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REGIONAL PATTERN AND CHARACTERISTICS OF ESSENTIAL ELEMENTS IN SEVERAL MEDICINAL PLANTS USING SPECTROMETRIC METHODS COMBINED WITH MULTIVARIATE STATISTICAL APPROACHES

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ABSTRACT. The aim of this study was to provide a regional pattern and characteristics of essential elements in several medicinal plants from North Macedonia and Romania. The content of Ca, Mg, Al, Fe, Cu, Ba and Zn was determined by ICP-OES while Na and K by FAES in some medicinal plants belonging to sixteen families. Similar profiles of elements with a high content of Ca, Mg and K were observed. Peppermint and blackberry from both countries showed extreme content in Al and Fe. A symmetric distribution for K, Ca and Zn and an asymmetric one for Na, Al, Fe and Ba were found in medicinal plants from both countries. Potassium, Ca, Mg, Al and Fe could be considered as markers for growing area. Principal Component Analysis highlighted that the variability of elements content was described by four factors (83.4%) in North Macedonia and three factors (70.0%) in Romania. The first factor could explain the influence of soil nature upon variability of elemental composition, calcareous in North Macedonia (Mg and Ca – 29.4% variance) and a rich one in hydroxides in Romania (Al and Fe – 33.1% variance).

Keywords: *essential element, medicinal plant, inductively coupled plasma optical emission spectrometry, flame atomic emission spectrometry, Principal Component Analysis, two-way joining Cluster Analysis*

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INTRODUCTION

The medicinal plants consumed preferable as teas or tinctures, have a long and rich history regarding their usage as therapeutics. The World Health Organization (WHO) estimated that 80% of the current population use medicinal plants for some aspect of primary healthcare [1]. It is thought that some medicinal plants contain elements of vital importance for human metabolism, disease prevention and healing [2]. Besides their use for treatment of diseases, the medicinal plants are also used as dietary supplements once they are found to be rich in one or more elements. So, their chemical composition in terms of nutrients, minerals, vitamins, and essential antioxidants need to be permanently monitored. Elemental content can vary in a wide range, depending on factors such as soil geochemical characteristics, the climate conditions, irrigation water, use of fertilizers and the ability of each plant species to selectively accumulate some of them [3, 4]. Metals, categorized as macro (primary) or micro (trace) elements play an essential role in the body some of them being crucial for many body functions. Essential elements such as sodium, potassium, calcium, magnesium, phosphorus, iron, zinc, copper, manganese and non-essential elements such as aluminium, barium, cadmium and lead were found in various medicinal plants [5]. Macroelements such as potassium, calcium, magnesium, phosphorus, and sodium are contained in concentration greater than 100 mg kg^{-1} while trace elements including iron, zinc, manganese, molybdenum, selenium, chromium, copper, cobalt and sulphur are usually contained in concentration less than 100 mg kg^{-1} [6].

The determination of elements in medicinal plants and their impact on human health is of great importance. Besides, their essential importance in the living system, several elements can be toxic when the concentrations exceed those necessary for metabolic functions [7, 8]. The level of elements permitted by the WHO should not endanger the health of consumers [9, 10]. Inductively coupled plasma-optical emission spectrometry (ICP-OES), atomic absorption spectrometry (AAS) and neutron activation analysis (NAA) methods have been used for the determination of elements concentration in medicinal plants and plant based multivitamin preparations [11, 12].

