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The Content of Bioactive Compounds in Highbush Blueberry *Vaccinium corymbosum* L. Leaves as a Potential Raw Material for the Food Technology or Pharmaceutical Industry

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Abstract: The study covered the leaves of 25 varieties of highbush blueberry *Vaccinium corymbosum L. Determinations were performed for* phenolic acid (chlorogenic, neochlorogenic, cryptochlorogenic, 3,5-dicaffeoylquinic and 4,5-dicaffeoylquinic acids), flavonoid (catechin, rutin, isoquercetin), arbutin and hydroquinone content. The content of phenolic acids and flavonoids in the test material suggests that highbush blueberry leaves can be a potentially good material for dietary supplement production, although the relatively high arbutin content (exceeding 4% in the case of the Bonus, Chantiklee and Herbert varieties) limits this possibility to herbal medicinal products applied in case of health issues for a limited time, similar to other over-the-counter arbutin products (bearberry *Arctostaphylos uva-ursi* and lingonberry *Vaccinium vitis-idaea* leaves).

Keywords: plant raw material; highbush blueberry; phenolic acids; flavonoids; arbutin

1. Introduction

Among numerous plants of the genus *Ericaceae*, bearberry *Arctostaphylos uva-ursi* (L.) Spreng. and lingonberry *Vaccinium vitis-idaea* L. [1–3] leaves are of particular importance due to their medicinal properties. An issue in harvesting herbal materials from these plants is that the former is a protected plant in numerous countries, while the range of the latter is limited. Consequently, our attention was drawn to highbush blueberry *V. corymbosum L.*. It is a plant grown for consumption in many regions of the world. Highbush blueberry fruits enjoy a fairly high demand due to their flavor and health-promoting properties [4]. Leaves, on the other hand, rarely attract interest, even though they can be easily obtained during bush screening and seedlings production.

Fruit bush leaves in horticultural production can find an alternative use as material for pharmaceutical purposes, although they need to meet strict pharmaceutical requirements [5]. In food production, the requirements imposed on materials could be slightly less strict, especially that the plants would be intended for producing plant-based concentrates of bioactive compounds used for food enrichment. Furthermore, as shown in other studies, it is also possible to selectively extract bioactive components without adversely affecting the health-promoting compounds, which makes it possible to effectively eliminate their presence in biocomponent-enriched products [6,7].

The leaves of orchard and forest plants already have use in nutrition as components of leaf infusions, which are a healthy alternative to teas and are made by fermenting the leaves of selected

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fruit bushes and/or medicinal plants. Through a process of wilting, rolling and drying, they not only obtain a higher concentration of ingredients beneficial to health, such as tannins, but also health-promoting ingredients, for example numerous phenolic compounds and glycosides. It needs to be added that food production is more liberal in using plant additives with a pro-health potential, often without considering the potential presence of harmful or toxic substances, such as plant hormones, saponins or alkaloids [8,9]. It is also known that other food ingredients and treatment processes applied to food containing pro-health additives, as well as food preservation processes can effectively limit the bioavailability of these pro-health or even harmful substances, but they should never be ruled out or ignored [10,11].

The sparse papers on biologically active compounds in highbush blueberry leaves indicate that the leaves contain chlorogenic acid and quercetin and its glycosides (rutin, hyperoside and isoquercetin) [12]. *V. corymbosum* leaf extracts above all exhibit a significant antibacterial and antioxidative activity [13]. The antioxidative activity of the compounds found in this plant's leaves have found use even in the production of functional packages [14].

These discoveries demonstrate that *V. corymbosum* leaf extracts can have a very broad range of applications in medicine and the food industry. However, the potentially high arbutin content in the leaves, and consequently hydroquinone as well, a compound suspected of carcinogenicity [15], can raise justified concerns. The aim of this study was therefore to investigate the qualitative and quantitative content of pro-health compounds in *V. corymbosum* leaves, and to determine their levels of arbutin and hydroquinone.

2. Materials and Methods

2.1. Plant Samples

The study material comprised 25 varieties of highbush blueberry leaves samples from plantation located in mountainous areas within the Carpathian Foothills region in south-eastern Poland, from clean areas with low industrial development. The Carpathian Foothills is the lowest portion of the Polish Carpathians, forming a group of low-altitude hills between 350 and 600 m a.s.l., with smooth and round slopes. The Carpathian Foothills are characterized by moderate, intermediate, and submontane climates. The leaves were taken from highbush blueberry *V. corymbosum* bushes after fruit harvest in August-September, so that defoliation could no longer have a negative effect of fruit crop. The varieties and harvest times are shown in Table 1.

Table 1. Characteristics of highbush blueberry varieties from which the leaves were collected.

Variety names	Fruit harvest time
Bonus, Chanticleer, Duke, Early Blue, Hannah Choice, Huron, Ivanhoe, Spartan, Sunrise	early
Blue Jay, Draper, Patriot, Toro	mid to early
Blue Crop, Chandler, Eliott, Herbert	mid to late
Aurora, Blue Gold, Darrow, Denis Blue, Elizabeth, Late Blue, Liberty, Nelson	late

The plant material was preserved by drying in a DanLAB (Poland) air circulation laboratory dryer at 35°C. Next, water content in the dried material was determined, which served as an indicator for the end of the drying process, as water content did not exceed 8%. Having considered the results of experiments involving test material available commercially in dried form [16] and the significant heterogeneity of the dried material, the rage of 6-8% water content was decided to be sufficient for preserving the test material and concluding the drying process. Portions of dried test material of each variety were fragmented in an IKA type A 11 Basic Analytical Mill (Germany), then sieved on a 0.18 mm sieve. For extraction, portions of 2 g of homogeneous dried plant material were moved to extraction tubes and covered with 20 ml solvent, sealed and placed for 60 min. in a Polsonic Sonic 22

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(Poland) ultrasound bath with a thermostat function at 40°C. Extraction was performed using ethanol with a 50% alcohol content. After the completion of the ultrasound-assisted extraction, the samples were transferred to the Biosan ES-20/60 rotary shaker (Poland) and mixed under similar conditions as before, for 30 minutes at 40°C at 180 revolutions per minute. After the extraction was concluded, the samples were purified under pressure on filter papers placed on the Buchner filter, ensuring that the extraction material was thoroughly dried from solvent, and then the filtrates were centrifuged in an Eppendorf 5702 laboratory centrifuge (Germany) for 10 minutes, RCF = 2600 g. After centrifuging, the supernatant was poured into separate clean tubes. Clear highbush blueberry extracts were applied in their entirety to conditioned Waters Sep-Pak (C18 500 mg) syringe filter cartridges (Ireland). Phenolic compounds were leached from the columns with methanol directly in a round-bottomed flask and were concentrated at 40 °C until the solvent was evaporated in a Hei-VAP Precision rotational vacuum evaporator from Heidolph (Schwabach, Germany). The leaves extracts in the round-bottomed flasks were dissolved with 50% hydrated acetonitrile and filtered through PTFE socket filters with a 0.45 µm pore size directly prior to chromatographic analysis.

