Research Article

Received: 4 July 2016 Revised: 6 October 2016 Accepted article published: 13 October 2016 Published online in Wiley Online Library: 10 November 2016

(wileyonlinelibrary.com) DOI 10.1002/jsfa.8085

The higher the better? Differences in phenolics and cyanogenic glycosides in *Sambucus nigra* leaves, flowers and berries from different altitudes

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Abstract

BACKGROUND: Elderberry (*Sambucus nigra* L.) possesses high antioxidant activity and has been used to treat numerous medicinal disorders. In addition to their antioxidant properties, elderberry parts accumulate toxic cyanogenic glycosides (CGG). It has been proven that altitude influences the biosynthesis of many secondary metabolites. In the present study we investigated the change of phenolics and CGG in elder leaves, flowers, and berries induced by different altitudes and locations.

RESULTS: The data indicate that the accumulation of CGG and phenolics is affected by the altitude of the growing site. An increase of anthocyanin content was recorded in elder berries collected at higher elevations in both locations. Fruit collected at the foothills of location 2 contained 3343 μg g^{-1} anthocyanins as opposed to fruit from the hilltop, which contained 7729 μg g^{-1}. Elder berries contained the lowest levels of harmful CGG compared to other analysed plant parts. However, more cyanogenic glycosides were always present in plant parts collected at the hilltop. Accordingly, berries accumulated 0.11 μg g^{-1} CGG at the foothills and 0.59 μg g^{-1} CGG at the hilltop.

CONCLUSION: Elder berries and flowers collected at the foothill were characterised by the lowest levels of both beneficial (phenolics) and harmful compounds (CGG) and are suitable for moderate consumption.
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Keywords: elderberry parts; phenolics; sambunigrin; altitude

INTRODUCTION

The synthesis and accumulation of specific secondary metabolites in plant species have been linked to plant–herbivore interactions[1] and adaptation to certain conditions, closely related to their ecological environment.[2–4] Many environmental factors like latitude, altitude, precipitation, temperature, light intensity, nutritional status, edaphic properties, wind speed, duration of snow cover, season, length of the vegetation period, the intensity of radiation under clear sky conditions, damage caused by pests as well as competition with other species have been proven to influence the biosynthesis of many secondary metabolites in a number of plant species.[2,5,6] Higher solar irradiation at higher altitudes and its impact on secondary metabolite profiles of higher plants has been confirmed in many studies.[2,3,5,6] It is well known that ultraviolet-absorbing compounds, mainly phenolics, are formed as a response to higher solar irradiance at higher altitudes and may protect plant cells against excessive ultraviolet (UV)-B radiation.[7,8] A popular traditional belief favours medicinal plants from higher altitudes as they proverbially contain more active ingredients than plants from lower sites.[2]

Sambucus nigra L., or elderberry (*Adoxaceae* family), is a tree-like shrub, distributed almost all over the world. Its flowers and berries are a popular natural source of bioactive compounds, which possess high antioxidant activity and have been used for medicinal purposes and traditionally consumed to prevent or diminish the effects of several diseases, such as respiratory tract infections, neuropathic pain, headache, arteriosclerosis, diabetes, arthritis and, also, cancer.[9,10] Elder berries and flowers are also utilised in the food industry to produce jams, pies, jellies and other dishes and beverages.[11] In addition to beneficial compounds and their antioxidant properties, black elder leaves, seeds, bark and unripe berries also accumulate potentially toxic secondary metabolites.[12] Black elder contains substantial amounts of sambunigrin, a cyanogenic glycoside (CGG), which is considered life-threatening due to its decomposition into chemically reactive hydrogen cyanide.[13] Ingestion of elderberry parts in larger doses may cause gastrointestinal disorders, such as nausea, vomiting, weakness and dizziness.[14] The presence of CGG in elder seeds may inhibit their microbial decay, prolonging their survival in adverse conditions.[15]

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The aim of this study was to identify and quantify phenolics and cyanogenic glycosides in elder leaves, flowers and berries collected at different altitudes and locations. We focused our study on *Sambucus nigra* because it is a generally known plant used in traditional medicine, widely distributed in Mediterranean and continental regions and can be found along a wide altitudinal gradient (from 0 to 1500 m). This is the first study on the comparison of cyanogenic glycosides and phenolics in different elderberry parts from diverse altitudes.

MATERIALS AND METHODS

Plant material

Elderberry leaves and flowers were collected in May and June and ripe elder berries (dark black colour of the fruit) in August at four different altitudes [between 200 and 1100 m above sea level (a.s.l.)] in two different locations, which were characterised by diverse climates. Location 1 is a hill between Dolenjske Toplice (latitude 45° 45′ N, longitude 15° 05′ E, altitude 180 m) and Črnomelj (latitude 45° 34′ N, longitude 15° 11′ E, altitude 175 m); location 2 is a hill between Idrija (latitude 46° 01′ N, longitude 14° 04′ E, altitude 335 m) and Tolmin (latitude 46° 11′ N, longitude 13° 44′ E, altitude 201 m) (Fig. 1). Elder shrubs were growing in their natural form and the samples were randomly harvested from different plant branches at each altitude. Elderberry parts (leaves, flowers and berries) were stored separately in paper bags, transported to the laboratory and kept at −20°C until further analyses. Meteorological data for both locations: average temperatures, temperatures for each sampling day and solar radiation for the year 2015 are presented in Table 1 (Slovenian Environment Agency).

Extraction of cyanogenic glycosides and phenolics from the samples

Elder leaves, flowers and berries were separately crushed in a mortar with liquid nitrogen and 1 g of elderberry paste was put into a 10 mL screw-cap tube. Sample was extracted with 5 mL of methanol/water (70:30, v/v, MeOH/H_2O) according to the method of Senica *et al.*[16] All treatments were performed in seven repetitions.

