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Lingonberry (*Vaccinium vitis-idaea*) and European Cranberry (*Vaccinium microcarpon*) Proanthocyanidins: Isolation, Identification, and Bioactivities

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ABSTRACT: European, small-fruited cranberries (*Vaccinium microcarpon*) and lingonberries (*Vaccinium vitis-idaea*) were characterized for their phenolic compounds and tested for antioxidant, antimicrobial, antiadhesive, and antiinflammatory effects. The main phenolic compounds in both lingonberries and cranberries were proanthocyanidins comprising 63−71% of the total phenolic content, but anthocyanins, hydroxycinnamic acids, hydroxybenzoic acids, and flavonols were also found. Proanthocyanidins are polymeric phenolic compounds consisting mainly of catechin, epicatechin, gallocatechin, and epigallocatechin units. In the present study, proanthocyanidins were divided into three groups: dimers and trimers, oligomers (mDP 4−10), and polymers (mDP > 10). Catechin, epicatechin, A-type dimers and trimers were found to be the terminal units of isolated proanthocyanidin fractions. Inhibitions of lipid oxidation in liposomes were over 70% and in emulsions over 85%, and in most cases the oligomeric or polymeric fraction was the most effective. Polymeric proanthocyanidin extracts of lingonberries and cranberries were strongly antimicrobial against *Staphylococcus aureus*, whereas they had no effect on other bacterial strains such as *Salmonella enterica* sv. Typhimurium, *Lactobacillus rhamnosus* and *Escherichia coli*. Polymeric fraction of cranberries and oligomeric fractions of both lingonberries and cranberries showed an inhibitory effect on hemagglutination of *E. coli*, which expresses the M hemagglutin. Cranberry phenolic extract inhibited LPS-induced NO production in a dose-dependent manner, but it had no major effect on iNOS of COX-2 expression. At a concentration of 100 μg/mL cranberry phenolic extract inhibited LPS-induced IL-6, IL-1β and TNF-α production. Lingonberry phenolics had no significant effect on IL-1β production but inhibited IL-6 and TNF-α production at a concentration of 100 μg/mL similarly to cranberry phenolic extract. In conclusion the phenolics, notably proanthocyanidins (oligomers and polymers), in both lingonberries and cranberries exert multiple bioactivities that may be exploited in food development.

KEYWORDS: lingonberry, European cranberry, proanthocyanidins, antioxidant, antimicrobial, antiadhesion, antiinflammatory

INTRODUCTION

Berries contain a large variety of different phenolic compounds such as anthocyanins, flavonols, and phenolic acids.[1] In some berries, such as cranberries and lingonberries, flavanols and proanthocyanidins are among the main constituents, but anthocyanidins, hydroxycinnamic acids, hydroxybenzoic acids, and flavonols can also be found.[2,3]

Proanthocyanidins consist of monomer units linked mainly through C4→C8 or C4→C6 interflavan bonds (B-type), but they can contain an additional ether bond O7→C2 (A-type). The isolation of proanthocyanidins is usually carried out with column chromatography using Sephadex LH-20 or XAD.[2,4] Proanthocyanidins may be analyzed by colorimetric methods using 4-(dimethylamino)cinnamaldehyde (DMAC). The condensation reaction of DMAC has been shown to be relatively specific to flavan-3-ols from monomeric catechins up to oligmers having DP 4.[5,6] In addition, more specific reversed or normal phase HPLC and LC−MS methods are used (reviewed in ref 7). A-type dimers and trimers were found predominantly in lingonberries (*Vaccinium vitis-idaea*) and European cranberries (*Vaccinium microcarpon*)[8] or American cranberries (*Vaccinium macrocarpon*).[4]

The ingestion of cranberry has traditionally been associated with the prevention of urinary tract infections (UTI). Kontiokari et al.[9] and Howell et al.[10] have found indications that the consumption of cranberry juice can decrease the incidence of recurrent UTI in women. Jepson and Craig reviewed four randomized controlled trials where cranberry products significantly reduced the occurrence of UTI in women.[11] However, there is no clear-cut evidence that the consumption of cranberry juice products prevents UTIs caused by *Escherichia coli*.[12] Consequently, in a recent scientific opinion on a health claim application concerning American cranberry products containing

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80 mg per daily dose of proanthocyanidins having A-type linkages between monomeric constituents, the European Food Safety Authority (EFSA) acknowledged that proanthocyanidins prevent the adhesion of bacteria (mostly *E. coli*) to the cell surface, a risk factor for urinary tract infections. However, according to EFSA, evidence provided was not sufficient to establish a cause and effect relationship between the consumption of cranberry products and the reduction of the risk of UTI in women.[13]

There are several reports of berry proanthocyanidins exhibiting antimicrobial effects,[1,3] and a number of studies have reported that the proanthocyanidins of American cranberry inhibit the attachment of uropathogenic *Escherichia coli* to uroepithelial and vaginal cells (reviewed in ref 12). The antiinflammatory effects of berries have been most often associated with flavonoids, which are usually present as glycosides in plants. Several of them have shown inhibitory effects on immunomodulatory mediators in various experimental systems. Both European cranberry and lingonberry contain quercetin,[14] which has shown antiinflammatory properties[15] in several studies. Myricetin, which is present in cranberry but not in lingonberry,[14] has been shown to exert inhibitory effects in phorbol ester-induced COX-2 expression in mouse epidermal cells[16] and interleukin-2 production in mouse EL-4 T-cells.[17] In inflammatory processes, nitric oxide (NO) is produced primarily by the enzyme inducible nitric oxide synthase (iNOS) in inflammatory cells such as macrophages. Aberrant iNOS induction and excessive NO production seem to be involved in the pathophysiology of human inflammatory diseases such as asthma, arthritis, and colitis. Inflammatory response is also enhanced by proinflammatory cytokines IL-1β, IL-6 and TNF-α and by enzyme cyclooxygenase-2 (COX-2).[18,19] Lipid oxidation was also shown to be inhibited by the cranberry and lingonberry phenolics, especially proanthocyanidins.[8,20] Among the oligomeric proanthocyanidins, A-type trimers have been reported to have the strongest antilipid peroxidation activity.[21] Data regarding bioactivities exist for a number of berry species including American cranberry (*Vaccinium macrocarpon*), but the bioactive properties of European cranberries and lingonberries have not been extensively characterized apart from a few studies.[8,20,22] Therefore, our aim was to characterize the phenolic composition of cranberries and lingonberries and to assess their different bioactivities, i.e. the antioxidant, antiinflammatory and antimicrobial activities as well as the effect on bacterial adhesion. More detailed information was sought with investigations of the bioactivities of different proanthocyanidin fractions.

MATERIALS AND METHODS

Materials. All solvents were of HPLC grade and purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). A Milli-Q water purification system was used (Millipore, Bedford, MA). Copper sulfate, Folin−Ciocalteu and α-tocopherol were from Merck (Darmstadt, Germany). Emultop emulgator was from Degussa Texturant Systems Deutschland GmbH & Co. KG (Hamburg, Germany). Lecithin from soybean was from Cargill Nordic (Espoo, Finland). Gallic acid, (+) catechin, (−)-epicatechin, procyanidins B1 and B2, caffeic acid, 3-caffeoylquinic acid (chlorogenic acid), ferulic acid, *p*-coumaric acid, sinapic acid, rutin and cyanidin-3-glucoside were purchased from Extrasynthese (Geney Cedex, France), benzylmercaptan from Fluka (Fluka Sigma-Aldrich Chemie Inc., Buchs, Switzerland) and concentrated hydrochloric acid (37−38%) from J.T.Baker (Mallinckrodt Baker Inc., Utrecht, Holland).