The aim of this paper was to provide a regional pattern and characteristics of essential elements in several medicinal plants from Romania and North Macedonia using spectrometric methods combined with multivariate statistical approaches. Romania and North Macedonia possesses a great vegetal genetic diversity, as for example Romania cover 30% of the European flora with a number of 3700 plant taxa, from which 283 were identified as medicinal plants [13, 14]. However, according to our best knowledge there are few studies related to determination of essential elements in medicinal plants from two countries. From this point of view, the study has relevance and is of general interest for both producers and consumers. The content of Ca, Mg, Cu, Fe, Zn, Al and Ba were determined by ICP-OES, while Na and K content was determined by flame atomic emission spectrometry (FAES) in different parts (root, leaves, flowers, fruits) of different variety of medicinal plants originating from two countries. Principal Component Analysis (PCA) and two-way joining Cluster Analysis were used to describe variability of chemical composition of the medicinal plants under study and recognize the parameters significantly different. It is expected that the elemental composition to be different depending of variety and region of provenience due to soil and climatic conditions influence. So far a similar complex approach has not been performed on medicinal plants from Romania and North Macedonia.

RESULTS AND DISCUSSION

Patterns of essential elements in medicinal plants

Results obtained in the determination of elements concentration in medicinal plants originated from North Macedonia and Romania are given in Supplementary material (Table S1). The sample code of medicinal plants is presented in the experimental part, Table 5.

The content of Co, Cr, Cd, Ag, Mn, Ni, Sr, Pb were below LOD in ICP-OES. The elemental profiles of the selected medicinal plants are presented in Figure 1, while summary statistics is provided in Table 1.

Figure 1 reveal that the selected medicinal plants from both countries show similar profiles but different content of macroelements and microelements depending of variety and the nature of the plant sample. Therefore, the analysed plants are mainly characterized by high content of Ca, K and Mg. Interestingly is the high content of Al and Fe for *Peppermint* (Pe) and *Blackberry* (Bl) from both countries.

Data in Table 1 shows either asymetric or symetric distribution of elements content around the mean.

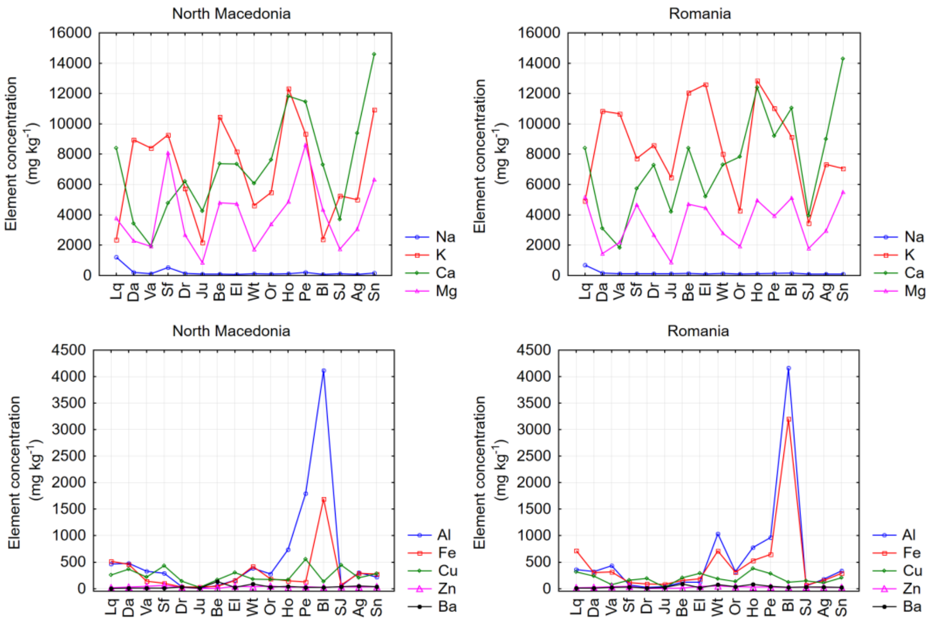

Figure 1. Profiles of the elemental content in the analysed medicinal plants from North Macedonia and Romania. The sample code of medicinal plants is presented in the experimental part, Table 5.

