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Food Grade Lingonberry Extract: Polyphenolic Composition and In Vivo Protective Effect against Oxidative Stress

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ABSTRACT: Fractionation of the polyphenols constituting a food grade lingonberry extract (*Vaccinium vitis-idaea*) highlighted a composition more complex than described until now in the berry. Procyanidins B1, B2, and A2 were identified by UPLC/ESI-MS^2 along with the presence of other flavanol oligomers. Processing induced the release of large amounts of aglycones for ferulic acid, *p*-coumaric acid, and quercetin. The described anthocyanic composition of lingonberry was completed with hexoside derivatives of peonidin, petunidin, malvidin, and delphinidin. Besides confirmation of in vitro antioxidant activity, in vivo study was performed on rats fed a diet inducing oxidative stress. Supplementation with lingonberry extract significantly decreased the total oxidant status and favorably affected antioxidant defense enzymes in red blood cells and liver. A drop in the serum reduced glutathione level was also prevented, and uric acid was maintained at low level, confirming the antioxidant activity of the extract (5% proanthocyanidins) from a dosage of 23 mg/kg of body weight.

KEYWORDS: lingonberry (*Vaccinium vitis-idaea*), polyphenols, anthocyanins, proanthocyanidins, UPLC/MS, oxidative stress

INTRODUCTION

Lingonberry (*Vaccinium vitis-idaea* L.) is a small red berry of the Ericaceae family originating from Nordic countries (Russia and Canada) as well as Scandinavia, where it is traditionally used in human diets. The double asset of lingonberry is to confer an appealing red-orange color to the food while being considered as a "good for health" berry. The fruit is actually not only rich in functional compounds such as fibers or minerals but also belongs to the family of these "superfruits" rich in antioxidants such as vitamins C, A, and E (tocopherol) and especially polyphenols. Major classes of polyphenols that contribute to the color and healthy effect are represented, reaching a total richness equivalent to that of the well-known cranberry (*Vaccinium macrocarpon*). Throughout the literature five anthocyanins have been characterized[1,2] as well as quercetin and kaempferol derivatives,[2] monomers, dimers, and trimers of flavanols,[3,4] phenolic acid derivatives,[4,5] and stilbenes.[5,6]

In the past decade, the antioxidant activity of lingonberry was highlighted through various chemical assays: 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) assay[5] (1.76 μM AEAC/100 g of fresh weight); 2,2-diphenyl-1-picrylhydrazyl assay, in particular for the evaluation of the antioxidant activity of monomeric flavanols and proanthocyanidic fractions of lingonberry;[3] oxygen radical absorbance capacity assay,[2,7] with results ranging from 38.1 to 320 μmol TE/g of fresh weight; oxidation of methyl linoleate assay;[8] and protection toward both protein and lipid oxidation.[9] Further in vitro assays on mouse epidermal cells confirmed the antioxidant activity of lingonberry.[10] Most of these evaluations were performed on organic or alcoholic extracts that are not allowed or rarely used in the food industry, and to our knowledge no study was performed on commercial products having been submitted to a complete process. The potential health benefits of lingonberry being brought to the consumers only through commercially available dietary supplements, it appears essential to control the antioxidant activity of the finished products rather than that of the originating berry. The type of extraction as well as the whole food process is thought to deeply affect the polyphenolic composition and thus the efficiency of the resulting lingonberry extract. For example, simple purification on a reverse-phase cartridge was shown to lower from 31.8 to 21.2 μmol of Trolox equiv/g of fresh weight the oxygen radical absorbance capacity value of a lingonberry extract.[2] Additionally, heat treatment of fruits largely affects their levels in flavonols and phenolic acids.[11,12] The antioxidant activity determined by chemical assays was shown to highly correlate with the total phenolic and anthocyanin contents,[2] whereas other health-promoting effects were also closely linked to a daily intake of proanthocyanidins.[13−16] This supposes a deep knowledge of the polyphenolic composition in lingonberry-based products and a robust dosage of active molecules and, in particular, of proanthocyanidins.

Recent studies with lingonberry (*V. vitis-idaea*) also associated health-promoting effects such as the decrease in proliferation of cancer cells by the fruit extract[17] or cancer cell death by an apoptotic mechanism in human leukemia HL-60 cells.[10] Even if it has not been demonstrated yet, lingonberry may also act as an inhibitor of bacteria adhesion in the urinary tract, like the American cranberry (*V. macrocarpon*).[18,19] The proanthocyanidin composition of lingonberry was actually demonstrated to be very close to that of cranberry, with a high content of A-type dimers,[3] which were described as key parameters in bacterial antiadhesion activity. An asset of lingonberry is its richness in

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proanthocyanidins, which may reach 4.5 times the concentration found in European cranberry (*Vaccinium oxycoccus*).[20]

The purpose of this study was to determine by UPLC/MS the polyphenolic composition of a food grade pure lingonberry extract titrated at 5% of proanthocyanidins and to evaluate the antioxidant activity of this lingonberry extract by the oxygen radical absorbance capacity assay and the ferric reducing ability of plasma assay as well as through in vivo tests on rats fed a diet inducing oxidative stress.

MATERIALS AND METHODS

Samples, Standards, and Chemicals. NutriPhy lingonberry and NutriPhy bilberry (*Vaccinium myrtillus*) extracts were obtained from Chr. Hansen SAS (Hoersholm, Danemark) as finished products derived from successive steps of extraction, purification, concentration, and spray-drying. Standard molecules of (+)-catechin and (−)-epicatechin as well as reagent were supplied by Sigma-Aldrich (St. Louis, MO); rutin and quercetin as well as acetic acid, hydrochloric acid, and anhydrous sodium carbonate were purchased from Merck (Darmstadt, Germany); cyanidin-3-*O*-galactoside (ideain chloride), cyanidin-3-*O*-glucoside (kuromanin), peonidin-3-*O*-glucoside, procyanidin B1, procyanidin B2, and procyanidin A2 were purchased from Extrasynthese (Genay, France); *p*-coumaric, gallic, chlorogenic, and vanillic acids were purchased from Fluka Chemika (Buchs, Switzerland). Peonidin-3-*O*-galactoside and cyanidin-3-*O*-arabinoside were purchased from Polyphenols AS (Sandnes, Norway). HPLC grade acetonitrile, as well as methanol, formic acid, and orthophosphoric acid, were purchased from Carlo Erba. The standard molecules of 4-glucosides of *p*-coumaric and caffeic acids were a kind gift from N. Mora (University of Avignon, France). Milli-Q water was obtained through MilliPore Milli-Q^{50} resins filtering at 0.22 μm.