2.2. Analytical Procedures

2.2.1. Profiles of Phenolic Compounds

The contents of phenolic compounds were determined according to the method described by Piechowiak et al. (2021) with own modifications [17]. Selected polyphenolic compounds were determined in highbush blueberry extracts by high-performance liquid chromatography (HPLC) using a SYKAM S600 analyser (Ersing, Germany) equipped with a photodiode detector (S 3210) operating within the 190 to 900 nm wavelength range, a pump (S 1132) and a column thermostat (S 4120). Before the analysis, the samples were filtered through a nylon syringe filter with a 0.22 µm pore diameter. The bioactive compounds were separated on a Bionacom Velocity STR C18 column (3.0 × 100 mm; 2.5 µm). Injection volume 20 µm, flow rate 0.5 mL/min., column thermostat temperature 25°C. Mobile phase composition was water acidified with formic acid (0.1% v/v) (A), acetonitrile (B). The separation was performed using the following gradient: 10% B (1.5 min); 10- 30% (1.5- 9 min); 30-50% (9-20 min); 50-10% (20- 25 min). Quantitative determinations were carried out using the calibration curves of individual standards ranging from 0.005 to 0.1 g L−1($R2 \geq 0.9998$).

2.2.2. Determination of Arbutin and Hydroquinone Content

Arbutin and hydroquinone determinations were performed based on the methodology described in the latest 7th edition of European Pharmacopoeia [18]. A 0.800 g weighed amount of powdered plant material was placed in a 100 mL round-bottom flask, 20 mL distilled water was added, and the flask was heated for 30 min. on a water bath under a reflux condenser. After cooling, the extract was filtered through a wad of cotton wool, which was again extracted together with the residue in the flask with another 20 ml distilled water portion for 30 minutes on a water bath under a reflux condenser. After cooling, all of the liquid was filtered through a paper filter and left to cool, then made up with 50 ml distilled water and again filtered, discarding the first 10 ml of the filtrate. The purified plant extracts were applied to conditioned Waters Sep-Pak (C18 500 mg) syringe filter cartridges (Ireland). Phenolic compounds were leached from the columns with methanol in volume 10 mL directly in a round-bottomed flask and were concentrated at 40 °C until the solvent was evaporated in a Hei-VAP Precision rotational vacuum evaporator from Heidolph (Schwabach, Germany). The leaves extracts in the round-bottomed flasks were dissolved with methanol and filtered through PTFE socket filters with a 0.45 µm pore size directly prior to chromatographic analysis. A standard solution was made by dissolving 50 mg arbutin in 50 ml mobile phase, and 2.5 mg hydroquinone in 10 ml mobile phase, then mixed at ratios specified in the methodology. The plant extracts were separated using a Thermo Dionex Ultimate 3000 liquid chromatograph with a UV detector and a DAD-3000 (RS) diode matrix (ESA Chlemsford. MA. USA). A HALO 90 A C18, 2.7 µm, 4.6 x 150 mm column was used for the separations at 25°C. The mobile phase was methanol and water (10:90 V/W). Flow rate 0.8 ml/ min, assay time was 30 minutes. Detection at wavelength 280

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nm. The injection volume was 10 µl. The average recovery for the highbush blueberry was 97%. The operation of the chromatographic set and processing of the obtained data were coordinated by the Chromeleon 7.2 software (Dionex).

2.2.3. Determination of Antioxidant Activity

The antioxidative activity was determined by FRAP (Ferring Ion Reducing Antioxidant Power), while DPPH (2,2-diphenyl-1-picrylhydrazyl) radical reduction was determined in accordance with the methodology described by Sowa et al. [19] The antioxidative activity results were expressed as Trolox equivalents (TE) calculated to g dry weight of plant material (µmol TE/g d.w.) based on a Trolox solution calibration curve within a 25-300 nmol/ml (FRAP) and 5-60 nmol (DPPH) range. The measurements were performed using a Spectrophotometer UV VIS UV6000 (Shanghai Metash Instruments Co., Shanghai, China).

2.2.4. Analysis of Total Phenolic Compounds (TPC)

The content of total phenolic compounds (TPC) was investigated by Folin–Cocialteu's method as described by Stratil et al. [20]. The results were expressed as 1 mg of gallic acid equivalents per l (mg GAE/L) of tested samples, using a calibration curve plotted for GAE solution in a concentration range of 25–250 µg/mL (R2 = 0.997). The measurements were performed using a Spectrophotometer UV VIS UV6000 (Shanghai Metash Instruments Co., Shanghai, China).

2.3. Statistical Analysis

All of the analyses were performed in three independent replications for each sample. The contents of phenolic compounds, arbutin hydroquinone content and antioxidant activity were expressed as the mean ± standard deviation. The acquired findings were subjected to statistical analyses with the use of Statistica ver. 13.1 (StatSoft, Inc., Tulsa, OK, USA). Significant differences between types of highbush blueberry leaves based on tested parameters were obtained through a one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. The multivariate statistical analysis was performed using cluster analysis (CA), combined with a heat map with the use of Statistica ver. 13.1 (StatSoft, Inc., Tulsa, OK, USA) and Principal Component Analysis (PCA) with the use of OriginPro 2023 (OriginLab Corporation, Northampton, Massachusetts). Correlations between tested parameters were established using Pearson's correlation test and presented as Triangle Heatmap with the use of OriginPro 2023 (OriginLab Corporation, Northampton, Massachusetts).

2.4. Chemicals and Reagents

For the purpose of determinations, analytical purity reagents (analytical standards) designed for liquid chromatography were used: hydrochloric acid, formic acid, ethanol, and acetonitrile from Sigma-Aldrich (Poznan, Poland), and methanol from J.T. Baker (Phillipsburg, USA). 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ),and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Poznan, Poland). Analytical standards of chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, catechin, rutin, isoquercetin for HPLC were obtained from Extrasynthese (Genay, France). Arbutin standard from CPAchem.Ltd (Bułgaria) and hydroquinone standard obtained from Sigma-Aldrich (Poznan, Poland). Deionised water obtained from a deioniser from Hydrolab Polska HLP 5P were used.

