

POLYPHENOLIC CONTENT, ANTIRADICAL ACTIVITY, STABILITY AND MICROBIOLOGICAL QUALITY OF ELDERBERRY (*SAMBUCUS NIGRA* L.) EXTRACTS

Barbara Pliszka✉

Department of Environment and Agriculture, University of Warmia and Mazury in Olsztyn
Łódzki Square 4, 10-957 Olsztyn, Poland

ABSTRACT

Background. The pharmaceutical and food industries expect detailed knowledge on the physicochemical properties of elderberry fruit extracts, their stability and microbiological quality, as well as the polyphenol content in elderberry cultivars. The characteristics of the extracts might be additionally modified by citric acid, which improves the stability of anthocyanins and protects processed fruits and syrups from pathogenic microorganisms. The choice of the method with citric acid was a consequence of the physicochemical characteristics of elderberry pigments, which are not stable under the effect of light in alcoholic solutions. The aim of study was to analyze the properties of elderberry fruit extracts regarding polyphenol content and antiradical activity, as well as their stability and microbiological quality.

Material and methods. The plant material consisted of fruit from four cultivars (Alleso, Korsor, Sampo, Samyl) of black elderberry (*Sambucus nigra* L.). The following were determined in fruit extracts: polyphenolic content (HPLC), antiradical activity (ABTS and DPPH) and stability and microbiological quality.

Results. The HPLC analysis of polyphenols demonstrated that the extracts from fruits collected from cv. Samyl had the highest 3-sambubioside cyanidin content and those from cv. Korsor contained the highest quantity of 3-glucoside cyanidin. The extracts from cv. Sampo fruit had a dominant 3-sambubioside-5-glucoside cyanidin and 3,5-diglucoside cyanidin content. The highest quercetin (5.92 mg 100 mg⁻¹ of extract) and caffeic acid (1.21 mg 100 mg⁻¹ of extract) content was found in fruit extracts from cv. Alleso. The cultivars Samyl and Korsor had a higher level of anthocyanins and higher antiradical activity (ABTS) in fruit extracts than cv. Alleso and Sampo. The antiradical activity (DPPH) of fruit extracts from elderberry cultivars assessed in this research was similar. The degradation index for all fruit extracts was similar (DI = 1.035). The microbiological species detected in extracts were classified as moulds (*Penicillium* sp., *Aspergillus* sp.) and yeasts (*Rhodotorula* sp., *Torulopsis* sp., *Trichosporon* sp., *Saccharomyces* sp.).

Conclusions. The research findings may support the selection of certain cultivars for industrial applications. The high stability of anthocyanins and low level of microbiological impurities in elderberry extracts ensure the high quality of such a raw material in food and pharmaceutical processing.

Keywords: ABTS, DPPH, elderberries, HPLC, microbiologia, stability

INTRODUCTION

Black elderberry (*Sambucus nigra* L.) belongs to the wide shrub with yellow-white flowers, which bloom in May and June. Its glossy black fruits are ripe in honeysuckle family *Caprifoliaceae*. It is a tall and

✉barbara.pliszka@uwm.edu.pl, phone +48 89 523 4447, fax 48 89 523 4801

August and September. Elderberry plants are native to Europe, North Africa and West Asia. Elderberry is a shrub cultivated in such countries as Denmark, the Czech Republic, Germany, Romania, the USA (Charlebois et al., 2010; Christensen et al., 2008). In Poland, Danish cultivars of black elderberry are distinguished by higher yields and superior morphological traits of fruit compared to wild forms (Ważbińska and Puczel, 2002). Elderberries are a valuable source of bioactive polyphenolic compounds, e.g. anthocyanins (3-glucoside cyanidin, 3-sambubioside cyanidin, 3-sambubioside-5-glucoside cyanidin, 3,5-diglucoside cyanidin, 3-rutinoside cyanidin), flavonols (quercetin, kaempferol, rutin), phenolic acids (e.g. cinnamic acid). The dominant anthocyanins in elderberries are cyanidin glycosides: 3-glucoside cyanidin and 3-sambubioside cyanidin (Dawidowicz, et al., 2006; Kaack and Austed, 1998; Kaack et al., 2008; Veberic et al., 2009). Figure 1 (drawn by the author) shows the structural formulas of the major cyanidin glycosides present in elderberries. Other compounds found in elderberries