EDTA-containing human erythrocytes of blood group O (Ortho-Clinical Diagnostics Inc., Raritan, NJ), α-methylmannoside (α-MM), and phosphate-buffered saline, pH 7.1 (PBS) were used. The microbial growth media MRS (de Man Rogosa Sharpe, Oxoid) and Luria agar and Nutrient (Difco) were used, and peptone saline (Maximal Recovery Diluent, Lab M, Amersham, U.K.) in antimicrobial activity analysis.

Rabbit polyclonal iNOS and actin antibodies, goat polyclonal COX-2 antibody, goat anti-rabbit and donkey anti-goat HRP-conjugated antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); all other reagents in the antiinflammatory tests were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Cell Cultures. J774 macrophages (American Type Culture Collection, Manassas, VA, USA) were cultured at 37 °C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with Ultraglutamine 1 (Lonza Verviers SPRL, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Lonza), 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B and harvested with trypsin-EDTA (all from Gibco, Paisley, U.K.).

Human THP-1 promonocytes (ATCC) were cultured at 37 °C in 5% CO_2 atmosphere in RPMI 1640 (Lonza) modified to complete growth medium as recommended by cell supplier and supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B. The cells were differentiated by adding 12-*O*-tetradecanoylphorbol-13-acetate (100 nM) at the time of seeding the cells on plates.

Cells were seeded on 6- or 24-well plates and grown for 72 h to confluence prior to the experiments. Tested compounds were first dissolved in DMSO, and the stock solution was diluted to 1:1000 in fresh culture medium to obtain the final concentrations, and the macrophage cell lines were activated through toll-like receptor-4 (TLR4) pathway by adding bacterial lipopolysaccharide (LPS, 10 ng/mL.) After 24 h incubations, the cell culture media and cell lysates were harvested for analysis of nitric oxide and cytokine production, and iNOS and COX-2 expression, respectively.

Berry Samples and Preparation of Phenolic Extracts. The isolation of phenolic compounds from cranberries (*Vaccinium microcarpon*) and lingonberries (*Vaccinium vitis-idaea*) that commonly grow in Finland, 1−2 kg purchased from a local producer, was carried out by the method outlined by Kähkönen et al.;[1] briefly, lyophilized berry material (2.0−3.0 g) was weighed into the centrifuge tube as 6 replicates, 20 mL of 70% aqueous acetone was added, and the sample was homogenized with Ultra-Turrax for 1 min. Samples were centrifuged (1570*g*, 15 min), and the supernatants were collected. The extraction was repeated from the pellets with another 20 mL of 70% aqueous acetone. Supernatants were combined, evaporated to dryness under vacuum, and dissolved in 25 mL of water. Samples were divided into 5 mL aliquots and were applied into the C_{18} solid phase extraction columns (Varian Mega Bond Elut, 5 g, 20 mL, loading capacity 250 mg). Sugars were eluted with 20 mL of 0.01 M HCl, and the phenolic compounds were subsequently eluted with 20 mL of methanol and evaporated by rotary evaporator. Dry phenolic material was reconstituted with 10 mL of water and lyophilized.

Isolation and Characterization of Proanthocyanidins. An accelerated solvent extractor ASE 200 System (Dionex, Sunnyvale, CA, USA) with 11 mL stainless steel ASE vessels was used for the accelerated solvent extraction. About 1.0 g of freeze-dried berry powder was mixed homogeneously with 0.25 g of diatomaceous earth and placed into an extraction cell. The total of 10 cells were used for extraction. The extraction was performed with 70% aqueous acetone. ASE settings were as follows: pressure 1500 psi, temperature 100 °C, heat time 5 min, static time 5 min, 1 static cycle. Each sample was extracted twice. After the

Table 1. Method Validation Results

component	retention time (RSD %)	peak area (RSD %)	range μg/mL	correlation coefficient R^2	LOD[a] μg/mL	LOQ[b] μg/mL	recovery (%)
gallic acid	0.04	0.8	0.32−306.6	1.0000	0.10	0.32	102.0
chlorogenic acid	0.03	2.0	0.23−193.8	1.0000	0.04	0.23	100.7
cyanidin-3-glucoside	0.02	1.2	1.87−94.0	0.9997	0.68	1.87	95.1
catechin	0.01	0.7	0.05−148.6	0.9993	0.02	0.05	89.0
rutin	0.01	0.6	2.13−47.9	1.0000	0.86	2.13	100.6

[a] Limit of detection. [b] Limit of quantification.

Table 2. Bacterial Strains Used in Analysis of Antimicrobial Activities of Phenolic Cranberry and Lingonberry Extracts

bacterial strain and catalogue numbers	origin	received from
Escherichia coli VTT E-94564^T (ATCC 11775)	human urine	DSM[a]
Lactobacillus rhamnosus VTT E-96666 (ATCC 53103)	human feces	ATCC[b]
Salmonella enterica sv. Typhimurium[c] VTT E-981151	human feces	National Public Health Institute, Finland
Staphylococcus aureus VTT E-70045[c] (ATCC 6538)	human lesion	Sanitized Testing Laboratory, Burgdorf, Switzerland

[a] Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany). [b] American Type Culture Collection (USA). [c] Virulent strain.

extraction, supernatants were evaporated to dryness and redissolved in 5 mL of methanol.

Proanthocyanidins from cranberry and lingonberry extracts were separated with a Sephadex LH-20 column described by Hellström et al.[2] Proanthocyanidin fractions were evaporated to dryness and dissolved in 5 mL of acetonitrile. Proanthocyanidin fractions were further purified and fractionated into 6 (cranberry) or 10 (lingonberry) fractions by Waters semipreparative NP-HPLC (Waters 2767 sample manager, Waters 2545 binary gradient module and, Waters system fluidic organizer) coupled with Waters 2998 DAD, Waters 474 fluorescence detector, and Waters ZQ mass spectrometer. Column was YMC-Pack Diol-120-NP, 250 × 20 mm, 5 μm. The mobile phases were as follows: A, dichloromethane/methanol/water (42:7:1); B, dichloromethane/methanol/water (5:44:1). Separation was started with 100% A, followed by 0−30% B 0−45 min, 30% B 45−65 min, 30−85% B 65−66 min, 85% B 66−70 min, 85−30% B 70−71 min, 30−0% B 71−73 min, 0% B 73−80 min. The injection volume was 6 mL, and the flow rate 15 mL/min.

The depolymerization of proanthocyanidins was carried out by the method outlined by Guyot et al.[23] and Hellström et al.[2] Procyanidins B1 and B2 were used for quantification of (epi)catechin benzylthioethers after thiolysis. Epicatechin and catechin were quantitated with authentic standards, and A-type dimers and trimers were quantified as procyanidin B2 equivalents.

