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ORIGINAL PAPER

Antioxidative properties of *Sambucus nigra* extracts

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Potential protective effects of elderberry (*Sambucus nigra*) extracts against oxidative degradation of hyaluronan (HA) were detected in vitro. To induce free-radical-mediated HA degradation, Weissberger's biogenic oxidative system, which mimics the situation of acute inflammation, was applied. Time- and dose-dependent changes of dynamic viscosity of the HA solutions in the presence and absence of two elderberry extracts produced in 2006 and 2012 were recorded by rotational viscometry (RV). Radical scavenging capacity of both extracts was investigated by the spectrocolorimetric ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] assay and the "inverted" ABTS assay. Oxygen consumption of the system oxidizing HA either in the absence or presence of the elderberry extracts was determined. The results of RV revealed that an addition of the newer extract (2012) promoted the inhibition of HA degradation more markedly compared to the older extract (2006). The same effect of both extracts on $ABTS^{\bullet+}$ scavenging was observed. Inverted ABTS assay demonstrated that colorful substances were not responsible for the radical-scavenging activity. Results of oximetry demonstrated that the effect of the extract from 2006 was more significant than that of the extract from 2012.

Keywords: ABTS assay, glycosaminoglycans, oximetry, rotational viscometry

Introduction

Sambucus is a flowering plant from the *Adoxaceae* family. It consists of 5 to 30 species of leafy shrubs, small trees and perennial herbs. The most often medicinally used species is *Sambucus nigra* (elderberry, black elder, European elder). *Sambucus* berries are often used as a food (wine and pies), flavoring or dye. The berries are edible after cooking (mildly poisonous when raw) and can be used to produce jam, jelly, chutney, etc. The flowerheads are frequently used in herbal extracts, delivering a refreshing drink. Elderberry brandy (Hungary) and wine can be produced from both flowers and berries. Elderflowers are used in liqueurs too, such as traditional snaps liqueur flavored with elderflower (South-Western Sweden) or St. Germainand – mildly alcoholic elderflower "champagne". Popularity of traditional elderberry drinks has stimulated some commercial producers to present elderflower-flavored drinks to the market. Also thanks to many already proven benefits of elderberry products, also food supplements can be found in pharmacies (Schmitzer et al., 2010, Schrøder-Aasen et al., 2012).

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Table 1. Composition of *Sambucus nigra* (Thorne Research, 2005; Veberic et al., 2009)

Components	Individual components
Anthocyanins	Mainly cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-sambubioside-5-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-sambubioside
Flavonoids	Quercetin, quercetin 3-rutinoside (rutin), quercetin 3-glucoside
Organic acids	Citric, malic, shikimic and fumaric acid
Saccharides	Mainly fructose and glucose
Hemagglutinin protein	*Sambucus nigra* agglutinin III (SNA-III)
Cyanogenic glycosides	Sambunigrin
Vitamins	A, C

At present, extracts of the berries are used mainly as antivirals for colds, influenza, and herpes virus infection. It was also demonstrated that elderberries have immuno-modulating, antioxidative and insulin-stimulating properties. The leaves, bark, flowers and berries have all been used in medicine throughout the history. Active ingredients in elderberry are found throughout the whole plant, but well-matured flowers are most commonly used. Composition of *Sambucus nigra* is described in Table 1 (Veberic et al., 2009).

One group of the elderberry components, anthocyanins, are the most widespread water-soluble plant pigments and belong to flavonoids present in polyphenols. Anthocyanins have been included into human nutrition for centuries and have been used as herbal remedies because of their various physiological abilities to treat conditions such as hypertension, pyrexia, liver disorders, dysentery and diarrhea, urinary problems, cold, etc.

They also participate in anti-diabetic actions such as lipid lowering, insulin secretion and vasoprotective effects. Moreover, studies have indicated that anthocyanin extracts can enhance sight acuteness, manifest antioxidative and radical-scavenging activity and that they are able to act as chemoprotective agents (Milbury et al., 2002; Shipp & El-Sayed, 2010).

Abuja et al. (1998) examined low concentrations (4 μg mL^{-1}) of elderberry anthocyanins. In copper-mediated oxidation, anthocyanins reduce α-tocopheroxyl radical to α-tocopherol. Youdim et al. (2000) showed incorporation of elderberry anthocyanin into endothelial cells, which led to an enhanced protection against oxidative stress. The elderberry extracts containing four anthocyanins were incorporated into the cytoplasmic membrane. The addition of elderberry anthocyanins into endothelial cells ensured significant protective effects against oxidative agents. A primary study on dietary anthocyanins has shown that the total antioxidant status of low-density lipoprotein (LDL) was increased and LDL cholesterol was decreased. The bioavailability of anthocyanins from elderberry in humans was shown by Cao and Prior (1999) and Murkovic et al. (2001).