are organic acids (e.g. malic, citric, shikimic, fumaric, malonic, valeric acids), sugars, pectins and vitamins C and B (Dawidowicz et al., 2006; Veberic et al., 2009). Elderberries have antibacterial, antifungal, anti-inflammatory, antiviral and pro-immunological properties (Zakay-Rones et al., 1995; Netzel et al., 2005). Fresh fruits are rarely consumed and they are most commonly processed into syrups and jams. Recently, there has been growing interest in elderberry extracts used as soft drinks and/or food additives (Netzel et al., 2005; Veberic et al., 2009). In the human body, the absorption of anthocyanins is probably more efficient when they are derived from extracts rather than from fresh fruits, in which these compounds are not extracted from plant cells (Wu et al., 2002). The pharmaceutical and food industries expect detailed knowledge on the physicochemical properties of elderberry fruit extracts, their stability and microbiological quality as well as the content of bioactive substances. It is also hoped that the concentrations of anthocyanins will remain stable during industrial processing. Presumably, the characteristics of the extracts might be additionally modified by citric acid, which improves the stability of anthocyanins and protects processed fruits and syrups from pathogenic microorganisms (Raybaudi-Massilia et al., 2009). Pliszka et al. (2016) documented the beneficial influence of citric acid on the polyphenol content and antiradical activity of extracts from berries. In this study, extraction of polyphenolic compounds from elderberry fruits was carried out in aqueous solutions of citric acid. The choice of this method was a consequence of the physicochemical characteristics of pigments in elderberry fruits. These pigments are not stable to the effect of light in alcoholic solutions (Pizło and Jankowska, 2001), which is why in this paper a water solution of citric acid was applied. The aim of the study was to analyze the properties of elderberry fruit extracts with regard to their level of polyphenolic compounds and their antiradical activity, as well as the stability and microbiological quality.

MATERIAL AND METHODS

Material

The plant material consisted of fruits from Danish cultivars (Alleso, Korsor, Sampo, Samyl) elderberry (*Sambucus nigra* L.) originating from a field

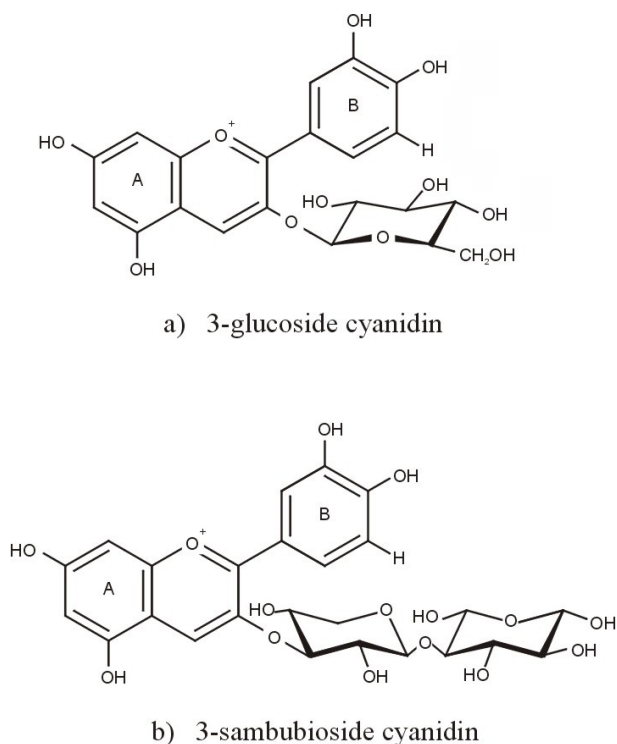


Fig. 1. Structure of cyanidin glycosides

experiment at the Research Station Garden, which belongs to the University of Warmia and Mazury in Olsztyn (Poland). These fruits were harvested 10 days after 90% of the fruits in umbels had been determined to be fully mature and black in colour. Samples of standards: 3-sambubioside cyanidin, 3-glucoside cyanidin, 3-sambubioside-5-glucoside cyanidin and 3,5-diglucoside cyanidin were obtained from the Department of Fruit and Vegetable Technology, Agricultural University of Wrocław (Poland). 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Merck. All other chemicals were of the highest grades from Merck.