3. Results and Discussion

In the analyzed highbush blueberry *Vaccinium corymbosum* L. leaf extracts, compounds belonging to phenolic acids were identified, mainly chlorogenic acid derivatives and flavonoids, primarily quercetin glycosides. The content of individual phenolic compounds varied depending on

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individual variety, and in most cases statistically significantly differed between varieties ($p<0.05$) (Tables 2 and 3). The results of our analyses confirm the information reported by Ferlemi et al. [12] that chlorogenic acid, rutin and isoquercetin are found in *V. corymbosum* leaves. Chlorogenic acid is the most abundant compound belonging to phenolic acids found in the plant material, and has a very broad range of pharmacological applications [21–24]. Its content in the analyzed leaf extracts ranged from 52.76 mg/g (Spartan variety) to 32.37 mg/g (Nelson variety). This acid was present in the highest concentration among all the analyzed phenolic acids, which is consistent with results produced by Wang et al. [25], who analyzed 104 blueberry varieties. In the leaves we tested, it was accompanied by cryptochlorogenic acid and neochlorogenic acid, which usually form a complex of phenolic acids in many traditional plant-based medicines Jie et al. [26]. Neochlorogenic acid content varied greatly. The highest content of this acid was found in the Bonus variety, which is classified as an early cultivar (9.03 mg/g), while in the Hannah Choice variety this acid was <LOQ. Cryptochlorogenic acid content also differed between varieties. The lowest levels were found in the Elizabeth and Nelson varieties (1.99 mg/g), while the highest content was identified in Darrow and Hannah Choice (4.31 mg/g and 4.30 mg/g, respectively). Additionally, we demonstrated the presence of 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid in the material, which are chlorogenic acid isomers of low stability, but also exhibit a broad range of therapeutic effects [27]. A significant variation was observed for these compounds as well, especially in the case of 4,5-dicaffeoylquinic acid, which was present at fairly high levels (above 7 mg/g) in the Bluejay and Chandler varieties, while in some others (Bluegold, Draper and Hannah Choice) it was below LOQ.

Table 2. Content of phenolic acids of the tested highbush blueberry varieties (mg/g s.m.).

Variety	Chlorogenic acid	Neochlorogenic acid	Cryptochlorogenic acid	3,5-dicaffeoyl quinic acid	4,5-dicaffeoyl quinic acid
Aurora	34.90 ± 1.21^{a}	8.43 ± 0.28	2.20 ± 0.18^{a}	6.21 ± 0.05	1.47 ± 0.04^{a}
Bluecrop	41.65 ± 0.21^{b}	2.94 ± 0.05^{a}	3.69 ± 0.13^{b}	2.46 ± 0.03^{b}	5.72 ± 0.01
Bluegold	33.39 ± 0.30^{c}	5.26 ± 0.32^{b}	2.75 ± 0.06^{c}	7.63 ± 0.03	<LOQ
Bluejay	40.53 ± 0.09^{b}	6.41 ± 0.03^{c}	3.17 ± 0.38^{d}	4.89 ± 0.04	7.79 ± 0.01^{b}
Bonus	41.02 ± 0.29^{b}	9.03 ± 0.21	3.65 ± 0.10^{b}	2.03 ± 0.04^{c}	5.04 ± 0.02
Chandler	45.88 ± 0.30^{d}	6.66 ± 0.11^{c}	4.00 ± 0.07^{e}	3.23 ± 0.09	7.83 ± 0.06^{b}
Chanticleer	44.88 ± 0.16^{e}	2.43 ± 0.13^{d}	3.72 ± 0.04^{b}	2.27 ± 0.01^{d}	2.72 ± 0.08
Darrow	46.67 ± 0.40^{f}	4.58 ± 0.00^{e}	4.31 ± 0.24^{f}	3.01 ± 0.03^{e}	4.65 ± 0.03
Denis Blue	39.99 ± 0.04	3.84 ± 0.03^{f}	2.38 ± 0.13^{a}	2.57 ± 0.04^{b}	1.01 ± 0.01^{c}
Draper	48.60 ± 0.13^{g}	3.02 ± 0.38^{a}	3.91 ± 0.04^{b,e,g}	2.25 ± 0.11^{d}	<LOQ
Duke	48.23 ± 0.38^{g,h}	7.05 ± 0.08	3.37 ± 0.06^{d}	2.48 ± 0.04^{b}	2.03 ± 0.07^{d}
Early Blue	40.04 ± 0.20^{b}	1.17± 0.01	2.16 ± 0.12^{a}	1.35 ± 0.07	<LOQ
Eliot	45.86 ± 0.00^{d}	7.80 ± 0.03	3.94 ± 0.02^{b,e,g}	2.00 ± 0.01^{c}	6.20 ± 0.01
Elizabeth	33.84 ± 0.24^{c,i}	4.00 ± 0.04^{f}	1.99 ± 0.08^{a}	3.76 ± 0.06	0.99 ± 0.01^{c}
Hannah Choice	47.63 ± 0.00^{h}	<LOQ	4.30 ± 0.03^{f}	1.74 ± 0.04	<LOQ
Herbert	40.95 ± 0.42^{b}	4.33 ± 0.09^{e,g}	2.14 ± 0.15^{a}	4.43 ± 0.14	2.00 ± 0.04^{d}
Huron	49.98±0.09^{f}	3.50 ± 0.11^{h}	3.03 ± 0.04^{d}	8.32 ± 0.14	1.71 ± 0.05
Ivanhoe	34.21±0.04^{a,i}	2.93 ± 0.13^{a}	2.08 ± 0.02^{a}	3.90 ± 0.06	2.46 ± 0.01^{e}
Late Blue	50.52±0.35^{j}	4.16 ± 0.08^{f,g}	4.12 ± 0.01^{e,f,g}	5.00 ± 0.06	4.10 ± 0.06
Liberty	44.44±0.21^{e}	5.82 ± 0.08	2.79 ± 0.06^{c}	7.97 ± 0.02	2.50 ± 0.01^{e}
Nelson	32.37±0.23	1.73 ± 0.04	1.99 ± 0.01^{a}	2.57 ± 0.01^{b}	3.87 ± 0.04
Patriot	50.26±0.15^{j}	5.09 ± 0.04^{b}	3.84 ± 0.06^{b,e,g}	3.05 ± 0.01^{e}	4.77 ± 0.03
Spartan	52.76±0.33	3.68 ± 0.21^{f,h}	4.14 ± 0.06^{e,f,g}	5.27 ± 0.01^{f}	1.39 ± 0.00^{a}
Sunrise	47.79±0.04^{h}	3.67 ±0.06^{f,h}	3.33 ± 0.11^{d}	5.99 ± 0.05	1.24 ± 0.12^{f}
Toro	36.99±0.06	2.42 ±0.04^{d}	2.34 ± 0.07^{a}	5.26 ± 0.08^{f}	1.20 ± 0.01^{f}

