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Sea Buckthorn Leaves as a Potential Source of Antioxidant Substances

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Abstract: Each year, agro-foods produce thousands of tonnes of by-products that contain high-value, unique nutrients. The application of plant by-products enables agro-food corporations to obtain value from them and avoid using natural resources. The idea of the bio economy protects against environmental pollution and leads to a cheap source of bioactive components, which can be transformed into value-added products for other industries. The numerous publications on the positive impact of sea buckthorn (SBT, *Hippophae rhamnoides* L., Elaeagnaceae family) and its products on human health mainly concern its fruits and seeds. However, there are few data relating to the properties of SBT leaves. The leaves of SBT can be a rich source of nutrients and biologically active substances. In this investigation, we report the study of the leaves of four SBT cultivars. We determined their antioxidant capacities, measured total polyphenols and total flavonoids, and quantified their major polyphenols and alkaloids. The results show that SBT leaves are a source of antioxidants and alkaloids. Additionally, in this raw material, we identified the presence of individual flavonols (rutoside and quercetin), individual anthocyanidins (cyaniding, delphinidin, and peonidin), and chelerythrine by high-performance thin-layer chromatography (HPTLC) for the first time. Through these types of studies, we aim to revalue this raw material, which is not well known in the world. Considering its nutritional properties, we seek to increase the use of its high-value, unique nutrients in food processing, medicine, and animal nutrition, in accordance with the goals of a closed-loop bioeconomy.

Keywords: alkaloids; anthocyanins; by-products; chelerythrine; flavonoids; flavonols; *Hippophae rhamnoides*; nutraceutical value; polyphenols; tannins

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1. Introduction

With increasing food/feed costs in the animal production industry, it is necessary to operate using alternative feed resources to conventional food/feeds, especially regarding agricultural and industrial by-products. The use of agro-industrial by-products as functional food/feed materials could be a promising strategy that would reduce feed costs while maintaining the nutritional qualities of the feed. Many recent studies report that various herbs, as rich sources of bioactive compounds with antioxidant properties, are added to food and feed and are widely used as antimicrobial, anti-inflammatory food/feed preservatives and for ingredient stabilization [1–4]. Herb residues and agro-food by-products still contain large amounts of bioactive components with high levels of antioxidant properties. The antioxidant potential of various by-products such as the solid distillation wastes of

Greek oregano, rosemary, Greek sage, lemon balm, and spearmint; olive pomace and leaves, spent coffee grounds, and brewer's spent grain; and fruit and vegetable leaves, pulp, peel, pomace, and seeds have been investigated [5–7].

Also, the twigs and leaves of sea buckthorn (*Hippophae rhamnoides* L., Elaeagnaceae family), previously regarded as waste material, were shown to be a valuable source of substances with promising antioxidant, antimicrobial activity [8,9]. Among the many health-promoting properties of this plant, a positive effect on the expression of aquaporins, and thus on the production and secretion of bile, can also be observed [10]. Common sea buckthorn (*Hippophae rhamnoides* L., Elaeagnaceae family), known as seaberry, Siberian pineapple, sandthorn and sallow thorn, is an ancient plant with modern uses, due to its nutritional and medicinal value. All parts of SBT, e.g., berries, leaves, and seeds or pulp oil, contain abundant nutrients and many bioactive compounds [11,12]. Documentation of indigenous ethnobotanical knowledge of *H. rhamnoides* reveals that this plant was traditionally used to treat a wide variety of illnesses. Sea buckthorn is a multi-purpose plant, and its berries are used in the production of food industry products (juices, drinks, smoothies, jams, sauces, oils) and alcohols (wine, liqueur, beer additives); additionally, its herbal leaf teas provide a high level of access to flavonoids and contain detoxifying properties, and sea buckthorn by-products can be used in the production of fodder supplements, cosmetics, pharmaceuticals, and fuel for firewood [13–15], although the leaves of SBT are not fully utilized. The leaves have attracted increasing attention because of their potential health value [16]. The development of food products often relies on ingredient sourcing and costs. For example, the value of animal-derived ingredients is most affected by the growth rate of these animals, which is clearly influenced by the type of composition of their feed, among other variables. Different feeding products may have a different impact on animal development and, ultimately, on the quality of the end-product or ingredient, leading to different end-point quality. Due to the interesting nutrient profiling of SBT products, several authors have investigated its effect on the final quality of different animal products. SBT leaves and other by-products can be used to feed livestock (both ruminant and monogastric animals), with a stimulatory effect on growth and performance [17–21]. Meat products enriched with nutrients, such as polyunsaturated fatty acids and antioxidants, are gaining much more interest among consumers. It has been reported that SBT leaves contributes positively to the nutritional quality and lipid oxidative stability of chicken breast and thigh meat [22]. For example, broiler production efficiency is quite high per se, because the growth and development of a broiler to its full potential does not take much longer than one month. However, different and more natural strategies are needed to achieve the same or higher levels of production efficiency, plainly because of the increasing consumer awareness of feeding products. Vlaicu et al. [18] compared three different diets in the growing and finishing stages of broiler chicks with a conventional diet, with one including rapeseeds and grape meal and the others including flaxseed meal and SBT meal. It showed that broilers who were fed a diet with SBT and flaxseed meals showed significantly higher concentrations of polyunsaturated fatty acids family n-3 when compared to the control or the group fed with rapeseeds and grape meal.

Sea buckthorn pomace (SBT_P) is an industrial by-product rich in a variety of functional compounds, but it has not yet been effectively used. Therefore, Yan et al. [23] concluded that SBT_P could improve the production, immune function, and antioxidant status of weaned piglets and that the appropriate level of SBP supplementation is from 1.0% to 2.0%

A study by Dannenberger et al. [24] investigated the effect of sea buckthorn pomace (SBT_P) supplementation in the diet of growing German Landrace pigs on fatty acids in the blood and hypothalamus, on peripheral immune parameters and mRNA expression of the corticotropin-releasing hormone, and on the mineralocorticoid receptor and glucocorticoid receptor in the hypothalamus and spleen. The fatty acid profiles in the blood plasma were significantly affected by SBT_P supplementation at a C18:2n-6 and n-6/n-3 PUFA ratio compared with the control group. And the authors pointed out that effects derived

from supplementation with a rich source of n-3 fatty acids (such as SBT_P) might be more appreciable on stressful situations.

Sea buckthorn leaves are a rich source of phytochemicals, such as phenolic compounds (including polyphenols and flavonoids), polysaccharides, carotenoids, and saponins [25–27]. Therefore, SBT leaves have various beneficial effects, like antioxidant, hepato-protective, immunomodulatory, antistress, cardioprotective, and antidiabetic actions [13,28–32]. In this regard, sea buckthorn leaves are a potential ingredient because of their nutritional and medical components that are beneficial to human and animal health. Modern research is needed for better utilization of this species for food/fodder and for their pharmaceutical potential.

Nowadays, traditionally grown plants are the centre of attention, and the demand for their production is increasing due to the strategy of inhibiting or delaying diseases using a natural diet [33]. Young branches with leaves are a by-product after the mechanical harvesting of sea buckthorn berries [33,34].

