ORIGINAL ARTICLE

MINERAL ELEMENTS PROFILE, BIOACTIVE COMPOUNDS AND ANTIOXIDANT CAPACITY OF WILD BLUEBERRY AND OF PHARMACEUTICAL PREPARATIONS FROM BLUEBERRY (*VACCINIUM MYRTILLUS*)

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Abstract

In this work, we investigated the mineral profile, the bioactive compounds (phenols, flavonoids) content and the antioxidant capacity of wild blueberries and their pharmaceutical preparations (tea, alcoholic extract and capsules). The highest content of phenols and flavonoids was recorded in the alcoholic sample (813.42 ± 15.55 mg GAE- gallic acid equivalent/100 g and 1425.44 ± 1.94 mg QE- quercetin equivalent/100 g), and the highest antioxidant capacity was recorded in fresh blueberries and in the tea derived from these fruits. The mineral elements composition analysis revealed high contents of manganese (8.7956 mg/kg) in the fresh fruits and the antioxidant capacity was strongly linked to the phenols and flavonoid content, as to the content of metals like Zn, Mn and Cu. Our results obtained in this study indicated that the pharmaceutical preparations derived from blueberry have antioxidant potential and high concentrations of bioactive compounds.

Rezumat

În această lucrare, a fost investigat profilul mineral, compușii bioactivi (fenoli, flavonoide) și capacitatea antioxidantă a fructelor de afin din flora spontană și a preparatelor farmaceutice (ceai cu afine, soluție alcoolică și capsule). Cel mai mare conținut de fenoli și flavonoide a fost înregistrat în proba de soluție alcoolică (813,42 ± 15,55 mg GAE/100 g și 1.425,44 ± 1,94 mg QE/100 g), în schimb cea mai mare capacitate antioxidantă a fost înregistrată în afinele proaspete și în forma de ceai de fructe de afin. Analiza compoziției elementelor minerale a demonstrat un conținut ridicat de mangan (8,7956 mg/kg) în fructe proaspete, zinc și mangan precum și o accentuată capacitate antioxidantă, legată de conținutul în polifenoli totali și flavonoide. Rezultatele noastre obținute în acest studiu indică faptul că preparatele farmaceutice derivate din afine au un potențial antioxidant și un nivel ridicat de compuși bioactivi.

Keywords: *Vaccinium myrtillus*, bioactive compounds, antioxidant capacity, mineral content

Introduction

Numerous independent investigations have demonstrated the direct correlation between consumption of fresh fruits and vegetables with the prevention, delay or onset of chronic degenerative diseases, including cancer. Fresh fruits and vegetables are rich sources of a large number of nutrients, including vitamins/antioxidants, trace minerals/ micronutrients, phytosterols, enzymes and dietary fibres [1, 2, 6]. These structurally diverse phyto-nutrients may possess complementary and over-lapping, or identical mechanisms of potential disease-preventing action. Such mechanism include antioxidant, antibacterial, antiviral, and antiangiogenic properties, enhancing the activity of detoxification enzymes, the immune system, reducing platelet aggregation, promoting a healthy lipid profile, reducing hypertension, and modulating hormone metabolism [7, 14, 19]. *Vaccinium myrtillus* has blue fruits, blueberries. Plants of the genus *Vaccinium* have edible blue, black or red berries, especially bilberry, whortleberry or European blueberry. Blue-berry fruits contain antibacterial and antiseptic substances, and most importantly, an herbal equivalent of insulin. Calcium, potassium, sodium and magnesium salts, along with vitamins such as A, C, B1, B2 and proteins that are found even after drying them transform this semi-shrub in a natural medicine, with many health effects [19].

The objectives of this study were to investigate the bioactive compounds such as total phenols, flavonoids content of fresh fruits and pharmaceutical preparations of blueberry (tea, alcoholic solutions and capsules) and to assess the antioxidant capacity.

Materials and Methods

Reagents

All reagents used were of analytical grade purity. 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), potassium persulfate, Folin-Ciocalteau's reagent,1,1-diphenyl-2-pycrylhydrazyl (DPPH), 2,4,6-Tri(2-pyridyl)-s-triazine (FRAP) were purchased from Sigma Aldrich (St. Louis, MO, USA). Gallic acid and sodium carbonate were purchased from Fluka (Switzerland).