Identification of phenolic compounds

Individual phenolics were identified on an Accela HPLC system (Thermo Scientific, San Jose, CA, USA), equipped with diode arrary detectors and controlled by CromQuest 4.0 chromatography workstation software (Thermo Fischer Scientific Institution, Waltham, MA, USA). The following parameters were applied in phenolic analysis: column, Gemini C_{18} (150 × 4.6 mm 3 µm; Phenomenex, Torrance, CA, USA), operated at 25°C; mobile phase A, formic acid/ACN/H_2O (0.1/3/96.9, v/v/v); mobile phase B, formic acid/ACN/H_2O (0.1/96.9/3, v/v/v); flow rate, 0.6 mL min^{-1}; injection volume, 20 µL; detection wavelength, 280 nm, 350 nm and 530 nm; linear gradient, 5–20% B 15 min, 20–30% B 5 min, then an isocratic mixture for 5 min, 30–90% B 5 min, and then an isocratic mixture for 15 min before returning to the initial conditions.[17]

Identification of cyanogenic glycosides

The separation of cyanogenic glycosides was performed on a HYPERSIL GOLD aQ column (Thermo Scientific) with the following parameters: operated at 25°C; flow rate 0.8 mL min^{-1}; sample injection volume 20 µL; mobile phase A, methanol/H_2O (3/97); mobile phase B methanol/H_2O (97/3); gradient elution: 0–1 min, 80% A; 1–8 min, 80% to 0% A; 8–10 min, 0% A; 10–14 min, 80% A.

Figure 1. Sampling locations.

The presence of cyanogenic glycosides was confirmed on a TSQ Quantum Access Max quadrupole mass spectrometer by Thermo Scientific applying the following parameters: (ESI) source in positive ion mode operating at 275°C, corona voltage 4.7 kV, sheat gas 60 L h^{-1}, auxiliary gas 10 L h^{-1}; mass spectra scanned in the range from *m/z* 70 to 650. Collision-induced dissociation was achieved using argon as the collision gas in the collision cell. Cyanogenic glycosides were analysed in selected reaction monitoring (SRM) mode. Data acquisition was performed using Xcalibur 2.2. Software (Thermo Fischer Scientific Institute, Waltham, MA, USA). Contents of sambunigrin were expressed in micrograms per gram of elderberry sample in fresh weight.

Reagents and standards

Commercially available standards for phenolics and a cyanogenic glycoside prunasin were obtained from Sigma–Aldrich (Steinheim, Germany). Quercetin-3-glucoside, catechin and *p*-coumaric acid were obtained from Fluka Chemie (Buch, Switzerland). Methanol and acetonitrile were purchased from Sigma–Aldrich. Double distilled water was produced with a Millipore purification system (Millipore, Bedford, MA, USA) and used to prepare all aqueous solutions.

Statistical analyses

Results are presented as mean ± standard error of seven replications. Significant differences among different elderberry samples from different altitudes were calculated by two-way analysis of variance (ANOVA). The differences in the content levels of each individual component were estimated with Duncan's multiple range test and were considered significant at $P < 0.05$. All statistical analyses were performed with R-Commander (R Formation for Statistical Computina, Anckland, New Zeland).

RESULTS

Content of phenolic compounds

In total, 28 phenolic compounds were detected in elder leaves, 31 in flowers and 31 in berries (Table 2). Phenolics were divided into 5 subgroups: hydroxycinnamic acids, flavanols, flavanones, and in berries additionally into flavanols and anthocyanins. The content of each phenolic group is presented separately for elder leaves (Table 3), elder flowers (Table 4) and elder berries (Table 5) from

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Table 1. Site specifics: location, altitude, collection date of elderberry parts, mean temperature and solar irradiation

Location	Latitude and longitude	Altitude (m a.s.l.)	Sampling date					Average temp.2015 (°C)	Solar irradiation (kWh m^{-2})
			Leaves	Flowers	Temp. (°C)	Berries	Temp. (°C)		
Location 1 (continental climate)	45° 44′ 28.2″ N; 15° 04′ 40.4″ E	210	31.5.2015	31.5.2015	20.5	8.8.2015	26.5	11.3	1243.203
	45° 40′ 24.2″ N; 15° 05′ 55.1″ E	420	31.5.2015	31.5.2015	18.5	8.8.2015	24.6	9.8	1328.862
	45° 39′ 14.6″ N; 15° 05′ 07.9″ E	850	7.6.2015	7.6.2015	17.8	12.8.2015	24.1	9.1	1345.465
	45° 37′ 45.8″ N; 15° 06′ 10.5″ E	1048	7.6.2015	7.6.2015	17.3	12.8.2015	23.2	8.2	1336.489
Location 2 (Mediterranean climate)	46° 05′ 30.4″ N; 13° 49′ 50.4″ E	209	23.5.2015	23.5.2015	17.6	9.8.2015	23.8	12.3	1334.364
	46° 02′ 14.1″ N; 13° 50′ 22.3″ E	450	23.5.2015	23.5.2015	16.3	9.8.2015	22.1	10.4	1306.329
	46° 00′ 25.9″ N; 13° 52′ 32.0″ E	858	23.5.2015	23.5.2015	15.3	21.8.2015	21.5	8.9	1348.955
	46° 01′ 24.1″ N; 13° 53′ 08.2″ E	1077	30.5.2015	30.5.2015	11.5	21.8.2015	19.3	7.9	1395.627

a.s.l., above sea level.

different altitudes of two sampled locations. Flavonols were the most abundant phenolic sub-group in elder leaves and flowers and anthocyanins comprised the majority of phenols in berries (Tables 3–5). A comparable pattern in total analysed phenolics has been determined in regard to altitude in all analysed elderberry parts and in both locations.

Altitudinal variation of phenolics in elder leaves:

The content of selected phenolics varied with altitude in both locations. Flavonols (isorhamnetin glycosides, kaempferol glycosides and quercetin glycosides) represented the highest share of total phenolic content in elder leaves. Total flavonol levels were in range from 838.74 to 1221.23 μg g^{-1} (Table 3). Kaempferol and iso-rhamnetin glycosides were slightly increased in leaves collected at higher elevations in both locations; however, the pattern of total flavonol increase was not always consistent with the altitudinal gradient. The contents increased from 1030.56 μg g^{-1} at the lowest altitude to 1221.23 μg g^{-1} at 850 m a.s.l. in location 1 and from 852.76 μg g^{-1} to 1058.17 μg g^{-1} at 858 m a.s.l. in location 2.