Sample preparations

For each analysis, 20 g portions of elderberries in three replications were weighed. Frozen fruits were stored at -25°C . Prior to analysis, samples of fruits were defrosted by keeping them at room temperature for 2 hours, after which they were crushed in a mortar. A solution of citric acid measuring 300 cm^3 and at a concentration of 0.1 mol dm^{-3} was poured to each portion of the homogenized material. The samples were left at a temperature of 2°C in the dark for 2 hours. Afterwards, beakers holding the samples were shaken in a water bath at 37°C for 15 minutes. Next, the samples were centrifuged for 15 min at RCF equal 1790 g in order to separate the solid parts of the fruits from the extract. The subsequent shaking and centrifugation of the samples was performed with portions of the solvent (100 cm^3). Extraction continued until the red colour had disappeared, thus ensuring complete leaching of the pigment from the fruits. The volumes of the resulting extracts were aggregated into single samples and subjected to analysis. The fruit extracts were concentrated in a vacuum evaporator at a temperature of 40°C , after which they are purified in an SPE system with a Bondecil C_{18} load. A sample loaded onto a column was rinsed with acidic water (0.01% HCl), and then eluted with acidic methanol (0.01% HCl). Fractions of polyphenolic compounds were washed on Sephadex LH-20100 gel, using a $\text{CH}_3\text{OH}:\text{H}_2\text{O}:\text{HCl}$ (100:100:0.1) (v/v/v) mixture as an eluant. The resulting elates were concentrated in a vacuum evaporator at 40°C and subsequently lyophilized.

The HPLC analysis of polyphenols

Each sample prepared as above was dissolved in 0.5 cm^3 of buffer at pH 1. The sample was then centrifuged and analyzed on a Hitachi L-7455 liquid chromatograph equipped with a diode detector. The separation was performed on a LiChroCART® 125-3 Purospher® RP-18 column at the flow rate of $1\text{ cm}^3\text{ min}^{-1}$. Phenolic compounds were eluted according to the elution gradient: A (4.5% formic acid), B (80% acetonitrile and 20% solution of A). First, 100% A solution was supplied on the column, after which the gradient was applied until 16 minute, decreasing A to 20% and increasing B to 80%. Subsequently, 100% B solution was supplied from 17 to 24 min and then, from 25 to 35 min, application of 100% A solution followed in order to achieve equilibrium. Detections were performed at the following wavelengths: 320 nm for caffeic acid derivatives, 360 nm for quercetin derivatives and 520 nm for monomers of anthocyanins. Compounds were identified according to the retention times of the peaks compared with samples of the standards. The column temperature was 25°C . The results were presented as $\text{mg } 100\text{ mg}^{-1}$ of extract. HPLC analysis of elderberry fruit extracts was performed at the Department of Fruit and Vegetable Technology, Agricultural University of Wrocław (Poland).

