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Antioxidant Activity of Elderberry Fruits during Maturation

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Abstract: Antioxidant activity (AOA) in fruits of *Sambucus nigra* and several new elderberry interspecific hybrids involving *S. javanica* (JA), *S. nigra* subsp. *nigra* (NI), *S. nigra* subsp. *nigra* 'Black Beauty' (BB), *S. nigra* subsp. *cerulea* (CER) and *S. racemosa* (*S. racemosa* subsp. *racemosa* (RAC), *S. racemosa* subsp. *racemosa* var. *miquelii* (MIQ), *S. racemosa* subsp. *tigranii* (TIG), *S. racemosa* subsp. kamtschatica—*coreana* (KOR) at five maturity stages were investigated by applying DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorption capacity) assays. The aim of the study was to compare different assays for estimating AOA in elderberries, to plan the appropriate harvest time and to determine whether the most promising genotypes could be predicted in advance. All assays used showed that AOA increased significantly during maturation. All assays were significantly correlated with each other and could be reliably used for the AOA determination of elderberries. In fully mature berries, significant correlations were also found between total phenolic (TP) content and AOA by all assays, but no significant correlations were found between total ascorbic acid (TAA) content and AOA by all assays. At the fully mature stage, (the most appropriate stage for harvesting), berries of (JA × NI) × BB and (JA × NI) × MIQ were found to be superior in AOA and TP content. Genotypes with higher/lower AOA and TP content could not be predicted in advance, while TAA showed the opposite.

Keywords: *Sambucus*; interspecific hybrids; maturation; ABTS; DPPH; ORAC; FRAP; total phenolic content; ascorbic acid; correlations

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1. Introduction

Antioxidants are compounds that retard or inhibit the production of free radicals produced by oxidation processes. Free radicals are reactive and unstable molecules or complexes whose chain reactions pass through the enzymatic system and can damage the cells of an organism and cause various diseases [1]. Naturally existing endogenous enzymatic and non-enzymatic antioxidants protect human organism from the reactive oxygen species and their harmful effects. When they are unable to provide complete protection, the need for exogenous antioxidants such as vitamins (E, C, D, K_3), ß-carotene, mineral Se and flavonoids increases [2]. Although many synthetic compounds are available in the market, there is an increasing interest in exogenous antioxidants of natural origin that have beneficial nutritional and therapeutic effects. Moreover, many synthetic antioxidants are no longer used in the food industry due to their potential adverse effects on human health and the stringent control that requires expensive testing to evaluate their safety [3]. The search for natural substitutes for synthetic antioxidants has led to the antioxidant evaluation of numerous plant sources. According to Halvorsen et al. [4], among 50 foods analysed that were high in antioxidants, five were berries. Considering typical portion sizes, the authors found that several berries (blackberries, strawberries, cranberries, raspberries and blueberries) and related products (e.g., juice from grape berries) were high on the list of foods high in antioxidants. According to Jakobek et al. [5], Jabłonska-Rys et al. [6] and Tarko et al. [7], elderberry fruits also ranked high in antioxidant activity compared to other

commonly used fruits and berries such as blackberries, strawberries, red raspberries, sweet and sour cherries, red and black currants, bilberries, apples, goji berries and cranberries. In addition, juices from some previously mentioned berries had lower antioxidant activity than elderberry fruit juice [8–12].

Elderberries (genus *Sambucus*) grow as small trees, shrubs or herbs and are widely distributed throughout the world, especially in temperate and subtropical parts of the northern hemisphere [13]. The genus is morphologically complex and characterised by great variability between and within the species. After the last available revision by Bolli [14], the number of recognised species was reduced from 30 to 9. The most widespread species in Europe, European black elderberry (*Sambucus nigra* subsp. *nigra*), was recognised as separate species with five subspecies, including the most widespread species in the United States, the American black elderberry (*S. nigra* subsp. *canadensis*). According to the available literature, the inflorescences and fruits of these two subspecies have been studied most frequently. In general, the chemical composition of elderberries varies among species and depends on environmental conditions and maturity stage [15–21]. However, they are known for their high content of some organic compounds (e.g., sugars, organic acids, ascorbic acid, phenolic compounds) [6,22–33] that could improve the nutritional value and organoleptic properties of the food. According to data from SPINScan Natural and Information Resources Inc., published by Cavaliere et al. [34], elderberry supplements ranked 8th (over $6.8 million) among the top 20 best-selling botanical dietary supplements in the United States. They increased nearly 50% in 2009 compared to 2008 in the health and natural food channels, including grocery stores, drugstores and mass market retailers, convenience stores, media and network sales and health professionals. Considering only grocery stores, drugstores and mass market retailers, the sales of elderberry supplements also remained relatively stable in 2009 compared to 2008. Increasing consumer awareness of their own health has sparked the interest in elderberry fruits, not only because of their favourable chemical composition but also due to their health benefits, including antibacterial, anti-inflammatory, antidiabetic, protective and antioxidant properties [35].

