

Antioxidant and radical oxygen species scavenging activities of 12 cultivars of blue honeysuckle fruit

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Abstract

ROP O., ŘEZNÍČEK V., MLČEK J., JURÍKOVÁ T., BALÍK J., SOCHOR J., KRAMÁŘOVÁ D., 2011. **Antioxidant and radical oxygen species scavenging activities of 12 cultivars of blue honeysuckle fruit.** Hort. Sci. (Prague), 38: 63–70.

The fruit of blue honeysuckle (*Lonicera caerulea* L. var. *kamtchatica* (Sevast.) Pojark.) belongs to the fruit of great value because of its unique chemical composition. The aim of our measurement of this fruit species was to select the cultivars which could be the most suitable food supplement in relation to strengthening human immunity system. The comparison of the fruit of 12 cultivars was made. The highest contents of polyphenolic substances were recorded in the cultivar Zolushka with the value of 9.03 g of gallic acid/kg fresh mass. In particular cultivars the DPPH (2,2-diphenyl-1-picrylhydrazyl) test was performed to determine antioxidant activity which ranged from 6.59–10.17 g of ascorbic acid equivalent/kg fresh mass. For comparison, scavenging activity of reactive oxygen species (superoxide anion, hydroxyl radical, and nitric oxide) was determined by using a 25% methanolic extract of fruit of particular cultivars. Antioxidant potential was also assessed using the rat liver slice model. The highest values of scavenging activity were found in the cultivars Zolushka, Goluboe vreteno, and Gerda. The cultivars Zolushka, Goluboe vreteno, and Gerda had also high values of flavonoids. The presented results may be used when popularizing this fruit species and also when studying the properties of new food and genetic resources.

Keywords: *Lonicera caerulea* L.; phenolics; antioxidant activity; lipid peroxidation; flavonoids

Free radicals cause the oxidation of biomolecules (e.g. protein, amino acids, lipid, and DNA), which can lead to cell injury and death (McCord 2000).

Their deteriorative effects can be diminished by natural antioxidants available in foods (ŠLOSÁR et al. 2009). Moreover, oxidative reactions limit the

Supported by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. MSM 7088352101 and the Ministry of Agriculture of the Czech Republic, Projects No. QH81142, QH82232.

shelf life of fresh and processed foodstuffs and are a serious concern in food industry. Antioxidants are substances that, when added to food products especially lipids and lipid-containing food, can increase shelf life of foods. Its mechanism is based on the retardation of lipid peroxidation, which is one of the major processes producing deterioration of food products during processing and storage (SINGH, MARIMUTHU 2006).

Various berries and fruit types of less common fruit species are known to contain antioxidants. Consumption of high amounts of antioxidant substances may have a positive impact on human health, particularly the prevention of cancer and inflammatory diseases (GAZDÍK et al. 2008). The fruit of blue honeysuckle (*Lonicera caerulea* L.) belong to fruit species with unique biological and chemical properties. Major positive features are extra early ripening, outstanding frost resistance of plants and flowers, a high content of flavonoids and other bioactive substances (PLEKHANOVA 2000). A high total polyphenolic content (TPC) and total antioxidant activity (TAA) are typical of blue honeysuckle berry (ZADERNOWSKI et al. 2005). For a long time berries have been harvested from wild plants in the regions of Russia, China, and Japan. During the past several decades, research in Russia and Japan has resulted in cultivars being selected for commercial production (THOMPSON, CHAOVANALIKIT 2003).

By morphological, anatomical and DNA analyses, as well as ploidy studies and geographical mapping of blue honeysuckle genetic resources, it was found that in Russia genetic diversity of the crop is represented by four species (PLEKHANOVA 2000). These include botanical varieties *edulis*, *kamtschatica*, *altaica*, and *boczkarovae* (THOMPSON, BARNEY 2007). The aim of our study was to investigate and compare 12 cultivars of *Lonicera caerulea* L. var. *kamtschatica* (Sevast.) Pojark. in relation to TPC, TAA, and flavonoid contents. Furthermore, scavenging activity of reactive oxygen species (superoxide anion, hydroxyl radical, and nitric oxide) and lipid peroxidation was determined. The presented results of the particular cultivars have not been introduced in literature, yet. Plants of *Lonicera caerulea* L. var. *kamtschatica* (Sevast.) Pojark. originate from the Kamchatka Peninsula and Eastern Siberia (PLEKHANOVA 1998). The berries could provide an additional opportunity for the cultivation of this species in a temperate zone and in high latitudes and colder climates (HUMMER 2006).