Current research on this plant is very important and can lead to the emergence of new avenues of its utilization, including food/feed and pharmaceutical potential [35]. The potential for the production and sustainable harvest of edible and other useful parts of this plant can also boost the local economy for farmers.

However, literature reports on the profiles of many bioactive components of the leaves of SBT are still very limited. The necessity to characterize secondary metabolites and nutrients also results from previous reports on SBT [36–38]. Hence, this study aimed to determine the antioxidant activity and content of bioactive components of the leaves of four cultivars of SBT collected in Poland.

2. Materials and Methods

2.1. Plant Material, Experiment Set-Up and Sample Preparation

The research material consisted of *Hippophae rhamnoides* L. leaves from an experiment conducted at the Agricultural Experimental Station in Lipnik, belonging to the West Pomeranian University of Technology in Szczecin (53°42′ N, 14°97′ S), in 2014–2016 (Figure 1). Poland is located in the temperate climate zone. Four cultivars (cv.), Ascola, Habego, Hergo, and Leikora were used in this study. The leaves for analysis were taken before the fruit reached technical maturity. The sample consisted of leaves from one-year-old shoots with no signs of damage or ageing. The leaves were weighed and dried at room temperature (18–22 °C) for 3–4 days. The samples were ground to 0.1 mm by use of a laboratory-mill-type KNIFETEC 1095 (Foss Tecator, Höganäs, Sweden). To determine dry matter (DM), the samples were dried at 105 °C by a constant-weight method based on AOAC [39] (method 945.15).

The remaining milled samples were then stored in individual sealed containers at 4 °C until they were required for further evaluation.

2.2. Extracts Preparation

Dried samples (1 g) were extracted with 95% ethanol for 2 h, and the extracts were stored in a refrigerator (−20 °C) and further determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays for the determination of total polyphenols, total flavonoids, flavonols, and total tannins. According to the methods, acidified methanol (1% v/v HCl) was used for the determination of anthocyanins, while 70% acetone was used for the determination of proanthocyanidins.

Extracts for high-performance thin-layer chromatography (HPTLC) were prepared using acidified methanol (a mixture of methanol and 25% hydrochloric acid, 4:1, v/v) as a solvent for the determination of rutoside, quercetin, cyaniding, delphinidin, pelargonidin, peonidin, and chelerythrine.

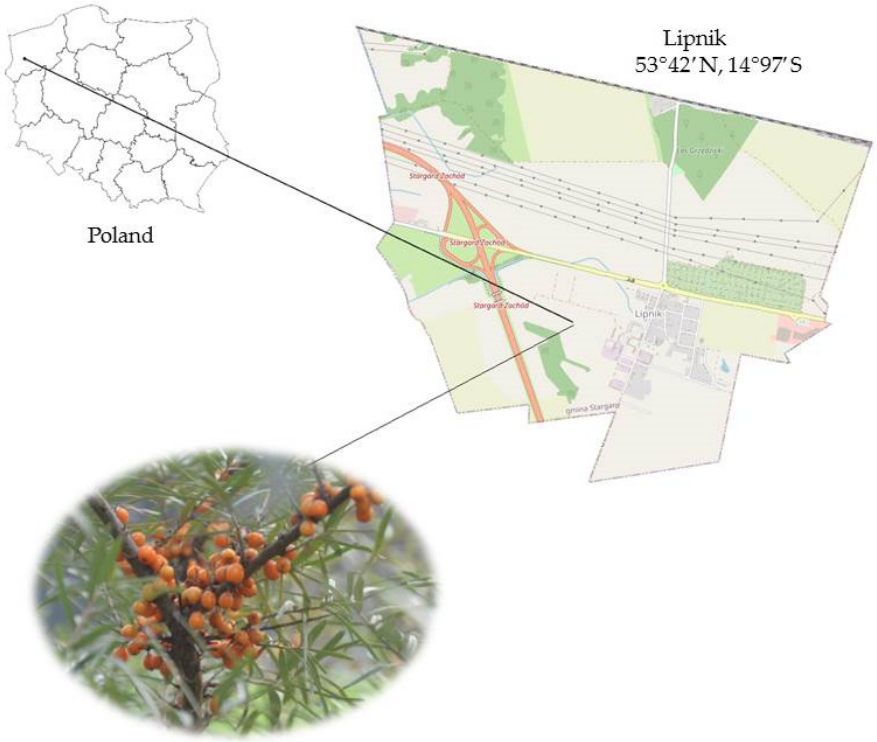


Figure 1. The map of the collection region and *Hippophae rhamnoides* L. leaves.

All the extracts used in the analyses were prepared according to an identical scheme. Approximately 0.5 g (weights to 4 decimal places) of powdered sea buckthorn leaves were weighed per 1 mL of solvent, and the samples thus prepared were shaken for 30 min in an MM 200 mixer mill (Retsch GmbH, Haan, Germany). After shaking, the samples were centrifuged for 15 min at 5.000 rpm at 4 °C in a Centrifuge 5415 R (Eppendorf AG, Hamburg, Germany). After centrifugation, the extracts were filtered through cellulose filters with a pore size of 0.45 µm and transferred to a new Eppendorf tube. The extracts were stored at −20 °C for further analysis.

2.3. Determination of Antioxidant Activity (AA)

2.3.1. DPPH Assay

Total AA was determined by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as described by Brand-Williams et al. [39]. The progress of the reduction reaction and, at the same time, the decolourisation of the radical solution was measured on a UV-VIS spectrophotometer (Analytik Jena, SPECORD® PLUS, Jena, Germany) at λ = 515 nm, 10 min after addition of the DPPH solution. The Trolox (TRX) equivalent was used to express antioxidant activity in µM TRX per 1 g of SBT leaf DM. For this purpose, Trolox solutions of various concentrations were prepared to determine a standard curve ($y = -0.0183x + 0.7762$; $R^2 = 0.9992$).

2.3.2. ABTS Assay

Antioxidant activity was determined by a spectrophotometric method using the $ABTS^{\bullet+}$ radical (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [40]. The progress of the reduction reaction and, at the same time, the decolourisation of the cation radical solution was measured on a UV-VIS spectrophotometer (Analytik Jena, SPECORD® PLUS, Jena, Germany) at λ = 734 nm, 6 min after addition of the ABTS solution. The Trolox equivalent was used to express antioxidant activity in µM TRX per 1 g of SBT leaf DM. For this purpose, Trolox solutions of various concentrations were prepared to determine a standard curve ($y = -0.0127x + 0.6292$; $R^2 = 0.9989$).

The percent inhibition of DPPH•+ radical and ABTS•+ radical generation was also calculated using Formula (1).

$$RSA\% = [(A0 - A1)/A0] \times 100 \quad (1)$$

where RSA—radical-scavenging activity, A0—the absorbance of control (for ABTS) and of the sample at the beginning of the reaction (for DPPH), and A1—the absorbance of the sample (in 6 min for ABTS; in 10 min for DPPH).

