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Regulation of the Phenolic Profile of Berries Can Increase Their Antioxidant Activity

JOZEF HUDEC,*,‡ RADKA KOCHANOVÁ,‡ MÁRIA BURDOVÁ,§ L'UBOMÍR KOBIDA,⊥ GRIGORIJ KOGAN,# IVAN TURIANICA,§ PETER CHLEBO,§ EVA HANÁČKOVÁ,‡ AND PAVOL SLAMKA‡

Department of Agrochemistry and Plant Nutrition, Department of Human Nutrition, and Department of Sustainable Agriculture and Herbology, Slovak Agricultural University, 949 76 Nitra, Slovakia, and Institute of Chemistry, Slovak Academy of Sciences, 845 38 Bratislava, Slovakia

The changes of the antioxidant activities (AOA), antiradical activities (ARA), low-density lipoprotein (LDL) oxidation, and total contents of phenolics, anthocyanins, flavonols, hydroxybenzoic acids, and hydroxycinnamic acids in black currant and black chokeberry, after treatment with ornithine decarboxylase inhibitor, a polyamine inhibitor (*O*-phosphoethanolamine, KF), and a phenol biosynthesis stimulator (carboxymethyl chitin glucan, CCHG), were analyzed spectrophotometrically. Gallic acid, hydroxycinnamic acids, and selected flavonol contents was analyzed by RP-HPLC. Both regulators increased the AOA measured as inhibition of peroxidation (IP) in black chokeberry, 1.71-fold after treatment with KF_1 and 1.74-fold after treatment with CCHG. In black currant IP was elevated after CCHG application only in lower dose ($CCHG_1$ 63.36% vs control 53.23%). In black chokeberry the total phenolics content was elevated 1.49-fold after KF_1 application and 1.31-fold after $CCHG_2$ application. The regulators had the lower effect on the phenolic accumulation in black currant. There was a strong relationship between the total phenolics in the both crops and anthocyanins, hydroxybenzoic acids, and hydroxycinnamic acids contents, respectively. Both regulators significantly changed the ratio of conjugated (rutin) to free (quercetin) flavonol mainly in black chokeberry. The antioxidant activities compared using LDL in vitro oxidation assay were increased more expressively after treatment with KF_2 in both crops.

KEYWORDS: Black chokeberry; black currant; antioxidant activity; polyphenols; hydroxybenzoic acids; hydroxycinnamic acids; polyamine inhibitor; carboxymethyl chitin glucan

INTRODUCTION

Antioxidants are protective agents that inactivate reactive oxygen species and thus significantly delay or prevent oxidative damage and can promote longevity by reducing oxidative stress symptoms. Small berries such as black currant (*Ribes nigrum* L.) and black chokeberry (*Aronia melanocarpa* (Michx) Elliot) represent one of the important sources of potential health-promoting phytochemicals because these fruits are rich in compounds with high antioxidant properties (*1*−*3*). As compared with fruits and vegetables, black berries possess a high antioxidant activity (*4*) owing to fact that the berries contain very high amounts of phenolic compounds.

Phenolics are an ecologically significant class of secondary metabolites expressed in all higher plants. The most common group of plant phenolics is the flavonoid family. Flavonoids with the high chemopreventive potential (*5*) are subdivided into several families according to molecular structure: flavonols, flavanols, flavones, isoflavones, and anthocyanidins. The other phenolic compounds occurring in plants are non-flavonoid compounds such as hydroxycinnamic acids, hydroxybenzoic acids, and stilbenes (*6*). Phenolic acids are present in plants mostly in bound form. The key enzyme for phenolic metabolism is phenylalanine ammonia-lyase (*7*). The antioxidant activities of plant phenolics have been studied using many different model system during the past few years. The different test systems used in determining the antioxidant activity of phenolics, including those involving human LDL or LDL plus VLDL as the oxidizing substrate, rather consistently show that many phenols and polyphenols are stronger antioxidants than the vitamins C, E, A, and F (*8*) or that the hydroxycinnamic acids have a higher antioxidant activity compared to the corresponding hydroxybenzoic acids (*9*).

* Author to whom correspodence should be addressed (telephone +421 37 6414386; fax +421 37 6414387; e-mail Jozef.Hudec.AF@uniag.sk).
‡ Department of Agrochemistry and Plant Nutrition, Slovak Agricultural University.
§ Department of Human Nutrition, Slovak Agricultural University.
⊥ Department of Sustainable Agriculture and Herbology, Slovak Agricultural University.
Institute of Chemistry, Slovak Academy of Sciences.

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Fresh black currant berries are particularly rich in anthocyanins. The two main anthocyanidins (aglycones) are delphinidin and cyanidin, and the major anthocyanins are the 3-rutinosides and 3-glucosides. According to Hollands et al. (*10*) fresh whole black currant contained 897 mg 100 g^{-1} of FW of total anthocyanins. The quercetin contents in black currant vary from 5.2 to 12.2 mg 100 g^{-1} of fresh weight (*12*). Other principal phenolics present in black currant but mainly in black chokeberry include flavonols, procyanidins, and various phenolic acids (*11*). Black chokeberry fruits have also a high content of vitamins P, PP, B_2, B_9, E, C, and provitamin A, mineral substances including microelements (boron, fluorine, iron, copper, zinc, manganese, molybdenum, and cobalt), and iodine (up to 400 mg kg^{-1}). A distinguishing feature is a high content of rutin. The total content of anthocyanins in the varieties Nero, Rubina, and Viking ranged from 6500 to 8500 mg kg^{-1} of dry weight (*13*). The total content of flavonols has been reported to be >71 mg 100 g^{-1} of fresh weight (*14*). Quercetin is the main component of flavonoids with an average content of 89 mg kg^{-1} of fresh weight (*15, 16*).