MATERIALS AND METHODS

Description of locality

Fruit were harvested in experimental germplasm orchard of the Mendel University in Brno, Czech Republic within the period of 2008–2010. This orchard is situated in the area of the Žabčice village, approximately 20 km southwards from Brno. The altitude is 184 m. The average annual temperature and a fifty-year average sum of precipitation are 9°C (during the growing season 15.6°C) and 553 mm (during the growing season 356 mm), respectively. Genetically, soils are classified as gleyed alluvial soils developed on the Holocene calciferous sediments with a marked accumulation of organic compounds. As far as the texture is concerned, the topsoil is loamy and the subsoil clayey-loamy (ANONYMOUS 2008).

Collection and processing of samples for chemical analyses

Fruit were harvested in full ripeness from three plants of each cultivar under study in the course of May. Forty randomly chosen fruit from each cultivar were mixed together and used for analyses (i.e. altogether 120 per each cultivar).

Fruit of individual cultivars were processed immediately after the harvest (not later than within two days). Harvested fruit were puréed in a mixer and the average sample was obtained by dividing into quarters. Each parameter was measured in five replications. The results were expressed as average of a three-year experiment.

The following cultivars of *Lonicera caerulea* L. var. *kamtschatica* (Sevast.) Pojark. were analyzed: Fialka, Gerda, Goluboe vreteno, Kamchadalka, Leningradskii velikan, Morena, Nimfa, Roksana, Vasilevskaya, Viola, Tomichka, and Zolushka. All cultivars are Russian in origin.

Sample preparation

Extraction and total phenolic content assay were performed according to the method described by KIM et al. (2003), using the following procedure: 10 g of fresh sample were homogenized for 10 s in 100 ml of methanol. The resulting paste was placed into Erlenmeyer flasks (120 ml) and let to stand in a

water bath with the temperature of 25°C for a period of 24 h. The residue was then extracted with two additional portions of methanol. The combined methanolic extracts were evaporated at 40°C to dryness and redissolved in methanol at a concentration of 100 mg/ml, and stored at 4°C for further use.

To measure total contents of phenolic substances, 0.5 ml of the sample was taken and diluted with water in a 50-ml volumetric flask. Thereafter, 2.5 ml of Folin-Ciocalteu reagent and 7.5 ml of a 20-percent solution of sodium carbonate were added. The resulting absorbance was measured in the spectrophotometer LIBRA S6 (Biochrom, Ltd., Cambridge, UK) at the wavelength of 765 nm against a blind sample, which was used as reference. The results were expressed as g of gallic acid/kg of fresh mass (FM).

Antioxidant activity by the DPPH test assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) was done according to the method of THAIPOONG et al. (2006). The stock solution was prepared by dissolving 24 mg of DPPH with 100 ml of methanol and then stored at -20°C until needed. The working solution was obtained by mixing 10 ml of stock solution with 45 ml of methanol to obtain the absorbance of 1.1 ± 0.02 units at 515 nm using the spectrophotometer LIBRA S6. Fruit extracts (150 µl) were allowed to react with 2,850 µl of the DPPH solution for 1 h in the dark. Then the absorbance was taken at 515 nm. The antioxidant activity was calculated as a decrease in absorbance value using the equation:

$$\text{Antioxidant activity (\%)} = (A_0 - A_1/A_0) \times 100$$

where:

A_0 – absorbance of the control (without the sample)

A_1 – absorbance of the mixture containing the sample

The results of absorbance were converted using a calibration curve of the standard and expressed in ascorbic acid equivalents (AAE) (RUPASINGHE et al. 2006). For comparison, the extract providing 50% of radical scavenging activity (IC_{50} , the concentration of the sample to scavenge 50% of the DPPH radicals) was calculated from the graph of radical scavenging activity percentage against extract concentration. For this purpose, dilution series (five different concentrations) were prepared for each cultivar extract. The results were calculated and expressed in µg/ml.

