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Influence of Seed Origin on Morphological Characteristics and Phytochemicals Levels in *Brassica oleracea* var. *acephala*

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Abstract: *Brassica oleracea* var. *acephala* production and seed selection in the Mediterranean region is traditionally limited to private, family needs or small enterprises. In recent years it became popular, especially in the US, and known as a superfood due to the presence of specialized metabolites associated with health benefits, mainly from polyphenols, glucosinolates, and carotenoids groups. With the increasing popularity of this plant, there is a growing interest in its commercial production. Therefore, in the present work we aimed to study how seed origin influences the content of specialized metabolites in *B. oleracea* var. *acephala*. We grew plants from six different seed producers, in a hydroponic system under controlled conditions, and determined seed germination percentage, morphological characteristics, pigments, polyphenols, glucosinolates, and carotenoids content, as well as antioxidant activity. Seed origin influenced germination percentage, yield, and slightly morphological characteristic, but did not influence pigments, total polyphenols, phenolic acids, glucosinolates, and carotenoids content. However, content of flavonoids, ferulic, sinapinic, and, consequently, antioxidant activity was slightly different.

Keywords: *Brassica oleracea* var. *acephala*; specialized metabolites; polyphenols; glucosinolates; carotenoids; pigments

1. Introduction

Cruciferous (Brassicaceae) vegetables have been grown and used by different cultures worldwide. They are known for their use in culinary, in traditional medicine, or as an industrial crop. A key agricultural genus of the Brassicaceae family, is *Brassica*, which includes many popular vegetables whose consumption, according to the epidemiological studies, may help in maintaining health and fighting chronic diseases [1]. In recent years, *Brassica* vegetables have become recognized as a functional food due to their antioxidant, anti-inflammatory, gastro-protective, and anti-obesity activities [1]. *Brassica* vegetables from *acephala* group, which includes leafy, non-heading cabbages with common names such as kale and collards, have become popular in the last 10 years, first in the US and then worldwide [2]. Kale and collards are higher in content of Ca, folate, riboflavin, and vitamins C, K, and A than other cruciferous vegetables [2]. They contain, same as other *Brassica* vegetables, phytochemicals from polyphenols, glucosinolates and carotenoids group whose presence in food is associated with antioxidant and anticancerogenic potential [2]. Production of *Brassica* vegetables from *acephala* group significantly increased, from 3994 to 6256 harvested acres, in the US in the period from 2007 to 2012, respectively [3]. Increased popularity and extensive consumption of *Brassica oleracea* var. *acephala* vegetables across Europe, Asia, and the US could be due to the fact that

these vegetables are easy and cheap for cultivation, and tolerant to unfavorable climate conditions, such as increased salinity, drought, high and low temperature, etc. [4–6].

B. oleracea var. *acephala* have a long history of extensive horticultural use that results with great genetic variability and a large number of populations/landraces across the world. This variability according to Cartea et al. [7] can be result of intrapopulation variability generated by cross-pollination of plants, and as inter-population variability resulted from farmer's selection and adaptations to local ecological conditions. Genetic variability directly influences morphological appearance, but also the level of specialized metabolites or phytochemicals [8–10]. In recent years, when the demand for "healthy food" is growing, agricultural production has became orientated towards food with increased levels of phytochemicals. Therefore, proper seed selection may be of crucial importance. Seeds from different producers potentially can be genetically different and result in products with different morphological characteristics and phytochemical levels [10]. Especially, proper seed selection is important in the case of vegetables, such as *B. oleracea* var. *acephala,* whose production, until recently, was limited to family and small producers in some parts of Europe. For example, flat leaf kale in the Croatian coastal region and in Herzegovina is a traditional vegetable whose use in known in many traditional meals, but production and seed selection is not well organized. The production of *B. oleracea* var. *acephala* has started to increase, as this species has been labeled as superfood [2]. However, it remains unclear how genetic variability (e.g., seed selection) influences morphology and phytochemical levels. In several studies, authors noticed differences between kale populations from different parts of Croatia [8] and Herzegovina [9] at the morphological level, mainly in leaf color, blade blistering, curling, and division, but it remains unclear if seed origin influences phytochemical levels.

In order to study the influence of seed origin on morphological characteristics and specialized metabolites levels in *B. oleracea* var. *acephala*, we have grown plants from six different seed producers— five from small farms of different parts of Croatia, Bosnia, and Herzegovina, and one from commercial seed producers, in a hydroponic system, under the identical environmental conditions. First, we evaluated morphological characteristics and yield. Then we assessed pigment content and level of phytochemicals associated with health benefits of *Brassica* vegetables: polyphenols, glucosinolates, and carotenoids, as well as antioxidant activity.

2. Materials and Methods

2.1. Seed Selection, Germination Test and Plant Growing

Seeds were purchased from one commercial producer and five local producers from different locations in Croatia and Herzegovina, representing different accessions: Pula, Gornja Brela, Mostar, Vrgorac, and Pelješac (Figure 1). After delivery to the laboratory, seeds were stored in a dark and cool place.

