses and preparation of the phenolic fraction. For determination of aliphatic and phenolic acids, flavonoids, anthocyanins, lipids, and sugars we used the freeze-dried fruits and phenolic fraction.

Preparation of Phenolic Fraction. Frozen fruits (4.84 kg) were percolated with an aqueous solution of phosphoric acid (0.1%; v/v; 50 °C) for 14 h (*11*). The obtained primary extract was purified on a column packed with nonionic polystyrene−divinylbenzene resin Sepabeads SP 207 (Mitsubishi Chemical Corp., Japan). The column was washed with deionized water; the phenolic compounds were then eluted by ethanol and concentrated by evaporation to yield 20.3 g of phenolic fraction.

Nutrition Analyses. The fresh berries were analyzed at the Department of Hygienic Laboratories Karviná of the Institute of Public Health in Ostrava (Czech Republic) using methods of analysis of AOAC International.

Saccharides. The frozen fruits were extracted twice by water (100 °C; 2 h) and by 0.1% H_3PO_4 at room temperature (pH 6). Extracts were freeze dried and analyzed by HPLC on a 250 mm × 4 mm i.d. Lichropher 100-5, NH_2 column (Watrex, Prag, Czech Republic), mobile phase water, flow rate 0.5 mL/min, column temperature 80 °C with RI and UV detection. Detection wavelength was 210 nm, scan 200−360 nm. The samples (0.953 g) were dissolved in water and separated using XAD-2 (9 × 5 cm), XAD-4 (16 × 6 cm), and Biogel P2 (80 × 2.5 cm) columns.

Lipids. Total lipids were analyzed after extraction with chloroform:methanol (2:1) from frozen fruits using the Folch method (*12*). This crude lipid extract was analyzed on an Iatroscan newMK-5 instrument (Iatron Laboratories, Tokyo, Japan) as follows: the extract was spread on Chromarods sticks, developed with the mobile phase (chloroform−methanol−water−25% ammonia (47:20:2.5:0.28; v/v)) at room temperature. After drying (110 °C; 3 min) subsequent development with the mobile phase hexane−diethyl ether (60:10; v/v) at 20 °C was used. The rods were charred with a flame (H_2 160 mL, air 1.5 L) and scanned at 526 nm; scan rate 50 s/stick. The extract of total lipids was evaporated to dryness and saponified by boiling for 2 h in 50 mL of 1 M sodium hydroxide solution in 50% ethanol. The nonsaponified portion was extracted with diethyl ether−hexane (1:1, v/v) and analyzed by GC-MS. The water solution was acidified to pH 2 with hydrochloric acid, and fatty acids were extracted with chloroform. Methyl esters of fatty acids were prepared by reaction with a 14% boron trifluoride solution in methanol at 50 °C for 10 min, then water (5 mL) was added, the esters were extracted with hexane (2 × 5 mL), and the hexane layer was washed with water (4 mL) and dried. The solution was filtered; the solvent was removed under reduced pressure and analyzed by GC-MS (*13*).

Sterols. Analysis was performed on an HP 6890 GC using a 5 m × 0.53 mm i.d. SPB-1701 capillary column (Supelco, Bellefonte, PA), temperature programmed from 50 to 300 at 15 °C/min, split ratio (1:50), constant He flow 3.2 mL/min.

Fatty Acids. A sample of the fatty acids methyl esters was dissolved in chloroform. Analysis was performed on an HP 6890 GC using a 30 m × 0.25 mm i.d. SPB35 capillary column (Supelco, Bellefonte, PA), temperature programmed at 15 °C/min, from 50 to 300 °C. Split was 1:50, constant He flow 1.2 mL/min. Detection was by Autospec Ultima MS (Micromass, Manchester, U.K.), scanned from 20 to 600 amu.

Organic Acids. Organic acids were determined by capillary zone electrophoresis with UV detection using an HP^3D instrument (Agilent Waldbronn, Germany) with diode array detection from 190 to 600 nm. A fused silica capillary with permanent polyacrylamide coating (effective length 40 cm) was used at 25 °C; separation voltage = −20 kV; running electrolyte = 10 mM salicylate/triethylamine pH 3.0; hydrodynamic injection 5000 Pa × 5/s. The detection wavelength was 238 nm. For analysis samples (1 mg) were dissolved in 1 mL of water, sonicated for 10 min, filtered, and analyzed using a calibration curve.

