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Superoxide anion radical scavenging activity of bilberry (*Vaccinium myrtillus* L.)

Vesna Tumbas[a,*], Jasna Čanadanović-Brunet[a], Lars Gille[b], Sonja Đilas[a] and Gordana Ćetković[a]
[a]*Faculty of Technology, University of Novi Sad, Novi Sad, Serbia*
[b]*Molecular Pharmacology and Toxicology Unit, Department of Biomedical Sciences, University of Veterinary Medicine Vienna, Vienna, Austria*

Abstract. Naturally occurring antioxidants present in bilberry were separated into three groups: vitamin C, phenolic acids and flavonoids using solid phase extraction (SPE). Chemical composition of bilberry extract fractions was obtained by spectrophotometry and high performance liquid chromatography (HPLC). The results of HPLC analysis point out the high content of vitamin C (1529 μg/g) in fraction Fr1, flavonoids (1328.58 μg/g) in fraction Fr2 and phenolic acids (494.31 μg/g) in fraction Fr3. The free radical scavenging activities of these antioxidant fractions on superoxide anion radicals and detection of free radical intermediates was studied using electron spin resonance (ESR) spectroscopy. Superoxide anion free radical assay demonstrated very potent free radical scavenging activity of bilberry extract fractions. The results of correlation analysis showed that the separated classes of antioxidants from bilberry (vitamin C, flavonoids and phenolic acids) are responsible for its antioxidant activity.

Keywords: Bilberry, ESR spectroscopy, superoxide anion radicals, phenolic acids, flavonoids, vitamin C

1. Introduction

Many of the human age-related degenerative diseases are associated with oxidative processes. Many edible plants are capable of producing natural chemopreventive compounds which have no synthetic counterparts and play a protective role in human health maintenance. It has been well established that many of the phytochemicals present in plant derived foods have antioxidant capacity, i.e. are able to remove damaging radical species, as shown by a range of *in vitro* assays. Fruits, vegetables and teas contain a wide range of antioxidant compounds, including phenolic compounds and vitamins [41]. Phenolic secondary metabolites play an important role in plant-derived food quality, as they affect quality characteristics such as appearance flavour and health-promoting properties.

Edible berries have been a part of man's diet for centuries. Berries are one of the most important dietary sources of fibre and essential vitamins, minerals and vast number of phytochemicals such as phenolic compounds, including anthocyanins, phenolic acids, flavonol glycosides and flavan-3-ols [26, 33]. It has been reported that berry extracts have cardioprotective effects and beneficial effects on platelet aggregation, they are very effective inhibitors of low density lipoprotein oxidation and inhibitors of the growth of cultured cancer cells [3]. Research at the Scottish Crop Research Institute and the University of Ulster has shown that berry extracts can inhibit the initiation, progression, and invasiveness of colon cancer cells [40]. Different berry components are responsible for the inhibition of α-glucosidase and α-amylase, which suggests considerable potentiation of effects on blood glucose levels [34]. Similar effects on lipid digestion have been noted [35].

*Corresponding author: V. Tumbas, Faculty of Technology, University of Novi Sad, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia. Tel.: +381 21 485 3652; Fax: +381 21 450 413; E-mail: vesnat@uns.ac.rs.

Compounds present in the fruits of *Vaccinium* species are reported to have beneficial influence on human health [38, 44]. Bilberry (*Vaccinium myrtillus* L.) is a member of the *Eriacaceae* family and is also known as European blueberry, huckleberry, whortleberry or blueberry. Fruits of wild bilberry have a well-established role in pharmacognosy and it has been used as food for centuries, due to its high nutritive value [48]. Bilberry is a rich source of anthocyanins, and its extracts are extensively used in food/dietary supplements and pharmaceutical products.

Biological properties of the bilberry fruit extract include antioxidant capacity, astringent and antiseptic properties, ability to decrease the permeability and fragility of capillaries, inhibition of platelet aggregation, inhibition of urinary tract infection and strengthening of collagen matrices via cross linkages [15]. Bilberry crude extracts are marketed as pharmaceutical preparations for the treatment of both ophthalmological diseases and blood vessel disorders. More recently, they have been used for the treatment of diarrhea, dysentery, and mouth and throat inflammations [48].

Bilberries may be eaten fresh or dried. Bilberry tea may also be made from fresh or dried berries, or from the leaves. Dried bilberries are high in tannin and pectin, which have an astringent action that controls the inflammation that causes diarrhea. Dried billberies are traditionally used as herbal tea remedy. Distribution and contents of antioxidant compounds (anthocyanins, flavonoids, phenolic acids) as well as antioxidant activity of fresh bilberries has already been confirmed in the literature [12, 19, 22, 37]. Laplaud et al. [28] reported that bilberry extract, characterised by 74.2 ± 4.9 mg of polyphenols/g, inhibited copperinduced oxidative modifcation of human LDL. In another study, Viljanen et al. [49] found that raspberry, bilberry, lingonberry and black currant extracts inhibited copper-induced protein and lipid oxidation in a lactalbumin-liposome oxidation system, bilberry extract being one of the most effective.

However, contents of phenolic compounds and vitamin C, as well as antioxidant activity of dried brilberry fruits has not been evaluated. Yue and Hue [52] examined changes of anthocyanins, anthocyanidins, and antioxidant activity in fresh bilberry extract during dry heating at 80°C, 100°C and 125°C. Conjugated sugars of anthocyanins were cleaved from the anthocyanins to produce their corresponding anthocyanidins or aglycones during heating and the heated extracts had higher free radical scavenging capability than unheated extract.

In this study we used dried bilberries as a potential source of naturally occuring antioxidants. Extract from dried bilberries was fractionated into three groups of naturally occuring antioxidants: vitamin C, neutral and acidic phenolics using solid phase extraction (SPE). Present study describes behaviour of obtained bilberry extract fractions in superoxide anion generating system – their free radical scavenging activities and free radical intermediates formed during this reaction. In addition, correlation matrix was conducted in order to evaluate the contribution of separated phytochemicals from bilberry and relationship with free radical scavenging activity.