Numerous diseases are determined by oxidative stress (cardiovascular disease, cancer, neurodegenerative disease, peripheral vascular disease, autoimmune diseases and multiple sclerosis). The protection ability of elderberry extract through inhibition of LDL oxidation and scavenging free radicals makes it a potentially worthy tool in the treatment of diseases characterized by oxidative stress (Youdim et al., 2000). The pharmaceutical and natural-product industries are also taking advantage of the high contents of phenolic compounds of elder, berries and flowers. A full range of products like syrups, herbal teas, extracts and supplements are also available, all offering diverse health benefits. The most popular one is Sambucol® (PharmaCare Europe, Horsham, UK) which is an elderberry extract formulation that activates the human health immune system (Barak et al., 2002).

Hyaluronan (HA), a linear natural polysaccharide made of *N*-acetyl-D-glucosamine and D-glucuronic acid connected by β-(1→4) and β-(1→3) linkages, is of molar mass ranging from hundreds thousands up to several millions of daltons. It was demonstrated that physico-chemical and biological features of HA are molar-mass-dependent. While high-molar-mass HA exhibits anti-angiogenic, anti-inflammatory, and immunosuppressive properties, intermediate-sized HA fragments act mostly in the opposite manner, i.e. they are angiogenic, pro-inflammatory, and immunostimulating (Šoltés et al., 2006, 2007; Stern et al., 2007). As study models, situation of acute inflammation were applied. To evoke free-radical-mediated HA degradation, the Weissberger biogenic oxidative system (WBOS) we applied (Fig. 1). Oxidation of ascorbate under catalytic action of Cu(II) ions generates hydrogen peroxide.

Antioxidative activity of elderberry samples was examined by several authors, e.g. Bratu et al. (2012), Jabłońska-Ryś et al. (2009) and Nussbaum and Levine (2008).

The aim of this study was to elucidate the mechanism of action of *Sambucus nigra* extracts produced in 2006 and 2012. For this reason, rotational viscometry was used to assess the protective effects of the extracts against oxidatively damaged hyaluronan ($H^{\bullet}$ donor properties). The ABTS assay was selected to examine the free-radical scavenging capacity of the extracts (electron donor properties). Moreover, antiox-

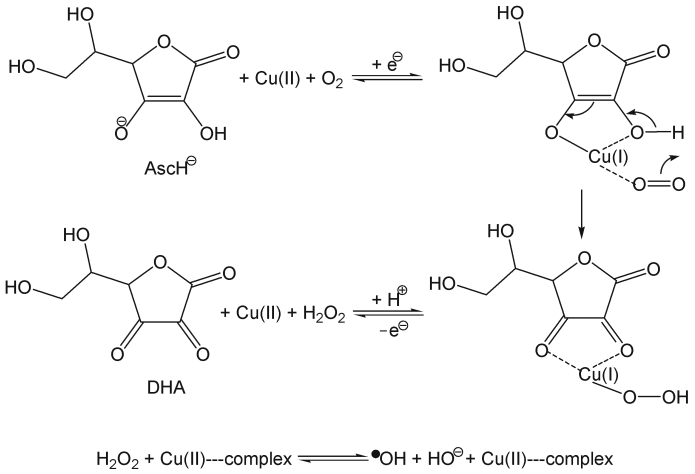

Fig. 1. Chemistry of the Weissberger biogenic oxidative system.

idative effect of the extracts was determined by measuring oxygen consumption in the hyaluronan reaction mixture.

The purpose of this study was to determine antioxidative capacity of elderberry extracts as a whole, in order to explore their potential as food supplement or as natural ingredient.

Experimental

A hyaluronan sample (sodium salt) of the molar mass of 970.4 kDa, coded P0207-1A, was obtained from Lifecore Biomedical (USA). Analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ were purchased from Slavus (Slovakia). Elderberry (*Sambucus nigra*) extracts made in 2006 and 2012 were the products of the Food Research Institute – Biocenter Modra (Slovakia). Ascorbic acid and potassium persulfate ($K_2S_2O_8$ p.a. purity, max. 0.001 mass % of nitrogen) were provided by Merck (Germany). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; purum, > 99 mass %) was obtained from Fluka (Germany). HCl p.a. 36 mass % and ethanol p.a. 96 vol. % were purchased from Slavus (Slovakia). Redistilled deionized high quality water, with the conductivity of < 0.055 μS cm^{-1}, was produced using the TKA water purification system from Water Purification Systems (Germany).