Various analytical assays are used to evaluate antioxidant activity (AOA). Those based on gas and liquid chromatography, electrochemistry (cyclic voltammetry, amperometry, biamperometry) and spectrometry (DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), FRAP (ferric reducing antioxidant power), PFRAP (potassium ferricyanide reducing power), CUPRAC (cupric reducing antioxidant capacity), ORAC (oxygen radical absorption capacity), HORAC (hydroxyl radical antioxidant capacity), TRAP (total radical trapping antioxidant potential), fluorimetry) are well known [2]. Most spectroscopic techniques are based on colour change, some on measurement of fluorescein fluorescence loos, while others quench chemiluminescence or detect excitation of fluorescence. FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to the intensely blue coloured iron and TPTZ complex $[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$. On the other hand, ABTS radical scavenging test and DPPH radical scavenging test are characterised by discoloration in the presence of antioxidants. After the reaction of ammonium persulfate, ABTS is converted to its radical cation, $ABTS^{\bullet+}$, which turns from dark to light green colour when antioxidants are added. On the other hand, the DPPH radical turns from dark purple to yellowish in the presence of antioxidants. ORAC assay is based on the measurement of antioxidant scavenging activity against peroxyl radical induced by 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) at 37 °C. The spectroscopic techniques mentioned above are generally not directly comparable. They are characterised by different reaction media, sensitivity and working mechanisms (electron transfer or hydrogen atom transfer). In addition, radicals have different antioxidant potentials when reacting with phenolic compounds, they may dissolve differently, and they may have different affinities for other compounds present in the samples. The methods also differ in their endpoint, quantification method and the possibility of measuring both lipophilic and hydrophilic antioxidants [36,37]. However, according to many authors [38–41], ABTS,

FRAP, DPPH and ORAC assays show similar trends and are significantly correlated with each other.

The AOA in fruit extracts of the most commonly used *Sambucus* species, *S. nigra* subsp. *nigra* [22,25,26] and *S. nigra* subsp. *canadensis* [29,30] has been studied previously. However, little is known about the antioxidant activity of maturing elderberry fruits [15,16] and even less about the antioxidant activity of fruits of elderberry interspecific hybrids [42]. Therefore, the purpose of the present study was to compare different assays (ABTS, DPPH, FRAP and ORAC) for estimating antioxidant activities in the extracts of maturing fruits of elderberry interspecific hybrids. The aim of this study was also to determine the superior genotypes in AOA and to investigate whether the most promising genetic combinations in terms of AOA, total phenolic and ascorbic content could be predicted in advance.

2. Materials and Methods

2.1. Plant Material

Twenty elderberry bushes from the Plant Gene Bank of the University of Maribor, Faculty of Agriculture and Life Sciences in Hoče near Maribor, Slovenia, were included in the present study. The plant material included the species *Sambucus nigra* (NI) and fourteen elderberry interspecific hybrids involving *S. javanica* (JA), *S. nigra* (NI), *S. nigra* 'Black Beauty' (BB), *S. cerulea* (CER) and *S. racemosa* (RAC). Some of them were represented by several bushes. The names *S. nigra*, *S. cerulea* and *S. racemosa* correspond to the names *S. nigra* subsp. *nigra*, *S. nigra* subsp. *cerulea* and *S. racemosa* subsp. *racemosa* respectively, according to the revised classification of Bolli [14]. *S. nigra* subsp. *nigra* also includes the cultivated variety named 'Black Beauty' (BB) (characterised by purple leaves and inflorescences). *S. racemosa* is represented by subsp. *racemosa* in the narrow sense (a local, wildly grown genotype (RAC), *S. racemosa* subsp. *racemosa* var. *miquelii* (MIQ), *S. racemosa* subsp. *tigranii* (TIG) and the taxon *coreana* (KOR) belonging to *S. racemosa* subsp. *kamtschatica*. C1 means the first clonal generation (plants developed by cuttings taken on the plant originated from seed).

Elderberries were collected in 2017 (between 7 September and 15 October) from three- to four-year-old bushes containing berries at different stages of maturity. The maturity stages were defined according to the fruit exterior colour and the percentage of fully coloured berries on the infructescence. Infructescences were classified into five groups (Figure 1): UR (containing unripe, green coloured berries that reached their final size); EM (containing berries at early stage of maturity—the exterior of approximately 30% of the berries exhibited the final (in most cases dark purple) colour); AM (containing almost mature berries—the exterior of approximately 70% of berries exhibited the final colour); FM (containing fully mature berries—the exterior of all berries exhibited the final colour (optimal maturity for harvest)); OM (containing overmature berries—the majority of berries were shrivelled due to the loss of moisture).