In the effort to improve dietary antioxidant content of crop plants, phenolics as antioxidant have also been the target for enhancement in crops (*17*). In recent years consumers have shown increasing interest in foodstuffs rich in natural ingredients, including natural pigments. Therefore, there is also interest in producing fruits and vegetables with enhanced levels of phenolics including anthocyanins, for example, grapes with enhanced levels of anthocyanins in the skin. Several strategies have proved to be successful in enhancing their level. One is the chemical treatment of plants with biologically active compounds acting on the molecular level (*18, 19*). Others use gene manipulations (*20*). Not only berries but also leaves and buds of some berry shrubs are already used in the preparation of some food supplements (*21*).

To potentially raise the levels of phenolics and antioxidant capacity of the studied small berries, we applied two regulators of the different biosynthesis pathways. The first compound was *O*-phosphoethanolamine (KF), which acts as the ornithine decarboxylase inhibitor, a polyamine biosynthesis inhibitor. KF indirectly influenced the phenolics content and significantly increased the total content of anthocyanins and total phenolics in black chokeberry (*19*). The second compound was water-soluble carboxymethyl chitin glucan (CCHG), because according to Vander et al. (*22*) and others (*23*−*25*) chitin and chitosan, like other elicitors, such as β-glucan and yeast extract, could induce secondary metabolites through the phenylpropanoid pathway in plants without any stresses and genetic modification. The major products of the phenylpropanoid pathway are the hydroxycinnamic acids, which can accumulate as esters or serve as precursors for other phenolic metabolites including flavonoids and lignin.

In the present study, we investigated the effect of KF and CCHG on the total content of phenolics, flavonols, anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, and certain individual phenolic compounds and antioxidant activities (linoleic acid/β-carotene system, DPPH, and inhibition of the copper-catalyzed oxidation of human LDL) of the leaves and berries of black currant and black chokeberry.

MATERIALS AND METHODS

Apparatus. The total phenolics, anthocyanins, flavonols, hydroxycinnamic acids, and hydroxybenzoic acids, and also antiradical activity and antioxidant activity, including in vitro LDL assay, were determined using a UV−vis spectrophotometer (model Mini 1240, Shimadzu Corp.). Some phenolics, for example, gallic acid, chlorogenic acid, caffeic acid, rutin, cinnamic acid, and quercetin, were determined using a Waters Breeze HPLC system.

Reagents and Standard Solutions. Linoleic acid (99%), Tween 20, β-carotene (95%), 2,2-diphenyl-1-picrylhydrazyl radicals (90%), quercetin dihydrate (98%), chlorogenic acid, gallic acid, caffeic acid (98%), cinnamic acid (99%), methanol (spectrophotometric grade), phosphate-buffered saline (PBS, pH 7.4), human LDL, and Folin−Ciocalteu's phenol reagent were obtained from Sigma-Aldrich. Kuromanin chloride (cyanidin-3-*O*-glucoside chloride, HPLC grade) was obtained from Extrasynthese. Rutin was obtained from Merck. Ethanol (96%) and natrium carbonate (p.a. grade) were obtained from Microchem Ltd. (Pezinok, Slovakia). *O*-Phosphoethanolamine (98%) was synthesized at the Petrochemistry Research Institute (Prievidza, Slovakia). Carboxymethyl chitin glucan was separated from *Penicillium chrizogenum* mycelia at the Institute of Chemistry, Slovak Academy of Sciences (Bratislava, Slovakia).

Plant Material. Eighteen shrubs of black chokeberry of the variety Nero were 14 years old and 18 shrubs of black currant of the variety Otelo were 5 years old at the beginning of the experiment. The plants were chosen at random, and the same plants were treated with the same compounds all two years (2006, 2007) and cultivated on the grounds of the Slovak Agricultural University in Nitra. Foliar treating was used for shrubs (3 shrubs/treatment/1.5 L of water or water solution of an active substance for black chokeberry or 0.75 L for black currant; 1 shrub equals one repetition of treatment) between July 10 and July 15 (green berries of black chokeberry) and between August 20 and August 25, 14 days before the harvest of chokeberries and leaves (the second spraying only in the sixth treatment). Black currants were treated between June 5 and June 10 in the first−sixth treatments and, again, the second spraying only in the sixth treatment between June 20 and June 25 (15 days before the harvest of black currant berries and leaves). The treatments were as follows: control treatment, water (500 L ha^{-1}); KF_1 treatment, O-phosphoethanolamine (5.6 g ha^{-1}, chokeberry; 7 g ha^{-1}, black currant); KF_2 treatment (56, 70 g ha^{-1}); $CCHG_1$ treatment, carboxymethyl chitin glucan (5, 6 g ha^{-1}); $CCHG_2$ treatment (350, 420 g ha^{-1}); KF_1-2× treatment (twice 5.6, 7 g ha^{-1}; berries and leaves were harvested 15 days after the second application). In all treatments the rate of active substance was dissolved in 500 L of water.