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<LOQ- limit od quantification. Data presented as mean value ± standard deviation (SD; n = 3). Due to the very high number of samples that statistically significantly differed, the results are shown as a-a, b-b, etc., with the same letters meaning no statistically significant differences (p> 0.05) between test samples.

A similarly broad range of potentially beneficial health effects is found in the flavonoids isoquercetin and rutin [28–30]. In the test material, isoquercetin was found in noticeably greater quantities. Particularly large levels of this substance were found in the Aurora, Ivanhoe and Toro varieties (28.40 mg/g, 26.24 mg/g and 21.57 mg/g, respectively). Nelson and Denis Blue contained the least amounts (5.13 mg/g and 8.75 mg/g, respectively). An exceptionally high rutin content ($p<0.05$) was found in the Ivanhoe variety (27.19 mg/g) as compared to the other varieties, where it ranged from 2.06 mg/g (Early Blue and Patriot varieties) to 10.55 mg/g (Bluejay variety). Among the flavonoids identified, catechin was present at the lowest levels, although as with the other compounds, a marked variation between the test varieties was observed ($p<0.05$ statistically significant differences in most cases). The highest catechin content was found in the Hannah Choice variety (4.15 mg/g), while the lowest was observed in the Duke variety (1.35 mg/g).

In general, a higher content of phenolic acids than flavonoids was observed in the analyzed varieties. A similar situation was noted in the study by Wu et al. [31]. However, the phenolic compound profile and the ratios of individual compounds they found were different than in the extracts we analyzed (for example, it was found that leaf extracts are more abundant in neochlorogenic acid). This is most likely a result of different extract preparation procedures, but it could also by affected by the time of harvesting, or geographical area [2]. Furthermore, as Riihinen et al. [32] observed in their study, the factor deciding the phytochemical composition of the blueberry is leaf tissue maturity. This study determined that read leaves of V. *corymbosum* have increased flavonol and hydroxycinnamic acid (quercetin, kaempferol, p-coumaric, caffeic, and ferulic) levels, compared to green leaves. In our study, we used a 50%v/v water-ethanol solution, which was dictated by the fact that water-ethanol solutions are very good at extracting bioactive compounds, and ethanol is used for preparing tinctures, as well as ethanol extracts and syrups in pharmacy. The study by Wu et al. [31] used an 85.00% methanol solution acidified with 0.1% formic acid. Similarly, in the Stefănescu et al. [2] study slightly different profiles for the analyzed compounds were produced for the six tested commercial blueberry (*Vaccinium corymbosum* L.) varieties. For example, chlorogenic acid was determined at much lower levels (between 0.44-1.23 mg/g), while rutin was found to be more abundant (14.44- 35.77 mg/g). However, like in our study, they observed significant differences between varieties. Varying content levels of individual compounds in the leaves and fruits of *Vaccinium corymbosum* L., depending on the extracting agent used, were shown in the study by Tenuta et al. [33].

Table 3. Content of probiotic flavonoids of the tested highbush blueberry varieties (mg/g s.m.).

Variety	Catechin	Rutin	Isoquercetin
Aurora	2.43 ± 0.28^{a}	6.79 ± 0.18^{a}	28.40 ± 0.17
Bluecrop	3.94 ± 0.16^{b}	6.09 ± 0.08	13.57 ± 0.02^{a}
Bluegold	2.48 ± 0.09^{a}	6.91 ± 0.13^{a}	19.89 ± 0.09
Bluejay	2.63 ± 0.11^{a,c}	10.55 ± 0.03	14.20 ± 0.13^{b}
Bonus	3.15 ± 0.08^{d}	4.52 ± 0.13^{b}	13.13 ± 0.08^{c}
Chandler	3.24 ± 0.19^{d}	7.08 ± 0.04^{a}	17.02 ± 0.06^{d}
Chanticleer	1.40 ± 0.12^{e}	4.04 ± 0.06^{c}	15.40 ± 0.08^{e}
Darrow	3.21 ± 0.06^{d}	5.51 ± 0.01^{d}	15.53 ± 0.32^{e}
Denis Blue	1.41 ± 0.05^{e}	3.86 ± 0.09^{c,e}	8.75 ± 0.07
Draper	3.19 ± 0.00^{d}	4.71 ± 0.07^{b}	14.20 ± 0.04^{b}
Duke	1.35 ± 0.03^{e}	8.07 ± 0.06	17.25 ± 0.00^{d}

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Early Blue	1.53 ± 0.00^{e}	2.06 ± 0.05^{f}	13.18 ± 0.02^{c}
Eliot	2.92 ± 0.32^{d}	4.49 ± 0.02^{b}	12.06 ± 0.05
Elizabeth	2.40 ± 0.05^{c}	4.16 ± 0.04^{c}	11.43 ± 0.07
Hannah Choice	4.15 ± 0.06^{b}	2.38 ± 0.05	13.00 ± 0.05^{c}
Herbert	2.90 ± 0.18^{c,d}	8.71 ± 0.12	17.07 ± 0.05^{d}
Huron	1.95 ± 0.05^{f}	4.08 ± 0.01^{c}	13.80 ± 0.22^{a}
Ivanhoe	2.06 ± 0.15^{f}	27.19 ± 0.59	26.24 ± 0.02
Late Blue	3.91 ± 0.01^{b}	9.06 ± 0.08	17.85 ± 0.02
Liberty	1.91 ± 0.03^{f}	4.67 ± 0.03^{b}	15.01 ± 0.00
Nelson	1.46 ± 0.16^{e}	3.61 ± 0.04^{e}	5.13 ± 0.04
Patriot	2.88 ± 0.13^{c,d}	2.06 ± 0.09^{f}	16.51 ± 0.29
Spartan	3.95 ± 0.04^{b}	5.14 ± 0.01^{g}	14.00 ± 0.02^{b}
Sunrise	3.60 ± 0.14	5.25 ± 0.13^{d,g}	17.34 ± 0.09^{d}
Toro	3.19 ± 0.02^{d}	4.27 ± 0.01^{b,c}	21.57 ± 0.55

<LOQ- limit od quantification. Data presented as mean value ± standard deviation (SD; n = 3). Due to the very high number of samples that statistically significantly differed, the results are shown as a-a, b-b, etc., with the same letters meaning no statistically significant differences (p> 0.05) between test samples.

