Chemical composition and antioxidant activity of leaves of mycorrhized sea-buckthorn (*Hippophae rhamnoides* L.)

Anna Jaroszewska[1], and Wioletta Biel[2*]

ABSTRACT

The leaves of sea-buckthorn (*Hippophae rhamnoides* L.) can be a rich source of nutrients and biologically active substances. Their levels depend on growing conditions, agricultural technology and climate. The studies however, mainly focus on nutritious value of fruit and seeds and there is shortage of the information regarding to buckthorn leaves. The aim of the experiment was to determine the effect of symbiotic mycorrhizal fungi on the chemical composition and antioxidant activity of sea-buckthorn leaves. The study was conducted in 2014 and 2015 at the Experimental Station in Lipnik, Poland. Mycorrhization improved the nutritional value of leaves of sea-buckthorn by increasing levels of total protein (3%), N free extract (1%), Ca (9%), Na (16%), Fe (18%), Cr (34%), and partially elevating antioxidant activity by increasing the concentration of polyphenols (7%). Leaves of 'Habego' had a higher nutritional value, containing more total protein (4%), crude fat (7%), crude fiber (30%), and all fractions of dietary fiber. 'Hergo' had more beneficial levels of minerals (P-27%, K-67%, Mg-4%, Na-30%, Zn-8%, and Cr-21%), polyphenols (51%), total flavonoids (35%), carotenoids (10%), and L-ascorbic acid (8%), as well as higher antioxidant activity (40%). The results of our study partly confirmed the earlier scientific reports on the impact of mycorrhiza on the chemical composition of plants. However, it is not possible to compare our results with data on berry plants, including sea-buckthorn, due to the lack of information in the literature.

Key words: Antioxidants, chemical composition, fiber fractions, mycorrhiza, vitamins.

[1]West Pomeranian University of Technology in Szczecin, Department of Agronomy, Papieża Pawła VI 3, 71-434 Szczecin, Poland.
[2]West Pomeranian University of Technology in Szczecin, Department of Pig Breeding, Animal Nutrition and Food, Judyma 10, 71-460 Szczecin, Poland. *Corresponding author (wioletta.biel@zut.edu.pl).
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INTRODUCTION

Natural products, either as extracts or as pure compounds, give limitless possibilities for the discovery of new drugs and improving food quality, also thanks to their wide availability. Sea-buckthorn (*Hippophae rhamnoides* L.) contains a series of compounds including, among others, carotenoids, tocopherols, sterols, flavonoids, lipids, ascorbic acid, and tannins. These compounds are of interest not only from the chemical point of view, but also because many of them exhibit biological and therapeutic activities, including antioxidant, antitumor, hepato-protective, and immunomodulatory properties (Saikia and Handique, 2013). Evaluation of the nutritional value of sea-buckthorn usually concerns its fruit and seeds, while the leaves are rather neglected. However, some studies confirm that also its leaves are a rich source of compounds with anti-inflammatory (Padwad et al., 2006) and antibacterial properties (Upadhyay et al., 2011). They contain large amounts of crude protein, relatively high crude fat, and are a good source of macroelements (Jaroszewska et al., 2016). Therefore, sea-buckthorn leaves may be an excellent source of biologically active phytochemicals in both medicine and human nutrition.

The arbuscular mycorrhizal symbiosis is recognized for its multiple positive effects on plant growth and for its important contribution to the maintenance of soil quality. In spite of these benefits to agriculture, the realization of the full potential of this symbiosis has not yet been reached (Karagiannidis et al., 2012). During the establishment of the arbuscular mycorrhizal symbiosis, a range of chemical and biological parameters is affected in plants, including the pattern of secondary compounds (Karagiannidis et al., 2012). Previous studies confirm that arbuscular mycorrhizal fungi (AMF) affects the content of phenols (Hazzoumi et al., 2015). Higher content of flavonoids has been demonstrated in mycorrhizal roots of alfalfa (*Medicago sativa* L.) (Larose et al., 2002). The AMF root colonization factor leads to significantly increased concentrations of most of the phenolics in purple coneflower (Araim et al., 2009) and in strawberry fruits (Castellanos-Morales et al., 2010).