A positive Skewness and leptokurtic distribution was observed for Na, Al, Fe and Ba in the samples from both countries. These distributions are consistent with extreme values observed for Na in *Liquorice (*Lq), Al and Fe in *Blackberry (*Bl) and Ba in *Wild thyme* (Wt) and *Bean* (Be) samples (Figure 2). An symetric repartition with platikurtic distribution could be observed for K, Ca and Zn in samples from both countries while Mg and Cu show a symetric distribution only for plants of Romanian provenience.

Therefore, the median and the mean values for asymetric and symetric distribution, respectively, were considered for an overall comparison of metals content in analysed plants. In case of plants from North Macedonia, the following decreasing order for macroelements Ca> K> Mg> Na, for microelements Al> Cu> Fe> Zn≈Ba respectively, was observed. In case on Romanian samples the following order was observed K>Ca>Mg>Na and Al≈Fe>Cu>Zn≈Ba. These differences could be explained by influence of the soil composition on metals bioavailability in medicinal plants.

Table 1. Summary statistics for elements content (mg kg $^{-1}$) in medicinal plants samples from North Macedonia (^{1}) and Romania (^{2}), described in the experimental part, Table 5.

Element	Min	Max	Mean	Median	STDEV	SKEW	KURT
Na^1	61.49	1443.92	253.44	108.70	342.34	2.63	6.80
Na^2	75.82	681.06	145.22	105.64	136.95	3.83	15.27
K^1	1804.47	12334.69	6818.36	5731.00	3178.30	0.05	-1.12
K^2	3440.42	13648.22	8702.09	8417.91	2968.28	0.00	-0.86
Mg^1	828.02	8594.53	3715.03	3419.53	1896.92	0.67	0.41
Mg^2	872.30	5504.87	3467.38	3369.00	1458.04	-0.23	-1.44
Al^1	9.29	4118.28	528.07	277.09	890.62	3.45	12.96
Al^2	21.64	4165.67	603.73	321.61	961.04	3.25	11.65
Ca^1	1941.08	16100.34	7606.94	7370.84	3387.57	0.63	0.51
Ca^2	1812.82	14285.71	7547.95	7820.90	3221.15	0.19	-0.21
Fe^1	28.34	1689.66	288.34	154.03	362.06	3.02	10.56
Fe^2	68.03	3203.59	500.14	305.08	716.79	3.41	12.72
Cu^1	23.91	584.18	267.33	222.58	137.99	0.82	0.42
Cu^2	23.98	380.97	196.12	189.16	92.48	0.23	-0.33
Zn^1	4.67	64.03	33.00	32.08	15.01	0.01	-0.31
Zn^2	2.07	47.55	25.94	25.63	12.38	-0.39	-0.26
Ba^1	2.85	134.45	33.14	21.42	32.12	1.93	3.90
Ba^2	9.45	90.39	34.46	27.37	24.47	1.33	0.60

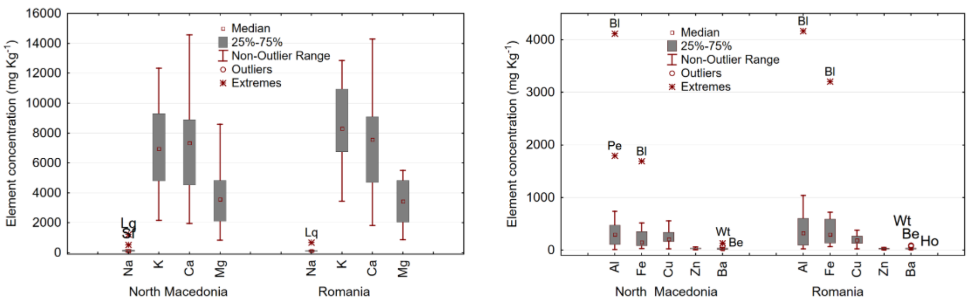

Figure 2. Box and whiskers plot for content of elements in medicinal plant samples. The sample code of medicinal plants is presented in the experimental part, Table 5.