For the germination test, seeds were sterilized in 3% Izosan® (Pliva, Zagreb, Croatia) and mixed for 10 min at 300 rpm. Seeds were then washed several times with sterile distilled water and transferred to Petri dishes (20 seeds per dish) on medium containing 1% agar (*w*/*v*), in a laminar flow hood. Plates were first placed in dark at 4 °C for 48 h, and then transferred to growing chamber and kept at 16/8 h photoperiod (light/dark) at 21 °C for three days. The germinated seeds were counted and the germination percentage calculated for each accession.

Germinated seedlings were then placed in a system for hydroponic growth, constructed at Ruđer Bošković Institute [5] and grown in a growing chamber with controlled environmental conditions at a temperature of 22 °C and 16/8 h photoperiod (light/dark) for 28 days. Each hydroponic tank was filled with 5.5 L of distilled water supplemented with commercially available nutrients for hydroponic growth: FloraBloom, FloraMicro, and FloraGro (General Hydroponics, France), according to the manufacturers' instructions, with submerged aeration pumps.

Figure 1. Seed origin locations.

2.2. Morphological Characteristic and Yield

After 28 days, we recorded morphological characteristics as was reported earlier for kale [8]. This includes observation of anthocyanin coloration, leaf shape and color, density of "curling", leaf blade blistering, and any other possible differences between samples. Then, plants were taken out of the hydroponic system, roots were removed, and we weighed the overhead part of the plants (6 plants per location). Leaves of each plant were placed in separate tubes, quickly frozen in liquid nitrogen, and freeze dried. Dried samples were stored in paper bags in a dry, dark, and cool place until further analysis.

2.3. Chlorophylls and Carotenoids

Content of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids were determined according to the method of Lichtenthaler and Buschmann [11] with modification [10]. Results are expressed as mgg^{-1} dw (dry weight).

2.4. Polyphenolic Compounds

For determination of polyphenolic compounds, we performed extraction using 60 mg of dry samples and 2 mL of 80% methanol as we reported earlier [10]. The level of polyphenolic compounds was determinate in 2 mL reaction volumes [10]. Total phenolic content was determinate using Folin–Ciocalteu reagent [12] and results are expressed as equivalents of gallic acid per dry weight (mg GAE g^{-1} dw). For total flavonoids determination, we used the method with $AlCl_3$ according to Zhishen et al. [13], and results are expressed as catechin equivalents per dry weight (mg CE g^{-1} dw). The total flavanol (TFL) content was determined using the p-dimethylaminocinnamaldehyde (DMACA) method [14] with modifications [10], and results are expressed as catechin equivalents per dry weight (μg CEg^{-1} dw). Total proanthocyanidins (TPA) were determined using vanillin–HCl methods [15] with modification [10], and results are expressed as catechin equivalents per dry weight (mg CE g^{-1} dw).

Total ferulic and sinapinic acids were determinate using HPLC-PDA (Knauer, Germany). A total of 50 mg of dry samples were extracted, using 80% methanol containing 20 μg mL^{-1} of anthracene-9-carboxylic acid as an internal standard, by homogenization, vortexing, and centrifugation as we described above. After centrifugation, supernatants were transferred to new tubes, filtered through 0.4 μm nylon filters, and injected to HPLC for determination of free ferulic and sinapinic acid. For determination of ferulic and sinapinic acid, which are part of complexes (bound phenolic acids), we performed hot alkaline hydrolysis [16]. To the pellets, we added 500 μL of 2 M NaOH and we kept

samples at 95 °C in a thermoblock for 1 h. Then, samples were cooled down on ice and in every sample, 500 μL of 2 M HCl was added and pH was adjusted to 2.0. Phenolic acids were extracted from the sample using 3 × 500 μL of ethyl acetate and solvents were evaporated. Before analysis, samples were dissolved in 200 μL of acetonitrile. For HPLC analysis, we used a C18 column (Kinetex Gemini, 2.6 × 100 × 50), acetonitrile containing 0.1% of formic acid as solvent A, and 0.1% formic acid as solvent B. We injected 50 μL, flow rate was 1 mL/min, and separation gradient was as follows: 0–10 min 2% B, 10–20 min 4% B, 20–25 min 10% B, 25–30 12% B, 30–35 min 13% B, 35–40 min 15% B, 40–50 min 30% B, 50–60 min 60% B, 60–70 min 98% B. Identification was done by comparing retention times and spectrum at 230 nm with those for commercial standards for ferulic and sinapinic acids.