Currently, there is little information on the chemical composition and antioxidant properties of sea-buckthorn (*Hippophae rhamnoides* L.) leaves. There is also a lack of data concerning the potential impact of symbiotic mycorrhizal fungi on the quality of berry plants, including sea-buckthorn. Therefore, in this study we conducted a field experiment to evaluate the impact of symbiotic mycorrhizal fungi on the chemical composition

and antioxidant activity of leaves of two varieties of sea-buckthorn.

MATERIALS AND METHODS

The study was conducted in 2014 and 2015 at the Experimental Station in Lipnik (53°20'35" N, 14°58'10" E, 7 m a.s.l.), Poland. The soil on which the experiment was carried out belonged to typical rusty soils (Polish Soil Classification, 2011), classified as Haplic Cambisol according to IUSS Working Group WRB (2015). At the Ap level, the soil has a slightly acidic loamy sand. The level of humus is formed from clay sands. The analysis of soil minerals showed high P levels and moderate Mg and K levels. The experiment was designed according to a completely randomized method in five replicates (one shrub = one repeat). Shrubs were planted in 4 × 3 m spacing. The size of single plot was 12 m^2. The total number of plots in the experiment was 20. The subject of the study included 2 and 3 yr old shrubs, female varieties Habego and Hergo. Mycorrhization was conducted with ectomicorrhizal mycelium, which is symbiotic for plants of the olive family. The isolate was obtained from the natural ecosystems in Croatia. It contains symbiotic mycorrhizal fungi (*Glomus* spp., *Gigaspora* spp., *Pochonia* spp., *Lecanicillium* spp.), and the root bacteria (*Bacillus* spp.) A dose of 15 mL was applied in two places the root zone of plants in the first year of experiment. The mycelium contained an addition of hydrogel (ensuring humidity essential for the initial fungi development). Leaves for the analysis were collected during harvest from shoots without fruiting. Leaves were collected from the outside of the bush, at half of their height. The leaves were taken from the 1 yr old shoots without any signs of aging or mechanical damage.

The experiment factors were mycorrhiza (half of the bushes from each variety were subject to mycorrhization, and half were used as a control group) and variety (Habego and Hergo).

All determinations were expressed on a dry weight (DW) basis. Dry matter, crude protein, ether extract, crude fiber, crude ash and N free extract (NFE) were determined by AOAC (2012). Dry matter was evaluated by drying at 105 °C to constant weight; ether extract by Soxhlet extraction with diethyl ether; crude ash by incineration in a muffle furnace at 580 °C for 8 h; crude protein (N × 6.25) by Kjeldahl method using a Büchi Distillation Unit B-324 (Büchi Labortechnik AG, Flawil, Switzerland); crude fiber was determined with a fiber analyzer ANKOM 220 (ANKOM Technology, Macedon, New York, USA); total carbohydrates were calculated as: N free extract (NFE) (%) = 100 – % (moisture + crude protein + crude fat + crude ash + crude fiber).

The fiber components were determined using the detergent method according to Van Soest et al. (1991) performed with a fiber analyzer ANKOM 220. Determination of neutral detergent fiber (NDF) was conducted on an ash-free basis and included sodium dodecyl sulfate ($NaC_{12}H_{25}SO_4$) (822050, Merck, Kenilworth, Nueva Jersey, USA). Determination of acid detergent fiber (ADF) included hexadecyltrimethylammonium bromide ($C_{16}H_{33}$) $N(CH_3)_3Br$ (Merck 102342), while acid detergent lignin (ADL) was determined by hydrolysis of ADF sample in 72% sulfuric acid (H_2SO_4). Hemicellulose (HCEL) was calculated as the difference between NDF and ADF, while cellulose (CEL) as the difference between ADF and ADL.

The material for the macro-components concentration analyses was subjected to mineralization in concentrated sulfuric acid (H_2SO_4) and perchloric acid ($HClO_4$), whilst the material for micro-components concentration analyses was digested in a mixture of nitric acid (HNO_3) and perchloric acid ($HClO_4$). The concentration of P was determined by the Egner-Riehm colorimetric method, with ammonium molybdate, at wavelength 660 nm, by using a Specol 221 apparatus (866287, Carl Zeiss Jena, Germany). An atomic absorption spectrometer (ASA) (iCE 3000 Series, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to determine K, Na, and Ca – by means of emulsion flame spectroscopy, and Mg, Zn, Fe, Pb, Cr and Cu – by means of absorption flame spectroscopy. The nitrate content was determined by potentiometry. Prior to assay Ca content, K and Mg trials were appropriately diluted. Other mineral compounds were determined in concentrated samples.