Preparation of stock and working solutions

Hyaluronan samples (20 mg) were dissolved in a 0.15 M aqueous NaCl solution for 24 h in the dark. HA sample solutions were prepared in two steps: first, 4.0 mL and after 6 h, 3.90 mL or 3.85 mL of the 0.15 M NaCl solution were added in the absence or in the presence of elderberry extracts, respectively. Solutions of ascorbate (16 mM), elderberry extracts 2006 and 2012 (each of 1.6 g mL^{-1}) and cupric chloride solution (160 μM) were prepared also in 0.15 M aqueous NaCl.

Uninhibited/inhibited hyaluronan degradation

First, HA degradation was induced by the oxidative system comprising $CuCl_2$ (1.0 μM) and ascorbic acid (100 μM). The procedure was as follows: 50 μL of a 160 μM $CuCl_2$ solution were added to the HA solution (7.90 mL) and the mixture was left to stand for 7 min and 30 s at room temperature after a 30 s stirring. Then, 50 μL of the ascorbic acid solution (16 mM) were added and the mixture was stirred for 30 s followed by its immediate addition into a viscometer Teflon® cup reservoir (made in the Institute of Experimental Pharmacology and Toxicology, Slovakia).

Procedures for elderberry extracts antioxidative effectiveness determination were as follows:

i) The volume of 50 μL of a 160 μM $CuCl_2$ solution was added to the HA solution (7.85 mL) and the mixture, after a 30 s stirring, was left to stand for 7 min and 30 s at room temperature. Then, 50 μL of an elderberry extract (100 mg mL^{-1}, 10 mg mL^{-1} or 1 mg mL^{-1}) were added to the solution, followed by stirring for 30 s. Finally, 50 μL of ascorbic acid solution (16 mM) were added to the solution which was then stirred for 30 s immediately added into a viscometer Teflon® cup reservoir.

ii) In the second experimental setting, a procedure similar to that described in *i*) was applied; however, after standing for 7 min and 30 s at room temperature, 50 μL of an ascorbic acid solution (16 mM) were added to the mixture and a 30 s stirring followed. After 1 h, finally 50 μL of the elderberry extract (100 mg mL^{-1}, 10 mg mL^{-1} or 1 mg mL^{-1}) were added to the solution, followed by 30 s stirring. The solution mixture was then immediately transferred into a viscometer Teflon® cup reservoir.

Rotational viscometry

Dynamic viscosity of the reaction mixture (8 mL;

0.15 M aqueous NaCl) containing HA (2.5 mg mL^{-1}), ascorbate (100 μM) plus Cu(II) ions (1 μM) in the absence and in the presence of elderberry extracts 2006 and 2012 (1 mg mL^{-1}, 10 mg mL^{-1} and 100 mg mL^{-1}) was monitored by a Brookfield LVDV-II+PRO digital rotational viscometer (Brookfield Engineering Labs., Middleboro, MA, USA) at (25.0 ± 0.1) ℃ and at the shear rate of 237.6 s^{-1} for 5 h in a Teflon® cup reservoir (Valachová et al., 2011). The elderberry extracts as potential protective agents were introduced into the HA reaction system before or 1 h after the HA degradation started.

ABTS assay

Radical-scavenging activity was measured according to a modified ABTS method (Re et al., 1999). To prepare the $ABTS^{\bullet+}$ cation radical, an aqueous solution of $K_2S_2O_8$ (3.3 mg of $K_2S_2O_8$ in 5 mL of H_2O) was added to ABTS (17.2 mg) and the resulting solution was freeze-stored for 14 h in the dark. Finally, the dark-green $ABTS^{\bullet+}$ radical cation solution (1 mL) was diluted with H_2O (60 mL) and used in the ABTS assays. The investigated samples were composed of 2 mL of the diluted $ABTS^{\bullet+}$ solution with an addition of 50 μL of the elderberry extracts (1 mg mL^{-1}). UV-VIS spectra were recorded in 1 min, 5 min, 10 min, 15 min and 20 min intervals using a UV-VIS S2000 spectrophotometer (Sentronic, Germany) (Rapta et al., 2009, Hrabárová et al., 2010).