The arbutin and hydroquinone content determined in our study is shown in Table 4. European Pharmacopoeia (Ph. Eur.) states that dried bearberry leaf as a herbal material should contain less than 7% European Pharmacopoeia 7.8 [34], while Polish Pharmacopoeia (Ph. Pol.) specifies a corresponding value of 4% arbutin for lingonberry [35]. Arbutin content in the leaves of all tested varieties exceeded 2%, so it can be concluded that they constitute a stable source of arbutin. Three varieties (Bonus, Chantiklee i Herbert) can be considered a potential alternative to bearberry and lingonberry leaves. Leaf harvest could therefore be an additional source of income for highbush blueberry growers. The content of this compound, calculated to dry plant weight, ranged from 19.94 mg/g in the Denis Blue variety, classified as a late variety, to 44.24 mg/g in the Bonus variety, which belongs to early varieties. However, no effect of growing season duration on the content of this compound was observed. Studies concerning arbutin content in *Vaccinium corymbosum* L. are scarce [36,37]. Hydroquinone content in the analyzed extracts was determined to be at a lower level. The lowest was found in the Bluegold, Darrow, Herbert, Liberty and Spartan varieties (an average of 0.26 mg/g), while the highest was noted for the Chandler variety (0.88 mg/g) ($p<0.05$). It is known that hydroquinone induces the formation of reactive forms of oxygen and quinones, which leads to oxidative damage to membrane lipids and proteins such as tyrosinase, while at the same time it inhibits the pigmentation process. Due to the risk of adverse effects, hydroquinone has been prohibited by the European Commission [38]. The majority of the scientific papers available analyzed extracts from *Vaccinium corymbosum* L. leaves in terms of health-promoting bioactive compounds and their pro-health activity (e.g. antioxidative activity, antimicrobial, anti-inflammatory, antidiabetic properties), and based on the result gave recommendations for using the leaf extracts from this species to supplement everyday diet, completely ignoring the content of a compound that can potentially be toxic, and products containing this compound should be used with care [1,2,13,31,39]. Hydroquinone content was confirmed in the study by Yavorska et al. [37], although no specific concentration in the test extracts was given. A study conducted by Garcia de Arriba et al. [40] assessed the potential toxicity of herbal preparations made from *Arctostaphylos Uva-ursi folium* (bearberry leaf), which contain high levels of arbutin, and at the same time a small concentration of free hydroquinone (<0.3 %).

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Table 4. Content of arbutin and hydroquinone of the tested highbush blueberry varieties.

Variety	Arbutin		Hydroquinone	
	mg/g d.w.	%	mg/g d.w.	%
Aurora	24.30±1.87[a]	2.43	0.62±0.01[a]	0.06
Bluecrop	34.21±1.56[b]	3.42	0.42±0.03[b]	0.04
Bluegold	25.41±0.84[a]	2.54	0.24±0.02[c]	0.02
Bluejoy	27.01±1.39[a]	2.70	0.59±0.06[a]	0.06
Bonus	44.24±3.84	4.42	0.61±0.04[a]	0.06
Chandler	30.18±1.61[c]	3.02	0.88±0.02	0.09
Chanticleer	40.01±1.23[d]	4.00	0.52±0.02[d]	0.05
Darrow	24.05±1.96[a]	2.40	0.26±0.02[c,e]	0.03
Denis Blue	19.94±1.63[e]	1.99	0.73±0.05[f]	0.07
Draper	30.27±1.07[c]	3.03	0.40±0.06[b,g]	0.04
Duke	24.97±1.39[a]	2.50	0.59±0.05[a]	0.06
Early Blue	31.51±1.15[c]	3.15	0.51±0.02[d]	0.05
Eliot	21.16±1.23[e]	2.12	0.41±0.01[b,g]	0.04
Elizabeth	30.38±1.71[c]	3.04	0.62±0.04[a]	0.06
Hannah Choise	35.69±1.63[b]	3.57	0.31±0.03[e,h]	0.03
Herbert	41.64±1.11[d]	4.16	0.26±0.01[c,e]	0.03
Huron	32.70±0.75[b,c]	3.27	0.52±0.02[d]	0.05
Ivanhoe	21.14±0.28[e]	2.11	0.60±0.03[a]	0.06
Late Blue	30.14±1.05[c]	3.01	0.36±0.01[e,g,h]	0.04
Liberty	21.15±0.97[e]	2.11	0.27±0.02[c]	0.03
Nelson	23.31±1.06[a]	2.33	0.71±0.03[f]	0.07
Patriot	26.52±1.32[a]	2.65	0.50±0.03[d,i]	0.05
Spartan	31.12±0.48[c]	3.11	0.28±0.01[c,e]	0.03
Sunrise	26.65±1.44[a]	2.66	0.45±0.01[b,g,i]	0.04
Toro	34.51±1.89[b]	3.45	0.34±0.02[g,h]	0.03

Data as mean value ± standard deviation (SD; n = 3). Due to the very high number of samples that statistically significantly differed, the results are shown as a-a, b-b, etc., with the same letters meaning no statistically significant differences ($p > 0.05$) between test samples.