Phenolics. The samples 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, 200 mg of sorbent/3 mL reservoir, Phenomenex, Torrance, CA). The column was washed with 3 mL of deionized water and eluted with 3 mL of methanol and subsequently with 3 mL of 1% HCl in methanol. Both fractions were analyzed, and the content of compounds eluted in both fractions was combined. The eluates were evaporated using a N_2 stream at 40 °C. The solid residue was dissolved in the mobile phase A and analyzed by μLC/MS/MS using gradient elution: mobile phase A, 10 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. Setup of ion source and ion optics was tuned using a methanolic solution of 4-hydroxybenzoic acid. A mixture of standards (protocatechuic, gentisic, 4-hydroxybenzoic, ellagic, salicylic, rosmarinic, ferulic, caffeic, dihydrocaffeic, chlorogenic, 3-hydroxycinnamic, coumaric, vanillic, and syringic acids) at four different concentrations (0.01−10 mg/L) was used for calibration.

Anthocyanins. The samples were dissolved in 1 mL of 0.01% HCl. The solutions (0.5 mL) were then applied on a conditioned Strata SDB-L column for solid-phase extraction (styrene−divinylbenzene copolymer, 500 mg of sorbent/3 mL reservoir, Phenomenex). The column was subsequently washed with 3 mL of 0.01% HCl, and the anthocyanins were eluted by 3 mL of 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 mobile phase A and analyzed by μLC/MS/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, 0−35 min, 10−90% B; 35−40 min, 90% B. Quantification of anthocyanins was done using standard solutions of malvidin-3-glucoside in the concentration range 5−100 mg/L. The calibration dependence was linear (R^2 = 0.994). The response factors of individual pigments were neglected. The sum of identified anthocyanins obtained using μLC/MS/MS corresponded acceptably with the pH differential absorbance method (relative difference 7%) performed as in ref *14*.

Antioxidant Activity. *Folin–Ciocalteu Assay.* Reducing capacity was evaluated using Folin–Ciocalteu reagent (*15*) with minor modifications. Briefly, 5 μL of samples or standard was mixed with 100 μL of reagent diluted 10 times with distilled water. After 5 min of incubation 100 μL of Na_2CO_3 (75 g/L) was added and the mixture further incubated for 90 min at RT, absorbance was measured at 725 nm using a Sunrise spectrophotometer (Schoeller Instruments, Praha, Czech Republic), and the reducing capacity was expressed as gallic acid equivalents (GAE).

DPPH Radical Scavenging. Antiradical activity was evaluated spectrophotometrically as the ability of the tested substances to reduce 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (*16, 17*) with minor modifications. Briefly, 90 μL of *L. caerulea* samples dissolved in methanol (0−100 μg/mL) was mixed with 180 μL of a freshly prepared methanolic DPPH solution (20 mg/L) in a microtitrate plate well. After incubation (30 min; RT) absorbance at 517 nm was measured with a Sunrise spectrophotometer, and IC_{50} values were obtained from the inhibition curves.

Superoxide Anion Scavenging. The superoxide-scavenging activity was determined spectrophotometrically by monitoring the effect of the

tested extract on the reduction of nitroblue tetrazolium chloride (2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-[3,3′-dimethoxy-4,4′-diphenylene]ditetrazolium chloride, NBT) to blue chromogen formazan by $O_2^{\bullet-}$. Superoxide radicals were generated by the nicotinamide−adenine−dinucleotide/phenazine methosulfate (NADH/PMS) system (*18*). Briefly, 50 μL of different dilutions of *L. caerulea* samples dissolved in phosphate buffered saline (PBS; 0−360 μg/mL) was added to the reaction mixture containing 150 μL of PMS in PBS (5.4 μmol/L), 60 μL of NBT in PBS (250 μmol/L), and 40 μL of NADH in PBS (996 μmol/L). After incubation (30 min; 37 °C) the mixture absorbance at 560 nm was measured (*19*). The test compound scavenging activity was calculated as the concentration that inhibited NADH oxidation by 50% as compared with a standard (IC_{50}).