2. Materials and methods

2.1. Plant material

Dried bilberry fruits were obtained in a local drugstore. The air dried fruits were ground using a coffee mill and passed through a 0.36 mm sieve.

2.2. Preparation, purification and fractionation of bilberry extract

20 g of dried and ground bilberry fruits were macerated with 500 ml of 80% acetone during 24 hours. The macerat was filtered (Whatman No. 4) and the maceration was repeated once more. Two macerates were mixed and evaporeted to dryness under reduced pressure. In order to separate vitamin C from the phenolic antioxidants and to remove organic acids, residual sugars, amino acids, proteins and other hydrophilic compounds as well as to exchange solvents, a cleanup of the bilberry extract by solid phase extraction (SPE) was performed according to Rigo et al. [39], with Chromabond C-18 (1000 mg, J.T. Baker, Holland). The dry bilberry extract was redissolved in 5 ml of 0.5 M H_2SO_4, filtered through 0.45 μm (pore size) membrane filters (Millipore, Bedford, MA) and loaded on the Chromabond C-18 preconditioned with 2 ml of methanol followed by 5 ml of 5 mM H_2SO_4. The polar substances, including vitamin C, were removed with 2 ml of 5 mM H_2SO_4 and this fraction was marked as Fr1. The phenolic compounds were eluted with 2 ml of methanol followed by 5 ml of distilled water and this solution was considered as purified

bilberry extract. Spectrophotometrical determiantion of total phenols, flavonoids and anthocyanins was conducted with purified bilberry extract. Further, extraction and fractionation of neutral and acidic phenolics was conducted according to Chen et al. [10]. The purified bilberry extract was adjusted to pH 7.0 with 2.0 M NaOH solution and loaded onto the Chromabond C-18 preconditioned for neutral phenolics with 8 ml of methanol followed by 4 ml of distilled-deionized water adjusted to pH 7.0. The column was washed with 10 ml of distilled-deionized water and eluted with 12 ml of methanol. Fraction eluted in this step contains neutral phenols and is used further as Fr2. The effluent portion was adjusted to pH 2.0 with 2.0 M HCl, passed through the preconditioned acidic column. For isolation of acidic phenolics, cartridge was preconditioned by passing 8 ml of methanol and 4 ml of 0.01 M HCl. Then the column was washed with 5 ml of 0.01 M HCl and the adsorbed fraction was eluted with 12 ml of methanol. This fraction contains acidic phenols and is labeled as Fr3. The obtained fractions of bilberry extract, Fr1, Fr2 and Fr3, were evaporated using a rotary evaporator until dryness at 35°C under reduced pressure.

2.3. Spectrophotometrical determination

2.3.1. Total phenol concentration in purified bilberry extract

The amount of total soluble phenolics in extracts was determined spectrophotometrically according to the Folin-Ciocalteu method [45]. The reaction mixture was prepared by mixing 0.1 ml of water solution (concentration 1 mg/ml) of purified bilberry extract, 7.9 ml of distilled water, 0.5 ml of Folin-Ciocalteu's reagent and 1.5 ml of 20% sodium carbonate. After 2 h, the absorbance at 750 nm (UV-1800 spectrophotometer, Shimadzu, Kyoto, Japan) was obtained against control that had been prepared in a similar manner, by replacing the extract with distilled water. The total phenolic content, expressed as mg of chlorogenic acid equivalents per g dry weight of purified bilberry extract, was determined using calibration curve of chlorogenic acid standard.

2.3.2. Total flavonoids in purified bilberry extract

Total flavonoids (expressed as mg rutin per g dry weight) in purified extract were estimated spectrophotometrically according to Markham [30]. Flavonoids from purified bilberry extract (0.5 ml) were extracted with 1 ml of extraction medium (70% [v/v] methanol, 5% [v/v] acetic acid and 25% [v/v] distilled water) at room temperature for 60 min. The resulting solution was filtered trough Whatman paper No. 4 and filtrate volume was adjusted to 100 ml. The probes were prepared by mixing: 5 ml of dilluted extract, 1 ml of distilled water and 2.5 ml of $AlCl_3$ solution (26.6 mg $AlCl_3 \cdot 6H_2O$ and 80 mg CH_3COONa dissolved in 20 ml distilled water). A blank probe was prepared by replacing $AlCl_3$ solution with distilled water. The absorbance of probes and blank probe were measured immediately at 430 nm (UV-1800 spectrophotometer, Shimadzu, Kyoto, Japan). Total flavonoid content, expressed as mg rutin per g dry weight of purified bilberry extract, was calculated from a calibration curve using rutin as standard.

2.3.3. Total and monomeric anthocyanins in purified bilberry extract

Total anthocyanin content in purified extract was estimated spectrophotometrically using the pH single and differential method [9]. The spectrophotometric single pH method was used to determine total (monomeric plus polymerized) anthocyanins and differential pH method for determination of monomeric anthocyanins in the extract. The aqueous extract was diluted with two buffer solutions at pH 1 and 4.5. The absorbance of each dilution was measured at 510 and 700 nm against a distilled water control using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The total anthocyanin concentration was obtained from equation: $C_{tot}(mg/l) = (A_{tot} \times MW \times DF \times 1000)/\varepsilon \times L$ while monomeric anthocyanin concentration was calculated from the equation: $C_{mon}(mg/l) = (A_{mon} \times MW \times DF \times 1000)/\varepsilon \times l$, where A_{tot} is calculated as $A_{tot} = A_{510} - A_{700}$, A_{mon} is calculated as $A_{mon} = (A_{515} - A_{700})_{pH\ 1} - (A_{515} - A_{700})_{pH\ 4,5}$, ε is cyanidin-3-glucoside molar absorbance coefficient (26900 l/(mol × cm)), MW is cyanidin-3-O-glucoside molecular weight (449.2 g/mol), DF is dilution factor and L is cell path length (1 cm). Total anthocyanin and monomeric anthocyanin content was expressed as mg cyanidin-3-O-glucoside equivalents per g of purified bilberry extract [1].