Immediately after harvest, infructescences were frozen in liquid nitrogen and the fruit stalks were removed. Approximately 100 g of berries were freeze-dried using Christ Alpha 1–2 LD (Vacuumbrand GMBH, Germany) freeze dryer, crushed into a fine powder, vacuum packed and stored at −80 °C until analysis.

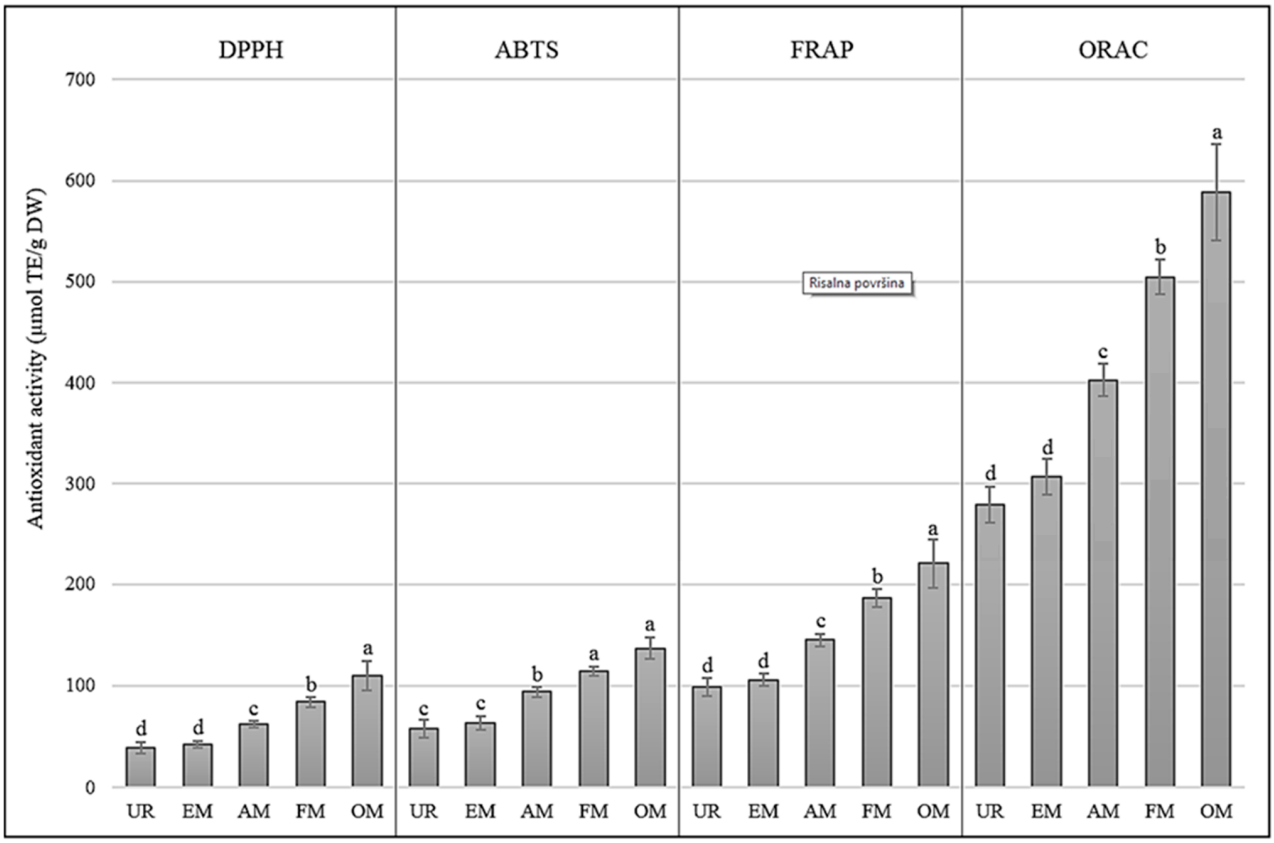

Figure 1. Means and standard errors of means for antioxidant activity of maturing fruits of elderberry interspecific hybrids determined by DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorption capacity) assays, respectively. Different letters (a, b, c, or d) above the bars of the same assay indicate significant differences between maturity stages (Tukey, $p \leq 0.05$). Maturity stages of infructescences were defined as UR (containing unripe green coloured berries that reached final size); EM (containing berries at early stage of maturity—the exterior of approximately 30% of berries exhibited the final colour); AM (containing almost mature berries—the exterior of approximately 70% of berries exhibited the final colour); FM (containing fully mature berries); OM (containing overmature berries).

