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Plant species from the vicinity of an abandoned As-Sb-Tl Allchar mine, Kožuf Mountains, with special reference to the bioavailability of endemic species – A review

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Abstract

The Allchar mine, located in the southern part of North Macedonia, has a unique mineral composition. This locality is world-famous for its thallium minerals, but is also known for its large number of arsenic and antimony minerals. The rare plant species found at the Allchar locality, are of particular interest to scientists working on this topic worldwide. The following plant species described from the Allchar locality which are local endemics (stenoendemics) for this area, are of particular importance: *Odontarrhena kavadarcensis* (Syn. *Alyssum kavadarcensis*), *Centaurea kavadarensis*, *Centaurea leucomalla*, *Galium kerneri*, *Knautia caroli-rechingeri*, *Onobrychis degenii*, *Thymus allchariensis*, *Viola alsharensis*, *Viola arsenica*, *and Viola* × *halacsyana*. Rare Balkan endemic or relict species are also present in this locality, such as *Alkanna noneiformis*, *Alkanna pulmonaria*, *Centaurea grbavacensis*, *Eryngium serbicum*, *Melampyrum heracleoticum*, *Ramonda nathaliae* and other, while species from Allchar that are on the CORINE list of Macedonia are *Viola arsenica* and *Ramonda nathaliae*. Hyperaccumulator plants are able to tolerate extremely high concentrations of metals/metalloids in the soil in which they grow and accumulate high concentrations in the plant organs (roots, shoots and their leaves). Due to the specificity and enriched content of these potentially toxic elements in the soil, the above-mentioned local endemic plant species grow at the Allchar locality. The aim of this work is to give an overview of the results of the studies on these endemic plants and on the bioaccumulation ability of certain potentially toxic elements (mainly As, Sb and Tl) in relation to their mobility in the endemic plant species. The studies were initiated to determine the uptake and distribution of arsenic, antimony, thallium, and other potentially toxic elements in different plant parts (roots, stems, leaves, flowers, and seeds) of these endemic species.

Keywords: Allchar locality, endemic plant species, bioaccumulation, arsenic, antimony, thallium

Introduction

Contamination of soils with toxic elements poses a serious threat to environmental quality and human health. While natural sources such as weathering of parent material and volcanic emissions contribute to soil pollution, anthropogenic activities, including industrialization and mining, are the main causes of soil pollution (Alloway 1995). Soil toxicity has become a worldwide problem due to increasing pollution from industrial and mining activities, which are often carried out without adequate pollution control measures.

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These activities release toxic metal(loid) residues into the environment that affect soil stability and pose risks to human health.

Certain plant species have developed mechanisms to accumulate both essential and non-essential metals/metalloids in their roots and shoots, often exceeding soil concentrations. These metal(loid)-accumulating plants, which are typically found in the soils (substrate) rich in metals(loids) such as ultramafic or gipsy substrates around the world, play a crucial role in phytoremediation, a technology in which plants are used to remediate polluted soils. Plants that thrive on substrates rich in metals and/or metalloids without showing significant toxicity symptoms are called metallophytes. They can be obligate or facultative, depending on whether they also occur on metal(loid)-poor substrates. Metallophytes can be excluders, indicators or accumulators, depending on their strategy to tolerate excessive trace elements. Hyperaccumulators are the extreme accumulators, capable of accumulating certain potentially toxic elements in their leaves at concentrations above threshold levels without showing symptoms of toxicity. These thresholds are element-specific and have recently been revised (Baker & Brooks 1989; van der Ent et al. 2013; van der Ent et al. 2015a; Reeves et al. 2018).

The physiological mechanisms underlying the accumulation of metals(loids) in plants include extracellular and intracellular chelation of metals, precipitation and translocation to the vascular system. Phytoremediation is a promising way to mitigate soil contamination, but detailed studies of soils and vegetation are essential to assess the impact of toxicity on herbivores and human health (Peer et al. 2006; Jimenez et al. 2013; Słomka et al., 2015, 2017, 2018).

Different plant species respond differently to metal(loid) contamination depending on their ability to take up or excrete different elements into their shoots during normal growth and reproduction. Recultivating areas contaminated with metals with native plant species helps stabilize soils and reduces the risk of metals spreading through erosion. Understanding the physiology of metal(loid) accumulation in plants is crucial. Studies have shown that metal ions are absorbed from the soil by the roots and transported into the stem tissue before being stored in the leaves. The composition of different plant species in different areas is influenced by the soil type and the underlying geology.

Among the most toxic elements released by mining are antimony, arsenic and lead, which often occur together in ore minerals. These elements have adverse effects on human health, and their accumulation in plants can lead to harmful effects when consumed. Further studies are needed to understand the environmental and health effects of metal(loid) contaminants and to develop appropriate remedial measures (Winship 1987; Lockitch 1993; Mandal & Suzuki 2002; Sundar & Chakravarty 2010). Studies have investigated the extraction and accumulation of toxic elements in plants, with extraction depending on various factors such as soil properties, plant species, and age and exposure time. For example, Viola species from the Allchar mine have been identified as arsenic accumulators, showing potential for phytoremediation (Stevanović et al. 2010).

Hyperaccumulation of thallium has been observed, albeit rarely, in certain plant taxa, including *Iberis* and *Biscutella* species in southern France and *Viola* species near the Allchar mine in North Macedonia. Further research is needed to confirm its status as a hyperaccumulator and to understand its ecological impact (LaCoste et al. 1999; Leblanc et al. 1999; Corzo Remigio et al. 2022; Bačeva et al. 2014, Jakovljević et al. 2023, 2024).

The aim of this work is to provide an overview of (i) the results of the studies of endemic plant species from the area of the abundant As-Sb-Tl mine Allchar on Kožuf Mountain, North Macedonia (Jovanovski et al. 2018) and (ii) the results of the studies on the bioaccumulation capacity of certain potentially toxic elements (mainly As, Sb and Tl) in relation to their mobility in seven endemic plant species *Viola allchariensis* G. Beck, *V. arsenica* G. Beck, *V. macedonica* Boiss. & Heldr. (Bačeva et al. 2014a), *Thymus alsarensis* Ronn. (Bačeva et al. 2016), *Centaurea leucomalla* Bornm (Bačeva et al. 2018a) *Onobrychis degenii* Dörfler, *Knautia caroli-rechingeri* Micev. and *Centaurea kavadarensis* Micev. (Bačeva et al. 2018b).

Material and methods

Study area

The Allchar locality is a volcanogenic hydrothermal mineral deposit in the northwestern part of the Kožuf Mountains in North Macedonia, near the border with Greece (Figure 1). Geologically and geotectonically, the ore mineralization at Allchar is associated with a Pliocene volcanic intrusive complex (Janković et al. 1997; Volkov et al. 2006; Boev & Jelenković 2012; Amthauer et al. 2012).

The geological formations at the Allchar locality include a complex of Precambrian metamorphic rocks, a complex of Mesozoic rocks, a complex of Upper Cretaceous sedimentary rocks, a complex of Pliocene sediments, pyroclastites and volcanic rocks, and a complex of Quaternary sediments. Several ore bodies are present within a zone about two kilometers long and 300–500 meters wide. The most important ore minerals found in this area include iron sulfides, arsenic and thallium-bearing minerals, cinnabarite and arsenic, lead- and antimony-bearing sulphosalts (Balić Žunić et al. 1993; Boev et al. 1993, 2001–2002; Volkov et al. 2006; Jelenković & Boev 2011). As a result of mineralization, the entire area, which extends over several square

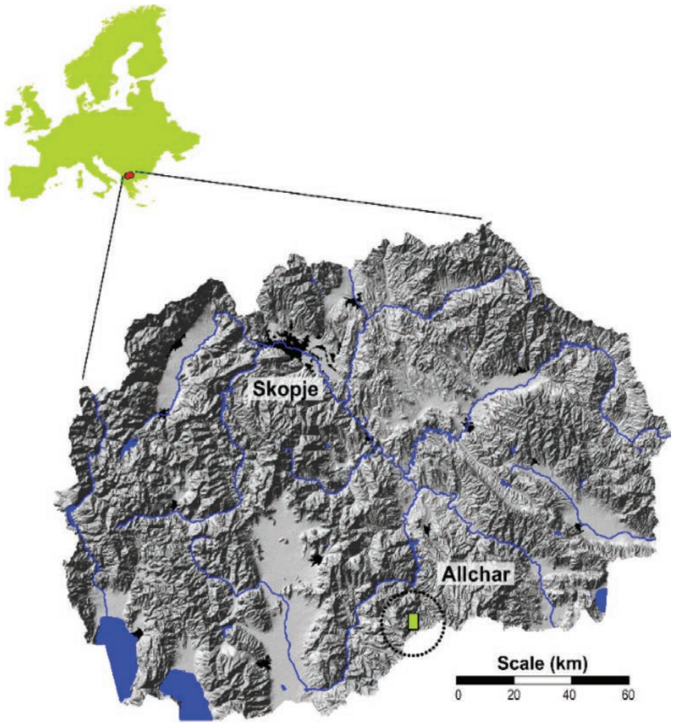

Figure 1. The location of the Allchar mine

kilometers, is enriched with toxic elements such as arsenic, thallium and antimony.

The overview of the flora studies at the Allchar locality (Jovanovski et al. 2018)

The territory of North Macedonia has a diverse flora that includes more than 3,200 species of vascular plants belonging to different plant associations, alliances, orders and classes. This region is one of the most attractive areas of the Balkan Peninsula and is home to numerous relicts and a large number of endemic plant species. This richness is primarily the result of geological, tectonic, climatic and other changes that have affected the Balkan Peninsula throughout history, especially from the Tertiary period to the present, as well as plant migrations throughout history. Research into the diversity and formation of the flora of North Macedonia began in the beginning of 19th century with the work of the German botanist and phytogeographer August Heinrich Rudolph Grisebach in his famous publication “Spicilegium florae rumelicae et bithynicae” I-II (Grisebach 1843–1844). Since then, Macedonia has attracted the attention of many European botanists, leading to the discovery of an immense number of new plant species and phytocoenoses that were previously unknown to science.