Antimicrobial Activities. The freeze-dried fruits of *L. caerulea* and its phenolic fraction were tested on antiadherence activity and inhibition of biofilm formation. The plant samples and D-(+)-mannose were diluted in distilled water to a concentration of 100 mg/mL and sodium hippurate to 5 mg/mL. Tested strains were *Enterococcus faecalis* NS123 (clinical strain, Collection of Microbiology Institute, Masaryk University and St. Anna University Hospital, Brno, Czech Republic), *Staphylococcus epidermidis* CCM 7221 (Czech Collection of Microorganisms, CCM), *Escherichia coli* FB27 (clinical strain, Collection of Microbiology Institute, Masaryk University and St. Anna University Hospital, Brno, Czech Republic), *Candida parapsilosis* BC 11 (clinical strain, Collection of Microbiology Institute, Masaryk University and St. Anna University Hospital, Brno, Czech Republic), *Streptococcus mutans* CCM 7409 (Czech Collection of Microorganisms, CCM). All isolates were biofilm positive. Bacteria were stored in cryotubes (ITEST plus, Czech Republic) at −76 °C.

Biofilm formation was detected using a modification of the adherence assay described by Christensen et al. (*20*). Innoculum preparation: Before each experiment, the strains were thawed quickly at 37 °C and cultivated on Muller-Hinton's agar (Bio-Rad, Marnes La Coquette, France) at 37 °C for 24 h. The microbial cultures were resuspended in physiological saline solution (PSS) to a density equal to McFarland 3 Standard (9×10^8 cells/mL). Wells of the 96-well flat-bottomed polystyrene tissue culture microtiter plates (GAMA Group, Trhove Sviny, Czech Republic) containing 160 μL of Brain Heart Infusion (BHI) broth (Hi-Media, Mumbai, India) with 4% glucose and 20 μL of stock solution of each tested substances were inoculated with 20 μL of microbial suspension. The positive control wells contained 180 μL of BHI broth with 4% glucose and 20 μL of microbial suspension. The negative control wells contained only sterile medium (160 μL) and PSS (40 μL). After 24 h of incubation at 37 °C the wells were emptied and washed three times with PSS. The biofilm layer on the wall of the wells was fixed by air drying. The adherent biofilm layer was stained with 1% crystal violet (180 μL) for 20 min and then washed three times with PSS. The plates were air dried, and for the spectrophotometric assessment the bound dye was eluted with 125 μL of 33% acetic acid per well. After 30 min, 100 μL of destaining solution was transferred to a new well and the absorbance of the well was measured at 595 nm, ref filter 690 nm, using an Anthos Labtec Instruments 2001 reader (Salzburg, Austria). The experiment was repeated three times.

The effect of tested samples on adhesion was evaluated on the basis of comparison of the number of colony forming units (CFU) on disks exposed to tested substances and untreated discs (adhesion positive control).

Innoculum Preparation: Before each experiment, the strains were thawed quickly at 37 °C and cultivated on Muller-Hinton's agar at 37 °C for 24 h. The microbial cultures were resuspended in PSS to a density equal to McFarland 2 Standard (6×10^8 cells/mL). Adhesion was tested on sterile discs, 5 mm in diameter, cut from Thermanox plastic coverslip, cell culture treated with Thermanox (Nunc, Roskilde, Denmark).The discs were submerged in the wells of 24-well Nunclon tissue culture plates (Nunc, Roskilde, Denmark) containing 100 μL of the inoculum, 800 μL of PSS, and 100 μL of the stock solution of each tested substance.

As control for microbial adhesion, the discs were transferred into wells containing 100 μL of the inoculum and 900 μL of PSS. As a negative control the discs were transferred into wells containing only 1 mL of PSS. The incubation lasted for 1 h at 37 °C. After incubation nonadherent microorganisms were removed by washing (three times with PSS). Washed disks were subsequently transferred into test tubes containing 1 mL of PSS, sonicated for 15 min, and vortexed. The mechanical effect of the ultrasound and vortexing disrupted the biofilm and released the microbial cells from the biofilm layer. Obtained bacterial suspension was serially diluted (1:10 to $1:10^6$) by transferring 100 μL of the suspension into 0.9 mL of sterile PSS. A 100 μL amount of all dilutions was inoculated on Muller−Hinton's agar (Bio-Rad, Marnes La Coquette, France) and incubated at 37 °C for 24 h. The number of CFU surviving on the discs was counted. The experiment was repeated three times.

Antibacterial activities were determined by the standard microdilution method. The samples were diluted with BHI broth (Becton Dickinson) to provide decreasing concentrations (geometric series, with a coefficient of 2) from a concentration *C* down to the concentration *C*/256. After incubation for 24−48 h in a thermostat at 37 °C the minimal inhibitory concentration (MIC) was evaluated as the lowest concentration of the test substance that inhibited growth of the bacterial strain. Standard reference bacterial strains (*S. aureus* CCM 3953 and CCM 4223, *P. aeruginosa* CCM 3955, *E. coli* CCM 4225 and CCM 3954) from the Czech Collection of Microorganisms (CCM), Faculty of Science, Masaryk University Brno, and *S. agalactiae* strain obtained from Olomouc Teaching Hospital were used.