2.5. Glucosinolates

Glucosinolates were determined using the method previously reported by Aghajanzaden et al. [17] with some modification [4]. Extracts were prepared by mixing 30 mg of frozen dried plant samples with 1 mL of 80% methanol. In order to deactivate myrosynase, samples were incubated at 90 °C for 2 min. Then, the extract was centrifuged for 3 min at 3000× *g* and supernatants were used for analysis. Total glucosinolates content was determined based on reaction with sodium tetrachloropalladate II (Na_2PdCl_4). The reaction containing 30 μL extract and 900 μL Na_2PdCl_4 was incubated for 1 h at room temperature, followed by absorbance measurement at 425 nm. The results were expressed as milligrams of sinigrin equivalent per gram of dry weight (mg sin g^{-1} dw).

2.6. Antioxidant Activity

Methanol extracts used for polyphenol content analysis were also used for determination of antioxidant activity, which was measured according to the DPPH (1,1-diphenyl-2-picrylhydrazyl) method [18]. Results were expressed as μmol Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) equivalents per gram dry weight (μmol TE g^{-1} dw).

2.7. Statistical analysis

All analyses were performed in 6 biological replicates and results are expressed as mean ± standard deviation (SD). All statistical analyses were done using Microsoft Office Excel 2010 upgraded with XLSTAT (ver. 2011.5.01.). One-way ANOVA and post hoc multiple mean comparison (Tukey's HSD test) were performed and the differences between measurements were considered to be significant at $p < 0.05$.

3. Results

3.1. Percentage of Germination, Morphological Characteristics and Yield

Photos of analyzed plants, information of seed origin, and results of seed germination percentage, yield per plant, as well as morphological characteristics are presented in Table 1. High germination rate (more than 80%) was observed for seeds from Mostar (90%), Vrgorac (85%), and Pula (82.5%), while seeds from Pelješac showed only 25% germination. Seeds from Mostar, which showed the highest percentage of germination, had also the highest average yield per plant (11.74 ± 4.20 g), while seeds from Pelješac, with the lowest germination percentage, had the lowest yield, only 3.45 ± 1.56 g.

In our samples we noticed different morphological characteristics, especially in leaf size, that influenced yield per plant (e.g., samples with bigger leaves showed higher yield per plant). All samples had lyrate leaf division (incision) and leaves without anthocyanin coloration, leaf curling, or leaf blade blistering. The plants derived from Pelješac seeds exhibited longer leaf petioles than in other plants.

Table 1. Photos of 28-day-old grown plants, information about seed origin, seed germination percentage, yield per plant, and morphological characteristics of analyzed samples. Value labeled with different letters differ significantly at $p < 0.05$.

	Seed Location	Seed Germination Percentage	Yield per Plant (g)	Morphological Characteristics (Batelja et al. (2009))
	Commercial seed producers, Zagreb county, Croatia	65%	9.60 ± 2.93 ^{b,c}	Anthocyanin coloration: Absent Leaf curling: Absent Leaf blade blistering: Absent Leaf division (incision): Lyrate
	**Pula**, Istria county, Croatia	82.5%	5.77 ± 4.06 ^{a,b}	Anthocyanin coloration: Absent Leaf curling: Absent Leaf blade blistering: Absent Leaf division (incision): Lyrate
	**Gornja Brela**, Split-Dalmatia County, Croatia	65%	9.04 ± 2.74 ^{b,c}	Anthocyanin coloration: Absent Leaf curling: Absent Leaf blade blistering: Absent Leaf division (incision): Lyrate

	**Mostar**, Federation of Bosnia and Herzegovina, Bosnia, and Herzegovina	90%	11.74 ± 4.20 [c]	Anthocyanin coloration: Absent Leaf curling: Absent Leaf blade blistering: Absent Leaf division (incision): Lyrate
	**Vrgorac**, Split-Dalmatia County, Croatia	85%	7.16 ± 1.86 [a,b]	Anthocyanin coloration: Absent Leaf curling: Absent Leaf blade blistering: Absent Leaf division (incision): Lyrate
	**Pelješac,** Dubrovnik-Neretva County, Croatia	25%	3.45 ± 1.56 [a]	Anthocyanin coloration: Absent Leaf curling: Absent Leaf blade blistering: Absent Leaf division (incision): Lyrate Evident longer leaf petiole

3.2. Content of Green Pigments

The main green pigments in *B. oleracea* var. *acephala* are chlorophylls whose content is present in Table 2. Chlorophyll *a* content was, in all samples, around 7 μg g^{-1} dw and did not differ between plants from different seed producers, while chlorophyll *b* content was less than 4 μg g^{-1} dw in plants from commercial seed producers (3.95 ± 0.34 μg g^{-1} dw) and Pula (3.94 ± 1.19 μg g^{-1} dw). Total chlorophyll content in all plants was around 12 μg g^{-1} dw and did not differ between samples.

Chlorophyll *a*/*b* ratio was around 2 in plants from commercial, Pula, and Mostar seed origins while the ones from Pelješac, Vrgorac, and Gornja Brela had lower ratio. The ratio of total chlorophyll/total carotenoids did not significantly differ between analyzed plants and it was around 8.

