PAPER

BIOLOGICALLY ACTIVE COMPOUNDS AND ANTIOXIDANT CAPACITY OF *CICHORIUM INTYBUS* L. LEAVES FROM MONTENEGRO

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ABSTRACT

The aim of this study was to determine biologically active substances (BAS) in the samples of *Cichorium intybus* L. leaves from different sources (wild and cultivated) in Montenegro and to investigate the potential influence of location and origin on the BAS. Fiber and fatty acid composition, amount of pigments, total phenols and flavonoids and some phenolic acids were analyzed. Antioxidant activity was also determined by three methods (DPPH, FRAP and ABTS) and the results obtained from all tests were used to calculate the antioxidant potency composite index (ACI). The dietary fiber profile confirmed chicory leaves as an important source of fiber. The majority of fats in chicory leaves consist-of unsaturated fatty acids, while saturated fatty acids were represented mainly by palmitic acid. Chlorophyll a and b, lutein and β-carotene were the main pigments in chicory leaves. ACI index had a good correlation with the total phenolic and total flavonoid content. All these features reinforced the interest of including chicory in modern diet as a healthy alternative to the variety of commonly used vegetables.

Keywords: antioxidant activity, chicory, fibres, fatty acids, pigments

1. INTRODUCTION

Chicory plant (*Cichorium intybus* L.) is a member of the *Asteraceae* family. It is an erect, glandular, biennial herb with a tuberous taproot and a rosette of 30-70 leaves, which grows up to 90 cm in height. The leaves of several *Cichorium* species have been used for centuries as part of traditional diet in the Mediterranean countries (as salads or cooked vegetable, and in meat dishes), while the roots (var. *sativum*) are baked, ground, and used as a substitute for coffee and inulin source. The bitter taste of chicory leaves is very well appreciated in certain Mediterranean cuisines (in Italy, Spain, Greece, Turkey, and so on). In the Montenegrin part of the Adriatic coast, especially in Boka Bay, wild chicory leaves are used in traditional diets, whereas, the cultivation of this vegetable crop just recently began to expand in certain areas of Montenegro. Most of the information available on plant chemical composition refers to the root and seed, while both the leaves and the differences between wild and cultivated plants have seldom been investigated (JAN *et al*., 2011; YING and GUI, 2012). Although, data exist on the differences in composition of some BAS in chicory leaves (SAHAN *et al*., 2017; D'ACUNZO *et al*., 2017), only data on nutrient composition and its difference between wild and cultivated plants from Montenegro is available (JANCIC *et al*., 2016). BAS include a group of nutritive components, which are naturally present in plants, fruits, and vegetables. Conducting a research on BAS is important due to their numerous health benefits. Biologically Active Substances can reduce the risks of vascular and renal diseases, lower glycemic index in diabetics, reduce risks of cancer and increase bifidobacteria population in the colon (GUHR and LaCHANCE, 1997; HASLER, 1998).
The aim of this study was to estimate the profile of biologically active ingredients in chicory leaves, growing in different locations throughout Montenegro. Within the category of BAS, the profile of dietary fiber (total, soluble and insoluble dietary fibers, hemicelluloses, cellulose, lignin, and fructan content), essential fatty acid profile, pigments and major antioxidant compounds were determined. This study is the first comprehensive study on the above named compounds in chicory leaves that are growing in Montenegro.

2. MATERIALS AND METHODS

2.1 Sample collection

Fresh materials (leaves) of the same chicory variety were collected from different locations in Montenegro. Out of the nine samples examined, seven were samples of wild plants (Zoganje, Risan, Podgor, Tivat, Pricelje, Plavnica and Pljevlja locations) and two were samples of greenhouse cultivated plants (Komani and Susanj). All plant leaves (ca. 2 kg) were sampled in their vegetative stage (before flowering) of growth between May and July, 2015. The leaves were collected in the morning.

2.2. Sample preparation

Fresh leaves were separated and all dirt was removed. A part of the leaves were immediately wrapped in aluminum foil to avoid degradation of pigments by light.

One part of the fresh plant material (ca. 1 kg) was milled using an electric grinder (IKA A11, Staufen, Germany) and stored in well-labeled air tight polyethylene bottles at -18 °C until the time for chemical testing in the laboratory. The remaining samples (for the purpose of polyphenol, pigment, and antioxidative capacity determinations) were lyophilized (Alpha 1-4LD, Christ, Germany) and grounded to a fine powder using a planetary ball mill (S100, Retsch, Germany) and stored at room temperature in tightly closed humidity-proof plastic containers until analysis. Before and after lyophilisation, samples were weighed in order to recalculate the data obtained from dry weight (DW) to fresh weight (FW).

2.3. Determination of content of total, soluble and insoluble dietary fibers, hemicelluloses, cellulose, lignin and fructan

For the determination of total (TDF), soluble (SDF) and insoluble (IDF) fractions of dietary fibers, samples were analyzed in accordance with AOAC 991.43 following the enzymatic-gravimetric procedure (AOAC, 1995) as described by LEE *et al*. (1992).
Determination of neutral detergent fibers (NDF), acid detergent fibers (ADF) and acid detergent lignin (ADL) was performed in order to obtain the content of hemicellulose, cellulose and lignin. NDF was determined gravimetrically as a fibrous residue (primarily cell wall components of plants as cellulose, hemicellulose, and lignin), which was formed after refluxing with a neutral detergent solution and heat-stable amylase. ADF was determined gravimetrically as the residue of cellulose, lignin, and heat damaged protein and a portion of cell wall protein and minerals (ash) remaining after extraction with an acidified quaternary detergent solution. ADL was determined gravimetrically upon treatment with an acid detergent solution, which includes cooking, filtering and drying. Hemicellulose, cellulose and lignin content were calculated as follow: a) Hemicellulose=NDF-ADF; b) Lignin=ADL; c) Cellulose=NDF-Hemicellulose-Lignin (GOERING and VAN SOEST, 1970; VAN SOEST *et al*., 1991; AOAC, 1990).
Fructan content was measured in accordance with the enzymatic/spectrophotometric AOAC 999.03 and AACC 32.32 methods using the enzyme assay kit K-FRUC (Megazyme, Bray, Ireland) (McCLEARY and BLAKENEY, 1999; AOAC, 2002; AACC INTERNATIONAL, 2000). In the process of fructan analysis, raffinose oligosaccharides were removed with the α-galactosidase treatment (Megazyme, Bray, Ireland) before degradation of starch, maltosaccharides and sucrose, as described in the kit.