2.3.3. Photochemiluminescence (PCL) Assay

A PCL assay was carried out to measure the antioxidant capacity of the hydrophilic extract with a Photochem apparatus (Analytik Jena, Leipzig, Germany) against superoxide anion radicals generated in a luminol reaction (photosensitizer) under exposure to UV light. The antioxidant activity of the extract was analysed using the ACW (Water-Soluble Antioxidant) kit and the manufacturer's measurement protocol [41]. Extract solutions in Milli-Q water were vortexed (30 s × 2), sonicated (30 s × 2), and centrifuged at 13,200 rpm for 30 min at 4 °C before analysis. The results were expressed in μM of the TRX equivalent per 1 g of SBT leaf DM.

2.4. Quantification of Antioxidant Compounds

2.4.1. Total Polyphenol (TP) Determination

The TP content was determined by UV-VIS spectroscopy (Analytik Jena, SPECORD® PLUS, Jena, Germany) using the Folin–Ciocalteu reagent [42] and using gallic acid as a standard. Absorbance was measured at λ = 765 nm against a blank sample. The results were expressed in mg gallic acid equivalents (GAE) per 1 g of SBT leaf DM. Gallic acid solutions of various concentrations were prepared to determine a standard curve ($y = 0.0115x + 0.0332$; $R^2 = 0.9997$).

2.4.2. Total Flavonoid (TF) Determination

The content of TFs was determined by UV-VIS spectroscopy (Analytik Jena, SPECORD® PLUS, Jena, Germany) using aluminium chloride ($AlCl_3$) and using quercetin (QEE) as a standard [40]. Absorbance was measured at λ = 420 nm against a blank. The results were expressed in mg QEE per 1 g of SBT leaf DM. Quercetin solutions of various concentrations were prepared to determine a standard curve ($y = 0.0187x + 0.0087$; $R^2 = 0.9964$).

2.4.3. Total Flavonol (TFL) Determination

The content of TFLs was determined by UV-VIS spectroscopy (Analytik Jena, SPECORD® PLUS, Jena, Germany) using aluminium chloride ($AlCl_3$) and using QEE as a standard [43]. Absorbance was measured at λ = 440 nm against a blank. The results were expressed in mg QEE per 1 g of SBT leaf DM. Quercetin solutions of various concentrations were prepared to determine a standard curve ($y = 0.5297x - 0.2227$; $R^2 = 0.9934$).

2.4.4. Evaluation of Individual Flavonols (Rutoside and Quercetin)

Evaluations of rutoside (R) and QE were performed on 10 × 10 cm aluminium plates (aluminium oxide 60 F254, type E, Merck, Darmstadt, Germany), dedicated to thin-layer chromatography (TLC) analyses. The plates were activated with methanol prior to use. They were then dried at 60 °C for 1 h. The samples and standards on the thus-prepared stationary phase were spotted with 1 or 2 μL glass capillaries using a Vomaticator Linomat (CAMAG, Muttenz, Switzerland), at a distance of 10 mm from the end of the plate, 10 mm from the edge, and with 10 mm of distance between the bands. Standards were applied first to determine the standard curve (1, 2, 3 and 4 μL), followed by the prepared extracts (2 μL each). The plates thus prepared were transferred to an automated chamber (CAMAG ADC, Muttenz, Switzerland) to develop the chromatograms. The mobile phase in the chamber consisted of a chloroform/methanol/acetone/ammonia mixture (10:22:53:0.2,

$v/v/v/v$), and the separation of the samples took 40 min in glass chromatographic chambers (17.5 × 16 × 8.2 cm, Sigma-Aldrich, Darmstadt, Germany). After drying in the dark, the plates were scanned using a Skaner TLC Scanner 3 (CAMAG, Muttenz, Switzerland). The results obtained were calculated using Visual Cats 1.3.4 software and expressed in µg per 1 g of SBT leaf DM.

2.4.5. Total Anthocyanin (TA) Determination

The TA content was determined using the UV-VIS spectroscopy method (Analytik Jena, SPECORD® PLUS, Jena, Germany) [44]. Absorbance of the upper phase was determined at λ = 530 and λ = 657 nm. Anthocyanin concentrations (mg/g dry mass) were calculated as follows (2):

$$\text{Total anthocyanins (mg/g DM)} = [A_{530} - 0.25 \times A_{657}]\ TV/[d_{wt} \times 1000] \quad (2)$$

where

A_{530}—absorbance of probe at λ = 530 nm;
A_{657}—absorbance of probe at λ = 657 nm;
TV—total volume of extract (mL);
d_{wt}—dried herb weight (g).

2.4.6. Evaluation of Individual Anthocyanidins

The content of cyaniding (C), delphinidin (DE), pelargonidin (PG), and peonidin (P) was measured using the HPTLC, following the same procedure given above in Section 2.4.4. The results of individual anthocyanidins were expressed in µg per 1 g of SBT leaf DM.

2.4.7. Proanthocyanidin (PAC) Determination

The PAC content of the extracts was determined by UV-VIS spectroscopy (Analytik Jena, SPECORD® PLUS, Jena, Germany), using butanoic acid according to the method reported by Porter et al. [45]. Absorbance was measured at λ = 550 nm. The percentage of total proanthocyanidins in the dry mass of sea buckthorn leaves was calculated according to the following Formula (3):

$$\text{Total proanthocyanidins (\%)} = A550 \times 78.26 \times df/\%\ DM \quad (3)$$

where

A_{550}—absorbance of probe at λ = 550 nm;
df—dilution factor;
% DM—percentage of dry mass in the test sample.

2.4.8. Total Tannin (TT) Determination

The TT content was determined using the Folin–Ciocalteu reagent [46], with tannic acid (TA) as the standard. Absorbance was measured at λ = 725 nm against a blank sample using a UV-VIS spectrophotometer (Analytik Jena, SPECORD® PLUS, Jena, Germany). The results were expressed in mg of tannic acid (TA) per 1 g of SBT leaf DM. Solutions of tannic acid of various concentrations were prepared to determine a standard curve ($y = 0.0009x + 0.0016$; $R^2 = 0.9961$).

2.4.9. Chelerythrine (CHE) Determination

Selected alkaloid CHE was analysed using the HPTLC, following the same procedure as described above in Section 2.4.4. The results of CHE were expressed in µg per 1 g of SBT leaf DM.

2.5. Statistical Analyses

A two-factorial analysis of variance (ANOVA) and principal component analysis (PCA) were carried out using the STATISTICA v13.30 software (TIBCO Software Inc. [47], Palo Alto, CA, USA). Before ANOVA was conducted, the homogeneity of variances was checked (Levene's test). Tukey's Honestly Significant Difference (HSD) at $p = 0.05$ was used to find the differences between means. The means denoted by different letters differed statistically.