To determine whether the colored substances in the extracts are responsible for their radical-scavenging activity, the so-called "inverted" ABTS assay was performed (Žemlička et al., 2014). In the inverted ABTS test, an appropriate volume of 0.05 mM $ABTS^{\bullet+}$ aqueous water solution (0.5 mL) was added to 1.5 mL of the elderberry extracts 2006 and 2012 (0.5 mg mL^{-1}).

Oximetry

The electrode chamber contained the oxidative system: HA solution (2.5 mg mL^{-1}), $CuCl_2$ (5.0 μM), ascorbic acid (100 μM) and elderberry extracts (100 μg mL^{-1}, 10 μg mL^{-1} or 1 μg mL^{-1}). The procedure of individual components addition was identical to that mentioned in *i*). Oxygen consumption of the HA oxidative system alone and in the presence of the extracts was monitored for 60 min at 25 ℃ using a Clark-type oxygen electrode (Model 782, Strathkelvin Instruments, UK) (Verner et al., 2014).

Anthocyanin concentrate preparation and determination of anthocyanin content in elderberry concentrates

An anthocyanin concentrate was obtained from the elderberry residues after pressing according to the patented procedure, which involves the extraction with an aqueous solution of ethanol (45 vol. %). After achieving the extraction equilibrium, the extract was left to sediment and a clear filtrate was concentrated in a vacuum evaporator. The content of anthocyanins in the concentrate was determined spectrophotometrically (Fuleki & Francis, 1968) and converted to a dominant anthocyanin, cyanidin 3-glucoside.

The concentrated extract (0.5 g) was dissolved in 100 mL of acidified ethanol (90 vol. %) (90 % ethanol was added to 20 mL of 36 mass % HCl to the final volume of 1 L). The solution was measured spectrophotometrically at the wavelength of 535 nm (Schimadzu, Japan) reaching the absorbance range from 0.2 to 0.8. The solution was prepared by dilution with 0.1 M HCl in 90 vol. % ethanol. As a background solution, 0.1 M HCl in 90 vol. % ethanol was used. Anthocyanin concentration (AC) was calculated according to the following formula:

$$\mathrm{AC} = AdM_{\mathrm{w}}V/\varepsilon wl \qquad (1)$$

where AC is the anthocyanin content expressed in grams per kilogram, A the absorbance at 535 nm, M_{w} the molecular mass of cyaniding 3-glucoside (449.0 g mol^{-1}), d the dilution factor, V volume of the solution with dissolved concentrate (100 mL), ε the molar absorption coefficient for cyaniding 3-glucoside (25740 L mol^{-1} cm^{-1}), w amount of the sample (g), and l the thickness of the cuvette (cm).

Results and discussion

Natural antioxidants which can be found in food and other biological materials have drawn significant interest because of their supposed safety and potential nutritional and therapeutic effects (Murkovic et al., 2001).

Potential effects of the elderberry extract 2006 against oxidative degradation of HA, which results are depicted in Figs. 2A and 2B. At first, HA was exposed to oxidative degradation by cupric ions in the presence of ascorbate, which resulted in the decrease in dynamic viscosity (η) of the HA solution from 11.5 mPa s to 7.6 mPa s (curve 0). To achieve the inhibition of HA degradation induced by $^{\bullet}OH$ radicals, the elderberry extract 2006 (100 mg mL^{-1}, 10 mg mL^{-1} and 1 mg mL^{-1}) was added to the HA reaction system. Curves 2 and 3, corresponding to the concentrations 10 mg mL^{-1} and 100 mg mL^{-1}, respectively, show the low efficacy of this extract, as they are similar as curve 0. Moreover, the use of the extract at the lowest concentration, i.e. 1 mg mL^{-1}, led to a more rapid HA degradation causing the decrease in the HA solution viscosity by 6.42 mPa s. The value of η decreased by 3.17 mPa s and 3.77 mPa s at the 100 mg mL^{-1} and 10 mg mL^{-1} concentrations of this extract, respectively.