Elder leaves contained significantly higher levels of total hydroxycinnamic acids (HCA) at the highest elevation (above 1000 m) than at the lowest altitude (approx. 200 m) (Table 3). The altitude gradient was significantly correlated with the contents of *p*-coumaric and caffeic acid derivatives, the prevalent HCA in elder leaves. Ferulic acid derivatives represented the lowest proportion of total HCA (less than 1%) (Table 3). Leaves collected at the lowest elevation in location 1 contained 75.12 μg g^{-1} HCA and 2.2-fold higher levels (167.36 μg g^{-1}) have been measured at the highest altitude. Similarly, at location 2, lowest levels of total HCA have been detected at the lowest altitude (84.37 μg g^{-1}) and highest at the highest altitude (136.77 μg g^{-1}). Similar contents of total HCA were measured in leaves, collected in both locations [$P = 0.0482$ (*)].

Higher altitude positively affected the content of flavanones (naringenin derivatives), a minor phenolic group in elder leaves in both locations (from 13.84 μg g^{-1} to 24.58 μg g^{-1} in location 1 and from 9.13 μg g^{-1} to 22.45 μg g^{-1} in location 2) (Table 3). Significant differences in flavanone levels have been detected between the two analysed locations [$P = 0.0249$(*)] but the altitude–location interaction was not significant ($P = 0.1730$) (Table 3).

Altitudinal variation of phenolics in elder flowers:

Flavonols were the major phenolic group in elder flowers, similar to the composition of elder leaves. The values ranged from 1005.50 to 4140.86 μg g^{-1} (Table 4) and were also highest compared to other analysed elderberry parts. An inconsistent response to the altitudinal gradient has only been recorded for iso-rhamnetin derivatives. The content of all other flavonol derivatives (kaempferol and quercetin glycosides) was significantly higher at the highest altitude compared to the lowest altitude, but the increase was not gradual. In location 1, elder flowers sampled at 210 m a.s.l. accumulated 2114.49 μg g^{-1} total flavonols and a two-fold increase has been recorded at 1048 m a.s.l. (4140.86 μg g^{-1} of total flavonols). Similarly, in location 2, 2742.14 μg g^{-1} of total flavonols have been measured in flowers collected at 1077 m a.s.l. as opposed to flowers from the lowest altitude (209 m a.s.l.), which only contained 1005.50 μg total flavonols g^{-1}.

A significant increase in caffeic acid derivatives, from HCA group has been recorded in elder flowers with rising altitude. Their contents increased from 191.70 μg g^{-1} to 359.41 μg g^{-1} at location 1 and from 141.22 μg g^{-1} to 293.04 μg g^{-1} at location 2 (Table 4). Correspondingly, total HCA increased with altitude (from 417 μg g^{-1} at the lowest elevation to 470.25 μg g^{-1} at the highest altitude in location 1 and from 209.17 μg g^{-1} to 367 μg g^{-1} at the highest altitude in location 2).

Total flavanone contents decreased with higher altitude in both locations (Table 4). At location 1 the contents decreased slightly (from 50.98 μg g^{-1} to 41.03 μg g^{-1}) and at location 2, greater differences in flavanone contents have been measured at various elevations. An initial increase has been recorded from 209 m (74.63 μg g^{-1}) to 850 m (96.15 μg g^{-1}) followed by a significant decrease of total flavanone content at 1077 m a.s.l. (37.80 μg g^{-1}) (Table 4).

Altitudinal variation of phenolics in elder berries

Anthocyanins represented the major part of total phenolics in elderberry fruits (approx. 90%) and were significantly increased with altitude in both locations. Cyanidin-3-glucoside and cyanidin-3-sambubioside represented the major share of anthocyanins and cyanidin-3-rutinoside, cyanidin-3-sambubioside-5-glucoside and cyanidin-3,5-diglucoside were minor contributors to total anthocyanin content (Table 5). The identified anthocyanins have previously been reported in different elderberry species.[16] Total anthocyanin content increased with higher altitude at both locations: from 3606.44 μg g^{-1} (210 m a.s.l.) to 5230.32 μg g^{-1} (1048 m a.s.l.) in location 1 and from 3343.47 μg g^{-1} (209 m a.s.l.) to 7728.53 μg g^{-1} (1077 m a.s.l.) in location 2.

Quercetin glycosides were the main flavonols in elder berries, followed by derivatives of kaempferol and isorhamnetin. The content of total flavonols decreased at the second analysed altitude and

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Table 2. Individual phenolics found in elder leaves, flowers and berries

Phenolics	Leaves	Flowers	Berries
Derivatives of caffeic acid			
Dicaffeoylquinic acid 1	X	X	X
Dicaffeoylquinic acid 2	X	X	X
Dicaffeoylquinic acid 3	X	X	X
Neochlorogenic acid	X	X	X
trans-5-Caffeoylquinic acid	X	X	X
4-Caffeoylquinic acid	X	X	X
cis-5-Caffeoylquinic acid	–	X	X
Derivatives of ferulic acid			
3-Feruloylquinic acid	X	X	X
5-Feruloylquinic acid	X	X	–
Derivatives of *p*-coumaric acid			
p-Coumaric acid	–	–	X
p-Coumaric acid hexoside	X	X	X
p-Coumaroylcaffeoylquinic acid 1	X	X	–
p-Coumaroylcaffeoylquinic acid 2	X	X	–
3-*p*-Coumaroylquinic acid	–	X	X
4-*p*-Coumaroylquinic acid	–	–	X
5-*p*-Coumaroylquinic acid 1	X	X	X
5-*p*-Coumaroylquinic acid 2	X	X	X
Iso-rhamnetin glycosides			
Iso-rhamnetin acetylhexoside	X	X	–
Iso-rhamnetin-hexoside	X	X	–
Iso-rhamnetin-dihexoside	–	X	–
Iso-rhamnetin-3-glucoside	–	X	–
Iso-rhamnetin-3-rutinoside	–	X	X
Kaemferol glycosides			
Kaempferol-acetylhexoside	X	X	–
Kaempferol-dihexoside	–	X	–
Kaempferol-3-galactoside	X	X	–
Kaempferol glycoside	X	X	–
Kaempferol-3-glucoside	X	–	–
Kaempferol-3-rutinoside	X	X	X
Quercetin glycosides			
Quercetin-acetylhexoside	X	X	X
Quercetin-dihexoside	X	X	–
Quercetin-3-glucoside	X	X	X
Quercetin-hexoside pentoside 1	X	X	X
Quercetin-hexoside pentoside 2	X	–	X
Quercetin-rhamnoside	X	–	–
Quercetin-rutinoside	X	X	X
Quercetin-3-xyloside	–	–	X
Flavanols			
Catechin	–	–	X
Epicatechin	–	–	X
Flavanones			
Naringenin	X	X	–
Naringenin hexoside 1	–	–	X
Naringenin hexoside 2	–	–	X
Anthocyanins			
Cyanidin-3-sambubioside	–	–	X
Cyanidin-3-glucoside	–	–	X
Cyanidin-3-rutinoside;	–	–	X
Cyanidin-3,5-diglucoside	–	–	X
Cyanidin-3-sambubioside-5-glucoside	–	–	X