2.2. Chemicals and Reagents

The following chemicals (Sigma-Aldrich, St. Louis, Missouri, USA) were used for antioxidant activity assays: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate ($K_2O_8S_2$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium acetate ($CH_3COONa \cdot 3H_2O$), acetic acid, sodium fluorescein salt, 2,2′-azobis(2-amindinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), Na_2HPO_4, NaH_2PO_4, hydrochloric acid and methanol.

2.3. Extraction

The extraction procedure was performed as described by Ochmian et al. [24], with slight modifications. To 0.40 g of sample, 4 mL of an extraction solution consisting of methanol: water: acetic acid (80:20:0.1, $v/v/v$) was added. The suspension was sonicated for 15 min and centrifuged at 7500 rpm. The extraction procedure was repeated twice, and the combined supernatants were diluted in a 10 mL volumetric flask.

2.4. Determination of Antioxidant Activity

The ABTS assay was performed according to the method of Mikulic-Petkovsek et al. [42] with slight modifications. First, 1 mL of diluted $ABTS^{\bullet+}$ solution was added to 50 µL of

diluted elderberry extract. The absorbance was measured after 6 min of incubation in the dark at room temperature at 734 nm. The results of the assay were expressed in µmol Trolox equvivalents per g of dry weight (µmol TE/g DW).

DPPH radical scavenging activity was determined as described by Duymus et al. [43] with slight modifications. Diluted elderberry extracts (100 µL) were reacted with 3.9 mL of freshly prepared methanol-DPPH solution. The mixture was incubated in the dark at room temperature for 30 min. After the incubation period, the absorbance at 517 nm was recorded. The inhibition percentage (IP) was calculated using the equation: IP = $[(A_{DPPH} - A_{sample})/(A_{DPPH})] \times 100$. According to IP, the antioxidant activity of each sample was calculated and expressed as µmol TE/g DW.

The FRAP assay was performed according to Granato et al. [8] with slight modifications. Three solutions (300 mM acetate buffer, 10 mM TPZT in 40 mM HCl and 20 mM ferric chloride hexahydrate) were prepared and combined (10:1:1, *v*/*v*/*v*) to prepare the FRAP reagent prior to analysis. FRAP reagent was incubated at 37 °C for 10 min and added to 100 µL of each sample extract. After 30 min of incubation at 37 °C in the dark, absorbance was measured at 593 nm. The results were expressed as µmol TE/g DW.

ORAC was performed according to Huang et al. [44] with slight modifications. A stock solution of fluorescein (FL_{ss}) (1.33×10^{-3} mM) was prepared in a 75 mM phosphate buffer and stored in the dark at 5 °C. Before analysis, FL_{ss} was diluted with phosphate buffer to 8.16×10^{-5} mM fluorescein working solution (FL_{ws}). The AAPH solution (153 mM) was prepared fresh daily in 75 mM phosphate buffer and stored in the dark in an ice bath. Before analysis, AAPH was warmed at 37 °C. When using the plates, the outer wells were not used for experimental determinations and were filled with 250 µL of water. The inner (experimental) wells were filled with standards (25 µL of Trolox dilution), samples (25 µL), blanks (25 µL of phosphate buffer), and controls (50 µL of phosphate buffer). To all experimental wells, 150 µL of FL_{ws} was added. The plate was then placed in the TECAN INFINITE M1000 PRO microplate reader and incubated for 10 min at 37 °C. After incubation, 25 µL of pre-warmed AAPH was added to all wells to initiate the reaction. Fluorescence was then monitored kinetically, with data recorded every minute for a period of 90 min. ORAC values were calculated according to Cao et al. [45] The results were expressed as µmol TE/g DW.

2.5. Determination of Total Phenolic Content and Total Ascorbic Acid Content

In maturing fruits of elderberry interspecific hybrids, the total phenolic content and total ascorbic content were determined as described in the work of Imenšek et al. in press [46]. In the present study, the obtained results were used only for the calculations of correlations.

2.6. Statistical Analysis

Statistical analysis was conducted using the software package R (Rx64 3.2.5) and IBM SPSS Statistics 25. Group means and standard errors of means were calculated for all numerical variables. To determine differences in AOA determined by different assays among five maturity stages of elderberries, we applied a linear mixed effects model, using the lmer function in the lme4 package [47]. "Maturity stage" was defined as a fixed factor, "plant-ID" as a random factor and time was considered as a covariate. Multiple comparisons were tested using Tukey's test in the emmeans package [48]. Correlations between observed variables were estimated using *Spearman's* rank correlation coefficient. Ward's method of hierarchical clustering was used to group genotypes into homogeneous subgroups for similarities of the quantitative variables.