Samples. A 0.5 kg amount of black currant berries (CB) and 0.1 kg of leaves (CL) from each shrub were harvested on July 4−8 of each year (mature berries), and 1 kg of black chokeberry fruits (CHB) and 0.2 kg of leaves (CHL) were harvested on September 3−7 of each year (mature berries). Leaves and berries were cut off and rinsed with water. After homogenization in a blender, 10 g of sample was taken and extracted with 68.5 mL of 70% ethanol, by maceration at room temperature in a sealed flask. All extractions from each repetition of treatments were done in duplicate (total of six analyses/treatment). Flasks were placed in darkness and shaken. After 6 h, the contents were centrifuged at 3000*g* for 15 min. The supernatants were filtered and extracts stored at −20 °C before analysis.

Analytical Procedures. On the basis of the chemical reactions involved, major antioxidant capacity assays are roughly divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (2) single-electron transfer (ET) reaction based assays (*26*). The extracts were examined for their phenolic contents (ET) and phenolic profiles as well as antioxidant activity in a β-carotene−linoleate model system (HAT), scavenging of DPPH radicals (ET) and using the human LDL in vitro oxidation assay. All methods give a measure of antioxidant activity. The values of antioxidant activity are classified as high (>70% inhibition), intermediate (40−70% inhibition), and low (<40% inhibition), at 11.68 mg of dry matter of *Aronia melanocarpa* samples (the extract was not diluted in a β-carotene−-linoleate model system, inhibition of peroxidation, IP). Each CB and CL extract was diluted to 1:9 with ethanol (70%) (analyzed 1.168 mg of dry matter) for determination of IP. All extracts of both crops were diluted to 1:49 with 70% ethanol for determination of antiradical activity (ARA) (analyzed on 0.234 mg of dry matter).

Inhibition of Peroxidation. The antioxidant activity test model according to Pratt (*27*) was used. One milliliter of β-carotene (0.2 mg/mL) dissolved in chloroform was added to an Erlenmeyer flask

containing linoleic acid (0.02 mL) and Tween 20 (0.2 mL). The mixture was then dosed with 0.168−0.380 mL of extracts and 1.832−1.620 mL of 70% ethanol. Fifty milliliters of distilled water, saturated for 15 min with oxygen, was added to the flask. The resulting mixture was shaken and kept for 2 h at 50 °C. The absorbance of the samples was measured spectrophotometrically at 470 nm, immediately after their preparation ($t = 0$ min) and at the end of the experiment ($t = 120$ min). The inhibition of peroxidation (IP) was calculated as percent inhibition of oxidation versus control sample (2 mL of 70% ethanol), using the equation

$$\% \text{ IP} = 100 \times [1 - (A_s^0 - A_s^{120})/(A_c^0 - A_c^{120})]$$

where A_s^0 is the absorbance of sample at 0 min, A_s^{120} is the absorbance of sample at 120 min, A_c^0 is the absorbance of control sample at 0 min, and A_c^{120} is the absorbance of control sample at 120 min. Not only in this method, but also in the DPPH method and LDL assay, radical scavenging takes place; the differences are in individual species of radicals.

Antiradical Activity Determination. Free radical scavenging potentials were tested in a methanolic solution of DPPH (*28*). The degree of decoloration of the solution indicates the scavenging efficiency of the added extract. Amounts from 0.168 to 0.380 mL of extracts and from 1.832 to 1.620 mL of 70% ethanol were added to 4 mL of DPPH solution (10 mg/L). Thirty minutes later, the absorbance was measured at 517 nm. A reference sample was prepared with 2 mL of 70% ethanol. The ARA was calculated as a percentage of DPPH decoloration using the equation

$$\% \text{ ARA} = 100 \times (1 - A_s/A_r)$$

where A_s is the absorbance of the sample and A_r is the absorbance of reference sample. All values of IP and ARA were expressed as mean ± standard deviation (SD).

Antioxidant Activity upon LDL Oxidation. Lipid oxidation of human LDL was assessed by spectrophotometric monitoring of conjugated diene lipid hydroperoxide formation at 234 nm during copper-induced oxidation (10 μM copper, 37 °C, pH 7.4) according to a slightly modified method of Esterbauer et al. (*29*). The antioxidant activities of the samples were then measured as the inhibition of the formation of the conjugated dienes relative to a control calculated as explained below. Berry extracts were evaluated and compared at the equal amount of dry matter (11.68 mg) in samples. For the LDL oxidation assay human LDL was diluted to a standard protein concentration of 0.2 mg/mL with 0.01 M PBS (0.15 M NaCl) (pH 7.4). Protein was measured according to the method of Bradford (*30*), using bovine serum albumin as a standard. For the assays 700 μL of diluted human LDL solution was diluted in a quartz cuvette with $(2080 - x)$ μL of PBS buffer and x μL of sample; $x = 324-365$ μL for black currant berry extracts and 230−280 μL for black chokeberry extracts. For blind samples additions of x μL of 70% ethanol was used. The oxidant reaction was initiated by the addition of 40 μL of 0.7 mM cupric sulfate solution dissolved in PBS buffer (pH 7.4), resulting in a final concentration of 10 μM copper in the assay mixture. Calculations were made to determine the lag time and the 50-factor. The lag time was defined as the interval (in minutes) between the intercept of the tangent of the slope of the propagation phase and the initial absorbance axis. The 50-factor was calculated from the time it took to attain 50% of maximum absorbance ($t_{50\%}$) in samples versus blind controls: [$t_{50\%}$ of sample]/[$t_{50\%}$ of blind control]. If the 50-factor was >1, the tested sample had antioxidant activity to retard LDL oxidation.