Analysis of Polyphenolic Pool. *Quantification.* Flavanols, flavonols, phenolic acids, and anthocyanins were quantified by liquid chromatography as described earlier.[21]

Fractionation of Phenolic Compounds. To facilitate the analysis of the polyphenolic composition by UPLC/MS, preliminary fractionation was performed. A lingonberry extract (200 mg) was dissolved in 0.3% aqueous hydrochloric acid (25 mL) and purified using a C-18 Sep-Pack cartridge (Waters, Milford, MA). Anthocyanins and other phenolic compounds were adsorbed onto the minicolumn, whereas water-soluble compounds were removed with 2 volumes of 0.01% aqueous hydrochloric acid. The less polar polyphenols were then eluted with 2 volumes of ethyl acetate, and the anthocyanic pool was finally eluted with methanol acidified with 0.01% hydrochloric acid. The ethyl acetate fraction was filtered through a Whatman 1PS filter and concentrated under vacuum at 40 °C. The dry residue mainly containing colorless phenolics was dissolved in methanol (3 mL) and stored at −10 °C.

Characterization of Phenolic Compounds by UPLC/MS. Separation and characterization of phenolic compounds were carried out using a Waters UPLC Acquity apparatus equipped with a photodiode array detector (detection at 280, 330, and 520 nm) and a reverse-phase column (50 mm × 2.1 mm i.d., 1.7 μm, Acquity BEH C18 protected by a guard column of the same material (Waters), in a 30 °C heated oven.

(a) Anthocyanin Composition A binary solvent system was used at a 0.17 mL/min flow rate with solvent A being water/formic acid (99:1 v/v), and solvent B being acetonitrile/formic acid (99:1 v/v). The elution gradient was as follows: 0−15 min, linear 0−20% B; 15−20 min, linear 20−40% B; 20−20.5 min, linear 40−100% B; 20.5−20.6 min, linear 100−0% B; 20.6−23.6 min, isocratic 0% B. The volume of injection was 2 μL.

(b) Other Polyphenolic Compounds A binary solvent system was used at a 1 mL/min flow rate with solvent A being water/formic acid (99.95:0.05 v/v) and solvent B being acetonitrile. The elution gradient was as follows: 0−2 min, linear 0−3% B; 2−3 min, isocratic 3% B; 3−6 min, linear 3−5% B; 6−7 min, linear 5−6% B; 7−12.5 min, linear 7−10%, 12.5−19.5 min, linear 10−30% B; 19.5−20.5 min, linear 30−60% B; 20.5−21 min, linear 60−100% B; 21−22 min, linear 100−0% B; 22−24 min, isocratic 0% B. The volume of injection was 3 μL.

Further characterization of phenolic and anthocyanin composition was achieved through the coupling of UPLC with an ion trap mass spectrometer Bruker Daltonics HCT ultra equipped with an electrospray ionization source. For polyphenol characterization, a capillary voltage of 2 kV was used in the negative ion mode. Nitrogen was used as drying and nebulizing gas with a flow rate of 12 L/min. The desolvation temperature was set at 365 °C and the nebulization pressure at 60 psi. The ion trap was operated in the Ultrascan mode from m/z 100 to 1000.

For anthocyanin characterization, a capillary voltage of 1.8 kV was used in the positive ion mode. Nitrogen was used as drying and nebulizing gas with a flow rate of 9 L/min. The desolvation temperature was set at 350 °C and the nebulization pressure at 40 psi. The ion trap was operated in the Ultrascan mode from m/z 100 to 1000.

Antioxidant Assays. *Oxygen Radical Absorbance Capacity (ORAC) Assay.* Evaluation of the antioxidant activity was first carried out through an optimized oxygen radical absorbance capacity procedure.[22] Lingonberry extract was diluted in an adjusted volume of fluorescein solution so as to be in the linearity range of the apparatus and kept at 37 °C in a spectrophotometer cell. Decrease of fluorescence was automatically measured every 100 s (exc, 493 nm; emi, 515 nm). Quantification of the antioxidant activity was done by taking into account the inhibition percentage and time in comparison with the Trolox curves. Results are expressed as micromoles per kilogram in Trolox equivalent.

Ferric Reducing Agent Power (FRAP) Assay. Antioxidant activity was evaluated through ferric reducing ability of plasma assay following the usual procedure slightly modified.[23] Lingonberry extract was diluted at 1 mg/mL in a water/methanol solution (10:90, v/v). Measurements were performed on a spectrophotomer at 593 nm after 4 min of incubation. Dilutions were performed in duplicate and dosages in triplicates. A calibration curve was built from standard aqueous solutions of $FeSO_4 \cdot 7H_2O$ at Fe^{2+} concentrations ranging from 250 to 3500 nmol/mL submitted to the same procedure as lingonberry extract. Results were thus expressed as ferric reducing ability of plasma millimoles per gram of powder.

Animal Study. *Diet.* Young male Albino Wistar rats, 5−6 weeks old and weighing 153.5 ± 0.9 g, were purchased from Charles River breeding center (L'Arbresle, France). Rats were housed in colonies (3 rats/cage) in a controlled environment (22 °C, 14 h−10 h light−dark cycle, humidity = 70%). For the 10 day acclimatization period in animal facilities, rats were fed ad libitum a commercial chow (Usine d'Alimentation Rationnelle, Villemoisson-sur-Orge, France). After this acclimatization period, 45 rats were randomly assigned into 5 groups (n = 9) fed during 42 days with one of the five diets detailed in Table 1. Proteins consisted of milk casein, lipids in lard and corn oil, and carbohydrates in starch and sucrose; vitamins and minerals were complex mixtures. Weight and food intakes were routinely checked during the feeding with diets inducing oxidative stress. The experimental protocol followed the procedures set out by the French Regulations for Animal Experimentation (Art. 19 Oct 1987, Ministry of Agriculture) and was conducted after approval by the Committee of Animal care at Faculty of Medicine "Université de la Méditerranée-Marseille".

Treatments of Biological Samples. At the end of the 42 day experimental period defined from literature overview, rats were food-deprived for 18 h and then anesthetized by inhalation of isofluorane (Abbott, Wiesbaden, Germany).