For total polyphenols, total flavonoids, and antioxidant activity determination, methanol extracts were prepared according to Kumaran and Karunakaran (2007). The total phenolic content of plant extracts was determined using Folin-Ciocalteu reagent (Yu et al., 2002); 0.1 cm^3 plant extract was mixed with 0.5 cm^3 Folin-Ciocalteu reagent and 1.5 cm^3 20% sodium carbonate. The mixture was shaken thoroughly and made up to 10 cm^3 using distilled water. The mixture was allowed to stand for 2 h. Then the absorbance at 765 nm was determined. These data were used to estimate phenolic content using a standard curve obtained from various concentrations of gallic acid. Total polyphenol content in samples was calculated as the amount of gallic acid equivalent (GAE) in mg kg^{-1} sample DW.

The total flavonoids content was determined by the method of Kumaran and Karunakaran (2007) using quercetin as a reference compound; 1 cm^3 plant extract in methanol was mixed with 1 cm^3 aluminum trichloride in methanol and a drop of acetic acid, and then diluted with ethanol to 25 cm^3. The absorption at 415 nm was read after 40 min. Blank samples were prepared from 1 cm^3 plant extract and a drop of acetic acid, and then diluted to 25 cm^3 with methanol.

The antioxidant capacity was assayed by Trolox Equivalent Antioxidant Capacity (TEAC) method (Re et al., 1999). This method is based on the generation of a stable colored free radical in aqueous solution and the measurement of antioxidant capacity, or free radical scavenging, as the decrease in the absorbance

of the colored solution in a UV-VIS spectrometer following addition of the antioxidant. The radical used is 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) with an absorption maximum of 734 nm. ABTS was dissolved in deionized water to give a stock solution with a concentration of 7 mM. The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 9 cm^3 ABTS stock solution with 1 cm^3 24.5 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature overnight before use. The $ABTS^{\bullet+}$ solution was diluted with deionized water (approximately 50-fold) to give an absorbance close to 0.70 at 734 nm. A 3 cm^3 aliquot of $ABTS^{\bullet+}$ solution was put into a cuvette in the spectrophotometer and absorbance measured. An aliquot of 30 mm^3 of methanol extract was then added and a second absorbance reading taken after 6 min, by which time the discoloration was effectively complete.

Carotenoids were extracted with 80% solution of acetone and determined according to Lichtenthaler and Wellburn (1983). These compounds were determined in wavelength 440 nm, after having subtracted the concentration of chlorophyll A and B, using wavelengths 663 and 645 nm, respectively, and corresponding absorption coefficients at which carotenoids do not absorb.

Tocopherols were extracted from plants samples with hexane and were determined by Prieto et al. (1999) method. A sample volume of 0.5 cm^3 hexane extract was mixed in a test tube with 5 cm^3 reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 37 °C for 90 min with vigorous shaking. Absorbance of the aqueous phase at 695 nm was measured against the appropriate blank. A typical blank contained 5 cm^3 reagent solution and 0.5 cm^3 pure hexane, and it was incubated under the same conditions as the samples. An UV-VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) was used.

Solution for extraction of water-soluble vitamins was made by mixing 50 cm^3 acetonitrile with 10 cm^3 glacial acetic acid and the volume was finally made up to 1000 cm^3 with double distilled water. A sample of 10 g was transferred into conical flasks and 25 cm^3 extraction solution was added, water bath was kept shaking at 70 °C for 40 min. Thereafter, the sample was cooled down, filtered, and finally the volume was made up to 50 mL with extraction solution (Aslam et al., 2013).

The contents of vitamins thiamine (B1), riboflavin (B2), ascorbic acid (C), and niacin (PP) in extracts were measured using a high-performance liquid chromatographic (HPLC) method described by Kłódka et al. (2008); using an HPLC system (Series 200, PerkinElmer, Shelton, Connecticut, USA) equipped with Hypersil ODS column (150 mm × 4.6 mm, 5 μm particle). The mobile phase was 50 mM KH_2PO_4 (A) and methanol (B) in gradient from 0% to 30% A in 8 min, then this ratio was maintained for 7 min. Wavelength was λ = 245 nm, flow rate 1 cm^3 min^{-1}, and injection of 20 mm^3. Retention times and recoveries for C, B1, PP, and B2 were 1.45, 5.50, 7.20, 9.80 min, and 96.20, 93.51, 89.53, and 92.41 respectively.