3. Results and Discussion

Phenolic compounds are a very large group of common, natural compounds and are secondary metabolites of plants. They belong to the group of antioxidants [48]. Evaluation of the antioxidant potential of raw materials is carried out by tests in different systems and using different reaction mechanisms [49,50]. In this study, tests involving scavenging of free stable radicals were used, $DPPH^{\bullet+}$ and $ABTS^{\bullet+}$. The use of the $DPPH^{\bullet+}$ radical to assess antioxidant potential only allows for the evaluation of hydrophobic antioxidants, in contrast to the $ABTS^{\bullet+}$ radical, which reacts with both hydrophobic and hydrophilic antioxidants. The PCL method was also used in this study. PCL was first used to assess antioxidant potential by Popov et al. [51]. This method has a high level of sensitivity and can therefore be used to determine the antioxidant properties of both antioxidant-rich and antioxidant-poor products. This method is used to test the antioxidant activity of both the hydrophilic fraction (water-soluble antioxidant capacity ACW) and the lipophilic fraction (lipid-soluble antioxidant capacity ACL) in plant samples.

Table 1 shows the total polyphenol (TP) content and antioxidant activity (AA) of sea buckthorn leaves. SBT leaves from different years of this study had statistically different levels of total polyphenols (from 186.64 to 330.16 mg GAE/1 g DM), confirming that the quality of the raw material (leaves) can be significantly modified by habitat conditions. A trend was also observed ($p = 0.239$) indicating a variation in the TP content of the leaves of the SBT cultivars tested. The TP content in the leaves of the Ascola cv. was 307.02 mg GAE/1 g DM, while in the leaves of the Hergo cv., it was only 195.80 mg GAE/1 g DM. In the study by Cho et al. [52], the TP content in sea buckhorn leaf tea and green tea extracts ranged only from 23 to 66 and from 33 to 118 mg GAE/1 g DM, respectively. Upadhyay et al. [53] also reported that 70% ethanol was optimal for extracting phenolic compounds from sea buckthorn leaves. The variability of TP has been recorded due to the influence of a number of factors, such as the type of locality, the year of harvest, and cultivars [11,24]. This wide variation in the compounds responsible for antioxidant properties was confirmed by the different results of the antioxidant assays in the studies presented (Tables 1 and S1). The free-radical-scavenging activity of SBT leaf extracts was studied for their ability to bleach stable $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ free radicals, providing information on the reactivity of compounds with a stable free radical. Extracts of SBT leaves exhibited AA potential, as analysed by DPPH and ABTS assays (Tables 1 and S1). The leaf extracts showed a significant reduction in both the $DPPH^{\bullet+}$ and the $ABTS^{\bullet+}$ radicals in the samples tested (RSA, from 56.33 to 98.25%). The AA of the SBT leaves measured with the $ABTS^{\bullet+}$ radical was dependent on the cultivar. SBT leaf extracts from different cultivars showed antioxidant activity ranging from 490.47 to 493.32 μM TRX/1 g DM, and these were the statistically different contents found in the leaves of the Ascola and Hego cv. Due to the lipophilic nature of the $DPPH^{\bullet+}$ radical, a slight difference in the experimental cultivars was observed in the AA measured by DPPH, in comparison to the ABTS assay. The simplicity of the ABTS method makes it a routinely used analysis for determining the AA of samples. However, it should be borne in mind that this radical has a much higher persistence than the radicals present in the raw materials, which may raise concerns as to whether such a method describes the actual free radical reactions well. On the other hand, a limitation of the DPPH method is that $DPPH^{\bullet+}$ is only soluble in organic solvents and does not allow for the determination of hydrophilic antioxidants [54]. Antioxidant properties can be influenced by many factors in addition to the plant species, including the freshness of the raw material

under investigation. The oxidative properties of raw materials may also change depending on the method and duration of storage. Alabri et al. [55] suggest that some compounds of botanical raw materials may volatilise or decompose during storage, while antioxidant efficacy depends on many factors, i.e., the type of raw material or the method of sample processing.

Table 1. The total polyphenol content and antioxidant activity of SBT [1] leaves.

Factor	Total Polyphenols (mg GAE [2]/1 g DM [3])	TEAC [4] ABTS•+ (µM TRX [5]/1 g DM)	TEAC DPPH•+ (µM TRX/1 g DM)	PCL µM TRX/1 g DM
		Year		
p [6]	=0.029	=0.071	<0.000	=0.905
2014	186.64 a [7]	491.86 a	340.57 b	348.49 a
2015	197.43 ab	493.36 a	350.04 b	313.23 a
2016	330.16 b	494.61 a	299.53 a	310.98 a
		Cultivar		
p	=0.239	=0.036	=0.095	=0.148
Ascola	307.02 a	490.47 a	323.49 a	290.76 a
Habego	216.26 a	492.82 ab	329.53 a	245.20 a
Hergo	195.80 a	493.32 b	332.94 a	254.41 a
Leikora	233.25 a	492.50 ab	334.23 a	506.57 a

[1] SBT—sea buckthorn; [2] GAE—gallic acid equivalent; [3] DM—dry matter; [4] TEAC—Trolox equivalent antioxidant capacity; [5] TRX—Trolox; [6] *p*—probability of null hypothesis rejection (H0; $m_1 = m_2 \ldots = m_n$); [7] means with at least one same letter (a, b) do not differ statistically at $p = 0.05$ (for all columns, separately).

In this study, the antioxidant potential of SBT leaves was also assessed using the PCL_ACW method. This method did not confirm the variation in AA of the raw material from the different growing seasons but, through the statistical trend shown ($p = 0.148$), indicates higher levels of AA in the Leikora cv. (506.57 µM TRX/1 g DM).

The methods used to measure the AA of sea buckthorn leaves did not provide a clear answer as to the variation in the quality of the raw material from different growing seasons; rather, they allow Leikora cv. leaves to be identified as having higher levels of AA. Taking into account the link between the development of civilisation diseases and long-term oxidative stress, the strong antioxidant properties of SBT leaves may indicate the potential use of this raw material not only in the prevention but also in the treatment of many diseases [56].

The factor coordinate plot for the variables (Figure 2A) shows the scatterplot of the set of factor loadings on a two-dimensional plane that includes the first two components. Figure 2B, on the other hand, shows the scatterplot of the data set on the plane of the first two components. The presented graphs confirm the particular variation in the AA and polyphenol content in the leaves of the Ascola cv., since the position of this cultivar on the plane of the components includes the quadrants I—year 2015, III—year 2014, and IV—year 2016. The graph also confirms the significant similarity of the Habego and Hergo cv. and a certain distinctiveness of the Leikora cv., which is also confirmed by the sensitive and precise PCL method. However, one surprising result of the PCA analysis is the negative correlation of AA (ABTS and DPPH) with TP and the lack of correlation between TP and AA (PCL). These results are confirmed by statistically significant correlation coefficients (Tables 2 and 3). Such an effect may indicate that not only labelled flavonols or tannins are responsible for the AA of sea buckthorn leaves but also, and perhaps to a greater extent, other unlabelled fractions such as phenolic acids, carotenoids, and sterols are [57]. Furthermore, the variability of AA in the plant material is determined not only by the course of the growing season but also by the physiological age of the raw material [58].