Antioxidative activity determined by two methods (FRAP and DPPH) for the analyzed leaf extracts differed between varieties (Table 5), similarly as with the preceding analyses. Most varieties differed statistically significantly ($p<0.05$). The highest antioxidative activity determined using the FRAP method characterised the Patriot variety (1086.15 µmol Trolox/g d.w.), while the lowest was noted for the Elizabeth variety (350.77 15 µmol Trolox/g d.w.). For antioxidative activity determined against the DPPH radical, the highest activity was observed for the Patriot (1124.17 µmol Trolox/g d.w.) and Huron varieties (1144.47 µmol Trolox/g d.w.), while the lowest was noted for Denis Blue (400.74 µmol Trolox/g s.m.) and Elizabeth (403.65 µmol Trolox/g d.w.). Antioxidative activity largely resulted from the presence of polyphenolic compounds. The total phenolic content (TPC) determined by spectrophotometry ranged from 48.11 mg GAE/g d.w. (Elizabeth variety) to 177.31 GAE/g d.w. (Patriot variety) (Table 5). A significant coincidence between the antioxidative activity measurement results and general phenolic compound content was observed for the analyzed leaf extracts. In the

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Wu et. al [31] study, antioxidative activity determined by FRAP was consistent with our results, ranging from 358.2 to 1600 µmol FEAC/g DW, while for DPPH lower values were noted, from 182.3 to 357.9 µmol TEAC /g DW , although antioxidative activity differed between varieties. The TPC value they determined was within the 32.18- 185.2 mg GAE/g d.w. range. In the Stefănescu et al. [2] study, the ability to inhibit DPPH radicals was determined as a % of free radical sweeping. High values were found for three varieties: Toro, Elliot and Nelson, with 70.41%, 68.42%, and 58.69%, respectively. The highest TPC was determined for the Nelson and Toro varieties (13.555 mg GAE/100 g leaf material and 13,292 mg GAE/100 g leaf material, respectively). The Routray and Orsat [41] study analyzed the impact of harvest time on phenolic compound content for two varieties, Nelson and Elliot. It was found that phenolic content began to rise in September and October, reaching 152.356 mgGAE/g d.w. (Nelson variety) and 155.830 mgGAE/g d.w. (Eliot variety).

Table 5. Antioxidant activity of the tested highbush blueberry varieties.

Variety	FRAP µmol Trolox/g s.m.	TPC mg GAE/g s.m.	DPPH µmol Trolox/g s.m.
Aurora	793.85±10.88^{a}	121.44±2.04^{a}	792.78±4.05^{a}
Bluecrop	758.08±16.86^{b}	129.01±1.02^{a,b}	751.21±16.22^{b}
Bluegold	718.08±14.69	118.74±5.35^{a,b,c}	730.43±5.12^{b,c}
Bluejay	549.23±1.09^{c}	89.73±1.53^{d}	573.47±24.33^{d}
Bonus	552.69±15.77^{c,d}	114.95±12.23^{a,e}	665.21±24.33^{e}
Chandler	537.31±2.72^{c}	94.77±1.05^{d,f}	579.21±4.05^{d}
Chanticleer	521.15±13.62^{e}	87.93±1.10^{d}	556.98±15.24^{d}
Darrow	661.54±17.41^{f}	101.62±1.15^{f,g}	606.44±4.05^{f}
Denis Blue	433.08±8.70	49.19±0.76^{h}	400.74±19.26^{g}
Draper	685. 00±2.72^{g}	114.05±1.78^{a,g}	681.69±3.04^{e}
Duke	541.15±20.13^{c,e}	90.45±12.74^{d}	661.62±1.01^{e}
Early Blue	513.85±4.35^{e}	87.39±9.94^{d}	599.99±23.31^{d,f}
Eliot	840.77±10.88^{h}	136.22±5.13^{b,h}	798.65±2.21^{a}
Elizabeth	350.77±6.53	48.11±0.76^{h}	403.65±8.83^{g}
Hannah Choice	676.54±4.92^{f,g}	107.75±4.59^{c,e,g,i}	715.93±24.29^{c,f}
Herbert	573.85±11.97^{d,i}	88.11±0.25^{d}	608.96±16.56
Huron	763.08±8.71^{b,j}	116.04±1.08^{a,e,i}	1144.47±12.14^{h}
Ivanhoe	628.85±7.07	106.49±7.39^{e,g,i}	642.52±4.42^{e}
Late Blue	809.62±13.6^{a}	118.21±2.55^{a,b,e,i}	736.98±3.31^{b,c}
Liberty	853.85±10.88^{h}	129.91±0.76^{a,b}	790.84±8.83^{a}
Nelson	583.08±2.18^{i}	83.60±3.57^{d}	574.61±7.73^{d}
Patriot	1086.15±8.7	177.31±3.57	1124.17±3.31^{h}
Spartan	938.46±1.09	141.98±2.57^{h}	809.58±6.62^{a}
Sunrise	885.77±7.07	138.56±5.35^{b,h}	814.26±4.42^{a}
Toro	783.46±5.98^{a,j}	98.23±4.84^{d,g,i}	711.22±24.29^{c}

Data presented as mean value ± standard deviation (SD; n = 3). Due to the very high number of samples that statistically significantly differed, the results are shown as a-a, b-b, etc., with the same letters meaning no statistically significant differences ($p > 0.05$) between test samples.

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In light of the obtained results of antioxidant activity, as well as the content of individual compounds and total phenolic compounds, a principal component analysis (PCA) and cluster analysis (CA), combined with a heat map, were conducted to determine the relationships between the investigated leaf extracts of different varieties of *Vaccinium corymbosum* L. This analysis aimed to unveil patterns and dependencies among the studied samples for potential insights into their characteristics.

Cluster analysis was performed using the Euclidean distance as the distance measure and the Ward method as the method of combining objects. The analyzed variables had a different unit, so the standardization of values was performed. A heat map was applied for visualization, and the data matrix was displayed (Figure 1). Based on the color scale of the heat map, values of individual parameters can be compared (where the darkest red indicates the highest value of a particular compound or antioxidant activity, and the darkest green represents the lowest value). Moreover, the importance of variables was established based on the C&RT model. The variable with the highest importance was the content of cryptochlorogenic acid (variable significance = 1), followed by the content of chlorogenic acid (0.85) and isoquercetin (0.76). The samples examined were divided into two main clusters. Extracts with the most similar values of the determined parameters are located closest to each other, but analyzing the heat map reveals how significantly the analyzed varieties differ from each other. For example, three varieties Huron, Bluegold and Liberty were characterized by the highest content of 3,5-DCQA (3,5-dicaffeoylquinic acid), while a high level of 4,5-DCQA (4,5-dicaffeoylquinic acid) was characteristic for Bluejay and Chandler. Furthermore, it can be observed how one Ivanhoe variety stands out in terms of rutin content compared to other varieties. Based on the results of chemometric analysis, it is also possible to quickly identify varieties with elevated levels of potentially toxic compounds, such Chandler with hydroquinone (HQ) content or with unique phenolics such as Bonus with arbutin (AR) content.