2.4. HPLC analysis of bilberry extract fractions

All analyte solutions and solvents were filtered prior to analysis through 0.45 μm (pore size) membrane filters (Millipore, Bedford, MA). Quantification of phenolics in Fr2 and Fr3 was done by HPLC analysis by a Waters Breeze chromatographic system (Waters, Milford, MA), which consisted of 1525 binary pumps, thermostat and 717+ autosampler connected to the Waters 2996 diode array detector (Waters, Milford, USA). Chromatograms were recorded in 3D mode. Separation was performed on a Symmetry C-18 RP column 125 × 4 mm with 5 μm particle size (Waters, Milford, USA) with an appropriate guard column. Two mobile phases, A (0.1% phosphoric acid) and B (acetonitrile), were used at flow rates of 1 ml/min with the following gradient profile: the first 20 m from 10 to 22% B; next 20 m of linear rise up to 40% B, and for last 10 m 55% of B, followed by 10 m reverse to initial 10% B with additional 5 m of equilibration time. The data acquisition and spectral evaluation for peak confirmation were carried out by the Waters Empower 2 Software (Waters, Milford, USA). Quantification of total phenolic compounds in bilberry fractions Fr2 and Fr3 were performed using quercetin and *p*-coumaric acid as secondary standards for flavonoids and phenolic acids, respectively.

HPLC analysis of vitamin C content in Fr1 was performed on a liquid chromatograph "Agilent 1100", USA, equiped with a ultraviolet diode array detector (UV-DAD). For separation a C-18 column with a 5 μm particle size was used at a flow rate of 0.4 ml/min and temperature 37°C. Reference substance (vitamin C) and samples were dissolved/extracted in solution of meta-phosphoric acid (3% w/w) in 8% acetic acid. Ammoniumacetate (0.1 mol/l, pH 5.1) was used as a mobile phase. The injected volume was 20 μl and the total running time was 6 min.

2.5. ESR measurements

2.5.1. Superoxide anion radical scavenging activity of bilberry extract fractions

A solution containing superoxide anion radicals was prepared by dissolving KO_2/crown ether (10 mM/20 mM) in dry dimethylsulfoxide (DMSO) and 0.005 ml of this solution were added to 0.5 ml of dry DMSO and 0.005 ml of an DMSO spin trap solution (DMPO, 5,5-dimethyl-1-pyrroline-N-oxide, 2 M). The influence of extracts on the formation of DMPO/$^{\bullet}$OOH adducts was studied by adding the N,N-dimethylformamide (DMF) solution of bilberry extract fraction to the superoxide anion reaction system at a final concentration range of 0.001–1 mg/ml. Synthetic antioxidant, butylated hydroxyanisole (BHA), was used for comparison. Then the solution was transferred to a quartz flat cell ER-160FT and 2 m after mixing ESR spectra were recorded on an EMX spectrometer from Bruker (Rheinstetten, Germany) using a TE_{102} cavity. The following instrument settings were used: field modulation 100 kHz, modulation amplitude 4.00 G, receiver gain 1×10^4, time constant 327.68 ms, conversion time 40.96 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23°C.

The extent of scavenging by antioxidant fractions was expressed as scavenfing activity (SA) values. The $SAo_2^{\bullet-}$ value of the extract was defined as: $SAo_2^{\bullet-}$ (%) = $100 \times (h_0–h_x)/h_0$, where h_0 and h_x are the hight of the second peak in the ESR spectrum of DMPO/$^{\bullet}$OOH spin adduct of the sample without and with antioxidant fractions, respectively.

2.5.2. Detection of antioxidant-derived radicals

The antioxidant-derived radicals were determined in the reaction system containing 0.5 ml of DMSO solution of KO_2/crown ether (10 mM/20 mM) and 0.5 ml of DMF solution of bilberry extract fraction (5 mg/ml). These solutions were aspirated from autosampler vial into the quartz flat cell, which was located in the TE_{102}-resonator of the EMX spectrometer (Bruker, Rheinstetten, Germany). The mixing of both solutions was performed in the lower part of the cell prior to reaching the active zone of the flat cell. ESR measurements were started 20 s after mixing. The following instrument settings were used: microwave frequency, 9.73 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; center field, 3491.1 G; sweep, 25 G; modulation amplitude, 0.48 G; receiver gain, 8×10^5; scan rate, 35.77 G/min; time constant, 0.163 s; scans 5. ESR spectral files were imported into the WINSIM program [16] for the analysis of the hyperfine splitting constants.

2.6. Statistical analysis

All analysis were run in triplicate and were expressed as means ± standard deviation (SD). Statistical analysis was done by using Statistica 8.0 software package (StatSoft Inc., 1984–2007). Significant differences were calculated by ANOVA test and then least significant difference (LSD) test ($p < 0.05$, unless noted otherwise).

3. Results

3.1. Chemical composition of bilberry

The contents of total phenols, flavonoids, anthocyanins and monomers of anthocyanins in purified bilberry extract are given in Table 1. According to these results, flavonoids are the most abundant class of phenolic compounds present in bilberry extract (82.24%). Anthocyanins are one of the main flavonoid subgroups in fruits and berries and they are responsible for their red, violet, purple and blue colours. The anthocyanins are considered the most important of the pharmacologically active constituents. Anthocyanin concentration in the fresh bilberry fruit is approximately 0.1–0.5%, while concentrated bilberry extracts are usually standardized to 25% anthocyanins [24, 53]. Valentová et al. [48] detected cyanidin, delphinidin, malvidin, paeonidin and petunidin and its glycosides in bilberry extract, summary 25% of extract. Presented results show that 35% of flavonoids and less than 30% of total phenols in bilberry extract represent anthocyanins.

