



МЕЂУНАРОДНА ИСТРАЖИВАЧКА
АКАДЕМИЈА НАУКА И УМЕТНОСТИ - МИАНУ
INTERNATIONAL RESEARCH ACADEMY OF
SCIENCE AND ART - IRASA

IRASA
International Scientific Conference
SCIENCE, EDUCATION,
TECHNOLOGY AND INNOVATION
SETI V 2023



Book of Proceedings

Belgrade,

October 14, 2023



IRASA International Scientific Conference
SCIENCE, EDUCATION, TECHNOLOGY AND INNOVATION

SETI V 2023

Book of Proceedings



Publisher

IRASA – International Research Academy of Science and Art

For the Publisher

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Editors

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Print run

150

Printed by

Instant system

Belgrade, 2023

ISBN 978-86-81512-11-1

Publication of the Book of Proceedings has been co-financed by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia.



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ANALYSIS OF SECONDARY METABOLITES OF *TEUCRIUM MONTANUM* (LAMIACEAE) WITH SERPENTINE GEOLOGICAL BACKGROUNDS

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Abstract

The influence of physical and chemical characteristics of the geological substrate have a significant role on the properties of the soil that is formed on a given substrate. The serpentine geological base is characterized by the presence of an increased amount of heavy metals in the soil. In serpentine soil, the amount of magnesium exceeds the amount of calcium. Such edaphic conditions of habitats impose and encourage the development of structural functional adaptations of plants serpentinophytes, which inhabit these habitats. It is represented on a limestone substrate, ultrabasic serpentines and on an acid silicate substrate. In response to adverse environmental conditions, plants produce a large number of secondary metabolites whose chemical composition depends on different environmental conditions. Therefore, the results of the research showed that the examined species has mechanisms that enable survival in stressful environmental conditions, in terms of physical and chemical characteristics of the soil.

Key words: *Teucrium montanum*, variability and adaptive significance of secondary metabolites, serpentine geological substrate.

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Introduction

General characteristics of the genus *Teucrium* L.

According to the classification of *Teucrium* it is classified in the family Lamiaceae. Representatives of plants from this genus are largely perennial, a smaller number of species are shrubs or semi-shrubs, annual herbaceous plants are less common (Diklić, 1974).

Plant species from the genus *Teucrium* are widespread in temperate areas, mostly in the Mediterranean and South-West Asia. Fewer representatives of the genus are represented in the tropical region and (Diklić, 1974). The species of this genus are widely distributed in Europe, Asia, America and Australia, but the Mediterranean represents the main distribution area of the genus, where about 96% of all species are found. There are 195 species and subspecies present in the Mediterranean, of which 83 inhabit the Iberian Peninsula, 72 the northwestern part of North Africa (Algeria and Morocco), 61 species are represented in Asia Minor, 41 on the Balkan Peninsula and 23 on the Apennine Peninsula (Lakušić et al., 2010). The flora of the Republic of Serbia is characterized by seven species of the genus *Teucrium* (Diklić, 1974).

Species of this genus most often inhabit open, rocky and dry habitats. They grow on all types of substrate - they inhabit ultrabasic serpentine, all types of limestone soils, neutral and acidic silicate soils. According to the life form, the majority of representatives of this genus are hemicryptophytes (Lakušić et al., 2010).

Representatives of the genus *Teucrium* are today the subject of many studies due to their medicinal effect, especially in terms of secondary metabolites produced by these plants to a large extent - iridoid glycosides and other terpene compounds, then flavonoids, tannins and essential oil (Ivanić et al., 1987). Medicinal species of the genus *Teucrium* are used in the treatment of diseases of the digestive tract, rheumatism, diabetes and inflammatory processes. The most common group of secondary metabolites represented in species of the genus *Teucrium* are phenolic acids, flavonoids, monoterpenes, diterpenes, sesquiterpenes and others. Because of the characteristic composition of secondary metabolites, plant species of the genus *Teucrium* exhibit different types of biological activity such as: anticancer, anti-inflammatory, antioxidant, antiviral and antimicrobial activity (Stanković and Zlatić, 2017).

General characteristics of *Teucrium species montanum* L.

Teucrium montanum (mountain dubčac, willow grass) is a perennial, bushy plant, of low growth, with a developed spindle root. It is characterized by a branched habit. The branches are thin, round in cross-section, 5 to 25 cm long (Diklić, 1974).