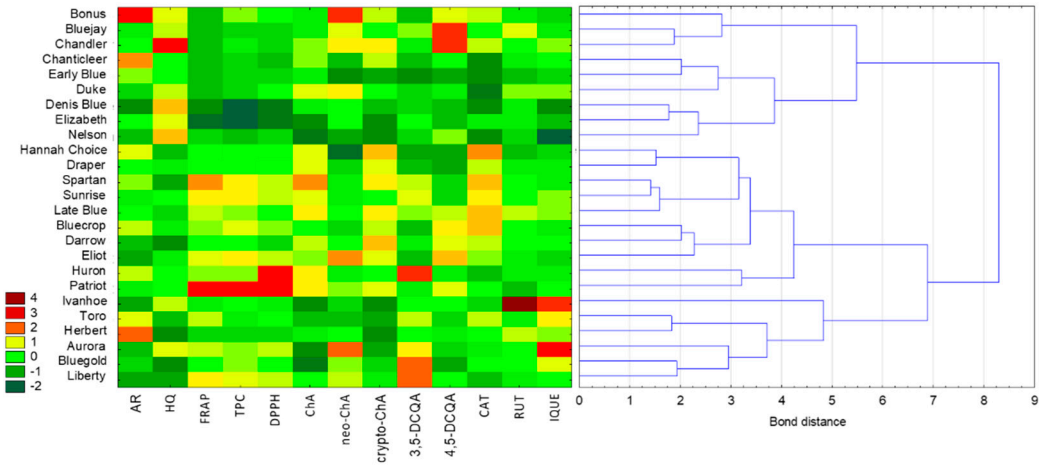

Figure 1. Analysis of similarity between varieties of leaf extracts of different varieties of *Vaccinium corymbosum* L. based on the content of individual identified compounds, the total content of phenolic compounds, and antioxidant activity using Cluster Analysis combined with a Heat Map. Variables were standardized. Colors on the heat map represent contents in respective extracts, with red indicating high content and dark green indicating low content. (AR- arbutin, HQ- hydroquinone, ChA- chlorogenic acid, neo-ChA- neochlorogenic acid, crytpo-ChA- cryptochlorogenic acid, 3,5-DCQA- 3,5-dicaffeoylquinic acid, 4,5-DCQA- 4,5-dicaffeoylquinic acid, CAT- catechin, RUT- rutin, IQUE- isoquercetin).

Principal component analysis (PCA) was performed to find relationships between variables (the content of individual compound, the total phenolic compounds content, antioxidant activity based on FRAP, and DPPH) and varieties of *Vaccinium corymbosum* L. Based on the Kaiser criterion, 13 variables were reduced to 4 main components, that explain 74.7 % of variance (PC1: 32.44 %, PC2: 18.36 %, PC3: 14.32 %, PC4: 9.58 %). Due to the fact that the first two principal components carry the most information about the analyzed set, the obtained results are presented as a 2D biplot (projection

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of PC1, and PC2) (Figure 2). The analyzed varieties have not been grouped into clear groups. Similarities can be observed within specific varieties, for example Elizabeth, Denis Blue and Nelson (left, lower part of the chart) or Hebert, Bluejay and Duke (left, upper part of the chart). Moreover, these varieties were characterized by a low content of the analyzed phenolic compounds or antioxidant activity. Varieties with a higher content of a specific compound or antioxidant activity are placed on the right side of the chart. Examining the obtained biplot, correlations between the analyzed variables can also be observed. However, considering that there are four principal components, direct analysis from this plot can be misleading. Therefore, correlation results between variables were presented in the form of a Triangle Heatmap illustrating the Pearson correlation matrix in graphical form (Figure 3). The antioxidant capacity of the tested extracts was highly correlated with the levels of the total phenolic content DPPH vs TPC r=0.84, FRAP vs TPC r=0.93, as well as the strong correlation between FRAP and DPPH methods was observed r=0.84. This confirms our previous observations [42,43]. Interestingly, only in the case of chlorogenic acid a mean correlation was observed with antioxidant activity (vs FRAP r=0.47, DPPH r= 0.49), suggesting that this compound may be primarily responsible for shaping the antioxidant activity of blueberry leaves. However, in general, the antioxidant activity of blueberry leaf extracts depends on the synergistic interaction of multiple polyphenolic compounds. Interestingly, a negative correlation was observed between the content of hydroquinone and the antioxidant activity determined by the FRAP method (r=- 0.5). Taking into account individual compounds, a correlation was observed between the content of chlorogenic acid and cryptochlogenic acid (r= 0.8).

To explore additional relationships between varieties, we further analyzed them in relation to the harvest date and presented the findings as a 3D biplot, considering three principal components (Figure 4). As can be seen in the three-dimensional graph taking into account the analyzed variables, whether the variety is early or late does not affect the similarity of the polyphenol composition or the antioxidant activity of *Vaccinium corymbosum* L. leaves. There is also no correlation between whether a variety is early or late and the content of some specific compound. Varieties differ in genotype and, consequently, chemical composition, including the content of individual phenolic compounds, although the overall profile is very similar. Chemometric analysis allows for identifying varieties rich in antioxidants, as well as varieties containing the highest content of potentially toxic compounds. High differences in the content of phenolic compounds, as well as antioxidant activity depending on the variety have been found in other studies [2,25,31] which confirms our results.

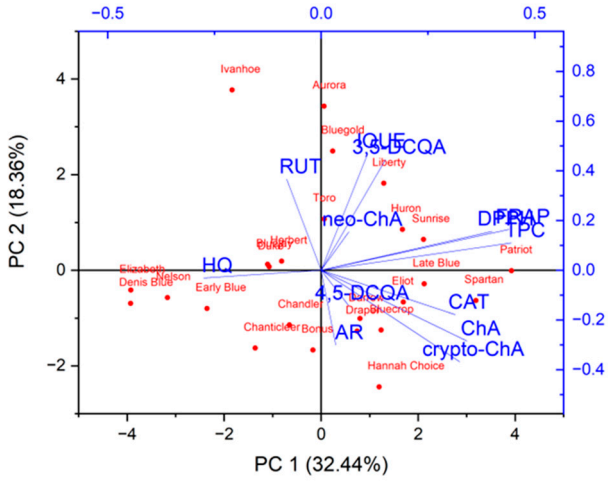

Figure 2. Determination of connections and relationships between varieties of leaf extracts of different varieties of *Vaccinium corymbosum* L. in relation to the analyzed parameters (content of individual compounds, total content of phenolic compounds and antioxidant activity) using Principal Components Analysis as a projection of PC1 vs PC2. (AR- arbutin, HQ- hydroquinone, ChA- chlorogenic acid, neo-ChA- neochlorogenic acid, crytpo-ChA- cryptochlorogenic acid, 3,5-DCQA- 3,5-dicaffeoylquinic acid, 4,5-DCQA- 4,5-dicaffeoylquinic acid, CAT- catechin, RUT- rutin, IQUE- isoquercetin).