Statistical Analyses. The difference between the tested samples and positive control was analyzed using a *t* test. All tests were analyzed at the significance level $p = 0.05$ in a statistical program Statistica CZ, version 7.1 (StatSoft, Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Nutritional Composition. The *L. caerulea* berries contained 82.7% water, 1.6% proteins, 1.5% lipids, 7.2% saccharides, 6.7% dietary fiber, and 0.5% ash. Glutamic acid and arginine were the most common amino acids. Among others, aspartic acid, leucine, phenylalanine, and glycine predominated. The energetic value of *L. caerulea* fruits was calculated to be 330 kcal/kg (1380 kJ/kg). The berries also contained quite large amounts of potassium, calcium, phosphorus, magnesium, and other minerals. The dominant vitamins were ascorbic acid, niacin, and tocopherols (**Table 1**).

Saccharides. Acidified water extract of *L. caerulea* fruit was freeze dried and analyzed by HPLC. Saccharides constituted 7.2% of the fruits. Free saccharides included 3.2% glucose and 2.9% fructose; bound saccharides were 0.8% glucose, 0.2% galactose, and 0.1% arabinose. The phenolic fraction contained only 5.4% glucose and 1.9% fructose.

Lipids. Chloroform−methanol extract of *L. caerulea* was analyzed, and steroids and fatty acids were analyzed by GC-MS. The total amount of lipids was determined as 1.52% of the fruit weight. Lipid fraction includes hydrocarbons, sterols, triacylglycerols, and phosphatidyl choline as major and free fatty acids, phosphatidic acid, phosphatidyl serine, phospholipids, digalactosyl diglycerol, and phosphatidyl ethanolamine as minor components; α-amyrin, β-amyrin, stigmasterol, and ursolic acids were the major sterols present. The total amount of unsaponifiable matter was 0.46% of fruit weight. Fatty acids were analyzed after dissolution in chloroform using GC-MS and constituted 0.89% of berries, and palmitic and oleic acids dominated. Small amounts of polyunsaturated fatty acids were also found.

Organic Acids. Malonic, quinic, citric, and malic acids were determined in the fresh fruits. Citric and malic acids were also found in the phenolic fraction.

Phenolic Compounds. *Phenolic Acids.* In *L. caerulea* fresh berries phenolic acids (protocatechuic, gentisic, ellagic, ferulic, caffeic, chlorogenic, and coumaric) were determined (0.025%

Table 1. Nutrition Analyses of Fresh *L. caerulea* Berries[a]

amino acid	value (mg/100 g)	vitamin	value (mg/100 g)
alanine	47.52	pantothenic acid	1.40
arginine	136.08	niacin	0.47
aspartic acid	86.26	vitamin B1	0.16
cystine	14.40	vitamin B2	0.02
phenylalanine	55.73	vitamin B6	0.02
glycine	55.44	folic acid	0.02
histidine	51.12	vitamin B12	0.02
isoleucine	49.68	vitamin A	<80.00
glutamic acid	161.21	tocopherol α	0.42
leucine	79.92	tocopherol $\beta + \gamma$	0.40
methionine	19.87	tocopherol δ	0.28
lysine	52.92	vitamin K1	0.05
serine	38.09	carotene α	<0.05
threonine	<35.86	carotene β	0.72
tyrosine	29.66	xanthophyll	0.16
valine	52.20	lycopene	<0.001
vitamin C	12.10	cryptoxanthin	0.01
nicotinamide	1.87	zeaxanthin	0.01

mineral	value (mg/kg)	mineral	value (mg/kg)
potassium	2110.0	iron	2.7
calcium	245.0	zinc	1.7
phosphorus	151.0	manganese	1.4
magnesium	135.0	copper	0.6
sodium	93.0		

[a] Determination of individual substances was made by methods of analysis of AOAC International.

in total). Among these chlorogenic (0.018%) and gentisic (0.005%) acids predominated.