One of the sites investigated in this research was Allchar and its surroundings, which were first studied by Formánek and Dörfler. During his floristic studies of the Balkans between 1890–1900, the Czech botanist Formánek (1894a, 1894b) published several papers on the flora of parts of the Balkan Peninsula, including Macedonia. These treatises contain data on the Kožuf Mountains (Dudica-Konopište, Mrežičko and Roždeń). His herbarium collection was additionally reviewed and supplemented by Vandas (1909). In 1890 and 1893, Ignac Dörfler from the Botanical Garden and Museum in Vienna also examined the flora of Macedonia. The herbarium material collected at that time was later studied and published by Wettstein (1892), Beck (1894), Degen and Dörfler (1897) and Ronniger (1924). Beck (1894) published an article on the three new species found in Allchar – *Viola arsenica*, *V. allchariensis* (Figure 2) and *V.* × *halacsiana* (*V. allchariensis* × *V. arsenica*), all of which are now botanical hallmarks of the territory. Degen & Dörfler (1897) published three new species, previously unknown to science, as, such as *Onobrychis degenii*, *Galium kerneri*, and *Campanula formanekiana*. There are also detailed illustrations and descriptions of the previously described violet species – *Viola arsenica*, *V. allchariensis* and *V.* × *halacsiana* Ronniger (1924) described the Allchar wild thyme – *Thymus alsarensis*.

At the beginning of the 20th century and in the first three decades, the following data on rare species from the Allchar locality were published: Borbás (1902) on the species *Hesperis theophrasti*, Degranc (1902) on the species *Daphne blagayana*, Domin (1904) on *Koeleria glaucovirens*, Handel-Mazzeti (1909) on *Onobrychis pindicola*, Hayek (1920) on *Tragopogon pratensis* subsp. *hayekii*, Košanin (1921) on *Ramonda nathaliae* (Figure 3), Herzog (1922) on *Thesium linophyllon*, etc.

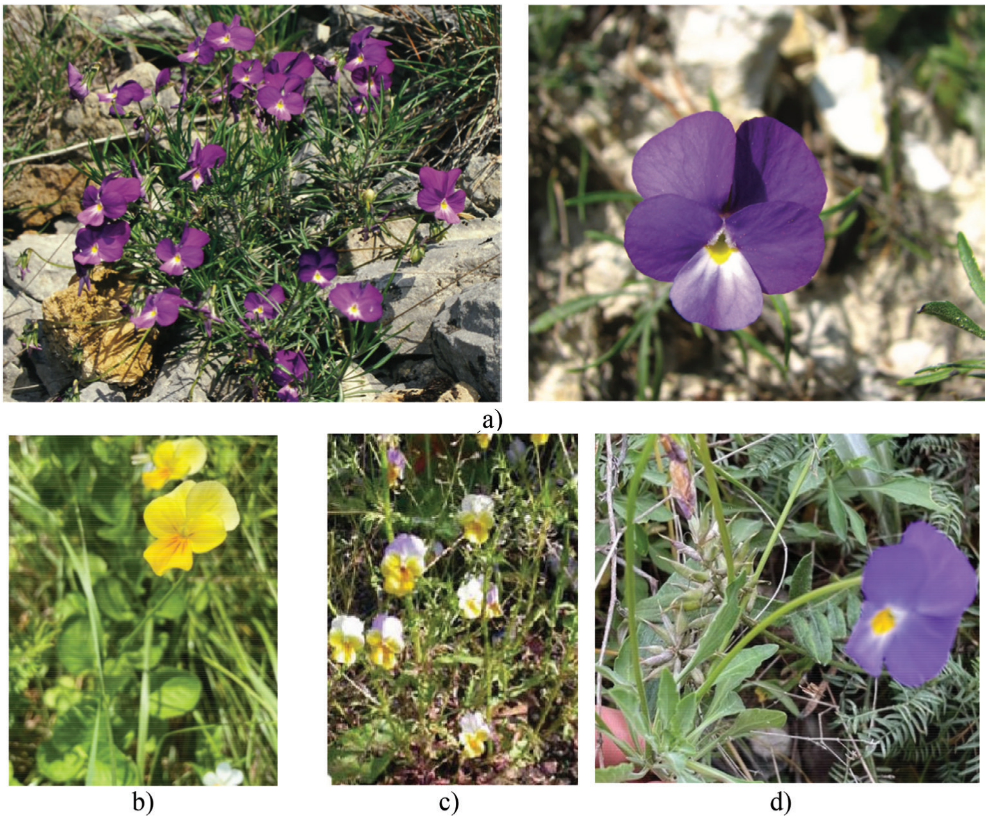

Figure 2. *Viola allchariensis* G. Beck (a), *V. arsenica* G. Beck (b) and *V. tricolor* subsp. *macedonica* Boiss. & Heldr. (c), V. x. *halacsyana* (*V. allchariensis* x *V. arsenica*) (d)

As far as the flora of Macedonia is concerned, the studies of the important German botanist Josef Bornmüller are particularly important. He described more than 70 new taxa whose type locality (locus classicus) lies in the territory of North Macedonia. During his field research, which took place between 1917 and 1918, he visited a large number of places on the Macedonian territory and consequently collected a rich herbarium. Bornmüller (1921, 1925, 1926, 1928, 1932) had published data on the Allchar locality, including the newly discovered species *Centaurea leucomala* (Figure 4a), the holotype of which belong from this locality.

Soška (1933, 1938/1939) confirms and expands the findings of Degen & Dörfler (1897) and Bornmüller (1921, 1925, 1926, 1928, 1932) on Allchar in his work on the gorges in Macedonia. More other important studies at

Figure 3. *Ramonda nathaliae*

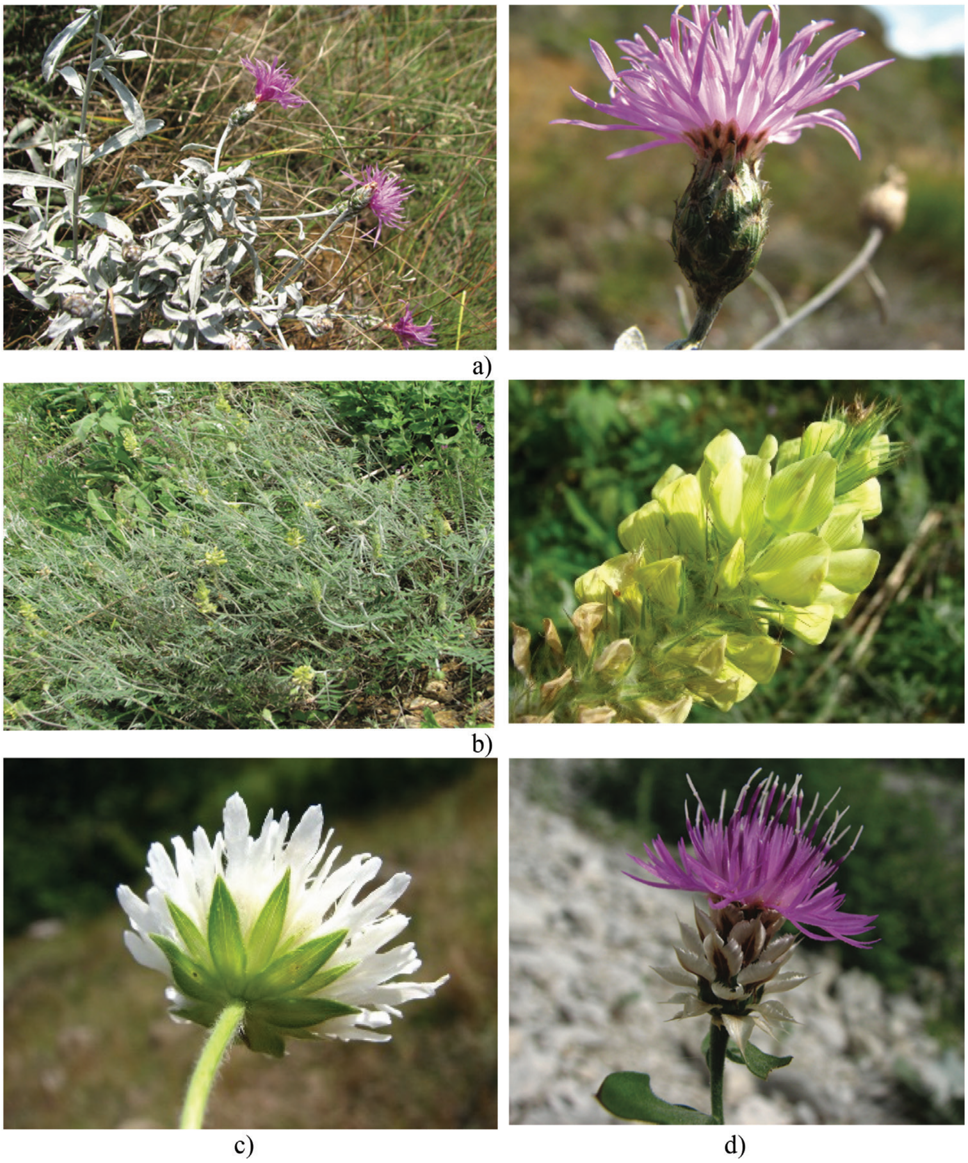

Figure 4. *Centaurea leucomalla* Bornm. (a), *Onobrychis degenii* (b), *Knautia caroli-rechingeri* (c) and *Centaurea kavadarensis* (d)

Allchar includes the work of Achtaroff and Lindtner (1940) on the species *Centaurea grisebachii*, Rechinger (1941) on *Salvia verticillata* var. *longipilosa,* Pulević (1979) on *Crocus veluchensis*, and Lakušić & Grgić (1971) on *Gymnadenia friwaldii*.