Two-factor ANOVA was carried out on the experimental results using the mycorrhization and genotype as independent variables, after assessing normality and homogeneity of variance. The significance of differences between means was compared by Tukey's multiple range tests (admissible error for determinations of chemical components was 5%). Results were presented as mean ± standard deviation of three independent determinations. Statistical significance was considered at $p \leq 0.05$. The results from the experiment were analyzed using the Statistica version 12.0 software (StatSoft, Tulsa, Oklahoma, USA).

RESULTS AND DISCUSSION

We found nonsignificant effect of mycorrhization on the content of dry weight, crude ash, and crude fat in sea-buckthorn leaves (Table 1). Moisture content of fresh leaves used in the experiment was 68 ± 7% of fresh weight (FW). It was lower than the range of 82% to 96% FW in most vegetables (lettuce, green beans, asparagus, green peppers, and spinach) reported by Granado et al. (1992), but comparable with that (67% FW) of tea leaf (Li et al., 2012). Morgenstern et al. (2014) found the highest DW content in leaves of sea-buckthorn collected at the end of July (32%). Our analysis did not distinguish a variety of sea-buckthorn that differs significantly in DW content in the dried leaves ($p \leq 0.05$). The average dry weight content was 95.3%, with a standard deviation of 0.9.

Mycorrhization resulted in an increase in protein content and NFE in sea-buckthorn leaves (by 2.63% and 1.21%, respectively, relative to the control) and a reduction in crude fiber content (by ~ 3.24%). In the present study variety had

Table 1. Chemical composition (n = 3) of sea buckthorn leaves (g kg^{-1} DW).

	Mycorrhizal effect			Genotype effect		
Item	Control	Mycorrhiza	P-value	Habego	Hergo	P-value
Moisture (g kg^{-1} FW)	680.0 ± 70.3a	625.9 ± 0.27a	0.223	638.2 ± 14.2a	667.7 ± 78.1a	0.476
Dry matter	953.4 ± 0.9a	953.1 ± 19.6a	0.540	953.3 ± 1.1a	953.2 ± 0.8a	0.900
Crude protein	140.9 ± 0.9b	144.7 ± 25.5a	0.006	145.4 ± 27.4a	140.3 ± 20.4b	0.002
Crude ash	40.9 ± 1.0a	39.1 ± 5.6a	0.081	37.2 ± 3.4b	42.9 ± 1.3a	0.000
Crude fat	54.4 ± 4.6a	55.4 ± 0.7a	0.376	56.7 ± 1.9a	53.1 ± 3.2b	0.021
Crude fiber	104.8 ± 10.6a	101.4 ± 20.2b	0.031	116.4 ± 6.9a	89.7 ± 3.1b	0.000
NFE	655.0 ± 14.8b	663.0 ± 49.2a	0.001	644.2 ± 36.7b	673.9 ± 27.3a	0.000

Mean values with the same letter in each line are non-significantly different at $p \leq 0.05$ according to the Tukey test, DW: Dry weight, FW: fresh weight, NFE: nitrogen free extract.

an impact on the level of crude ash, crude protein, crude fat, crude fiber, and NFE.

The leaves of the studied varieties of sea-buckthorn showed a considerable diversity in mineral content measured by crude ash. Its average level was 4% DW and ranged from 3.72% to 4.29% DW, with a standard deviation of 0.39. 'Habego' contained 13.3% more ash than 'Hergo'. This study confirmed sea-buckthorn leaves as a rich source of protein. 'Habego' leaves had 145.4 g protein kg^{-1} DW, that is more than the level reported by Kashif and Ullah (2013) (120.35 g kg^{-1} DW). The difference is probably due to the genotype and the impact of environmental conditions (Zheng et al., 2012). It is worth noting that the protein content in sea-buckthorn fruit (more frequently used as food) stands at a more than 2.5 times lower level of 47 g kg^{-1} DW (Selvamuthukumaran and Farhath, 2014).

The leaves of the studied varieties of sea-buckthorn differed in crude fat content. Similar to protein, the highest crude fat levels were found in 'Habego' (56.7 g kg^{-1} DW). This confirms the results of other studies, which show that its leaves are a good source of lipids. Fulkerson et al. (2008) report that leaves of fodder radish, rape, chicory, and plantain contain on average 55.6% less crude fat than sea-buckthorn.