(a) Blood. Blood was collected by cardiac puncture in ethylenediaminetetraacetic acid triphosphate and dry tubes. Red blood cells and plasma were isolated by centrifugation at 3000 rpm and 4 °C for 15 min. A total blood sample was immediately stored at −80 °C and kept aside

Table 1. Stress-Inducing Diets Fed to the Different Groups of Rats

diet/ group	comments	proteins (%)	total carbohydrates (%)	lipids (%)	vitamins and minerals (%)	cholesterol (%)	lingonberry extract (mg/100 g)
C	cholesterol-free low-fat diet	25	66.7	3.3	5	0	0
D0	stress-induced diet with cholesterol and fat	20	53.9	20	5	1.1	0
D1	stress-induced diet with cholesterol and fat + lingonberry extract (41.7 mg/100 g)	20	53.9	20	5	1.1	41.7
D2	stress-induced diet with cholesterol and fat + lingonberry extract (83.3 mg/100 g)	20	53.9	20	5	1.1	83.3
D3	stress-induced diet with cholesterol and fat + lingonberry extract (250 mg/100 g)	20	53.6	20	5	1.1	250

for red blood cell measurements of either reduced or oxidized glutathione after the addition of 1-methyl-2-vinylpyridium trifluoromethane sulfonate scavenger (10% v/v). White blood cells were removed, and the red ones were collected and lysed at 4 °C in bidistilled water. Red blood cells were then placed at −80 °C before determination of antioxidant defense enzyme activities (superoxide dismutase, glutathione reductase, catalase).

(b) Liver. Just after collection, livers were rinsed with phosphate-buffered saline (0.01 M, pH 7.4)/heparin (0.6 mg/mL) solution maintained at 4 °C, until complete removal of residual blood. For superoxide dismutase assay, the liver was blended at 4 °C in a buffer of 20 mM hepes, pH 7.2/1 mM ethyleneglycoltetraacetic acid/210 mM mannitol/70 mM sucrose. For glutathione reductase assay, the liver was blended at 4 °C in a buffer of 50 mM Tris-HCl, pH 7.5/5 mM ethylenediaminetetraacetic acid triphosphate/1 mM dithiothreitol For catalase, the liver was blended at 4 °C in a buffer of 50 mM K_2HPO_4, pH 7.0/1 mM ethylenediaminetetraacetic acid triphosphate.

Total Oxidant Status. Determination of total oxidant status is based on the ability of oxidants present in plasma to oxidize the ferrous ion *o*-dianisidine complex into ferric ion. The latter then forms a colored complex with xylenol orange in an acidic medium that can be evaluated by spectrophotometry through change of absorbance at 593 nm. The assay was calibrated with hydrogen peroxide, and the results were expressed in equivalents of micromoles of hydrogen peroxide per liter of plasma.

Antioxidant Enzyme Activities in Erythrocytes and Liver. Antioxidant enzymes in erythrocytes and liver were assessed using specific kits: superoxide dismutase assay kit, glutathione reductase assay kit, and catalase assay kit (Cayman, Ann Arbor, MI). Enzyme activities were expressed as International Units per milliliter in erythrocytes and International Units per milligram of proteins in the liver. Hepatic proteins were assessed using a colorimetric kit BCA (Pierce, Rockford, IL). For superoxide dismutase evaluation, erythrocyte lysate and liver samples were diluted up to 1/1000 before the addition of free radical detecting and xanthine oxidase. The reaction was measured at 450 nm after a 20 min incubation at room temperature. In glutathione reductase measurement, erythrocyte lysate and liver samples were diluted at 1/50 and then incubated in reactional buffer and cumene hydroperoxide. Absorbance was measured after 5 min at 340 nm. Finally, for catalase determination, methanol was mixed with erythrocyte lysate and liver samples diluted at 1/250 and supplemented with hydrogen peroxide. After a 20 min incubation at room temperature, this mixture was incubated with chromogene product in potassic media. After a 10 min incubation with potassium periodate, absorbance was read at 540 nm.

Antioxidant Molecules. . (a) Glutathione Reduced glutathione and oxidized glutathione were quantified using a specific kit, the reduced/oxidized glutathione ratio assay kit (Oxford Biomedical Research, Rochester Hill, MI). Metaphosphoric acid (5% final concentration) was added to total blood sample before centrifugation. Chromogene product (dithio-5,5′-bis(nitro-2-benzoic) acid) and glutathione reductase were added to the supernatant and incubated for 5 min at room temperature prior to the addition of the coenzyme nicotinamide adenine dinucleotide phosphate oxidase. Absorbance was read for 3 min at 412 nm. Oxidized glutathione and reduced glutathione were expressed as micromoles per liter of plasma.

(b) Uric Acid. Plasma uric acid was measured using enzymatic and colorimetric kits (BioMerieux, Marcy-L'Etoile, France). Results were expressed as millimoles per liter.

Statistical Treatment. Values were expressed as the mean (M) ± standard error (SE). Each group was composed of nine rats receiving one of the five dietary treatments. In these conditions, results were analyzed using a nonparametric test for small samples ($n < 30$) and unbound samples, the Kruskal−Wallis test at the probability of 5%. For a given parameter, when a significant difference was observed, groups were compared two by two using another nonparametric test for unpaired samples, the Mann−Whitney test at the probability of 5%.

RESULTS AND DISCUSSION

Polyphenolic Composition of Food Grade Lingonberry Extract. On the basis of chromatographic analysis, flavanols are found to be the major class of polyphenols in lingonberry extract, representing around 5.8% (w/w), followed by flavonols (2.9%), phenolic acids (1.9%), and finally anthocyanins (1.5%).

Phenolic Acids, Flavonols, and Flavanols. UPLC/MS analysis of the ethyl acetate fraction gave access to the detailed composition in phenolic acid, flavanol, and flavonol derivatives characterizing the processed lingonberry extract. The chromatographic profile extracted at 280 nm (Figure 1A) reflects a complex mixture of 39 identified phenolics that include 7 major molecules belonging to phenolic acid and flavonol classes and corresponding to peaks **4**, **8**, **9**, **13**, **21**, **30**, and **31**.

Molecular ions, fragmentation patterns, and parallel chromatographic runs of the molecular standards (Table 2) lead to the identification of compound **4** as being caffeic acid (RT = 3.2 min, m/z 179), compound **8** as *p*-coumaric acid (RT = 3.6 min, m/z 163), and compound **13** as ferulic acid (RT = 7.2 min, m/z 193). *p*-Coumaric acid is the predominant hydroxycinnamic acid in this processed lingonberry extract, in agreement with a previous study on lingonberry polyphenolics analyzed by capillary electrophoresis,[5] but in contrast with other lingonberry extracts, where *p*-coumaric acid was present in amounts similar to those of caffeic acid.[2] Free ferulic acid is identified for the first time in a lingonberry extract. The presence of two ferulic hexosides (compounds **15** and **20**) in agreement with their detection in