The interest of botanists for this region has not waned at all to date. Micevski (1981, 1985, 1990, 1993, 1995, 1998, 2001), Micevski and Matevski (1987, 2000, 2005) and Matevski (2009, 2010, 2013) provide a wealth of data on Allchar and give a review of all data obtained from the territory of Macedonia, including data on Allchar and its surroundings. Thus, three new species have been described for science: *Knautia caroli-rechingeri* (Figure 4c), *Centaurea kavadarensis* (Figure 4d) and *Odontarrhena kavadarcensis* (syn. *Alyssum kavadarcense*) from this site (Micevski, 1981, 1990).

Endemic plants from Allchar

Endemism is a phenomenon in which a unique biological diversity occurs in a specific area. The flora of Macedonia is rich in Balkan endemics, which have different origins from a floro-genetic point of view. They can be found in regions throughout the Balkan Peninsula.

According to Micevski and Matevski (1987), Allchar has the greatest importance, as several newly discovered species [*Odontarrhena kavadarcensis* (syn. *Alyssum kavadarcensis*), *Centaurea kavadarensis* (Figure 4d), *Centaurea leucomalla* (Figure 4a), *Knautia caroli-rechingeri* (Figure 4c), *Viola allcharensis* (Figure 2a), *Viola arsenica* (Figure 2b), *Viola* × *halacsyana* (Figure 2d)] which are local endemic species (stenoendemics), characterized by a very low chorological capacity and

Figure 5. *Campanula formanekiana* (a) and *Galium kerneri* (b)

Figure 6. *Thymus alsarensis*

a low ecological value. Therefore, their distribution is very limited.

Allchar and its surroundings are characterized by the locus classicus of some other species described in this region: *Asyneuma canescens* subsp. *cordifolium, Campanula formanekiana* (Figure 5a), *Galium kerneri* (Figure 5b), *Hesperis theophrasti, Onobrychis degenii* (Figure 4b), *Thymus alsarensis* (Figure 6), which are, however, more widespread in the territory of North Macedonia as well as in some countries of the Balkan Peninsula.

The extended area around Allchar is also distinguished for the high diversity of flora and vegetation units. The most important floristic sites in this region are the following:

The surroundings of the village of Majdan – Allchar, Crven Dol. From a botanical point of view, this is the most important site in this area, where mining studies were carried out in the past, as well as the exploitation of antimony ore from the Allchar mine. Due to the long-term cessation of mining activities in this area, the main plant species near the village of Majdan – Allchar and Crven Dol are characterized by relatively high biological vitality. The resumption of mining activities in this region could definitely have a negative impact on the local endemic species in this area, threatening in particular the following species: *Viola allchariensis*, *V. arsenica*, *V. × halacsyana*, *Centaurea leucomalla*, *Knautia caroli-rechingeri* and *Onobrychis degenii*, whose populations thrive in the immediate vicinity of the abandoned mines, where the growing habitats of these species are located. Modern concepts for the protection of globally and locally important but endangered plant species today pay special attention to the protection of the type localities (classic sites), because in this way the original populations of the species, i.e. the original genetic material that served as the basis for the description of a particular species or other lower taxon, are preserved and protected.

Special protection measures must therefore be taken, especially in the Allchar and Crven Dol regions, where there are still heavily degraded habitats around the old mining pits and their surroundings, but in the vicinity of which occurs the huge populations of endemic species described from this area, such as *Asyneuma canescens* subsp. *cordifolium, Centaurea leucomalla, Hesperis theophrasti, Knautia caroli-rechingeri, Onobrychis degenii, Thymus alsarensis, Viola allchariensis, V. arsenica, V. × halacsyana* (Figure 2), as well as other important species with community interest.

The gorge on the Majdanska River (Mrežičko-Roždeн-Majdan). The authentic appearance of this gorge has been significantly affected by the construction of the road to the Ržanovo mine and the road to the villages of Rožden and Majdan. Heavy vehicle traffic for the needs of the Ržanovo mine, where intensive construction and mining activities took place for a long time, as well as activities related to the needs of the Kavadarci forestry economy pose an additional threat to the species thriving in this region, which is the locus classicus for the species *Odontarrhena kavadarcensis* (syn. *Alyssum kavadarcense*), *Centaurea kavadarensis* and *Galium kerneri*.

Plant species which are floristic rarities for this region and for the territory of North Macedonia in general are *Astragalus monspessulanus, Campanula formanekiana, Hypericum cerastioides, Ophioglossum vulgatum, Ranunculus lateriflorus, Saxifraga grisebachii* and others).

The area between the village of Majdan and mountain pass Tribor is one of the best-preserved forest areas with black (*Pinus nigra*) and white (*Pinus sylvestris*) pines in North Macedonia. There are quite well preserved beech and fir-beech forests here. Sustainable management of these forests will lead to the preservation of the vital forest potential of this region, which must be considered when preparing future forest management plans for this region and for the wider area of the Mariovo region. Particularly important species in this area are *Pinus nigra, Pinus sylvestris, Abies × borisii-regis, Daphne blagayana, D. laureola, Hypericum cerastioides, H. hirsutum, Festuca gigantea, Veronica urticifolia, Chenopodium foliosum, Ranunculus ophioglossifolius, Paris quadrifolia, Gentiana lutea* subsp. *symphyandra, Anemone ranunculoides, Laser trilobum* and others.

Study species

Seven local endemic plant species were collected in the locality of Allchar: *Viola allchariensis* G. Beck, *V. arsenica* G. Beck, *V. macedonica* Boiss. & Heldr. (Bačeva et al. 2014a), *Thymus alsarensis* Ronn. (Bačeva et al. 2016), *Centaurea leucomalla* Bornm (Bačeva, 2018a), *Onobrychis degenii* Dörfler, *Knautia caroli-rechingeri*, and *Centaurea kavadarensis* (Bačeva et al. 2018b).

Three *Viola* species occur at the same locality, near the abandoned As-Sb-Tl mine of Allchar (Figure 2). These species are part of the section *Melanium* Ging. within the family *Violaceae*. Erben (1985) documented 50 species of sect. *Melanium* in south-eastern Europe, of which 28 species occur in North Macedonia. *V. arsenica* and *V. allchariensis* occur exclusively at the Allchar locality and are considered as local endemics, while *V. tricolor* subsp. *macedonica* is distributed across the central and southern Balkans and is classified as a member of the Balkan endemic group (Micevski, 1995). Another *Viola* taxon, *Viola × halacsyana*, is present at the Allchar site (Figure 2d). It is a hybrid of *V. alshariensis* and *V. arsenica* and is not always easy to find.

The genus *Thymus* is one of the most polymorphic genera in the *Lamiaceae* family. The species within this genus exhibit remarkable polymorphism and are characterized by the presence of numerous subspecies, varieties, and forms. The *Thymus* flora is particularly rich and diverse on the territory of North Macedonia (Matevski 1991). *Thymus alsarensis* Ronn. (Figure 6) which belongs to the genus *Thymus* L., sect. *Marginati* (A. Kerner) A. Kerner Subsect. Verticillati (Klok. ET Shots.) Menisci, is an endemic aromatic and medicinal plant species found in the Allchar locality (Runnier 1924). Its holotype is conserved in the Herbarium of the Natural History Museum in Vienna. Wild thyme is used for its expectorant, antiseptic, antispasmodic, and anthelmintic properties (Kulevanova et al. 1996).

The species *Centaurea leucomalla* Bornm. grows near the abandoned Allchar As–Sb–Tl mine. *Centaurea leucomalla* Bornm. (family Compositae, section *Phalolepis*) was described at the beginning of the 20th century by Bornmüller (1921), who at that time was one of the leading botanist on the flora of the southern Balkan Peninsula. This autochthonous plant species grows exclusively at the Allchar locality and is considered a local endemic species (Figure 4a). *Centaurea leucomalla* Bornm. is regarded as taxonomically stable and is accepted in the contemporary floristic literature (Euro+Med Plant Base, https://www.emplantbase.org/home.html).

Onobrychis degenii (Figure 4b) occurs exclusively in the Allchar locality near the village of Majdan. While it can be considered a Macedonian (sub)endemic species, it is also mentioned in the floristic literature for Bulgaria (Kozhukharov, 1976, 1992).

Knautia caroli-rechingeri (Figure 4c) is a local endemic plant species from Macedonia, described by Micevski (1981) and discovered only near the village of Majdan locality of Allchar area, which serves as locus classicus. This plant occupies an isolated position compared to other species of the genus *Knautia* in North Macedonia and is taxonomically closer to the species *Knautia dinarica*. At present, this species is known to occur in only two places near the village of Majdan.

Centaurea kavadarensis (Figure 4d) belongs to the *Centaurea deusta* group and is a local endemic plant species occurring near the village of Majdan in the locality of Allchar (Micevski 1987).

Soil and plant collection and sample preparation

Plant and soil samples were collected in the summer of 2011 at the Majdan locality around the abandoned Allchar mine, as described above. Soil samples were selected according to the presence of endemic plant species. Soil samples (5-10 replicates representing composite material from one sample for analysis) were collected from the rhizosphere of plant specimens at each study site. *Viola* species were collected from six sampling sites, and plant and soil samples were collected from the vicinity of the abandoned mine as described above. It should be noted that only three samples had seeds at the time the plant samples were collected. *Thymus* species and soil samples were collected from 14 locations in the study area, while samples of *Centaurea leucomalla* species were collected from eight sampling points. In summer 2011, the species *Onobrychis degenii, Knautia caroli-rechingeri* and *Centaurea kavadarensis* were collected at one sampling site.