UPLC and LC−MS Analyses. The phenolic profiles of berry extracts were determined by ultraperformance liquid chromatography (UPLC) diode array (PDA) and fluorescence (FLR) and LC−MS methods. The UPLC-PDA-FLR method was used for quantitative and the LC−MS method for qualitative analyses of proanthocyanidin fractions. Phenolic compounds were analyzed by Waters ACQUITY UPLC coupled with eλ PDA diode array and FLR detectors. The method described in ref 24 was scaled to the UPLC, and the separation was done using a Waters HSS T3 C_{18}, 1.7 μm, 2.1 × 150 mm column heated to 40 °C. The mobile phase consisted of gradient performed with water/0.5% formic acid (solvent A) and acetonitrile/0.5% formic acid (solvent B) at a constant flow rate of 0.5 mL/min. Gradient (v/v) of B was as follows: 0−1 min, 0% B; 1−3.5 min, 0−6% B; 3.5−9.8 min, 6−10% B; 9.8−16 min, 10−16% B; 16−19 min, 16% B; 19−21 min, 16−24% B; 21−23 min, 24−32% B; 23−25 min, 32−64% B; 25−27 min, 64% B; 27−28 min, 64−0% B, next inject delay 3 min. PDA settings were as follows: wavelength range 200−600 nm, 5 data points/s (>15 points across the narrowest peak), resolution 3.6 nm. Excitation and emission wavelengths for FLR were 280 and 325 nm, respectively. Phenolic compounds were tentatively identified on their UV spectrum and were clustered into five subclasses: anthocyanins as cyanidin-3-glucosides, 520 nm; flavonols as rutin, 365 nm; hydroxycinnamates as chlorogenic acid, 320 nm; hydroxybenzoates as gallic acid, 280 nm; catechins and flavan-3-ols as catechin, FLR. The detection limit was calculated from the concentration that gave a signal-to-noise ratio (S/N) > 3 and the quantification limit the concentration that gave $S/N > 10$. Recoveries of the reference compounds were tested by spiking berry extract which had been analyzed several times. Method validation parameters are presented in Table 1.

An Agilent 1100 LC instrument, including diode array and fluorescence detectors, equipped with Bruker Esquire-LC ion trap ESI-MS (Bruker Daltonics, Bremen, Germany) was used for identification of proanthocyanidin fractions. Separation conditions were the same as previously.[20] ESI-MS analyses were performed in positive mode, and the parameters were as follows: dry gas 10.0 L/min, nebulizer 20 psi, ESI interface temperature 350 °C, capillary voltage 3 kV, end-plate offset −500 V, skimmer (I) 15.0 V, skimmer (II) 6.0 V, lens (I) −5.0 V, lens (II) −60 V, trap drive value 45.0.

Liposome and Emulsion Oxidation. Liposomes and emulsions were prepared as described in our previous study.[25] The concentrations of the phenolic extracts (based on the total phenolic content as determined by HPLC) in liposomes were 2.1, 4.2, and 8.4 μg/mL of a total sample volume and in the emulsions 50 and 100 μg/g of lipid. Oxidation was followed by the formation of conjugated diene hydroperoxides and hexanal. Samples (25−100 μL) for conjugated diene hydroperoxide analysis were dissolved in methanol (5 mL) and analyzed spectrophotometrically at 234 nm (Lambda 25 UV/vis spectrometer, Norwalk, CT). Samples (500 μL) were analyzed for hexanal content using static headspace gas chromatography (Autosystem XL gas chromatograph equipped with an HS40XL headspace sampler, Perkin-Elmer, Shelton, CT, column NB-54, Nordion).

Antimicrobial Activity. Antimicrobial activities of proanthocyanidin fractions of cranberry and lingonberry were measured on *Lactobacillus rhamnosus* VTT E-96666 (probiotic strain), *Escherichia coli* VTT E-94564^T, *Salmonella enterica* sv. Typhimurium VTT E-981151, and *Staphylococcus aureus* VTT E-70045. The bacterial strains used are introduced in Table 2. Liquid culture method was used as was described earlier,[26] and the cultures were dosed with proanthocyanidin extract of 1 mg/mL.

Inhibition of Bacterial Hemagglutination and Yeast Cell Agglutination by Phenolic Extracts. The recombinant *E. coli* strains used are listed in Table 3. These strains, which all express a single

type of fimbria, were available from previous work.[24] Bacteria were grown for 18 h at 37 °C on Luria agar plates supplemented with appropriate antibiotics, collected and suspended in PBS. Yeast cell agglutination (YA), inhibition of YA (IYA), hemagglutination (HA) and inhibition of HA (IHA) were performed as previously.[24] The phenolic extracts and proanthocyanidin fractions of cranberries and lingonberries were dissolved in PBS to obtain a stock solution of 0.3 mg of total phenolics/mL (pH 6.5) and tested for inhibitory effect on bacteria-mediated HA and YA at concentrations between 0.5 and 30 μg of total phenolics/mL. In inhibition, analysis bacteria were mixed with the berry sample and incubated for 30 min prior to the addition of erythrocytes or yeast cells.

Antiinflammatory Testing. *Cytotoxicity.* Cytotoxicity of tested samples was assessed by measuring cell viability using XTT test (Cell Proliferation Kit II, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Nitric Oxide Production. NO production was determined by measuring the accumulation of its stable metabolite nitrite in the culture medium of J774 macrophages. The culture medium was collected after 24 h incubation with LPS ± tested compounds, and the nitrite concentration was measured by Griess reaction.

Cytokine Measurements. Production of cytokines TNF-α, IL-6 and IL-1β was studied in cultures of human THP-1 macrophages. After 24 h incubation with LPS ± tested compounds, culture medium was collected, and cytokine concentrations were determined by enzyme immunoassay using the following commercial reagents: TNF-α (DuoSet ELISA kit, R&D Systems Inc., Minneapolis, MN, USA), IL-1β (IL-1β ELISA Set II, Biosciences, San Diego, CA, USA) and IL-6 (PeliPair, Sanquin Reagents, Amsterdam, The Netherlands).

Western Blot Analysis. Expression of the inflammatory enzymes iNOS and COX-2 was studied in J774 macrophages by Western blotting as described earlier.[27] The protein samples (20 μg) were separated by SDS−PAGE on 8% polyacrylamide gel and transferred to a nitrocellulose membrane. iNOS and COX-2 proteins were detected and identified by using specific primary and HRP-conjugated secondary antibodies. Bound antibodies were detected using SuperSignal West Pico chemiluminescent substrate (Pierce, Cheshire, U.K.) and FluorChem 8800 imaging system (Alpha Innotech, San Leandro, CA, USA).

Statistical Analysis. Statistical differences among antioxidant activities were tested by multivariance analysis using PASW 17.0.2 (SPSS Inc., Chicago, IL). One-way ANOVA with Dunnett's or Bonferri's post test was used for antiinflammatory tests. The significance level was set at $p < 0.05$.