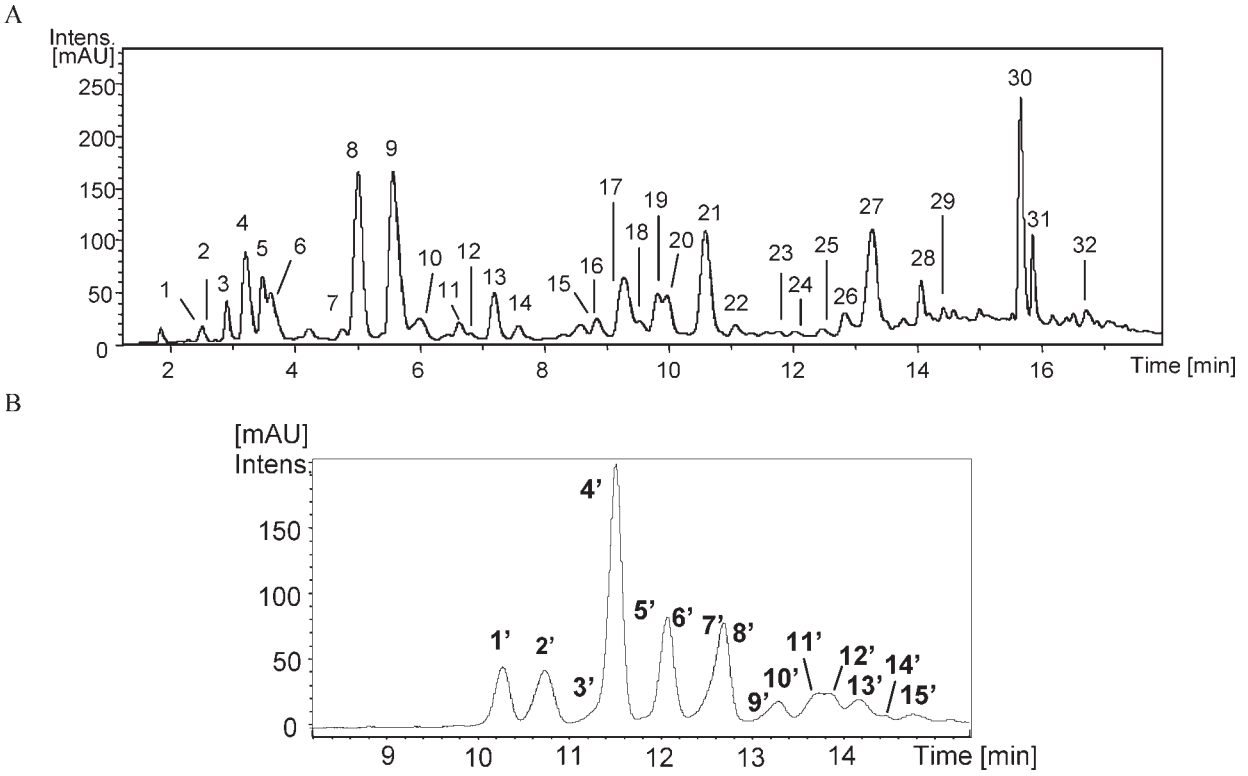

Figure 1. Chromatographic profiles extracted at 280 nm (A) and 520 nm (B) of lingonberry extract.

lingonberries in earlier literature[4] suggests that ferulic hexosides have been partially hydrolyzed during the industrial process, releasing this large quantity of ferulic acid. Two other derivatives of ferulic acid were characterized for the first time as major compounds. Compound **9** (m/z 195, RT = 5.7 min) is associated with a maximum absorption wavelength at 275 nm. MS^2 fragmentation of molecular ion yielding even fragments (m/z 136 ($[M - H]^-$ − 59 amu) and m/z 108 ($[M - H]^-$ − 87 amu)) is usually associated with the presence of methoxyl groups, and peak 9 was thus tentatively identified as dihydroferulic acid. Compound **21** (m/z 193, RT = 10.6 min) presents a similar UV−vis spectrum and molecular weight as ferulic acid, although there is a major difference in the fragmentation pattern (m/z 149 is replaced by ion m/z 161). In the literature, positional isomers exhibit this kind of distinction,[24] and compound **21** was thus tentatively identified as (*E*)-isoferulic acid. Among other phenolic acids represented in lower amount, we may mention esters of caffeic acid, that is, chlorogenic acid (**6**, m/z 353, RT = 3.6 min), already known in the berry,[5] and cryptochlorogenic acid (**7**, m/z 353, RT = 4.8 min), newly identified through parallel chromatographic run of molecular standard. The 4-glucosides of *p*-coumaric (**1**, m/z 325, RT = 2.5 min) and caffeic (**2**, m/z 341, RT = 2.6 min) acids were also detected for the first time in a lingonberry extract according to parallel analysis of the synthesized standard molecules. MS^2 fragmentation of the 4-glucoside of caffeic acid yields fragments m/z 179 ($[M - H]^-$ − 162 amu) typical for caffeic acid moiety associated with the loss of a hexose, and m/z 135, which is a fragment ion commonly encountered in caffeic acid fragmentation.[25] MS^2 fragmentation of the 4-glucoside of *p*-coumaric acid yields fragments m/z *187* and 163 ($[M - H]^-$ − 162 amu), typical for *p*-coumaric acid moiety associated with the loss of a hexose.

Flavonols are also present in large amounts in the processed extract. A major one (**30**, m/z 301, RT = 15.6 min) is characterized by maximum absorption wavelengths at 270 and 371 nm. Further MS^2 fragmentation leads to fragments m/z 179 and 151, typical for quercetin. Its presence in a quite high amount, although never seen in the raw material until now, might be related to linkage breakdown of quercetin derivatives during the process. Among these latter, quercetin-3-*O*-(3-hydroxy-3-methylglutaroyl)rhamnoside (**31**, m/z 591, RT = 15.8 min) was identified through mass fingerprint and comparison with the literature.[4] Other pentosides and hexosides of quercetin were also identified in lower amount in the processed extract and marked as **23**, **24**, **25**, **27**, **28** (Figure 1A; Table 2). The presence of quercetin-3-*O*-galactoside (**23**, m/z 463, RT = 11.7 min), 3-*O*-glucoside (**25**, m/z 463, RT = 12.5 min), and 3-*O*-rhamnoside (**28**, m/z 447, RT = 14.0 min) was assessed by the injection of standard molecules, confirming previous studies.[2] Tentative identification of quercetin-3-*O*-glucuronide (**24**, m/z 477, RT = 12.0 min) was then deduced from molecular weight, relative molecular polarity compared to other quercetin hexosides, and mass fingerprint showing the fragment m/z 301. Finally, all kaempferol derivatives found in low amount were already known in the raw material:[4] kaempferol-3-*O*-rhamnoside (**29**, m/z 431, RT = 15.0 min) and kaempferol-3-*O*-(3-hydroxy-3-methylglutaroyl)rhamnoside (**32**, m/z 575, RT = 15.7 min).