Table 2. Content of pigments (mg/g dw) in *Brassica oleracea* var. *acephala* grown from different seed sources. Value labeled with different letters differ significantly at $p < 0.05$.

	Chlorophyll *a* (μg g^{-1} dw)	**Chlorophyll *b* (μg g^{-1} dw)**	**Total Chlorophyll (μg g^{-1} dw)**	**Chlorophyll *a*/Chlorophyll *b***	**Total Chlorophyll/Total Carotenoids**
commercial	7.96 ± 0.96 ^{a}	3.95 ± 0.34 ^{a}	11.91 ± 1.05 ^{a}	2.11 ± 0.27 ^{b}	7.49 ± 0.53 ^{a}
Pelješac	6.77 ± 0.53 ^{a}	4.28 ± 0.75 ^{a,b}	11.06 ± 0.82 ^{a}	1.65 ± 0.34 ^{a,b}	8.16 ± 0.95 ^{a}
Vrgorac	7.23 ± 059 ^{a}	5.43 ± 0.63 ^{b}	12.66 ± 0.59 ^{a}	1.43 ± 0.21 ^{a}	8.49 ± 1.27 ^{a}
Pula	7.76 ± 1.66 ^{a}	3.94 ± 1.19 ^{a}	11.70 ± 2.67 ^{a}	1.95 ± 0.40 ^{b}	7.69 ± 1.89 ^{a}
Gornja Brela	6.54 ± 0.70 ^{a}	4.40 ± 0.84 ^{a,b}	10.94 ± 1.13 ^{a}	1.60 ± 0.38 ^{a,b}	8.22 ± 1.32 ^{a}
Mostar	8.28 ± 1.45 ^{a}	4.14 ± 0.62 ^{a,b}	12.43 ± 1.96 ^{a}	2.01 ± 0.25 ^{b}	7.41 ± 0.49 ^{a}

3.3. Specialized Metabolites Level

3.3.1. Polyphenolic Compounds

The content of total phenols, total phenolic acids, flavonoids, flavanols, and proanthocyanidins are presented in Figure 2.

Total polyphenols content was between 9.34 ± 2.72 (Pula) and 11.44 ± 1.21 (Vrgorac) mg GAE g^{-1} dw and did not significantly differ between samples. The same trend was observed for phenolic acids, of which the content was between 9.34 ± 0.47 (Pula) and 10.73 ± 0.64 (Pelješac) μg CAE mg^{-1} dw. Unlike total phenols and phenolic acid content, flavonoids content was different between samples. Interestingly, plants grown from seeds from Pelješac, which had the highest total flavonoid content (2.51 ± 0.33 mg CE g^{-1} dw), contained the lowest content of flavonoid group flavanols (18.03 ± 1.16 μg CE g^{-1}dw) and proanthocyanidins (1.23 ± 0.03 mg CE g^{-1} dw).

In addition, we optimized the HPLC method, which allowed us the ability to separate ferulic and sinapinic acids (Figure 3a), and then we compared total ferulic (Figure 3b) and sinapinic (Figure 3c) acids in analyzed samples. We analyzed free and total content of those phenolic acids. Under our experimental condition we were unable to detect free ferulic and sinapinic acids, but after hydrolysis there were predominantly phenolic acids (Figure 3a). Although spectrophotometric data did not show significant differences in total phenolic acids between samples the content of individual total ferulic and sinapinic acids varied significantly between samples (Figure 3b,c). Plants from commercial seed producers and those from Pelješac and Pula had a significantly lower amount of ferulic acid than plants grown from Vrgorac, Gornja Brela, and Mostar seeds. Plants grown from seeds from Vrgorac also had the highest amount of sinapinic acid, significantly higher than plants grown from seeds from Pelješac.