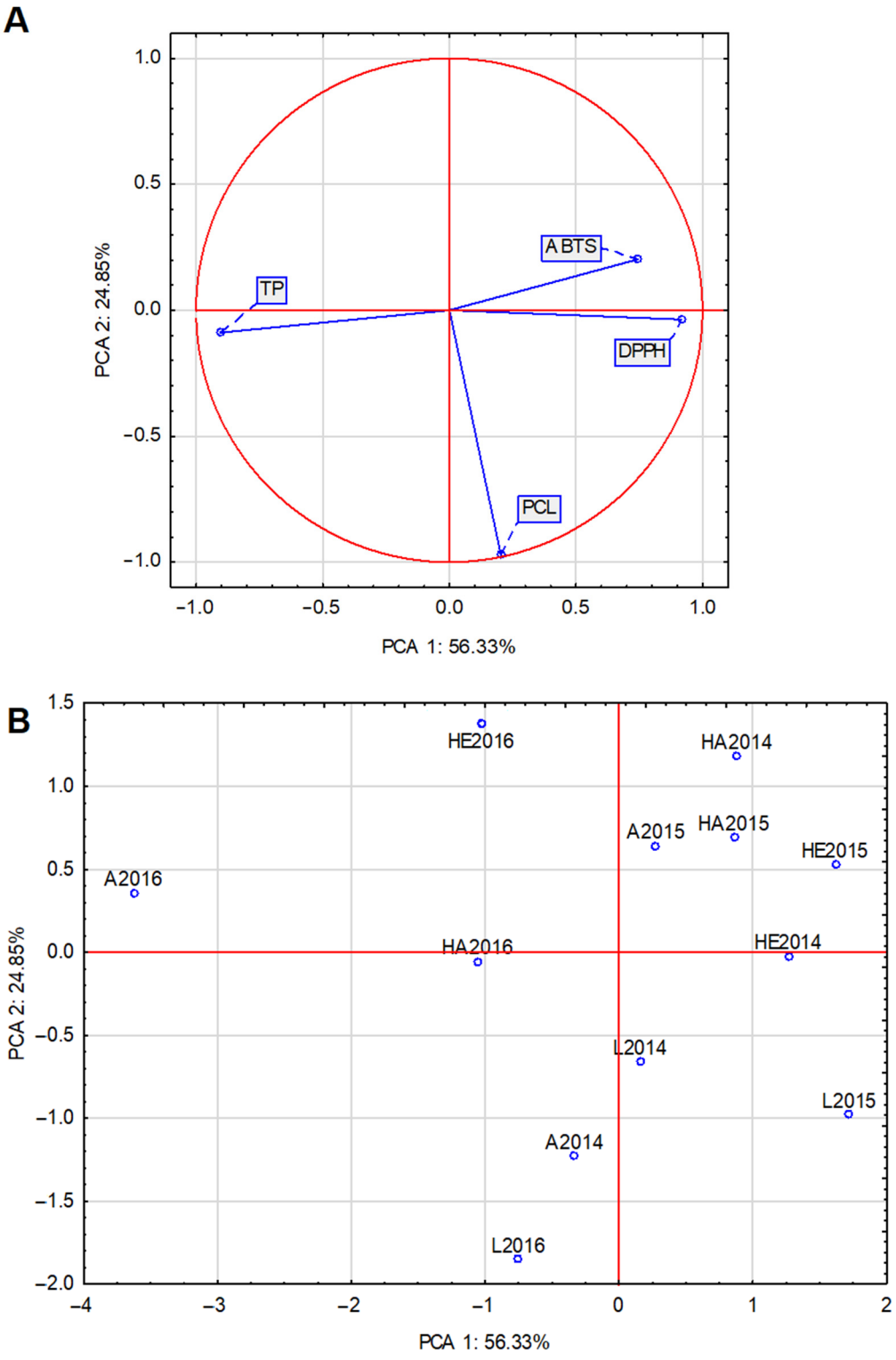


Figure 2. The first two principal component axes for polyphenol content and antioxidant activity in SBT leaves for the variables (**A**) and for the scores (**B**), (ABTS—2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; DPPH—1,1-diphenyl-2-picrylhydrazyl; PCL—photochemiluminescence assay; TP—Total Polyphenol; A—cv. Ascola; HA—cv. Habego; HE—cv. Hergo; L—cv. Leikora).

Table 2. Statistical significance (p [1]) of Pearson's r correlation coefficients (n = 12) for feature [2].

Feature	DPPH	ABTS	RSA ABTS	RSA DPPH	PCL	TP	TF	TFL	PAC	TA	TT	DE	R	QE	P	C	CHE
DPPH	1.000																
ABTS	0.091	1.000															
RSA ABTS	0.105	0.382	1.000														
RSA DPPH	0.001	0.105	0.285	1.000													
PCL	0.576	0.930	0.470	0.759	1.000												
TP	0.001	0.091	0.345	0.054	0.814	1.000											
TF	0.407	0.443	0.403	0.966	0.149	0.285	1.000										
TFL	0.513	0.921	0.470	0.591	0.050	0.357	0.000	1.000									
PAC	0.001	0.417	0.012	0.015	0.962	0.018	0.638	0.666	1.000								
TA	0.255	0.174	0.691	0.670	0.557	0.241	0.400	0.166	0.219	1.000							
TT	0.011	0.198	0.782	0.105	0.873	0.004	0.206	0.344	0.098	0.142	1.000						
DE	0.281	0.306	0.978	0.111	0.566	0.883	0.588	0.481	0.240	0.186	0.195	1.000					
R	0.040	0.756	0.942	0.115	0.681	0.050	0.752	0.942	0.014	0.003	0.089	0.126	1.000				
QE	0.050	0.393	0.709	0.190	0.754	0.113	0.981	0.531	0.018	0.000	0.167	0.104	0.000	1.000			
P	0.123	0.080	0.027	0.390	0.458	0.068	0.168	0.158	0.188	0.743	0.282	0.318	0.731	0.816	1.000		
C	0.021	0.893	0.570	0.071	0.321	0.053	0.602	0.559	0.014	0.019	0.099	0.131	0.003	0.006	0.708	1.000	
CHE	0.158	0.355	0.658	0.104	0.236	0.269	0.490	0.547	0.072	0.008	0.192	0.010	0.000	0.002	0.321	0.002	1.000

[1] Probability of null hypothesis rejection (H0; r = 0); [2] DPPH—1,1-diphenyl-2-picrylhydrazyl; ABTS—2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; RSA—radical-scavenging activity; PCL—photochemiluminescence assay; TP—Total Polyphenol; TF—Total Flavonoid; TFL—Total Flavonol; PAC—Proanthocyanidin; TA—Total Anthocyanin; TT—Total Tannin; DE—Delphinidin; R—Rutoside; QE—Quercetin; P—Peonidin; C—Cyanidin; CHE—Chelerythrine.

Table 3. Pearson's correlation coefficients (n = 12) for feature [1].