A broad diversity of flavanols is detected in the processed lingonberry extract, which could highly contribute to the antioxidant activity of the extract.[8] So far, the literature has described four dimers and two trimers of B-type and two dimers and four trimers of A-type [3,4] besides catechin and epicatechin monomers.[4] Our study goes a step further with identification of procyanidins B1 (**5**, m/z 577, RT = 3.5 min), B2 (**10**, m/z 577, RT = 6.1 min), and A2 (**26**, m/z 575, RT = 12.8 min) through separate

Table 2. Identification of Polyphenols in the Studied Lingonberry Extract

					method of identification	
peak	name	RT (min)	$[M - H]^- m/z$	$MS^2 m/z$	standard	literature
1	4-Glc-*p*-coumaric acid	2.5	325	187, 163	x[a]	
2	4-Glc-caffeic acid	2.6	341	179, 135	x	
3	B-type dimer	3.1	577	451, 425, 407, 289		
4	caffeic acid	3.2	179		x	Zheng et al.
5	(+)-catechin	3.5	289	245, 205	x	Ek et al.
	procyanidin B1	3.5	577	451, 425, 407, 289	x	
	caffeic acid hexoside	3.5	341	179, 135		
6	chlorogenic acid	3.6	353	191	x	Ehala et al.
7	cryptochlorogenic acid	4.8	353	191, 179, 173	x	
	B-type dimer	4.9	577	451, 425, 407, 289		
8	*p*-coumaric acid	5	163	119	x	Zheng et al.
	B-type trimer	5.4	865	739, 695, 577, 525, 287		
9	dihydroferulic acid	5.7	195	167, 136, 108		
10	procyanidin B2	6.1	577	451, 425, 407, 289	x	
11	(−)-epicatechin	6.7	289	245, 205	x	Ek et al.
12	caffeoylshikimic acid	6.8	335	179, 161, 135		
13	ferulic acid	7.2	193	178, 149, 134	x	
14	A-type trimer	7.6	863	711, 693, 559, 451, 411		
15	ferulic acid hexoside	8.9	355	193		
16	A-type trimer	8.9	863	711, 693, 559, 451, 411		
17	gentisic acid derivative	9.2	319	183, 165, 153, 109		
18	B-type dimer	9.5	577	451, 425, 407, 289		
19	A-type dimer	9.8	575	539, 449, 407, 289, 245		
20	caffeoylarbutin	10.0	433	323, 161		Ek et al.
	ferulic acid hexoside	10.0	355	193		
21	(*E*)-isoferulic acid	10.6	193	178, 161, 134		
22	A-type trimer	11.1	863	711, 693, 575, 449		
23	quercetin-3-*O*-galactoside	11.7	463	301	x	Zheng et al., Ek et al.
24	quercetin-3-*O*-glucuronide	12.0	477	301		
25	quercetin-3-*O*-glucoside	12.5	463	301	x	Ek et al.
26	procyanidin A2	12.8	575	539, 449, 423, 289	x	
27	quercetin pentoside	13.3	433	343, 271		
	A-type trimer	13.4	863	737, 711, 693, 575, 449		
	quercetin pentoside	13.7	433	397, 301, 271		
28	quercetin-3-*O*-rhamnoside	14.0	447	301	x	Zheng et al., Ek et al.
29	kaempferol-3-*O*-rhamnoside	15.0	431	285, 255	x	Ek et al.
30	quercetin	15.6	301	273, 179, 151		
31	quercetin-3-*O*-(HMG)-rhamnoside	15.8	591	529, 489, 447		Ek et al.
32	kaempferol-3-*O*-(HMG)-rhamnoside	16.7	575	513, 473, 431, 285		Ek et al.

[a] x, chromatographic run of standard.

chromatographic runs of molecular standards and comparison of fragmentation patterns. Three additional B-type dimers (**3**, RT = 3.1 min; **7**, RT = 4.9 min; **18**, RT = 9.5 min) were characterized through their molecular ion m/z 577 and fragmentation pattern yielding fragments m/z 451, 425, 407, 289 as well as a single B-type trimer (**8**, RT = 5.4 min) corresponding to molecular ion m/z 865 and fragmentation pattern yielding fragments m/z 739, 695, 577, 543, 525, and 287. Finally, one additional A-type dimer (**19**, RT = 9.8 min) was characterized through its molecular ion m/z 575 and fragmentation pattern yielding fragments m/z 539, 449, 407, and 289, and four A-type trimers (**14**, RT = 7.6 min; **16**, RT = 8.9 min; **22**, RT = 11.1 min; **27** RT = 13.4 min) were characterized through their molecular ion m/z 863 and fragmentation pattern yielding fragments m/z 711, 693, 559, 451, and 411. Lack of structural elucidations by nuclear magnetic resonance in earlier literature on lingonberry and in this study does not allow concluding if these oligomers are the ones already detected in previous studies or not.

Anthocyanins. The bright red-orange color of lingonberry extract is based on a complex pool of 15 anthocyanins as reflected by the profile at 520 nm (Figure 1B). The presence of cyanidin-3-*O*-galactoside (**4′**, m/z 449, RT = 11.6 min), cyanidin-3-*O*-glucoside (**5′**, m/z 449, RT = 12.2 min), cyanidin-3-*O*-arabinoside (**8′**, m/z 419, RT = 12.8 min), and peonidin-3-*O*-glucoside (**12′**, m/z

Table 3. Identification of Anthocyanins in the Lingonberry Extract

					method of identification	
peak	name	RT (min)	$[M]^+ m/z$	$MS^2 m/z$	standard	literature
1′	delphinidin-3-*O*-galactoside	10.4	465	303	xx[a]	
2′	delphinidin-3-*O*-glucoside	10.8	465	303	xx	Andersen
3′	delphinidin-3-*O*-arabinoside	11.6	435	303	xx	
4′	cyanidin-3-*O*-galactoside	11.6	449	287	x[b]	Andersen, Zheng et al., Ek et al.
5′	cyanidin-3-*O*-glucoside	12.2	449	287	x	Andersen, Zheng et al., Ek et al.
6′	petunidin-3-*O*-galactoside	12.2	479	317	xx	
7′	petunidin-3-*O*-glucoside	12.8	479	317	xx	
8′	cyanidin-3-*O*-arabinoside	12.8	419	287	x	Andersen, Zheng et al., Ek et al.
9′	petunidin-3-*O*-arabinoside	13.4	449	317	xx	
10′	peonidin-3-*O*-galactoside	13.4	463	301	x	
11′	malvidin-3-*O*-galactoside	13.8	493	331	x	
12′	peonidin-3-*O*-glucoside	14	463	301	x	Zheng et al.
13′	malvidin-3-*O*-glucoside	14.3	493	331	x	
14′	peonidin-3-*O*-arabinoside	14.6	433	301	xx	
15′	malvidin-3-*O*-arabinoside	14.9	463	331	xx	

[a] xx, chromatographic run of *Vaccinium myrtillus* extract. [b] x, chromatographic run of molecular standard.