The compounds of interest in this study were vitamin C, flavonoids and phenolic acids. Total phenolic content measured by the Folin-Ciocalteu method may give false and overestimated results because possible interference from other chemical components present in the extract (sugars, aromatic amines, sulfur dioxide, ascorbic acid, organic acids, Fe(II), and other nonphenolic organic substances that react with Folin-Ciocalteu reagent) [42, 45]. High performance liquid chromatography (HPLC) method was used for further quantification of total phenolic compounds, flavonoids in Fr2 and phenolic acids in Fr3, as well as vitamin C in Fr1.

As presented in Table 2. bilberry extract contains two times higher levels of flavonoids than phenolic acids. Herrmann [23] and Wildanger and Herrmann [51] reported that the far predominant phenolic compound in bilberry is quercetin. Beside quercetin, the detectable levels of myricetin and kaempferol were found in several European bilberry varieties [46]. High contents of quercetin were confirmed also by Häkkinen et al. [20] where quercetin was abundant in 21.4% of total phenolic compounds detected in bilberry. In that study the main phenolic acid in bilberry was *p*-coumaric acid followed by ferulic and caffeic acids. Also, total contents of phenolic compounds obtained in this study were lower comparing to the values obtained in the study of Prior et al. [37] probably due to the variability of plant material used for extraction. Their data are based on fresh fruit flesh and we used dried plant material. According to the study of Häkkinen [21] quercetin content in bilberry decreased markedly during 9 months of storage, the lower values we observed seem to be reasonable. Also, the contents of vitamin C, as expected, were much lower than reported [2] due to the the sensitivity and instability of this vitamin during storage and processing

Table 1
Total contents of phenols, flavonoids and anthocyanins in purified bilberry extract

Compounds	Total content	
	mg/g dry weight	mg/g plant material
Total phenols[a]	273.25 ± 10.69	136.29 ± 4.28
Total flavonoids[b]	224.71 ± 7.34	112.08 ± 3.87
Total anthocyanins[c]	78.50 ± 3.34	39.15 ± 1.34
Monomeric anthocyanins[c]	58.45 ± 2.28	29.15 ± 0.98

[a]mg chlorogenic acid/g.
[b]mg rutin/g.
[c]mg cyanidin-3-glycoside/g.

Table 2
Contents of phenolic compounds and vitamin C in bilberry extract fractions

Compounds	Total content		
	Expressed as mg per 100 g of bilberry extract fraction	Expressed as mg per 100 g of bilberry extract	Expressed as mg per 100 g of bilberry sample extracted
Vitamin C (Fr1)	152.90 ± 14.60	2.02 ± 0.19	1.01 ± 0.09
Flavonoids (Fr2)	132.86 ± 0.05	0.77 ± 0.01	0.38 ± 0.01
Phenolic acids (Fr3)	49.43 ± 0.02	0.36 ± 0.00	0.18 ± 0.00

[17]. In general, vitamin C content of berries declines during storage by up to 56% depending on duration and temperature [4]. Finally, actual levels of antioxidants in plants depend greatly on the variety of plant analysed, the way in which it was cultivated (since synthesis of antioxidants can be a response to stress), and how it is harvested and stored [11].

3.2. Superoxide anion radical scavenging activity of bilberry extract fractions

Although the superoxide anion radical is a weak oxidant, it gives rise to the generation of more powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to the oxidative stress [29]. Superoxide anion radical can be produced enzymatically, in xanthine/xanthine oxidase system or chemically, as in this study. The advantage of the latter system for determination of superoxide anion radical scavenging activity is the simplicity in interpretation of results, since possible inhibition of the xanthine oxidase enzyme by antioxidant fractions is not a problem.

The superoxide adduct of 5,5-dimethyl-l-pyrroline-N-oxide (DMPO) has been detected by ESR spectroscopy using KO_2 solubilized in crown ether as a source of superoxide (Fig. 1). The ESR hyperfine splitting constants of the DMPO/$^{\bullet}$OOH adduct were: $a_N = 12.65$ G; $a_{H\beta} = 10.4$ G; $a_{H\gamma} = 1.3$G, which is in accordance with literature data [8].

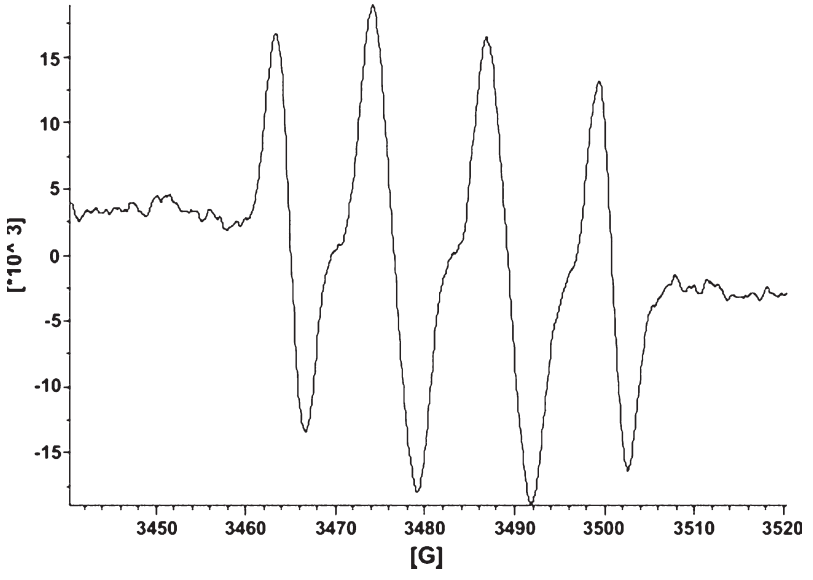

Fig. 1. ESR spectrum of DMPO/$^{\bullet}$OOH spin adduct (blank) recorded 2 min in after mixing of 0.005 ml of KO_2/crown ether (10 mM/20 mM) dissolved in dry DMSO, 0.5 ml of dry DMSO and 0.005 ml of an DMSO spin trap solution (DMPO, 2 M).