Total flavonoid content assay

The total flavonoid content was determined following SINGLETON et al. (1999). In a 10-ml Eppendorf tube, 0.3 ml of the fruit extract, 3.4 ml of 30% ethanol, 0.15 ml of $NaNO_2$ ($c = 0.5$ mol/l) and 0.15 ml of $AlCl_3 \cdot 6H_2O$ ($c = 0.3$ mol/l) were added and mixed. After 5 min, 1 ml of $NaOH$ ($c = 1$ mol/l) was added, and the mixture was measured at the wavelength of 506 nm. The total flavonoid concentration was calculated from a calibration curve using rutin as the standard. The results were expressed in g/kg FM.

Reactive oxygen species scavenging activity assay

For measurement of reactive oxygen species activity a 25% extract was prepared in phosphate buffer ($c = 50$ mmol/l, pH 7.0). The hydroxyl radical scavenging activity was assayed according to the method of GHISELLI et al. (1998). One ml of the extract was mixed with 0.8 ml of reaction buffer ($KH_2PO_4 \cdot KOH$, $c = 0.2$ mol/l, pH 7.4; deoxyribose, $c = 1.75$ µmol/l; iron ammonium sulphate, $c = 0.1$ µmol/l; and EDTA, $c = 0.1$ µmol/l). Then was added 0.1 ml of H_2O_2 ($c = 0.01$ mol/l) to the reaction solution. The solution was incubated for 10 min at 37°C prior to the addition of 0.5 ml of 1% thiobarbituric acid and 1 ml of 2.8% trichloroacetic acid. The mixture was boiled for 10 min and cooled rapidly. The absorbance of the mixture was measured at 532 nm (apparatus LIBRA S6).

The assay of nitric oxide scavenging activity was done according to the method described by GREEN et al. (1982). One ml of the extract was mixed with 1 ml of the reaction solution containing sodium nitroprusside ($c = 10$ mmol/l) in phosphate buffer ($c = 50$ mmol/l, pH 7.0). The incubation at 37°C for 1 h followed and 0.5 ml of aliquot was then mixed with 0.5 ml of Griess reagent. The absorbance at 540 nm was measured.

The superoxide anion scavenging activity was done by the method described by BEISSENHIRTZ et al. (2004) and it is based on the reduction of cytochrome *c*. One ml of the extract was mixed with 1 ml of the solution containing 0.07 units per ml of xanthine oxidase, xanthine ($c = 100$ µmol/l), and cytochrome *c* ($c = 50$ µmol/l). After incubation at 20°C for 3 min, the absorbance at 550 nm was determined.

All tests were performed in triplicate. The hydroxyl radical scavenging activity, nitric oxide scavenging activity, and superoxide anion scavenging activity were calculated as follows:

$$\text{Scavenging activity (\%)} = (A_0 - A_1/A_0) \times 100$$

where:

A_0 – absorbance of the control (without the sample)

A_1 – absorbance of the mixture containing the sample

The blank was performed by substituting Tris-HCl buffer ($c = 50$ mmol/l, pH 7.0) for the sample. The inhibition percentage of the formation of TBA-reactive substances was calculated as:

$$\text{Inhibition activity (\%)} = (A_0 - A_1/A_0) \times 100$$

where:

A_0 – absorbance of the control (without the sample)

A_1 – absorbance of the mixture containing the sample

Lipid peroxidation inhibition activity

The inhibition of lipid peroxidation was assayed by the method of SRIVASTAVA et al. (2006). Five μg of rat liver were homogenized in 20 ml of Tris-HCl buffer ($c = 40$ mmol/l, pH 7.0). The liver homogenate (0.1 ml) was incubated with the sample (0.2 ml of a 5% extract), 0.1 ml of KCl ($c = 30$ mmol/l), 0.1 ml of FeSO_4 ($c = 0.16$ mmol/l), and 0.1 ml of ascorbic acid ($c = 0.06$ mmol/l) at 37°C for 1 h. Thereafter, 1 ml of 1% thiobarbituric acid (TBA) and 1 ml of 15% trichloroacetic acid were added. The final solution was heated at 100°C in a boiling water bath for 15 min, cooled with ice for 10 min, and then centrifuged at 5,000 r.p.m. for 10 min. The absorbance of the supernatant was measured at 532 nm, using a LIBRA S6 spectrophotometer.

Statistical analysis

The data obtained were analyzed statistically by the analysis of variance (ANOVA) and Tukey's multiple range test for comparison of means (SNEDECOR, COCHRAN 1967). Correlation functions were calculated using the statistical package Unistat, v. 5.1 (Unistat, Ltd., London, UK) and Office Excel® Microsoft.