2.4. Determination of fatty acids profile

The method recommended by the Association of Official Analytical Chemists AOAC 930.09 was used for the determination of crude lipid content (AOAC, 1990).
Fatty acids were determined by gas chromatography (GC) after the following trans-esterification procedure: fatty acids in crude fat with added hexane were methylated by shaking for 20 s with 5 mL of 2 M KOH. Sample was heated for 60 s on water bath (60°C) and additionally shaken up for 20 s. After addition of 10 mL of 1 N HCl, the mixture was shaken up well again and another portion of hexane was added. In the separated phases, fatty acid methyl esters (FAME) were in the upper hexane layer. FAMEs were analyzed using gas chromatograph with FID detector (Shimadzu GC-17A, Japan) and Supelco SP-2560 Fused Silica Capillary Column (100 m, id 0.25 mm, deb.

phase: 0.20 μm, Sigma-Aldrich, Germany). Fatty acid identification was performed by comparing the relative retention times of FAME peaks from samples with the standards. FAME standard (Supelco 37 Component FAME Mix, Sigma Co, St Louis, MO, USA) was used for the identification process. All solvents and chemicals were of analytical grade.

2.5. Determination of pigments

Extraction of pigments was carried out according to ZNIDARCIC *et al*. (2011): 100 mg of the dry leaf powder with 5 mL of ice-cold acetone on an ice bath, using T-25 Ultra-Turrax (Ika-Labortechnik, Staufen, Germany) homogenizer for 25 s. All extraction procedures were performed in dim light. Acetone extracts were filtered through 0.2 μm Minisart SRP 15 filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and then subjected to HPLC gradient analysis (a Spherisorb S5 ODS-2 250x4.6 mm column with an S5 ODS-2 50x4.6 mm precolumn, Alltech Associaties, Inc., Deerfield, USA), using the following solvents: solvent A: acetonitrile/methanol/water (100/10/5, v/v/v); solvent B: acetone/ethylacetate (2/1, v/v), at a flow rate of 1 mL/min, employing linear gradient from 10% solvent B to 70% solvent B in 18 min, with a run time of 30 min, and photometric detection at 440 nm. The HPLC analysis was performed on a Spectra-Physics HPLC system with Spectra Focus UV-VIS detector (Fremont, USA). Pigments were quantified by determining peak areas under the curve in the high-performance liquid chromatograms calibrated against known amounts of standards. Each peak was confirmed by the retention time and characteristic spectra of the standards. The following standards were used for the determination of photosynthetic pigments: neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, chlorophyll a and b, pheophytin a and b, and α-, β-carotene, all from DHI LAB products (Hoersholm, Denmark). All standards were highly purified. The solvents acetone, ethylacetate, methanol, and acetonitrile were from Merck and HPLC grade.

2.6. Determination of total polyphenol, total flavonoid, chlorogenic and caffeic acid content

Plant extracts were prepared as described by WEN *et al*., 2005. One gram of each lyophilized sample was taken in a measuring flask and dissolved in methanol/water/trifluoroacetic acid (50/50/0.1, v/v/v) mixed solvent, and then the volume of the turbid fluid was adjusted to 10 mL accurately. The mixture was sonicated for 30 min at room temperature, centrifuged at 3000 rpm for 5 min, and finally filtered through a 0.45 μm nylon filter.

Total polyphenol content (TPC) of plant extracts was determined spectrophotometrically according to the Follin-Ciocalteau method as described by TODOROVIC *et al*. (2015). Briefly, to a 0.5 mL aliquot of samples, 2.5 mL Folin-Ciocalteu's reagent, 30 mL distilled water and 7.5 mL of 20% Na_2CO_3 were added and filled up to 50 mL with distilled water. The absorbance of blue coloration was measured at 765 nm against a blank sample, after 2 h storage in the dark. Gallic acid (Sigma Aldrich, Germany) was used as the standard and the results were expressed as mg Gallic Acid Equivalents (GAE) per gram of fresh sample. A calibration curve was developed for the working solutions of gallic acid in the concentration range of 0-80 mg/mL and it showed good linearity.

For the purpose of determination of total flavonoid content (TFC), the following procedure was followed (TODOROVIC *et al*., 2015): 0.5 mL of each extract was transferred into a 5 mL volumetric flask and 0.15 mL of the 5% $NaNO_2$ was added for 6 min and evenly mixed. After that 0.15 mL of the 10% $AlCl_3$ solution was added and shaken up. Six minutes later 1 mL of the 1 M NaOH solution was added. The mixture was diluted with distilled water up to 5 mL. Absorbance was measured on a UV-VIS spectrophotometer (J.P. Selecta, Barcelona, Spain) at 510 nm and compared to the blank solution. A standard curve was prepared using a 1000 mM solution of catechin (Sigma Aldrich, Germany) at intervals of 200 mM catechin concentration. The results were expressed as μmol Catechin Equivalents (CE)/g FW.