The properties of dietary fiber and its value depend on the source and the mutual proportions of the respective fractions. Dietary fiber contains many structures with diverse physical and chemical properties, and is capable of inducing physiological effects on the human body (Mann and Cummings, 2009). The leaves of 'Habego' contained ~ 23% more crude fiber than 'Hergo'. Mycorrhization significantly reduced levels of crude fiber (Table 1) and neutral detergent fiber (NDF) (Figure 1). We also observed some downward trends in the levels of acid detergent fraction (ADF), hemicellulose (HCEL), and cellulose (CEL) in the mycorrhized leaves of sea-buckthorn, although these were nonsignificant. No such trend was observed for acid detergent lignin (ADL). The leaves of 'Habego' contained significantly more crude fiber (23%, Table 1) and all evaluated dietary fiber fractions (Figure 1). In the literature there are no data on the composition of the dietary fiber fractions obtained by the detergent method. In this study, the average content of NDF ranged from 219.3 to 278.7 g kg^{-1} DW, and again, it is impossible to compare this data to literature due to the lack of reports relating directly to sea-buckthorn. Fulkerson et al. (2008) examined NDF in fodder radish (*Raphanus sativus* L. var. *oleiformis* Pers.), rape (*Brassica napus* L.), chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.), which ranged from 156 to 489 g kg^{-1} DW.

The determined average ADF, consisting of cellulose and lignin, ranged from 167.9 to 205.1 g kg^{-1} DW. 'Habego' leaves contained ~ 18% of this fraction than those of 'Hergo'. ADF level, just like NDF, was similar to fodder radish, rape, chicory, and plantain in the study by Fulkerson et al. (2008).

Acid detergent lignin (ADL), which to some extent influences the hardness of sea-buckthorn leaves, differed between 'Habego' (71.8 g) and 'Hergo' (65.9 g) by as much as 8%.

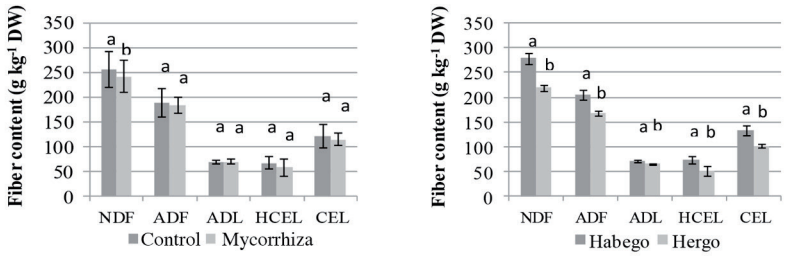
Hemicellulose content was in the range of 73.6 to 51.4 to g kg^{-1} DW. A common significant source of HCEL are cereal grains, herbs, and vegetables (Biel and Jacyno, 2014; Černiauskiene et al., 2014). Schädel et al. (2010) examined four herb species (*Geum urbanum* L., *Leontodon hispidus* L., *Salvia pratensis* L., *Silene flos-cuculi* (L.) Greuter & Burdet) and reported HCEL content between the range of 60-220 g kg^{-1} DW. These authors found higher concentrations in silene, while the lowest HCEL concentrations were measured in leaves of geum, leontodon, and salvia, at less than 10% DW.

In this study, the average CEL content was 117.6 g kg^{-1} DW and varied from 102 to 133.2 g kg^{-1} DW. Fraser and Rowarth (1996) found a similar CEL level in the herbs of chicory and plantain. In the present study 'Habego' leaves contained significantly more CEL (~ 23%) than 'Hergo'.

Sea-buckthorn leaves are a rich source of minerals (Jaroszewska et al., 2016), but their levels depend on many factors, including genetic characteristics, climate, soil conditions, maturity of the plant, and the time of harvesting, which was confirmed in our study (Table 2). Compared to the levels found in berries of sea-buckthorn (Ercisli et al., 2007) the average mineral content of the leaves was similar, with the exception of K (116% greater), Mg (31%), Ca (94%), and Fe (574%).

Mycorrhization significantly differentiated macro- and microelements in leaves of sea-buckthorn. Compared to control, leaves of the mycorrhized sea-buckthorn had significantly lower concentrations of P (by 0.5 g kg^{-1} DW,

Figure 1. Fiber components (n = 3) of sea-buckthorn leaves.



