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Research Article

Chemometric and ICP-OES analyses of *Forsythia europaea* Degen & Bald. and its extracts

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Abstract

Forsythia represents a group of plants originating mainly from China and Japan, but one species is endemic and grows in the Balkans (Balkan forsythia, *Forsythia europaea* Degen & Bald.). Our previous studies on polyphenols in investigated extracts of Balkan forsythia showed that this plant is a good source of polyphenols. Analysis of the various extracts of Balkan forsythia (*Forsythia europaea* Degen & Bald.) by the application of ICP-OES method showed that they are rich in different macro and microelements. The abundance order of macroelements is K>Ca>P>Mg>Na in all extracts. Among the transition metals iron, manganese, zinc and copper are particularly important, and the order of abundance is Zn>Fe>Cu>Mn. Heavy metals which are the most frequent contaminants of food are lead, cadmium and arsenic, and the determination of their contents is of special importance on the safe use of plant species. The determination shows that aqueous extracts contain the highest quantity of elements, which is especially important. The contents of toxic elements are significantly lower than the permitted values. Statistical methods (Principal Component Analysis (PCA) and Agglomerative Hierarchical Clustering (AHC)) are useful tools for the grouping of samples and determining relations between investigated elements. This analysis shows that when higher quantities of Cr and Ba are present, the lower quantities of V are present, and *vice versa*. Based on our studies on polyphenols and minerals, we can expect the anti-inflammatory effects of extracts of Balkan forsythia.

Keywords: Balkan forsythia; chemometrics; ICP-OES; macroelements; microelements

Introduction

Forsythia is a genus belonging to the olive family (Oleaceae). It is a shrub with usually yellow flowers, but the species with white and pink flowers can be found as well, particularly in Asia. *Forsythia* covers a group of plants originating mainly from Asia (China, Japan), but one species is endemic and grows in the Balkans (Balkan forsythia, *Forsythia europaea* Degen & Bald.).

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Balkan forsythia is the only European representative. It is an endemic species of the *Forsythia* genus whose range of distribution covers northern Albania and areas of the former Yugoslavia. This species is thermophilic and heliophilic, *i.e.*, it requires heat and light.

The fruit of this herb has anti-inflammatory, antipyretic and antiviral properties and is used to treat respiratory infections. It can slow blood coagulation so it should be avoided before surgery or if a person is already using a drug that has the same effect. Forsythia is not toxic if used in moderate quantities. However, it is not recommended for pregnant women. The list of poisonous plants for pets and humans does not contain forsythia. However, there is a difference between non-toxicity and edibility. Some people use flowers of forsythia in their diet, but not in large quantities because petals can have a bitter taste. The flowers of the plant are also used as a salad decoration. It is safe to have it in the garden, but it is necessary to find out more about its chemical composition and its effects on humans (Ha *et al*., 2018)

Phytochemical studies have shown that the major components of this plant, accumulated mainly in the fruit, are triterpenoids, lignans, flavonoids, phenylethanoid glycosides. Studies have shown that phenolic components, including lignans, flavonoids, phenylethanoid glycosides, are responsible for the diverse biological activity of this species (Zheng and Wang, 2001).

Human beings require a number of organic and inorganic compounds in order to meet the requirements for daily activities. Minerals and vitamins form comparatively smaller part and are consumed in much smaller amounts. Mineral elements can be therapeutic or can contribute to normal health. 25 elements have been identified as essential for keeping human health. Therefore, the study of elements in food and medicinal plants is of high interest. Plant materials form a major portion of diet, and their nutritive value is important. Mineral elements play an important role in the normal physiology of the body and their absence or insufficiency leads to adverse effects on the body. Minerals serve as components of enzymes, regulate cellular energy transduction, gas transport, antioxidant defense, membrane receptor functions, second-messenger systems, and integration of physiological functions. Hence, mineral elements are involved in the regulation of use of macronutrients (Indrayan *et al.*, 2005; Lukaski, 2004; Petenatti *et al.*, 2011).

The leaves and flowers of this plant are a source of valuable compounds such as polyphenols. Previously, we performed research on the quantity of total phenols and flavonoids in the bloom of this plant (Kostic *et al.*, 2020). These compounds have potential pharmacological activity and anti-inflammatory properties. Inflammation causes many chronic diseases, such as atherosclerosis, diabetes mellitus, obesity, osteoporosis, rheumatoid arthritis, inflammatory bowel disease, asthma, and others.

The research question in this study was the content of macro and microelements in *Forsythia europaea* Degen & Bald. and the different extracts (aqueous, methanol, acetone, ethyl-acetate, 50% ethanol, 50% acetone and ethanol) of this plant. The objective of the study was to check if the quantity of all investigated elements in aqueous extract are in ranges which enable from this point safe consumption of tea made from this plant.