Feature	DPPH	ABTS	RSA ABTS	RSA DPPH	PCL	TP	TF	TFL	PAC	TA	TT	DE	R	QE	P	C	CHE
DPPH	1.000																
ABTS	0.509	1.000															
RSA ABTS	0.491	0.278	1.000														
RSA DPPH	−0.836	−0.491	−0.337	1.000													

Table 3. *Cont.*

Feature	DPPH	ABTS	RSA ABTS	RSA DPPH	PCL	TP	TF	TFL	PAC	TA	TT	DE	R	QE	P	C	CHE
PCL	0.180	0.029	0.231	0.099	1.000												
TP	−0.808	−0.509	−0.299	0.568	−0.076	1.000											
TF	−0.264	−0.245	−0.266	0.014	−0.443	0.336	1.000										
TFL	−0.209	−0.032	−0.231	−0.173	−0.575	0.292	0.900	1.000									
PAC	−0.815	−0.258	−0.694	0.678	0.015	0.667	0.152	0.139	1.000								
TA	0.357	−0.420	−0.128	−0.137	0.189	−0.366	−0.268	−0.427	−0.383	1.000							
TT	−0.699	−0.400	−0.090	0.492	−0.052	0.763	0.393	0.300	0.500	−0.450	1.000						
DE	−0.339	0.323	0.009	0.484	0.184	0.048	−0.174	−0.226	0.368	−0.410	0.402	1.000					
R	−0.599	0.101	−0.024	0.479	0.133	0.575	-0.102	−0.024	0.686	−0.780	0.512	0.467	1.000				
QE	−0.577	0.271	−0.121	0.407	−0.102	0.481	−0.008	0.201	0.665	−0.858	0.426	0.492	0.878	1.000			
P	0.470	0524	0.635	−0.273	0.237	−0.543	−0.426	−0.435	−0.408	−0.106	−0.338	0.315	0.111	−0.075	1.000		
C	−0.656	0.043	−0.183	0.538	0.314	0569	0.168	0.188	0.683	−0.661	0.498	0.461	0.772	0.735	−0.121	1.000	
CHE	−0.435	0.293	0.143	0.492	0.370	0.347	−0.221	−0.193	0.537	−0.724	0.405	0.705	0.902	0.800	0.313	0.793	1.000

[1] DPPH—1,1-diphenyl-2-picrylhydrazyl; ABTS—2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; RSA—radical-scavenging activity; PCL—photochemiluminescence assay; TP—Total Polyphenol; TF—Total Flavonoid; TFL—Total Flavonol; PAC—Proanthocyanidin; TA—Total Anthocyanin; TT—Total Tannin; DE—Delphinidin; R—Rutoside; QE—Quercetin; P—Peonidin; C—Cyanidin; CHE—Chelerythrine.

The largest group of plant polyphenols (TPs) are flavonoids (TFs). Within this group, several subclasses have been distinguished, differing in structure and resulting antioxidant activity. The phenolic compounds quantified are generally considered to be the major determinants for the antioxidant capacity of plants. The total flavonoid (TF) content in the leaves of sea buckthorn are shown in Table 4. The average TF content of SBT leaves was high at 281.76 mg QEE/1 g DM. However, there was no significant effect of growing season or cultivar on the TF content of sea buckthorn leaves. The total flavonoid content of 36.58 mg of mg QEE per 1 g of extract was found in the leaves of SBT grown in Romania by Criste et al. [11]. This value is relatively lower than our results, but our results are expressed on 1 g of DM, and this is not clearly stated in the referenced study. Wang et al. [59] found TF content in SBT leaves ranging from 24.57 to 34.83 mg rutin/g DM. The content of these compounds in sea buckthorn leaves was not affected by the factors studied, but the presence of rutoside and quercetin was found to be significant. The content of these compounds in the leaves of the tested cultivars was the same, but the raw material from different growing seasons contained statistically different contents. The average flavonol content was 80.19 mg QEE/1 g DM (Table 4). The flavonoid content in the leaves and fruit of sea buckthorn has been reported to range from 310 to 2100 mg/100 g of dried leaf and 120 to 1000 mg/100 g of fresh fruit, respectively [60–62]. Due to the different methods of determination and the different standards used, it is impossible to compare the results obtained. However, it has been widely confirmed that SBTs are rich in phenolic compounds. The content of individual flavonoids in the studied SBT leaves was statistically differentiated by the experimental factors analysed. The content of proanthocyanidin, anthocyanidin, and total tannins was significantly influenced by the origin of the raw material (growing season). PACs are a group of condensed tannins consisting of catechin and epicatechin monomers that differ from the hydrolysable tannin structure of ellagitannins. PACs have attracted increasing amounts of attention in the fields of nutrition and medicine because of their various bioactivities, such as their antioxidant, anti-diabetic, anti-obesity, anticancer, anti-inflammatory, and cardioprotective effects [63,64]. SBT leaves are a rich source of PACs, and indeed, the highest amount of these compounds was found in SBT leaves from the 2016 growing season (18.517 mg/1 g DM). Although the leaves of the tested cultivars did not differ statistically in their PAC content, a trend towards a slightly higher content level of these compounds can be observed in the leaves of the Habego cv. (14.547 mg/1 g DM). Recent years have provided evidence that PACs are present in the extracts of various plants and have antimicrobial potential, including antifungal effects. For example, Luiz et al. [65] evaluated the effect of proanthocyanidin polymer-rich fractions from *Stryphnodendron adstringens* and successfully showed the inhibition of *Candida albican* (ATCC 10231) planktonic growth and biofilm development. According to the study of Fan et al. [66] and Yang et al. [67], sea buckthorn seeds contain a substantial number of PACs, but little is known about their antimicrobial activity. SBT twigs, previously regarded as a by-product, were shown to be also a valuable source of substances with promising antimicrobial activity. Sadowska et al. [9] reported that SBT-derived twig and leaf extracts rich in PACs exhibit antifungal activity and affect important *Candida* virulence factors, thus having good potential for the development of novel antifungal products supporting traditionally used drugs. From the point of view of the nutritional properties of the raw material, it is also important to bear the interaction of PACs with proteins in mind, especially those rich in proline. The authors of this study found an average of 4 g of proline in 100 g of SBT leaf protein in the raw material assessed, which represents only 5.28% of the sum of all 20 amino acids determined (unpublished data). Biologically important effects of this interaction include blocking the activity of enzymes, particularly proteolytic enzymes. Another effect of the presence of PACs may be to reduce the bioavailability of certain minerals.

Table 4. Total and individual flavonoids in SBT [1] leaves.

Factor	Total Flavonoids (mg QEE [2]/1 g DM [3]	Flavonols mg QEE/1 g DM	Anthocyanins mg/g DM	Proanthocyanidins mg/g DM	Total Tannins mg TA [4]/1 g DM
			Year		
p [5]	=0.931	=0.868	=0.004	=0.015	=0.029
2014	281.05 a [6]	79.29 a	10.928 b	8.634 a	1.429 a
2015	281.66 a	80.71 a	6.932 a	7.860 a	1.445 ab
2016	282.58 a	80.70 a	6.375 a	18.517 b	1.516 b
			Cultivar		
p	=0.833	=0.892	=0.430	=0.570	=0.128
Ascola	283.54 a	80.53 a	9.202 a	11.136 a	1.491 a
Habego	282.72 a	81.37 a	7.710 a	14.547 a	1.413 a
Hergo	279.51 a	79.18 a	7.794 a	10.426 a	1.471 a
Leikora	281.28 a	79.56 a	7.608 a	10.573 a	1.479 a

[1] SBT—sea buckthorn; [2] QEE—quercetin equivalent; [3] DM—dry matter; [4] TA—tannic acid; [5] *p*—probability of null hypothesis rejection (H0; $m_1 = m_2 \ldots = m_n$); [6] means with at least one same letter (a, b) do not differ statistically at $p = 0.05$ (for all columns, separately).