463, RT = 14.0 min) in the processed extract of lingonberry was assessed by comparison with separate chromatographic runs of molecular standards (Table 3). This result supports previous studies describing the anthocyanic composition of lingonberry,[2,4] and cyanidin-3-*O*-galactoside was confirmed to be the major anthocyanin, as already highlighted.[2] Delphinidin-3-*O*-glucoside detected only once so far in the literature as a minor compound in lingonberry[1] was also identified in the processed extract as compound **2′** (RT = 10.8 min) through molecular ion m/z 465 and fragmentation pattern m/z 303 [M^+ − 162 amu] typical for delphinidin hexoside. Comparison with a parallel chromatographic run of *V. myrtillus* L. extract in which all peaks reflecting the anthocyanic pool have been assigned, and particularly delphinidin-3-*O*-glucoside,[26] completed the identification.

Ten anthocyanins detected in the processed extract were characterized for the first time in lingonberry. 3-*O*-Galactoside derivatives of peonidin and malvidin, respectively, compounds **10′** (RT = 13.4 min) and **11′** (RT = 13.8 min), as well as malvidin-3-*O*-glucoside (**13′**, RT = 14.3 min) were identified by comparison with separate chromatographic runs of molecular standards. In a first step the seven remaining anthocyanins were partially characterized through molecular ions and fragmentation patterns. MS^2 fragmentations giving rise to fragments m/z 301, 303, 317, and 331 were actually linked to anthocyanins containing peonidin, delphinidin, petunidin, and malvidin, respectively. The corresponding losses of 162 or 132 amu were associated with the presence of hexose or pentose in the molecule. Final comparison with retention times and UV−vis spectra of anthocyanins in *V. myrtillus* L. extract expected to be close to the one of lingonberry (*V. vitis-idaea*) resulting to the identification of the following anthocyanins: delphinidin-3-*O*-galactoside (**1′**, m/z 465, RT = 10.4 min), delphinidin-3-*O*-arabinoside (**3′**, m/z 435, RT = 11.6 min), petunidin-3-*O*-galactoside (**6′**, m/z 479, RT = 12.2 min), petunidin-3-*O*-glucoside (**7′**, m/z 479, RT = 12.8 min), petunidin-3-*O*-arabinoside (**9′**, m/z 449, RT = 13.4 min), peonidin-3-*O*-arabinoside (compound **14′**, m/z 433, RT = 14.6 min), and malvidin-3-*O*-arabinoside (**15′**, m/z 463, RT = 14.9 min).

Animal Study. Traditional in vitro evaluations highlighted the great antioxidant activity of this lingonberry extract titrated at 5% of proanthocyanidins (4610 ± 230 μmol TE/g for oxygen radical absorbance capacity value and 2.11 ± 0.09 mmol AC/g for ferric reducing ability of plasma value). As a comparison, juices made from different varieties of lingonberries were shown to exhibit lower ORAC values ranging from 54 to 136 μmol TE/g,[10] and ORAC value in fresh berries was evaluated at 38.1 μmol TE/g.[2] Further animal study was performed on rats to confirm the antioxidant potential of this pure dried extract having undergone an industrial processing. Besides a control low-fat diet, a high-fat/high-cholesterol diet was chosen as an inducer of oxidative stress, and processed lingonberry extract was supplemented at different dosages according to the groups (Table 1).

Influence of Lingonberry Extract on Energy Intake and Growth Parameters. Besides aiming at protecting against oxidative stress, diet supplementation with the lingonberry extract should not lead to specific metabolic disorders and particularly not affect growth and energy intake of the rats. Mean daily energy intake, lingonberry extract intake, and weight gain of each group of rats fed the various diets during 42 days are summarized in Table 4.

A first observation is that daily energy intakes in groups fed high-fat/high-cholesterol diets containing or not lingonberry extracts (D0, D1, D2, D3) are significantly higher than observed for the group fed the low-fat diet (C): high-fat diets actually bring 91.4−96.0 kcal/day, meaning 8−13% more than low-fat diet. Nevertheless, there is no influence of lingonberry extract dosage on this parameter. Significant discrepancies between daily energy intakes may be related to the amount of food intake ingested by the high-fat/high-cholesterol diet groups that does not counterbalance the supplementary energy brought by such diets: the C group actually ingested 23.8 ± 0.2 g/day, whereas the D1 or D3 group had slightly lower food intakes with, respectively, 21.4 ± 0.2 and 22.5 ± 0.2 g/day, but the latter diets provide 20% more energy. The fact that rats do not adjust their food intakes to the energy intake appeared unusual compared to previous studies[27−29] but remains unexplained. Daily lingonberry extract

Table 4. Daily Dose of Lingonberry Extract, Daily Energy Intake, and Body Weight Gain after 42 Days of Feeding with the Different Diets

diet/group	daily dose of lingonberry extract (mg/day)	daily energy intake[a] (kcal/day)	body weight gain in 42 days[a] (g)
C		84.9 ± 0.7	298.8 ± 9.7
D0		$93.9 \pm 0.9^{*\ D1,\ D3}$	$357.3 \pm 11.7^{*\ D1,\ D2}$
D1	8.02 ± 0.06	$91.4 \pm 0.7^{*\ D0,\ D2,\ D3}$	$321.6 \pm 8.1^{\ D0}$
D2	16.72 ± 0.34	$95.2 \pm 1.9^{*\ D1}$	$312.5 \pm 11.3^{\ D0}$
D3	50.57 ± 0.43	$96.0 \pm 0.8^{*\ D0,\ D1}$	336.6 ± 14.4

[a] *, Significant difference between control and either diet D0, D1, D2, or D3 ($p < 0.05$). Group names indicate a significant difference between D0, D1, D2, and D3 ($p < 0,05$).