Shoots while young are herbaceous, overgrown with short, gray hairs on all sides. The leaves are linear-lanceolate, cross-arranged, slightly narrowed into a short petiole. The length of the leaves is from 0.5 to 2 cm, and the width is from 2 to 4 mm. The leaves



are toothless, the peripheral edges are slightly curled, and narrowed at the top. The back side of the leaf is overgrown with white hairs, while the face of the leaf is dark green with or without hairs (Diklić, 1974).

The flowers are yellow, 12 to 15 mm long, and are located on a short flower stem. The inflorescence grows from the axils of the leaves and are arranged in dichiums of 1 to 3 flowers. Together with the tips of the branches, the flowers form apparent headlike inflorescences. The calyx is tubular, expanded at the base, with five triangular teeth around the rim, often covered with hairs or bare. The corolla is twice as long as the cup, yellowish in color. It consists of five lobes, with the upper two forming the upper lip, and the lower three forming the lower lip, and it is tubular at the base. The middle lobe of the lower lip is bent downwards, much longer than the upper lobes and is egg-shaped. After flowering, the crown falls off, and the cup remains and is green in color. Anthers are longer than the corolla and are located between the upper corolla lobes. The anthers are pale yellow to dark purple in color. The fruits are nuts, up to 2 mm long with a net surface (Diklić, 1974).

Teucrium species montanum is a facultative serpentinophyte, which means it can grow on serpentine soil and but can also live on other types of land. Represented is on a limestone base, ultrabasic serpentinites as well as acid silicates. It is widespread in the southern parts of Europe, but it can also be found in the southern parts of Western and Eastern Europe, Asia Minor, as well as in the northern parts of Africa. It is also widespread in the Mediterranean area as a mountain plant species (Zlatić & Stanković, 2019).

This species is represented in habitats from 30 to 2000 m above sea level, however, the largest number of sites was recorded at an altitude between 500 and 1000 m above sea level (Lakusić, 2010). It grows in communities of deciduous, mixed, broadleaf-coniferous and conifer forests, in communities of serpentine rocks and continental limestone scarp, gorges and canyons, in communities of the sub-Mediterranean region, Mediterranean communities of the xerophilic type and slopes of hilly-mountainous regions. In Serbia, the species *Teucrium montanum* is most widespread on dry limestone and serpentine rock fields, stony meadows and dry pastures around light pine forests (Horvat et al., 1974; Diklić, 1974).

Secondary metabolites of plants

Plants are the primary producers of organic compounds. These compounds are products of primary and secondary metabolism of plant cells (Yazaki et al., 2008).

The products of primary metabolism are called primary metabolites, and they play a role in the growth and development of plants (Kovačević, 2004). The role of primary metabolism is that it allows the plant to use water, minerals and carbon dioxide for the synthesis of essential primary metabolites such as sugar, fatty acids, nucleic acids and amino acids (Klibenstein and Osbourn, 2012).

Products of secondary metabolism, i.e. secondary metabolites, represent the continuation of primary metabolic pathways that are associated with catabolism and transformation of sugars, amino acids or fatty acids (Kovačević, 2004). Secondary



metabolites play a role in the adaptation of plants to unfavorable conditions, but also enable interactions with the external environment (Klibenstein, 2013). Secondary metabolites allow the plant to interact with the external environment (Klibenstein and Osbourn, 2012). They have a protective role, which is reflected in the fact that their activity prevents diseases, protects plants from increased concentrations of ultraviolet radiation, excessive transpiration and other factors that adversely affect plants (Stanković, 2016).

Unlike primary metabolites, the absence of secondary metabolites does not cause the death of the plant, but the absence of secondary metabolites for a long period of time can reduce the ability of plants to survive, can lead to the infertility of the organism, but can also pass without any significant changes in the plant (Tiwari and Rana, 2015). Compounds that are necessary for the synthesis of secondary metabolites come from primary metabolism. The products produced in the process of photosynthesis, glycolysis and in the Krebs cycle are used as biosynthetic intermediates. The number of these components is very small, but there is a wide range of compounds that can be formed from them. The most important components used in the biosynthesis of secondary metabolites are derived from intermediates: acetyl coenzyme A (acetyl-CoA), shikimic acid, mevalonic acid and 1-deoxyxylulose-5-phosphate (Dewick, 2002).

Plants have a wide spectrum of secondary metabolites that have different functions. That function is reflected in: defense against microorganisms and herbivores, some secondary metabolites attract pollinating insects or zoochory animals, others have a role in protection against ultraviolet radiation. According to their chemical composition, secondary metabolites can be divided into two groups (Wink, 2004):

- molecules that contain nitrogen in their composition - alkaloids, glucosinolates, lecithins, amines, non-protein amino acids, cyanogens, glycosides;
- molecules that do not contain nitrogen in their composition - phenolic compounds, terpenoids, polyketides and polyacetylenes.