The soil samples were dried at room temperature for five to seven days. After foreign material (stones, plants) had been removed, the soil samples were sieved using a plastic sieve with a mesh size of 2 mm. A certain amount of the sieved material was ground in an agate mill until a powder with a particle size of 0.125 mm was obtained (Salminen et al. 2005). The prepared samples were then collected in polyethylene bags and prepared for chemical analysis. Digestion of the soil samples was carried out in a PTFE digestion vessel and digestion was carried out on a hot plate at about 100°C. The digestion was carried out with HNO_3, HF, $HClO_4$ and HCl. The digested solution was quantitatively transferred to a 25 ml volumetric flask and then analyzed (Bačeva et al. 2016).

The plant material was collected based on the presence of endemic species, with 10–20 plant specimens representing one composite sample for analysis). The entire plant was carefully washed with distilled water to remove accumulated impurities from the soil and then separated into root, stem, leaves and flower parts. The plant material was dried at room temperature for seven to fourteen days until a constant mass was achieved, then pulverized, homogenized and prepared for complete digestion. For chemical analysis, a certain mass (0.5000 g) of each plant sample was weighed into a PTFE container and digested in a microwave digestion system with concentrated HNO_3 and H_2O_2 (Bačeva et al. 2016).

The bioavailability of the analyzed elements was investigated using soil extraction methods. Soil extraction with water was used to obtain information on the actual availability of elements from the soil solution (Pansu & Gautheyrou, 2006). In addition, sequestration reagents for selective solubilization, such as DTPA-$CaCl_2$-TEA, were used (Risser & Baker 1990; Pansu & Gautheyrou 2006). Acidic reagents were also used to displace potentially available forms that are not easily extracted (Risser & Baker 1990; Pansu & Gautheyrou 2006), using a solution of 0.1 M HCl. These extractions allowed us to determine the content of plant-available elements and the conditions in living plants.

Thus, three different extraction solutions were used to determine the degree of extraction (bioavailability) of the analyzed elements from the soil samples (Pansu and Gautheyrou, 2006):

- Extraction with water: this extraction simulated the natural conditions for the extraction of toxic elements from the soil to determine which elements can be extracted from the soil under normal precipitation conditions.
- Extraction with diethylenetriamine pentaacetic acid-calcium chloride-triethanolamine reagent (DTPA-$CaCl_2$-TEA):

Table 1. The content of As, Sb and Tl in different parts of the *Viola species*. Values are given in mg/kg. (Bačeva et al. 2014a)

Element	Root		Steam		Leaf		Flower		Seed	
Viola allchariensis										
	Med	Min-Max	Med	Min-Max	Med	Min-Max	Med	Min-Max	Med	Min-Max
As	24.3	4.1-373	3.40	2.08-23.1	4.26	3.12-71.5	2.96	1.45-7.19	108	1.45-215
Sb	0.55	0.25-0.57	0.25	0.25-0.52	0.50	0.25-0.63	0.25	0.25-0.25	0.40	0.25-0.55
Tl	311	15-1215	459	9.72-842	2192	18.9-4013	975	36-2907	1690	472-2907
Viola arsenica										
As	211	77.8-343	21.9	4.82-38.9	32.3	2.41-62.3	34.3	10.6-58.0	1392	6.3-2776
Sb	1.06	0.98-1.14	0.25	0.25-0.25	0.72	0.50-0.93	0.47	0.25-0.69	0.91	0.25-1.57
Tl	1877	1653-2102	2831	2649-3013	9092	7780-10404	2669	2566-2771	1877	1388-2367
*Viola macedonica**										
As	158		0.25		1.46		1.38			
Sb	0.25		0.25		0.25		0.25			
Tl	356		567		4292		2310			

Med – Median, Min – Minimum, Max – maximum

This extraction solution is often used to determine the extraction of trace elements or biogenic elements that plants commonly require for their growth and development.
- Extraction with reagent with low pH value: This was used to determine potentially accessible forms that were difficult to extract from the soil. In this case, a 0.1 M HCl solution was used.

By simulating these extraction solutions, the degree of availability of elements from the soil by the plants could be determined.

Instrumentation

All analyzed elements were determined using inductively coupled plasma – atomic emission spectrometry (ICP-AES) (Varian 715-ES) equipped with an additional ultrasonic nebulizer CETAC (ICP/U-5000AT+). To validate the method, certified reference materials were used for all elements considered to ensure that the difference between measured and certified values was within 15%. The standard moss reference materials M2 and M3 (Steinnes et al. 1997) and the soil reference material JSAC 0401 were used for quality control. The measured concentrations showed good agreement with the recommended values (Bačeva et al. 2016).

Results and discussion

Viola species (Bačeva et al. 2014a)

The content of 27 elements (Ag, Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Ga, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Sb, Sr, Tl, V, and Zn) in all three endemic *Viola* species (V. *allchariensis, V. arsenica,* and *V. tricolor* subsp. *macedonica*) was determined (Bačeva et al. 2014a). Since all three *Viola* species grow at the Allchar locality, and these endemic plant species are exposed to excessive amounts of As, Sb, and Tl in the soil (Volkov et al. 2006; Boev & Jelenković, 2012; Bačeva et al. 2014b), special attention was paid to the content of As, Sb and Tl. The data on the content of these elements in different parts of *Viola* species (roots, stems, leaves, flowers and seeds) collected from all six sites are presented in Table 1. The mass fractions of As, Sb and Tl in different plant parts of *Viola* species collected from all six sites are shown in Figure 7.

The highest accumulation of As was found in the seeds of *V. arsenica* with a median value of 1392 mg/kg (mass fraction of 2.5-88.5%), and in the roots of *V. arsenica* with a median value of 211 mg/kg (mass fraction of 10-31%). The median value was 158 mg/kg for the roots of *V. tricolor* subsp. *macedonica* and the lowest content is in the roots of *V. allchariensis* (24.3 mg/kg). For the aerial parts, the accumulation of As in the stems was found to be highest in *V. arsenica* with a median value of 21.9 mg/kg (mass fraction of 16%), followed by *V. allchariensis*, with a median value of 3.40 mg/kg (mass fraction of 25%), and lowest in the stems of *V. tricolor* subsp. *macedonica* with 0.25 mg/kg (mass fraction of 0.16%). The accumulation of As in the leaves

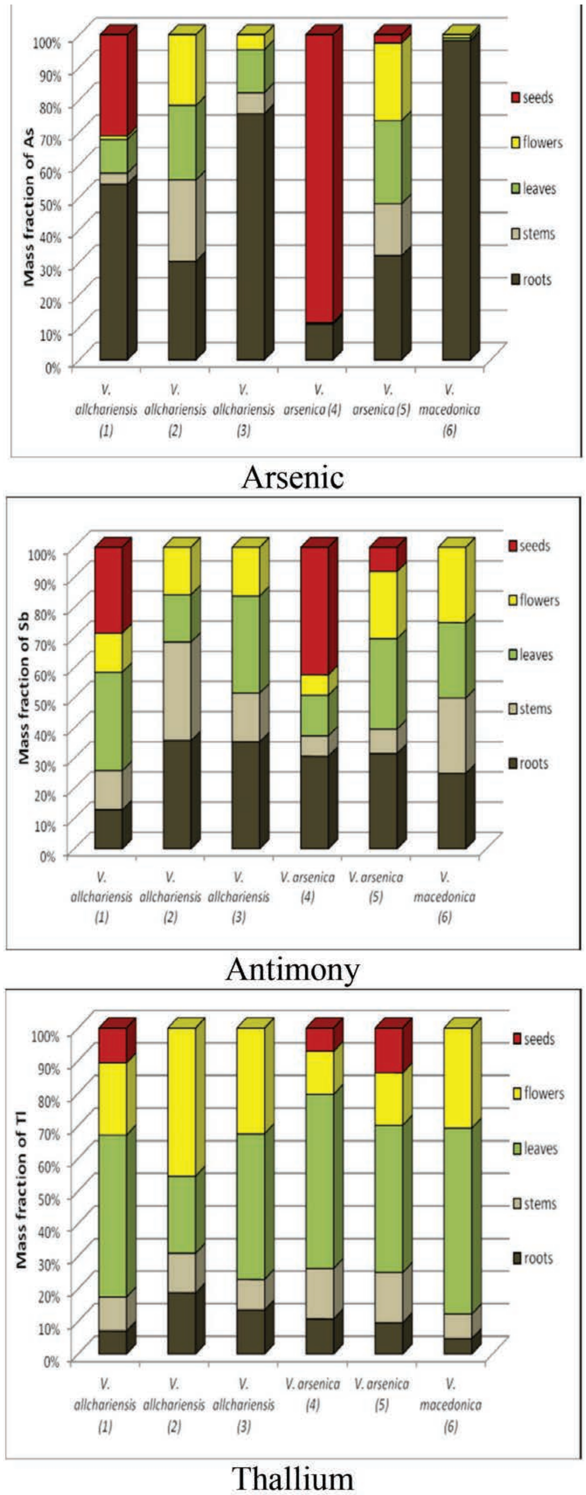

Figure 7. Mass fraction of As, Sb and Tl in different plant parts of *Viola* species (Bačeva et al. 2014a)

and flowers was low in all three plant species (<40 mg/kg), but the accumulation of As was higher in the seeds; in *V. arsenica* the values ranged from 6.34 to 2776 mg/kg (median of 1392 mg/kg) and lowest in *V. allchariensis* with a median value of 108 mg/kg (mass fraction of 31.2%). For *V. tricolor* subsp. *macedonica*, no seeds were available during the period in which the samples were collected. Although the plants grew in places with higher As supply, the content of this toxic element in the roots and seeds was significantly higher.