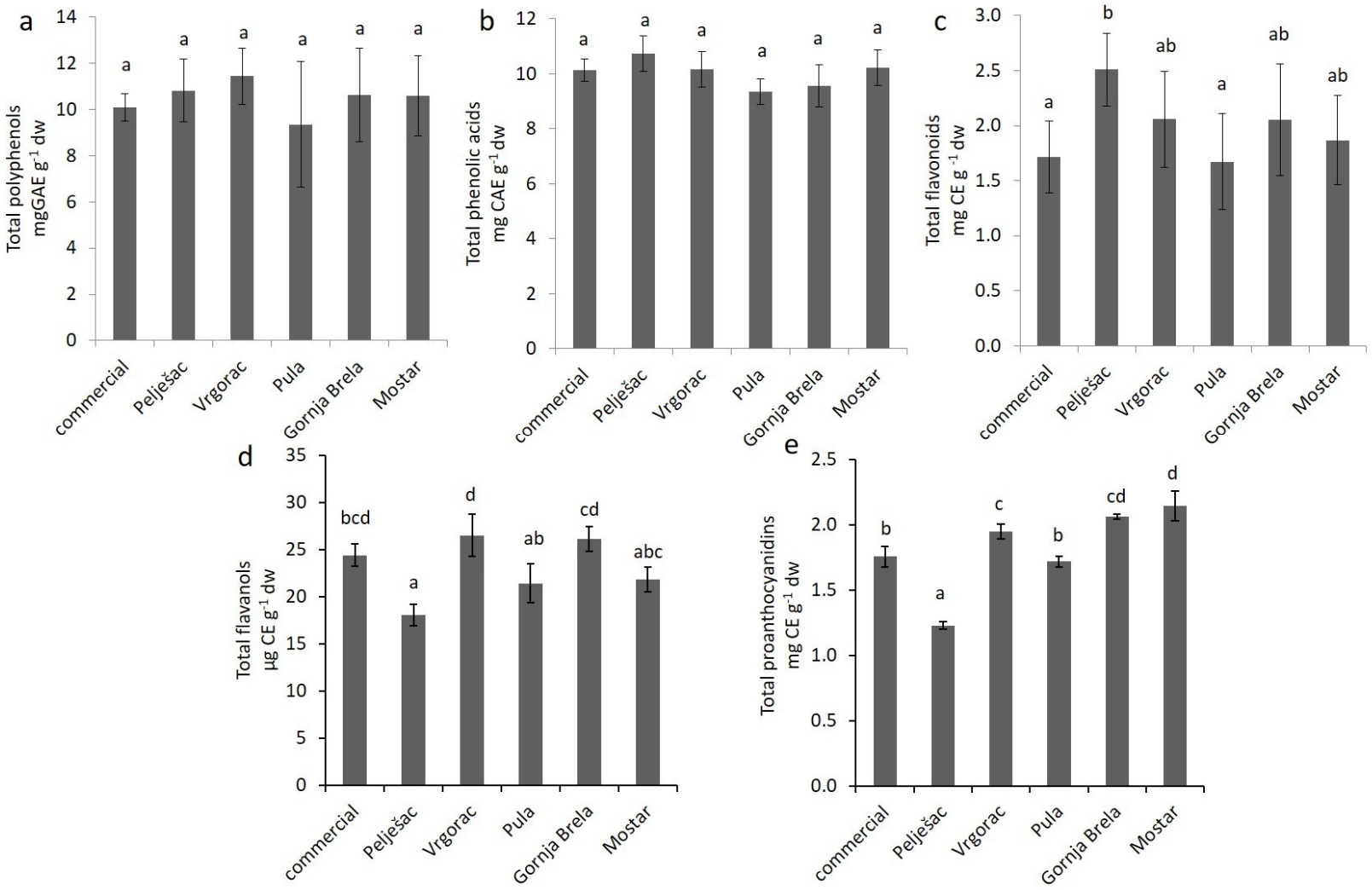

Figure 2. Content of polyphenolic compounds in *Brassica oleracea* var. *acephala* grown from different seed sources. Value labeled with different letters differ significantly at $p < 0.05$.