Plant materials and pharmaceutical preparations

The fruits of *Myrtillum vaccinum* were collected from spontaneous flora of Bihor County, Romania, in July 2014.

After harvesting, the blueberry fruits were dried, at an average temperature of 40°C, for 96 hours in an oven and were grinded in order to obtain a powder.

The extract preparation for the evaluation of phytochemical content and antioxidant capacity

The bioactive compounds from the dried fruits (sample **a1**) and the pharmaceutical preparations containing blueberry (tea, sample **a2**, capsules, sample **a4**,) were extracted with 70% aqueous ethanol (1:10 w/v) using a stirrer for 20 min and sonicated for 5 min. The samples including the alcoholic blueberry extract (sample **a5**) and tincture of blueberry-pharmaceutical preparation (sample **a3**) were centrifuged and the supernatants were vacuum evaporated to dryness on a rotary evaporator.

The dried extract was transferred to a Petri plate with 10 mL of water and frozen overnight (-25°C). The extract was lyophilized and the residue was weighed and transferred to a sample vial.

Determination of phytochemical content from blueberries samples

Total phenols. The total phenols content (TPh) was determined by the Folin-Ciocalteu method using the method proposed by Vicaș *et al.* [25]. Briefly, the blueberry sample (100 µL) was mixed with 1750 µL distilled water, 200 µL Folin-Ciocalteu reagent (dilution 1:10, v/v) and 1000 µL of 15% Na_2CO_3 solution. The mixture was incubated at room temperature, in the dark, for 2 hours. Total polyphenols content of extracts was expressed as mg gallic acid equivalents (GAE)/100 g, using the following equation based on the calibration curve: y =1.9735x + 0.0261 (R^2 = 0.9928), where x was the absorbance recorded at 765 nm and y was the gallic acid equivalent [24, 25].

Total flavonoids. The total flavonoids content (TFLAV) was determined using a colorimetric method. The absorbance was recorded at 510 nm, using a spectrophotometer Shimadzu mini UV-Vis and the results were expressed as mg quercetin equivalent (QE)/100 g. The equation based on the calibration curve was: y = 56.818571x - 0.066498 (R^2 = 0.9983), where x was the absorbance and y was the mg quercetin equivalent [13].

Determination of antioxidant capacity of blueberry extracts

DPPH method. Radical scavenging activity of plant extracts against stable 2,2-diphenyl-2-picryl-hydrazyl-hydrate (DPPH) was determined by the slightly modified method of Brand-Williams *et al.* [8]. The experiment was carried out in triplicate. The radical scavenging activity was calculated by the following formula:

$$\%\text{Inhibition} = \frac{(A_B - A_A)}{A_A} \times 100,$$

where A_B = absorption of blank sample (t = 0 min) and A_A = absorption of sample extract solution (t = 15 min) [24, 28].

FRAP (ferric reducing antioxidant power) method. It is a simple spectrophotometric method that assesses the antioxidant power of the studied samples, being based on the reduction of ferric tripyridyltriazine complex [Fe(III)-TPTZ] by a reductant, at an acid pH. The Trolox was used as a standard solution, the method had demonstrated to be linear in the range 0 - 300 mg/mL, having a correlation coefficient R^2 = 0.9956 and, the anti-oxidant capacity from the extracts being calculated from the regression equation (y = 0.0017x + 0.0848), where y represents the absorbance registered at 595 nm, and x represents µmol Trolox equivalents (TE)/g [2, 8, 11].

TEAC (Trolox equivalent antioxidant capacity) method. This method is based on the ability of antioxidants to decrease the cation-radical life ($ABTS^{\cdot+}$), a blue-green chromophore that absorbs at 734 nm, compared to Trolox. The results are expressed as µmol Trolox equivalents (TE)/g.

Minerals profile assessment

Elements concentrations (Al, B, Ba, Cr, Cu, Fe, Li, Mn, Ni, Pb, Sr, Zn) in the blueberries samples were determined by an Agilent 4100 inductively coupled plasma (ICP) mass spectrometer. Samples were digested by acid assisted microwave irradiation method using a Microwave Milestone MLS-1200 system.