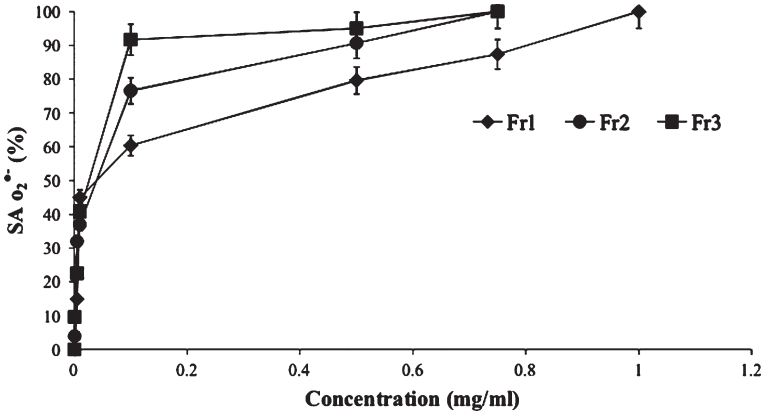

Fig. 2. Superoxide anion radical scavenging activity of bilberry extract fractions: ◆Fr1; ●Fr2; ■Fr3. Each value is presented as mean ± SD of three determinations.

All three fractions of bilberry extract have efficiently scavenged superoxide anion radicals (Fig. 2). These fractions showed similar, not significantly different ($p < 0.05$) scavenging activity, when applied at lower concentrations (0.001–0.01 mg/ml). Complete elimination of superoxide anion radicals ($SAo_2^{\bullet-} = 100\%$) was achieved with the same concentration, 0.75 mg/ml, of Fr2 and Fr3, and with 1 mg/ml of Fr1. The $SAo_2^{\bullet-}$ of bilberry extract fractions decreased in the following order: Fr3 > Fr2 > Fr1.

In the present study bilberry fractions scavenged superoxide anion radicals with an efficiancy higher than BHA. Calculated IC_{50} values for Fr1 (49 μg/ml), Fr2 (21 μg/ml) and Fr3 (15 μg/ml) were much lower than IC_{50} value for BHA (2680 μg/ml). IC_{50} values of bilberry extract fractions were comparable to the superoxide anion radical scavenging activity values of bilberry extract obtained in the study of Valentová et al. [48] (final concentration 47.6 μg/ml scavenged 25.7% of superoxide anion radicals formed) and Martin-Aragon et al. [32] (final concentration 50 μg/ml scavenged 63% of superoxide anion radicals formed). Difference in reactivity is due to the difference in the contents of active compounds, resulting from different extraction methods, and different experimental conditions, especially because in these studies superoxide anion radical was produced enzymatically.

The scavenging properties of antioxidant compounds are often associated with their ability to form stable radicals. It is well known that aromatic compounds containing hydroxyl groups, especially those having *ortho* di- or trihydroxy-function, can give rise to radicals stable enough to be directly detected by ESR spectroscopy [14]. Seyoum et al. [43] proposed a mechanism of antioxidant reaction of flavonoids, via hydrogen donation leading to formation of a flavonoid radical and termination of flavonoid radicals by further loss of a hydrogen atom via disproportionation or other subsequent reactions. An ESR signal assignable to a flavonoid semiquinone radical was found in the superoxide generating system in the presence of Fr2 and without adding the spin trap (Fig. 3). After simulation of the obtained ESR spectrum (Fig. 3) and comparing the obtained hyperfine constants ($a_H^{2'} = 1.7$ G; $a_H^{5'} = 0.8$ G and $a_H^{6'} = 2.8$ G) with the reports of Canadanovic-Brunet et al. [6] ($a_H^{2'} = 1.5$ G; $a_H^{5'} = 0.7$ G and $a_H^{6'} = 2.7$ G), it was determined that the observed radical was quercetin semiquinone radical. This is consistent with the findings of Herrmann [51] and Wildanger and Herrmann [46] who determined that the dominant bilberry flavonoid is quercetin. The stability of flavonoid, i.e. quercetin is proposed to be due to the large conjugated system providing delocalization of the unpaired electron. However, the reaction of superoxide anion radical with Fr3 without presence of a spin trap did not result in an ESR visible product, probably due to the low stability of the radicals of phenolic acids. This could be caused by the lack of stabilization by resonance due to the smaller conjugated system comparing to the flavonoid molecule. On the other hand, the free-radical product obtained by oxidation of Fr1 with superoxide anion radicals was characterized by ESR spectroscopy as a simple doublet showing coupling constants of $a_H = 1.84$ G (Fig. 4). According to literature data and due to the high content of vitamin C confirmed by HPLC analysis (Table 2), this can be assigned to an ascorbyl radical [18, 36]. It has been reported that the reaction of ascorbic acid with more aggressive radicals results in the production of an intermediate radical, ascorbyl, of low reactivity. The lower activity comes from the ability of ascorbate to delocalize the radical electron through its π-system.