Statistical evaluation of analysed medicinal plants

The correlations between the content of elements found in medicinal plants from North Macedonia and Romania are presented in Table 2.

Table 2. Correlations between content of elements found in medicinal plants from North Macedonia ([1]) and Romania ([2]). Bold values show significant correlations ($p < 0.05$).

Element	Na[1]	K[1]	Ca[1]	Mg[1]	Al[1]	Fe[1]	Cu[1]	Zn[1]	Ba[1]
Na[2]	**0.92**	-0.32	0.02	0.35	-0.13	0.00	0.35	-0.08	-0.27
K[2]	-0.37	0.62	0.21	0.34	-0.29	-0.36	0.49	0.26	0.41
Ca[2]	0.07	0.13	**0.92**	0.66	0.12	0.11	0.53	0.02	0.26
Mg[2]	-0.02	0.30	0.50	0.72	0.11	0.10	0.46	0.24	0.13
Al[2]	0.07	0.18	0.37	0.36	**0.96**	**0.95**	0.01	0.03	-0.06
Fe[2]	0.25	-0.01	0.31	0.31	**0.92**	**0.95**	-0.07	0.02	-0.17
Cu[2]	0.01	0.02	-0.13	0.13	-0.16	-0.17	0.35	0.39	-0.16
Zn[2]	-0.29	-0.13	-0.18	-0.01	-0.17	-0.21	0.01	0.74	-0.24
Ba[2]	-0.21	0.23	0.25	0.16	-0.06	-0.10	0.06	0.02	**0.82**

Statistical significant correlations were observed for Na, Ca, Al, Fe and Ba. For Mg, Cu and Zn (elements with a symmetric distribution in the analysed plants) a lower correlation was observed.

The two-way joining Cluster Analysis and PCA methods were applied to better reveal the variability of profile for content of elements in medicinal plants under study. Heat maps (Figure 3) were depicted by choosing a colour graduation starting with the green tone for the low values below the mean content, up to the red tones above these values. Based on similarity pattern approaches, in both countries the concentration of elements depends on the variety of medicinal plants. Significant differences between medicinal plants from North Macedonia and Romania were observed especially for K, Ca, Mg, Al and Fe content which can be considered as important constituents for originating growth area of the plants. Also, in both countries *Stinging nettle* (Sn), *Horsetail* (Ho), *Peppermint* (Pe), *Bean* (Be) and *Elderberry* (El) have a high content in Ca and Mg while *Blackberry* (Bl) is characterized by high content in Al. Plants as *Elderberry* (El), *Dandelion* (Da), *Valerian* (Va) and *Blackberry* (Bl) from Romania were observed to have a high content in K comparative with content in samples from North Macedonia.