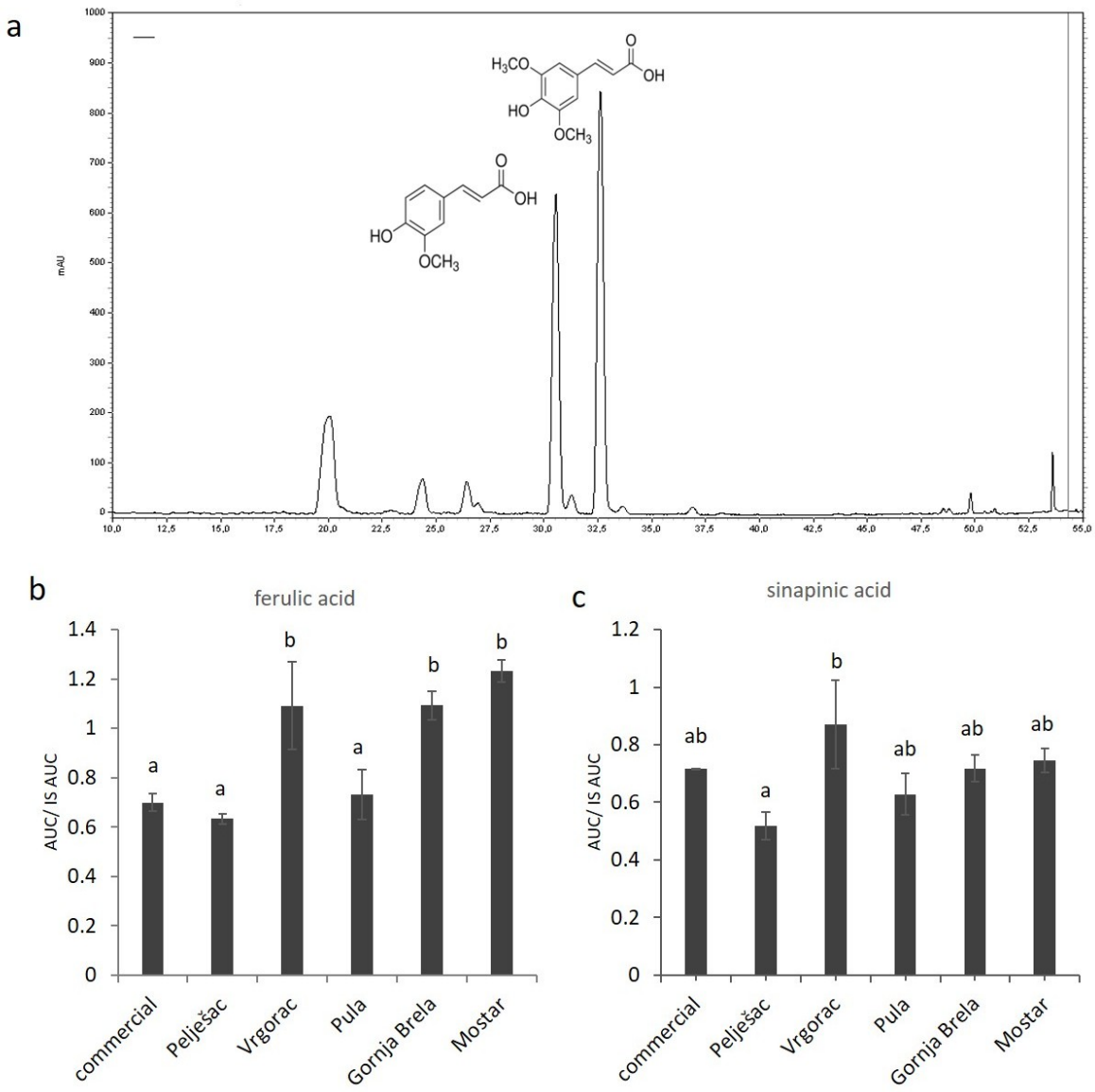

Figure 3. Representative chromatogram at 230 nm (**a**) and content of total ferulic (**b**) and sinapinic (**c**) acids in *Brassica oleracea* var. *acephala* grown from different seed sources. Value labeled with different letters differ significantly at $p < 0.05$.

3.3.2. Carotenoids and Glucosinolates

Beside polyphenols, important specialized metabolites or phytochemicals in *Brassica* plants are glucosinolates and carotenoids. These analytes are presented in Figure 4. As it is evident, total carotenoids and total glucosinolates content did not significantly differ between analyzed *B. oleracea* var. *acephala* samples. Total carotenoids content in samples was around 1.5 μg mg^{-1} dw, while total glucosinolates content was around 55 μg mg^{-1}.

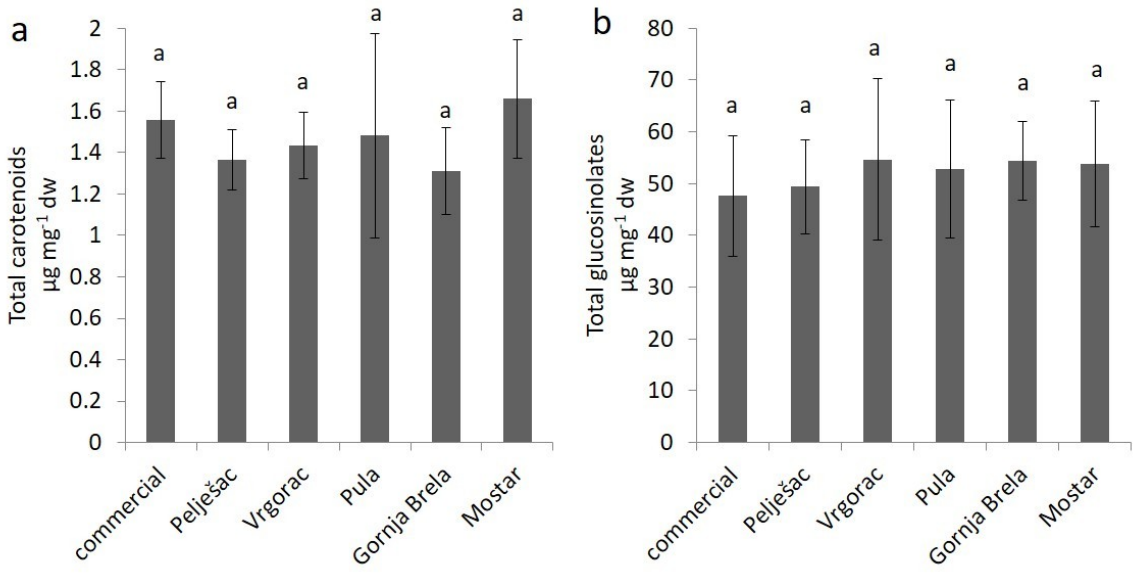

Figure 4. Content of total carotenoids (**a**) and total glucosinolates (**b**) in *Brassica oleracea* var. *acephala* grown from different seed sources.