Materials and Methods

Material

The petals of the Balkan forsythia plant harvested from the Niš area (Čair Park) in March 2020 were used for this work. The plant was identified and recorded in the herbarium of the Faculty of Sciences and Mathematics in Niš and was given voucher number 11982 (*Forsythia europaea* Degen & Bald., 11982).

Mineralization

Samples (1 g) of previously dried plant petals were transferred into the porcelain crucible, then to the oven where the temperature was gradually increased by 50 °C. When the temperature reached 450 °C, heating was continued on that temperature for the next 24 h. The cooled sample was treated with 5 cm^3 HCl (1:1),

transferred into volumetric flask (50 cm^3) and filled up with HNO_3 (0.1 mol dm^{-3}). After the sample was cooled, the filtration of the sample was performed. Afterward, 10 cm^3 from each extract was taken and evaporated to dryness. Obtained dry residues were dissolved with 50 cm^3 of demineralized water, filtrated, and transferred into volumetric flasks (50 cm^3) (AOAC, 2000).

Preparation of the extracts

2 g of dried plant petals was weighed. The extraction was performed using the ultrasound bath thrice for 15 min with 30 cm^3 of the following solvents: distilled water, methanol, acetone, ethyl-acetate, 50% ethanol, 50% acetone and ethanol. The suspension was filtrated through Buchner funnel and Whatman No. 1 filter paper. The extracts were transferred into volumetric flasks (100 cm^3) and filled up with the proper solvent, and then put in the fridge until the analysis.

Reagents

The multi-element standard solution (Ultra Scientific, USA, Item ICM-240, EPA Method 200.7 LPC Solution of 30 analytes) of 20.00±0.10 mg dm^{-3} was used as a stock solution for the calibration. Matrix of multi-standard was 2 % HNO_3 with traces of tartaric acid in deionized water with low values of total organic carbon (TOC<50 mg dm^{-3}). Analytical grade 65% nitric acid (Merck, Darmstadt, Germany) was used for complete mineralization of analyzed samples. All the solutions were prepared using high purity deionized water.

Instrumentation

The iCAP 6000 inductively coupled plasma-optical emission spectrometer which combines an Echelle optical design, and a charge injection device (CID) solid state detector (Thermo Scientific, Cambridge, United Kingdom) was used for the analysis of the contents of investigated elements. Analyses were made in triplicate and the mean values are reported. The blank sample involving the addition of all used reagents except sample was also processed to make corrections during calculation of elemental concentrations. iTEVA operating software series was used to control all functions of the instrument. Analytical balance Mettler Toledo (Switzerland) was used to measure the mass. High purity water (conductivity 0.05 μS cm^{-1}) was obtained using MicroMed high purity water system, Thermo Electron LED GmbH (Germany). Under the optimal operating conditions for the instrument (radio frequency power-1150 W; analysis pump rate-50 rpm; flush pump rate-100 rpm; nebulizer gas flow-0.7 dm^3 min^{-1}; coolant gas flow-12 dm^3 min^{-1} and auxiliary gas flow-0.5 dm^3 min^{-1}), analytical emission lines for each of the element were selected based upon the tables of known interferences, baseline shifts and the background correction (the highest signal-to-background ratio) which was manually selected for the quantitative measurements. The observation axe is axial/radial, and the rinsing time is 30 s. Three probes were used for each measurement.

For the determination of the content of elements in the investigated samples, analytical methods were created for each element. For each element, it was chosen four wavelengths with the highest relative intensities of the emission. First the calibration curves were constructed using three standards; two of three standards were prepared by diluting the referent multi-standard with the concentrations 2 ppm and 5 ppm, and the third standard was a deionized water. The working wavelength was chosen based on the relative intensity of the emission, standard deviation of the slope, standard deviation of the intercept, correlation coefficient, the interference on the wavelengths left and right from the chosen. Table 1 contains the chosen wavelengths for each element, parameters of the calibration line for the studied elements, correlation coefficient (R), limit of detection (LOD) and limit of the quantification (LOQ).

Statistical analysis

All ICP-OES measurements were carried out in triplicate, presented as mean ± standard deviation (SD) and the results were subjected to statistical analysis (PCA and AHC) using XLSTAT 2020 (Lumivero, New York, USA).