The separation of chlorogenic and caffeic acid was performed using LC-MS/MS Quattro Micro™ API tandem quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with SunFire C18 column (3.5 μm; 3.0 x 100.0 mm, Waters, Milford, MA, USA). The elution was carried out at a flow rate of 0.5 mL/min with 0.1% formic acid in water (eluent A) and acetonitrile (eluent B). The gradient started with 5% B, reached 31% B in 28 minutes, and 71% B after 35 minutes; while 76% B was kept for 2 minutes. The temperature of column was controlled at 40°C. Injection volume was 5 μL.

The components were detected by negative electrospray ionization (ESI-): capillary voltage, 3.2 kV; ion source temperature, 120°C; desolvation gas temperature, 450°C; desolvation gas flow rate, 600 L/h; and cone gas flow rate, 50 L/h. The multiple reactions monitoring (MRM) was used to confirm the detected substances. Characteristic masses for component identification and their retention times (MRM parameters) are presented in Table 1. Calibration curves were developed using standard solution prepared from chlorogenic (≥95%, Sigma Aldrich, Germany) and caffeic acid (≥98%, Sigma Aldrich, Germany).

Table 1. Characteristic mass for chlorogenic and caffeic acid identification and their retention times (MRM parameters).

Component	Parent ion (m/z)	Daughter ion (m/z)	Dwell (s)	Cone voltage (V)	Collision energy (V)
Chlorogenic acid	353.00	191.00	0.2	25	22
Caffeic acid	179.00	135.00	0.2	18	19

2.7. Antioxidant activity determination

Antioxidant capacity of extracts was determined by running 3 tests.

In DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, every diluted sample (0.2 mL) was added to the DPPH working solution (2.8 mL). DPPH solution was prepared as a mixture of 1.86×10^{-4} mol/L DPPH in ethanol and 0.1 M acetate buffer (pH 4.3) in volume ratio 2:1. The free radical scavenging capacity was evaluated at room temperature by measuring the absorbance at 525 nm after 1 h of reaction in the dark. Calibration curve, in the range of 0.2–0.7 mmol Trolox l^{-1} was used for the quantification of antioxidant activity. The results are expressed as μM Trolox Equivalents (TE)/g FW (BRAND-WILLIAMS *et al*., 1995).

In FRAP (ferric ion reducing antioxidant power) assay, stock solutions were prepared by mixing 300 mM of acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \times 6H_2O$ solution. The fresh working solution was made using 25 mL of acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3 \times 6H_2O$ solution and then warmed at 37°C before using. Methanol/water/trifluoroacetic acid diluted samples (300 μL) were allowed to react with 3 mL of the FRAP solution for 40 minutes under dark conditions. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. The antioxidant activity was calculated from the calibration curve using the range 0.1-0.8 mmol Trolox l^{-1}. Results are expressed in μM Trolox Equivalents (TE)/g of FW (BENZIE and STRAIN, 1996).
The Trolox equivalent antioxidant capacity (TEAC or ABTS assay) of chicory extracts was estimated by the ABTS radical action decolorization assay. Stock solutions of ABTS (14 mM) and potassium peroxodisulfate (4.9 mM) in phosphate buffer (pH 7.4) were prepared, and mixed together in equal volumes. The mixture was left to react overnight (12-16 h) in the dark, at room temperature. On the day of analysis, the ABTS radical solution was diluted with phosphate buffer to an absorbance of 0.70 ($\pm$0.02) at 734 nm. Exactly 30 μL of aliquoted samples were added to 3.0 mL of the ABTS radical solution, and after 6 min at 30°C the absorbance readings were taken. Instead of the sample, the reagent blank used was 30 μL of phosphate buffer. Calibration curve was developed using a range of 0.2-1.5 mmol Trolox l^{-1}. The results were expressed as μM Trolox Equivalents (TE)/g of fresh weight (RE *et al*., 1999).
An overall antioxidant potency composite index was determined according to SEERAM *et al*. (2008). An index value of 100 was assigned to the best score for each test and an index score was calculated for all other samples within the test as follows: Antioxidant index score = [(sample score/best score) × 100]. The overall mean index value was determined by dividing the sum of the individual index by the number of tests (three assays in total: DPPH, FRAP and ABTS).

2.8. Statistical analyses

Analyses were performed in triplicate. Results are expressed as mean values with the corresponding standard deviation (SD).
Statistical difference between means of the two groups (wild and cultivated plants) was determined using Student's t-test, *two sample assuming unequal variances*, and a p value <0.05 was considered statistically significant.
Analysis of variance, one-way ANOVA was performed to test the significance of the observed differences between wild plants locations. When the observed differences were significant ($p<0.05$) the mean values were compared by the Least Significant Difference (LSD) Post hoc multiple comparison test.
Correlation analysis was performed using Pearson's. All statistical analyses were performed using the software SPSS ver. 19 (BRYMAN and CRAMER, 2012).

3. RESULTS AND DISCUSSIONS

3.1. Fiber profile

The results for total, soluble and insoluble dietary fibers, hemicelluloses, cellulose, lignin and fructan in analyzed samples are presented in Table 2. The results are expressed on fresh weight (FW) basis.

Table 2. Fiber profile in leaves of wild and cultivated chicory*.