NDF: Neutral detergent fiber, ADF: acid detergent fiber, ADL: acid detergent lignin, HCEL: hemicelluloses, CEL: cellulose, DW: dry weight.
Mean values with the same letter are not significantly different at $p \leq 0.05$ according to the Tukey test.

Table 2. Macro- and microelements (n = 3) and nitrates in sea buckthorn leaves.

Item	Mycorrhizal effect			Genotype effect		
	Control	Mycorrhiza	P-value	Habego	Hergo	P-value
			Macroelements (g kg^{-1} DW)			
P	4.75 ± 0.4a	4.25 ± 0.9b	0.001	3.96 ± 0.5b	5.04 ± 0.1a	0.000
K	14.9 ± 3.8a	12.2 ± 4.1b	0.000	10.2 ± 1.8b	17.0 ± 1.4a	0.000
Mg	2.35 ± 0.1a	1.57 ± 0.0b	0.000	1.92 ± 0.4b	1.99 ± 0.5a	0.000
Ca	3.45 ± 0.3b	3.77 ± 0.2a	0.001	3.69 ± 0.1a	3.57 ± 0.4b	0.026
Na, mg kg^{-1} DW	84.2 ± 16.9b	98.1 ± 10.8a	0.000	79.1 ± 11.0b	103.2 ± 5.0a	0.000
NO_3, mg kg^{-1} DW	152.1 ± 16.2b	191.5 ± 9.2a	0.000	173.1 ± 30.4a	170.5 ± 14.9b	0.000
			Microelements (mg kg^{-1} DW)			
Fe	49.5 ± 3.9b	58.3 ± 1.1a	0.000	56.0 ± 3.6a	51.7 ± 6.5b	0.000
Zn	33.7 ± 4.5a	30.1 ± 1.7b	0.000	30.6 ± 1.0b	33.1 ± 5.2a	0.000
Cr	0.94 ± 0.0b	1.26 ± 0.2a	0.000	1.00 ± 0.1b	1.21 ± 0.3a	0.000
Cu	3.82 ± 0.2a	3.66 ± 0.3a	0.294	3.82 ± 0.1a	3.66 ± 0.3a	0.293
Pb	nf	nf		nf	nf	

Mean values with the same letter in each line are not significantly different at $p \leq 0.05$ according to the Tukey test, nf: not found, DW: dry weight.

i.e. 12%), K (by ~ 2.7 g kg^{-1} DW, i.e. 18%), Mg (by ~ 0.78 g kg^{-1} DW, i.e. 33%), and Zn (by ~ 3.6 g kg^{-1} DW, i.e. 11%). Although there was nonsignificant effect of mycorrhiza on the Cu concentration, we observed a downward trend in Cu levels, which confirms the results of Kowalska et al. (2015), stating that the mycorrhiza significantly reduced content of Cu and Zn in leaves of mycorrhized lettuce.

The tested leaves of mycorrhized sea-buckthorn contained significantly more Ca (by ~ 0.32 g kg^{-1} DW, 9%), Na (by 13.9 g kg^{-1} DW, 16%), Fe (8.8 g kg^{-1} DW, 18%), and Cr (by 0.32 g kg^{-1} DW, 34%) than control. Lower K and higher Ca levels in leaves of mycorrhized sea-buckthorn were reported by Jaroszewska et al. (2016). Similarly, Baslam et al. (2011) reports an increase in the concentration of Ca and Fe in fresh weight of mycorrhized lettuce. Sea-buckthorn leaves grown in mycorrhized plots were also characterized by higher nitrate (by ~ 39.4 g kg^{-1} DW, i.e. 26%), probably due to the mycorrhiza enabling better uptake of N by plant roots, and also by the soil pH. An increase in acidity increases the intensity of nitrate uptake by plants ($N\text{-}NO_3^-$).

Lead belongs to the group of metals hazardous to health. It can cause severe anemia, brain damage, neurological disorders, reproductive problems, and reduced intelligence. Plants normally contain trace amounts of lead which increase with the contamination of soil and air. Significantly, in the analyzed samples of sea buckthorn leaves, we found no lead.