Table 1. Equation of the line for the determination of the content of the investigated elements, parameters of the calibration line for the studied elements

Number	Element	Wavelength (nm)	Intercept	Slope	R	R^2	LOD	LOQ
1	Al	308.215	850.02197	2132.73943	0.99989	0.99978	0.00452	0.01507
2	As	189.042	1.01309	281.03537	0.99951	0.99902	0.00275	0.00915
3	B	249.773	96.09794	5762.50796	0.99967	0.99934	0.00069	0.00232
4	Ba	455.403	215.92940	379462.46123	0.99990	0.9998	0.00005	0.00016
5	Ca	422.673	4.09876	358.49178	0.99990	0.9990	0.01698	0.05661
6	Cd	228.802	2.01461	7690.87184	0.99935	0.99870	0.00022	0.00073
7	Co	237.862	-2.64248	2517.72544	0.99962	0.99924	0.00171	0.00569
8	Cr	283.563	155.44079	10848.23911	0.99945	0.99890	0.00059	0.00195
9	Cu	324.754	46.11921	11635.05554	0.99957	0.99871	0.00075	0.00249
10	Fe	259.940	12.01229	8889.64397	0.99919	0.99757	0.00057	0.00189
11	K	766.490	0.17985	393.54264	0.99140	0.98287	0.03943	0.13144
12	Mg	279.553	47.71326	18104.47426	0.99999	0.99998	0.00012	0.00041
13	Mn	257.610	16.77733	34201.84179	0.99888	0.99776	0.00013	0.00042
14	Na	588.995	-5996.4742	57100.13438	1	1	0.00053	0.00177
15	Ni	231.604	-3.15857	3249.00756	0.99969	0.99938	0.00055	0.00182
16	P	213.618	-2.75798	151.24053	0.99952	0.99904	0.00693	0.02310
17	Pb	220.353	-2.14240	536.04003	0.99966	0.99932	0.00244	0.00812
18	Se	196.090	-0.95279	210.18735	0.99919	0.99838	0.00382	0.01274
19	Si	251.611	56.63052	2419.86496	0.99998	0.99996	0.00174	0.00579
20	V	310.230	5171.94828	11964.01821	0.99972	0.99944	0.00061	0.00202
21	Zn	213.856	12.25222	7708.81461	0.99867	0.99734	0.00014	0.00047

Results and Discussion

In this paper it was investigated the content of micro and macroelements in different extracts of the plant *Forsythia europaea* Degen and Bald. as well as in the oven-treated plant petals. C, O, H, N, P, K, Ca, Mg, S, Na and Cl belong to macroelements and their content, if C, O and H, are omitted, in the dry matter of the plant the average goes from 2-60 mg g^{-1}. The content of microelements (Cu, Zn, B, Mn, *etc.*) in dry matter of the plant is smaller than 1 mg g^{-1} and usually it is higher than 1 μg g^{-1} (Mihaljev *et al.*, 2015).

The content of macro and microelements is presented in Tables 2-4, in the following extracts and the oven-treated plant sample:

1. Oven-treated sample
2. Water-HCl (99:1)
3. Methanol-HCl (99:1)
4. Acetone-HCl (99:1)
5. Ethyl acetate-HCl (99:1)
6. Ethanol-water-HCl (50:49:1)
7. Acetone-water-HCl (50:49:1)
8. Ethanol-HCl (99:1).

The analysis of different extracts of Balkan forsythia using ICP-OES method shows that they are rich in various macro and microelements. Among macroelements, the order of abundance is K>Ca>P>Mg>Na, in all extracts.

In oven-treated petals of Balkan forsythia the most abundant is potassium (14155 μg g^{-1}), and the least abundant is sodium (151.17 μg g^{-1}).

Table 2. The content of essential macroelements in *F. europaea* Degen and Bald.

Sample	Content (μg g^{-1} DW)				
	K	Na	Ca	Mg	P
Oven-treated	14155.0 ±25.23	151.17 ±0.74	12942.5 ±8.63	1599.5 ±5.36	11865.5 ±9.63
Water	5670 ± 13.5	43.275 ± 0.23	2236.75 ± 9.42	489.75 ± 3.30	1784 ±12.3
Methanol	5087.5 ± 18.9	55.6 ± 0.48	3115.0 ± 8.38	361.0 ± 3.15	1902.5 ±11.02
Acetone	1498.0± 20.5	47.25 ± 0.22	1645.75 ± 9.52	227.0 ± 2.19	1481.25 ±13.20
Ethyl-acetate	156.5 ± 32.2	43.42 ± 0.17	858.5± 9.48	107.5 ± 1.28	51.8 ±0.66
Ethanol 50%	4930.0 ± 17.8	50.8 ± 0.15	3205.0 ± 9.28	524 ± 5.22	1503 ±13.20
Acetone 50%	6122.5 ±41.2	54.275 ±0.56	3630.0 ±7.89	641.25 ±3.20	2090 ±11.30
Ethanol	2687.5 ±18.2	53.1 ±0.65	1643.75 ±5.98	418.5 ±2.20	4652.5± 20.10

In the extracts, in general, the highest content was shown by potassium (6122.5 μg g^{-1}) in the acetone 50 % extract, while the lowest content was shown by sodium (43.275 μg g^{-1}) in the aqueous extract. The content of potassium goes from 156.5 μg g^{-1} to 6122.5 μg g^{-1} in ethyl-acetate and acetone 50 % extracts, respectively. The recommended daily intake of potassium goes from 0.4 g for infants, 3.8 g for children from 4-8 years up to 4.7 g for adolescents, women and men (Razic *et al*., 2013).