The data in Table 1 show that the Sb content was low in all plant parts and that there were no significant differences between the different plant parts. Regarding the accumulation of Sb in all three *Viola* species, we can conclude that most of the accumulated Sb was contained in the roots, seeds and leaves of *V. arsenica* with median values of 1.06, 0.91 and 0.72 mg/kg, respectively (mass fractions of 30, 42 and 30%). The accumulation of Sb in *V. allchariensis* and *V. tricolor* subsp. *macedonica* was very low (<0.60 mg/kg).

One of the main objectives was also to study the accumulation of Tl in *Viola* species in relation to its mobility. It was found that the Tl content was significantly higher in the leaves and flowers (Table 1, Figure 7). The highest Tl accumulation was found in the leaves, flowers, and seeds of *V. arsenica*, with median values of 9092 mg/kg, 2669 mg/kg, and 1877 mg/kg, respectively (mass fractions of 45-53%, 13-16% and 13.7%). For *V. allchariensis*, the median values were 2192 mg/kg 975 mg/kg, and 1690 mg/kg, respectively (mass fractions of 23.7-49.7%, 22-45.4% and 10%). For *V. tricolor* subsp. *macedonica* the median values of Tl in leaves and flowers were 4292 mg/kg and 2310 mg/kg (mass fractions of 57% and 30.7%), respectively. The content of Tl in the roots and stems of all *Viola* plant species was also very high. Thus, the median content in the roots and stems of *V. arsenica* was 1877 mg/kg and 2831 mg/kg, in *V. allchariensis* 312 mg/kg and 459 mg/kg and in *V. tricolor* subsp. *macedonica* 365 mg/kg and 567 mg/kg. The mass fractions of Tl in roots and stems were appropriate, with 10% and 15.5% for *V. arsenica*, 7-18.7% and 9.4-12.2% for *V. allchariensis*, and 4.7% and 7.5% for *V. tricolor* subsp. *macedonica,* respectively.

For animals and humans, the most important exposure pathway is the uptake of plant species growing on Tl-contaminated soils (Scheckel et al. 2004). Thallium can easily be taken up by plants if it is present in soils as thermodynamically stable Tl(I), an analogue of potassium (Madejon et al. 2007). Thallium(I) has a low stability that is constant with both organic and inorganic ligands (Kaplan and Mattigod, 1998; Nriagu, 1998), making it easy to extract from leaves (Scheckel et al. 2004). However, the transport of K and Tl appears to be different, as Tremel et al. (1997) determined that there is no relation between Tl and K in seeds. This suggests that the behaviour of Tl in this study may be similar to these findings.

The results in Table 1 and Figure 7 show that all *Viola* species are able to accumulate toxic elements and distribute them to all parts of the plant, especially with regard to the accumulation of As and Tl. The As content was significantly higher in the roots and seeds, while the Sb content was significantly lower compared to As and Tl. Interestingly, the Tl content was significantly higher in the leaves and flowers of all *Viola* species, with no significant differences between the different plant parts.

Previous studies on endemic *Viola* species from Allchar locality focused only on the accumulated As and indicated a significantly higher As content in the roots (Stevanović et al. 2010). It is worth mentioning that Stevanović et al. (2010) divided the plant species into roots and shoots and analyzed only for As. They concluded that *Viola* species from the abandoned As mine of Allchar can be classified as As accumulators. They found a clear difference in As concentrations between the three *Viola* species growing in the same habitat near the mine. As accumulation in the above-ground plant organs ranged from 29 to 440 mg/kg and in the roots from 780 to 2200 mg/kg. Such accumulation may make these plants valuable bioindicators and potential tools for phytoremediation (phytostabilization) of As in anthropogenically contaminated soils (Stevanović et al. 2010).

All soil samples collected from the same locations as the *Viola* species were also analyzed for the same elements (Table 2). It is evident that all three *Viola* species were exposed to an excess amount of As present in the soil (average of 5464 mg/kg), resulting in a significantly high accumulation of As in these endemic species. The results presented in Table 2 show that the As content in the soil samples ranged from 86 to 12775 mg/kg, with a median value of 5875 mg/kg. The Sb content in the same soil samples ranged from 21 to 116 mg/kg (median 37 mg/kg), while the Tl content ranged from 14 to 2140 mg/kg, with a median of 431 mg/kg. Previous geological studies of this site indicate that these high contents of these elements are primarily due to natural phenomena rather than anthropogenic activities (Volkov et al. 2006; Boev and Jelenković, 2012). According to (Volkov et al. 2006) the content of thallium in these area ranges between 0.1–0.5%, of Sb is up to 2.5 and of As about 1.5%.

Table 2. The content of As, Sb and Tl in soil samples from the locations were *Viola* species were collected (in mg/kg) (Bačeva et al. 2014a)

Element	Average	Median	Minimum	Maximum
As	5464	5875	86	12775
Sb	47	37	21	116
Tl	595	431	14	2140

The degree of extraction or the content of plant-available As, Sb and Tl from the soil samples taken from sites where *Viola* species were extracted is shown in

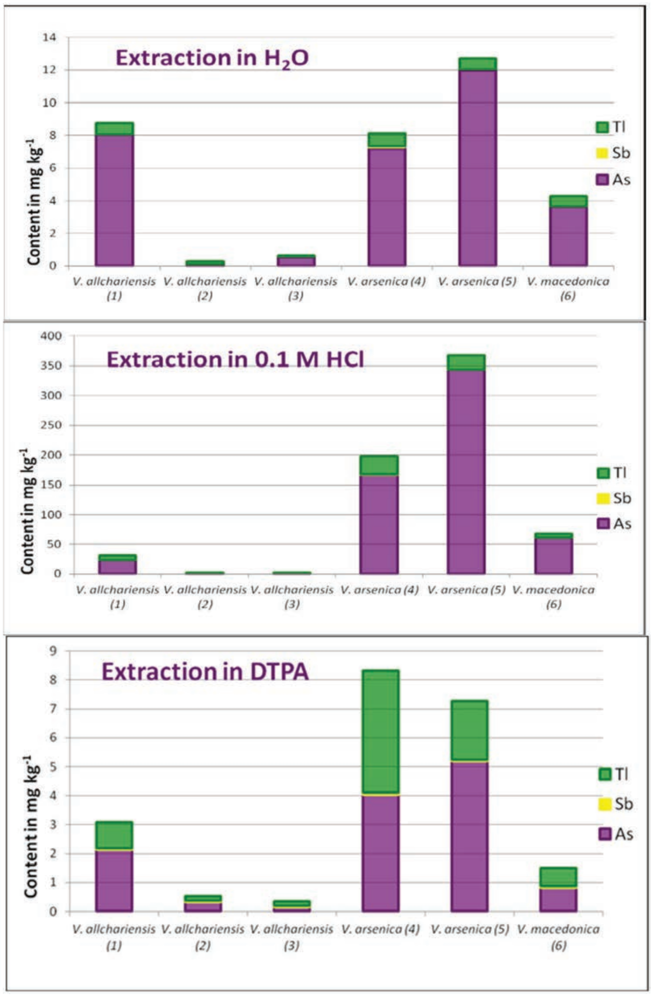

Figure 8. Extraction for plant-available As, Sb and Tl in H_2O, 0.1 M HCl and DTPA-TEA-$CaCl_2$ extraction solutions (Bačeva et al. 2014a)

Figure 8. It was found that As was most readily available in the soil samples in the HCl extraction solution. For example, soil from sites with *V. arsenica* extracted about 344 mg/kg As, which corresponds to about 2.5% of the As present in the soil. Similar trends were observed for Tl, with an extractability of about 2 % for all *Viola* species sites. However, the extractability of Sb from the soil samples was lower, ranging from 0.2 % to 0.82 % at the different species sites.

In a recent study led by Jakovljević et al. (2023), XRF analysis of herbarium specimens from two endemic *Viola* species, *V. allchariensis* and *V. arsenica*, and the widespread *V. tricolor* subsp. *macedonica* from Allchar locality, showed increased concentrations of As in leaf tissue, although they did not exceed the hyperaccumulation threshold. Noteworthy differences were observed both between and within the same taxa, with *V. allchariensis* and *V. arsenica* having similar mean As concentrations (~180 mg/kg), while *V. tricolor* subsp. *macedonica* had significantly lower values. Despite lower mean and absolute Tl concentrations in herbarium specimens, hyperaccumulation of Tl was observed in some individuals, especially in *V. arsenica*.

The results of the chemical analysis showed significantly lower As values for all three Viola taxa compared to the XRF analyses, particularly pronounced in *V. allchariensis* and *V. tricolor* subsp. *macedonica*. While As concentrations remained below the hyperaccumulation threshold (<1000 mg/kg), hyperaccumulation of Tl was observed in all samples, with the highest concentrations found in *V. arsenica*, exceeding those previously reported for some strong hyperaccumulators of this element (Jakovljević et al. 2023).

Significant differences were found in the uptake of As and Tl in the Allchar area, with As concentrations in soil being significantly higher than those of Tl. However, Tl concentrations in leaves were several times higher than those of As, suggesting that plant affinity is a key factor for accumulation. Although surface contamination with soil particles could contribute to the extreme metal(loid) concentrations in the leaves, Tl was found to be endogenous in Viola taxa, while As appeared to be influenced by contamination, which was particularly evident in *V. tricolor* subsp. *macedonica* and *V. allchariensis* (Jakovljević et al. 2023).