gradually increased at higher altitudes in both locations (Table 5). Elder berries contained 270.61 μg g^{-1} of total flavonols at the lowest altitude and 315.12 μg g^{-1} of total flavonols at the highest altitude in location 1. In location 2, elder fruits accumulated 439.74 μg of total flavonols per g at the lowest elevation and 554.57 μg g^{-1} at the highest altitude (Table 5). Generally, significantly higher levels of total flavonols have been measured in fruits, collected at location 2 compared to location 1.

In location 1, elder berries contained significantly highest levels of total HCA at the highest altitude compared to the lowest altitude (55.48 μg g^{-1} at 210 m a.s.l. and 88.92 μg g^{-1} at 1048 m a.s.l.) (Table 5). A similar pattern has been detected in location 2; however, the increase was moderate (83.58 μg g^{-1} of total HCA at 209 m a.s.l. and 101.03 μg g^{-1} at 1077 m a.s.l.). Caffeic acid derivatives were the major contributors to total HCA contents, as already reported for leaves and flowers.

Total flavanones (naringenin derivatives) increased in elder berries at higher altitudes in both locations. In location 1, elder berries accumulated 0.18 μg g^{-1} total flavanones at 210 m a.s.l. and almost two-fold levels at 1048 m a.s.l. (0.31 μg g^{-1}). The pattern was similar at location 2; elder fruit contained 0.25 μg g^{-1} total flavanones at 209 m a.s.l. and 0.36 μg g^{-1} total flavanones at 1077 m a.s.l.(Table 4).The interaction between location and altitude was not significant ($P = 0.8634$).

Altitude positively affected the content of flavanols (catechin and epicatechin) in elder berries at location 1. The amount of total flavanols ranged from 19.30 μg g^{-1} at 210 m a.s.l. to 63.11 μg g^{-1} at 1048 m a.s.l. (Table 5). Only a slight increase in total flavanol levels has been detected in berries collected at 450 m a.s.l. compared to other altitudes in location 2.

Content of cyanogenic glycosides

Sambunigrin has been identified from the group of cyanogenic glycosides (CGG) in elder leaves (Table 3), elder flowers (Table 4) and elder berries (Table 5) collected at different altitudes in two locations.

Altitudinal variation of CGG in elder leaves

The content of cyanogenic glycosides was highest at the hilltop compared to the foothill in both locations (Table 3), but the increase was not gradual with the rising altitude (from 28.82 μg g^{-1} to 75.49 μg g^{-1} at location 1 and from 153.31 μg g^{-1} to 209.61 μg g^{-1} at location 2). Elder leaves collected at location 2 generally accumulated more CGG than leaves at location 1.

Altitudinal variation of CGG in elder flowers

Elder flowers contained lower CGG contents compared to leaves. Similarly, their values were highest at the highest altitude compared to the lowest at both locations and did not increase gradually (Table 4). Sambunigrin content ranged from 7.02 μg g^{-1} (210 m a.s.l.) to 18.88 μg g^{-1} at 1048 m a.s.l. in location 1 and from 1.23 μg g^{-1} at 209 m a.s.l. to 15.72 μg g^{-1} at 1077 m a.s.l. in location 2. Significant differences in sambunigrin contents have been determined between the analysed locations.

Altitudinal variation of CGG in elder berries

Elder berries contained the lowest contents of CGG in comparison with elder leaves and flowers. Moreover, the pattern of sambunigrin accumulation was not distinguishable among different altitudes in any location (Table 5). Sambunigrin levels ranged from

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Table 3. The content of phenolic compounds and sambunigrin (cyanogenic glycoside) in elder leaves (mean ± standard error in μg g^{-1} FW) collected at various altitudes in two different locations

	Location 1 (continental climate) (μg g^{-1})				Location 2 (Mediterranean climate) (μg g^{-1})				Interaction		
Compounds	210 m	420 m	800 m	1048 m	209 m	450 m	858 m	1077 m	Alt.	Loc.	Int.
Phenolics											
Derivatives of caffeic acid	26.19 ± 3.01^{d}	9.41 ± 4.52^{f}	56.70 ± 8.46^{c}	83.14 ± 3.25^{a}	26.39 ± 2.50^{d}	15.85 ± 1.65^{e}	70.48 ± 2.81^{b}	75.43 ± 4.54^{b}	***	*	***
Derivatives of ferulic acid	0.73 ± 0.04^{e}	0.43 ± 0.06^{d}	0.77 ± 0.10^{a}	0.43 ± 0.04^{c}	0.63 ± 0.05^{ab}	0.50 ± 0.04^{c}	0.62 ± 0.06^{b}	0.46 ± 0.05^{a}	***	*	**
Derivatives of *p*-coumaric acid	48.20 ± 2.66^{e}	39.23 ± 6.01^{f}	68.11 ± 8.22^{b}	83.79 ± 2.58^{a}	57.35 ± 2.48^{cd}	49.71 ± 3.63^{de}	76.77 ± 8.17^{a}	60.87 ± 7.89^{bc}	***	***	***
Total hydroxycinnamic acids	75.12 ± 4.94^{de}	49.06 ± 6.10^{f}	125.59 ± 16.64^{c}	167.36 ± 4.94^{a}	84.37 ± 3.88^{d}	66.06 ± 4.88^{e}	147.87 ± 9.19^{b}	136.77 ± 11.02^{bc}	***		***
Iso-rhamnetin glycosides	19.70 ± 1.15^{d}	22.84 ± 1.60^{c}	14.69 ± 1.82^{e}	28.22 ± 1.73^{b}	8.38 ± 0.82^{g}	23.25 ± 2.04^{c}	11.21 ± 2.34^{f}	35.95 ± 0.88^{a}	***	**	***
Kaempferol glycosides	64.77 ± 3.64^{b}	17.01 ± 1.10^{e}	53.89 ± 8.02^{c}	81.50 ± 1.52^{a}	25.23 ± 1.00^{d}	27.63 ± 1.93^{d}	70.13 ± 9.46^{b}	47.81 ± 3.67^{c}	***	***	***
Quercetin glycosides	483.69 ± 50.25^{d}	246.46 ± 46.62^{e}	715.34 ± 96.81^{ab}	629.33 ± 102.84^{bc}	539.04 ± 62.33^{cd}	448.52 ± 71.80^{d}	815.68 ± 62.97^{a}	496.27 ± 37.21^{d}	***	*	***
Quercetin rutinoside	712.40 ± 28.34^{a}	228.11 ± 43.15^{d}	437.32 ± 60.51^{c}	758.05 ± 24.32^{a}	514.78 ± 62.97^{b}	423.36 ± 70.86^{c}	386.14 ± 42.73^{c}	258.71 ± 36.42^{d}	***	***	**
Total flavonols	1030.56 ± 29.92^{b}	889.43 ± 43.75^{c}	1221.23 ± 45.52^{a}	1197.10 ± 38.26^{a}	1087.44 ± 95.23^{b}	852.76 ± 76.31^{c}	1058.17 ± 23.56^{b}	838.74 ± 54.62^{c}	***	***	***
Total flavanones	13.84 ± 1.06^{b}	13.49 ± 0.05^{b}	15.38 ± 1.99^{b}	24.58 ± 2.34^{a}	9.13 ± 3.10^{c}	12.97 ± 2.64^{b}	15.29 ± 3.02^{b}	22.45 ± 1.48^{a}	***	*	
Cyanogenic glycosides											
Sambunigrin	28.82 ± 1.10^{d}	60.84 ± 6.37^{c}	31.06 ± 5.53^{d}	75.49 ± 9.72^{c}	153.31 ± 3.26^{b}	27.68 ± 6.48^{d}	67.57 ± 8.26^{c}	209.61 ± 15.67^{a}	***	***	***