The aqueous extract contains the lowest quantity of sodium (43.275 μg g^{-1}), while it is in the highest quantity in methanol extract (55.6 μg g^{-1}). The recommended daily intake of sodium for infants is 0.12 g, for children from 4-8 years 1.2 mg and for adolescents, men and women around 1.5 g (Razic *et al*., 2013).

It was observed that the content of Ca is the lowest in the ethyl-acetate extract (858.5 μg g^{-1}), and the highest in acetone 50 % extract (3630 μg g^{-1}). The recommended daily intake of Ca for infants is 210 mg, for children from 4-8 years 800 mg, for adolescents 1300 mg and for men and women depending on the age from 1000 to 1200 mg. The content of Mg goes from 107.5 μg g^{-1} in the ethyl-acetate extract to 641.25 μg g^{-1} in acetone 50 % extract. The recommended daily intake of Mg for infants is 30 mg, from children from 4-8 years 130 mg and for adolescents, men, and women around 400 mg (Razic *et al*., 2013).

With this analysis it was determined that the phosphorus is present in the lowest quantity in the ethyl acetate extract (51.8 μg g^{-1}), and the highest in the ethanol extract (4652.5 μg g^{-1}). Mihaljev *et al.* (2015) were investigating the content of these macroelements in dry samples of plants used in Serbia as teas. In St. John's wort, the content of Ca is almost identical to our result (oven-treated sample), while the other elements present are in higher quantities. The content of P in dry samples of thymus, flower of hibiscus, and fruit of briar is very similar to ours. The content of other macroelements is generally significantly higher compared to our values.

The similar situation is with the results obtained by Razic *et al.* (2013) in the investigation of samples of medicinal plants grown in Serbia. The higher content of elements in their case can be explained by the geographically different areas, different characteristics of soil, application of agrotechnical measures and the different methods for the analysis.

The content of microelements in the extracts of the plant *Forsythia europaea* Degen & Bald. (μg g^{-1} of dry mass) is presented in Tables 3 and 4. In the oven-treated sample it is present the lowest concentration of cadmium (1.25 μg g^{-1}), which is good, taking into account that it is a toxic metal, while the highest content of silicon is present there (286.6 μg g^{-1}). In the dried petals of the plant, generally, there is the lowest amount of cadmium, and the highest of aluminum. Cadmium is present in the lowest quantity in ethanol 50% extract (0.075 μg g^{-1}), while the aluminum is present in the highest quantity in the ethanol extract (101.77 μg g^{-1}).

The importance of microelements in the organism of humans can be observed in the right functioning of the enzymes, because metalloenzymes in their composition contain the transition metals. Among the transition metals, particularly iron, manganese, zinc, and copper are important. The order of abundance is Zn>Fe>Cu>Mn.

Table 3. The content of microelements in *Forsythia europaea* Degen & Bald.

Element / Sample	Content (μg g^{-1} DW)							
	B	Ba	Co	Cr	Cu	Fe	Mn	Ni
Oven-treated	234.6±0.06	19.87±0.31	3.32±0.02	9.97±0.02	35.07±0.07	230.3±0.15	83.7±0.41	14.17±0.08
Water	62.50±0.75	6.25±0.24	1.00±0.05	4.25±0.05	13.07±0.40	44.37±0.18	11.37±0.7	2.9± 0.05
Methanol	40.4 ± 0.09	5.87±0.045	1.63±0.00	4.70±0.03	15.37±0.05	41.7± 0.13	12.02±0.03	3.72±0.04
Acetone	52.6± 0.18	2.35±0.045	1.25±0.01	4.17±0.04	13.85±0.034	34.5± 0.19	9.87±0.06	2.05±0.02
Ethyl-acetate	57.5± 0.17	1.9± 0.021	1.10± 0.01	4.10±0.03	2.33± 0.054	3.75± 0.16	2.25± 0.07	0.57±0.02
Ethanol 50%	39.67±0.18	5.33±0.05	1.25±0.02	4.35±0.05	12.12±0.05	7.48±0.16	10.87±0.02	3.32±0.01
Acetone 50%	53.3± 0.13	6.50±0.02	1.07±0.03	4.40±0.03	21.60±0.04	29.30±1.15	14.05±0.03	3.2± 0.01
Ethanol	64.42±0.11	3.75±0.07	1.20±0.000	4.67±0.04	7.10± 0.06	23.62±0.17	7.25± 0.04	2.37±0.01