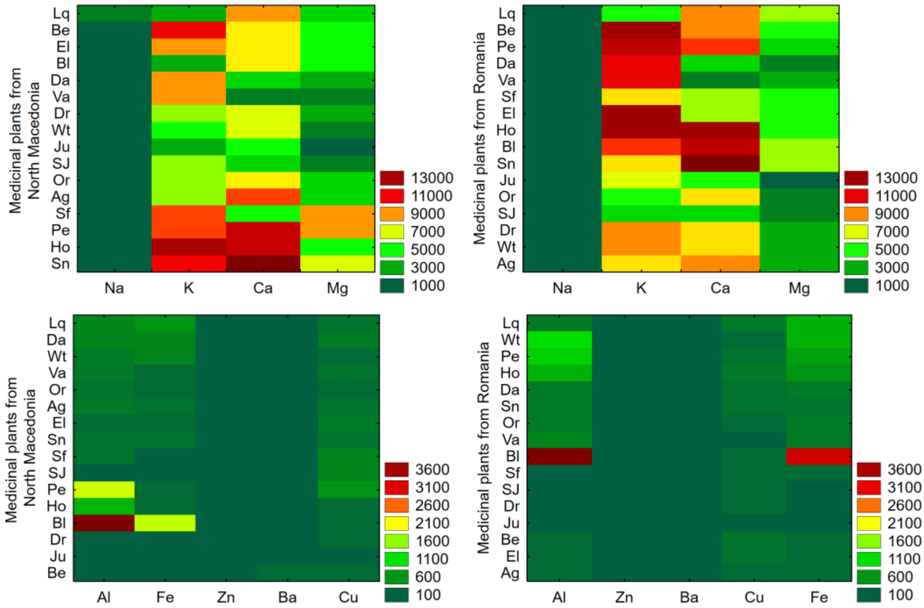

Figure 3. Two-way joining heat map revealing the variation of macroelements and microelements content (mg kg^{-1}) in medicinal plants from North Macedonia and Romania. The sample code of medicinal plants is presented in the experimental part, Table 5.

Based on the heat maps from the auto-scaled values of determinations (Figure 4), some markers were revealed as differentiating the elemental profile of some of the analysed medicinal plants from two countries. Among the analysed elements potassium differentiate *Stinging nettle* (Sn) and *Elderberry* (El), cupper differentiate *Horsetail* (Ho), *St.John's wort* (SJ) and *Sweet fennel* (Sf) while magnesium is an important element that differentiate the elemental profile of *Liquorice* (Lq), *Blackberry* (Bl), *Bean* (Be), *Elderberry* (El) and *Horsetail* (Ho) from North Macedonia and Romania.

The PCA analysis (Table 3) shows that variability of the characteristics in the medicinal plants under study from North Macedonia is described in proportion of 83.4% by four factors with a strong contribution (loadings > 0.700) for Mg, Ca, Al, Fe, Na, Cu, Zn and moderate contribution (0.500 < loadings < 0.700) for K and Ba. The first factor describing 29.4 % of elemental content variation is mainly due to Mg and Ca, but also to K and has been associated with soil carbonates, the main source of Ca and Mg. The second factor (23.3% variability) is due to Al and Fe and has been associated with the hydroxides contained in soil, the main source of these elements.

The third factor (18.0% variability) is mainly due to Na but also to Ba which has a medium influence. Copper and Zn, two essential microelements for plants, describe 12.7% of the characteristics variability of the plants under study.

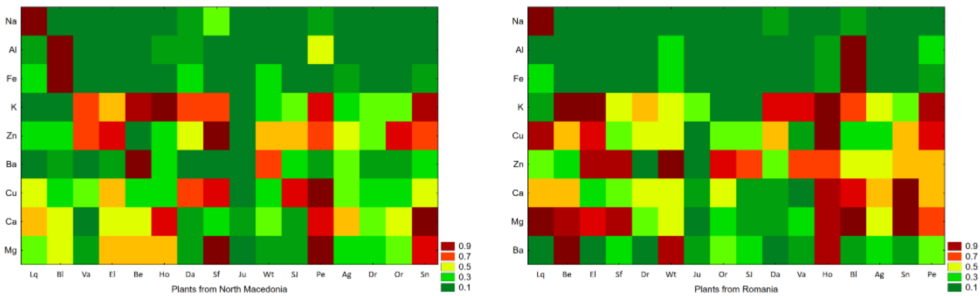

Figure 4. Two-way joining heat map revealing the elemental markers in medicinal plants from North Macedonia and Romania. The sample code of medicinal plants is presented in the experimental part, Table 5.

Table 3. Factor loadings after Varimax rotation of the first PCs with eigenvalue >1.0 reflecting the contribution of each variable in the characteristics of medicinal plants from North Macedonia. (Bold face loadings values > 0.700, strong contribution; Italic loadings values >0.500, moderate contribution).