Another group of phenolic compounds labelled in SBT leaves were anthocyanins, which also have diverse effects, including antioxidant, antimutagenic, anti-inflammatory, and anticancer activity [68]. They are mainly found in berries and give the fruit attractive colours. Berries such as cranberries, bilberries, raspberries, strawberries, grapes, elderberries, and blueberries have the highest anthocyanin content and antioxidant activity. Their presence in other plant species (including other morphological parts of plants), which have not been previously identified, is also highlighted. The leaves from many species are remarkably polymorphic for anthocyanin expression [69]. For example, the yam, in addition to the edible root tuber, also has edible leaves and above-ground shoots, which contain numerous polyphenolic compounds and, among others, anthocyanins [70,71]. The anthocyanidin content of the SBT leaves studied ranged from 6.375 to 10.928 mg/g DM, confirming, at the same time, the variation in their content in the raw material depending on the year of harvest ($p = 0.004$). The content of these compounds in the leaves of the cultivars tested was not statistically differentiated ($p = 0.430$), although the leaves of the Ascola cv. tended to have a slightly higher concentration of this component (9.202 mg/g DM). Many studies have investigated the anthocyanidin content of the fruit. It should be noted that their content depends on the colour of the fruit. For example, relatively red berries were found to contain more anthocyanins than yellow and light-yellow berries [72]. A qualitative assessment of flavonoids was also made by determining the amount of rutoside and quercetin in SBT leaves (Table 5).

In the case of delphinidin as well as cyanidin, variation was observed in the content of these compounds in SBT by the experimental factors studied. The raw material obtained in the 2016 growing season was characterised by a significantly higher content of these compounds, and, among the cultivars, Hergo and Leikora were characterised by a higher content of delphinidin. The presence of pelargonidin, which is common in plant raw materials, was not found in the tested raw material.

Previous studies have confirmed the presence of a few alkaloids in the biomass of sea buckthorn, e.g., hyppophamide (seeds), cauilexin (peels of fruits), or harmane (leaves and branches) [57,73]. This study confirmed the presence of benzophenanthridine alkaloidchelerythrine. This compound shows properties that make it likely that it will be a component of many new drugs, especially anticancer drugs [74].

Table 5. Content of secondary metabolites in SBT [1] leaves.

Factor	Rutoside	Quercetin	Delphinidin	Peonidin	Cyanidin	Chelerythrine
			µg/1 g DM [2]			
			Year			
p [3]	=0.001	=0.003	=0.045	=0.335	=0.005	=0.005
2014	0.246 a [4]	0.178 a	0.794 a	36.668 a	0.681 a	0.681 a
2015	0.295 b	0.203 b	0.815 a	41.141 a	0.687 a	0.885 ab
2016	0.335 c	0.223 b	0.972 b	35.942 a	0.723 b	1.043 b
			Cultivar			
p	=0.402	=0.270	=0.017	=0.298	=0.052	=0.096
Ascola	0.280 a	0.190 a	0.723 a	33.034 a	0.685 a	0.749 a
Habego	0.296 a	0.207 a	0.783 a	37.863 a	0.700 a	0.845 a
Hergo	0.287 a	0.207 a	1.026 b	39.662 a	0.687 a	0.898 a
Leikora	0.305 a	0.200 a	0.909 ab	41.108 a	0.716 a	0.988 a

[1] SBT—sea buckthorn; [2] DM—dry matter; [3] *p*—probability of null hypothesis rejection (H0; $m_1 = m_2 \ldots = m_n$); [4] means with at least one same letter (a, b, c) do not differ statistically at $p = 0.05$ (for all columns, separately).

The content of chelerythrine in SBT leaves was determined by the climatic conditions of the growing season. By far, the highest content of the alkaloid was found in the 2016 raw material. The chelerythrine content in the raw material of the tested cultivars was close to statistical variation. The Ascola cv. showed a clear tendency to accumulate less of this valuable alkaloid. Extracts from medicinal plants with chelerythrine are components of veterinary and human phytopreparations. They display distinct antibacterial and anti-inflammatory properties. Kosina et al. [75] showed that an average daily oral dose of alkaloids up to 5 mg per 1 kg of animal body weight is safe for piglets. Hu et al. [76] conducted a detailed evaluation of the pharmacokinetic characteristics of a mixture of sanguinarine and CHE in broiler chickens following p.o. and showed that this addition to feed is safe because of the first-pass effect after intestinal and liver metabolism.

The state of knowledge on the biological activity of flavonoid compounds clearly indicates that their positive effects on the body are mainly due to their antioxidant properties. This is possible due to the presence of hydroxyl groups in their structure. It has been shown that the more hydroxyl groups in the molecule, the stronger the antioxidant effect [77,78]. Of the labelled compounds, rutoside has the most -OH groups—10, delphinidin has 6, quercetin and cyanidin have 5, and peonidin has the least—4. The abundance of phenolic compounds in SBT leaves confirmed previous studies by other authors, including Wei et al. [79].

The PCA analysis confirmed the significant variation in leaf quality of the studied SBT cultivars harvested in different growing seasons (Figure 3). This is not a positive finding from the point of view of obtaining stable quality raw material. The change in the quality of the raw material of the Ascola cv. can be taken as an example. There is a clear decrease in the anthocyanin content in favour of other active components (especially flavonoids, flavonols, and total tannins) in the following years of this study. In the Leikora cv., the content of mainly chelerythrine and delphinidin increased with successive growing seasons.

The analysis of the correlations between the determined raw material properties showed a correlation coefficient close to statistical significance ($p = 0.091$) ($r = 0.509$) between the DPPH and ABTS methods but, at the same time, confirmed the lack of correlation between these methods and the PCL-ACW method ($r = 0.180$ and 0.029, respectively) (Tables 2 and 3). PCL methods are less frequently used to assess the quality of plant material, so this lack of correlation is difficult to discuss with respect to sea buckthorn AA analyses. Nevertheless, Witkowicz et al. [80], in their study on the AA assessment of buckwheat sprouts, showed a correlation of sprout AA measured by DPPH with PCL-ACW methods ($r = 0.81$). The correlations of the analysed properties with chelerythrine are also interesting. The content of this alkaloid does not show a correlative relationship with AA, but statistically significant relationships were observed between its content and the

content of anthocyanins and flavonols (rutin, quercetin). The correlation relationship with anthocyanins is negative (r = −0.724), and with flavonoids, it is positive (r = 0.902 and 0.800, respectively) (Tables 2 and 3).