Two-way ANOVA for Altitude (Alt.), Location (Loc.) and their interaction (Int.) is presented.
Different superscript letters (a–d) in rows denote statistically significant interaction between altitude and location for individual phenolics and sambunigrin levels in elderberry leaves by Duncan's multiple range test ($P < 0.05$). Significant differences in phenolic and sambunigrin levels among altitudes (Alt.)/locations (Loc) are denoted with an asterisk.
*Statistically significant differences at P value <0.05;
**statistically significant differences at P value <0.001;
***statistically significant differences at P value <0.0001.
The altitudes of each location are given as metres above sea level.

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Table 4. The content of phenolic compounds and sambunigrin (cyanogenic glycoside) in elder flowers (mean ± standard error in μg g^{-1} FW) collected at various altitudes in two different locations

	Location 1 (continental climate) (μg g^{-1})				Location 2 (Mediterrannean climate) (μg g^{-1})				Interaction		
Compounds	210 m	420 m	800 m	1048 m	209 m	450 m	858 m	1077 m	Alt.	Loc.	Int.
Phenolics											
Derivatives of caffeic acid	249.49 ± 28.91^{bc}	191.70 ± 22.64^{d}	226.72 ± 56.19^{cd}	359.41 ± 33.99^{a}	141.22 ± 19.21^{e}	262.05 ± 20.89^{bc}	247.55 ± 23.51^{bc}	293.04 ± 37.13^{b}	***		***
Derivatives of ferulic acid	0.90 ± 0.06^{c}	1.19 ± 0.03^{a}	0.45 ± 0.06^{g}	0.61 ± 0.08^{f}	0.70 ± 0.05^{e}	0.83 ± 0.02^{cd}	1.07 ± 0.03^{b}	0.78 ± 0.06^{de}	***	*	***
Derivatives of *p*-coumaric acid	166.79 ± 10.05^{a}	133.24 ± 2.40^{b}	124.26 ± 6.28^{c}	110.24 ± 6.52^{d}	67.25 ± 3.91^{g}	83.00 ± 1.82^{f}	92.79 ± 4.44^{e}	73.71 ± 5.85^{g}	***	***	***
Total hydroxycinnamic acids	417.18 ± 34.11^{b}	326.13 ± 22.71^{f}	351.43 ± 59.38^{c}	470.25 ± 27.74^{a}	209.17 ± 22.34^{d}	345.88 ± 20.59^{c}	341.40 ± 19.97^{c}	367.53 ± 42.85^{bc}	***	***	***
Iso-rhamnetin glycosides	494.74 ± 44.14^{a}	463.15 ± 12.33^{ab}	408.37 ± 74.13^{bc}	476.45 ± 49.90^{a}	216.57 ± 16.89^{d}	377.21 ± 10.47^{c}	484.30 ± 18.03^{a}	207.84 ± 19.90^{d}	**	***	***
Kaempferol glycosides	85.71 ± 3.81^{d}	194.76 ± 4.07^{ab}	182.49 ± 16.83^{b}	214.79 ± 7.67^{a}	108.91 ± 8.07^{c}	119.66 ± 0.37^{c}	213.63 ± 29.25^{a}	111.90 ± 26.91^{c}	***	***	***
Quercetin glycosides	848.70 ± 10.30^{d}	1235.72 ± 41.99^{b}	1107.71 ± 99.83^{c}	1794.71 ± 47.48^{a}	366.41 ± 51.65^{f}	741.76 ± 35.57^{e}	1145.03 ± 37.15^{c}	1226.45 ± 20.47^{b}	***	***	***
Quercetin rutinoside	685.35 ± 87.94^{c}	1129.31 ± 53.62^{b}	1050.48 ± 96.90^{b}	1654.90 ± 109.51^{a}	313.62 ± 47.75^{d}	679.76 ± 37.42^{c}	1616.07 ± 257.86^{a}	1195.95 ± 33.15^{b}	***	**	***
Total flavonols	2114.49 ± 124.44^{e}	3022.94 ± 94.48^{c}	2749.05 ± 251.41^{d}	4140.86 ± 105.66^{a}	1005.50 ± 122.17^{g}	1868.40 ± 35.59^{f}	3459.03 ± 270.11^{b}	2742.14 ± 53.17^{d}	***	***	***
Total flavanones	50.98 ± 2.79^{c}	51.80 ± 2.32^{c}	33.71 ± 4.87^{d}	41.03 ± 11.26^{d}	74.63 ± 7.81^{b}	82.47 ± 4.05^{b}	96.15 ± 4.01^{a}	37.80 ± 3.18^{d}	**	***	***
Cyanogenic glycosides											
Sambunigrin	7.02 ± 1.24^{d}	4.82 ± 1.12^{e}	11.09 ± 1.32^{c}	18.88 ± 0.40^{a}	1.23 ± 0.88^{f}	6.88 ± 2.03^{d}	6.17 ± 2.06^{d}	15.72 ± 1.73^{b}	***	***	***