The content of the tested mineral components in the two examined varieties of sea-buckthorn leaves were significantly different, which points to the various capability to absorb and to cumulate in the biomass the mentioned components, by specific varieties cultivated in the same habitat conditions. A significantly higher concentration of the analyzed elements was found in 'Hergo'. Its leaves had more P (1.08 g kg^{-1} DW, i.e. 27%), K (6.8 g kg^{-1} DW, i.e. 67%), Mg (0.7 g kg^{-1} DW, i.e. 4 %), Na (24.1 g kg^{-1} DW, i.e. 30%), Zn (2.5 mg kg^{-1}, i.e. 8%), and Cr (0.21 mg kg^{-1} DW, i.e. 21%) than 'Habego'. 'Hergo' leaves also had less nitrate (~ 2.6 mg kg^{-1} DW, i.e. 1%). The results correspond with previous reports by Jaroszewska et al. (2016), which showed significant differences in mineral content between varieties of sea-buckthorn.

Sea-buckthorn is a natural source of bioactive compounds with antibacterial and anti-stress properties, used in the treatment of cancer, cardiovascular diseases, and diabetes. The average concentrations of antioxidants tested (Table 3) were higher than those in sea-buckthorn berries which contain 530 to 970 mg carotenoids kg^{-1} DW (Pop et al., 2014) and 380 mg flavonoids kg^{-1} DW (Chu et al., 2003).

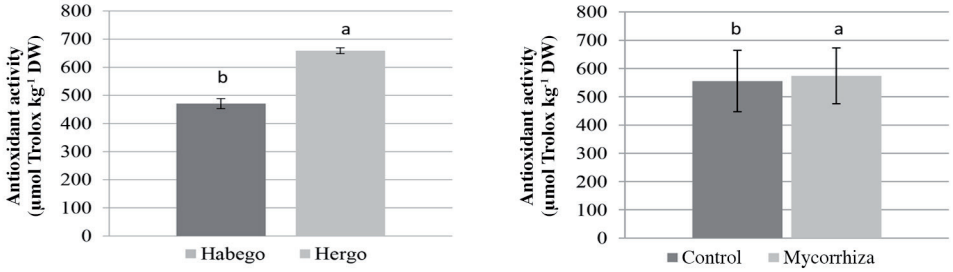
In our study, leaves of the mycorrhized sea-buckthorn contained significantly more polyphenols (by 590.2 mg GAE kg^{-1} DW, i.e. 7%), compared to plants from control plots. However, they had fewer total flavonoids (261.3 mg quercetin equivalent [QE] kg^{-1} DW, i.e. 16%) and carotenoids (58.2 mg kg^{-1} DW, i.e. 6%). Previous studies on the influence of mycorrhization on the content of secondary metabolites in plants (purple coneflower and lettuce) (Araim et al., 2009; Baslam et al., 2011) confirm the significant role of AMF in increasing their concentration. The leaves of the mycorrhized sea-buckthorn had significantly higher antioxidant activity (by 18.2 µmol Trolox kg^{-1} DW, i.e. 3%) (Figure 2), which is reflected in the research of Ordookhani et al. (2010), reporting increased antioxidant activity in fruits mycorrhized tomatoes.

Table 3. Content of antioxidants (n = 3) in sea buckthorn leaves.

Item	Mycorrhizal effect			Genotype effect		
	Control	Mycorrhiza	P-value	Habego	Hergo	P-value
Polyphenols, mg GAE kg^{-1} DW	8584.7 ± 2191.9b	9174.6 ± 1834.9a	0.023	7067 ± 570.3b	10691.7 ± 417.9a	0.000
Total flavonoids, mg QE kg^{-1} DW	2546.2 ± 386.3a	2284.9 ± 560.8a	0.171	2054.1 ± 243.5b	2777.1 ± 362.9a	0.003
Carotenoids, mg kg^{-1} DW	1004.3 ± 8.3a	946.1 ± 92.9b	0.000	929.9 ± 74.9b	1020.5 ± 14.2a	0.000

Mean values with the same letter in each line are not significantly different at $p \leq 0.05$ according to the Tukey test, GAE: Gallic acid equivalent, DW: dry weight, QE: quercetin equivalent.

Figure 2. Antioxidant activities (n = 3) measured by ABTS assay in sea-buckthorn leaves.