Anthocyanins. The fresh fruits contained 0.31% anthocyanins, cyanidin-3-glucoside predominated (83.3% using internal normalization), followed by peonidin-3-glucoside (5.9%) and cyanidin-3-rutinoside (3.3%). Paeonidin and pelargonidin glycosides (i.e., 3-glucoside, 3,5-diglucoside, and 3-rutinoside) were identified. As minor anthocyanins, some glycosylated derivatives of delphinidin and petunidin were also found (**Table 2**). The collision spectrum of monoglycosides of anthocyanins obtained (MS/MS) was simple, and the signal of the related aglycone dominated. Cleavage of dehydrated sugars of anthocyanins substituted with two (undifferentiated) hexoses ($\Delta m/z$ 162) and rutinoses (6-*O*-L-rhamnosyl-D-glucoses, $\Delta m/z$ 162 + 146 = 308) was a dominant process during fragmentation, resulting in either monoglycosylated fragments or aglycones. Among identified anthocyanins, two isomeric pairs occurred, i.e., cyanidin-3,5-dihexoside/delphinidin-3-rutinoside and pelargonidin-3,5-dihexoside/cyanidin-3-rutinoside. These compounds were well separated chromatographically. Minor isomers found were delphinidin-3-rutinoside and pelargonidin-3,5-dihexoside. Although the quality of the collision spectra of delphinidin-3-rutinoside is somewhat lower, it still allowed determination of delphinidin as the aglycone (m/z 303.05), and thus, the identity of the pigment could be deduced. Structures of the main anthocyanins are shown in **Figure 1**. The analysis of anthocyanins is displayed in **Figure 2a** and **2b**.

The phenolic fraction (0.4% of fresh fruits) contained anthocyanins (77%), flavonoids, and phenolic acids. Among anthocyanins, cyanidin-3-glucoside predominated.

Other Phenolics. Quercetin (0.10%), its 3-glycoside (0.06%), and 3-rutinoside (0.75%) as well as catechin (0.27%) and epicatechin (~0.02%) were found in the phenolic fraction. In fresh fruit, the amounts of quercetin, rutin, catechin, and epicatechin were 0.004%, 0.006%, 0.003%, and 0.003%, respectively. In the collision spectra of the glycosylated flavonoids the fragments corresponding to cleavage of sugar moieties dominated. Among other minor compounds, apigenin was also identified (data not shown).

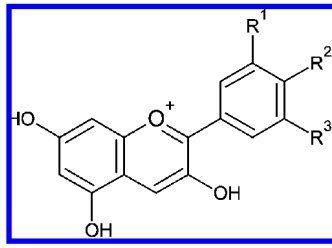
Table 2. Anthocyanins of *L. caerulea*, *Vaccinium macrocarpon, Vaccinium myrtillus*, and *Rubus occidentalis*

	relative content (% w/w)			
compound	*L. caerulea*	*V. macrocarpon* (*25*)	*V. myrtillus* (*26*)	*R. occidentalis* (*27*)
cyanidin			1.2	
cyanidin-3-arabinoside		5.5	7.1	
cyanidin-3-galactoside		7.1	**19.0**	
cyanidin-3-glucoside	**83.3**	12.3	**10.0**	**7.1**
cyanidin-3(6′-acetyl)-glucoside				
cyanidin-3,5-diglucoside	1.9			
cyanidin-3-pentoside		2.9		
cyanidin-3-rutinoside	3.3			51.8
cyanidin-3-sambubioside				**3.5**
cyanidin-3-xylosylrutinoside				**36.8**
delphinidin		1.8	1.4	
delphinidin-3-arabinoside-hexoside	0.3			
delphinidin-3-galactoside			**11.6**	
delphinidin-3-glucoside	2.1		**13.3**	
delphinidin-3-rutinoside	1.2			
pelargonidin-3-glucoside	0.6			
pelargonidin-3,5-diglucoside	0.5			
pelargonidin-3-rutinoside	0.1			**0.8**
peonidin			0.2	
peonidin-3-arabinoside		5.7	0.5	
peonidin-3-galactoside		10.2	0.9	
peonidin-3,5-digalactoside		24.1		
peonidin-3-glucoside	5.9	**60.7**	4.1	
peonidin-3,5-diglucoside	0.4			
peonidin-3(6′-coumaroyl)-diglucoside				
peonidin-3-rutinoside	0.4			
petunidin			0.5	
petunidin-3-arabinoside			2.7	
petunidin-3-galactoside			4.0	
petunidin-3-glucoside	0.1		9.0	
malvidin			0.5	
malvidin-3-arabinoside			1.9	
malvidin-3-galactoside			3.2	
malvidin-3-glucoside			8.9	
malvidin-3(6′-acetyl)-glucoside				
malvidin-3(6′-caffeoyl)-glucoside				
malvidin-3(6′-coumaroyl)-glucoside				
total anthocyanins	**100**	**100**	**100**	**100**