Two-way ANOVA for Altitude (Alt.), Location (Loc.) and their interaction (Int.) is presented.
Different superscript letters (a–d) in rows denote statistically significant interaction between altitude and location for individual phenolics and sambunigrin levels in elderberry leaves by Duncan's multiple range test ($P < 0.05$).
Significant differences in phenolic and sambunigrin levels among altitudes (Alt.)/locations (Loc) are denoted with an asterisk.
*Statistically significant differences at P value <0.05;
**statistically significant differences at P value <0.001;
***statistically significant differences at P value <0.0001.
The altitudes of each location are given as metres above sea level.

Table 5. The content of phenolic compounds and sambunigrin (cyanogenic glycoside) in elder berries (mean ± standard error in μg g^{-1} FW) collected at various altitudes in two different locations

	Location 1 (continental climate) (μg g^{-1})				Location 2 (Mediterrannean climate) (μg g^{-1})				Interaction		
Compounds	210 m	420 m	800 m	1048 m	209 m	450 m	858 m	1077 m	Alt.	Loc.	Int.
Phenolics											
Derivatives of caffeic acid	42.56 ± 2.57^{c}	26.22 ± 3.78^{d}	39.80 ± 3.95^{c}	53.66 ± 4.56^{b}	48.45 ± 3.48^{b}	62.49 ± 4.53^{a}	65.54 ± 2.60^{a}	60.23 ± 5.40^{a}	***	***	***
Derivatives of ferulic acid	0.04 ± 0.02^{e}	0.07 ± 0.01^{d}	0.15 ± 0.01^{a}	0.11 ± 0.01^{c}	0.14 ± 0.01^{ab}	0.12 ± 0.01^{c}	0.13 ± 0.01^{b}	0.15 ± 0.02^{a}	***	***	***
Derivatives of p-coumaric acid	12.89 ± 1.77^{f}	16.91 ± 2.46^{e}	30.37 ± 2.25^{d}	35.15 ± 2.44^{c}	34.99 ± 2.23^{c}	47.65 ± 3.44^{a}	38.85 ± 2.30^{bc}	40.64 ± 2.61^{b}	***	***	***
Total hydroxycinnamic acids	55.48 ± 1.70^{e}	43.20 ± 6.21^{f}	70.33 ± 6.00^{d}	88.92 ± 6.78^{c}	83.58 ± 5.55^{c}	110.25 ± 6.20^{a}	104.52 ± 4.71^{ab}	101.03 ± 7.80^{b}	***	***	***
Isorhamnetin glycosides	0.09 ± 0.01^{c}	0.15 ± 0.01^{c}	0.04 ± 0.02^{c}	0.09 ± 0.01^{c}	0.03 ± 0.01^{c}	0.14 ± 0.02^{b}	0.37 ± 0.07^{b}	0.61 ± 0.11^{a}	***	***	***
Kaempferol glycosides	10.06 ± 1.03^{bc}	8.49 ± 1.51^{c}	13.69 ± 1.14^{a}	11.03 ± 0.49^{b}	6.06 ± 0.51^{d}	13.73 ± 1.38^{a}	8.87 ± 1.98^{c}	10.50 ± 1.97^{bc}	***	*	***
Quercetin glycosides	260.47 ± 10.30^{d}	82.41 ± 22.46^{e}	361.89 ± 7.46^{c}	304.00 ± 18.35^{d}	433.65 ± 30.88^{b}	313.46 ± 53.03^{cd}	458.50 ± 66.39^{b}	543.46 ± 39.17^{a}	***	***	**
Total flavonols	270.61 ± 11.19^{f}	91.04 ± 23.44^{g}	375.61 ± 7.56^{d}	315.12 ± 17.94^{e}	439.74 ± 30.95^{c}	327.33 ± 53.84^{e}	492.75 ± 17.24^{b}	554.57 ± 37.20^{a}	***	***	***
Total flavanols	19.30 ± 2.22^{e}	39.71 ± 5.58^{d}	57.72 ± 4.78^{c}	63.11 ± 4.31^{c}	63.42 ± 3.43^{c}	96.70 ± 6.59^{a}	66.35 ± 5.99^{b}	65.06 ± 5.32^{b}	***	***	***
Total flavanones	0.18 ± 0.08^{cd}	0.09 ± 0.01^{e}	0.18 ± 0.06^{cd}	0.31 ± 0.03^{ab}	0.25 ± 0.02^{bc}	0.15 ± 0.02^{de}	0.27 ± 0.03^{b}	0.36 ± 0.10^{a}	***	***	–
Cyanidin-3-sambubioside	1141.39 ± 82.90^{d}	1410.76 ± 325.34^{cd}	2397.10 ± 235.30^{b}	2548.35 ± 360.27^{b}	1709.82 ± 116.97^{c}	2265.64 ± 487.08^{b}	3816.30 ± 142.82^{a}	3633.76 ± 247.10^{a}	***	***	*
Cyanidin-3-glucoside	1744.30 ± 233.04^{a}	1241.47 ± 286.30^{e}	2109.44 ± 207.06^{bc}	2242.55 ± 317.03^{b}	1446.34 ± 95.19^{de}	1821.65 ± 390.81^{cd}	3358.34 ± 125.68^{a}	3257.71 ± 160.43^{a}	***	***	***
Cyanidin-3-rutinoside	227.57 ± 744.99^{c}	143.03 ± 32.98^{d}	243.02 ± 23.8^{bc}	283.36 ± 35.07^{b}	164.79 ± 24.60^{d}	255.39 ± 37.05^{bc}	386.91 ± 14.48^{a}	367.71 ± 25.75^{a}	***	***	***
Cyanidin-3,5-diglucoside	41.24 ± 2.72^{bcd}	16.31 ± 2.80^{ef}	50.57 ± 4.87^{bc}	24.92 ± 1.20^{de}	3.60 ± 0.50^{f}	37.93 ± 4.74^{cd}	58.28 ± 26.50^{b}	109.69 ± 15.97^{a}	***	**	***
Cyanidin-3-sambubioside-5-glucoside	214.44 ± 16.61^{d}	85.82 ± 14.73^{f}	266.16 ± 25.65^{e}	131.15 ± 6.32^{de}	18.93 ± 2.61^{a}	199.63 ± 24.97^{d}	381.76 ± 68.93^{b}	644.40 ± 22.97^{a}	**	**	***
Total anthocyanins	3606.44 ± 311.60^{c}	2897.39 ± 661.75^{c}	5066.30 ± 492.26^{b}	5230.32 ± 712.58^{b}	3343.47 ± 218.42^{c}	4580.25 ± 857.88^{b}	7926.59 ± 279.71^{a}	7728.53 ± 320.70^{a}	***	***	***
Cyanogenic glycosides											
Sambunigrin	0.24 ± 0.08^{cd}	0.12 ± 0.02^{de}	0.08 ± 0.01^{e}	0.35 ± 0.03^{c}	0.11 ± 0.02^{b}	0.77 ± 0.08^{a}	0.36 ± 0.14^{c}	0.59 ± 0.12^{b}	***	***	***