Mean values with the same letter are not significantly different at $p \leq 0.05$ according to the Tukey test.
DW: Dry weight, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

The studied 'Hergo' had clearly superior antioxidant properties, with significantly more polyphenols (3624.7 mg GAE kg^{-1} DW, i.e. 51%), total flavonoids (723 mg QE kg^{-1} DW, i.e. 35%), and carotenoids (90.6 mg kg^{-1} DW, i.e. 10%). The leaves of this variety were also distinguished by higher antioxidant activities (by ~ 188.3 µmol $Trolox^{-1}$ DW, 40%) (Figure 2). Morgenstern et al. (2015) showed that sea buckthorn leaves are very rich in beneficial phenolic compounds. The significant differences in antioxidant levels between the two varieties of sea-buckthorn were probably due to the varietal characteristics, as the plants were grown under the same conditions of climate and soil. The results as well as other analysis (Alfaro et al., 2013) highlight the importance of genotype as a crucial factor affecting the content of antioxidants in berry plants.

The harmful effects of free radicals are counteracted by antioxidants, which include, among others, vitamins A, C, and E, abundant in fruits and vegetables. In the studied sea-buckthorn leaves, mycorrhization and variety significantly differentiated only the level of ascorbic acid (Table 4). Depending on the ecotype, sea-buckthorn berries are reported to contain L-ascorbic acid at levels ranging from 360 to 2500 mg 100 g^{-1} (Bal et al., 2011). The examined leaves contained an average of 2139.6 mg kg^{-1} DW, compared to 155.3 mg kg^{-1} DW (7%) in control. Baslam et al. (2011) demonstrated a significant increase in L-ascorbic acid in fresh weight in mycorrhized lettuce. The leaves of 'Hergo' sea-buckthorn had a 164.1 mg kg^{-1} DW (8%) higher concentration of L-ascorbic acid than leaves of 'Habego'. Although we did not find significant differences in the concentrations of the remaining vitamins (tocopherols, thiamine, riboflavin, and niacin) in sea-buckthorn leaves, there was a tendency towards their higher levels in the leaves of non-mycorrhized sea-buckthorn and 'Hergo' leaves.

CONCLUSION

The leaves of mycorrhized sea-buckthorn had a higher content of total protein, N free extract, Ca, Na, Fe, Cr, and polyphenols, which means that the cultivation of mycorrhized sea-buckthorn may improve the intake of these compounds with diet without the need to increase their consumption. Leaves collected from mycorrhized sea-buckthorn also showed an increased antioxidant activity.

The concentration of test compounds in the leaves of different varieties was clearly dependent on the varietal characteristics. The Habego variety was characterized by a higher nutritional value, containing more total protein, crude fat, crude fiber, and all tested fractions of dietary fiber. On the other hand, leaves of 'Hergo' sea-buckthorn had more minerals (P, K, Mg, Na, Zn, and Cr), polyphenols, total flavonoids, carotenoids, and L-ascorbic acid. The leaves of 'Hergo' also showed higher antioxidant activity.

Summing up the results of the experiment is difficult to assess the impact of arbuscular mycorrhizal fungi (AMF) on the chemical composition of sea buckthorn leaves and their antioxidant activity. The results of our study partly confirmed the earlier scientific reports on the impact of mycorrhiza on the chemical composition of plants (fruits, leaves). However, it is not possible to compare our results with data on berry plants, including sea-buckthorn, due to the lack of information in the literature. Therefore, further research in this area seems necessary.

Table 4. Content of vitamins (n = 3) in sea buckthorn leaves.

	Mycorrhizal effect			Genotype effect		
Item	Control	Mycorrhiza	P-value	Habego	Hergo	P-value
	mg kg^{-1} DW			mg kg^{-1} DW		
Tocopherols	40.98 ± 4.9a	38.19 ± 2.7a	0.094	38.1 ± 2.4a	41.0 ± 5.1a	0.080
L-ascorbic acid	2217.3 ± 188a	2062.0 ± 16.0b	0.007	2057.6 ± 17.4b	2221.7 ± 184a	0.000
Thiamine	1.40 ± 0.1a	1.36 ± 0.2a	0.609	1.40 ± 0.1a	1.38 ± 0.2a	0.849
Riboflavin	5.86 ± 0.3a	5.68 ± 0.2a	0.143	5.67 ± 0.2a	5.87 ± 0.3a	0.113
Niacin	4.86 ± 0.4a	4.57 ± 0.2a	0.187	4.66 ± 0.3a	4.77 ± 0.4a	0.583

Mean values with the same letter in each line are not significantly different at $p \leq 0.05$ according to the Tukey test.

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