Location	Content (g/100 g)						
	Total fiber	Insoluble fiber	Soluble fiber	Hemicelluloses	Lignin	Cellulose	Fructan
Wild chicory (*Cichorium intybus* L.)							
Zoganje	5.0±0.1^{b}	3.7±0.1^{a}	1.26±0.15^{b}	1.14±0.11^{bc}	0.24±0.10	1.60±0.25^{a}	0.23±0.05^{a}
Risan	4.3±0.2^{c}	3.3±0.3^{b}	1.10±0.09^{c}	1.10±0.20^{bc}	0.20±0.12	1.49±0.17^{a}	0.06±0.03^{cf}
Podgor	4.1±0.2^{c}	2.9±0.2^{c}	1.17±0.14^{bc}	1.31±0.16^{ac}	0.17±0.08	1.31±0.22^{ab}	0.15±0.07^{b}
Tivat	3.0±0.2^{e}	2.1±0.2^{d}	0.93±0.13^{d}	1.07±0.18^{bc}	0.09±0.14	0.77±0.26^{cd}	0.07±0.02^{cd}
Pricelje	3.4±0.1^{d}	2.8±0.2^{c}	0.64±0.12^{e}	1.51±0.19^{a}	0.11±0.06	1.06±0.38^{bc}	0.12±0.04^{bc}
Plavnica	3.3±0.1^{de}	2.1±0.2^{d}	1.14±0.12^{bc}	1.34±0.22^{ab}	0.19±0.06	0.41±0.14^{d}	0.07±0.^{01ce}
Pljevlja	6.2±0.2^{a}	3.8±0.3^{a}	2.35±0.11^{a}	1.56±0.19^{a}	0.21±0.08	1.56±0.25^{a}	0.07±0.02^{bdef}
Average	4.2±0.1	3.0±0.1	1.23±0.02	1.29±0.04	0.17±0.03	1.17±0.08	0.11±0.02
Cultivated chicory (*Cichorium intybus* L.)							
Komani	4.3±0.2	3.2±0.2	1.08±0.10	0.80±0.16	0.19±0.08	1.11±0.19	0.10±0.03
Susanj	2.9±0.1	2.2±0.1	0.67±0.08	0.58±0.12	0.18±0.11	1.30±0.16	0.06±0.02
Average	3.6±0.1	2.7±0.1	0.88±0.01	0.69±0.03$^{\#}$	0.19±0.02	1.21±0.02	0.08±0.01

*data are expressed on 100 g fresh weight and presented as mean±SD of three independent determinations.

$^{\#}$statistically significant difference between wild and cultivated chicory, $p<0.05$.
Data sharing the same letter (a, b, c, d, e, f) in the same column are not significantly different, $p>0.05$.

Determined average values for total fiber were 4.2 g/100 g for the leaves of wild plants and 3.6 g/100 g of the cultivated plant leaves. Results indicate that the amount of soluble fiber was 1.6 to 4.4 times higher than the content of soluble fiber (DODEVSKA *et al*., 2015). Hemicellulose and cellulose were the main insoluble fiber in chicory leaves (average values of 1% and 1.19%, respectively), while lignin was present in small amounts (ca. 0.2%). Fructan is an important fiber in chicory plant, but its content in leaves is quite low (on average around 0.1%). Fructan is a fructose polymer that stores carbohydrate in a large number of plant species. The content of inulin and other fructan in chicory root is 15-20% and these compounds comprise more than 70% of total carbohydrates in fresh chicory roots (GUPTA *et al*., 2003). Chicory fructan is known as inulin and the chicory root, together with Jerusalem artichoke root, are the most important sources for industrial inulin production. Since fructan is not digested in the small intestine because of the b (2-1) bonds between fructose molecules, it belongs to the

soluble dietary fiber fraction. There are several known positive nutritional effects of fructan and inulin: when consumed in adequate quantities they increase stool frequency, have beneficial effects on blood lipids, and have prebiotic effects (stimulate growth of "good" bifidobacteria in the intestine) (ROBERFORID, 1999). Unfortunately, although the root of chicory plants is rich in fructan, the leaves are very poor in this prebiotic carbohydrate and the result of our investigation showed that fructans represented only ca. 4% of insoluble and ca. 2% of total fiber fraction. MILALA *et al*. (2009) also detected low amounts of fructan in chicory leaves, although their results were slightly higher.

Results for dietary fiber profile showed that wild plants had higher amounts of almost all fiber fractions in comparison to cultivated plants, but significant difference was identified only in the hemicellulose content ($p<0.05$). The location influenced the total fiber content both in wild and cultivated plants, which was obvious from the wide range of results (3.0-6.2% of total fiber in wild leaf samples, and 2.9-4.3% in cultivated ones). After statistical analysis, it was seen from the results obtained that there was a significant statistical difference in the content of TDF, IDF, SDF, cellulose, and fructan depending on the wild plants' sampling locations.

In comparison with other leafy vegetables, chicory leaves are significantly better fiber sources. Our results for cellulose content were about twenty times higher than results published for chard and even forty times higher than those published for lettuce. As far as hemicellulose content is concerned, wild chicory was twice richer than spinach, 3.5 times than chard, and 7.5 times richer than lettuce (HERRANZ *et al*., 1981).

3.2. Total fat content and fatty acid composition

Composition of fatty acids (FA) and total fat content of analyzed samples of wild and cultivated chicory are presented in Table 3. The results for total fat content are presented on FW basis, while results for FA composition are expressed as percentage of total fatty acids.

All studied samples showed fat content to be lower than 0.5 g/100 g, ranging between 0.22-0.49g/100 g, with no difference between cultivated and wild plants. Low level of fat is common in leafy vegetables.

Ten different fatty acids were identified and quantified. The main fatty acids found were α-linolenic (C18:3n-3), linoleic (C18:2n-6c), and palmitic acid (C16:0) and they comprised more than 95% of all fatty acids. These results are in good correlation with the data obtained for cultivated chicory leaves in Slovenia and Holland (SINKOVIC *et al*., 2015(a); WARNER *et al*., 2010). Alpha-linolenic acid was the most abundant fatty acid in chicory leaves and its content varied between 60.0 and 75.3% for the wild plants leaves, while the average content in cultivated plants was 75.8%. The linoleic acid content ranged from 11.0% in the samples from Komani to 17.4% in the samples from Podgor. Oleic acid (C18:1n-9c) was the only determined unsaturated fatty acid in all chicory samples. The difference between the contents of linoleic acid and α-linolenic acid in the leaves of wild and cultivated plants was significant ($p<0.05$). Wild plants were richer in linoleic, while cultivated ones had higher content of α-linolenic acid. Furthermore, obvious differences in the content of palmitic, linoleic and α-linolenic acid was noticed between wild plants sampled from different locations.