Total Phenolics Determination. The Folin−Ciocalteu (FC) method (*31*) was used to determine phenolic compounds. Briefly, 5 mL of water, 0.02 mL (CHB and CHL), 0.1 mL (CB), 0.05 mL (CL) of extracts, and 1.0 mL of FC reagent were added to a 25 mL volumetric flask. The contents were mixed and allowed to stand for 5 min at room temperature. Next, 10 mL of a 7.5% of sodium carbonate solution and water filled to volume was added. After 2.5 h of standing at room temperature, the absorption at 765 nm was measured. The total phenolic content was expressed as gallic acid equivalents in miligrams per gram of dry matter.

Phenolic Profile Determination. A modified version of the Glories' method (*32*) was used to estimate the phenolic profiles of the same samples as were used for the FC method. The absorbance (A) at 280 nm was used to estimate hydroxybenzoic acids, A_{320nm} was used to estimate hydroxycinnamic acids, A_{360nm} was used to estimate flavonols, and A_{520nm} was used to estimate anthocyanins. Used standards were gallic acid in 10% ethanol for hydroxybenzoic acids, caffeic acid in 10% ethanol for hydroxycinnamic acids, quercetin in 95% ethanol for flavonols, and cyanidin-3-glucoside in 10% ethanol for anthocyanins. Phenolic subclasses were expressed as milligrams per gram of dry matter.

High-Performance Liquid Chromatography (HPLC). Analysis of several phenolic compounds in black chokeberry and black currant extracts was performed using a Waters Breeze HPLC system equipped with a Waters UV-2487 detector. Separation was achieved on a reverse phase Spherisorb ODS 2 column of 250 × 4.6 mm i.d. The column temperature was maintained at 35 °C, and a flow rate of 1.0 mL/min was used. Solvent A was 0.2% (v/v) phosphoric acid, and solvent B was methanol. The elution conditions were as follows: linear gradient from 90% A to 10% A/90% B, 0−25 min; to 90% A/10% B, 25−27 min; isocratic elution 90% A/10% B, 27−30 min; post time 15 min before next injection. Identification of individual compounds was based on retention times of original standards and their UV absorbance spectra. The retention times for gallic acid, chlorogenic acid, caffeic acid, rutin, cinnamic acid, and quercetin measured at 324 and 254 nm were 6.48, 13.08, 13.42, 17.68, 18.92, and 20.51 min, respectively. All quantifications were carried out with external standard.

Data Analysis. Three repetition of treatments, and two different samples from each repetition produced six values per year. All values of antioxidant and antiradical activity in tables are expressed as mean (from six analyses) ± SD. The LSD method was used to determine the significant differences of phenolic contents between the treatments at the $\alpha = 0.05$ level. Correlations between the total phenolic contents and IR or ARA were calculated using analysis of variance (ANOVA test).

RESULTS AND DISCUSSION

Several epidemiological studies have been published showing an inverse correlation between phenolic-rich diets and the incidence of several diseases. Therefore, phenolics as antioxidants have also been targets for enhancement in crops. Our two phenol biosynthesis regulators significantly influenced the various studied parameters.

Antioxidant Activity. Fruit extracts, including berries, have shown high antioxidant potential in several studies (*1, 33*). The antioxidant activity of extracts measured by the β-carotene bleaching method (IP) was observed to be highest in CB and CL (between them were insignificant differences) (**Tables 1** and **2**). In control plants IP then followed by CHB (**Table 3**) and CHL in decreasing order. An overall antioxidant activity of CB and CL measured as IP was high, because only these extracts were before analysis diluted to 1:9, intermediate in other extracts except for CHL, where IP was low (**Table 4**). The IP of black currant leaves at 1.168 mg of dry matter of samples was determined to be 53.51%, CB 53.23%, whereas for CHB and CHL at 10 times higher concentration of IP were 46.67 and 20.00%, respectively. The high antioxidant activity of black currant berries is in accordance with Heinonen et al. (*33*) but somewhat surprising was the only intermediate IP of black chokeberry, in contrast to our results in years 2004 and 2005 or those of Kähkönen et al. (*34*).

The regulators had an expressively different effect on IP of black chokeberry and black currant. Both regulators significantly increased IP of *A. melanocarpa* berries and leaves. They increased IP in berries by 1.71-fold (KF_1) and after treatment with $CCHG_1$ by 1.74-fold, in leaves after treatment with KF_2 by 2.93-fold, and by 4.13-fold after treatment with $CCHG_2$. The maximum elevation of IP in CHB (**Table 3**) was from 46.67 to 84.01% after treatment with KF_1-2×, where the second ap-

Table 1. Effect of Phenolic Metabolism Regulators on Antioxidant and Antiradical Activities and Phenolics Content[a] of Black Currant Berries Var. Otelo

treatment	hba[b]	hca[c]	flavonols[d]	anthocyanins[e]	total phenols[f]	inhibition of peroxidation[g]	antiradical activity[h]
control	122.0 a	403.5 a	260.6 a	2010.8 a	2368.0 a	53.23 ± 1.63	49.99 ± 1.82
KF_1	168.8 b	433.0 a	277.0 ab	2022.0 a	2341.0 a	53.19 ± 1.74	45.16 ± 1.70
KF_2	172.5 b	529.4 b	267.0 a	2203.9 b	2710.9 b	21.37 ± 1.51	46.64 ± 1.73
$CCHG_1$	134.4 a	517.3 b	259.6 a	2069.4 a	2461.0 a	61.36 ± 2.11	49.63 ± 1.91
$CCHG_2$	191.4 b	491.0 b	286.0 b	2157.0 b	2675.8 b	17.61 ± 1.49	50.75 ± 1.78
KF_1-2×	134.2 a	371.7 c	234.9 c	2048.9 a	2196.6 a	13.07 ± 1.73	42.54 ± 1.42