Table 3. Bacterial Strains Used in the Analysis of Inhibition of Agglutination[20]

E. coli strain	fimbria or adhesion (serotype)
HB101 (pPIL291-15)	P ($F7_1$)
HB101 (pPIL110-75)	P (F_{11})
HB101 (pRR7)	M
HB101 (pBJN406)	Dr
HB101	no fimbria
LE392 (pANN801-13)	S
LE392 (pPKL4)	type 1
LE392 (pBR322)	no fimbria

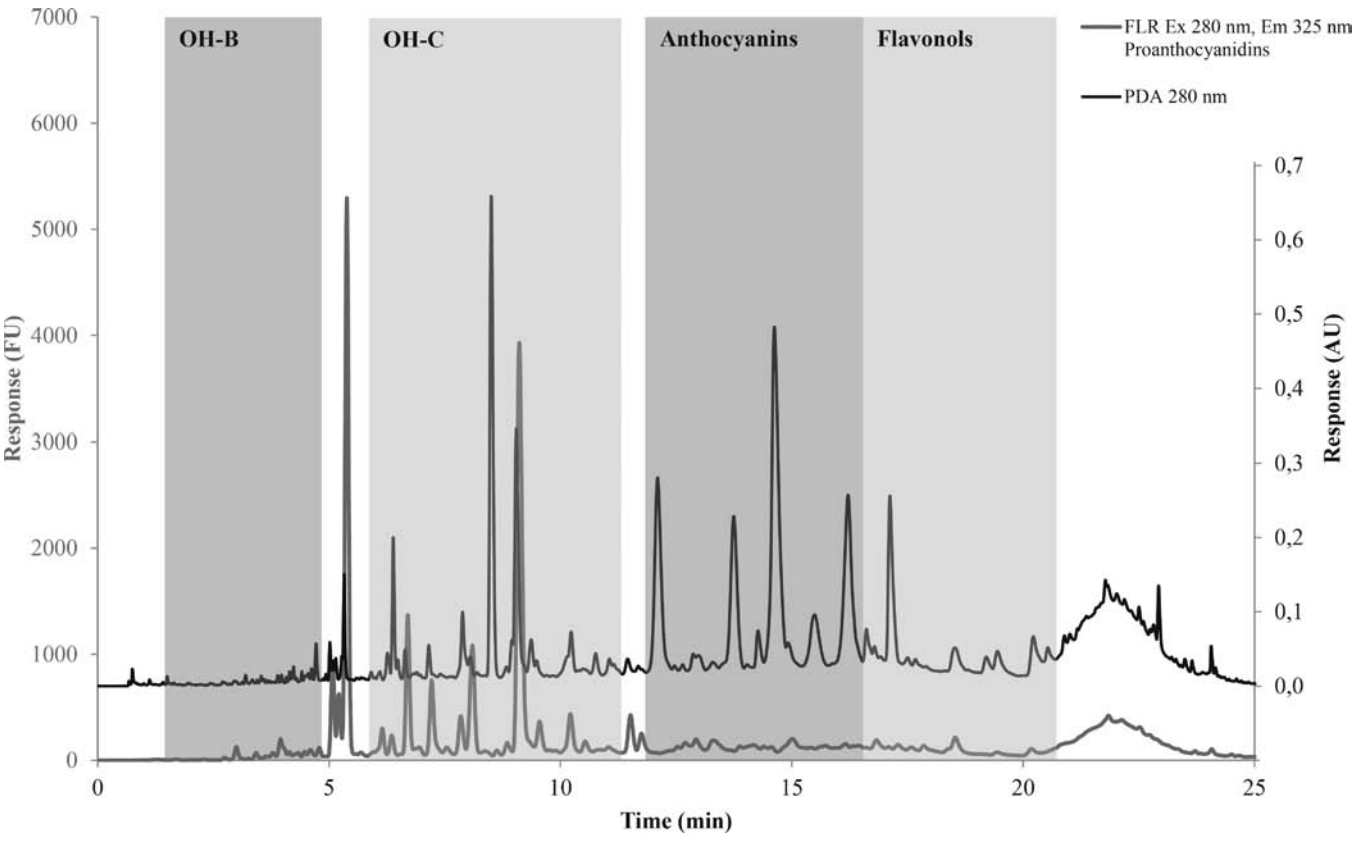

Figure 1. UPLC-PDA-FLR chromatogram of the European cranberry presenting the phenolic subclasses. Hydroxybenzoic acids (OHB), hydroxycinnamic acids (OHC), anthocyanins, and flavonols were detected from PDA at their discrete wavelengths 280, 320, 520, and 365 nm, respectively. Proanthocyanidins were detected from FLR. Peaks overlapping on both chromatograms were proanthocyanidins.

Table 4. Phenolic Composition of Cranberries and Lingonberries (mg/100 g of Dry Weight, Mean ± SD)[a]

cultivar	anthocyanins	flavonols	OH-C[b]	OH-B[c]	flavan-3-ols and proanthocyanidins	moisture%
cranberry	299.3 ± 2.8	259.6 ± 2.0	129.1 ± 1.2	0.9 ± 0.6	1172.5 ± 58.6	80.2
lingonberry	257.0 ± 0.01	163.4 ± 1.8	77.7 ± 2.9	8.6 ± 0.8	1230.3 ± 61.5	78.8

[a] Moisture contents from www.fineli.fi Finnish Food Composition Database. [b] Hydroxycinnamic acids. [c] Hydroxybenzoic acids.

Table 5. Structural Composition (Percent in Moles) of Lingonberry and Cranberry Proanthocyanidin Fractions

	terminal unit					
fraction	catechin (%)	epicatechin (%)	A-type dimer (%)	A-type trimer (%)	extension unit (%)	mDP[a]
			Lingonberry			
1	19.2	3.4	20.3		57.2	2.3
2	14.8	2.3	7.9	0.5	74.5	3.9
3	12.5	1.9	5.4		80.2	5.0
4	10.5	1.4	5.0		83.1	5.9
5	9.3	1.1	4.6		85.0	6.7
6	10.4	1.4	3.3	0.3	84.6	6.5
7	8.7	0.8	2.9		87.6	8.1
8	7.5	1.1	3.3		88.2	8.4
9	7.6	0.8	1.9		89.7	9.8
10	2.5	0.5	0.1		96.9	32.0
			Cranberry			
1	21.6	12.1	0.8	7.9	57.6	2.4
2	16.0	8.7	0.2	0.7	74.5	3.9
3	12.8	7.1		0.2	79.8	5.0
4	11.1	6.4		0.1	82.5	5.7
5	9.6	5.3			85.1	6.7
6	2.1	0.7			97.1	35.0

[a] Mean degree of polymerization.

RESULTS AND DISCUSSION

Phenolic Profiles. The UPLC-PDA-FLR chromatogram of the European cranberry is presented in Figure 1. The main phenolic compounds in lingonberries and cranberries were proanthocyanidins, respectively representing 71 and 63% of the total phenolic compounds. Proanthocyanidins were detected and quantified by the fluorescence detector coupled to the UPLC. Anthocyanins (15 and 16%), flavonols (9 and 14%), hydroxycinnamic acids (5 and 7%) and hydroxybenzoic acids (0.5 and 0.05%) were also found (Table 4). Therefore, lingonberries contained the same amount of proanthocyanidins as in cranberries, whereas cranberries contained more anthocyanins, flavanols, and hydroxycinnamic acids. The results are of the same order of magnitude and in accordance with the previous results obtained by Kähkönen et al. and Määttä-Riihinen et al.,[3,28] except for the flavanol and proanthocyanidin content in cranberries. Kähkönen et al.[3] reported that the proanthocyanidin content of cranberries was 219 mg/100 g dw, while we determined the content at 1.2 g/100 g dw. Also Määttä-Riihinen et al.[28] had detected lower amounts of proanthocyanidins. This may be due to the ethyl acetate they used as an extraction solvent. Solubility of the higher molecular weight proanthocyanidins in ethyl acetate is lower than in acetone or methanol.[29] In another study, the proanthocyanidin content of the lingonberries was determined to be 158 mg/100 g fw (ca. 1000 mg/100 g dw), which is slightly less than reported here.[30]