Table 3. Total fat content* and fatty acid composition** in wild and cultivated chicory leaves.

	Wild chicory (*Cichorium intybus* L.)								Cultivated chicory (*Cichorium intybus* L.)		
%	**Zoganje**	**Risan**	**Podgor**	**Tivat**	**Pricelje**	**Plavnica**	**Pljevlja**	***Average***	**Komani**	**Susanj**	***Average***
C14:0	0.5±0.1	0.2±0.1	0.4±0.1	0.2±0.1	0.3±0.1	0.3±0.1	0.2±0.1	0.3±0.1	0.4±0.1	0.2±0.1	0.3±0.1
C15:0	nd	nd	0.2±0.1	nd	nd	0.2±0.1	nd	-	nd	nd	-
C16:0	17.5±0.6^{a}	9.6±0.5^{d}	13.6±0.7^{b}	10.0±0.3^{d}	12.6±0.4^{c}	12.2±0.7^{c}	10.2±0.4^{d}	12.2±0.5	10.2±0.5	10.4±0.1	10.3±0.3
C18:0	2.0±0.1	0.7±0.1	1.0±0.1	0.8±0.2	0.8±0.2	1.1±0.2	1.4±0.3	1.1±0.2	0.9±0.2	0.6±0.2	0.8±0.2
C18:1n-9c	2.8±0.2	0.8±0.2	1.1±0.2	0.7±0.2	1.4±0.4	1.1±0.2	1.2±0.4	1.3±0.3	0.7±0.1	0.6±0.1	0.7±0.1
C18:2n-6c	15.7±0.4^{b}	12.1±0.6^{d}	17.4±0.8^{a}	14.4±0.5^{c}	15.5±0.8^{b}	15.5±0.5^{b}	13.8±0.8^{c}	14.9±0.6	11.0±0.6	11.2±0.8	11.1±0.7$^{\#}$
C18:3n-3	60.0±0.8^{e}	75.3±0.9^{c}	64.8±1.0^{d}	72.5±1.1^{b}	68.1±1.2^{c}	67.7±0.9^{c}	71.9±0.8^{b}	68.6±1.0	75.5±1.0	76.0±1.2	75.8±1.1$^{\#}$
C20:0	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
C22:0	0.5±0.1	0.4±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.7±0.2	0.3±0.1	0.5±0.1	0.5±0.1	0.3±0.1	0.4±0.1
C24:0	0.8±0.2	0.7±0.2	0.8±0.2	0.7±0.1	0.7±0.1	0.9±0.1	0.8±0.2	0.8±0.1	0.6±0.1	0.5±0.1	0.6±0.1
Total fat	**0.45±0.09**	**0.49±0.10**	**0.35±0.05**	**0.41±0.11**	**0.45±0.10**	**0.33±0.14**	**0.22±0.07**	**0.39±0.09**	**0.48±0.06**	**0.39±0.10**	**0.44±0.06**

*data are expressed on 100 g fresh weight and presented as mean±SD of three independent determinations.
**expressed as relative percentage of total fatty acids.

$^{\#}$statistically significant difference between wild and cultivated chicory, $p<0.05$.
Data sharing the same letter (a, b, c, d) in the same row are not significantly different, $p>0.05$.
myristic acid (C14:0); pentadecanoic acid (C15:0); palmitic acid (C16:0); stearic acid (C18:0); oleic acid (C18:1n-9c); linoleic acid (C18:2n-6c); α-linolenic acid (C18:3n-3); arachidic acid (C20:0); behenic acid (C22:0); lignoceric acid (C24:0); nd: not detected.

The ratio of unsaturated and saturated acids in wild plants was 6:1, while in cultivated ones it was 7:1. Alpha-linolenic acid as a ω-3 polyunsaturated fatty acid has many nutritional and health benefits (it contributes to lowering the level of LDL cholesterol, reducing triglyceride levels and platelet aggregation, vasoconstriction and ventricular arrhythmia) and increasing its intake in diet have been widely suggested (BRADBERRY and HILLEMAN, 2013). It is not unusual that plant leaf lipids contain significant amounts of C18:3n-3, which is a component of chloroplast membrane lipids. Through history, it has been observed that wild plants are very important sources of this essential omega-3 fatty acid (SIMOPOULOS, 2004).
Compared to data from literature on fatty acid composition in the most-commonly consumed leafy vegetable species, chicory from Montenegro was richer in *α*-linolenic acid than lettuce by almost 20% (VIDRIH *et al*., 2009) and by 40% than spinach (NARSING RAO *et al*., 2015).

3.3. Pigments

Three classes of pigments namely: xanthophylls, chlorophylls and carotenes were identified and quantified (Table 4). The results are presented on FW basis.

Table 4. Pigments in wild and cultivated chicory leaves*.