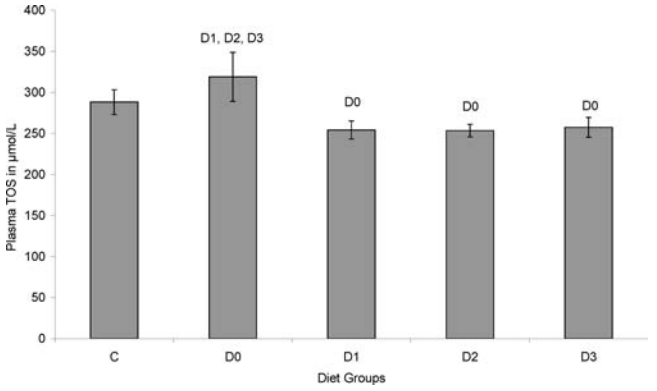

Figure 2. Total oxidant status in relation to the diet. Group names on histograms indicate a significant difference between groups ($P < 0.05$).

intakes of 8, 16, and 50 mg were targeted with diets D1, D2, and D3. The medium value corresponds to the daily consumption of proanthocyanidins of 36 mg/day and was shown to promote health effects.[19] As a beneficial consequence of the nonalteration of food intake by high-fat/high-cholesterol and lingonberry extract enrichment, the daily dose of lingonberry extract conformed with the nutritional doses expected in this experiment with intakes of 8.02 ± 0.06 mg in the D1 diet, 16.7 ± 0.3 mg in the D2 diet, and 50.6 ± 0.4 mg in the D3 diet.

Compared with low-fat diet C, enrichment of the diet with 20% fat (diet D0) led to a significant increase in body weight gain (1.2-fold). Additional lingonberry extract in high-fat/high-cholesterol diets lowered this effect whatever the dose as reflected by no significant difference between groups D1, D2, and D3, on the one hand, and group C, on the other hand. Decrease was evaluated at −11% for the lower dose of lingonberry extract (D1) compared to the high-fat diet group D0 and at −14% for the medium-dose D2 but was not significant with the higher dose D3. As a conclusion, no adverse effect on the growth was observed through supplementation with lingonberry extract.

Lingonberry Extract and Total Oxidant Status. Total oxidant status corresponding to the resulting effect of antioxidant defenses and oxidative stress related to the diets are presented in Figure 2. The total oxidant status in control fed rats (group C) displays higher values (288.1 ± 15.1 µmol/L) than those found in other studies using similar methods.[30] The explanation may lay in the methodology used. Moreover, chronic ingestion of high-fat/high-cholesterol diet D0 leads to an 11% increase of antioxidant status that remains nonsignificant compared to the low-fat diet C and was unexpected. A higher increase of total oxidant status was actually reported for a similar high-fat diet.[27]

Nevertheless, conclusions can be drawn on the efficacy of lingonberry extract. As compared to the D0 diet, diets D1, D2, and D3 enriched with lingonberry extract exhibit a significant antioxidant protective effect and total antioxidant status is lowered by 25% whatever the dosage. Although not statistically different, the total oxidant status is 13% lower in animals consuming diets D1, D2, and D3 compared to the low-fat control diet C.

Antioxidant Effect of the Lingonberry Extract on Antioxidant Enzymes. The antioxidant effect of lingonberry extract was first evaluated considering activities in erythrocytes and liver of three antioxidant defense enzymes (superoxide dismutase, glutathione reductase, and catalase) after the 42 day feeding with oxidative stress induced diets. Considering superoxide dismutase at first, activity in the liver is significantly different between the control diet, for which superoxide dismutase activity is 0.9 UI/mg protein, and the high-fat diet D0, resulting in an increase of activity until 3.2 UI/mg protein as already seen in a previous study[31] (Figure 3A). Additional intake of lingonberry in diets D1, D2, and D3 does not lead to a significant lowering of hepatic superoxide dismutase activity due to large individual variations within the groups (until 16% in D2 group). Nevertheless, the two highest dosages seem to have a beneficial effect on hepatic superoxide dismutase activity reflected by a decreasing trend from 3.2 to 2.2 and 2.5 UI/mg protein for diets D2 and D3, respectively. Similar observations can be made for erythrocyte superoxide dismutase with the high-fat diet, largely increasing the superoxide dismutase activity to 137 mUI/mg protein compared to 32 mUI/mg protein in the control diet. However, in this system, supplementation of the high-fat/high-cholesterol diet with lingonberry extract at 83.3 or 250 mg/100 g of diet (D2 and D3) results in a significant decrease of erythrocyte superoxide dismutase activity estimated at 32 and 35%, respectively. In both cases the activity remains at least 3 times higher than the one associated with the control diet.

Ingestion of a high-fat/high-cholesterol diet leads into a significant increase of hepatic glutathione reductase activity from 2.7 mUI/mg protein in the control diet to 7.3 mUI/mg protein in diet D0, respectively (Figure 3B). Addition of lingonberry extract at the highest dosage is shown to significantly stimulate glutathione reductase activity (+50% compared to D0 diet), whereas medium and low dosages have no significant impact. Such effects on hepatic glutathione reductase activity have already been described for another class of antioxidant molecules, that is, carotenoids, applied to rats fed with a high-fat/high-cholesterol diet.[31] In erythrocytes glutathione reductase activity is not significantly increased through high-fat/high-cholesterol diet (128 vs 110 mUI/mg protein in the control diet), but supplementation of diet with high doses of lingonberry extract