Two-way ANOVA for Altitude (Alt.), Location (Loc.) and their interaction (Int.) is presented.
Different superscript letters (a–d) in rows denote statistically significant interaction between altitude and location for individual phenolics and sambunigrin levels in elderberry leaves by Duncan's multiple range test ($P < 0.05$).
Significant differences in phenolic and sambunigrin levels among altitudes (Alt.)/locations (Loc) are denoted with an asterisk.
*Statistically significant differences at P value <0.05;
**statistically significant differences at P value <0.001;
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The altitudes of each location are given as metres above sea level.

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0.24 μg g^{-1} (210 m a.s.l.) to 0.35 μg g^{-1} (1048 m a.s.l.) in location 1 and from 0.11 μg g^{-1} (209 m a.s.l.) to 0.59 μg g^{-1} (1077 m a.s.l) in location 2.

DISCUSSION

Phenolic compounds

The content of phenolics in different elderberry parts was significantly affected by the altitude. Their composition and contents can be modified by several meteorological factors,[18] such as temperature, precipitation and light, which are closely related to altitudinal gradient of a specific site.[6,7] Long days with cool night temperatures and intense solar irradiation during the growing season positively impact the biosynthesis of phenolics in plants, although variations in plant response has been detected among species and within individual phenolic groups.[3,19] Different climatic environments, duration of solar irradiation and plant phenophase at specific altitudes caused the discrepancy in the amounts of active compounds in elder leaves, flowers and berries. Consequently, a delay of flowering and ripening has been monitored at higher altitudes compared to lower elevation.[2,3,20,21] Therefore, elderberry samples have to be collected at different periods (Table 1) to ensure similar conditions. Nevertheless, differences in the contents of phenolic compounds have been detected among different altitudes and locations and to some extent the composition of flavanols (catechin and epicatechin) may have been affected by slight variation in elderberry maturity. Flavanols are generally the constituents of unripe fruits with the function to prevent herbivores to consume and disseminate immature seed.[22] Total flavanols at location 1 accounted for approximately 69% of the total increase in response to environmental factors related to the altitude of the growing site. Contrary, the pattern of flavanol turnover was not as clear in location 2 as highest levels of this phenolic group were measured in fruit collected at 450 m a.s.l. Yang *et al.*[23] and Bakhshi and Arakawa[24] reported significant differences in flavanone levels among various maturity stages of *Ribes* and apple fruit regardless of irradiation and temperature variables.[2] It can be summarised that the ripening stage, phenophase delay and sun exposure significantly alter the content of flavonols in elder berries.

Hydroxycinnamic acids (HCA) represented approximately four-fold higher levels in elder flowers as in leaves and berries. The increase of HCA was not gradual along the altitudinal gradient, but nonetheless their levels were higher at the hilltop than at the foothill in all elderberry parts. The content of total HCA in elder leaves was 55% and 38% higher at the hilltop compared to foothill in locations 1 and 2, respectively. Similarly, higher HCA content was measured in elder flowers (11% more in location 1 and 43% more in location 2) and elder berries (37% more in location 1 and 17% more in location 2) sampled at the highest altitude compared to the lowest site. Differences in HCA values among different altitudes may be a result of the long-term abiotic stress effect.[2] HCA (particularly caffeic acid derivatives) increased with the rising altitude as a response of higher light intensity (especially UV radiation). It is known, that UV-B irradiation negatively impacts the plant life at higher altitudes and increases the content of phenolics, which are accumulated in epidermal cells lessening the effects of UV-irradiation on sensitive processes in mesophyll cells.[1,2,6,8,20,21] Flavonols, particularly anthocyanins, quercetin and kaempferol derivatives act as photoprotective compounds and respond to increased UV-B irradiation.[25] Flavonols represented the most abundant phenolic group in elder leaves and flowers and the latter were particularly rich in this phenolic group. Quercetin glycosides were the major contributors to total flavonols in all analysed elderberry parts. In general, an increase of total flavonol content was recorded along the altitudinal gradient, with a slight decrease at intermediate altitudes. Total flavonol levels were higher at the highest altitude compared to the lowest elevation in all analysed elderberry parts at location 1. However, the altitudinal effect on flavonol levels in elder leaves was not coherent in location 2. Fifty-one % and 63% higher total flavonol levels were measured in elder flowers and 14% and 21% more flavonols were detected in elder berries at higher altitudes in comparison to the lowest elevation in locations 1 and 2, respectively. A moderate increase in the levels of isorhamnetin and kaempferol glycosides was detected at higher altitudes in both locations, which is in accordance with previous studies.[20,21,25,26]