Essential elements	**Factor 1**	**Factor 2**	**Factor 3**	**Factor 4**
Na	0.12	0.08	**0.92**	-0.09
K	*0.62*	0.31	-0.43	0.37
Mg	**0.92**	-0.15	0.06	0.23
Al	0.14	**-0.96**	-0.03	-0.00
Ca	**0.93**	-0.05	0.05	-0.04
Fe	-0.04	**-0.93**	0.09	-0.18
Cu	0.17	0.07	0.14	**0.82**
Zn	0.07	0.11	-0.06	**0.86**
Ba	0.12	0.18	*-0.66*	-0.41
Total variance (%)	29.4	23.3	18.0	12.7
Cumulative variance (%)	29.4	52.7	70.6	83.4

The variability of medicinal plants in Romania (Table 4) is described in proportion of 70.0% by the first three factors, similar to 70.6% for plants from North Macedonia. Instead of this, there are differences between the contributions of elements on the characteristics variability. Thus, 33.1% is due to Al and Fe variability associated with the hydroxides contained soil. A variability of 20.3% is due to Ba but also Na, K and Zn, and 16.5% is due to Mg, Cu and Na. In both countries the influence of macroelements in high concentration, as is for example K, was not revealed by a single factor. A similar observation was revealed for Na in case of plants from Romania.

Table 4. Factor loadings after Varimax rotation of the first PCs with eigenvalue >1.0 reflecting the contribution of each variable in the characteristics of medicinal plants from Romania. (Bold face loadings values > 0.700, strong contribution; Italic loadings values >0.500, moderate contribution).

Essential elements	**Factor 1**	**Factor 2**	**Factor 3**
Na	0.02	*-0.65*	*0.57*
K	0.05	*0.64*	0.31
Mg	0.30	0.10	0.81
Al	**0.98**	0.09	0.09
Ca	0.38	0.13	0.66
Fe	**0.97**	-0.04	0.14
Cu	-0.17	0.25	0.85
Zn	0.05	*0.56*	0.00
Ba	-0.03	**0.73**	0.26
Total variance (%)	33.1	20.3	16.5
Cumulative variance (%)	33.1	53.4	70.0

The differences between the factors variability for plants in two countries could be explained by differences between the soils on which they grew, namely a mountainous calcareous soil with higher carbonate content in North Macedonia, and one dominated by Fe and Al hydroxides in Romania. It would also explain the differences between plant varieties in these two countries.

CONCLUSIONS

The existence of a regional pattern of medicinal plants originating in North Macedonia and Romania has been demonstrated based on the macroelements and microelements content. Among the studied elements, the content of K, Ca, Mg, Al and Fe can be considered as a marker parameter for medicinal plants. Medicinal plants such as blackberry and peppermint have been found to bio-accumulate the Al and Fe. The content of Na, Al, Fe and Ba showed an asymmetric distribution while that of K, Ca and Zn showed a symmetric distribution for plants in both countries. Some elements such as Mg showed a symmetric distribution only in plants from Romania. In general, a significant correlation was observed between elements with asymmetric distribution. The

PCA analysis showed that the first four factors and the first three factors describe over 83.4% for North Macedonia and 70.0% for Romania with a strong influence from Ca, Mg, Al and Fe content. These were associated with the different soil composition of these two countries. The first factor in plants from North Macedonia was described by Ca and Mg in accordance with calcareous soil while the first factor in plants from Romania was described by Al and Fe in accordance with presence of hydroxides in the soil.

The PCA analysis showed that indeed the content of Ca, Mg, Al and Fe can be used for discriminating the medicinal plants from these two countries, which was also highlighted by the two-way joining Cluster Analysis. Obviously, the combination of elemental analysis and chemometric methods is an efficient approach in the study of regional pattern and characterization of the medicinal plants from different countries.