Thioacidolysis provides more detailed information on cranberry and lingonberry proanthocyanidin fractions. Table 5 presents the structural composition of lingonberry and cranberry proanthocyanidin fractions as the molar percentages. Catechin, epicatechin, A-type dimers and A-type trimers were found as terminal units, and in the positive ion mode the m/z values of the ions of these terminal units were 291, 291, 577, and 865, respectively. The MS^2 fragmentation was also similar to previously described.[31,32] The extension unit was detected as epicatechin benzylthioether as it exhibited an $[M + H]^+$ ion at m/z 413 and an MS^2 fragment at m/z 289 on MS. Gallocatechin benzylthioethers ($[M + H]^+$ at m/z 429, MS^2 fragment at m/z 307) were also detected as an extension unit, but they were below quantification limit. The first fraction of cranberry and lingonberry contained dimers and trimers, as can be seen from the mean degree of polymerization (mDP), which was 2.4 and 2.3, respectively. In lingonberries, the first fraction contains about 20% A-type dimers as terminal units, as well as catechins and only 3.4% of epicatechins. In cranberries, the proportion of catechins was the same as in lingonberries, but it contained more epicatechin units (12.1%), A-type trimers (7.9%) and less than 1% A-type dimers. The proportions of catechin, epicatechin and A-type dimers in fractions 2−9 remained the same as the DP increased. The mDP of fractions 2−9 in lingonberries increased from 3.9 to 9.8, that is from tetramers to decamers. In cranberries, the trend was similar. The proportions of catechins and

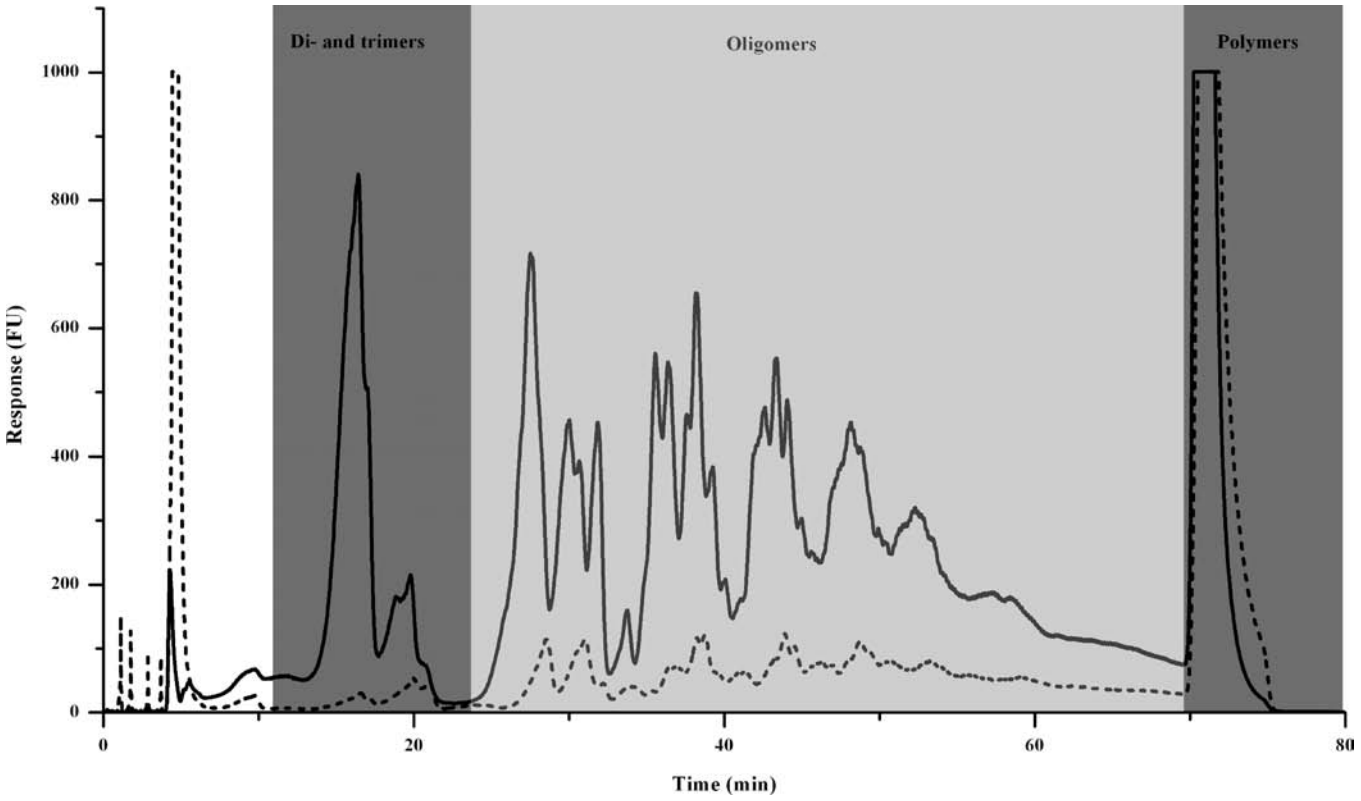

Figure 2. Semipreparative-HPLC chromatogram of cranberry [- - -] and lingonberry [—] procyanidins. Dimers and trimers: fraction 1. Oligomers: cranberry fractions 2−5, lingonberry fractions 2−9. Polymers: cranberry fraction 6, lingonberry fraction 10.

epicatechins in fractions 2−5 remained the same, and the mDP increased from 3.9 to 6.7. Fraction 5 contained oligomers from DP 6 to 10, as we were not able to fractionate it further. The quite low mDP of fraction 5 indicates a relatively low concentration of DPs 7−10. Overall, European cranberry contained more epicatechins and less A-type dimers as terminal units than lingonberry. In a previous study, lingonberries and European cranberries were noted to contain A-type dimers and trimers.[8] The mDP of the polymeric fraction for lingonberry was 32.0 and 35.0 for cranberry. Both of these polymeric fractions comprised over 60% of the total proanthocyanidin content. Hellström et al.[30] reported the proanthocyanidins of lingonberry to be predominantly of small oligomers and containing significant portions of A-type linkages. The degree of polymerization of an American cranberry has been previously determined by Gu et al.[4] to be 15.

Lingonberry fractions 2−9 were combined into one oligomeric fraction containing proanthocyanidins having mDP 4−10, and cranberry fractions 2−5 into another oligomeric fraction. Lingonberry fractions 1 (dimers and trimers) and 10 (polymers) and cranberry fractions 1 (dimers and trimers) and 6 (polymers) were kept as such (Figure 2). The purity of the fractions was analyzed by determining impurities by HPLC. The purity of the fractions was high, for example the polymeric fraction contained less than 1% other phenolics than procyanidins.