Antioxidant Activity. The reducing capacity of the phenolic fraction determined using Folin−Ciocalteu reagent (*15*) was 335 ± 15 μg of GAE/mg, which equals 140.5 mg/100 g fresh weight. The phenolic fraction scavenged the DPPH radical; 50% inhibition was achieved at 4.40 ± 0.50 μg/mL (IC_{50}). The antioxidant activity of the phenolic fraction was also assessed in the NADH/PMS system. The fraction scavenged the superoxide radical in a concentration-dependent manner with IC_{50} = 115.3 ± 11.4 μg/mL.

Antimicrobial Properties. Suppression of the biofilm formation by the freeze-dried fruit was statistically significant only in the case of *C. parapsilosis, S. epidermidis, E. faecalis*, and *S. mutans* ($p < 0.05$) (**Table 3**). We found no effect of these samples on biofilm formation in the case of *E. coli* strain. We were unable to evaluate the results of the tested phenolic fraction due to coagulation of the sample with the culture medium and adhesion to the polystyrene wells.

Both the freeze-dried fruit and its phenolic fraction significantly reduced adhesion to the artificial surface ($p < 0.05$) for *S. epidermidis*, *E. coli*, *E. faecalis*, and *S. mutans*. D-(+)-Mannose displayed a similar effect only in the case of *C.*



Name	R^1	R^2	R^3
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Pelargonidin	H	OH	H
Peonidin	OCH_3	OH	H
Petunidin	OCH_3	OH	OH
Malvidin	OCH_3	OH	OCH_3

Figure 1. Structures of the main anthocyanidins.

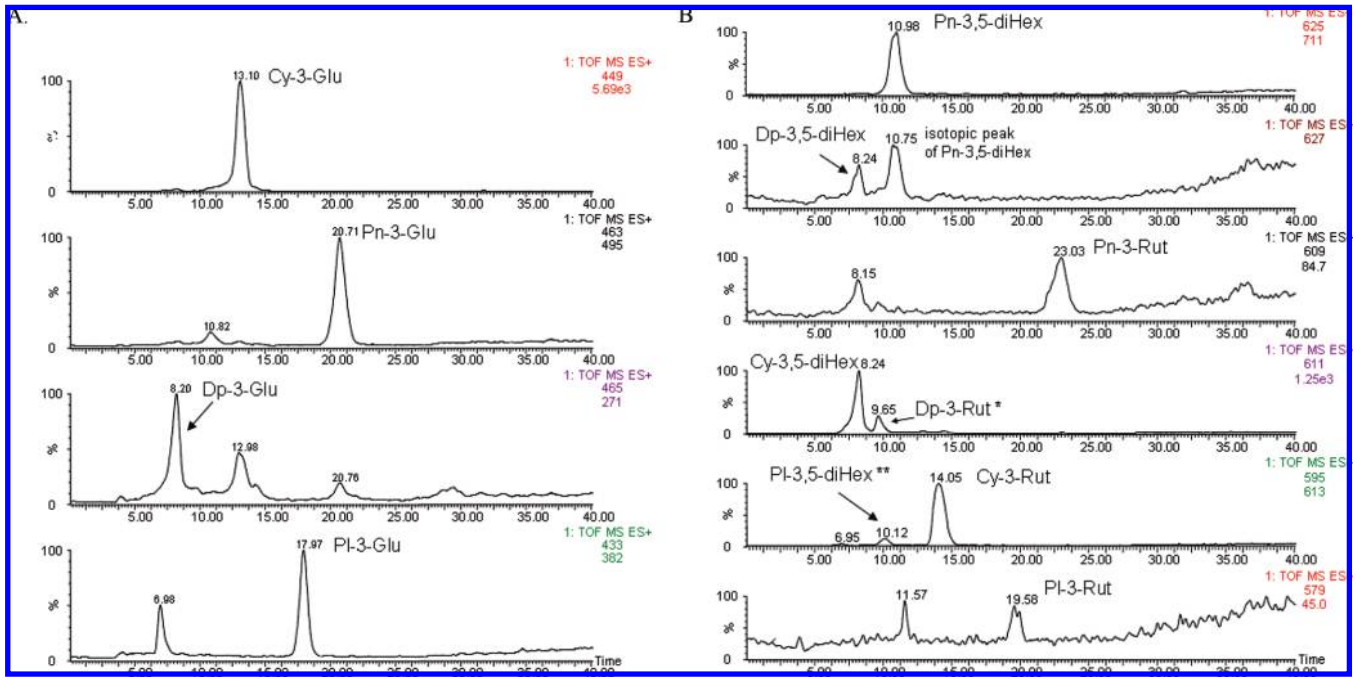


Figure 2. (**A**) LC/MS analysis of anthocyanins substituted with one hexose. (**B**) LC/MS analysis of anthocyanins substituted with rutinose or two hexose.