Table 4. The content of microelements in *Forsythia europaea* Degen & Bald. (μg g^{-1} DW)

	Se	Si	V	Zn	Al	As	Cd	Pb
Oven-treated sample	25.00±0.02	286.6±0.37	120.25±0.04	163.65±0.33	278.18±0.5	5.6± 0.09	1.25±0.01	8.8± 0.06
Water	7.21±0.20	66.42±0.41	18.05±0.18	57.62±0.4	98.75±0.96	2.72±0.23	0.25±0.01	3.75±0.01
Methanol	1.32±0.02	81.20±0.28	25.17±0.07	77.45± 0.28	85.55±0.82	0.85± 0.03	0.125±0.01	2.45±0.22
Acetone	3.00±0.09	63.67±0.35	23.5± 0.08	52.5± 0.02	67.75±0.75	1.57±0.33	0.12± 0.01	3.0± 0.35
Ethyl-acetate	0.5± 0.01	23.92±0.33	23.4± 0.08	8.17± 0.02	66.27±0.82	0.75± 0.02	0.175±0.02	0.7± 0.005
Ethanol 50%	1.00±0.1	67.67±0.45	27.60±0.08	40.80± 0.45	84.22±0.63	1.0± 0.12	0.075±0.01	3.15±0.09
Acetone 50%	1.27±0.04	105.12±1.20	27.95±0.08	50.76± 0.23	83.8± 0.69	1.5± 0.08	0.1±0.0008	2.325±0.23
Ethanol	1.0± 0.01	262± 0.58	27.07±0.04	12.75± 0.24	101.77±1.1	1.075±0.07	0.25±0.01	0.25±0.01

The lowest content of iron is found in ethyl-acetate extract (3.75 μg g^{-1}). The highest quantity of Fe is determined in aqueous (44.37 μg g^{-1}) and methanol (41.7 μg g^{-1}) solution. The recommended daily intake of Fe goes from 0.27 mg for infants, 10 mg for children from 4-8 years, 11 mg for adolescents, up to 8 mg for men and 18 mg for women.

The content of manganese is also the lowest in the ethyl-acetate extract (2.25 μg g^{-1}), while it is the highest in acetone 50 % extract (14.05 μg g^{-1}). The recommended daily intake of Mn goes from 0.003 mg for infants, 1.5 mg for children from 4-8 years, 2.2 mg for adolescents to 2.3 mg for men and 1.8 mg for women. During the pregnancy, the recommended daily intake of Mn is 2.0 mg.

The content of zinc goes from 8.17 μg g^{-1} to 77.45 μg g^{-1} in ethyl-acetate and methanol extracts, respectively. The recommended daily intake of Zn goes from 2 mg for infants, 5 mg for children from 4-8 years, 11 mg for adolescents, 11 mg for men and 8 mg for women. During pregnancy, the recommended daily intake of Zn is 11 mg (Trumbo *et al.*, 2001).

As in the previous cases, the content of copper is the lowest in the ethyl-acetate extract (2.33 μg g^{-1}), and the highest in acetone 50 % extract (21.6 μg g^{-1}). The recommended daily intake of Cu goes from 0.2 mg for infants, 0.44 mg for children from 4-8 years, 0.89 mg for adolescents to 0.90 mg for men and women. During pregnancy, the recommended daily intake of Cu is 1.0 mg (Trumbo *et al.*, 2001).

Plants can be easily contaminated with heavy metals during the cultivation or later during the processing, and because of this, the determination of the content of heavy metals is of high importance. The presence of heavy metals in high quantities in the body can have a toxic effect (Jabeen *et al.*, 2010; Khan *et al.*, 2008; Sharma *et al.*, 2009). Heavy metals (Pb, Cd, Cu, Cr and others), as pollutants at work and in the environment are serious health and ecological problems, because they are toxic, they are not biodegradable, and they have a long half-time in the soil. The content of heavy metals is one of the criteria for the use of plant material in the production of traditional medicines. Heavy metals have considerable toxicity for humans, animals, microorganisms, and plants (WHO, 1998). Lead and cadmium are very harmful elements for the human body, especially in high concentrations (Fotakis and Timbrell, 2006).