Antioxidant Activity. The oxidation of liposomes was carried out at concentrations of 2.1, 4.2, and 8.4 μg/mL of di- and trimeric, oligomeric and polymeric fractions of lingonberries and cranberries (Table 6). The di- and trimeric fraction of cranberry proanthocyanidins was the most effective (inhibition of formation of conjugated diene hydroperoxides and hexanal, 47.9−80.3% and 86.6−98.0%, respectively) in inhibiting the liposome oxidation at all concentrations, whereas the oligomeric fraction of lingonberry at concentrations of 2.1 and 4.8 μg/mL was the most effective (conjugated diene hydroperoxides 58.2 and 66.3% and hexanal 84.8 and 88.9%, respectively). The greater inhibition of lipid oxidation may be due to the presence of A-type trimeric structures in cranberries. Ho et al.[22] have previously reported the strongest antilipid peroxidation activity of a proanthocyanidin trimer containing an A-type linkage. In addition, A-type dimer showed strong superoxide scavenging activity in their study. However, the antioxidant activity toward hydroxyl free radical scavenging of oligomeric proanthocyanidin B3 was reported to be slightly higher than the two A-type proanthocyanidins (A2 epicatechin-(2β→7,4β→8)-epicatechin and trimer epicatechin-(4β→8,2β→O→7)-epicatechin-(4β→8)-epicatechin) from *Litchi chinensis* pericarp.[33]

The di- and trimeric fraction at 8.4 μg/mL concentration was the most effective of lingonberry fractions (conjugated diene hydroperoxides 77.9% and hexanal 96.3%). The polymeric fractions of lingonberry and cranberry proanthocyanidins at 2.1 μg/mL concentration had the lowest inhibition levels on liposome oxidation, although at higher concentration the inhibition was good. Overall, lingonberry fractions were slightly more effective than cranberry fractions in inhibiting liposome oxidation. The inhibition rates of lipid oxidation in emulsions are presented in Table 7. The inhibition was over 85%, in almost all fractions and concentrations. Differences between fractions were small, but in most cases the oligomeric or polymeric fraction was the most effective. Little information is available concerning the antioxidant activity of cranberry and lingonberry proanthocyanidins.

Table 6. Inhibition of Lipid Oxidation as Measured by Formation of Conjugated Dienes and Hexanal in Lecithin Liposome Oxidation Model System with 2.1, 4.2, and 8.4 μg/mL Phenolic Compounds (Percent Inhibition, Mean ± SD)[a]

	conjugated diene hydroperoxides			hexanal		
	2.1 μg/mL	4.2 μg/mL	8.4 μg/mL	2.1 μg/mL	4.2 μg/mL	8.4 μg/mL
			Lingonberry			
dimers and trimers	50.6 ± 4.0 a	65.3 ± 0.8 bc	77.9 ± 0.1 ab	76.1 ± 0.6 ab	88.0 ± 1.0 abc	96.3 ± 0.5 ab
oligomers	58.2 ± 5.0 a	66.3 ± 4.2 abc	72.6 ± 2.6 c	84.8 ± 4.4 a	88.9 ± 2.4 abc	93.0 ± 1.0 bcd
polymers	30.8 ± 5.1 bc	68.7 ± 4.9 ab	54.1 ± 0.6 f	74.7 ± 5.4 ab	90.2 ± 7.7 ab	93.6 ± 0.6 bc
extract	49.0 ± 4.71a	73.5 ± 0.4 a	74.1 ± 1.8 bc	83.9 ± 5.4 a	92.5 ± 3.4 ab	94.4 ± 0.6 abc
			Cranberry			
dimers and trimers	47.9 ± 5.0 a	69.8 ± 2.2 ab	80.3 ± 0.3 a	86.6 ± 6.5 a	95.4 ± 0.3 a	98.0 ± 0.1 a
oligomers	46.4 ± 2.3 ab	58.8 ± 1.3 cd	64.8 ± 0.7 d	64.1 ± 4.6 b	79.7 ± 1.4 c	85.3 ± 0.8 e
polymers	24.6 ± 15.3 c	50.8 ± 1.5 e	60.1 ± 0.2 e	64.9 ± 0.8 b	84.5 ± 6.2 bc	89.2 ± 1.0 de
extract	56.4 ± 4.7 a	54.4 ± 2.9 de	63.0 ± 1.0 de	83.2 ± 3.5 a	84.4 ± 3.5 bc	90.9 ± 2.5 cd

[a] Different letters in the concentration row denote significant difference ($p < 0.05$) in the inhibition between the concentrations.

Table 7. Inhibition of Lipid Oxidation as Measured by Formation of Conjugated Dienes and Hexanal in Emulsion Oxidation Model System with 50 and 100 μg/g Phenolic Compounds (Percent Inhibition, Mean ± SD)[a]

	conjugated diene hydroperoxides		hexanal	
	50 μg/g	100 μg/g	50 μg/g	100 μg/g
		Lingonberry		
dimers and trimers	90.5 ± 1.4 a	89.7 ± 0.5 c	84.9 ± 2.9 c	98.1 ± 0.2 b
oligomers	90.5 ± 0.4 a	89.9 ± 0.1 c	93.4 ± 1.2 ab	100.0 ± 0.0 a
polymers	92.6 ± 0.3 a	93.0 ± 0.4 a	98.3 ± 0.1 ab	98.9 ± 0.1 ab
		Cranberry		
dimers and trimers	69.8 ± 2.9 b	94.1 ± 0.5 a	94.7 ± 1.1 ab	93.2 ± 1.4 c
oligomers	26.8 ± 10.6 c	93.5 ± 0.1 a	91.5 ± 5.9 bc	98.7 ± 0.3 ab
polymers	92.6 ± 0.4 a	91.9 ± 0.8 b	99.7 ± 0.0 a	99.4 ± 0.2 ab

[a] Different letters in the concentration row denote significant difference ($p < 0.05$) in the inhibition between the concentrations.

Viljanen et al.[21,34] tested the commercially available procyanidin B1 (epicatechin-(4β→8)catechin) and B2 (epicatechin-(4β→8)epicatechin) dimer standards and isolated lingonberry proanthocyanidins for their antioxidant activity in lecithin liposomes. Lingonberry proanthocyanidins were divided into two fractions: one contained mainly (92%) monomeric proanthocyanidins (catechin and epicatechin) and the other dimers and trimers. The standard compounds inhibited conjugated diene hydroperoxide formation by 83−89% and the lingonberry monomeric fraction by 69% at 4.2 μg/mL and 82% at 8.4 μg/mL concentrations. The di- and trimeric fraction was a slightly more effective antioxidant. Our results are in agreement with these previous results. Määttä-Riihinen et al.[8] tested the antioxidant activity of monomeric and oligomeric fraction of lingonberry and cranberry proanthocyanidins in an emulsion model system. The effects of lingonberry fractions were equal; the inhibition of lipid oxidation was 84−85%, while the oligomeric fraction of cranberries was more potent (87%) compared to the monomeric fraction (80%).