RESULTS AND DISCUSSION

The aim of our work was to compare the fruit of 12 cultivars of *Lonicera caerulea* L. var. *kamtschatica* (Sevast.) Pojark. in relation to antioxidant activity, total content of phenolic substances and

Table 1. Total phenolic content (g of gallic acid/kg of fresh mass), antioxidant activity (g of ascorbic acid/kg of fresh mass)

Cultivar	Total phenolic content	Antioxidant activity	IC ₅₀
Fialka	6.00 ± 0.15 ^a	7.41 ± 0.29 ^a	123.38 ± 4.20 ^a
Gerda	8.94 ± 0.24 ^b	9.92 ± 0.37 ^b	84.15 ± 5.17 ^b
Goluboe vreteno	8.90 ± 0.39 ^b	9.86 ± 0.42 ^b	83.24 ± 5.59 ^b
Kamchadalka	7.33 ± 0.48 ^c	8.78 ± 0.34 ^c	90.17 ± 4.60 ^b
Leningradskii velikan	5.85 ± 0.18 ^a	6.59 ± 0.36 ^d	134.92 ± 4.62 ^c
Morena	5.75 ± 0.20 ^a	6.96 ± 0.44 ^{ad}	132.31 ± 4.73 ^c
Nimfa	5.79 ± 0.19 ^a	6.75 ± 0.38 ^d	134.06 ± 5.92 ^c
Roksana	7.47 ± 0.27 ^c	8.75 ± 0.28 ^c	101.18 ± 4.31 ^d
Vasilevskaya	6.41 ± 0.19 ^d	7.70 ± 0.30 ^a	116.82 ± 6.71 ^a
Viola	6.75 ± 0.14 ^d	7.81 ± 0.37 ^a	115.94 ± 4.92 ^a
Tomichka	7.95 ± 0.36 ^c	8.77 ± 0.40 ^c	91.44 ± 3.80 ^b
Zolushka	9.03 ± 0.28 ^b	10.17 ± 0.38 ^b	76.14 ± 4.04 ^b

Different superscripts in each column indicate the significant differences in the mean at $P < 0.05$; IC₅₀ – 50% scavenging activity (μg of extract/ml) of free radicals of fruit of different cultivars of blue honeysuckle

Table 2. Flavonoid content (g/kg of fresh mass), of fruit of different cultivars of blue honeysuckle

Cultivar	Flavonoid content
Fialka	3.37 ± 0.10 ^a
Gerda	3.98 ± 0.12 ^b
Goluboe vreteno	3.79 ± 0.12 ^b
Kamchadalka	3.41 ± 0.11 ^a
Leningradskii velikan	3.27 ± 0.14 ^a
Morena	3.06 ± 0.15 ^a
Nimfa	3.11 ± 0.10 ^a
Roksana	3.42 ± 0.15 ^{ab}
Vasilevskaya	3.42 ± 0.14 ^{ab}
Viola	3.68 ± 0.12 ^b
Tomichka	3.78 ± 0.11 ^b
Zolushka	4.01 ± 0.12 ^c

Different superscripts in each column indicate the significant differences in the mean at $P < 0.05$

flavonoids. For completeness of the results, reactive oxygen species scavenging activity assay (hydroxyl radical, nitric oxide, and superoxide anion) and lipid peroxidation scavenging activity using the rat liver slice model were used.

The results of these chemical parameters are presented in Tables 1–3.

The fruit of blue honeysuckle are unique because of their composition and a high content of substances having antioxidant activity (POKORNÁ-JURÍKOVÁ, MATUŠKOVIČ 2007). In our measurement very high contents of polyphenolics were observed. The values of their contents ranged from 5.75–9.03 g of gallic acid/kg FM. These contents correspond to the values which were found out in blue honeysuckle by other authors (THOMPSON, CHAOVANALIKIT 2003; ZADERNOWSKI et al. 2005), and some cultivars can even show values higher than 11 g of gallic acid/kg FM. For most fruit species lower contents are typical (KOPÉC, BALÍK 2008). For example, in apples the common content is between 0.6–2.1 g of gallic acid/kg FM (VRHOVSEK et al. 2004) or in plums it is 2.2–5.0 g of gallic acid/kg FM (ROP et al. 2009). Phenolic compounds are the major contributors of antioxidant activity in fruit and vegetables. Phenolic compounds are effective hydrogen donors, which makes them good antioxidant sources (BANERJEE et al. 2005).