3. Results and Discussion

3.1. Antioxidant Activity during Elderberry Maturation Process

To determine the AOA of elderberry fruits at five maturity stages, ABTS, DPPH, FRAP and ORAC assays were applied, and the results are shown in Figure 1. For all assays,

AOA increased during maturation of elderberry fruit. The lowest average values at all maturity stages were obtained with the DPPH assay (from 38.8 ± 5.8 in the extracts of unripe berries to 109 ± 14 μmol TE/g DW in overmature berries), followed by ABTS (from 57.4 ± 8.8 to 137 ± 11 μmol TE/g DW) and FRAP values (from 99.1 ± 8.8 to 221 ± 24 μmol TE/g DW). The highest values were obtained with the ORAC assay (from 279 ± 18 to 588 ± 48 μmol TE/g DW). For each assay used, unripe green (UR) elderberries and those at early stage of maturity (EM) were characterised by significantly lower AOA compared to berries at later maturity stages. Almost mature berries (AM) showed significantly lower AOA compared to fully mature (FM) berries for each assay, and overmature berries (OM) were characterised by significantly higher AOA compared to fully mature (FM) berries according to DPPH, FRAP and ORAC assays, while ABTS assay showed no differences. A similar increasing pattern of AOA during maturation was previously reported for cherry fruit [49], mandarin melon berries [50], ginseng berries [51], black and red currants [52] and blackberries [53,54]. The authors explained the increase in AOA during fruit maturation by the concomitant increase in anthocyanins and other phenolic compounds that contribute strongly to AOA [55]. Elderberries are known for their high content of phenolic compounds, especially anthocyanins [56,57]. Since these compounds showed an increasing pattern during the maturation process of elderberries [15–17,19,46], a similar pattern could be explained for AOA.

Comparing the AOA of fully mature elderberries with some previous reports on elderberries, our results, when expressed on fresh weight (22.6 μmol TE/g for ABTS, 16.6 μmol TE/g for DPPH, 36.9 μmol TE/g for FRAP and 100 μmol TE/g for ORAC) are in agreement with those reported by Mikulic-Petkovsek et al. [42], Jabłonska-Rys et al. [6], Jakobek et al. [5] for the ABTS assay, Tarko et al. [7] for the DPPH assay, Akbulut et al. [22], Perkins-Veazie et al. [30] for the FRAP assay and Wu et al. [58] for the ORAC assay. The conclusions of the authors were similar, describing elderberries as a raw material with relatively high AOA that could be suitable for use in dietary supplements and the pharmaceutical industry.

The results of the present work showed that the AOA of elderberries was found at each maturity stage in ascending order of DPPH, ABTS, FRAP and ORAC values. The same results have been reported for the AOA of kiwifruit [38] and some Mediterranean edible plants [59]. Our results are also in partial agreement with those of Jimenez et al. [60] who reported higher ORAC values compared to ABTS, DPPH and FRAP values of buriti, but lower FRAP values compared to DPPH and ABTS values. Similar findings were reported by Candido et al. [39] for some medicinal plants. Regarding elderberries, our results are in agreement with those reported by Ozgen et al. [29] who found that FRAP values of fully mature elderberries were proportionally higher than DPPH assay values. The AOA of the samples depends not only on the composition of the extract but also on the conditions of the assay used [61]. DPPH, ABTS and FRAP are based on SET reaction mechanisms (Single Electron Transfer), which could help explain the similarity of their results. However, each method only provides an estimate of AOA based on their reaction reagents and conditions [41]. The redox potential of $ABTS^{\bullet+}$ is similar to that of Fe (III)-TPTZ, so similar compounds react in the ABTS and FRAP assays. On the other hand, their reaction pH is different (3.6 for FRAP and neutral for ABTS). Since lower pH causes the increase of the redox potential and facilitates electron transfer, FRAP assay values could be higher than ABTS assay values, although ABTS can determine the AOA of hydrophilic and lipophilic antioxidants [62]. Differences between assays may also occur due to different times for radical generation and reaction competition. $ABTS^{\bullet+}$ radicals require up to 16 h to be generated, whereas $DPPH^{\bullet}$ and Fe (III)-TPTZ can be generated just prior to assay. The reaction of the ABTS assay is complete in 4–6 min, while FRAP and DPPH assay usually require a longer time (30 min) [36]. The DPPH assay might have a lower yield compared to the ABTS assay, probably due to the slower reaction of $DPPH^{\bullet}$ with most antioxidants and lower sensitivity [63]. Although both the ABTS and ORAC assays allow the measurement of AOA of hydrophilic and lipophilic fractions, the ORAC assay values were expected to

be the highest because this assay is based on the HAT (Hydrogen Atom Transfer) reaction mechanism, which is pH and solvent independent [36] and is considered to be more sensitive and accurate than other assays studied [39]. Even though DPPH, ABTS, FRAP and ORAC assays have different reaction mechanisms and do not necessarily measure the same activity [36], they clearly showed that the examined fruits of elderberry interspecific hybrids possess variable but considerable antioxidant activities.