EXPERIMENTAL SECTION

Reagents and standards

Nitric acid (69%) and hydrogen peroxide (30%) of analytical reagent grade used for samples digestion were purchased from Merck (Darmstadt, Germany). Double distilled water was used through this study. An ICP multielement standard solution IV (CertiPUR IV, traceable to NIST, Merck, Darmstadt, Germany) contained 23 elements (1000 mg L^{-1}) in diluted nitric acid was used for preparation of calibration standards in the range 0–1.0 mg L^{-1} element (n= 5) in 2% (v/v) diluted nitric acid, necessary for ICP -OES method. The calibration standards in the range 0-5.0 mg L^{-1} Na and K (n=6) in 2% v/v nitric acid were used in FAES method.

Description of medicinal plants and samples

The study was carried out on different vegetal parts (root, leave, flower, fruit and seeds) of 38 medicinal plants from North Macedonia and Romania (Table 5). The plants originated from North Macedonia were collected from the south-eastern part of the country, from three localities in the basin of the Osogovo Mountains. They were identified by determination key using the data from Matevski [15], a specimen being kept in the herbarium at the Department of Plant Production, Faculty of Agriculture, Goce Delcev University in Štip, North Macedonia. Samples of medicinal plants of Romania were purchased from a local specialized market.

Table 5. Description and provenience of investigated medicinal plants.

No.	Common name	Genus (taxonomic rank)	Familly	Part of the plant	Code of sample
1	Liquorice	*Glycyrrhiza glabra L.*	Fabaceae	*radix*	Lq^{1a}
				radix	Lq^{1b}
				radix	Lq^{2}
2	Dandelion	*Taraxacum officinale F.H.Wigg.*	Asteraceae	*radix*	Da^{1}
				radix	Da^{2}
3	Valerian	*Valeriana officinalis L.*	Caprifoliaceae	*radix*	Va^{1}
				radix	Va^{2}
4	Sweet fennel	*Feniculum vulgare var. dulcis Mill.*	Apiaceae	*fructus*	Sf^{1a}
				fructus	Sf^{1b}
				fructus	Sw^{2}
5	Dog rose	*Rosa canina L.*	Rosaceae	*fructus*	Dr^{1}
				fructus	Dr^{2}
6	Juniper	*Juniperus communis L.*	Cupresaceae	*fructus*	Ju^{1}
				fructus	Ju^{2}
7	Bean	*Phaseolus vulgaris L.*	Fabaceae	*pericarpum*	Be^{1}
				pericarpum	Be^{2}
8	Elderberry	*Sambucus nigra L.*	Adoxaceae	*flos*	El^{1}
				flos	El^{2}
9	Wild thyme	*Thymus serpyllum L.*	Lamiaceae	*herba*	Wt^{1a}
				herba	Wt^{1b}
				herba	Wt^{2}
10	Oregano	*Origanum vulgare L.*	Lamiaceae	*herba*	Or^{1}
				herba	Or^{2}
11	Horsetail	*Equisetum arvense L.*	Equisetaceae	*herba*	Ho^{1}
				herba	Ho^{2}
12	Peppermint	*Mentha x piperita L.*	Lamiaceae	*folium*	Pe^{1}
				herba	Pe^{2a}
				folium	Pe^{2b}
13	Blackberry	*Rubus fructicosus L.*	Rosaceae	*folium*	Bl^{1}
				folium	Bl^{2}
14	St.John's wort	*Hypericum perforatum L.*	Hypericaceae	*herba*	SJ^{1a}
				herba	SJ^{1b}
				herba	SJ^{2}
15	Agrimony	*Agrimonia eupatoria L.*	Rosaceae	*herba*	Ag^{1}
				herba	Ag^{2}
16	Stinging nettle	*Urtica dioica L.*	Urticaceae	*semen*	Sn^{1a}
				folium	Sn^{1b}
				herba	Sn^{2}

[1] - Medicinal plants from North Macedonia; [2] - Medicinal plants from Romania

Samples preparation

Amounts of 0.50 g powdered vegetal material (n = 3 paralel samples) weighed were overnight predigested in 10 mL of HNO_3 (69%), and subjected to microwave-assisted digestion after 2 ml of H_2O_2 (30%) addition. The microwave digestion program is provided in Supplementary material Table S2.