Different factors, such as exhaust fumes, industrial waste, increase the content of heavy metals in fruits and other edible parts of the plants. The greatest pollutant of areas beside roads is exhaust fumes (Hamurcu *et al.*, 2010). Lead, cadmium, and arsenic are heavy metals which are the most frequent contaminants of food. Conditionally accepted weekly intake for the toxic metals upon the recommendation of FAO/WHO is 0.025 for lead, 0.007 for cadmium, 0.015 μg g^{-1} for arsenic, for adults (WHO, 1986; 1989).

Therefore, it is very important to know the coefficient of the extraction of metals from plants using various solvents, especially water, because the plants are usually consumed in the form of teas. The coefficients of metal extraction from the plant with different solvents are shown in Figures 1 and 2.

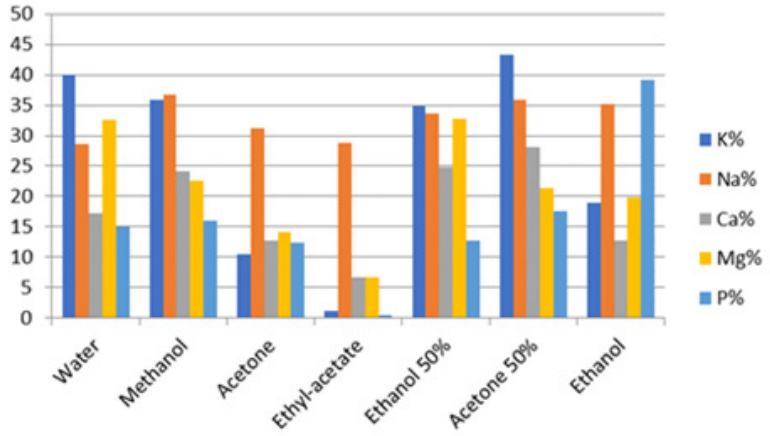

Figure 1. Coefficients of the extraction of macroelements using different solvents

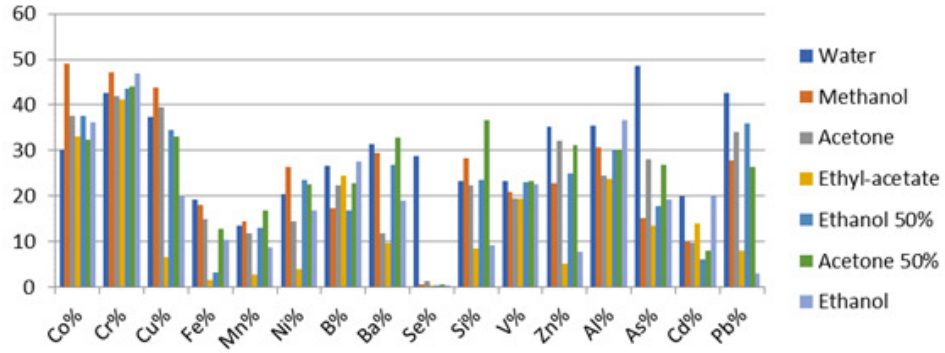

Figure 2. Coefficients of the extraction of microelements using different solvents

Maximal allowed values of heavy metals in food are regulated by the Policy on the quantities of pesticides, metals, metalloids, and other poisonous substances, hemotherapeutics, anabolics and other substances which can be found in foodstuff.

Allowed concentrations of Pb in tea go from 2-5 μg g^{-1}, As 1 μg g^{-1}. For the rest of toxic metals, there is no determined normative. In oven-treated sample, the content of cadmium is the lowest (1.25 μg g^{-1}), and the content of lead is the highest (8.8 μg g^{-1}), while the content of arsenic is somewhat lower, and it is equal to 5.6 μg g^{-1}. The content of lead is smaller than the maximal allowed value in the tea; however, the content of As, although lower than the content of lead, exceeds the maximal allowed values.

In the dried petals of the plant, the highest content of arsenic is in aqueous extract (2.72 μg g^{-1}), then it goes lead with the highest concentration in the aqueous extract (3.75 μg g^{-1}), while the content of cadmium is equally abundant in aqueous and ethanol extracts (0.25 μg g^{-1}). On the basis of these results, it was shown that

the aqueous extracts contain the highest content of heavy metals. It is very useful information when it is known that the plant species are the most frequently consumed in the form of teas.

The investigation conducted by Mihaljev *et al.* (2015) on the determination of heavy metals in dry samples of herbal teas, also show that the content of heavy metals in our samples is higher than theirs in most samples. Literature data on the concentration of heavy metals in aromatic herbs (pepper, basil, oregano, ground red pepper, parsley, rosemary) show that the content of chromium in our samples is lower compared to those data, while the content of copper in some cases crosses a little bit the limits. The content of the lead is mostly within the permitted limits (WHO, 1989).