Fig. 2B, curve 3 illustrates the results for extract

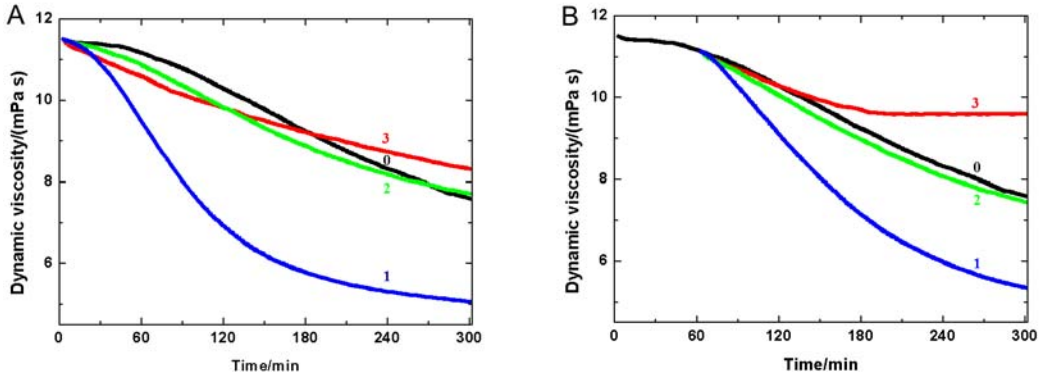

Fig. 2. Effect of elderberry extract 2006 on HA degradation induced by WBOS. Concentrations of the extract in the system before the start of HA degradation: 0 mg mL^{-1} (0); 1 mg mL^{-1} (1); 10 mg mL^{-1} (2); 100 mg mL^{-1} (3) (A). Concentrations of the elderberry extract in the system after 1 h of HA degradation: 0 mg mL^{-1} (0); 1 mg mL^{-1} (1); 10 mg mL^{-1} (2); 100 mg mL^{-1} (3) (B).

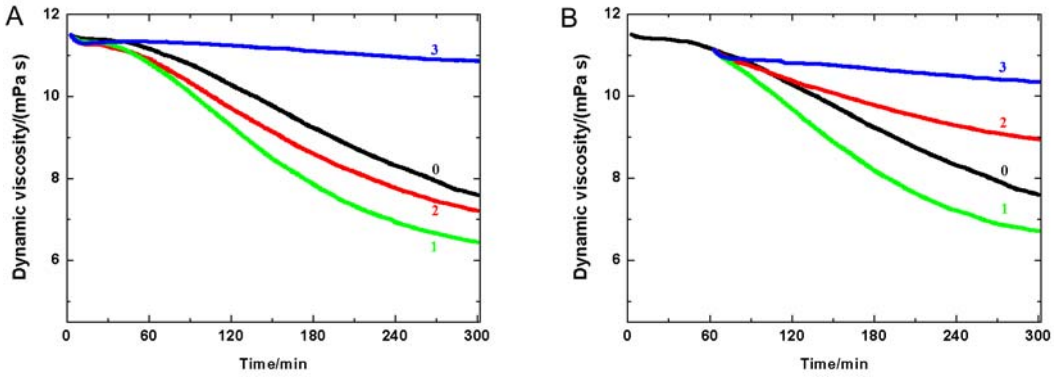

Fig. 3. Effect of elderberry extract 2012 on HA degradation induced by WBOS. Concentrations of the extract in the system before the start of HA degradation: 0 mg mL^{-1} (0); 1 mg mL^{-1} (1); 10 mg mL^{-1} (2); 100 mg mL^{-1} (3) (A). Concentrations of the extract in the system after 1 h of HA degradation: 0 mg mL^{-1} (0); 1 mg mL^{-1} (1); 10 mg mL^{-1} (2); 100 mg mL^{-1} (3).

2006 at the concentration of 100 mg mL^{-1} admixed with the HA reaction system already in the phase of HA degradation – after 1 h. This extract was demonstrated to suppress HA degradation, which was evident especially after 3 h of the experiment (curve 3). When applying the 10 mg mL^{-1} concentration of the extract (curve 2), η of the HA solutions decreased slightly faster compared to the reference (curve 0). In addition, the extract at the 1 mg mL^{-1} concentration pronounced the HA degradation more significantly, whereas η of the HA solution was lower by 5.78 mPa s after 5 h (curve 1).