[a] Means followed by the same letter are not significantly different by LSD test at the 5% level. [b] Hydroxybenzoic acids (mg of gallic acid 100 g^{-1} of DW[l], 280 nm). [c] Hydroxycinnamic acids (mg of caffeic acid 100 g^{-1} of DW, 330 nm). [d] Milligrams of quercetin 100 g^{-1} of DW, 360 nm. [e] Milligrams of cyanidin-3-glucoside 100 g^{-1} of DW, 520 nm. [f] Spectrophotometrically (mg of gallic acid 100 g^{-1} of DW, 765 nm). [g] Percent (analyzed 1.168 mg of DW). [h] Percent (analyzed 0.2336 mg of DW). [l] Dry weight. KF_1, lower dose of *O*-phosphoethanolamine; KF_2, higher dose of KF; $CCHG_1$, lower dose of carboxymethyl chitin glucan; $CCHG_2$, higher dose of CCHG; KF_1-2×, KF_1 was twice applied. Details are given under Plant Material.

Table 2. Effect of Phenolic Metabolism Regulators on Antioxidant and Antiradical Activities and Phenolics Content[a] of Black Currant Leaves Var. Otelo

treatment	hba[b]	hca[c]	flavonols[d]	anthocyanins[e]	total phenols[f]	inhibition of peroxidation[g]	antiradical activity[h]
control	163.9 a	453.9 a	350.2 a	1182.8 a	1404.1 a	53.51 ± 1.77	92.54 ± 2.86
KF_1	159.0 a	611.9 b	377.2 ab	1207.3 a	1516.9 b	39.47 ± 1.98	85.07 ± 2.81
KF_2	151.2 a	581.7 b	391.6 b	1433.6 b	1801.4 d	57.02 ± 2.23	90.30 ± 3.27
$CCHG_1$	154.0 a	431.9 a	333.2 a	1263.0 a	1692.2 c	47.24 ± 2.04	89.55 ± 2.62
$CCHG_2$	150.0 a	300.5 c	197.8 d	635.2 d	805.0 f	57.48 ± 1.90	46.29 ± 1.87
KF_1-2×	161.3 a	451.7 a	285.5 c	896.1 c	1185.3 e	56.14 ± 1.80	68.66 ± 2.18

[a] See footnotes of **Table 1**.

Table 3. Effect of Phenolic Metabolism Regulators on Antioxidant and Antiradical Activities and Phenolics Content[a] of Black Chokeberry Fruits Var. Nero

treatment	hba[b]	hca[c]	flavonols[d]	anthocyanins[e]	total phenols[f]	inhibition of peroxidation[g]	antiradical activity[h]
control	348.1 a	828.9 a	312.3 a	1822.0 a	2780.7 a	46.67 ± 1.92	87.04 ± 3.02
KF_1	459.0 b	1127.5 b	302.8 a	3329.2 c	4139.5 c	80.01 ± 2.64	83.34 ± 2.77
KF_2	536.3 c	1166.7 b	308.1 a	2568.8 b	3310.0 b	76.67 ± 2.41	88.89 ± 2.81
$CCHG_1$	400.8 ab	968.2 a	342.7 a	3287.6 c	3438.6 b	81.34 ± 2.72	77.78 ± 2.51
$CCHG_2$	638.2 d	852.1 a	357.8 ab	2737.9 b	3641.8 b	71.34 ± 2.36	85.18 ± 2.60
KF_1-2×	559.4 c	1201.2 b	385.5 b	4648.5 d	4819.4 d	84.01 ± 2.90	87.04 ± 2.72

[a] See footnotes of **Table 1** with the exception of [g], percent (analyzed 11.68 mg of DW).

Table 4. Effect of Phenolic Metabolism Regulators on Antioxidant and Antiradical Activities and Phenolics Content[a] of Black Chokeberry Leaves Var. Nero

treatment	hba[b]	hca[c]	flavonols[d]	anthocyanins[e]	total phenols[f]	inhibition of peroxidation[g]	antiradical activity[h]
control	127.4 a	725.2 a	363.3 a	1082.3 a	1393.8 a	20.00 ± 1.02	74.07 ± 2.28
KF_1	133.2 a	758.4 ab	379.9 a	1452.6 c	1678.4 b	21.00 ± 0.80	78.52 ± 2.84
KF_2	130.4 a	742.5 a	743.8 c	982.0 a	1233.2 a	58.67 ± 2.07	70.37 ± 2.05
$CCHG_1$	195.3 b	741.3 a	412.8 b	1263.1 b	1357.4 a	50.67 ± 1.91	85.19 ± 2.89
$CCHG_2$	194.9 b	739.7 a	823.8 d	1322.8 b	1510.6 a	82.67 ± 2.49	88.89 ± 2.62
KF_1-2×	138.5 a	788.5 b	439.0 b	1009.5 a	1230.2 c	53.34 ± 1.97	88.89 ± 2.46

[a] See footnotes of **Table 1** with the exception of [g], percent (analyzed 11.68 mg of DW).

plication of KF_1 (low dose) was realized 15 days before the harvest. According to this quality parameter of berries the lower dose application of both regulators is suitable. Quoted is also valid for CB, where only CCHG in low dose had the more expressively positive effect on the elevation of IP ($CCHG_1$ 61.36% vs 53.23% of control berries). On the contrary, IP of both crop leaves after treatment with regulators was higher if their higher dose was applied. The fact that both regulators influenced positively the various sections of crops (leaves, fruits) is in accordance with our results (*35*), of course with herbs that do not have fruits. Therefore, the IP elevation of fruits after treatment with regulators is the minimum benefit contribution for pharmaceutical producers.