3.2. Correlations of AOA Based on Different Assays with Total Phenolic (TP) Content and Total Ascorbic Acid (TAA) Content

Correlations between DPPH, ABTS, FRAP and ORAC assays were calculated for unripe (UR) and fully mature (FM) elderberries to determine if the assays used provide comparable results for AOA in elderberry fruits. Correlations between AOA, the total phenolic (TP) content and total ascorbic acid (TAA) content were also reported for both maturity stages. The results showed that for UR berries the correlations between DPPH, ABTS, FRAP and ORAC were significant and positive (Table 1). On the other hand, only AOA determined by FRAP assay was significantly correlated with TP content in the extracts of UR berries (correlation coefficient 0.734), and AOA determined by ABTS assay was significantly correlated with TAA content. The results were slightly different for FM elderberries (Table 2). We also found significant and positive correlations between all assays, but the correlation coefficients were generally higher than those for UR berries. For FM berries, the results also showed that AOA determined by DPPH, ABTS, FRAP and ORAC assays were significantly correlated with TP content (correlation coefficients 0.544, 0.641, 0.635 and 0.626, respectively), while no significant correlations were found between TAA content and AOA determined by all assays. These results are in agreement with some other reports. Namely, DPPH, ABTS, FRAP and ORAC assays were used for AOA determination in the extracts of buriti fruits [39], sorghum [64], some medicinal plants [60] and other plant extracts [61]. Studies revealed that all the assays used showed a similar pattern and were significantly correlated with each other. In most cases, the authors also agreed that the correlations between assays based on similar mechanisms were stronger than those between assays based on different mechanisms.

Table 1. Spearman correlation coefficients between antioxidant activity (AOA) assays, total phenolic (TP) content and total ascorbic acid (TAA) of unripe green (UR) fruits of elderberry interspecific hybrids.

	DPPH	ABTS	FRAP	ORAC	TP
ABTS	0.633 **				
FRAP	0.529 *	0.698 **			
ORAC	0.524 *	0.851 **	0.576 **		
TP	0.070	0.259	0.734 **	0.254	
TAA	0.409	0.594 **	0.352	0.286	0.183

* sig. < 0.05, ** sig. < 0.01.

Table 2. Spearman correlation coefficients between antioxidant activity (AOA) assays, total phenolic (TP) content and total ascorbic acid (TAA) content of fully mature (FM) fruits of elderberry interspecific hybrids.

	DPPH	ABTS	FRAP	ORAC	TP
ABTS	0.764 **				
FRAP	0.832 **	0.885 **			
ORAC	0.621 **	0.720 **	0.868 **		
TP	0.544 *	0.641 **	0.635 **	0.626 **	
TAA	0.385	0.214	0.348	0.377	0.023

* sig. < 0.05, ** sig. < 0.01.

For both maturity stages, UR and FM berries, the difference in the results obtained regarding the correlations between AOA, TP and TAA could be partly explained by the

difference in chemical composition between these maturity stages. Indeed, the UR berries are characterised by a higher TAA [46] and a lower TP content (mainly due to the absence of anthocyanins) [15,16] than the FM berries. Although phenolic compounds and total ascorbic acid contribute more to antioxidant activity [65], the contribution of phenolic compounds, especially flavonoids, to the total antioxidant activity of fruits is significantly higher than that of total ascorbic acid, which contributes less than 15% [55,65]. Therefore, a high TAA content (such as in UR berries) does not necessarily reflect a high AOA. On the other hand, high/low TP content also does not always result in high/low AOA values, as possible interactions between different types of phenolic compounds and other compounds in fruit extract may cause the incomplete reactions of antioxidants with radicals [66]. However, this was not the case in FM berries, where similar to some other authors [39,64–66], significant correlations between AOA assays and TP content were observed. Moreover, our results regarding FM berries are in agreement with those reported by Ma et al. [41], who found significant and positive correlations between TP content and DPPH, ABTS, FRAP and ORAC antioxidant abilities but no significant correlations between AOA and TAA content in mango fruits. Similar results (significant correlation between AOA and TP and no significant correlation between AOA and TAA) were also obtained for some common fruits regarding FRAP assay [67] and some tropical fruits regarding DPPH and ORAC assay [66].