Statistical analysis

The differences between the elements and the bio-components content from blueberries samples (n = 6) were investigated with one-way analysis (ANOVA) test and Tukey's multi-comparisons test (p = 0.05). The software used for analysis of variance was GraphPad Prism v5.03.

Multivariate analysis principal component analysis (PCA), hierarchical cluster analysis (HCA) and one-way analysis of similarities (ANOSIM) were used to investigate the blueberries' clustering. One-way analysis of similarities represents a non-

parametric test to compare two or more groups for significant differences based on certain distance measure - in our case the Euclidean measure was chosen. The MANOVA and ANOSIM results are analogous, including the pair wise comparison significance table. From this table, one can decide the number of the clusters with 95% statistical confidence, and consequently, from the HCA dendrogram it can be extracted the dissimilarity threshold distance that generates this number of clusters. The software used for multivariate analysis was PAST v3.07 (PAleontologicalSTatistics) [12].

Results and Discussion

The comparative data about bioactive compounds such as total polyphenols, flavonoids and antioxidant capacity in fresh fruits and pharmaceutical preparations containing blueberries are presented in Table I.

Table I

Content of bioactive compounds and antioxidant capacity determined by three different methods from blueberries samples (n = 6)

	Extracts				
	a1	**a2**	**a3**	**a4**	**a5**
TPh (mg GAE/100 g)	$343.90^{b} \pm 7.32$	$344.60^{b} \pm 4.96$	$811.26^{a} \pm 14.53$	$341.51^{b} \pm 9.11$	$813.42^{a} \pm 15.55$
TFLAV (mg QE/100 g)	$1.182.95^{b} \pm 6.46$	$1.180.55^{b} \pm 6.47$	$1.426.00^{a} \pm 1.80$	$1.179.19^{b} \pm 7.90$	$1.425.44^{a} \pm 1.94$
DPPH (%)	$82.38^{a} \pm 17.56$	$86.36^{a} \pm 13.67$	$3.12^{a} \pm 0.33$	$78.50^{b} \pm 16.85$	$3.24^{b} \pm 0.40$
TEAC (µmol TE/g)	$326.36^{a} \pm 3.88$	$327.63^{a} \pm 3.42$	$275.55^{b} \pm 21.10$	$324.66^{a} \pm 7.41$	$283.51^{b} \pm 19.59$
FRAP(µmol TE/g)	$73.16^{ab} \pm 1.86$	$74.78^{a} \pm 4.86$	$3.39^{c} \pm 0.16$	$69.12^{b} \pm 3.19$	$3.46^{c} \pm 0.22$

*Different letters along the rows describe statistical significant differences. TPh = total phenols content; TFLAV = the total flavonoids content; DPPH = 2,2-diphenyl-2-picryl-hydrazyl-hydrate; FRAP = ferric reducing antioxidant power; TEAC = trolox equivalent antioxidant capacity; µmol TE = trolox equivalents

Values, presented as mean ± standard deviation, were compared with one-way analysis of variance (ANOVA) and the multi-comparison Tukey's test ($p = 0.05$).

The TPh values in the blueberry extracts analysed were in the range 341.60 - 813.42 mg GAE/100 g DW dry weight. Among all the samples analysed, the a5 sample revealed the highest TPh concentration at 813.42 mg GAE/100 g DW (dry weight) followed by a3 sample of blueberries (811.26 mg GAE/100g DW). The TPh data's obtained are comparable to previous findings which reported values between 251 - 310 mg GAE/100 g DW for cultivated blueberries and between 577 and 614 mg GAE/100 DW g for wild Italian blueberries [10].

The total flavonoids content ranged from 1182.95 mg QE/100 g DW in a1 sample, to 1426.00 mg QE/100 g DW in a3 sample. In blueberry varieties, the TFLAV content range between 29.07 - 82.21 mg QE/100 g DW [22].