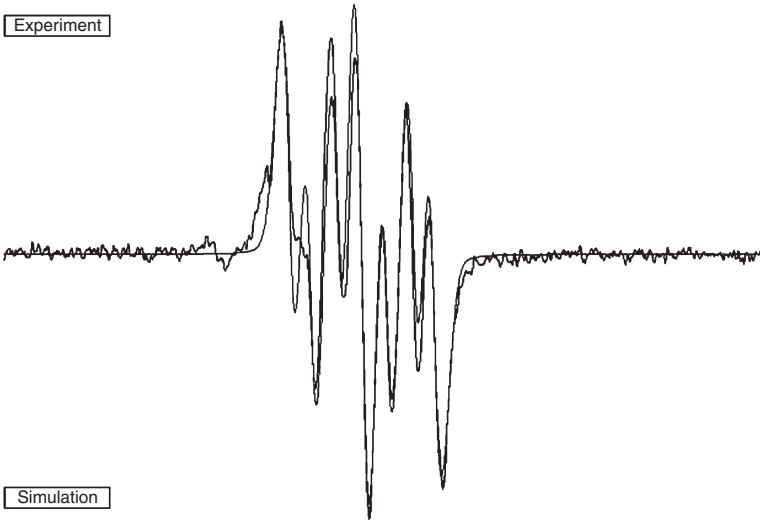

Fig. 3. ESR spectrum of antioxidant-derived radical obtained in the reaction system containing 0.5 ml of DMSO solution of KO_2/crown ether (10 mM/20 mM) and 0.5 ml of DMF solution of bilberry extract fraction Fr2 (5 mg/ml) 20 s after mixing. Simulated spectrum was obtained using WINSIM program.

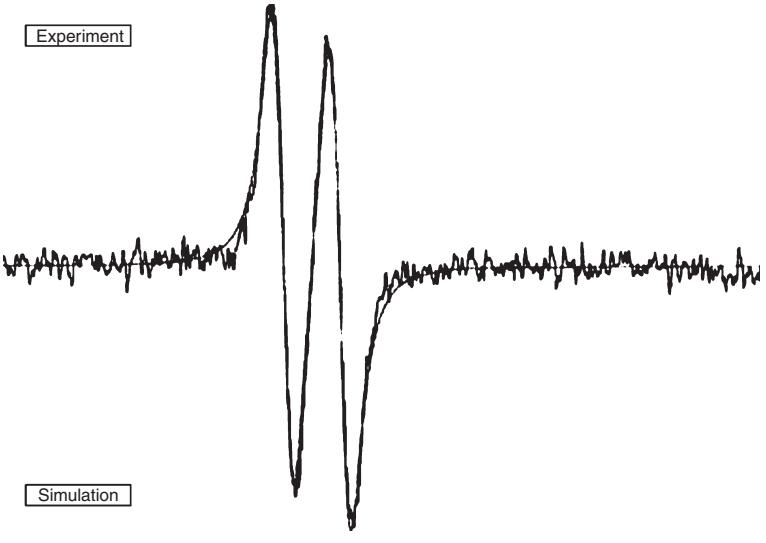

Fig. 4. ESR spectrum of antioxidant-derived radical obtained in the reaction system containing 0.5 ml of DMSO solution of KO_2/crown ether (10 mM/20 mM) and 0.5 ml of DMF solution of bilberry extract fraction Fr1 (5 mg/ml) 20 s after mixing. Simulated spectrum was obtained using WINSIM program.

Berries of the genus *Vaccinium* have been reported to express antioxidative and anticarcinogenic effects *in vitro*, which are partly proposed to be due to phenolic compounds in these berries [2, 13, 20, 27]. In these studies berry extracts, including bilberry, had high total phenolic contents and high antioxidant activity when compared to other plant materials. Kay et al. [25] reported that ingestion of 100 g of freeze dried blueberries (100 g) and berry juices increases the antioxidant capacity of blood plasma by 14–30%. A commercial extract of bilberry scavenged superoxide anion and hydroxyl radicals, inhibited lipid peroxidation in rat liver microsomes and liver lipid peroxidation *in vivo* in mice [31].

Understanding the link between the antioxidant capacity of individual components and the bioactivities of different berries may direct the biotechnological improvement of new berry varieties [3]. Many researchers have found a strong correlation between antioxidant activity and total phenolics [7, 20, 50]. Beekwilder et al. [5] reported that about half of antioxidant activity of raspberries was due to ellagitannins, 20% due to vitamin C and 25% to anthocyanins, while Tulipani et al. [47] found greater contribution of vitamin C (30–35%) in some strawberry varieties. Positive linear correlation between $SAo_2^{\bullet-}$ and total conents of vitamin C ($r^2 = 0.810$), flavonoids ($r^2 = 0.707$) and phenolic acids ($r^2 = 0.608$) obtained in this study indicate that these phytochemicals are one of the main components responsible for antioxidant behaviour of bilberry.

4. Conclusion

Analysis of bilberry extract fractions containing vitamin C (Fr1), neutral phenols (Fr2) and acidic phenols (Fr3) showed high superoxide anion radical scavenging activities, better than synthetic antioxidant BHA. The highest activity was achieved with Fr3. HPLC analysis confirmed significant levels of vitamin C, flavonoids and phenolic acids in investigated bilberry extract fractions. Correlation analysis proved the involvement of these compounds in antioxidative activity. Further research is focused in the investigation of exact and comlete polyphenol profile of bilberry extract, especially anthocyanin contents, and other biological activities contributing the well known beneficial health effects of bilberry.

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References

[1] AOAC Official Method 2005.02, Total monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines, pH Differential Method, AOAC International, 2006.

[2] N. Artik and A. Eksi, Studies on chemical composition of some wild fruits (*Rosa canina*, *Crataegus monogyna*, *Crataegus aronia*, *Vaccinium myrtillus* and *Berberis vulgaris*), *Food Industry* **9** (1988), 33–34.

[3] M. Battino, J. Beekwilder, B. Denoyes-Rothan, M. Laimer, G.J. McDougall and B. Mezzetti, Bioactive compounds in berries relevant to human health, *Nutrition Reviews* **67**(1) (2009), S145–S150.

[4] J. Beattie, A. Crozier and G. Duthie, Potential health benefits of berries, *Current Nutrition & Food Science* **1** (2005), 71–86.