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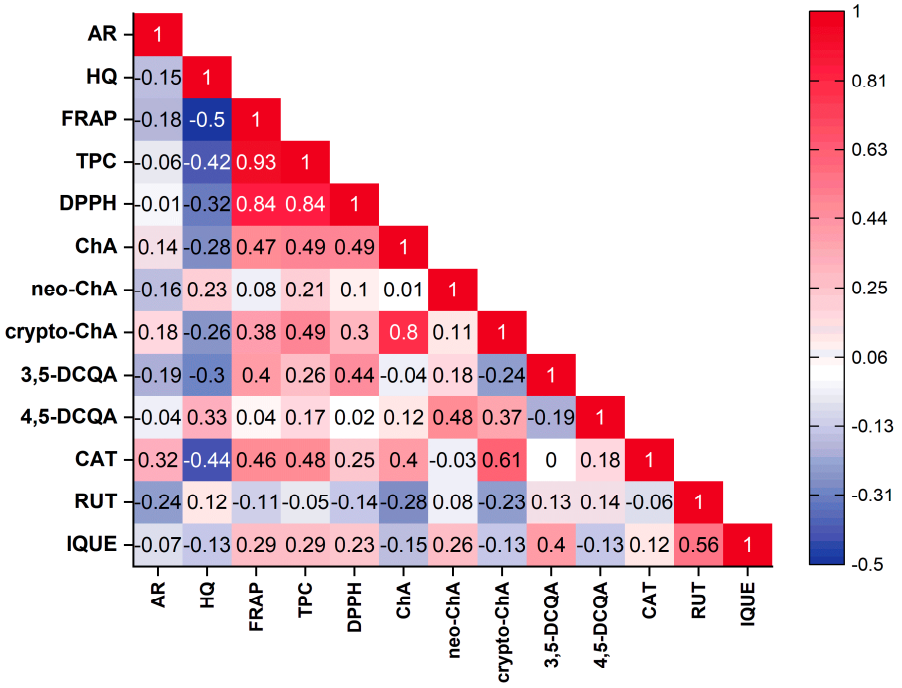

Figure 3. Pearson's correlation matrix between the analyzed parameters presented as Triangle Heatmap. (AR- arbutin, HQ- hydroquinone, ChA- chlorogenic acid, neo-ChA- neochlorogenic acid, crytpo-ChA- cryptochlorogenic acid, 3,5-DCQA- 3,5-dicaffeoylquinic acid, 4,5-DCQA- 4,5-dicaffeoylquinic acid, CAT- catechin, RUT- rutin, IQUE- isoquercetin).

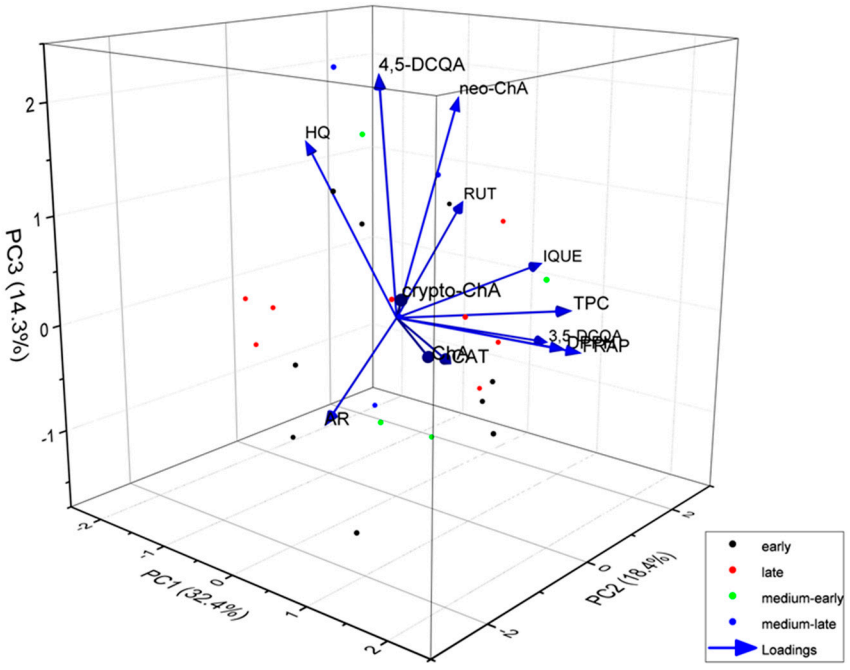

Figure 4. Biplot 3D Principal Component Analysis based on the variables (content of individual compounds, total phenolic compounds content and antioxidant activity) and analyzed leaf extracts of different varieties of *Vaccinium corymbosum L.* The colors of the dots indicate the type of variety, respectively early, late, medium-early, and medium-late varieties (AR- arbutin, HQ- hydroquinone, ChA- chlorogenic acid, neo-ChA- neochlorogenic acid, crytpo-ChA- cryptochlorogenic acid, 3,5-DCQA- 3,5-dicaffeoylquinic acid, 4,5-DCQA- 4,5-dicaffeoylquinic acid, CAT- catechin, RUT- rutin, IQUE- isoquercetin).

5. Conclusions

The content of chlorogenic acid and its isomers and the flavonoids listed above in highbush blueberry leaves points to a major probiotic potential of this material. The significant levels of arbutin in *V. corymbosum* leaves must be noted, however. Consumption of plant products containing arbutin results in a rapid increase in hydroquinone levels in blood plasma [44,45]. Toxicity studies of hydroquinone administered to animals over prolonged periods of time demonstrated that it is a genotoxic compounds with carcinogenic potential, although there is no basis for determining a permissible concentration in biological material for hydroquinone [46]. For this reason, *V. corymbosum* leaves can be considered an interesting herbal material for use in traditional herbal medicinal products utilised for relief of symptoms of mild recurrent lower urinary tract infections [47,48]. However, due to the relatively high arbutin levels, despite the content of valuable probiotic substances, primarily antioxidants, the leaves of the investigated highbush blueberry varieties in our opinion are not suitable for food products and dietary supplements.

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

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