Location	Content (mg/100 g)				
	Lutein	Violaxanthin	Antheraxanthin	VAZ**	Neoxanthin
			Wild chicory (*Cichorium intybus* L.)		
Zoganje	7.0±0.5[f]	3.0±0.4[c]	0.25±0.04[a]	3.2±0.2[b]	3.3±0.2[d]
Risan	10.7±0.9[a]	5.2±0.5[b]	0.14±0.02[bc]	5.4±0.4[b]	5.4±0.5[a]
Podgor	9.5±0.6[bd]	3.1±0.4[c]	0.11±0.02[bc]	3.2±0.3[b]	3.5±0.2[cd]
Tivat	10.3±0.6[ab]	3.5±0.3[c]	0.10±0.02[c]	3.6±0.2[b]	4.9±0.4[a]
Pricelje	9.6±0.6[bc]	6.5±0.6[a]	0.29±0.05[a]	6.8±0.4[a]	4.0±0.3[bc]
Plavnica	8.9±0.6[cde]	6.3±0.6[a]	0.15±0.04[bc]	6.5±0.4[a]	4.0±0.3[bc]
Pljevlja	9.5±0.6[b,e]	5.5±0.5[b]	0.16±0.05[b]	5.7±0.3[b]	4.2±0.4[b]
Average	9.4±0.6	4.7±0.5	0.17±0.03	4.9±0.3	4.2±0.3
			Cultivated chicory (*Cichorium intybus* L.)		
Komani	13.1±0.2	5.8±0.5	0.05±0.01	5.8±0.3	6.6±0.6
Susanj	11.9±0.2	3.3±0.3	0.06±0.02	3.3±0.2	6.5±0.7
Average	12.5±0.2	4.6±0.4	0.05±0.01[#]	4.6±0.2	6.6±0.7[#]
Location	**Content (mg/100 g)**				
	Chlorophyll a	**Chlorophyll b**	**Pheophytin a**	**Pheophytin b**	***β*-carotene**
			Wild chicory (*Cichorium intybus* L.)		
Zoganje	45.0±0.9[f]	13.7±0.9[f]	1.6±0.2[c]	20.1±0.4[b]	3.7±0.5[c]
Risan	97.2±1.3[a]	30.2±1.0[a]	2.1±0.1[b]	13.4±0.3[c]	6.1±0.7[a]
Podgor	74.0±1.1[e]	23.4±1.0[de]	2.1±0.2[b]	19.8±0.2[b]	6.2±0.6[a]
Tivat	77.6±1.0[d]	28.2±1.0[b]	2.4±0.4[a]	22.9±0.4[a]	6.1±0.7[a]
Pricelje	92.5±1.2[b]	25.5±1.0[c]	1.7±0.1[c]	10.4±0.2[d]	5.8±0.4[ab]
Plavnica	84.3±1.0[c]	24.1±1.0[ce]	0.1±0.1[d]	8.7±0.2[e]	6.2±0.5[a]
Pljevlja	77.9±1.2[d]	24.2±1.1[cd]	1.7±0.2[c]	10.4±0.3[d]	4.8±0.5[b]
Average	78.4±0.1	24.2±1.0	1.7±0.2	15.1±0.3	5.6±0.6
			Cultivated chicory (*Cichorium intybus* L.)		
Komani	115.0±1.3	36.9±0.9	2.2±0.2	13.9±0.9	8.2±0.5
Susanj	107.9±1.5	36.0±0.9	0.6±0.1	3.5±0.7	6.5±0.6
Average	111.5±1.4[#]	36.4±0.9[#]	1.4±0.2	8.7±0.8	7.4±0.6

*data are expressed on the original weight basis and presented as mean±SD of three independent determinations.
**Content of xanthophyll cycle pigments (violaxanthin, antheraxanthin, zeaxanthin).
[#]statistically significant difference between wild and cultivated chicory, $p<0.05$.
Data sharing the same letter (a, b, c, d, e, f) in the same column are not significantly different, $p>0.05$.

Four xanthophyll pigments – lutein, violaxanthin, antheraxanthin, and neoxanthin were identified and quantified in the leaves of chicory. Zeaxantin was not found in any of the samples. Based on concentration, the major xanthophyll was lutein representing on average 50% of the total xanthophylls, which is in agreement with the results of ZNIDARCIC *et al*. (2011). The lowest lutein content was measured in chicory from Zoganje (7.0 mg/100 g), while the highest was measured in chicory from Komani (13.1 mg/100 g). Lutein was confirmed as the main xanthophyll in the edible portion of wild and cultivated chicory varieties grown in the South of Italy as supported by MONTEFUSCO *et al*. (2015). Although, the reported concentrations in the tissues were much lower (0.8-3.0 mg/100 g FW). The content of xanthophyll cycle pigments - VAZ (violaxanthin, antheraxanthin and zeaxanthin) in analyzed chicory varied at intervals 3.2-6.8 mg/100 g FW. The major cycle pigment was violaxanthin, with antheraxanthin representing only 0.9-7.8% of the VAZ pool. The difference between wild and cultivated chicory was significant in the case of antheraxanthin ($p<0.05$). The average values for the only non-VAZ pigment neoxantin content in chicory leaves were 4.2 mg/100 g in wild plants and 6.6 mg/100 g FW in cultivated plants, and there was a significant difference between them ($p<0.05$). A significant difference for all pigments in wild samples from different locations was noticed.

Dietary carotenoids, especially xanthophylls, enjoyed significant scientific attention because of their characteristic biological activities, including anti-allergic, anti-cancer, and anti-obese actions. Lutein is one of the major xanthophylls present in green leafy vegetables and it is known to selectively accumulate in the macula of the human retina (KOTAKE-NARA and NAGAO, 2011). As an antioxidant (MILLER *et al*., 1996; DI MASCIO *et al*., 1989) and as a blue light filter (JUNGHANS *et al*., 2001), lutein can protect the eyes from oxidative stress, of which a non-protectioncan lead to age-related macular degeneration and cataracts. When compared with data from literature, our results for lutein content in chicory leaves were twice higher than in spinach and even sixty times higher than in lettuce (PERRY *et al*., 2009).