Free radical scavenging ability by the use ABTS radical cation

The antiradical activity (ABTS) was determined with the Miller et al. method (1993) with certain modifications. The ABTS method involved generating $\text{ABTS}^{\bullet+}$ cation radical, the formation of which was inhibited by adding an antioxidant. The fruit extract (20 mL) was mixed with 1 cm^3 reagent ($\text{ABTS} - 610\text{ mmol/L}^{-1}$, metmyoglobin – $6.1\text{ mmol}\times\text{L}^{-1}$ and buffer – $5\text{ mmol}\times\text{L}^{-1}$) and the reaction was initiated by the addition 200mL of hydrogen peroxide ($250\text{ mmol}\times\text{L}^{-1}$). The absorbance was measured at 37°C , after a 6-minute incubation, at $\lambda = 734\text{ nm}$, using a Shimadzu UV-1800 spectrophotometer. The determinations were performed with reference to the control reagent (deionized water) and Trolox standard ($1.65\text{ mmol}\times\text{L}^{-1}$). The results were converted to a Trolox equivalent ($\text{mmol Trolox}\times\text{dm}^{-3}$).

Free radical scavenging ability by DPPH radical

The antiradical activity (DPPH) was determined by the Yen and Hung method (2000) with a slight modification. A fruit extract (0.5 cm³) in 4 cm³ of distilled water/methanol (1:3, v/v) was added to a 1 cm³ solution of DPPH (2.5 mmol×L⁻¹) in methanol. The mixture was shaken and left to stand in the dark at room temperature. Absorbance was measured after 30 minutes at $\lambda = 517$ nm using a Shimadzu UV-1800 spectrophotometer. The results were expressed as an inhibition percentage. Inhibition of free radical DPPH in percent (I%) was calculated as follows:

$$I, \% = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100\%$$

Analysis of anthocyanin stability

Extracts from fruits were kept in cooling conditions (2°C) for 0, 7, 14 days. After 14 days, microorganisms appeared (turbidity). Samples were prepared by diluting 1 cm³ of the extract to 25 cm³ with a buffer at pH 1, and by diluting 1 cm³ of the extract to 5 cm³ with a buffer at pH 4.5. Diluted extracts were maintained in the dark at room temperature for 2 hours. Absorbance was measured in extracts at $\lambda_{\text{max}} = 508$ nm using a Shimadzu UV-1800 spectrophotometer. The degradation index (DI) of these extracts was calculated using the formula presented by Fuleki and Francis (1968).

Analysis of microbiological impurities 5 cm³ volumes of extracts were placed on a YFC agar selective medium and the cultures were incubated at a temperature of +30°C for 4 days. The cultures obtained were transferred onto multiplication media. Detection of bacteria was accomplished based on: the morphology of colonies, morphology of cells, staining (the Gram method) and the ability to form endospores (Krieg and Holt, 1994). The type of mould was identified by microscopic observation of the morphology of colonies, coloration of mycelia, formation of chlamydo-spores, structure of conidiophores, shape and colour of spores, and by comparing the observations with the descriptions and drawings available in relevant literature (Fassatióva, 1983). The determination of the total count of yeast fungi was performed according to the morphological traits of cells, i.e. type of multiplication, ability to form sacs with spores and the number

of pores, as well as the ability to form pseudomycelia (Rose and Harrison, 1970).

Statistical analysis

The results were verified in three replications. The data regarding the polyphenolic compound content, the antiradical activity and the degradation index (DI) of the extract were submitted to statistical analysis of variance for univariate experiments (the Duncan test) using Statistica 12 PL (StatSoft PL) software ($\alpha = 0.05$).

RESULTS

Results pertaining to the polyphenol content of the extracts from the analyzed fruit cultivars are presented in Table 1. The HPLC analysis demonstrated the dominant contribution of cyanidin glycosides to the general composition of anthocyanins: 3-sambubioside cyanidin, 3-glucoside cyanidin, 3-sambubioside-5-glucoside cyanidin, 3,5-diglucoside cyanidin. The extracts from fruits collected from cv. Samyl had the highest 3-sambubioside cyanidin content, while extracts from fruits of cv. Korsor contained the lowest content of this compound. The extracts from fruits harvested from cv. Korsor contained the highest level of 3-glucoside cyanidin, while extracts from fruits picked from cv. Alleso and Sampo had the lowest content of this compound. The extracts from cv. Sampo fruit had a dominant content of 3-sambubioside-5-glucoside cyanidin and 3,5-diglucoside cyanidin. The Samyl and Korsor cultivars were classified as producing fruits with a higher anthocyanin content in extracts (*ca* 40 mg 100 mg⁻¹ of extract). Extracts from fruit yielded by the Alleso and Sampo cultivars were classified into the group with a lower anthocyanin content (*ca* 30 mg 100 mg⁻¹ of extract). The highest content of quercetin and caffeic acid derivatives was found in fruit extracts from cv. Alleso, while extracts from cv. Sampo fruits had the lowest quercetin content and extracts from cv. Samyl fruit contained the least caffeic acid (Table 1).