Regarding the fact that all cultivars were grown under identical conditions and in the same locality, it is possible to conclude that one can clearly see the cultivar variability, which is quite typical of fruit (SZAJDEK, BOROWSKA 2008). This variability became evident in case of total antioxidant activity (Table 1). Antioxidant activity was determined by the DPPH test. In the cultivar Gerda it was 9.92 g of ascorbic acid (AAE)/kg FM, in the cultivar Goluboe vreteno it was 9.86 g of AAE/kg FM. Antioxidant activity was

Table 3. Scavenging effect of blue honeysuckle fruit methanol extract (25%) on nitric oxide, superoxide anion, hydroxyl radical, and lipid peroxidation (% of inhibition)

Cultivar	Nitric oxide	Superoxide anion	Hydroxyl radical	Lipid peroxidation
Fialka	28.37 ± 0.93 ^a	31.53 ± 1.44 ^a	27.02 ± 0.84 ^a	17.31 ± 0.28 ^a
Gerda	38.94 ± 1.29 ^b	40.99 ± 0.48 ^b	36.85 ± 0.87 ^b	22.34 ± 0.36 ^b
Goluboe vreteno	38.85 ± 0.65 ^b	40.80 ± 1.03 ^b	36.90 ± 0.79 ^b	22.15 ± 0.37 ^b
Kamchadalka	35.12 ± 1.11 ^c	37.11 ± 1.26 ^c	33.25 ± 0.83 ^c	20.43 ± 0.41 ^c
Leningradskii velikan	26.58 ± 1.17 ^a	31.04 ± 0.85 ^a	25.94 ± 0.90 ^a	16.75 ± 0.29 ^d
Morena	27.53 ± 0.91 ^a	31.60 ± 1.19 ^a	26.92 ± 0.96 ^a	17.03 ± 0.25 ^{ad}
Nimfa	27.70 ± 0.88 ^a	31.27 ± 1.50 ^a	26.70 ± 1.13 ^a	17.29 ± 0.28 ^a
Roksana	35.18 ± 0.94 ^c	37.02 ± 0.67 ^c	33.06 ± 1.65 ^c	20.94 ± 0.30 ^c
Vasilevskaya	31.02 ± 0.93 ^d	34.17 ± 1.18 ^d	29.74 ± 0.64 ^d	20.50 ± 0.28 ^c
Viola	31.19 ± 0.86 ^d	34.84 ± 1.42 ^d	29.94 ± 0.59 ^d	20.68 ± 0.30 ^c
Tomichka	34.97 ± 0.86 ^c	36.45 ± 0.90 ^c	32.94 ± 1.41 ^c	21.01 ± 0.29 ^c
Zolushka	39.18 ± 1.57 ^b	41.15 ± 1.25 ^b	37.16 ± 0.95 ^b	22.95 ± 0.29 ^b

Different superscripts in each column indicate the significant differences in the mean at $P < 0.05$

the highest in the cultivar Zolushka with the value of 10.17 g of AAE/kg FM. In other fruit species, including apples (HUBER, RUPASINGHE 2009), small berries, and stone fruit (CHEN et al. 2006), the values of antioxidant activity are mostly much lower. For example, in cherries there are values of up to 0.9 g of AAE/kg FM (USENIK et al. 2008) and in plums the values can reach up to 6 g of AAE/kg FM (ROP et al. 2009). For confirmation of these results the plot of scavenging activity on the DPPH radical was done and IC_{50} was calculated (Table 1). The concentration of the extract needed for reaching the IC_{50} value was the lowest in the cultivars with the highest antioxidant activity value. Generally, high scavenging effectiveness of blue honeysuckle extract became evident in comparison with other species of berry plants (BAE, SUH 2007).

In general, in our measurement of chemical parameters the highest values were observed in the cultivars Gerda, Goluboe vreteno, and Zolushka. It is interesting that all three mentioned cultivars originate from the Tomsk region. On the contrary, the lowest values were measured in the cultivars which are from the Saint Petersburg region. As far as statistical evaluation of the results is concerned, the highest values of correlation coefficient between antioxidant activity and the total amount of phenolic substances were obtained (in case of the DPPH test $R^2 = 0.9689$, $y = 0.9946x - 1.0637$) (Fig. 1). Many authors notice a high correlation between TPC and antioxidant activity in fruit (THOMPSON, CHAOVANALIKIT 2003; POKORNÁ-JURÍKOVÁ, MATUŠKOVIČ 2007; ROP et al. 2009).