Also, the newer elderberry extract produced in 2012 was examined to compare the influence of the extract storage (the extract produced in 2006 represents an eight-year stored product). The elderberry extract 2012 (Fig. 3A) showed higher ability to scavenge $^{\bullet}OH$ radicals, which is more apparent at the highest and lowest concentrations of this extract, compared to the older extract produced in 2006 (Fig. 2A). The elderberry extract 2012 (100 mg mL^{-1}) was observed to trigger the inhibitory activity towards $^{\bullet}OH$ radicals, which is demonstrated by the minimal slope of the curve representing the decrease in η of the HA solution only by 0.62 mPa s at the end of the experiment (Fig. 3A, curve 3). Likewise, curve 1 (1 mg mL^{-1}) showed a less rapid HA degradation. Although η of the HA solution reached the value of 6.42 mPa s, its change after 5 h expressed the decrease by 5.03 mPa s, representing a higher inhibitory activity if compared to extract 2006 (Fig. 3A, curve 1).

Fig. 3A displays the results of the extract addition to the HA solution before HA degradation initiated by ascorbate in the presence of cupric ions forming $^{\bullet}OH$ radicals. The radicals were generated up to 1 h as shown by Stankovská et al. (2006). Panel B illustrates the results of the extract addition to the HA reaction mixture during the HA degradation, i.e. after 1 h, when peroxy-type radicals are supposed to prevail. The exposition of HA to ROS (reactive oxygen species) is expressed by the reactions below.

The reaction of ascorbate and Cu(II) ions is the decisive step in hydrogen peroxide formation (Fig. 1). Shortly, the sequence of the reactions can be described

as follows: removal of H$^\bullet$ from the HA chain leads to the formation of A$^\bullet$, i.e. a C-centered (macro) radical species Eq. (2). In the presence of oxygen, the A$^\bullet$ (macro) radical is converted into a peroxy-type radical reacting with HA and forming hydroperoxides (Eqs. (3) and (4)). AOOH can then be decomposed in a reaction catalyzed by cuprous ions forming AO$^\bullet$, the so-called alkoxy (macro) radicals., Eq. (5).

$$\mathrm{HA} + {}^\bullet\mathrm{OH} \rightarrow \mathrm{A}^\bullet + \mathrm{H_2O} \quad (2)$$

$$\mathrm{A}^\bullet + \mathrm{O_2} \rightarrow \mathrm{AOO}^\bullet \quad (3)$$

$$\mathrm{AOO}^\bullet + \mathrm{HA} \rightarrow \mathrm{AOOH} + \mathrm{A}^\bullet \quad (4)$$

$$\mathrm{AOOH} + \mathrm{Cu(I)} \rightarrow \mathrm{AO}^\bullet + \mathrm{Cu(II)} + \mathrm{OH}^- \quad (5)$$

Then, the oxygen-centered AOO$^\bullet$ and AO$^\bullet$ radicals promote reactions finally resulting in the biopolymer degradation indicated by a decrease in the dynamic viscosity of the HA reaction mixture. As published by Hrabárová et al. (2009), the longer the HA polymer chain is, the more it is susceptible to degradation by radicals generated.

To model the HA degradation, WBOS consisting of ascorbic acid and Cu(II) ions in the presence of oxygen was employed. Ascorbic acid (vitamin C) was completely consumed during the first hour of the reaction, while $^\bullet$OH radicals were generated. An apparent pro-oxidative effect of the elderberry extracts at the lower concentrations can be due to the high concentration of ascorbic acid (vitamin C) in the extracts, 28–34 mg per 100 mL^{-1} as published by Mustafa et al. (2009).

After the addition of the lowest concentration (1 mg mL^{-1}) of the elderberry extract, ascorbic acid was introduced into the system to promote, based on WBOS (see above), the generation of radicals. When adding elderberry extracts at the highest concentration (100 mg mL^{-1}), protective effects of other antioxidants present in the extracts, e.g. anthocyanins, which can act as a H$^\bullet$ donor, were expected. In our opinion, with the increasing concentration of the extract added to the system, the effect of the antioxidative components of the extracts can surpass the effect of ascorbic acid.

Radical scavenging capacity of both elderberry extracts was analyzed using the standard ABTS decolorization assay. The ABTS$^{\bullet+}$ radical cation is reactive towards most antioxidants and during these reactions, the blue ABTS$^{\bullet+}$ radical cation was transformed back to its colorless neutral form.