Similarly, regarding the radical scavenging activity of other proanthocyanidin sources such as cocoa and almonds[35,36] and grape seeds,[37] a higher degree of polymerization resulted in a higher level of efficacy. da Silva Porto et al.[38] also reported a higher degree of polymerization enhances the antioxidant activity of proanthocyanidins in low-density lipoprotein (LDL). This is possibly due to the increased number of OH groups.[39] On the other hand, it may be because of the interaction of proanthocyanidins with phopholipid polar head groups, which has been associated with the inhibition of lipid oxidation in a chain length-dependent manner,[40] i.e. a higher DP of proanthocyanidins provides more protection. It has been postulated that the greater antioxidant activity of dimeric and oligomeric proanthocyanidins is due to the increasing electron delocalization of the phenyl radical by the interflavan linkage.[41]

Antimicrobial Effect. The proanthocyanidin fractions of lingonberries and cranberries were strongly antimicrobial against *Staphylococcus aureus* VTT E-70045, whereas they had no effect on other tested bacterial strains (*Salmonella enterica* sv. Typhimurium VTT E-981151, *Lactobacillus rhamnosus* VTT E-96666 and *E. coli* VTT E-94564^T) (Table 8 and Figure 3 a,b). The studied fractions did not affect the pH of the culture, and therefore bacteriocidic activity on *Staphylococcus aureus* was caused by the proanthocyanidin fraction of cranberry and lingonberry. These results are in line with our earlier studies with the phenolic extracts of lingonberry and cranberry, which showed the antibacterial effect of phenolic berry extracts on Gram-positive pathogens including *Staphylococcus*, *Bacillus*, *Clostridium*, but showed only weak or no antimicrobial activity on Gram-negative strains of virulent *Salmonella*

Table 8. Antimicrobial Activities of Phenolic Cranberry and Lingonberry Extracts (1 mg/mL) on Selected Microbial Strains in Liquid Culture[a]

fraction	*L. rhamnosus* E-96666	*S. aureus* E-70045	*S. enterica T.* E-981151	*E. coli* E-94564T
cranberry polymeric proanthocyanidins pH 6.0−6.5[b]	−	++++	−	−
lingonberry polymeric proanthocyanidins pH 6.2−6.5[b]	−	++++	−	−
pH of the growth medium	5.5−6.0	~6.5	~6.5	~6.5

[a] Inhibition is indicated from − (no inhibition) to +++++ (strong bacteriocidic effect with decrease of number of cultivable microbial cells below detection limit of 1000 cfu/mL). [b] pH of the growth medium with berry proanthocyanidin of 1 mg/mL.

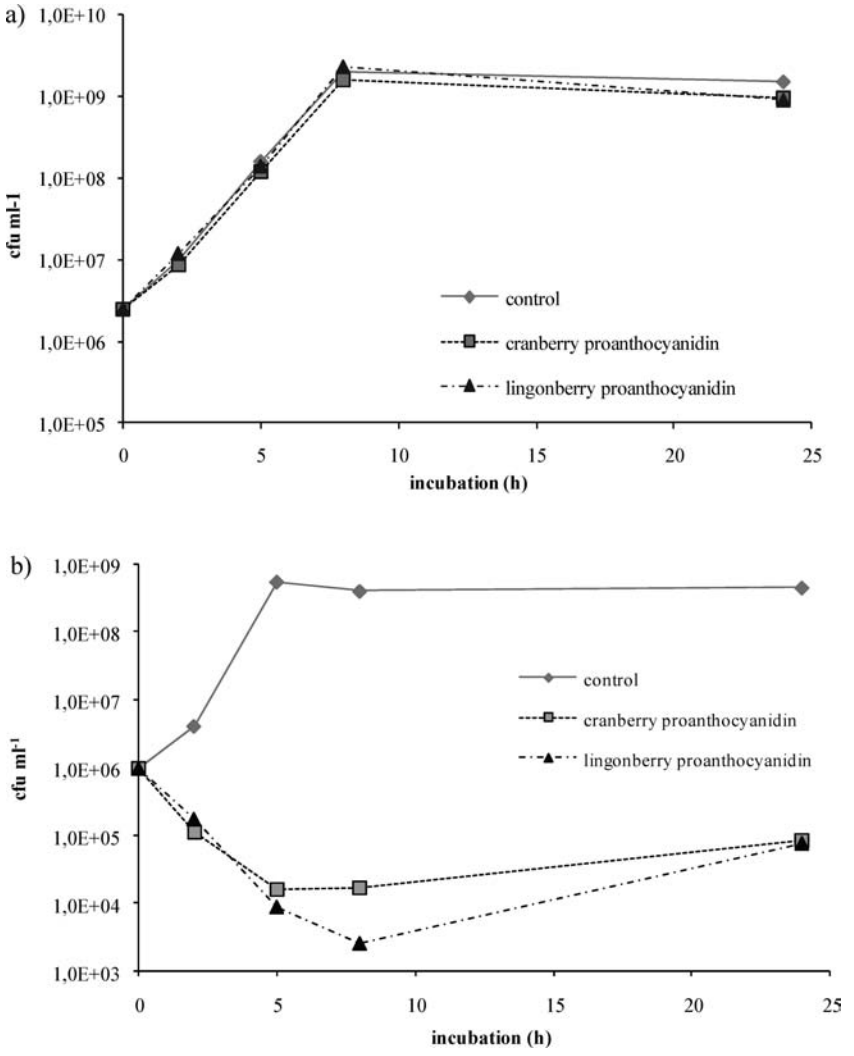

Figure 3. Antimicrobial effects of proanthocyanidin fractions of cranberry and lingonberry on *Salmonella enterica* sv. Typhimurium VTT E-981151 (a) and *Staphylococcus aureus* VTT E-70045 (b).

species, *Campylobacter jejuni, Escherichia coli* and *Helicobacter pyroli*.[26,42,43] Mayer et al.[44] observed similar behavior with the oligomeric procyanidin fraction of grape seeds. Sivakumaran et al.[45] studied the antimicrobial effects of different proanthocyanidin fractions from the leaves of forage legume *Dorycnium rectum* against rumen bacteria and reported general susceptibility of Gram-positive bacteria to proanthocyanidins. On the other hand, as we have shown in this study and also earlier, Gram-positive probiotic lactobacillus and bifidobacterial species as well as *Listeria monocytogenes* and *L. innocua* were not inhibited by lingonberry and cranberry extracts.[42,43] There are a few studies on the antimicrobial mechanism of action by proanthocyanidins (reviewed by Buzzini et al.[46]), and the hypothesis of mechanisms includes the effect of iron depletion, as well as the inhibition of cell-associated proteolysis and cell wall synthesis. However, further research on the mechanism of inhibition would be of great importance to unravel differences in susceptibility between especially Gram-positive genera. Sivakumaran et al.[45] also showed the significance of chemical structure of proanthocyanidin fraction on antimicrobial activity against selected rumen bacterial species, but for the other rumen species studied all the proanthocyanin

Table 9. Inhibition of Agglutination Tested at Equal Concentrations of Total Phenolics

	type of *E. coli* adhesion					
type of berry sample	P	type 1	S	Dr	M	autoagglutination of berry sample
			Cranberry			
phenolic extract	–[a]	–	–	–	–	no
polymeric proanthocyanidins	–	–	–	–	1–15[b]	30[c]
ditrimeric proanthocyanidins	–	–	–	–	–	no
oligomeric proanthocyanidins	–	–	–	–	7.5–30[b]	>30[c]
			Lingonberry			
phenolic extract	–	–	–	–	–	no
polymeric proanthocyanidins	–	–	–	–	–	no
ditrimeric proanthocyanidins	–	–	–	–	–	no
oligomeric proanthocyanidins	–	–	–	–	3.5–30[b]	>30[c]

[a] –: no inhibition of agglutination. [b] Inhibitory concentration (μg/mL). [c] Autoagglutinating concentration (μg/mL).