The antiradical activity of fruit extracts established with the ABTS and DPPH methods is presented in Table 2. Extracts of fruits from cv. Samyl and Korsor had a higher antiradical activity than extracts of fruits from cv. Alleso and Sampo. Extracts from elderberry fruit

Table 1. Content of polyphenolic compounds in elderberry fruit extracts

Cultivar	Anthocyanins				Quercetin derivatives Caffeic acid derivatives	
	Total	3-sambubioside cyanidin	3-glucoside cyanidin	3-sambubioside- 5-glucoside cyanidin and 3,5-diglucoside cyanidin		
mg×100 mg ⁻¹ extract						
Alleso	31.86 b	16.84 b	14.22 c	0.80 b	5.92 a	1.21 a
Korsor	40.69 a	14.13 bc	25.86 a	0.70 c	1.61 b	1.01 b
Sampo	30.34 b	15.78 b	13.52 c	1.04 a	0.27 c	0.56 c
Samyl	41.78 a	20.83 a	20.11 b	0.84 b	1.54 b	0.36 d

Mean values denoted by the same letters in the column do not differ significantly at $\alpha = 0.05$.

Table 2. Antiradical activity in elderberry fruit extracts

Cultivar	Antiradical activity	
	ABTS	DPPH
	mmol Trolox×dm ⁻³	% inhibition
Alleso	2.68 b	88.17 a
Korsor	2.83 a	88.50 a
Sampo	2.64 b	88.22 a
Samyl	2.88 a	88.47 a

Mean values denoted by the same letters in the column do not differ significantly at $\alpha = 0.05$.

were characterized by high scavenging of DPPH free radicals, from 88.17% to 88.50%.

The degradation index (DI) of anthocyanins in fruit extracts is illustrated in Figure 2. The DI value increased from 1.034 to 1.036, indicating that the changes associated with the degradation of anthocyanins in the extracts were relatively small. The average value for all fruit extracts was similar (DI = 1.035). In this study on the stability of anthocyanins in cool-stored elderberry fruit extracts, colonies of microorganisms began to appear, which inclined us to submit these extracts to microbiological analysis.

Microbiological species found in the extracts are presented in Table 3. Cultures from incubated extracts

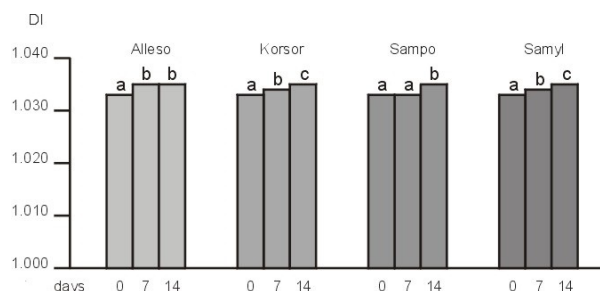


Fig. 2. Degradation index of anthocyanins (DI) in elderberry fruit extracts stored under cooling conditions. Explanation: mean values denoted by same letters in the column do not differ significant at $\alpha = 0.05$

did not reveal any live vegetative forms of bacteria (*Bacillus* sp.). In the repeated cultures of incubated extracts from elderberries, microorganisms were isolated that were able to develop and multiply in a highly acidic environment, which caused turbidity of the extracts and the formation of a gelatinous sediment. The microorganisms detected were classified as moulds (*Paecilomyces* sp., *Penicillium* sp. and *Aspergillus* sp.) and yeasts (*Rhodotorula* sp., *Torulopsis* sp., *Trichosporon* sp. and *Saccharomyces* sp.). No moulds of the genus *Paecilomyces* sp. were isolated from the cultures of incubated extracts, and mostly yeasts (*Rhodotorula* sp., *Torulopsis* sp., *Trichosporon* sp. and *Saccharomyces* sp.) were able to develop in the extracts.