The results of the ABTS assay demonstrate that the elderberry extract 2006 (1 mg mL^{-1}) (Fig. 4, curve 1) scavenged ABTS$^{\bullet+}$ almost identically as the elderberry extract 2012 at the same concentration (Fig. 4, curve 2), i.e. the former extract was a slightly more effective electron donor. According to the results of

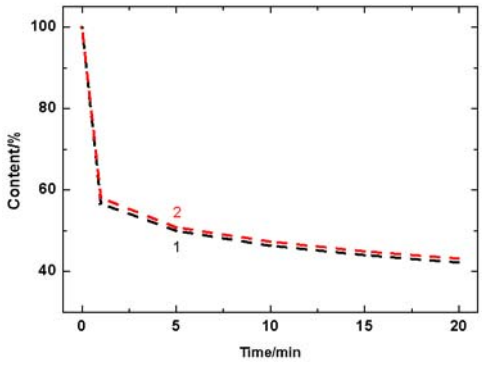

Fig. 4. Content of ABTS$^{\bullet+}$ radical cations in elderberry extract 2006 (1) and elderberry extract 2012 (2). Measurements were performed at the wavelength of 730 nm and the concentration of 1 mg mL^{-1} for both extracts.

rotational viscometry (RV), extract 2012 inhibited the HA degradation more significantly than extract 2006. It means that extract 2012 is a more suitable H$^\bullet$ donor. These observations support the rule that usually more than one single test should be done. While the RV method provides the H$^\bullet$ radicals for $^\bullet$OH, and/or peroxy- and alkoxy-type radicals, the ABTS assay displays the "reductive" property of the screened sample or more clearly the electron donating property of the investigated sample.

To determine whether colored substances present in the extracts are responsible for the radical-scavenging activity, the so-called inverted ABTS assay was performed. In this case, no decrease in the absorbance was observed when the ABTS$^{\bullet+}$ solution was added to the solutions of the elderberry extracts (Fig. 5), which shows the negligible influence of the colored substances, e.g. anthocyanins, on the ABTS$^{\bullet+}$ cation radical scavenging. These results suggest that anthocyanins are not dominantly responsible for the antioxidative effect in elderberry extracts. Antioxidative properties of elderberry extracts can originate in other polyphenol compounds.

Results in Fig. 6 display the link between oxygen consumption and elderberry extracts concentration. In oximetry, 1000-fold lower concentrations of the extracts were used compared to those employed in rotational viscometry and the ABTS assay. The addition procedure for each component was identical to that in *i*) in the Experimental section.

In a 1 h measurement, both elderberry extracts significantly lowered the oxygen consumption of the HA reactive system, whereas their effects were higher after applying higher concentrations of the extracts. It was predictable because oxygen consumption is connected with HA degradation which is prevented by some components of the extract. However, the effect of extract 2006 was more significant than that of extract 2012 probably due to the fact that extract 2012 contains more oxidizable compounds and oxygen is con-

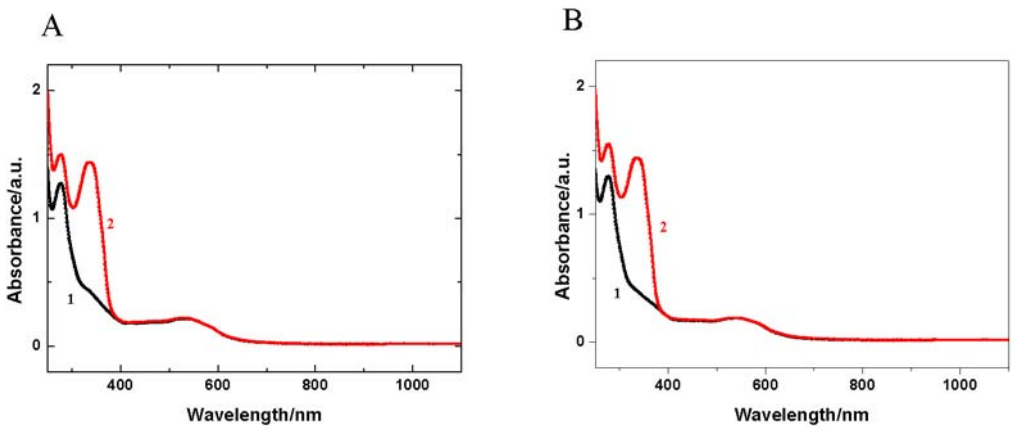

Fig. 5. Inverted ABTS test. UV-VIS spectra of the mixture of elderberry extracts 2006 (0.5 mg mL^{-1}) measured before (1) and after the addition of an aqueous solution of the $ABTS^{\bullet+}$ radical cation (2) (A). UV-VIS spectra of the mixture of elderberry extracts 2012 (0.5 mg mL^{-1}) measured before (1) and after the addition of an aqueous solution of the $ABTS^{\bullet+}$ radical cation (2) (B).