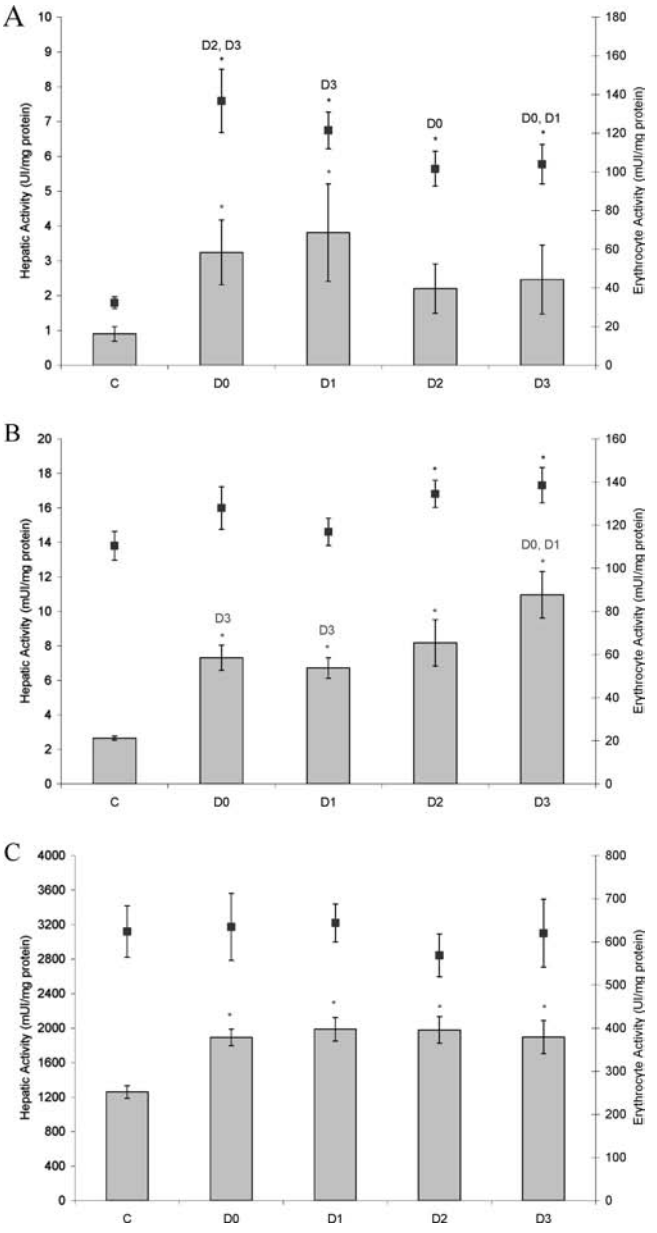

Figure 3. (A) Superoxide dismutase activity in liver (gray bar) and red blood cells (dark point). (B) Glutathione reductase activity in liver (gray bar) and red blood cells (dark point). (C) Catalase activity in liver (gray bar) and red blood cells (dark point). ∗ indicates a significant difference between either D0, D1, D2, or D3 and C group ($P < 0.05$). Group names on histograms indicate a significant difference between groups ($P < 0.05$).

leads to a slight but significant stimulation of glutathione reductase activity: the latter is evaluated at 135 and 139 mUI/mg protein through diets D2 and D3, respectively.

Finally, catalase activity in the liver is increased through a high-fat diet with 1900 mUI/mg protein versus 1260 mUI/mg protein associated with the control diet (Figure 3C). Nevertheless, supplementation of the diet with lingonberry extract does not affect hepatic catalase activity resulting from a high-fat/high-cholesterol diet whatever the dose. As to the catalase activity in red blood cells, it is neither modified by a high-fat/high-cholesterol diet nor influenced by lingonberry extract.

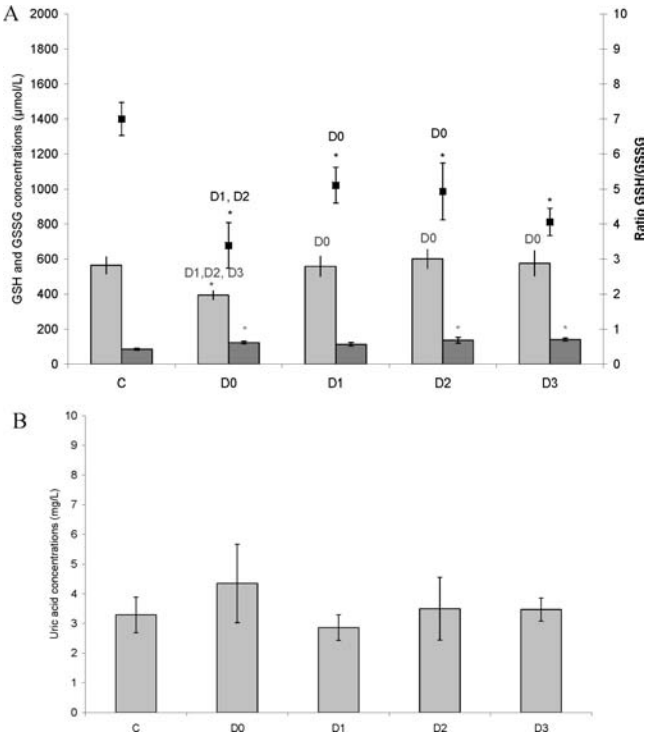

Figure 4. (A) Concentrations in reduced (light gray) and oxidized (dark gray) glutathione and corresponding ratio (black square). (B) Concentrations in uric acid after 42 days of feeding in relation with the diets. ∗ indicates a significant difference between either D0, D1, D2, or D3 and C group ($P < 0.05$). Group names on histograms indicate a significant difference between groups ($P < 0.05$).

Antioxidant Effect of the Lingonberry Extract on Antioxidant Molecules. Fasting plasma concentrations of reduced glutathione, oxidized glutathione, and the corresponding ratio reduced/oxidized glutathione as well as uric acid concentrations after 42 days of feeding with experimental diets are presented in Figure 4. Data show that high-fat/high-cholesterol diet D0 significantly lowers reduced glutathione concentration to 394 μmol/L compared to 564 μmol/L for the low-fat control diet C. In parallel, oxidized glutathione level increases from 86 to 123 μmol/L, leading to a significant decrease in the reduced/oxidized glutathione ratio from 7.0 to 3.4. These trends are congruent with a previous study where a 32% decrease in reduced glutathione was observed consecutively with oxidative stress induced by a 14% fat diet.[27] Compared to the rats in group D0, rats supplemented with every lingonberry dosage present significantly higher reduced glutathione amounts from 559 to 601 μmol/L, supporting the antioxidant effect of lingonberry extract. It is worth noting that reduced glutathione levels are maintained at the same level in these groups fed with supplementary lingonberry extract as the one associated with the control group C. Surprisingly, serum oxidized glutathione levels are also increased in rats fed every dose of lingonberry extract as compared to the control diet, and the difference is not significant with group D0. As a consequence, the two lowest doses of lingonberry extract are efficient to maintain a reduced/oxidized glutathione ratio not drastically reduced in comparison to diet C, but significantly increased compared to diet D0. Finally, the total glutathione level in control group C (650 μmol/L) is recovered by supplementing high-fat/high-cholesterol diets whatever the dose of lingonberry

extract, reflecting the capacity conferred to the organism to respond to oxidative stress. Despite no dose−effect relationship being clearly highlighted, our results suggest that lingonberry extract provides optimal antioxidant protection with the intermediate (0.83% of diet) or even the low (0.41% of diet) dose.

For the last antioxidant molecule studied, uric acid, the high-fat diet (D0) leads to a 32% increase in concentration compared to control diet C. By contrast, plasma uric acid concentrations are lower in groups fed the high-fat diet containing lingonberry extract, suggesting for this latter a beneficial effect on oxidative stress. Unsuccessfully large variation in responses observed in each group limits the statistical analysis.

Finally, lingonberry extract titrated at 5% proanthocyanidins promotes an in vivo antioxidant protective effect in rats even when used at the minimal dose of 23 mg/kg of body weight, whereas the intermediate dose (48 mg/kg) demonstrates the most interesting efficacy. Besides, the different metabolic disorders induced by high-fat/high-cholesterol diets do not occur in the animals fed this diet supplemented with lingonberry extract. From this study, the use of lingonberry extract as a dietary supplement may be considered in the future to improve the antioxidant activity in human health while minimizing the active volume to be ingested compared to berries.

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