3.3. Clustering of Elderberry Interspecific Hybrids

All analysed genotypes were clustered into four groups (Figure 2). The characteristics of each group (average AOA, TP and TAA content of fully mature berries) are shown in Table 3. The genotypes of groups 4 and 3 were characterised with the lowest AOA and TP contents compared to the other groups. Moreover, JA × (JA × MIQ), (JA × NI) × RAC, (JA × NI) × CER, JA × TIG, JA × KOR and ((JA × NI) × CER) × MIQ from group 4 showed the lowest average AOA according to FRAP and ABTS assays and lowest TP content among the groups. Similar but slightly better results were obtained for the genotypes JA × CER No3 C1, (JA × CER) × (JA × NI) and *S. nigra* (NI) from group 3, which were found as poorest in AOA according to DPPH and ORAC assays but dominated in TAA content among all groups. More desirable characteristics compared to the previously mentioned genotypes in terms of AOA and TP were obtained for JA × NI, JA × (JA× NI), (JA × NI) × NI and ((JA × NI) × MIQ) × (CER × NI) from group 1. These genotypes had higher AOA according to DPPH, FRAP and ORAC assay and higher average TP content. However, the genotypes (JA × NI) × BB and (JA × NI) × MIQ from group 2 were found to be superior in terms of AOA as per DPPH, FRAP and ORAC assay and TP content but showed a lower average TAA content than the genotypes from group 3 and 4. Nowadays, since there is a great demand for raw materials with high AOA and TP content, the genotypes from groups 1 and 2 could be recommended for further inclusion in breeding processes or use in the food industry. Due to the high TAA content, the genotypes from group 3 may also be useful in breeding programme.