[5] J. Beekwilder, H. Jonker, P. Meesters, R.D. Hall, I.M. Van der Meer and C.H.R. de Vos, Antioxidants in raspberry: on-line analysis links antioxidant activity to a diversity of individual metabolites, *Journal of Agriculture and Food Chemistry* **53** (2005), 3313–3320.

[6] J.M. Canadanovic-Brunet, S.M. Djilas, G.S. Cetkovic and V.T. Tumbas, Free radical scavenging activity of wormwood (*Artemisia absinthium* L.) extracts, *Journal of Food Science and Agriculture* **85** (2005), 265–272.

[7] G. Cao, E. Srife and R.L. Prior, Antioxidant capacity of tea and common vegetables, *Journal of Agricultural Food Chemistry* **44** (1996), 3425–3431.

[8] A.J. Carmichael, A. Samuni and P. Riesz, Photogeneration of superoxide and decarboxylated peptide radicals by carboquone, mitomycin C and streptonigrin, an electron spin resonance and spin trapping study, *Photochemistry Photobiology* **41** (1985), 635.

[9] G.W. Cheng and P.J. Breen, Activity of Phenylalanine Ammonialyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruits, *Journal of the American Society for Horticultural Science* **116** (1991), 865–869.

[10] H. Chen, Y. Zuo, and Y. Deng, Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography, *Journal of Chromatography A* **913** (2001), 387–395.

[11] A.R. Collins, Antioxidant intervention as a route to cancer prevention, *European Journal of Cancer* **41** (2005) 1923–1930.

[12] A.M. Connor, J.J. Luby, J.F. Hancock, S. Berkheimer and E.J. Hanson, Changes in fruit antioxidant activity among blueberry cultivars during cold-temperature storage, *Journal of Agricultural and Food Chemistry* **46** (1998), 4107–4112.

[13] L. Constantino, A. Albasini, G. Rastelli and S. Benvenuti, Activity of polyphenolic crude extracts as scavengers of superoxide radicals and inhibitors of xanthine oxidase, *Planta Medica* **58** (1992) 342–344.

[14] N. Cotelle, J.-L. Bernier, J.-P. Catteau, J. Pommery, J.-C. Wallet and E.M. Gaydou, Antioxidant properties of hydroxy flavones, *Free Radical Biology and Medicine* **20** (1996), 35–43.

[15] Q. Du, G. Jerz and P. Winterhalter, Isolation of two anthocyanin sambubiosides from bilberry (*Vaccinium myrtillus*) by high-speed counter-current chromatography, *Journal of Chromatography A* **1045** (2004), 59–63.

[16] D.R. Duling, Simulation of multiple isotropic spin-trap EPR spectra, *Journal of Magnetic Resonance* **104**(2) (1994), 105–110.

[17] S. Erenturk, M.S. Gulaboglu and S. Gultekin, The effects of cutting and drying medium on the vitamin C content of rosehip during drying, *Journal of Food Engineering* **68** (2005), 513–518.

[18] L. Gille, M. Kleiter, M. Willmann and H. Nohl, Paramagnetic species in the plasma of dogs with lymphoma prior to and after treatment with doxorubicin, an ESR study, *Biochemical Pharmacology* **64** (2002), 1737–1744.

[19] B.L. Halvorsen, K. Holte, M.C.W. Myhrstad, I. Barikmo, E. Hvattum, S.F. Remberg, A.-B. Wold, K. Haffner, H. Baugerød, L.F. Andersen, Ø. Moskaug, D.R. Jacobs, Jr. and R. Blomhoff, A systematic screening of total antioxidants in dietary plants, *Journal of Nutrition* **132** (2001), 461–471.

[20] S. Häkkinen, M. Heinonen, S. Kärenlampi, H. Mykkänen, J. Ruuskanen and R. Törrönen, Screening of selected flavonoids and phenolic acids in 19 berries, *Food Research International* **32** (1999), 345–353.

[21] S.H. Häkkinen and A.R. Törrönen, Content of flavonols and selected phenolic acids in strawberries and *Vaccinium* species: influence of cultivar, cultivation site and technique, *Food Research International* **33** (2000), 517–524.

[22] I.M. Heinonen, A.S. Meyer and E.N. Frankel, Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation, *Journal of Agricultural and Food Chemistry* **46** (1998), 4107–4112.

[23] K. Herrmann, Flavonols and flavones in food plants: a review, *Journal of Food Technology* **11** (1976), 433–448.

[24] T. Ichiyanagi, Y. Kashiwada, Y. Ikeshiro, Y. Hatano, Y. Shida and M. Horie, Complete assignment of bilberry (*Vaccinium myrtillus* L.) anthocyanins separated by capillary zone electrophoresis, *Chemical & Pharmaceutical Bulletin (Tokyo)* **52**(2) (2004), 226–229.

[25] C.D. Kay and B.J. Holub, The effect of wild blueberry (*Vaccinium angustifolium*) consumption on postprandial serum antioxidant status in human subjects, *British Journal of Nutrition* **88** (2002), 389–397.

[26] M.P. Kähkönen, A.I. Hopia and M. Heinonen, Berry phenolics and their antioxidant activity, *Journal of Agricultural and Food Chemistry* **49** (2001), 4076–4082.

[27] M.P. Kähkönen, A.I. Hopia, H.J. Vuorela, J.-P. Rauha, K. Pihlaja, T.S. Kujala and M. Heinonen, Antioxidant activity of plant extracts containing phenolic compounds, *Journal of Agricultural and Food Chemistry* **47** (1999), 3954–3962.

[28] P.M. Laplaud, A. Lelubre and M.J. Chapman, Antioxidant action of vaccinium myrtillus extract on human low density lipoproteins in vitro: initial observations, *Fundamental & Clinical Pharmacology* **11**(1) (1997), 35–40.

[29] X. Liu, M. Zhao, J. Wang, B. Yang and Y. Jiang, Antioxidant activity of methanolic extract of emblica fruit (*Phyllanthus emblica* L.) from six regions in China, *Journal of Food Composition and Analysis* **21**(3) (2008), 219–228.