Table 3. Effect of Freeze-Dried Fruits and Phenolic Fraction on Biofilm Formation[a]

	C. parapsilosis BC 11 A_{595} (SD)	*S. epidermidis* CCM 7221 A_{595} (SD)	*E. coli* FB 27 A_{595} (SD)	*E. faecalis* NS123 A_{595} (SD)	*S. mutans* CCM7409 A_{595} (SD)
positive control (BHI)	3.074 (0.050)	2.937 (0.141)	0.403 (0.005)	1.449 (0.19)	0.494 (0.031)
freeze-dried fruits	2.901 (0.07)	0.307 (0.005)	0.438 (0.052)	1.146 (0.078)	0.381 (0.021)
phenolic fraction[b]	not evaluated	not evaluated	not evaluated	not evaluated	not evaluated
D-(+)-mannose	2.777 (0.103)	2.571 (0.06)	0.431 (0.019)	1.516 (0.143)	0.639 (0.214)
sodium hippurate	3.074 (0.054)	2.325 (0.11)	0.364 (0.041)	1.387 (0.3)	0.464 (0.027)

[a] Negative control, A_{595} (SD) = 0.157 (0.006). [b] It was impossible to evaluate the results of tested phenolic fraction due to coagulation of the sample with the culture medium and adhesion of its substances to polystyrene wells.

Table 4. Effect of Freeze-Dried Fruits and Phenolic Fraction on Microbial Adhesion (CFU)

	C. parapsilosis BC 11	*S. epidermidis* A117	*E. coli* FB 27	*E. faecalis* NS123	*S. mutans* CCM7409
positive control (BHI)	5.26×10^3	2.59×10^3	1.6×10^4	6.1×10^4	5.44×10^4
freeze-dried fruits	8.45×10^2	0	4.55×10^3	6.9×10^3	4.48×10^1
phenolic fraction	1.79×10^3	0	1.35×10^1	4.5×10^0	0
D-(+)-mannose	1.86×10^3	2.45×10^3	2.2×10^2	3.6×10^4	1.14×10^5
sodium hippurate	3.386×10^3	2.6×10^3	3.2×10^3	5.27×10^4	5.75×10^4

parapsilosis, *S. epidermidis*, and *E. coli* (**Table 4**). No statistically significant effect of phenolic fraction was found for the *C. parapsilosis* strain.

The minimal inhibitory concentrations of *L. caerulea* berries and phenolic fraction on *S. aureus* CCM 3953 and CCM 4223, *P. aeruginosa* CCM 3955, *E. coli* CCM 4225, *E. coli* 3954, and *S. agalactiae* were 250, 250, 500, 250, 125, and 125 μg/mL, respectively.

A large number of recently published investigations have proved the therapeutic effects of various fruits and vegetables in prevention of a number of chronic diseases. In this respect the chemopreventive, antimicrobial, antiadherence, and antioxidant benefits of *L. caerulea* berries have also been described (*5, 6*).

In this study, the major constituents, mainly saccharides, lipids, and polyphenolics of *L. caerulea* var. *kamtschatica* were

identified by μLC/MS/MS, GC, and UV data. *L. caerulea* contained 7.2% saccharides. Free saccharides included 3.2% glucose and 2.9% fructose; bound saccharides are 0.8% glucose, 0.2% galactose, and 0.1% arabinose. The fruit contained 1.5% lipids, mainly hydrocarbons, sterols, triacylglycerols, and phosphatidyl-choline. The weight of fatty acids was less than 1% of the total weight of the berries. The main acids included palmitic and oleic acids; stearic, myristic, linolic, palmitoleic, and lauric acids were minor acids. The entire weight of the unsaponifiable proportion (sterols, alcohols, and hydrocarbons) was only 0.5% and included α-amyrin, β-amyrin, stigmasterol, and ursolic acid as major constituents. The fruit content included 14.6% dry matter of which 14.8% is soluble fiber. Organic acids were represented by citric, malic, and other acids. The complete composition including the spectrum of saccharides and lipids in *L. caerulea* berries is published here for the first time. To date, only the content and variation of saccharides and proteins in the shoot apices have been studied (*9, 10*).