The chlorophyll a/b ratio was found to be similar in all analyzed samples, although the chlorophyll a and b contents varied greatly (chlorophyll a 45.0-115.0 mg/100 g and chlorophyll b 13.7–36.9 mg/100 g). There was a statistical difference in chlorophyll a and b contents between leaves of cultivated and wild plants, with cultivated ones being richer than the wild ones. The results also indicated that significant amounts of chlorophyll a and b were converted into pheophytin a and b, probably during the lyophilization process. Chlorophyll gives a characteristic coloration to the green leafy plants and its content correlate with the photosynthetic potential, giving some indication of the plant physiological status. There are indications that chlorophyll can play an important role in the prevention of various diseases associated with oxidative stress and certain environmental contaminants, such as cancer, cardiovascular diseases and other chronic diseases (GAMON and SURFUS, 1999; SANGEETHA and BASKARAN, 2010). The mechanism underlying the supposed chlorophyll suppression of *in vitro* mutagenicity of certain environmental contaminants could be the trapping of the carcinogenic molecules (SARKAR *et al*., 1994). In comparison with lettuce, chicory contained about 7 times more chlorophyll a and chlorophyll b. The obtained results confirm the similarity between chicory and spinach in chlorophyll a and chlorophyll b content (DUMA *et al*., 2014).

Beta-carotene was found in all samples (3.7–8.2 mg/100 g), while α-carotene was below detection limit. These results were similar to the results obtained by MONTEFUSCO *et al*. (2015). Compared to spinach and chard, chicory leaves contained 10-30% and 500%, respectively, more β-carotene (PERRY *et al*., 2009; RAJU *et al*., 2007). An increased intake of β-carotene rich food in daily diet may be one of the strategies for improving vitamin A status instead of synthetic vitamin A (GOPALAN, 1992). Obtained results for β-carotene

showed that consumption of 100 g of chicory leaves satisfied up to 135% of the referenced daily intake values (RDI) of vitamin A.

3.4. Total polyphenols, total flavonoids, chlorogenic and caffeic acid

The total amount of polyphenols and flavonoids in different chicory samples ranged between 0.65 and 3.73 mg GAE/g FW and 1.57-4.42 μmol CE/g FW, respectively (Table 5). The flavonoids content followed the content of total polyphenols in all samples. Our results showed that plants grown on different locations had different TPC and TFC.

Table 5. Content of polyphenols (TPC), flavonoids (TFC), chlorogenic and caffeic acid in wild and cultivated chicory leaves*.

Location	TPC (mg GAE/g)	TFC (μM CE/g)	Chlorogenic acid (μg/100 g)	Caffeic acid (μg/100 g)
Wild chicory (*Cichorium intybus* L.)				
Zoganje	3.73±0.04^{a}	4.42±0.01^{a}	1034±18^{a}	7.0±0.6^{a}
Risan	1.17±0.02^{d}	2.54±0.12^{e}	251±9^{f}	3.2±0.1^{d}
Podgor	2.90±0.08^{b}	3.81±0.01^{b}	908±14^{b}	6.6±0.1^{a}
Tivat	1.45±0.02^{c}	2.67±0.03^{d}	437±9^{d}	4.6±0.8^{bc}
Pricelje	1.45±0.03^{c}	3.00±0.09^{c}	440±10^{d}	4.4±0.6^{bc}
Plavnica	1.15±0.02^{d}	2.50±0.02^{e}	378±10^{e}	3.1±0.8^{d}
Pljevlja	1.05±0.02^{e}	2.29±0.04^{f}	526±8^{c}	3.8±0.5^{cd}
Average	1.84±0.03	3.03±0.05	568±4	4.7±0.3
Cultivated chicory (*Cichorium intybus* L.)				
Komani	0.82±0.01	2.01±0.03	104±4	2.1±0.5
Susanj	0.65±0.01	1.57±0.02	104±6	1.4±0.6
Average	0.74±0.01$^{\#}$	1.79±0.01$^{\#}$	104±1$^{\#}$	1.7±0.5$^{\#}$

*data are expressed on the original weight basis and presented as mean±SD of three independent determinations.

$^{\#}$statistically significant difference between wild and cultivated chicory, $p<0.05$.

Data sharing the same letter (a, b, c, d, e, f) in the same column are not significantly different, $p>0.05$.

TPC - Total Polyphenol Content; TFC - Total Flavonoid Content; GAE - Galic Acid Equivalents; CE - Catechin Equivalent.

Similar range of TPC was obtained for "Catalogna" landraces (*C. intybus*) in the study carried out by D'ACUNZO *et al*. (2017). The average value for content of total polyphenols was lower than in the work of SAHAN *et al*. (2017) and MILALA *et al*. (2009), but at the same time was significantly higher than in all chicory cultivars analyzed in the study of SINKOVIC *et al*. (2014) Also, our results for TFC were higher than those obtained by MONTEFUSCO *et al*. (2015).

The most important phenolic compounds in chicory leaves are hydroxycinnamic acid derivatives, such as chlorogenic and chicoric acids (MILALA *et al*., 2009; SINKOVIC *et al*., 2015(b)). The chlorogenic acid content (sum of isomers) in our study was the highest in the sample from Zoganje (1034 μg/100 g FW) and the lowest in those samples cultivated in Komani and Susanj (104 μg/100 g FW). Overall range for caffeic acid content was 1.4-7.0 μg/100 g FW.

Difference in TPC, TFC and analyzed polyphenol acids between wild and cultivated plants, as well as between wild samples from different locations, was significant ($p<0.05$). The wild plants were 2.5 times richer in TPC and 1.7 times in TFC than the cultivated ones.

3.5. Antioxidant capacity

Antioxidant activities of obtained extracts from chicory leaves, their abilities to scavenge the synthetic DPPH and ABTS radicals, as well as their power to reduce ferric (FRAP) ions were examined. The analysis were performed in triplicates and expressed as μM TE/g of fresh weight (Table 6).
The FRAP assay is quick and simple to perform, the reaction is reproducible and the reducing power that is measured in this assay is linearly related to the molar concentration of the antioxidants (MÜLLER *et al.*, 2010). Therefore, total phenolic content represents a reliable indicator of the antioxidant activity of analyzed plant. The antioxidant activity measured with FRAP test and total polyphenol content was almost 3 times lower in cultivated than in wild chicory samples.