3.4. Antioxidant Activity

Many health benefits properties of specialized metabolites are associated with antioxidant activity. In our samples we measured antioxidant activity using DPPH method (Figure 5) and results are expressed as Trolox equivalents. The highest antioxidant activity had plants from Vrgorac seeds, while the lowest antioxidant activity showed plants grown from commercial seed producers.

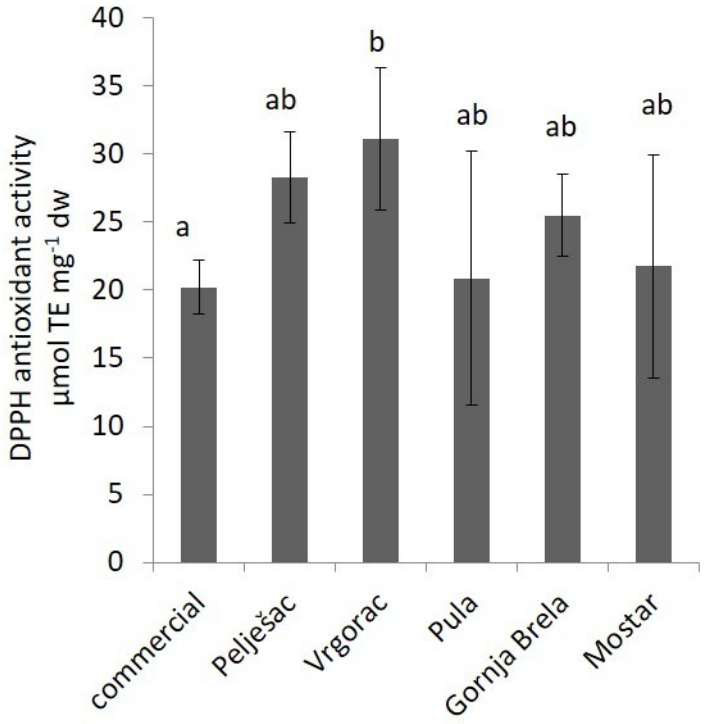

Figure 5. Antioxidant capacity of *Brassica oleracea* var. *acephala* grown from different seed sources measured by DPPH method. . Value labeled with different letters differ significantly at $p < 0.05$.

4. Discussion

B. olerace var. *acephala* is originated from eastern Mediterranean and, according to some authors, it is an indigenous plant in Croatian costal region and Herzegovina [8,9]. However, due to unorganized seed production there are differences in seed quality and morphological characteristics between populations from different parts of Croatia. As is evident from our results, germination rate greatly varied between samples. Seeds from Mostar had the highest germination percentage and also the highest yield per plant compared to seeds from Pelješac, of which germination percentage was only 25% and produced the lowest yield. Therefore, in order to reach maximum quality and yield, proper seed collecting and storing is important.

Batelja et al. [8], who analyzed morphological characteristics of 15 populations of *B. oleracea* var. *acephala* from different parts of Croatia, grown on natural locations, reported that plants may differ in morphological characteristics such as anthocyanin coloration, distribution of anthocyanin coloration, color of young leaves, density of curling, leaf blade blistering, and leaf division. In our samples we did not notice anthocyanin coloration. Anthocyanin presence in the green varieties of *Brassica* plants may be associated with unfavorable environmental conditions such as high irradiance [19] or with deficiency of nutrients in the soil [20]. Since all our plants were grown under identical and controlled environmental condition, the absence of anthocyanin is not surprising. The absence of environmental stress in the plants is evident from total chlorophyll/total carotenoids ratio, which is in all our samples around 8 (Table 2). The ratio of chlorophyll *a* and chlorophyll *b*, as well as total chlorophyll and total carotenoids ratio can indicate some physiological processes in plants [11]. Values lower than 4.2 for the total chlorophyll/total carotenoids ratio are an indicator of senescence, stress, and damage to the plant and the photosynthetic apparatus, which is expressed by a faster breakdown of chlorophylls than carotenoids [11]. This ratio is, in addition, an indicator of the greenness of plants [11], higher values are associated with greener plants and our data suggest that our plants have dark green color, which is not significantly different between analyzed samples. All our plants had lyrate leaf division what is the most common shape of Croatian *Brassica olerace* var. *acephala* populations [8]. However, some of our samples had slightly bigger leaves, which influenced yield per plant.