The aim of the study was to evaluate the potential hyperaccumulation of As and Tl in the leaves of three *Viola* taxa. Hyperaccumulation of Tl was found in all taxa, with exceptionally high concentrations found in *V. arsenica*. Synchrotron µXRF analysis indicated that As was strongly influenced by contamination in some taxa, but appeared to be endogenous in *V. arsenica*. These results lay the foundation for further controlled studies, including plant dosing experiments, to better understand the mechanisms of metal(loid) accumulation (Jakovljević et al. 2023).

Thymus alsarensis **Ronniger (Bačeva et al. 2016)**

Since the endemic plant species thyme grows on the Allchar deposit and is exposed to excess amounts of As, Sb and Tl in the soil (Volkov et al. 2006; Boev & Jelenković, 2012; Bačeva et al. 2014b), special attention was paid to the content of these elements in plant samples. The content of some other heavy metals(loids) in *Thymus alsarensis* Ronniger was previously determined in the whole plant (Kadifkova Panovska et al. 1996; Bačeva et al. 2016). As these are the most common toxic elements in the soil of the Allchar locality (Bačeva et al. 2014b), the data on the content of As, Sb and Tl in *T. alsarensis* and the surrounding soil are presented in Tables 3 and 4.

The median value of As, Sb and Tl at the different sampling sites was 215, 23 and 15 mg/kg, respectively. The total As content in soil samples from different sites where *Thymus* species were collected varied remarkably between 34.7 and 5290 mg/kg, with a median of 215 mg/kg. The total Sb content in the soil where samples of *T. alsarensis* were collected varied from 6.3 to 130 mg/kg, with a median value of 23 mg/kg (Table 3). The total Tl content in the soils sampled by *T. alsarensis* varied between 2.0 and 330 mg/kg, with a median of 15.3 mg/kg.

Table 4. Average values for As, Sb and Tl in different parts of the plant *Thymus alsarensis* Ronniger (in mg/kg) (Bačeva et al. 2016)

Element	Root	Steam	Leaf	Flower
As	62.7	10.6	14	4.45
Sb	0.89	0.29	0.43	0.3
Tl	147	60.7	104	131

The results presented in Tables 3 and 4 show that the content of Tl and As is significantly increased in some plant samples collected in this area (up to 140 mg/kg and 496 mg/kg for As and Tl, respectively). Tables 3 and 4 and Figure 9 show that the samples of *T. alsarensis*

Table 3. The content of As, Sb and Tl in *Thymus alsarensis* Ronniger. Values are given in mg/kg. Samples were collected from 14 locations. (Bačeva et al. 2016)

Element	*Thymus alsarensis* Ronn.			Soil (0– 5 cm)		
	Average	Med	Min-Max	Average	Med	Min- Max
As	16.1	3	0.25–140	809	215	35–5290
Sb	0.4	0.25	0.25–1.51	34.7	23	6.3–130
Tl	95.4	20.7	0.10–496	58.8	15.3	2.0–330

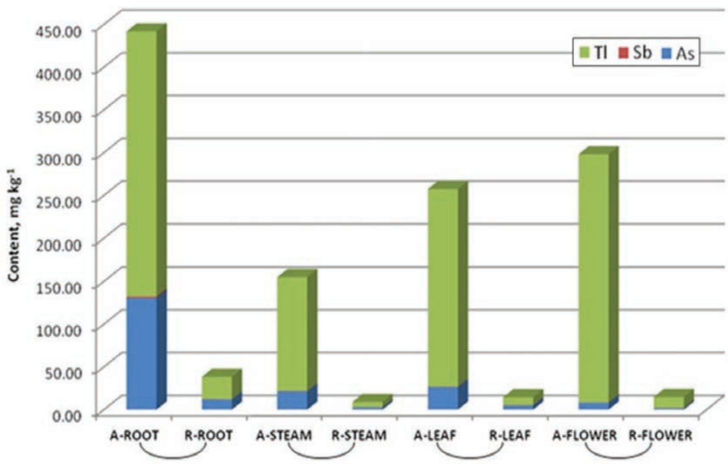

Figure 9. Comparison between samples of *Thymus alsarensis* Ronn. from the Allchar area (A – area around the Allchar mine; R – rest of the Allchar area). (Bačeva et al. 2016)

Ronn. are able to accumulate toxic elements, especially As and Tl, and distribute them in all parts of the plant. The As content was significantly higher in roots and leaves, but the Sb content was significantly lower than the As and Tl content, with no significant differences between the contents in the different parts of the plant. The Tl content is much higher in all parts of *T. alsarensis* Ronn.

All *T. alsarensis* samples appear to be exposed to excessive amounts of As in the soil (Table 3). Significant accumulation of As was observed in this endemic species. The highest accumulation of As in *T. alsarensis* was found in the roots, with an average value of 62.7 mg/kg (Table 4). In the aerial parts of these endemic plant species, the highest average value of 14 mg/kg was found in the leaves of the plant, while the average value for the steams was 10.6 mg/kg. The lowest As content was observed in the flowers.

It is also evident that *T. alsarensis* from this area is exposed to soils containing elevated amounts of Sb (Table 4), but much less than As and Tl. Moreover, no significant differences were found between the different parts of the plant. Most of the Sb accumulated in the roots and leaves, with average values for these parts of 0.89 mg/kg and 0.43 mg/kg, respectively. The accumulation of Sb in the flowers and stems was minimal, <0.30 mg/kg. When comparing the Tl content in the different plant parts, a significantly higher Tl content was found in the roots and flowers (Table 4). The accumulation of Tl in roots, flowers and leaves of *T. alsarensis* had an average value of 147 mg/kg, 131 mg/kg and 104 mg/kg, respectively. In the stems, the average value was 60.7 mg/kg.

The comparison of the contents of As and Tl in samples collected near the Allchar mine (n=5) with those from the rest of the area (n=9) shows differences in the content of As and Tl (Figure 9). Thus, the average As content in the roots (A-root) of the samples from the vicinity of the Allchar mine was 139 mg/kg, almost seven times higher than the values of the samples from the rest of the area (average value of 20.1 mg/kg). Differences between the As content in stems and leaves of samples from the vicinity of the Allchar mine (19.7 mg/kg and 22.7 mg/kg, respectively) compared to samples from the rest of the area (5.61 mg/kg in stems and 9.15 mg/kg in leaves) were also evident.

The content of Tl was significantly higher compared to As and Sb in all parts of the plant. The accumulation of Tl was significantly higher in the roots from the (A-R) area with an average content of 317 mg/kg than in the roots from the (R-R) area, where the average value was 53.6 mg/kg. Remarkable differences between the Tl levels in the aerial parts of plants (flowers, leaves and stems) were found in samples from the vicinity of the Allchar mine (262, 172 and 89.7 mg/kg, respectively) compared to samples from the rest of the area (58.8, 66.5 and 44.5 mg/kg, respectively). The enrichment of antimony was significantly lower compared to As and Tl, and there were no significant differences between the different parts of *T. alsarensis* from the Allchar locality (Figure 9). The lower enrichment and content of Sb in the samples of *T. alsarensis* is due to the fact that the Sb mineralization (mainly the mineral stibnite) was distributed in deeper layers of the mine deposit (Jelenković and Boev, 2011).

Extraction tests of the soil showed that Tl was extracted with extraction solutions of HCl amounting to about 1.2% of the Tl present in the soil (Figure 10). Smaller amounts of As were extracted from the soil (0.84%), while Sb was the least extractable (0.81% extractability). The extractability of As, Sb, and Tl in an extraction solution of H_2O (pH=7) and DTPA–$CaCl_2$–TEA was low (below 0.2% for all elements).

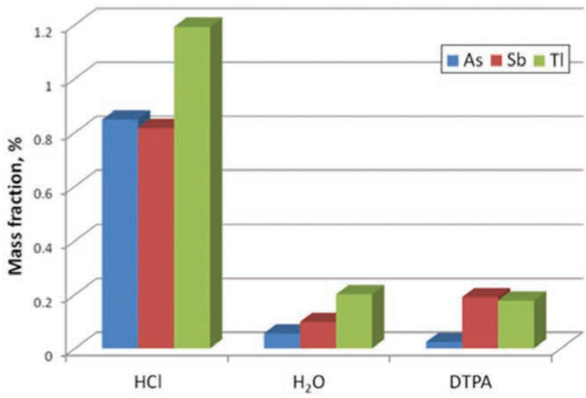

Figure 10. Plant-availability of As, Sb and Tl in 0.1 M HCl, H_2O and DTPA+TEA+$CaCl_2$ extraction solutions (Bačeva et al. 2016)

Table 5. Results of the analysis of As, Sb and Tl in the plant *Centaurea leucomalla* Bornm. and the corresponding soil (in mg/kg) (Bačeva et al. 2018a)

Element	*Centaurea leucomalla* Bornm.			Soil (0-5 cm)		
	Average	Median	Min-Max	Average	Median	Min-Max
As	59.48	28.4	0.55-210	1801	1655	26.9-5288
Sb	6.94	0.74	0.25-58.2	2079	65.6	8.66-8816
Tl	42.1	20.9	0.10-238	271	289	22.2-530

Table 6. Data on the content of As, Sb and Tl in different parts of *Centaurea leucomalla* Bornm. (in mg/kg) (Bačeva et al. 2018a)

Element	Root			Steam			Leaf		
	Average	Median	Min-Max	Average	Median	Min-Max	Average	Median	Min-Max
As	92.3	103	2.39-204	22.1	8.85	0.55-91.5	63.9	20.6	1.59-210
Sb	7.57	1.14	0.25-54.4	5.52	0.74	0.25-37.7	7.74	0.38	0.25-58.2
Tl	86.3	63.1	10.3-238	17.4	9.93	1.86-69.1	22.5	13.8	0.10-75.2

*Centaurea species (**Bačeva et al. 2018a, 2018b**)*

Centaurea leucomalla Bornm.