Table 6. Antioxidant capacities of wild and cultivated chicory leaves*.

Location	FRAP (μM TE/ 1g)	DPPH (μM TE/1 g)	ABTS (μM TE/ 1g)
Wild chicory (*Cichorium intybus* L.)			
Zoganje	46.90±1.08^{a}	11.66±0.02^{a}	32.81±0.15^{a}
Risan	13.31±0.42^{e}	6.85±0.26^{e}	16.82±0.09^{c}
Podgor	36.45±0.56^{b}	10.29±0.03^{b}	26.32±0.15^{b}
Tivat	19.44±0.36^{c}	7.64±0.08^{d}	16.20±0.11^{d}
Pricelje	17.43±0.50^{d}	8.41±0.27^{c}	15.12±0.17^{e}
Plavnica	13.65±0.09^{e}	6.90±0.29^{e}	10.61±0.08^{f}
Pljevlja	13.33±0.27^{e}	6.19±0.22^{f}	10.80±0.09^{f}
Average	22.93±0.47	8.28±0.17	18.38±0.12
Cultivated chicory (*Cichorium intybus* L.)			
Komani	8.80±0.52	5.05±0.27	11.07±0.07
Susanj	6.75±0.13	3.76±0.17	10.46±0.09
Average	7.77±0.33$^{\#}$	4.40±0.22$^{\#}$	10.77±0.08

*data are expressed on the original weight basis and presented as mean±SD of three independent determinations.
$^{\#}$statistically significant difference between wild and cultivated chicory, $p<0.05$.
Data sharing the same letter (a, b, c, d, e, f) in the same column are not significantly different, $p>0.05$.
DPPH - 2,2-diphenyl-1-picrylhydrazyl; FRAP - Ferric ion Reducing Antioxidant Power; ABTS (TEAC) - Trolox Equivalent Antioxidant Capacity; TE - Trolox Equivalent.

For evaluation of free radical scavenging properties of the extracts, we used two assays: the DPPH radical and the ABTS radical cation assay.
The relatively stable organic radical DPPH has been widely used in the determination of antioxidant activity of different plant extracts (COSTA *et al.*, 2009). The free radical scavenging ability varied between 3.76 μM TE/g in cultivated sample from Susanj and 11.66 μM TE/g in wild sample from Zoganje, using the DPPH antioxidant method.

In accordance with the obtained data for the ABTS radical cation, it is interesting to note that the reduced antioxidant activity of cultivated samples is in agreement with the lower total flavonoid content of the same samples compared to the wild ones (40% lower on average). The reason for this pattern could be that flavonoids are the phenol class that specifically reflect and are better indicators of the segment of antioxidant activity obtained in ABTS test.

Obtained results for all three assays have shown that the antioxidant capacity of analyzed extracts, as presumed, followed the pattern of TP and TF contents. FRAP and DPPH assays showed significantly different results for wild and cultivated plants ($p<0.05$), as well as for wild plants sampled from different locations. Correlations among results obtained with all three antioxidant assays were positively high as well as correlation between antioxidant activity with TP and TF contents ($r\sim0.99$, $p<0.05$).

When compared with data of SAHAN *et al*. (2017), the obtained results for ABTS and DPPH assay were lower.

The overall antioxidant activity of analyzed samples is expressed as antioxidant composite index (ACI). ACI value is followed by one statistical-mathematical model, which is a modern ranking tool for the representation of antioxidant activity of various plants. One basic advantage of using ACI is the value being expressed in percent, which covers all segments of antioxidant action obtained in different antioxidant assays. Another advantage of using ACI index instead of three assays is because it simplifies the process of comparison between antioxidant properties of different foods. The antioxidant composite index (ACI) of chicory samples (Table 7) was in the following decreasing order: Zoganje > Podgor > Tivat > Pricelje > Risan > Plavnica > Pljevlja > Komani > Susanj. All samples of wild plants had higher ACI than the cultivated plants.

Table 7. Antioxidant composite index (ACI) of wild and cultivated chicory leaves.

Location	FRAP index	DPPH index	ABTS index	ACI (%)
Wild chicory (*Cichorium intybus* L.)				
Zoganje	100.0	100.0	100.0	100.0
Risan	28.4	58.7	51.3	46.1
Podgor	77.7	88.2	80.2	82.1
Tivat	41.4	65.5	49.2	52.1
Pricelje	37.2	72.1	46.1	51.8
Plavnica	29.1	59.2	32.3	40.2
Pljevlja	28.4	53.1	32.9	38.1
Cultivated chicory (*Cichorium intybus* L.)				
Komani	18.8	43.3	33.7	31.9
Susanj	14.4	32.2	31.9	26.2

FRAP - ferric ion reducing antioxidant power; DPPH - 2,2-diphenyl-1-picrylhydrazyl; ABTS (TEAC) - Trolox equivalent antioxidant capacity; ACI - Antioxidant composite index.

4. CONCLUSIONS

This study is the first comprehensive study offering detailed information on fibers, fatty acids, pigments, phenolics, flavonoids, and antioxidant activity of chicory (*Cichorium intybus* L.) leaves grown in Montenegro. Chicory leaves are rich sources of fiber, polyphenols, flavonoids, and almost all analyzed pigments. The lipid content in chicory

leaves is quite low, but they have high nutritional value due to their favorable balance of fatty acids with the omega-3 fatty acids as the dominant ones. The antioxidant potential of chicory leaves extract was positively correlated with their phenolic and flavonoid content. Accordingly, this composition of biologically active substances offer a number of different ways of using chicory leaves in the field of nutrition and production of healthy food. Wild plant samples had higher content of the majority of analyzed BAS in comparison with the cultivated ones.

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