Table 3. Microbiological species in elderberry fruit extracts

Cultivar	Bacteria	Moulds			Yeasts			
	<i>Bacillus</i> sp.	<i>Paecilomyces</i> sp.	<i>Penicillium</i> sp.	<i>Aspergillus</i> sp.	<i>Rhodotorula</i> sp.	<i>Torulopsis</i> sp.	<i>Trichosporon</i> sp.	<i>Saccharomyces</i> sp.
Alleso	–	–	–	+	+	–	–	–
Korsor	–	–	+	–	–	+	+	–
Sampo	–	–	–	–	–	–	+	+
Samyl	–	–	+	+	–	+	–	–

Explanation: – no microbiological species in 1 cm³ of extract, + microbiological species in 1 cm³ of extract.

DISCUSSION

The results of this study suggest that extracts from elderberry fruits contained varied levels of cyanidin glycosides and caffeic and quercetin derivatives. The Samyl and Korsor cultivars were classified as producing fruits with a higher anthocyanin content in extracts. The study by Kaack and Austed (1998) resulted in a different specification of cyanidin glycosides and different total content of anthocyanins in fruit of cv. Alleso, Korsor, Sampo, Samyl. These authors reported that the prevalent content of 3-sambubioside cyanidin was determined only in fruits of cv. Alleso, while fruits of other elderberry varieties had a dominant 3-glucoside cyanidin content. In turn, higher content of anthocyanins were detected in the fruits of the Sampo and Samyl cultivars than in the fruits of the Korsor and Alleso cultivars. The presence of anthocyanins in elderberry fruits, in the form of cyanidin glycosides (3-sambubioside cyanidin, 3-glucoside cyanidin, 3-sambubioside-5-glucoside cyanidin and 3,5-diglucoside cyanidin), has been verified by other authors (Lee and Finn, 2007; Veberic et al., 2009). On the other hand, Lee and Finn (2007) detected trace quantities of 3-glucoside pelargonidin in the fruits of cv. Korsor, which was not confirmed in this study. In this research, the highest quercetin and caffeic acid derivative content was found in fruit extracts from cv. Alleso (5.92 mg and 1.21 mg 100 mg⁻¹ of extract, for quercetin and caffeic acid, respectively). In general, a small content of these compounds was determined in extracts from fruit collected from cv. Sampo shrubs. These results support the findings reported by Kaack

and Austed (1998), who analyzed quercetin in elderberry fruits. Lee and Finn (2007) indicated the presence of cinnamic acids in the fruits of cv. Korsor. Jakobek et al. (2007), who carried out research on *Sambucus nigra*, concluded that fruits contained small quantities of quercetin, caffeic acid and *p*-coumaric acid.

In this study, the HPLC analysis of polyphenolic compounds demonstrated the presence of cyanidin glycosides, as well as quercetin and caffeic acid derivatives in extracts from elderberry fruits. All these compounds are known for their antioxidant properties (Jakobek et al., 2007). The antiradical activity (ABTS) of fruits is directly connected with the content of anthocyanins in elderberry fruits. The Samyl and Korsor cultivars were classified as producing fruits with a higher content of anthocyanins and antiradical activity in extracts. The antiradical activity (DPPH) of fruit extracts from elderberry cultivars assessed in this research was similar. However, the available literature lacks information on the antiradical activity of the fruits of the elderberry cultivars analyzed in this research. The antiradical activity (ABTS) of wild forms of elderberry fruits was 2.37 mmol TE 1 g⁻¹ extract (Salvador et al., 2017). Research conducted by Jakobek et al. (2007) suggests that wild elderberry possesses strong antiradical activity (100.16 mmol TE 1 g⁻¹) in the DPPH method.