Higher solar irradiation at higher altitudes positively affected anthocyanin biosynthesis.[3,20,23,25] Anthocyanins were the major contributors of total phenolic content in elder berries and increased significantly at higher altitudes in both locations (Fig. 1). Elderberries contain high levels of anthocyanins, but all are not absorbed into the plasma. Some studies monitored the presence of anthocyanins in human plasma and urine after dietary intake and suggested their low bioavailability (less than 1%). The degree of absorption of anthocyanins into the system is highly dependent on their structure.[27,28] Bacteria in the human colon promote the cleavage of sugars linked to the anthocyanin molecule and, consequently, its transformation into lower-weight phenolic components. Some of these newly formed compounds can be more active as the initial anthocyanin molecule.[29] Cyanidin-3-glucoside and cyanidin-3-sambubioside were the most abundant anthocyanins in elder berries and a two-fold higher content of these pigments was measured at the highest altitude compared to the lowest elevation in both locations. Correspondingly, a 31% and 57% increase of total anthocyanins was detected in fruit from highest altitudes as opposed to lowest sites in locations 1 and 2. The increase of total anthocyanin content in elder fruit collected at higher altitudes can be linked to higher UV-irradiation and decreased temperatures of the site (Table 1). Stiles *et al.*[30] and Choi *et al.*[31] measured higher anthocyanin content in plants subjected to low temperatures compared to plants growing at higher temperatures. Choi *et al.*[31] also reported that anthocyanin accumulation is light dependent at low temperatures and the synthesis is down-regulated at low temperatures and poor solar irradiation and vice versa. Higher anthocyanin contents at higher altitudes can therefore be ascribed to a combined effect of intense solar irradiation and temperature decrease. Flavonols were also affected by the temperature and altitude of the site, but less prominent as anthocyanins. Albert *et al.*[32] reported that the content of selected flavonols increased at lower temperatures and Jaakola and Hohtola[3] determined that higher temperatures may inhibit flavonol biosynthesis and cause degradation of selected flavonols. A decrease of temperatures was measured at higher altitudes in both locations (Table 1), which could be reflected in an increase of flavonol levels in analysed elderberry parts.

Total flavanones (naringenin glycosides) represented a minor share of total phenolics in analysed elderberry parts. Their content was affected by the altitude but a diverse pattern of their turnover has been detected in different elderberry parts. Elderberry leaves and berries collected at higher altitudes contained 44% and 42% more flavanones in location 1 and 59% more flavanones in location 2 compared to lowest elevations, respectively. Stark *et al.*[33] determined a positive correlation between naringenin content and temperature sum and slope.

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Cyanogenic glycosides

The variation in the content of CGG is a response of individual plants to environmental factors related to altitude or genetic adaption of the populations growing at specific environments.[5] During the periods of low temperatures and slow vegetative growth clover plants defend themselves against herbivores by a drastic increase of CGG synthesis. When the environmental conditions for rapid regrowth are improved and grazing creates little hazard for the plant, the synthesis of cyanogenic glycosides is strongly reduced.[34] A similar protective strategy can be observed in plants at higher altitudes and shorter lengths of vegetation periods as they rapidly synthetise more toxins for defence against herbivores. Furthermore, the contents of CGG can be greatly affected by seasonal, nutritional and genetic factors.[35]

In general, increased amounts of CGG have been measured in elderberry parts collected at higher altitudes. Elder leaves were the richest in sambunigrin in comparison with other plant parts and 63% and 27% more sambunigrin was measured in leaves collected at highest altitude compared to the lowest site at locations 1 and 2. Nevertheless, almost three-fold higher content of cyanogenic glycosides was recorded in elder leaves from location 2 compared to the other location. Elder flowers accumulated four-fold lower sambunigrin levels compared to the leaves. At locations 1 and 2, 62% and 92% more sambunigrin was detected in flowers from the highest altitudes compared to the lowest elevations Temperature is probably the critical factor affecting the contents of CGG (Table 1), which corresponds to the data on white clover reported by Stochmal and Oleszek.[34] They measured the highest content of CGG during the spring months (May to June), followed by a decrease in August (synchronised with temperature above 15°C) and a gradual increase during autumn (September to October). Even a short warm period in the middle of September resulted in a decrease of cyanogenic glycosides.[34] Similarly, elder leaves and flowers contained highest levels of CGG on samplings, when temperatures were below 15°C, suggesting that CGG increase is linked with low temperatures. Lower temperatures at the time of sampling were characteristic of location 2 compared to location 1, which is probably the reason for significantly higher levels of CGG in elder leaves collected at location 2. The content of CGG was lowest in elder berries compared to elder leaves and flowers, which can partly be explained by highest temperature at August samplings (Table 1). The results on CGG in different elderberry parts related to temperature increase are not consistent with the data reported by Niedźwiedź-Siegień and Gierasimiuk,[36] who measured more CGG in flax seedlings subjected to 30°C compared to seedlings grown at 15°C.

Differences in sambunigrin content levels have been detected in elder berries collected at different altitudes in both locations. Elder berries generally accumulated more CGG at highest altitudes, but the pattern was not consistent. Lidroht *et al.*[37] and Niedźwiedź-Siegień and Gierasimiuk[36] reported that higher light intensity increases the content of CGG in plants, which could be the reason for their increase in various parts of elderberry at higher altitudes.

CONCLUSION

A similar response of elder plants to environmental factors (altitude, location) in terms of the composition of secondary metabolites has been detected in the analysed elder parts. It seems that the populations growing at different altitudes and locations have adapted to their specific environmental conditions, which is reflected in biosynthesis of phenolics and cyanogenic glycosides. Different plant parts (leaves, flowers and berries) accumulate diverse levels of both analysed groups of secondary metabolites, irrespective of the altitude. Their synthesis was mostly affected by solar irradiation and changes of temperature. Hydroxycinnamic acids, flavonols and anthocyanins responded as UV absorbing secondary metabolites and their content levels increased at higher altitudes. Furthermore, flavonols, flavanones, anthocyanins and cyanogenic glycosides were affected by the environmental temperature. To sum up, elder berries and flowers collected at the foothill were characterised by lowest levels of both beneficial (phenolics) and harmful compounds (CGG) and as such seem adequate for human consumption. However, in order to maximise the content of phenolics (and with certain measures eliminate the negative effects of cyanogenic glycosides) in elder products it may be advantageous to collect elder plants at different altitudes as the variability and genetic adaptation to the specific environment positively influences their contents of beneficial secondary metabolites.

ACKNOWLEDGEMENT

The research is part of program Horticulture No. P4-0013-0481 funded by the Slovenian Research Agency (ARRS).

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