PCA and AHC analyses of macroelements

Kolmogorov-Smirnov test (the significance level α was 0.05) was used to evaluate the normality of the distribution of concentrations of each investigated element. The test showed that the original data set was normally distributed, so they were used for further analysis. Before PCA analysis, the data were checked for outliers. Applied Grubbs test to the experimental data, resulted in the detection of one outlier in all cases (the critical value for α=0.05 and n=7 was 2.020). The outliers were removed from PCA analysis (Grubbs, 1969).

PCA showed the presence of two components exceeding one (3.086 and 1.141) explaining 84.554 % (Table 5). To get better information about the data, the correlation matrix was subjected to rotation with Kaiser normalization (Kaiser, 1960).

Table 5. Factor loadings after rotation for macroelements

	D1	D2
K	0.903	-0.410
Na	-0.004	0.999
Ca	0.999	0.036
Mg	0.604	-0.793
P	-0.973	0.210

The first factor explains the largest proportion of variance (62.498%). The representatives of this factor are potassium, calcium and phosphorus with high loading values, magnesium with moderate, and sodium with low value of the loading. Except sodium and phosphorus, all the elements have positive values in this factor. The key variables in the second factor are sodium and magnesium. This second factor is responsible for 36.793% of the total variance. The analysis shows that when higher quantities of K and Ca are present, the quantity of phosphorus is lower, and *vice versa*. Sodium and magnesium are also correlated in such a way that when the quantity of sodium is high, the quantity of magnesium is low, and *vice versa*. These data gave us information about the connections between elements in the metabolic pathways of the plant.

AHC analysis of the variables using the Ward method and the squared Euclidean distance was performed. The dendrogram of the samples is presented in Figure 3. Cluster I consist of Z1 (oven-treated sample), cluster II of Z2 (aqueous extract), Z3 (methanol extract), Z6 (ethanol 50% extract) and Z7 (acetone 50% extract). Cluster III consists of Z4 (acetone extract) and Z5 (ethyl-acetate extract), and cluster IV of Z8 (ethanol extract). Obviously, clustering is based on the polarity of the used solvent systems. Cluster I of course represent only oven sample as no solvent was used. Cluster II consists of extracts of polar solvent systems. Cluster III represents the extracts with the least polar solvent systems used. Therefore, AHC represents good tool for the differentiation of the investigated samples based on the macroelements.

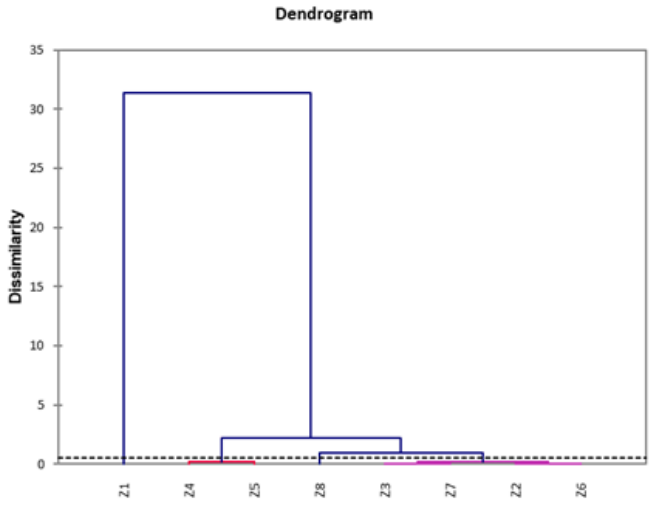

Figure 3. Dendrogram obtained after AHC analysis for macroelements

PCA and AHC analyses of microelements

Kolmogorov-Smirnov test (the significance level α was 0.05) was used to evaluate the normality of the distribution of concentrations of each investigated element. The test showed that the original data set was not normally distributed, so they were transformed using the sinus function in order to get the data showing the normal distribution. Before PCA analysis, the data were checked for outliers. Applied Grubbs test (Grubbs, 1969) to experimental data, resulted in the detection of one outlier in case of Co, Cr, As and Cd (the critical value for α=0.05 and n=7 was 2.020), and no outliers in all other cases (the critical value for α=0.05 and n=8 was 2.127). The outliers were removed from PCA analysis.

PCA showed the presence of five components exceeding one (5.796, 3.438, 2.510, 2.254, and 1.335) explaining 95.839%. Based on the Kaiser criterion, five components should be used for the explanation of variances. The contribution of the first component is 36.223%, the second 21.489%, the third 15.690%, the fourth 14.090%, and the fifth component 8.347%. To get better information about the data, the correlation matrix was subjected to rotation with Kaiser normalization (Table 6).