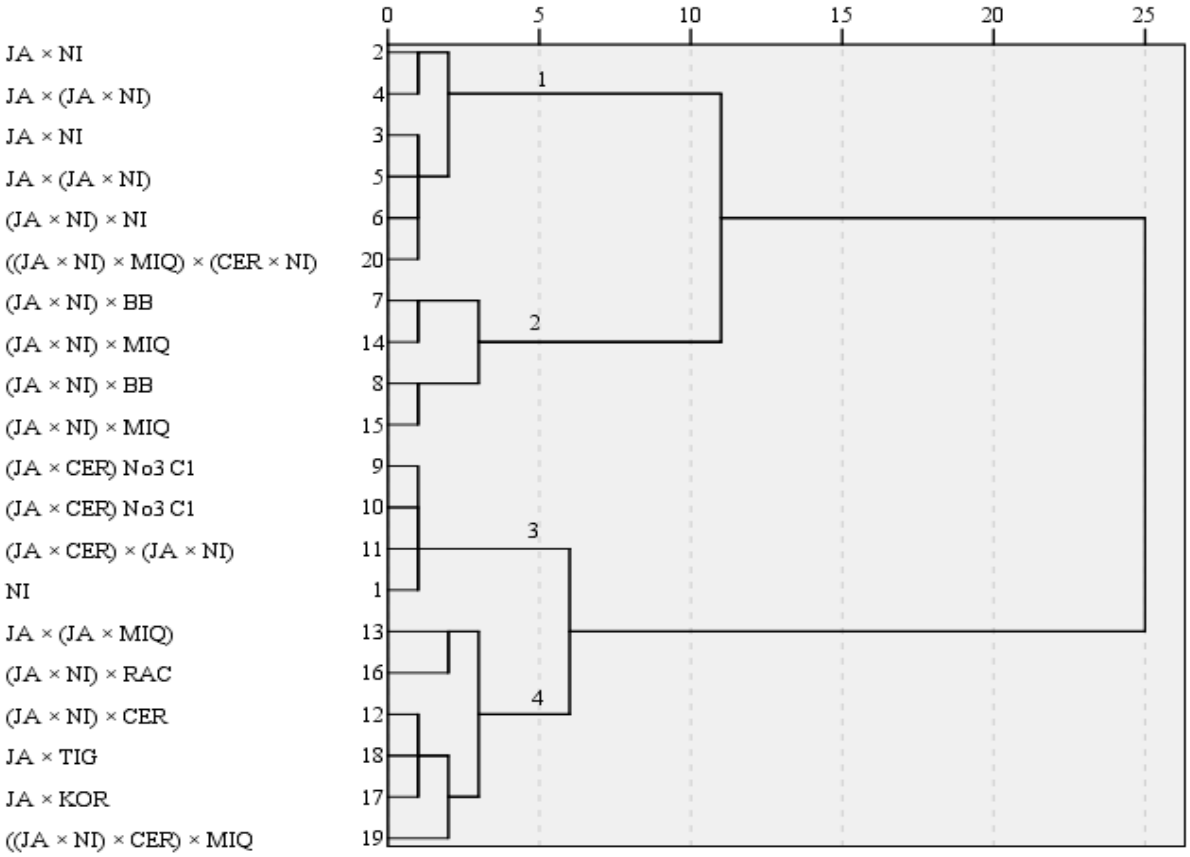

Figure 2. Dendrogram of fully mature berries of the 20 elderberry interspecific hybrids studied, obtained by Ward's method. Abbreviations correspond to *S. nigra* subsp. *nigra* (NI), *S. nigra* subsp. *nigra* 'Black Beauty' (BB), *S. nigra* subsp. *cerulea* (CER), *S. racemosa* subsp. *racemosa* (RAC), *S. racemosa* subsp. *coreana* (KOR), *S. racemosa* subsp. *tigranii* (TIG), *S. racemosa* var. *miquelii* (MIQ) and *S. javanica* (JA).

Table 3. Antioxidant activity of four groups of fully mature berries of elderberry interspecific hybrids evaluated by different assays, total phenolic (TP) content and total ascorbic acid (TAA).

Group	DPPH	FRAP	ORAC	ABTS	TP	TAA
1	96 ± 14	208 ± 25	527 ± 60	130 ± 14	1130 ± 81	158 ± 59
2	100 ± 16	214 ± 33	589 ± 89	124 ±13	2801 ± 246	170 ±45
3	66 ± 21	172 ± 26	450 ± 24	106 ± 25	1762 ± 76	205 ±75
4	73 ± 19	156 ± 36	463 ± 59	98 ± 17	1361 ± 170	183 ± 125

Values are expressed as mean ± std. deviation of means in µmol TE/g for DPPH, FRAP, ABTS and ORAC, and mg/100 g DW for total ascorbic acid (TAA) content and total phenolic (TP) content. Groups were determined by the Ward's method and are defined in the dendrogram (Figure 2).

To investigate whether genotypes with favourable chemical composition (AO activity, TP content and TAA content) of fully mature (FM) berries could be predicted in advance based of the known chemical composition of unripe (UR) berries, the correlation coefficients for above mentioned parameters between unripe (UR) and fully mature (FM) were calculated (Table 4). The majority of correlations were not significant, the only exception was TAA (correlation coefficient 0.827). In conclusion, the genotype with higher AO activity and TP content in unripe berries (UR) was, in general, not characterised with higher AO activity and TP content in fully mature (FM) berries, while the opposite results were found for TAA content.

Table 4. Spearman correlation coefficients for antioxidant activity assays, total phenolic (TP) content and total ascorbic acid (TAA) content between unripe (UR) and fully mature (FM) fruits of elderberry interspecific hybrids.

FM \ UR	DPPH	ABTS	FRAP	ORAC	TP	TAA
DPPH	0.384					
ABTS		0.300				
FRAP			0.232			
ORAC				−0.155		
TP					0.265	
TAA						0.827 **

** sig. < 0.01.

4. Conclusions

DPPH, ABTS, FRAP and ORAC assays showed different AOA levels but a similar increasing pattern of antioxidant activity during elderberry fruit maturation. Moreover, significant and positive correlations were observed between all the methods used for unripe green (UR) and fully mature (FM) stage of elderberry infructescences. The results also showed that total phenolic content strongly contributed to antioxidant activity (positive and significant correlations), while the contribution of total ascorbic acid was lower (no significant correlations). Based on the results, we can conclude that the antioxidant activity of elderberries could be reliably measured using any of the four methods, however, to obtain more comprehensive information on antioxidant properties, the use of more than one method could be recommended.

All the assays used showed significant differences in antioxidant activity between most important stages when planning the harvest time (almost mature (AM), fully mature (FM) and overmature (OM) berries). Considering these results, berries could not be harvested before reaching full maturity (e.g., at the early stage (EM) of maturity). Even though overmature berries showed the highest antioxidant activity, harvesting at this maturity stage could not be recommended due to the overall reduction in yield and quality.

Among the genotypes analysed, berries of (JA × NI) × BB and (JA × NI) × MIQ were found to be superior in antioxidant activity and total phenolic content at full maturity. The results also showed that the most promising genetic combinations (genotypes) regarding antioxidant activity and total phenolic content at full maturity could not be predicted in advance based on the known chemical composition of berries at the beginning of ripening (unripe stage). On the other hand, the results showed that interspecific hybrids with higher total ascorbic acid content in unripe berries were, in general, characterised by a higher total ascorbic acid content in fully mature berries.

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