Antiradical Activity. The radical scavenging activity in water−ethanolic extracts was very high, because extracts were before analyses diluted to 1:49 (**Tables 1**−**4**). Extracts from control CHB possessed the higher ARA (87.04%) than CHL (74.07%) contrary to black currant control extracts (berries, 49.99; leaves, 92.54%, respectively.).

The regulators practically did not change ARA of berries unlike leaves. After treatment with the higher dose of regulators, IP decreased and slightly increased ARA of berries compared to the lower dose application of regulators. This inverse relationship between IP and ARA can be seen with the evaluation in both sorts of berries (**Tables 1** and **3**). Of course, many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH. According to Joubert et al. (*36*), this apparent discrepancy could be explained if pro-oxidation, due to the high concentration of flavonoids, dihydrochalcones, and/or total polyphenols, is considered. Frankel et al. (*37*) also showed that high concentration of green tea extracts resulted in pro-oxidation, but Burda and Oleszek (*38*) refer especially to the significant effect of the structure of phenolic compounds in the plant. Our target is not to compare the antioxidant activity measured as IP or ARA because both represent the results of the antioxidant activity assays achieved in two different systems.

Total Phenol and Phenolic Fractions Content. The average total phenol contents in *Aronia* and currant control berries were found to be 2780.7 and 2368.0 mg 100 g^{-1} of dry weight, respectively, consequently higher in CHB. The total phenolic contents in CHL and CL were lower (1393.8 and 1404.1 mg 100 g^{-1} of dry weight, respectively). Tabart et al. (*39*) determined the pretty higher phenolics content in CL (90 mg g^{-1} of DW). Of course, the content of phenolics not only in berries but also in all sections of plants is affected by the degree of maturity at harvest, preharvest environmental conditions, postharvest storage conditions, growing season, genetic differences (cultivar), and processing, including the used extraction method and solvent.

The major phenolic fraction in control berries and leaves was anthocyanins. The percentage of anthocyanins was expressively high in CB (84.9%) and CL (84.2%). The content of flavonols in the berries of both crops was more expressively lower (5.8-fold in CHB and 7.71-fold in CB) compared to the content of anthocyanins. This is an accordance with Slimestad et al. (*14*), Anttonen and Karjalainen (*40*), and Benvenuti et al. (*11*). The difference between the total hydroxyacids content was not so expressive. The content of hydroxycinnamic acids (828.9 mg 100 g^{-1} of dry weight) was 2.38-fold higher than the content of hydroxybenzoic (348.1 mg 100 g^{-1} of DW) in CHB and 3.31-fold in CB. The more detailed identification of hydroxyacids composition could play a part in the answer of which berries are more suitable for consumption not only for their potential antioxidant properties but also for their ability to increase or decrease the mutation frequency to antibiotic resistance (*41*), because not all phenolic compounds and not all of their actions are beneficial.

Both regulators, KF indirectly and CCHG directly, induced the accumulation of phenolic compounds. Both regulators changed the conditions of synthesis or translocation of phenolic compounds in both small-berry crops. They increased the total phenol content in *A. melanocarpa* by 1.49-fold after treatment with KF_1 and 1.31-fold after treatment with $CCHG_2$. The 2-fold application of KF (KF_1-2×) had the most expressive effect on the phenolic content in CHB (1.73-fold increase). The regulators had the lower effect on the elevation of the phenolic compounds accumulation in CB (maximally was increased 1.14-fold after treatment with KF_2) and both crop leaves. The high correlation between total phenolic content and antioxidant activity (DPPH) has already been observed in CL and bud extracts (*42*), as in different berries (*43*) and other common foods (*44*). Some authors (*45*) have shown a high correlation between antioxidant activity and phenolic content, whereas other authors (*46*) have found a low correlation or no correlation (*47*). Furthermore, berries and leaves contain a wide array of chemical compounds working together. Most often there is a rather incomplete knowledge of their common effects, and some of the active components may have remained undetected, but some of them may create an inhibiting or stimulating effect from the point of different analyzed parameters, which complicates the understanding of the mechanisms and relations. According to our results, the total phenolic contents were in a tighter relationship with ARA than IP in both crops, more in leaves than in berries, after treatment with both regulators (the correlations CL-IP, $r^2 = 0.22$; CL-ARA, $r^2 = 0.68$; CB-IP, $r^2 = 0.43$; CB-ARA, $r^2 = 0.76$; CHL-IP, $r^2 = 0.28$; CHL-ARA, $r^2 = 0.48$; CHB-IP, $r^2 = 0.75$, and CHB-ARA, $r^2 = 0.42$), which support the notion that phenolics act through an electron transfer mechanism and that their content reflects the reducing capacity of the antioxidatively affecting source (*26*, *48*). The total phenolic contents were in the simple relationship with an elevated contents of anthocyanins, hydroxybenzoic acids, and hydroxycinnamic acids only in berries after treatment with regulators.