Table 6. Factor loadings after rotation for microelements

	D1	D2	D3	D4
Co	-0.413	-0.039	0.009	0.885
Cr	0.929	-0.203	0.051	-0.280
Cu	0.596	-0.187	0.755	-0.087
Fe	0.168	-0.930	-0.123	-0.146
Mn	0.034	0.994	-0.052	-0.012
Ni	0.704	0.035	0.622	-0.226
B	-0.171	0.201	0.148	0.891
Ba	0.961	0.171	0.067	-0.199
Se	-0.744	0.396	-0.372	0.383
Si	0.080	-0.032	0.962	0.179
V	-0.987	0.055	-0.077	-0.024
Zn	0.826	-0.064	0.182	-0.514
Al	-0.730	0.500	-0.304	0.191
As	-0.210	0.490	-0.216	0.755
Cd	0.094	-0.004	0.723	-0.673
Pb	-0.071	0.747	-0.378	0.511

The first factor explains the largest proportion of variance (35.463%). The representatives of this factor are chromium, nickel, barium, selenium, vanadium, aluminum, and zinc with high loading value; cobalt, and copper with moderate; iron, manganese, boron, silicon, arsenic, cadmium, and lead with low value of the loading. Except cobalt, boron, selenium, vanadium, aluminum, arsenic, and lead, all the elements have positive values in this factor. The key variables in the second factor are iron and manganese. This second factor is responsible for 20.094% of the total variance, the third 18.206%, and the fourth 22.129%. This analysis shows that when higher quantities of Cr and Ba are present, the lower quantities of V are present, and *vice versa*. Based on the obtained results, it is obvious that toxic non-essential element-Ba can be incorporated into the plant metabolism having an influence on the concentration of V, and that Cr and V are connected within plant metabolism.

AHC analysis of variables was performed using Ward method and the squared Euclidean distance. The obtained dendrogram is presented in Figure 4.

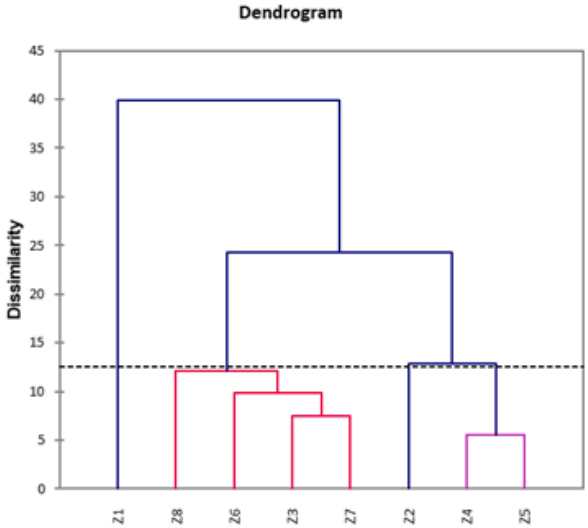

Figure 4. The obtained dendrogram after the performed AHC analysis for microelements

Cluster I consists again of only Z1 (oven-treated sample), cluster II of Z2 (aqueous extract), cluster III of Z3 (methanol extract), Z6 (ethanol 50 % extract), Z7 (acetone 50 % extract) and Z8 (ethanol extract). At the end, cluster IV consists of Z4 (acetone extract) and Z5 (ethyl-acetate extract). Again, as in the case of macroelements, clustering is based on the solvent system used with the difference in Z8 (ethanol extract).

Conclusions

Balkan forsythia (*Forsythia europaea* Degen & Bald.) is an endemic plant not investigated enough. The determination shows that aqueous extracts contain the highest quantity of elements, which is especially important, because the plants are usually consumed in the form of teas. The contents of toxic elements are significantly lower than the permitted values. Statistical analysis, such as PCA and AHC, are useful tools for the grouping of samples, and relations between investigated elements. Due to the reason that this plant is a good source of polyphenols and minerals, it can act anti-inflammatory. Our future studies will be directed towards *in vitro* investigation of the various extracts of Balkan forsythia.

Authors' Contributions

Conceptualization: D.K., B.B.A.; Data curation: D.K., B.B.A., S.T., A.P.; Formal analysis: B.B.A, S.T., A.P., M.T.; Methodology: D.K., B.B.A., S.T., A.P., G.S.; Supervision: D.K.; Validation: D.K., B.B.A., S.T., A.P., G.S.; Writing - original draft: D.K., B.B.A.; Writing - review and editing: D.K., B.B.A., S.T., A.P., E.P.M., M.T., G.S. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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