Table 10. Antiinflammatory Effects of Phenolic Extracts of Lingonberry and Cranberry[a]

	nitric oxide (μM)	iNOS (%)	COX-2 (%)	IL-6 (ng/mL)	IL-1β (ng/mL)	TNF-α (ng/mL)
			Lingonberry			
untreated	0.26 ± 0.01	6.82 ± 1.17	9.56 ± 3.16	0.01 ± 0.003	0.06 ± 0.01	0.18 ± 0.10
LPS 10 ng/mL	23.37 ± 1.29	100 ± 0	100 ± 0	3.27 ± 0.02	4.87 ± 0.06	61.51 ± 0.56
+ lingonberry 30 μg/mL	25.35 ± 1.27	104.66 ± 6.34	111.84 ± 12.19	2.83 ± 0.26	6.05 ± 0.77	65.11 ± 6.58
+ lingonberry 100 μg/mL	19.12 ± 1.61	114.68 ± 14.90	nd[b]	1.41 ± 0.31**[c]	5.41 ± 0.88	23.73 ± 3.10**
			Cranberry			
untreated	0.52 ± 0.12	16.54 ± 3.72	16.54 ± 3.72	0.02 ± 0.001	0.04 ± 0.001	0.13 ± 0.01
LPS 10 ng/mL	30.96 ± 1.13	100 ± 0	100 ± 0	3.20 ± 0.07	4.50 ± 0.05	53.84 ± 2.12
+ cranberry 30 μg/mL	23.52 ± 0.81**	92.16 ± 17.03	94.66 ± 4.66	3.35 ± 0.33	5.06 ± 0.41	52.05 ± 5.99
+ cranberry 100 μg/mL	12.11 ± 1.30**	125.74 ± 14.29	nd	1.43 ± 0.42**	2.01 ± 0.49**	9.56 ± 2.09**

[a] Results are expressed as mean ± SEM, $n = 3–6$. [b] Not determined. [c] ** indicates $p < 0.01$ as compared to cells treated with LPS (10 ng/mL) alone.

fractions were equally active. Sivakumaran et al.[45] concluded that polymeric composition and the size of the polymer may have an effect on the antimicrobial activity. The proanthocyanidin fractions of the leaves of forage legume *Dorycnium rectum* were mainly composed of prodelphinidins, while our fractions were mainly formed of procyanidins. In the present study, the polymeric fractions of lingonberries and cranberries correspond to the medium molecular weight proanthocyanidin fraction of the leaves of forage legumes.

Effect of Phenolic Extracts on Bacterial Hemagglutination (HA) and Yeast Cell Agglutination (YA). The phenolic extracts of cranberry and lingonberry were analyzed for their inhibitory effect on bacterial HA and YA. The HA of *E. coli* HB101 (pRR7), which expresses the M hemagglutinin, was inhibited by cranberry polymeric and oligomeric proanthocyanidin fractions at concentrations of 1–15 μg and 7.5–30 μg total phenolics per mL, respectively. The lingonberry oligomeric proanthocyanidin fraction also inhibited the HA of *E. coli* HB101 (pRR7) at concentrations of 3.5–30 μg total phenolics per mL (Table 9). The cranberry and lingonberry phenolic extracts did not affect the HA of strains HB101 (pPIL110-75), HB101 (pPIL291-15), HB101 (pBJN406), and LE392 (pANN801-13) expressing P, Dr, and S fimbriae or the YA of the type 1 fimbriated strain LE392 (pPKL4). The control strains HB101 and LE392 (pBR322), which do not express fimbriae, were included in all the analyses. The phenolic extracts agglutinated erythrocytes alone as well as the nonfimbriated control strains when used at concentrations higher than 30 μg total phenolics per mL (for cranberry polymeric and oligomeric proanthocyanidins as well as the oligomeric fraction of lingonberry proanthocyanidins), which hampered the evaluation of the inhibitory activity of the extracts at high concentrations.

A number of reports have shown that the proanthocyanidins of American cranberry inhibit the attachment of uropathogenic *Escherichia coli* to uroepithelial and vaginal cells (reviewed by Howell et al.[12]), whereas lingonberry is much less studied in this aspect.[9] In comparison to the P fimbria, which is a potent virulence factor in urinary tract infections caused by *E. coli*, the M hemagglutinin is found very rarely on uropathogenic *E. coli* strains and a homologue of M hemagglutinin named Afa-8 fimbria is highly prevalent in bovine isolates.[47] Thus the biological relevance of the inhibitory effect of cranberry and lingonberry on the M hemagglutinin remains unclear and does not give rise to further speculations regarding a potential therapeutic application of these berries in inhibition of *E. coli* attachment to the urinary tract. The lack of an effect on P-fimbriae mediated HA seen in our analysis may be due to the use of the domestic European cranberry (*Vaccinium microcarpon*) instead of American cranberry (*Vaccinium macrocarpon*). American cranberries contain more A-type proanthocyanidins compared to European cranberries.[48] Thus, European cranberry products may not be effective at preventing bacterial adhesion.

Antiinflammatory Testing. *Effects on Nitric Oxide Production, and on iNOS and COX-2 Expression.* Bacterial endotoxin lipopolysaccharide induced iNOS and COX-2 expression as well as NO production in J774 macrophages. Cranberry phenolic extract inhibited LPS-induced NO production in a dose-dependent manner when used at concentrations of 30 μg/mL and 100 μg/mL, but it had no significant effect on iNOS of COX-2 expression (Table 10). Anthocyanin content in lingonberry phenolic extract was lower than in cranberry phenolic extract. Consistent with this difference in the anthocyanin content, lingonberry phenolic extract showed weaker effect on LPS-induced NO production and it had no significant effect on iNOS or COX-2 expression in J774 macrophages (Table 10).

Effects on Cytokine Production. To study the effects of cranberry and lingonberry phenolic extracts on cytokine production, we activated human THP-1 macrophages with LPS in the presence and in the absence of the tested extracts. At the very high concentration of 100 μg/mL cranberry phenolic extract inhibited LPS-induced IL-6, IL-1β and TNF-α production (Table 10). Lingonberry phenolic extract had no significant effect on IL-1β production, but it inhibited IL-6 and TNF-α production at concentration 100 μg/mL similarly as cranberry phenolic extract (Table 10).

The detected inhibitory effects of cranberry extracts on NO-production could well be explained by the earlier reported inhibitory effects of anthocyanins on NO-production.[49] According to Pergola et al.[50] anthocyanins inhibit NO production in murine macrophages by decreasing LPS-induced NF-κB activation through inhibition of IκBα degradation and reduced ERK1/2 phosphorylation. In addition, anthocyanins have been shown to inhibit the expression of proinflammatory cytokines and of COX-2 in a murine asthma model.[51] Interestingly, Terra et al.[52] reported that the degree of polymerization is important in determining proanthocyanidin effects. They showed that trimeric and longer oligomeric-rich proanthocyanidin fractions, but not the monomeric forms catechin and epicatechin, inhibited iNOS expression. Our results concerning the inhibition of IL-6 and TNF-α production by both extracts and the inhibition of the production of IL-1β by the cranberry extract are in agreement with Bodet et al.,[53] who have reported inhibitory effects of an American cranberry fraction (containing mostly anthocyanins and proanthocyanins but devoid of acids and sugars) on the production of cytokines IL-1β, IL-6 and TNF-α.

In summary, lingonberries and cranberries are rich in proanthocyanidins comprising 63−71% of the total phenolic content. These proanthocyanidins are effective toward the inhibition of lipid oxidation both in emulsions and in liposomes, exert antimicrobial properties against *Staphylococcus aureus*, show an inhibitory effect on hemagglutination of *E. coli*, which expresses the M hemagglutin, and inhibit LPS-induced IL-6 and TNF-α production. The strongest effects were attributed to the polymers and oligomers of proanthocyanidins. No significant differences were found between the effects of lingonberries and European cranberries.

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