After 1-fold treatment with regulators, the highest contents of hydroxybenzoic acids and flavonols were in both crop berries after treatment with $CCHG_2$; the highest content of hydroxycinnamic acids in berries was after treatment with KF_2. The content of anthocyanins was observed to be highest in *A. melanocarpa* after treatment with KF_1 (3329.2 mg 100 g^{-1} of DW) but in black currant after treatment with KF_2 (2203.9 mg 100 g^{-1} of DW). The 2-fold treatment with KF_1 (KF_1-2×) increased the content of an all analyzed phenolic fraction only in CHB compared to the 1-fold application of KF_1. Therefore, the KF_1-2× treatment increased the content of hydroxybenzoic acids by 1.22-fold, hydroxycinnamic acids by 1.07-fold, flavonols by 1.27-fold, and anthocyanins by 39.6% (but in comparison with control berries, too, by 2.55-fold). The findings presented in this study that KF more markedly prefers an anthocyanin synthesis and accumulation in black chokeberry are in accordance with Hudec et al. (*19*) and were confirmed in both crops, in *A. melanocarpa* expressively after the lower dose treatment and in CB expressively after the higher dose treatment. CCHG slightly prefers the flavonol formation. Because this text is the first fruit of study of the quoted regulators in berries, we can only hypothesize their possible mechanism. Both regulators are able to alter flavonoid metabolism. They increased the total phenols content. The key enzyme for the phenolic metabolism is phenylalanine ammonia-lyase (PAL) (*7*). Here it is possible that both regulators increase either PAL synthesis or activity in plants or further enzymes of the starting phase of the major phenylpropanoid pathway (cinnamate 4-hydroxylase, chalcone synthase, or chalcone isomerase). CCHG, similar to chitin, chitosan, β-glucan, or yeast extract (*22*, *23*), probably induced secondary metabolites through the phenylpropanoid pathway. The alternation of flavonoid composition after treatment with CCHG is proposed to be related to enhanced resistance of plants. Its use had an antimutagenic, anti-infective, and antiviral activity (*24*), so phenolic compounds can be involved in the resistance effect. Because the hydroxycinnamic acids serve as precursors for other phenolic compounds including flavonoids, their higher content after application of regulators is in accordance with that indicated. KF is an ornithine decarboxylase and glutamic acid decarboxylase activity inhibitor and, consequently, nonspecific inhibitor. According Atiénzar et al. (*50*) the enzyme polyphenol oxidase was shown to be activated by polyamines. Activation of the enzyme increased with increasing polyamine concentrations. KF decreases polyamine concentrations and therefore indirectly decreases the polyphenol oxidase activity. This can be one reason why the KF application increases the total phenolics content. Of course, KF increased also the ratio between anthocyanins and flavonols. Maybe KF stimulates in certain plants dihydroflavonol 4-reductase, because the content of flavonols was not statistically changed but the anthocyanins content was statistically increased. The definite answer can be found only through detailed research using more exact instrumental methods and the analysis of enzymes in the phenylpropanoid pathway. With the evaluation of the values in this paper there is a need to take into consideration that the contents of total phenols and phenolic fractions were obtained by different methods and using spectrophotometry analysis. According to Koponen et al. (*49*), black currant contained flavonols at the level of 156 mg kg^{-1} of FW (naturally the lower content than our results) after HPLC-DAD-ESI-MS detection. The results ascribed to the individual phenolic fractions are not the absolute

Table 5. Content (Milligrams per 100 g of DW) of Phenolic Compounds[a] in Black Chokeberry and Black Currant Berries after Treatment with Phenolic Metabolism Regulators

	black chokeberry						black currant					
compound	control	KF_1	KF_2	$CCHG_1$	$CCHG_2$	KF_1-2×	control	KF_1	KF_2	$CCHG_1$	$CCHG_2$	KF_1-2×
gallic acid	1.6 a	0.8 b	1.4 a	1.4 a	0.7 b	0.0 c	12.8 a	12.5 a	14.1 a	8.8 b	15.4 c	13.7 a
chlorogenic acid	54.2 a	56.4 a	60.1 ab	52.7 a	62.3 b	70.4 c	10.5 a	9.7 a	10.3 a	10.0 a	10.7 a	10.1 a
caffeic acid	48.1 a	56.4 b	54.6 ab	60.9 c	52.2 a	53.9 ab	5.8 a	9.2 b	9.7 b	11.0 c	9.9 b	8.8 b
rutin	220.1 a	229.2 a	309.6 b	290.9 b	281.3 b	220.7 a	121.5 a	127.5 a	112.2 a	111.0 a	115.9 a	112.5 a
cinnamic acid	33.5 a	28.2 b	40.4 c	38.3 c	46.6 d	31.6 ab	11.4 a	10.5 a	4.1 c	7.6 b	9.6 a	15.2 d
quercetin	10.8 a	6.6 b	8.1 b	19.5 c	22.5 c	10.0 a	10.0 a	7.2 b	10.9 a	4.3 c	4.4 c	11.3 a

[a] Determined by HPLC. Means followed by the same letter for compared treatments are not significantly different by LSD test at the 5% level.