Bačeva et al. (2018a) presented the contents of 22 elements (As, Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, Pb, Sb, Sr, Tl, V and Zn) in the endemic species of *Centaurea leucomalla* Bornm. collected from 8 sub-sites of the Allchar locality. The average, median, minimum and maximum values of the content of As, Sb and Tl in plant and soil samples are presented in Table 5, while their content in different plant parts (root, stem and leaf) of *C. leucomalla* is given in Table 6. It can be seen that the average content of As in the soil samples was 1801 mg/kg and ranged from 26.9 to 5288 mg/kg (Table 5). The average Sb content in soil samples from the same locations where the *C. leucomalla* samples were collected was 2079 mg/kg and ranged from 8.66 to 8816 mg/kg. Based on the soil sample data, it was found that the average content of Tl was 271 mg/kg and ranged from 22.2 to 530 mg/kg.

The results obtained (Tables 5 and 6) show that the content of Tl and As in the plant samples was significantly high (up to 210 mg/kg for As and up to 238 mg/kg for Tl). These results confirm that *C. leucomalla* was able to accumulate toxic elements, especially As and Tl, and distribute them in all parts of the plant. The content of As was significantly higher in the roots and leaves. Although the Sb content in the soil was high, its content in the plant samples was low compared to As and Tl and showed no significant differences between the different plant parts.

The results of the extraction values of As, Sb and Tl from the soil samples from sites where *Centaurea* samples were taken are shown in Figure 11. It can be seen that the highest extractability of these elements was achieved with the extraction solution of 0.1 M HCl, with an extractable content of 6.27 mg/kg, which corresponds to 2.3 % of the total content of Tl in the soil. The extraction values of As were 0.36% (6.5 mg/kg), while the extractable content of Sb was the lowest (6.08 mg/kg or 0.29%). The extractability of As and Sb in water (pH 7) and DTPA–$CaCl_2$–TEA was very low (less than 0.1% for As and Sb), while the extraction of Tl in these extraction solvents was slightly higher than that of other elements (0.32% and 0.84%, respectively).

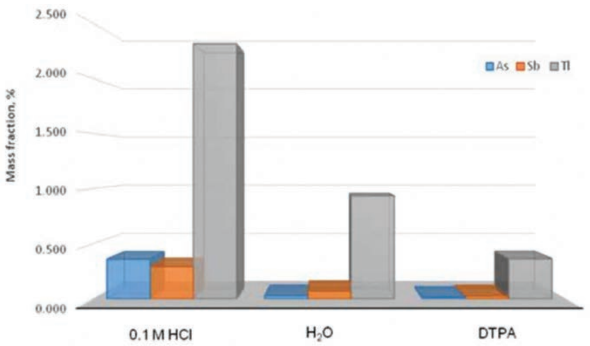

Figure 11. Plant-available As, Sb and Tl in 0.1 M HCl, H_2O and DTPA+TEA+$CaCl_2$ extraction solutions of soil from locations where *Centaurea* samples were taken (Bačeva et al. 2018a)

The biotransfer of these elements in *C. leucomalla* was remarkably high, with the average content in the plant reaching 59.5 mg/kg and ranging from 0.55 to 210 mg/kg. Among the different parts of the plant, the roots had the highest content (average value of 92.3 mg/kg, ranging from 2.39 to 204 mg/kg), followed by the leaves (average value of 63.9 mg/kg, ranging from 1.59 to 210 mg/kg), while the stems had the lowest average content (22.1 mg/kg, ranging from 0.55 to 91.5 mg/kg). Despite the high Sb content in the soil, the Sb content in the plant was relatively low compared to the values for As and Tl in the same plants (average content of 6.94 mg/kg, with a range of 0.25 to 58.2 mg/kg).

Special attention was paid to the accumulation and transfer of Tl in different parts of *C. leucomalla*. A high bioaccumulation was observed, with an average content in the plant of 42.1 mg/kg, ranging from 0.1 to 238 mg/kg. The accumulation of Tl followed a similar pattern to that of As, with high levels in all parts of the plant, highest in the roots (86.3 mg/kg, ranging from 10.3 to 238 mg/kg), followed by the leaves (average 22.5 mg/kg, ranging from 0.1 to 75.2 mg/kg) and lowest in the stems (average level of 17.4 mg/kg, ranging from 1.86 to 69.1 mg/kg).

Centaurea kavadarensis Micev.

The results presented in Table 7 show that the levels of As, Sb and Tl in the soil sample from the site where samples of *Centaurea kavadarensis* Micev, were collected were significantly lower compared to the soil samples from the sites where the samples off *Centaurea leucomalla* Bornm. were collected. Consequently, the accumulation of these elements in *C. kavadarensis* was much lower than in the other plant species from the Allchar site. This difference could possibly be due to the fact that the plant samples of the other endemic species were collected near the mine or in an area of hydrothermally modified rock where mineralization of As and Tl was predominant. In contrast, the plant samples of *C kavadarensis* were collected 5.5 km away from the hydrothermally altered rocks along the Majdanska River.

Table 7. The content of As, Sb and Tl in different plant parts of *Centaurea kavadarensis* and corresponding soil (in mg/kg) (Bačeva et al. 2018b)

Element	Soil	Root	Stem	Leaf	Flower
As	102	<0.50	<0.50	<0.50	<0.50
Sb	8.40	<0.50	<0.50	<0.50	<0.50
Tl	4.45	4.31	4.13	5.38	3.18

Onobrychis degenii Dörfler and *Knautia caroli-rechingeri* Micev.

Statistical analysis of the analyzed results for plant samples of the endemic plant species *Onobrychis degenii* and *Knautia caroli-rechingeri* and the corresponding soil samples provided data on the content of 27 elements in different parts of the plant (root, stem, leaf and flower) (Bačeva et al. 2018b). In particular, Table 8 shows the data for the content of As, Sb and Tl. These plant species were collected in the Crven Dol region, which is characterized by hydrothermally modified rock in which the mineralization of As and Tl was predominant.

The As content in the root of *O. degenii* was 114 mg/kg, whereas in *K. caroli-rechingeri* it was 354 mg/kg. In the aerial parts of *O. degenii*, the As content was 4.03 mg/kg in the leaf and 3.49 mg/kg in the flower. In *K. caroli-rechingeri*, the As content in the leaf was 3.78 mg/kg (Table 8). In addition, the Tl content in the roots of these plants was also notably high in the roots of *O. degenii* (86.7 mg/kg) and in the roots of *K. caroli-rechingeri* (216 mg/kg). In other plant parts (stem, leaf, and flower), the Tl content in *O. degenii* ranged from 6.34 mg/kg to 6.19 mg/kg, while it was higher in *K. caroli-rechingeri*, ranging from 10.4 mg/kg to 26.3 mg/kg. However, the Sb content was significantly lower in the different plant parts compared to the As and Tl content, probably due to the lower Sb content in the Crven Dol area and the resulting lower accumulation by the plants.

Extraction tests of As, Sb and Tl from the soil samples showed that the highest As content was extracted with an extraction solution of 0.1 M HCl, extracting 7.0% of As compared to 1.2% of Sb and 1.2% of Tl.

Assimilation by plants (Bačeva et al. 2014a, 2014b, 2018a, 2018b)

The Biological Accumulation Factor (BAF) and the Biological Transfer Factor (BTF) provide valuable insights into the bioaccumulation and translocation of toxic elements in plant species. Using the results of the analysis of As, Sb and Tl in different plant parts of the studied endemic species from the Allchar area, these factors were calculated.

For *Viola* species (Table 9), the average biological accumulation factor (BAF) for As was 0.17, indicating an increased accumulation compared to previous data. In particular, *V. arsenica* and *V. allchariensis* had significantly high translocation factors, indicating efficient movement of As from roots to flowers or seeds. However, BAF and BTF for Sb were insignificant due to its negligible extractability from soil.

Remarkably, *V. allchariensis* exhibited a high BAF of 33.8 for Tl, suggesting that the plant is capable of accumulating significant amounts of Tl. Furthermore, the BTF values of 3.29 emphasize the efficient translocation of Tl from the roots into the flowers of *V. allchariensis*. These results emphasize the importance of understanding the bioaccumulation dynamics of toxic elements in endemic plant species to support risk assessment for human health and environmental effects.

The assessment of the distribution and accumulation of arsenic (As), antimony (Sb) and thallium (Tl) in *Thymus* plants is crucial for environmental monitoring,

Table 8. The content of As, Sb and Tl in different plant parts of *Onobrychis degenii* and *Knautia caroli-rechingeri* and corresponding soil (in mg/kg) (Bačeva et al. 2018b)

	Onobrychis degenii					*Knautia caroli-rechingeri*			
Element	Soil	Root	Stem	Leaf	Flower	Soil	Root	Stem	Leaf
As	5288	114	0.58	4.03	3.49	4932	354	1.66	3.78
Sb	80.7	<0.50	<0.50	<0.50	<0.50	30.9	0.68	<0.50	<0.50
Tl	330	86.7	6.34	6.83	6.19	409	216	26.3	10.4

Table 9. The mean content of As, Sb and Tl in plant parts of *Viola* species and the calculated bioaccumulation factor (BAF) and biotransfer factor (BTF) (in mg/kg) (Bačeva et al. 2014)

Species	Root	Steam	Leaves	Flowers	Seeds	Sum	Soil	BAF	BTF
			As						
V. allchariensis	134	9.54	26.3	3.87	108	282	2573	0.11	0.81
V. arsenica	210	21.9	32.3	34.3	1391	1691	10066	0.17	6.61
V. tricolor subsp. *macedonica*	158	0.25	1.46	1.38	-	161	4932	0.03	0.01
			Sb						
V. allchariensis	0.46	0.34	0.46	0.25	0.40	1.91	33.8	0.06	0.87
V. arsenica	1.06	0.25	0.72	0.47	0.91	3.40	70.8	0.05	0.86
V. tricolor subsp. *macedonica*	0.25	0.25	0.25	0.25	-	1.00	30.9	0.03	1.00
			Tl						
V. allchariensis	514	437	2075	1306	1690	6022	178	33.80	3.29
V. arsenica	1877	2831	9092	2669	1877	18346	1312	13.98	1.00
V. tricolor subsp. *macedonica*	356	567	4292	2310	-	7525	409	18.40	6.49

especially considering their frequent use for medicinal and culinary purposes. Based on the results of the analysis of these elements in different plant parts (roots, stems, leaves and flowers) of *Thymus* species growing in the Allchar mining area, BAF and BTF were calculated (Table 10). These calculations provide valuable insights into how these elements are absorbed and transferred in the *Thymus* plants, which is crucial for assessing environmental risks and ensuring the safety of plant-derived products used in various applications.