The a2 sample showed the highest antioxidant activity based on FRAP and ABTS assay (74.78 µmol TE/g and 327.63 µmol TE/g). The lowest level in both assays was obtained for a5 sample. The antioxidant capacity in decreasing order in both assays, FRAP and ABTS, was: a2 > a1 > a4 > a3 > a5. The range of FRAP values in the present study was generally higher than the one reported by Giovanelli and Burrati [10] (7.41 - 13.69 µmol/g for cultivated and 34.45 - 57.92 µmol/g for wild blueberries). The ability of the tested samples to decrease the cation-radical life ($ABTS^{\cdot+}$) was higher compared to the samples reported by Zadernowski R. and Naczk M. [27] (8.11 - 38.29 µmol/g).

The $DPPH^{\cdot}$ scavenging activity of blueberry extracts for the a2 sample was 86.36%, while for the a5 sample was only 3.24%. Our data are in accordance with other studies [3, 17]. In Table II there are shown the Pearson's coefficients, which indicate the correlation between phenols and flavonoid content with antioxidant activity values.

Table II

Correlation matrix with the Pearson coefficient (r) values for the bioactive compounds and antioxidant capacity of analysed samples

r	**DPPH**	**TEAC**	**FRAP**	**TPh**	**TFLAV**
DPPH	-	**0.840**	**0.944**	**-0.962**	**-0.947**
TEAC		-	**0.874**	**-0.888**	**-0.872**
FRAP			-	**-0.993**	**-0.996**
TPh				-	**0.997**
TFLAV					-

TPh = total phenols content; TFLAV = the total flavonoids content; DPPH = 2,2-diphenyl-2-picryl-hydrazyl-hydrate; FRAP = ferric reducing antioxidant power; TEAC = trolox equivalent antioxidant capacity

The multivariate analysis, PCA, of the phyto-chemicals (TPh and TFLAV) and values of antioxidant assay (ABTS, FRAP, DPPH) are presented in Figure 1.

The first two major components from the PCA of phytochemicals levels from analysed samples explain 98.44% of the total variance – 94.59% for first principal component and 3.855% for the second one. The PCA biplot (Figure 1) shows only two samples groups and three variables groups. The first sample group consists of aqueous extracts: a1, a2, a4 and the second group of alcoholic extracts: a3 and a5. The first sample group has the highest value of antioxidant capacity (determined by FRAP

and DPPH) and the second sample group has the highest level of phytochemicals (TPh and TFLAV). The values obtaining by ABTS assay has the same level in both sample groups.

The multivariate analysis of the phytochemicals and antioxidant capacity of analysed samples demonstrates that these parameters generate two clusters, thus they can be considered as discriminators of the tested samples. The concentrations of different mineral elements that include Al, B, Ba, Cr, Cu, Fe, Li, Mn, Ni, Pb, Sr, and Zn are presented in Figure 2.

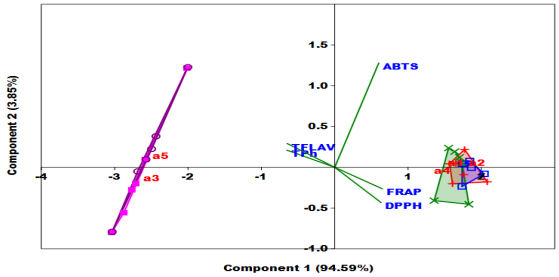

Figure 1.

PCA biplot of phenols (TPh) and flavonoids (TFLAV) and values of antioxidant assay (DPPH, FRAP, ABTS) content from tested samples (a1-a5)

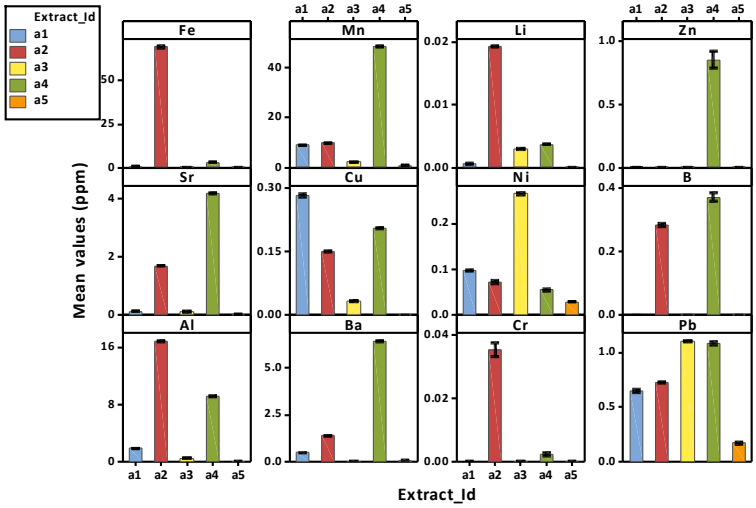

Figure 2.