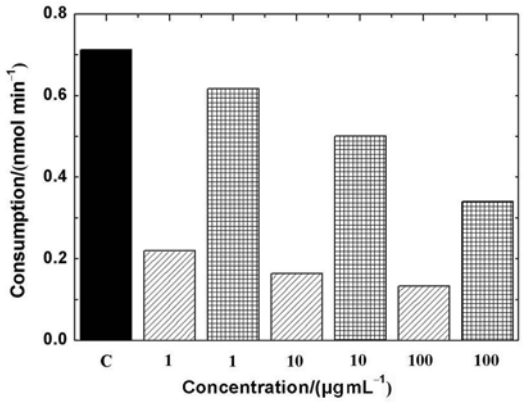

Fig. 6. Consumption of oxygen. Reaction mixture containing HA (2.5 mg mL^{-1}), 5.0 μM $CuCl_2$ and 100 μM ascorbate (C), reaction mixture after the addition of elderberry extracts 2012 (checked pattern) and 2006 (striped pattern).

Table 2. Contents of dry mass and anthocyanins in elderberry extracts

Sample	Dry mass	Anthocyanins in dry mass
	%	g kg^{-1}
Elderberry extract 2006	81.7 ± 0.1	57.0 ± 6.08
Elderberry extract 2012	78.8 ± 0.1	89.7 ± 10.7

sumed by the extract itself. Extract 2006, stored for a longer period of time, has lower content of readily oxidizable compounds, e.g. polyphenols such as anthocyanins, whose concentration is higher in extract 2012 than in extract 2006 (Table 2).

The content of both extracts can vary for a number of reasons. Besides the storage period, the extracts differ in the time of harvest and climate conditions, which results in a different content of components, not only in terms of the content of vitamin C (Mustafa et al., 2009) but also e.g. of anthocyanins, etc. (Table 2). However, it seems to be clear that components responsible for the antioxidative activities of the elderberry extracts can be stored (conditions of storage: cool, dry place) for a relatively long time.

Our study proved the antioxidative properties of elderberry extracts, which means that the extracts are suitable for use in health-enhancing foods. All results obtained are summarized in Table 3.

Conclusions

Several principles of measuring antioxidative activity of elderberry extract can be considered. 1) Scavenging of $^{\bullet}OH$ and peroxy- and/or alkoxy-type radicals by means of a relevant sensitive probe such as high-molar-mass hyaluronan with an advantage of monitoring the changes of the HA solution dynamic viscosity. Solutions of a high-molar-mass HA are of high viscosity and thus rotational viscometry is the most favored and simple method for monitoring changes in their dynamic viscosity. It reflects also the changes in the molar mass of HA. Hyaluronan degradation testing is significantly closer to real living systems than other antioxidant indirect tests (e.g. ABTS, DPPH, FRAP). HA is a substance known to be present in human organisms, for example in joints etc. RV gives an important information on the real situation during oxidation processes. 2) Measuring electron donor properties of the samples. 3) Measuring oxygen consumption of the investigated samples by an appropriate method.

Properties of extract 2012 ascribed to the scavenging of free radicals such as $^{\bullet}OH$ and peroxy- type radicals determined by RV, can be related with the higher content of anthocyanins (Table 1), which triggers the complexation of cupric ions, when applying WBOS.

Table 3. Results overview

Used method	Principle of the method	Results		
		Elderberry extract 2006		Elderberry extract 2012
Rotational viscometry	Determination of H$^\bullet$ donor properties	Effective H$^\bullet$ donor	$<$	Effective H$^\bullet$ donor
ABTS assay	Determination of electron donor properties	Effective electron donor	$\geq$	Effective electron donor
Inverted ABTS assay	Determination of electron donor properties of colored components	No effect	$=$	No effect
Oximetry	Measuring oxygen consumption	Significant decrease of oxygen consumption	$>$	Significant decrease of oxygen consumption

Extract 2006 was proved to be slightly more effective in the ABTS assay.

It can be concluded that fresh extracts act properly in free radicals scavenging, namely those of hydroxyl-, peroxy- and alkoxy-type, and that they effectively provide H$^\bullet$.

These results support the fact that elderberry extracts are good anti-inflammatory agents, which is useful for patients suffering from diseases such as arthritis as consumption of food or supplements containing elderberry can improve their treatment.

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