[30] K.R. Markham, Flavones, flavonols and their glycosides, in: *Methods in Plant Biochemistry*, P.M. Dey and J.B. Harborne, eds., Academic Press, London, UK, 1989, pp. 193–237.

[31] S. Martín-Aragon, B. Basabe, J. Benedi and A. Villar, Antioxidant action of *Vaccinium myrtillus* L., *Phytotherapy Research* **12** (1998), S104–S106.

[32] S. Martín-Aragon, B. Basabe, J. Benedi and A. Villar, *In vitro* and *in vivo* antioxidant properties of *Vaccinium myrtillus*, *Pharmaceutical Biology* **37** (1999), 109–113.

[33] K.R. Määtä-Riihinen, A. Kamal-Eldin, P.H. Mattila, A.M. González-Paramás and A.R. Törrönen, Distribution and contents of phenolic compounds in 18 Scandinavian berry species, *Journal of Agricultural and Food Chemistry* **52** (2004), 4477–4486.

[34] G.J. McDougall, F. Shapiro, P. Dobson, P. Smith, A. Blake and D. Stewart, Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase, *Journal of Agricultural Food Chemistry* **53** (2005), 2760–2766.

[35] G.J. McDougall and D. Stewart, The inhibitory effects of berry extracts on digestive enzymes, *Biofactors* **23** (2005), 189–195.

[36] M. Minetti, T. Forte, M. Soriani, M. Quaresima, A. Menditto and M. Ferrari, Iron-induced ascorbate oxidation in plasma as monitored by ascorbate free radical formation, *Biochemistry Journal* **282** (1992), 459–465.

[37] R.L. Prior, G. Cao, A. Martin, E. Sofic, J. McEwen, C. O'Brien, N. Lischner, M. Ehlenfeldt, W. Kalt, G. Krewer and C.M. Mainland, Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species, *Journal of Agricultural and Food Chemistry* **46** (1998) 2686–2693.

[38] M.R. Ramirez, I. Izquierdo, M.C.B. Raseira, J.Â. Zuanazzi, D. Barros and A.T. Henriques, Effect of lyophilised *Vaccinium* berries on memory, anxiety and locomotion in adult rats, *Pharmacological Research* **52** (2005), 457–462.

[39] A. Rigo, F. Vianello, and G. Clementi, Contribution of proanthocyanidins to the peroxy radical scavenging capacity of some italian red wines, *Journal of Agricultural and Food Chemistry* **48** (2000) 1996–2002.

[40] H.A. Ross, G.J. McDougall and D. Stewart, Antiproliferative activity is predominantly associated with ellagitannins in raspberry extracts, *Phytochemistry* **68** (2007), 218–228.

[41] J.-M. Rouanet, K. Décordé, D. Del Rio, C. Auger, G. Borges, J.-P. Cristol, M.E.J. Lean and A. Crozier, Berry juices, teas, antioxidants and the prevention of atherosclerosis in hamsters, *Food Chemistry* **118** (2010), 266–271.

[42] E. Roura, C. Andres-Lacueva, R. Estruch and R.M. Lamuela-Raventos, Total polyphenol intake estimated by a modified Folin–Ciocalteu assay of urine, *Clinical Chemistry* **52** (2006), 749–752.

[43] A. Seyoum, K. Asres and F. Kandeel El-Fiky, Structure radical scavenging activity relation-ships of flavonoids, *Phytochemistry* **67**(18) (2006), 2058–2070.

[44] B. Shukitt-Hale, A.N. Carey, D. Jenkins, B.M. Rabin and J.A. Joseph, Beneficial effects of fruit extracts on neuronal function and behavior in a rodent model of accelerated aging, *Neurobiology of Aging* **28**(8) (2007), 1187–1194.

[45] V.L. Singelton, R. Orthofer and R.M. Lamuela-Raventos, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent, *Methods in Enzymology* **299** (1999), 52–178.

[46] H. Starke and K. Herrmann, The phenolics of fruits, VIII. Changes of flavonol concentrations during fruit development, *Zeitschrift für Lebensmittel-Untersuchung und–Forschung* **161** (1976), 131–135.

[47] S. Tulipani, B. Mezzetti, F. Capocasa, S. Bompadre, J. Beekwilder, C.H.R. de Vos, E. Capanoglu, A. Bovy and M. Battino, Antioxidants, phenolic compounds and nutritional quality in different strawberry genotypes, *Journal of Agricultural and Food Chemistry* **56** (2008), 696–704.

[48] K. Valentová, J. Ulrichová, L. Cvak and V. Šimánek, Cytoprotective effect of a bilberry extract against oxidative damage of rat hepatocytes, *Food Chemistry* **101** (2007), 912–917.

[49] K. Viljanen, P. Kylli, R. Kivikari and M. Heinonen, Inhibition of protein and lipid oxidation in liposomes by berry phenolics, *Journal of Agricultural and Food Chemistry* **52**(24) (2004), 7419–7424.

[50] H. Wang, G. Cao and R.L. Prior, Total antioxidant capacity of fruits, *Journal of Agricultural Food Chemistry* **44** (1996) 701–705.

[51] W. Wildanger and K. Herrmann, The phenolics of fruits. II. The flavonols of fruits, *Zeitschrift für Lebensmittel-Untersuchung und–Forschung* **151** (1973), 103–108.

[52] X. Yue and Z. Xu, Changes of anthocyanins, anthocyanidins, and antioxidant activity in bilberry extract during dry heating, *Journal of Food Science* **73**(6) (2008), C494–C499.

[53] Z. Zhang, X. Kou, K. Fugal and J. McLaughlin, Comparison of HPLC methods for determination of anthocyanins and anthocyanidins in bilberry extracts, *Journal of Agricultural and Food Chemistry* **52**(4) (2004), 688–691.