Results of the elements content analysis from analysed samples (a1 - a5) (n = 6) represented as mean with 95% confidence

Currently, there are 17 elements known to be required by all plants. Nine of them are macro-nutrients (C, N, O, H, K, Ca, Mg, P and S). An additional eight elements are defined as micro-nutrients (B, Cl, Cu, Fe, Mn, Mo, Ni and Zn). Other elements may be essential for certain plant species grown under specific environmental conditions [8]. Zn, Mn and Fe are important as coenzymes; Cu is bound to amino acids, while some elements, such as Mn, Nd and Ce, are bound to some bio-macromolecules forming coordination compounds. Fe, Cu, Mn, Co etc., are important components of many antioxidant processes, a deficiency of any of these essential elements may impair the function of the overall oxidative balance [23]. A common characteristic feature is the relatively high Li and Cr concentration compared to the average concentration present in other plants, as reported for Cr in an earlier publication [16].

Copper (Cu) is an important micronutrient, but also quite toxic at higher concentrations [20, 21]. In this work, minimum Cu concentration found to be as 0.033 mg/kg, whereas maximum was found to be 0.2833 mg/kg.

There is little reliable information about the content of Al in human and animal food. The content in fruits and vegetables varies with a range of 0.1 to 5.0 mg/kg. Concentrations of 1.211 ± 86 mg/kg DW and 611 ± 13 mg/kg DW were determined in tobacco leaves and tomato leaves, respectively [5]. The content of aluminium in all sample ranged between 0.473 and 9.268 mg/kg DW in our study.

The highest Fe concentration in the examined samples was to be 69.342 mg/kg DW, while the lowest was found to be 0.0524 mg/kg DW. Lead (Pb), toxic for plants, ranges from 1.1107 to 0.1717 mg/kg DW in this study. Reimenn *et al.* reported that a high Pb concentration (4.13 mg/kg DW) is observed for bryophytes [21].

Manganese is an essential element for plant growth, though it is required in smaller quantities. It is also an important element from the point of view of biochemical activity, since it associates with an antioxidant enzyme, namely superoxide dismutase (Mn-SOD) [15].

The mineral element composition analysis revealed high contents of manganese (8.7956 - 48.3540 mg/kg DW) in fresh fruits and capsules. The

blueberry is good sources for some elements, since they contain 12 analysed essential elements in higher quantity, which means 15% or a higher rate of the Recommended Dietary Allowances (RDA) or Dietary Reference Intake (DRI) [5].

Zinc (Zn) is an important micronutrient. It is known that plants vary widely in their optimum requirement for Zn. There may be large differences considering the Zn concentrations found in different plant leaves. For example, while Zn concentration in birch was found to be 205 mg/kg DW, in willow, it was found to be 125 mg/kg DW, whereas it was 14 mg/kg DW in blueberries [18]. The range of zinc in the examined plants was found to be 0.852 mg/kg DW.

The PCA analysis is presented in Figure 3 for the trace elements content. The scores of samples and the loadings of the variables are jointly represented using the first two principal axes. The first principal component, Component 1, explains 49.62% from the total variance of the analysed samples and the second principal component, Component 2, 30.40%, both gathering 81.02% variance explained. The first two principal components are statistically enough to describe the variability of the trace elements content from the analysed samples and prescribes a good statistical accuracy for the conclusions that can be drawn from the PCA biplot.