The phenolic fraction of *L. caerulea* var. *kamtschatica* (0.4% of fresh fruits) contained anthocyanins, flavonoids,, and phenolic acids as major compounds. Phenolic acids are present in free and bound forms. Bound phenolic acids may be linked through ester, ether, acetal, or other bonds (*22*). The most abundants in phenolic fraction were chlorogenic (0.018%) and gentisic (0.005%) acids. The contents of protocatechuic, ellagic, ferulic, caffeic, and coumaric acids were very low. Other hydroxycinnamic acid and hydroxybenzoic acid derivatives are mentioned in the literature, especially *m*-coumaric and *p*-coumaric acids (*23*). Dimethoxycinnamic, hydroxycaffeic, gallic, *o*-pyrocatechuic, protocatechuic, salicylic, *p*-hydroxyphenylacetic, and *p*-hydroxyphenyllactic acids have been reported (*24*), but we did not confirm them.

A comparison of the contents of anthocyanins in *L. caerulea*, *V. macrocarpon* (*25*), *V. myrtillus* (*26*), and *R. occidentalis* (*27*) is shown in **Table 2**. It can be clearly seen that the dominant anthocyanins differ in particular berries. While cyanidin glycosides dominate in *L. caerulea* and *R. occidentalis*, cyanidin and delphinidin glycosides are prevalent dyes in *V. myrtillus* and peonidin glycosides in *V. macrocarpon*.

The presence of paeonidin-3,5-diglucoside, delphinidin-3-glucoside, delphinidin-3-rutinoside, pelargonidin-3,5-diglucoside, and pelargonidin-3-rutinoside and antioxidant activity of the *L. caerulea* fruit phenolic fraction have not been reported before. The presence of cyanidin-3-glucoside as the major pigment confirms an earlier study (*23*). In addition, Terahara et al. (*28*) reported the presence of malvidin-3-glucoside and cyanidin-3-gentiobioside that were not detected in our study.

A number of publications consider Folin−Ciocalteu assay as a total phenol assay. Thompson and Chaovanalikit (*29*) reported total phenolics as a range from 427 to 1.142 mg of GAE/100 g of fresh weight among 11 samples of *L. caerulea*. We determined only an antioxidant capacity of 140 mg of GAE/100 g of fresh weight.

The antimicrobial activity of *L. caerule*a may be related to adhesion of the microorganisms to tissue cells and/or implant surfaces which is a prerequisite for colonization and infection of a number of pathogens in the urinary tract and mouth. The polyphenolic components of *L. caerulea* fruits may have important applications in the future as natural antimicrobial agents in the food industry as well as for medicine and oral hygiene.

The quantitative and qualitative variation in the content of secondary metabolites of *L. caerulea* is influenced by ecological and environmental effects as well as physiological and genetic factors. Phenolic compounds such as anthocyanin dyes, other flavonoids (notably quercetin and its derivatives), as well as phenolic acids and proanthocyanidins display potential health promoting effects (*1*). Several berry dietary supplements are currently available on the market. Due to the high quantity of health promoting phytoceuticals, low concentration of lipids, and acceptable nutritional value, *L. caerulea* appears to be a prospective target for new food supplement research.

In conclusion, *L. caerulea* berries seem to be a prospective source of health supporting phytochemicals, especially phenolic compounds that exhibit beneficial activities such as antiadherence, antioxidant, and chemoprotective. Thus, natural antioxidants, natural colorants, and an ingredient of functional foods based on *L. caerulea* berries look promising as a useful addition in the prevention of a number of chronic conditions, e.g., cancer, diabetes mellitus, tumor growth, and cardiovascular diseases.

ABBREVIATIONS USED

BHI, brain heart infusion; CFU, colony forming units; CZE, capillary zone electrophoresis; DAD, diode array detector; DPPH, 1,1-diphenyl-2-picrylhydrazyl; GAE, gallic acid equivalents; MIC, minimal antimicrobial concentration; μLC/MS/MS, microliquid chromatography/mass spectrometry; NADH, nicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium chloride; PBS, phosphate-buffered saline; PMS, phenazine methosulfate; PSS, physiological saline solution; SOP, standard operating protocols; SPE, solid-phase extraction.

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