The Biological Accumulation Factor (BAF) for arsenic (As) and antimony (Sb) in the endemic *Thymus* species was found to be insignificant, indicating minimal accumulation of these elements. However, for thallium (Tl), the BAF was 7.5, indicating a significantly higher accumulation compared to As and Sb. In addition, the BTF for Tl in *Thymus* samples was 0.89, indicating slightly lower transfer efficiency from roots to flowers compared to its accumulation. These results indicate that *T. alsarensis* is more efficient in accumulating Tl compared to As and Sb.

Similarly, in *Centaurea leucomalla* Bornm., the BAF values for As and Sb were insignificant, while the BAF for Tl was the highest at 0.466 (Table 11). This indicates that this endemic species from Allchar accumulates thallium much more intensively than arsenic and antimony. On the other hand, BTF was higher for As and Sb, suggesting that these elements are transferred from the roots to the flowers to a greater extent compared to Tl. Overall, these results provide valuable insights into the accumulation and transfer dynamics of As, Sb, and Tl in *C. leucomalla*.

Table 12 shows the results of the contents of arsenic, antimony, and thallium in the soil and in all

Table 10. The content of As, Sb and Tl in different parts of *T. alsarensis* Ronniger and the calculated BAF and BTF (Bačeva et al. 2016)

Element	Root	Stem	Leaves	Flowers	Sum	Soil	BAF	BTF
As	62.7	10.6	14.0	4.45	91.8	809	0.114	0.071
Sb	0.89	0.29	0.43	0.30	1.90	34.7	0.055	0.336
Tl	147	60.7	104	131	444	58.8	7.556	0.891

Table 11. The content of As, Sb and Tl and the values of BAF and BTF for the different parts of *Centaurea leucomalla* Bornm. (in mg/kg) (Bačeva et al. 2018a)

Element	Root	Stem	Leaf	Sum	Soil	BAF	BTF
As	92.3	22.1	63.9	178	1801	0.099	0.693
Sb	7.57	5.52	7.74	20.8	2079	0.010	1.022
Tl	86.3	17.4	22.5	126	271	0.466	0.261

Table 12. Results of the analysis of different parts of *Onobrychis degenii*, *Knautia caroli-rechingeri* and *Centaurea kavadarensis* and calculations for bioaccumulation and biotransfer factor (in mg/kg)

Species	Root	Stem	Leaf	Flower	Sum	Soil	BAF	BTF
				As				
Onobrychis degenii	114	0.58	4.03	3.49	122	5288	0.023	0.031
Knautia caroli-rechingeri	354	1.66	3.78	-	360	4932	0.073	0.011
Centaurea kavadarensis	<0.50	<0.50	<0.50	<0.50	-	102	-	-
				Sb				
Onobrychis degenii	<0.50	<0.50	<0.50	<0.50	-	80.7	-	-
Knautia caroli-rechingeri	0.68	<0.50	<0.50	-	0.68	30.9	0.022	-
Centaurea kavadarensis	<0.50	<0.50	<0.50	<0.50	-	8.40	-	-
				Tl				
Onobrychis degenii	86.7	6.34	6.83	6.19	106	330	0.322	0.071
Knautia caroli-rechingeri	216	26.3	10.4	-	253	409	0.618	0.048
Centaurea kavadarensis	4.31	4.13	5.38	3.18	17.0	4.45	3.819	0.737

parts of *Centaurea kavadarensis, Onobrychis degenii*, and *Knautia caroli-rechingeri*, together with the calculations for the BAF and BTF. From these results it can be concluded that the bioaccumulation factor (transfer of the element from soil to all plant parts) for As was very low (0.023 for *Onobrychis degenii* and 0.073 for *Knautia caroli-rechingeri*), while it could not be calculated for *Centaurea kavadarensis*, as the As contents in the plant parts were below the detection limit. The BAF for antimony (Sb) was also very low. On the other hand, the BAF for thallium was much higher, ranging from 0.322 in *Onobrychis degenii* to 0.618 in *Knautia caroli-rechingeri*, with the highest value of 3.819 found for *Centaurea kavadarensis*. These results indicate that although the bioaccumulation of As and Sb is minimal in these species, they have a higher capacity to accumulate thallium, especially *Centaurea kavadarensis*.

The biological transfer factor (BTF) calculated as ratio between the content of the elements in the flower (or seeds) and in the root, was the highest for Tl (from 0.048 in *Knautia caroli-rechingeri* to 0.737 in *Centaurea kavadarensis*). This indicates their capability to accumulate high amounts of Tl. This behavior of thallium could possibly be explained by the interpretations of Madejon et al. (2007), which state that Tl, when present in soils, is easily taken up by plants because it is generally present as thermodynamically stable Tl(I), an analogue to potassium. Another explanation is that thallium(I) has very low stability constants with organic and inorganic ligands, allowing it to be easily extracted from leaves (Kaplan and Mattigod 1998; Nriagu 1998; Scheckel et al. 2004; Bačeva et al. 2014b).

Recently, Jakovljević et al. (2024) published the results on the characteristics of metal(loid) accumulation in selected native plant species naturally colonizing tailings at Allchar. They focused on known pseudo-metallophytes such as *Minuartia verna, Plantago lanceolata* and *Silene vulgaris*, as well as *Knautia caroli-rechingeri*, which is endemic to the area. They also analyzed *Odontarrhena kavadarcensis* (syn. *Alyssum kavadarcense*) for the first time in this context to assess its potential suitability for phytoremediation. The study examined five pseudo-metallophyte species that naturally colonize the spoil heaps of Allchar: *Odontarrhena kavadarcensis* (syn. *Alyssum kavadarcense*), *Knautia caroli-rechingeri*, *Minuartia verna*, *Plantago lanceolata* and *Silene vulgaris*. Soil, roots and leaves were collected from each individual and subjected to elemental analysis to determine the concentrations of macro- and microelements. Hyperaccumulation of Tl was observed in *M. verna* and *P. lanceolata*, with mean concentrations of 1770 and 1030 mg/kg, respectively. As concentrations exceeded the hyperaccumulation threshold with a leaf-to-root ratio of >1 in *M. verna, S. vulgaris* and *A. kavadarcensis*, reaching up to 1340, 2870, and 1330 mg/kg, respectively. These results underline the potential of the investigated plants for phytoremediation of mine waste contaminated with As and Tl.

Conclusion

The unique mineral and soil composition of the Allchar study area has resulted in a distinct flora characterized by numerous edaphic endemic species that are closely tied to specific substrate types, favored by the mountainous terrain. Seven endemic taxa have been identified in the vicinity of the abandoned mine of Allchar, *Centaurea kavadarensis, C. leucomalla, Knautia caroli-rechingeri, Onobrychis degenii, Thymus alsarensis, Viola allchariensis*, and *V. arsenica*. The accumulation of the metal(loid)s in the plant tissues revealed different behavior of As and Tl uptake, whereby it was established that Tl is accumulated in higher concentrations. Most species, such as *Centaurea kavadarensis, C. leucomalla,* *Knautia caroli-rechingeri* and *Onobrychis degenii* exclude metal(loid)s, with the highest metal(loid) concentrations found in the roots but remaining below the thresholds for over-accumulation. Exclusion was also the predominant strategy in *Thymus alsarensis*, although Tl concentrations in foliage were slightly above the threshold of 100 mg/kg. In *Thymus* species, As content was significantly higher in roots, leaves and stems, while Tl content was significantly increased in roots, flowers and leaves in all *T. alsarensis* specimens tested. This trend was confirmed by extraction tests with different solvents on soil samples taken from the same locations as the *Thymus* specimens, where Tl proved to be the most easily extractable element.

Higher concentrations of As and Tl were observed in *Viola* species. Among them, *Viola tricolor* subsp. *macedonica, V. allchariensis,* and *V. arsenica* differed significantly in their characteristics of metal/metalloid (hyper)accumulation. Thus, *V. tricolor* subsp. *macedonica* contained up to 1.46 mg/kg As and 4290 mg/kg Tl in its leaves, while *V. allchariensis* and *V. arsenica* had even higher concentrations. Soil extraction tests with water, HCl, and buffered DTPA solutions showed that As was most easily extractable from the HCl solution, while the extractability of Tl was the same for all *Viola* species and Sb showed lower extractability compared to As and Tl. In addition, differences in metal(loid) accumulation were observed in different plant tissues, both below and above ground. Thallium was accumulated mainly in the leaves, with lower concentrations in the flowers and seeds, while the highest concentrations of As were found in the seeds of *V. allchariensis* and *V. arsenica*. Overall, these results shed light on the different strategies of metal(loid) accumulation among endemic plant species in the Allchar mining area.

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