Table 6. Indices of LDL Oxidation in Black Chokeberry and Black Currant after Treatment with Phenolic Metabolism Regulators[a]

	black chokeberry			black currant			
LDL oxidation index	control	KF_2	$CCHG_2$	control	KF_2	$CCHG_2$	blind sample
lag time[b]	18 ± 3	13 ± 3	13 ± 3	13 ± 3	19 ± 3	18 ± 3	7 ± 3
antioxidant activity[c]	1.38 ± 0.1	1.52 ± 0.1	1.23 ± 0.1	1.38 ± 0.1	1.62 ± 0.1	1.43 ± 0.1	1.00

[a] All values are mean ± SE. [b] Minutes. [c] 50-factor (see Analytical Procedures).

values, but the higher one, because it reflects contribution from a wider range of polyphenols (flavonols, hydroxycinnamates, etc.). The effect of both regulators on the analyzed parameters is unambiguous.

HPLC Analysis. Black chokeberry control fruits possessed with the exception of gallic acid the higher content of chlorogenic acid (5.16-fold; an absolute value in mg 100 g^{-1} of DW of 54.2), caffeic acid (8.29-fold; 48.1), cinnamic acid (2.94-fold; 33.5), rutin (1.81-fold; 220.1), and quercetin (1.08-fold; 10.8) than black currant control fruits (**Table 5**). According to Slimestad et al. (*14*) the amount of flavonols (four quercetin conjugates) in black chokeberry exceeds 71 mg g^{-1} of FW and was higher compared to the content of chlorogenic acid. The same result was obtained in our study. The content of rutin (quercetin-3-rutinoside) was 4.06-fold higher than the chlorogenic acid content. Quercetin was predominantly presented in conjugated form. Nikkonen et al. (*12*) detected that the flavonol myricetin was the most abundant flavonol in 10 black currant cultivar. The quercetin level in black currant was slightly lower and varied widely among cultivars, from 5.2 to 12.2 mg 100 g^{-1} of FW, in accordance with our results.

Both regulators expressively changed the content of individual phenolic compounds. KF in the higher dose increased most expressively the content of rutin by 40.7% compared to the control, free cinnamic acid by 20.6% in black chokeberry, and free caffeic acid by 67.2% in black currant berries, but decreased the content of free quercetin by 25%. The 2-fold KF treatment increased most expressively the content of chlorogenic acid by 29.9% in black chokeberry, free caffeic acid by 51.7%, and free cinnamic acid by 33.3% in black currant. After treatment with CCHG in the lower dose, the content of free caffeic acid increased by 26.9% in black chokeberry and that increased by 89.7% in black currant, the content of rutin and free quercetin by 32.2 and 80.6%, respectively, versus control in black chokeberry. After treatment with CCHG in higher dose the content of quercetin increased most expressively by 108.3% in black chokeberry. Both regulators significantly changed the ratio of conjugated (rutin) to free flavonol (quercetin) mainly in black chokeberry. The value of this ratio in the control was 20.37. These ratios were 34.7, 38.2, 14.9, and 12.5 in berries treated with KF_1, KF_2, $CCHG_1$, and $CCHG_2$, respectively. Carboxymethyl chitin glucan prefers the free flavonol (quercetin) formation, and *O*-phosphoethanolamine prefers flavonol conjugates (rutin). These results could be explained by differences in the mechanisms of the action of regulators. It should be noted that the kind of crop answers by the different positive measure on the treatment with regulators.

Antioxidant Activity of Berry Extracts on Copper-Induced LDL Oxidation. The in vitro peroxidation of human low-density lipoprotein (LDL) was used as a model to study the free radical-induced damage of biological membranes and the protective effect of black chokeberry and black currant extracts. The antioxidant activities were compared using the Esterbauer LDL in vitro oxidation assay with Cu^{2+}-catalyzed oxidation and spectroscopic monitoring of conjugated diene hydroperoxides development (*29*).

Although caffeic acid, chlorogenic acid, and rutin were analyzed relatively in great amount (our HPLC analysis), their concentration in LDL assay was considerably lower than 1 μM, and therefore their antioxidant effect was low. Mayer et al. (*51*), Cheng et al. (*52*), and Andreasen et al. (*53*) used the minimal concentration of individual compounds of 5 μM. If the antioxidant effect of extracts was calculated according to the 50-factor method, a low antioxidant activity was seen in all samples. The antioxidant effect was increased more expressively after treatment with KF_2 in both crops (**Table 6**). The extracts of berries after treatment with both regulators prolonged the induction time of LDL oxidation only in the case of black currant compared to the control berry extracts, but overall all extracts showed a weak inhibitory effect on the oxidation of LDL compared to the blind sample. Of course, a very low concentration of the active substances used in the LDL test can skew the real effect of berry extracts. The results of the LDL test are not in full accordance with that of IP or with ARA.

Differences in pigment content between specific years and specific treatments were not significant, and differences in antioxidant activities had the same tendency.

These results are important from the perspective of utilization in practice where the treated berries might be potential sources of higher antioxidant and phenolics content and owing to the higher amount of pigments in the food, pharmaceutical, cosmetics, etc., industries. Therefore, it is important that KF was already synthesized in semioperation conditions, in metric ton amounts for other uses. CCHG was microbiologically produced only in several hundred grams, but the preparation in large amounts is without problems.

ABBREVIATIONS USED

AOA, antioxidant activity; ARA, antiradical activity; LDL, low-density lipoprotein; IP, inhibition of peroxidation; CCHG, carboxymethyl chitin glucan; KF, *O*-phosphoethanolamine; HPLC, high-performance liquid chromatography; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; FC, Folin−Ciocalteu; PBS, phosphate-buffered saline; DW, dry weight; FW, fresh weight, CL, black currant leaves; CB, black currant berries; CHL, black chokeberry leaves; CHB, black chokeberry fruits.

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