Degradation index (DI) of anthocyanins is a parameter which determines the stability of anthocyanins in extracts. It is defined as a ratio of anthocyanins which have undergone degradation to all anthocyanins in a sample. A higher DI value indicates the decreased stability of anthocyanins in an analyzed sample

(Fuleki and Francis, 1968). In this paper, the degradation index (average values of $DI = 1.035$) was similar for all fruit extracts, thus indicating that the stability of anthocyanins in the extracts did not depend on the cultivars of elderberry. Pliszka et al. (2009), while analyzing the stability of anthocyanins in extracts from red cabbage, found that the DI value depended on red cabbage cultivars. The authors attributed this relationship to a slightly different composition and structure of anthocyanins in red cabbage cultivars. Conversely, the current study showed that elderberry fruit extracts, irrespective of the elderberry cultivar, contained anthocyanins of a similar chemical structure (cyanidin glycosides), which may have affected the DI. The DI value may also depend on the fruit species and methods applied to prepare the material to be analyzed. The DI value determined in cranberry concentrates was 1.70, while in grape concentrates it was 1.16 (Sapers et al., 1981). The findings reported by Vatai et al. (2009) prove that the DI was lowered by performing extraction with a solvent containing a natural antioxidant, which meant a higher stability of anthocyanins, for example, in grape extracts. In this paper, the results show that the DI of extracts from elderberry fruit was closest to the DI determined in extracts of chokeberry, highbush blueberry and European blueberry determined by Pliszka et al. (2013). Additionally, the results of HPLC assays reported in this paper demonstrated that 3-sambubioside cyanidin and 3-glucoside cyanidin were the dominant anthocyanins in extracts of elderberry fruits. They were probably responsible for the stability of anthocyanin dyes. Most fruits contain a mixture of several anthocyanins, one or two of which dominate over the others and play a decisive role in the stability of the colour of fruits. Research carried out by Drdak and Daucik (1990) suggests that 3-sambubioside cyanidin was the most stable anthocyanin during technological processes, whereas 3-glucoside cyanidin disintegrated, for example, during the alcoholic fermentation of elderberries.

In this study, our analysis of the extracts showed a very low degree of contamination with microbiological species (moulds and yeasts). Spores of *Penicillium* sp. and *Aspergillus* sp. are airborne and represent the most common type of microbiological contaminants in raw food products, as well as in the materials, packaging and rooms used for food processing. Both fresh

and processed fruits can contain moulds of the genera *Penicillium* sp., *Aspergillus* sp., *Paecilomyces* sp., *Eurotium* sp., *Alternaria* sp., *Cladosporium* sp. and *Botrytis* sp., as well as yeasts such as *Saccharomyces* sp., *Rhodotorula* sp. and *Cryptococcus* sp. (Jay et al., 2005). Pliszka et al. (2013) obtained similar results when analyzing microbiological contamination of extracts from fruits of chokeberry, highbush blueberry and European blueberry. Regarding this study, it can be supposed that elderberry fruit extracts inhibited the development of bacteria (including *Bacillus* sp.). The antibacterial properties of polyphenolic compounds in plant extracts depend on the type of microorganisms (Karou et al., 2005). Puupponen-Pimiä et al. (2005) concluded that fruits rich in polyphenolic compounds, e.g. cranberry, raspberry, strawberry and European blueberry prevented the spread of *Salmonella* and *Staphylococcus*. Accessed references did not provide us with any information about the antimicrobial properties of extracts from elderberry fruits.

CONCLUSIONS

The research findings may support the selection of certain elderberry cultivars for industrial applications. The high stability of anthocyanins and low degree of microbiological impurities in elderberry extracts ensures the high quality of such raw material in food and pharmaceutical processing. Further and more in-depth research into the improvement of analytical methods for determining the stability and microbiological quality of extracts is required before they are implemented in the food production and food preservation.

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