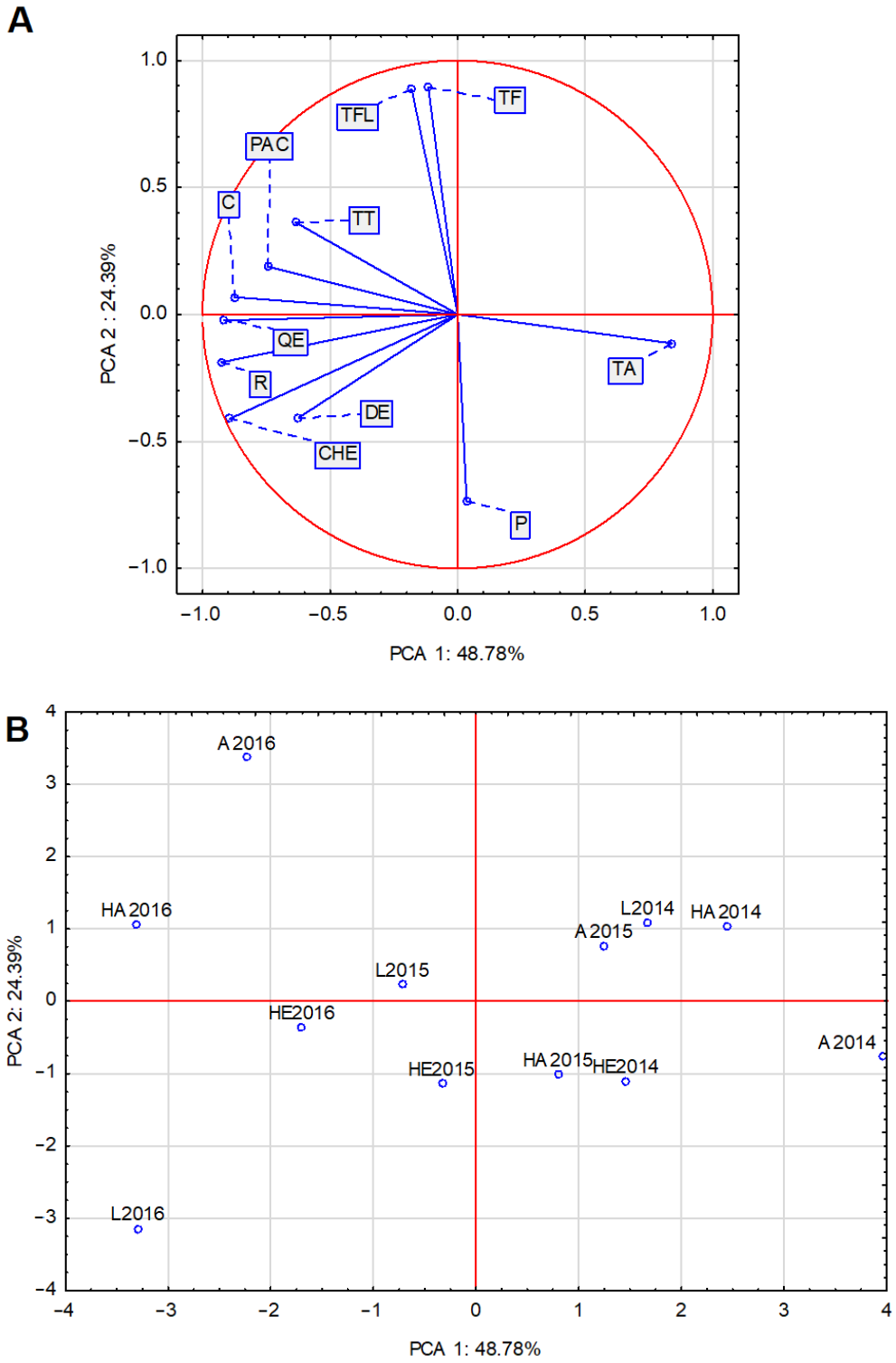


Figure 3. The first two principal component axes for bioactive compound content in SBT leaves for variables (**A**) and for the scores (**B**), (C—Cyanidin; CHE—Chelerythrine; DE—Delphinidin; P—Peonidin; PAC—Proanthocyanidin; QE—Quercetin; R—Rutoside; TA—Total Anthocyanin; TF—Total Flavonoid; TFL—Total Flavonol; TT—Total Tannin; A—cv. Ascola; HA—cv. Habego; HE—cv. Hergo; L—cv. Leikora).

Diet, along with genetic and environmental factors, is considered a major aspect affecting longevity, as well as vascular disease outcome. Cardiovascular disease (CVD) is the major cause of mortality and morbidity in the world [81]. Therefore, the prevention of and reduction in risk factors, which are associated with CVD, are the major tasks of health care professionals and scientists. Several investigators have studied the role of different bioactive components such as antioxidants and flavonoids in the prevention and management of CVD. Since synthetic vitamin E and vitamin C have shown inconclusive results regarding their role in cardiovascular health and diseases, various investigators are presently searching for alternate sources of vitamins and other bioactive components such as flavonoids from natural sources like SBT by-products. The effect of the major components of SBT on the cardiovascular system is well documented in scientific research [82–84]. Most prospective cohort studies have indicated some degree of inverse association between flavonoid intake and coronary heart disease. Animal and human studies suggest that sea buckthorn flavonoids may scavenge free radicals, lower blood viscosity, and enhance cardiac function. Suomela et al. [85] studied the effects of flavonol aglycones derived from SBT on the risk factors of cardiovascular disease, as well as their absorption in humans. The flavonols, ingested with oatmeal porridge, did not have a significant effect on the levels of oxidized low-density lipoprotein, C-reactive protein, and homocysteine; on plasma antioxidant potential; or on paraoxonase activity. Total flavonols at two dosages (1.78 mg and 2.39 mg) in oatmeal porridge were rapidly absorbed, and a relatively small amount of sea buckthorn oil added to the porridge seemed to have increased the bioavailability of SBT flavonols consumed at a higher dose. Sea buckthorn by-products appear to be promising ingredients for health benefits, and their inclusion in daily diets should therefore be encouraged.

4. Conclusions

Sea buckthorn leaves have great potential as a source of healthy food/feed, as well as raw materials and ingredients for health care products and animal nutrition. The majority of sea buckthorn research has been conducted in Asia. It is a promising crop for Europe, and recently, it has attracted considerable attention of researchers, producers, and industries. This study has proved that the leaves of sea buckthorn are excellent sources of bioactive compounds, such as rutoside, quercetin, and individual anthocyanidins—compounds with significant antioxidant activity. The isoquinoline alkaloid chelerythrine was also found in sea buckthorn leaves. Therefore, SBT leaves can be used as a source of new antioxidant compounds. These natural antioxidants have attracted considerable attention because of their extensive biological functions and regulatory effects on mammals. Our finding clearly support the notion that SBT leaves, as a natural and non-polluting resource, could be a better choice as a healthy food/feed resource. In addition, it would be beneficial to use SBT leaves with high antioxidant potential as food/feed additives, as well as in-feed growth enhancers as alternatives to antibiotics after the latter's ban in the EU.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app14125038/s1, Table S1: The antioxidant activity of SBT [1] leaves (RSA%).

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