The main group of bioactive compounds in *Brassica* plants associated with health benefits are polyphenols, glucosinolates, and carotenoids [1]. As is evident from Figure 2, total polyphenols and total phenolic acid content did not differ between analyzed samples, while we noticed differences in flavonoid compounds. This observation is different than in our previous study on four-week-old white cabbages grown from different seed producers, where we noticed significant differences in total polyphenols, flavonoids, and flavanols, and moderate differences in total phenolic acid content [10]. A previous study on Polish *B. oleracea* var. *acephala* showed that kale contain 359 mg of total polyphenols per 100 g of fresh matter [21]. In the same paper, the author compared total phenol content in three kale varieties, and found that red-leaved varieties had the highest level of total phenols, Another Polish study, in which authors analyzed total polyphenols content in *B. oleracea.* var. *acephala* for three consecutive years, showed that average polyphenol content is 574.95 ± 90.35 mg of chlorogenic acid per 100 g of fresh matter [22]. In *B. olerace* var. *acephala,* polyphenolic compounds are present as complex conjugates, where one to five sugar moieties are bound to the aglycone, and they are often acylated with hydroxycinnamic acids [23]. In our experiment, alkaline hydrolysis was performed to reduce the complexity of the naturally-occurring compounds present in analyzed samples, due to the release of the hydroxycinnamic acids by cleavage of the ester linkage between the acids and the glycosides. We optimized method which allow us separation of ferulic and sinapinic acids, two predominant phenolic acids evident in chromatogram after hydrolysis (Figure 3a). Samples from Vrgorac, Gornja Brela, and Mostar had a significantly higher amount of ferulic acid, while Vrgorac had the highest amount of sinapinic acid (Figure 3b,c). Previously, sinapinic acid was found to be the most abundant phenolic acid in *B. oleracea* cv. *acephala* seeds, while in leaves much more ferulic than sinapinic acid was reported [24]. Derivatives of sinapinic acid are characteristic compounds for *Brassica* plants associated with antioxidant, antimicrobial, anti-inflammatory, anticancer, and anti-anxiety activities [25].

Characteristic compounds for *Brassica* plants are also glucosinolates, of which the hydrolysis products are associated with numerous health benefits [1]. Each type of *Brassica* shows a characteristic glucosinolates profile that includes more than ten different glucosinolates in each species/variety, although only three to four are predominant [2]. Previous studies have shown that vegetables from *acephala* group contain higher total glucosinolates content that other *Brassica oleracea* vegetables [26]. Content of total glucosinolates in *B. oleracea* var. *acephala* is reported to be from 3–35 μmol g^{-1} dw, depending upon geographical origin [2]. In our samples, total glucosinolates content did not show differences between analyzed samples and was 55 μg mg^{-1} dw (Figure 4b). It is reported that glucosinolates content in *B. oleracea* var. *acephala* is dependent upon the environmental factors, plant part examined, phenological stage of plant growth, and level of insect damage [27]. Therefore, in our experiment, where all plants were grown under identical, controlled conditions, it is not surprising that glucosinolates content did not differ between samples.

Bioactive compounds in *Brassica* plants known for strong antioxidant activity are carotenoids. Their presence in green *Brassica* plants is masked by chlorophylls, but level of total carotenoids is reported to be around 0.5 mg g^{-1} dw or 0.2 mg g^{-1} fw in *Brassica olerace* var. *acephala* seedlings. In older leaves, total carotenoids content usually increases [28]. Similar as for total phenols, total phenolic acids, and total glucosinolates content, total carotenoids content did not differ between samples (Figure 4a). As was reported previously, carotenoids level in *Brassica oleracea* var. *acephala* are affected by pre-harvest effects such as maturity, climate, and farming practice [28]; therefore, low variation between our samples is expected.

All analyzed specialized metabolites (or their hydrolysis products) had antioxidant activity; thus, in addition to specialized metabolites content, we measured antioxidant activity using the DPPH method (Figure 5). The highest was observed for plants grown from seeds from Vrgorac samples, which showed the highest content of ferulic and sinapinic acids. Those phenolic acids, and their derivatives, are known as a strong antioxidant [29]. Derivatives of these phenolic acids further increase their antioxidant activity, for example esterification of ferulic acid resulted in increasing activity [29]. As we mention above, ferulic and sinapinic acids in *Brassica* are present as complexes and alkane hydrolysis releases the hydroxycinnamic acids by cleavage of the ester linkage between the acids and the glycosides. Therefore, plants from seeds originated from Vrgorac, where we found the highest amount of ferulic and sinapinic acids, probably contain the highest amount of complex forms with antioxidant activity.

Author Contributions: D.Š. designed the research. P.I. grew the plants, determined germination rate and morphological characteristics, and measured pigments, total polyphenols, total phenolic acids, total flavonoids, total glucosinolates content, and antioxidant activity under the supervision of D.Š. V.K. measured total flavanols and proanthocyanidins content and determinate total ferulic and sinapinic acids using HPLC-PDA under the supervision of D.Š. D.Š. conducted the statistical analysis, designed the tables and figures, drafted the manuscript, and was responsible for project administration and funding acquisition.

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Conflicts of Interest: The authors declare no conflicts of interest.

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