The content of flavonoids ranged from 3.06 to 4.01 g/kg FM (Table 2). Nevertheless, in fruit lower values are mostly presented (LUGASI, HOVARI 2002; WOLFE, LIU 2003). Flavonoids are known to

retain free radical scavenging activity by forming complexes with metal ions. Potent chelating activities with metal ions might come from abundant contents of kaempferol and quercetin (CHEN et al. 2006). Generally, flavonoids identify possessed antioxidant activity including scavenging free radicals (CROZIER et al. 2009).

High scavenging activity was observed in case of nitric oxide, superoxide anion and hydroxyl radical (Table 3). A 25% methanol extract of the cultivar Zolushka caused the percentage inhibition of nitric oxide by 39.18%, superoxide anion by 41.15%, and hydroxyl radical by 37.16%. Nitric oxide has many physiological functions including vasodilatation or synaptic plasticity in the central nervous system. On the other hand, the nitric oxide radical is implicated in pathogenesis of several diseases (SUMANONT et al. 2004). Nitric oxide belongs to reactive oxygen species (ROS) including free radicals such as superoxide anion (O_2^-) and hydroxyl radical species ($OH\bullet$) (WANG et al. 2009). These ROS are known to cause aging, cancer, and many other mal-effects on the human body (ARUOMA 1994). In the present paper, the blue honeysuckle fruit extract was evaluated for high ability to scavenge hydroxyl radical using the deoxyribose degradation assay. Scavenging activity of superoxide anion in the extracts of particular cultivars was also demonstrated in the xanthine/xanthine oxidase system. Using extracts of blue honeysuckle fruit was more effective than in other fruit species, e.g. mulberry (BAE, SUH 2007), apples (MAFFEI et al. 2007) or fruits of *Prunus* species (JUNG et al. 2002).

ROS attacks biomolecules such as lipids to initiate free radical chain reactions and cause lipid peroxidation (ČEKEY et al. 2009). Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids. In a biological system, lipid peroxidation

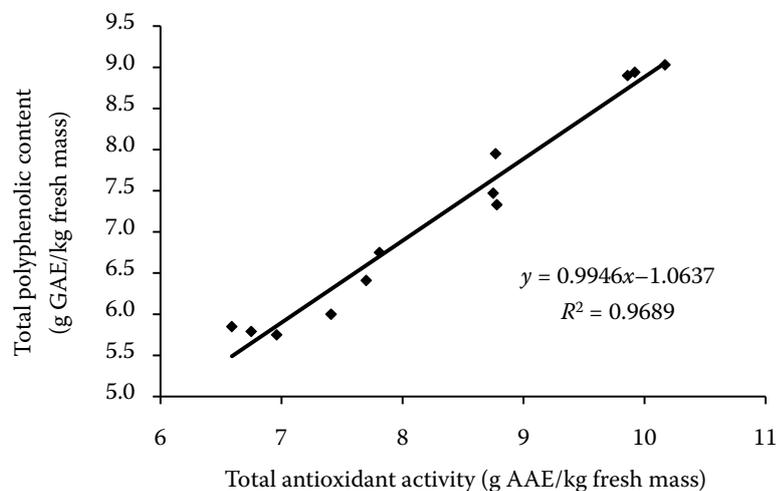


Fig. 1. Relationship between total phenolic content and total antioxidant activity in 12 cultivars of blue honeysuckle

generates a number of degradation products and it is found to be an important cause of cell membrane destruction and cell damage (WANG et al. 2009). The antioxidant activity of flavonoids against lipid peroxidation was described by many authors (e.g. TAKAO et al. 1994; GRACIA-ALONSO et al. 2004; BANERJEE et al. 2005). The highest values of lipid peroxidation activities were observed in the cultivars Gerda, Goluboe vreteno, and Zolushka, which confirms biological efficiency of these cultivars.

The results of the cultivars under investigation and their mutual comparison have not been published, yet, and in this way the work is a contribution to a further selection of the most suitable cultivars which could become a part of nutrition participating in strengthening immune system of human organism.

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Received for publication October 15, 2010

Accepted after corrections December 20, 2010

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