After cooling, the digest was transferred into 100 ml volumetric flask, filtered and stored in polyethylene bottles at room temperature until analysis.

Instrumentation

The SPECTRO CIROS^{CCD} spectrometer (Spectro, Kleve, Germany) was used for the ICP-OES measurements. Instrumental details and operating conditions are summarized in Supplementary material Table S3. Determination of elements was based on external calibration using a 5-point linear calibration curve over the range 0 – 1.0 mg L^{-1} element in 2% HNO_3 (v/v). The emission signals as peak heights were measured using the best signal-to-noise ration SNR strategy for 48 s integration time.

The two points model approach was used for background correction. The most sensitive emission lines free of spectral interferences were selected.

For Na and K determination the FAES instrument using methane-air flame was interfaced with a low resolution microspectrometrer HR 4000 200-1100 nm spectral range Ocean Optix (Dunedin, USA). The flame was operated so that a stable burning was obtained. The emission spectrum was recorded using 500 ms integration time. The selected wavelengths were Na 588.995 nm and K 766.49 nm.

A MW3S+ Berghof model closed-vessel microwave digestion system (Berghof, Germany) with temperature monitoring option up to 210 °C was used for sample mineralization.

Analytical performance and methods validation

The analytical performances of the ICP-OES and FAES methods were evaluated in terms of the limit of detection (LOD), limit of quantification (LOQ), precision and accuracy. LOD was calculated using the (3σ) criterion and parameters of the calibration curve [16].

$$LOD = \frac{3s_b}{m} \quad (1)$$

where (s_b) is the standard deviation of background assessed from 11 measurements of reagent blank 2% V/V HNO_3, while (m) the slope of the calibration curve.

LOQ was considered as 3xLOD. Values of LOD and LOQ in solid sample were calculated taking into account the sample preparation protocol. Statistical evaluation of the linear calibration function was assessed according to SR ISO 8466-1 [17]. The parameters of the calibration curves (LODs and LOQs) for ICP-OES and FAES methods are presented in Supplementary material Table S4.

A good linearity of the calibration curves with determination coefficients in the range 0.9949 – 1.0000 was obtained. The LODs were in the range 0.068 (Ba) – 8.471 (Pb) mg kg^{-1} while LOQs were in the range 0.205 – 25.413 mg kg^{-1} in ICP-OES (Table 1). The LODs in FAES were 10.27 mg kg^{-1} for Na and 0.051 mg kg^{-1} for K.

The ICP-OES and FAES methods were previously validated by analysing a several materials of vegetables and fruits providing recovery in the range 87-108% [18]. The relative expanded uncertainly (k=2, 95%) was in the range 9-25%. The major contribution to combine standard uncertainty come from replicate analysis, fitting and standard preparation.

Statistical analysis

Two-way joining cluster analysis was used to reveal the elements significantly different in medicinal plants samples and to better explore variability in elemental composition on samples from North Macedonia and Romania. Two-way clustering is a common multivariate technique used to analyse complex data matrices and produces a heat map graph (two-way display of a data matrix) ordering cases and variables based on similarity patterns. The individual cells in the heat map are displayed as colored rectangles where the color of a cell is proportional to its position along a color gradient. The elements were shown as column variables of the data matrix while the variety medicinal plants were shown as rows.

The PCA with varimax rotation approach was used to maximize the variation expressed by the principal components (PCs). The varimax rotation algorithm rearrange the amount of variance among principal components, so each new factor has only few variables with significant contribution (large loadings) and many others with no significant contribution (low loadings). In this study only PCs with eigenvalue >1 were retained for varimax rotation method.

Correlations and multivariate statistical analyses were performed using the Software Package Statistica 8.0 (StatSoft inc. 1984-2007, USA).

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