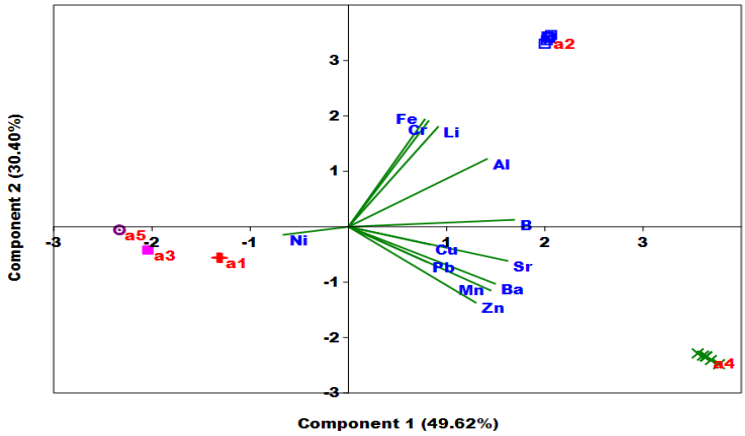

Figure 3.
PCA biplot of trace elements content

Table III
Correlation matrix with the Pearson coefficient (r) values for the trace elements of blueberry samples (green rows describe statistical significant differences)

r	Fe	Mn	Li	Zn	Sr	Cu	Ni	B	Al	Ba	Cr	Pb
Fe	-	-0.084	**0.986**	-0.214	0.181	0.090	-0.201	0.501	**0.877**	-0.023	**0.998**	-0.015
Mn		-	0.011	**0.976**	**0.955**	0.495	-0.330	**0.808**	0.405	**0.994**	-0.058	0.521
Li			-	-0.115	0.277	0.094	-0.110	0.580	**0.910**	0.072	**0.987**	0.143
Zn				-	**0.919**	0.334	-0.290	**0.736**	0.271	**0.976**	-0.188	0.493
Sr					-	0.386	-0.360	**0.941**	0.623	**0.977**	0.207	0.508
Cu						-	-0.253	0.358	0.337	0.422	0.099	0.279
Ni							-	-0.397	-0.337	-0.361	-0.210	0.598
B								-	**0.847**	**0.851**	0.523	0.427
Al									-	0.456	**0.888**	0.244
Ba										-	0.004	0.494
Cr											-	-0.002
Pb												-

The element B has the highest positive correlation with the first principal component. The elements: Al, Li, Cr and Fe, are positive moderate loadings with both principal axes, and the elements: Zn, Mn, Ba, Pb, Sr and Cu, are positive moderate loadings for first principal axis and negative loadings for the second one. The element Ni, has negative loadings for both principal axes, but higher for the first one than the second. All these results are in concordance with the variables Pearson correlations coefficient high values (higher than 0.700), all with significance higher than $p = 0.05$.

The blueberries alcoholic extracts samples, a3 and a5, are located with negative scores on both principal axes. Near these samples is located sample a1. The aqueous extract sample a2, has positive scores for both principal axes and the aqueous extract sample a4, has positive scores for on the first axis and

negative for the second one. Thus, the sample a2 has the highest abundance of second variables group: Al, Li, Cr and Fe elements. The sample a4 has the highest abundance of third variables group: Zn, Mn, Ba, Pb, Sr and Cu. The samples a1, a3 and a5 have the highest abundance of Ni and the lowest of all the rest of the assessed elements.

Conclusions

Alcoholic extracts and tinctures have the highest level of phenolic and flavonoid compounds. For the first time there was performed a comparative study between fresh blueberries and pharmaceutical products containing blueberries found on the market.
In the fresh blueberries and the a1, a2 and a4 samples, the same quantity of phenols was found, meanwhile in the a3 and a5 alcoholic extracts, the concentration was about 2.5 times higher. Similar results were obtained in the case of flavonoids, however the extracted quantity was 1.2 times higher than in the alcoholic ones. Our results proved that in the aqueous extracts, the antioxidant capacity is greater in comparison with the alcoholic ones, possibly because of the presence of hydrophilic compounds. The antioxidant capacity is strongly linked to the phenols and flavonoid content, as to the content of metals like Zn, Mn and Cu.
By comparing the fresh fruits with the pharmaceutical products, we can observe that the antioxidant properties and the bioactive compounds are present in both of them. We can affirm that the blueberry extract ought to be included in dietary supplements, having an anti-oxidant effect and being helpful in preventing different diseases.

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