Phenolic compounds

Phenolic compounds include a large number of plant substances characterized by an aromatic ring with one or more hydroxyl groups. The property of these compounds is that they are soluble in water, and most often occur in combination with sugar as glycosides, which are usually located in the vacuole of the plant cell (Harborne, 1973). Phenolic compounds can be divided based on (Ka beraet al., 2014):

- number of hydroxyl groups (1-, 2- or polyatomic phenols);
- chemical structures (mono-, di-, oligo- and polyphenols);
- the number of aromatic rings and the number of carbon atoms in the side chain (phenols with one aromatic ring, with two aromatic rings, quinones and polymers).

The most important pathway of biosynthesis of phenolic compounds in higher plants is the geranyl phenylpropanoid biosynthetic pathway, i.e. shikimic acid cycle. In the geranyl phenylpropanoid biosynthetic pathway, aromatic amino acids such as



tyrosine, phenylalanine and tryptophan are produced, which are precursors of many secondary metabolites. Deamination of phenylalanine produces cinnamic acid and all compounds that have a phenyl-propane structure. Hydroxylation of cinnamic acid using the enzyme cinnamate-4-hydroxylase (CAH) produces 4-coumaric acid, and in the next step, under the action of the enzyme hydroxycinnamate CoA ligase (4CL), the formation of CoA thioester occurs, i.e. 4-coumaroyl-CoA as well as other cinnamic acid esters (eg chlorogenic acid). From tyrosine, transamination using the enzyme tyrosine aminotransferase (TAT) produces 4-hydroxyphenyl pyruvate, whose reduction, in a reaction catalyzed by hydroxyphenylpyruvate reductase (HPPR), produces 4-hydroxyphenyl acetate. By releasing CoA from the 4-coumaroyl-CoA complex, an ester is formed between the carboxyl group of coumaric acid and the hydroxyl group of 4-hydroxyphenyl acetate, resulting in 4-coumaroyl-4-hydroxyphenyl acetate. Hydroxylation of the resulting ester at one place, in the part of coumaric acid, produces caffeic acid ester, caffeoyl-4-hydroxyphenyl acetate, while hydroxylation of 4-coumaroyl-4-hydroxyphenyl acetate at two places in position 3 and 3' produces 4-coumaroyl-3, 4-dihydroxyphenyl acetate. Rosmarinic acid is formed from the two resulting esters (Nestorović-Živković, 2013).

Phenolic acids

The term phenolic acid represents a phenolic compound that has at least one carboxyl functional group. On the other hand, when we talk about phenolic acids as secondary metabolites of plants, we mean a certain group of organic acids that are further divided according to their structure into: benzoic acid derivatives and cinnamic acid derivatives. Both groups of compounds consist of a phenolic nucleus and a side chain containing one (benzoic acid derivatives) or three (cinnamic acid derivatives) carbon atoms (Jakovetić- Tanasković, 2016).

Phenolic acids have multiple biological roles as secondary metabolites in plants. The processes in which these secondary metabolites are involved are protein synthesis, nutrient absorption, photosynthesis, activity of various enzymes, etc. In addition, phenolic acids, in interaction with plant pathogens, play the role of signaling molecules. The synthesis of phenolic acids increases during biotic or abiotic stress, which means that these compounds also play a role in the adaptation processes of the organism. Also, phenolic acids exhibit antioxidant activity, depending on the position of hydroxyl groups in the molecule (Pereira et al., 2009; Goleniowski et al., 2013).

Flavonoids

Flavonoids represent an important class of secondary plant metabolites that have a polyphenolic structure. They are widely represented by plants. They have various beneficial biochemical and antioxidant effects for plants (Pancheet al., 2016). Flavonoids play a significant role in plant survival - attracting animal vectors for pollination and seed dispersal, stimulating the growth of pollen tubes, stimulating nitrogen-fixing bacteria, increasing tolerance to various adverse abiotic factors, etc. (Gould and Lister, 2006).



Flavonoids belong to the class of phenolic compounds with low molecular weight and are widely represented in the plant kingdom. Many flavonoids are easily recognizable as flower pigments in a large number of angiosperm families. Their distribution is not limited to flowers, but can be found in all parts of plants (Dewick, 2002).

All flavonoids are characterized by the basic structural skeleton ($S_6-C_3-C_6$), which consists of two aromatic rings with six carbon atoms each and one heterocyclic ring with three carbon atoms and one oxygen atom (Ghasemzadeh and Ghasemzadeh, 2011).

Flavonoids can be classified in several ways, and the basic division is made, based on origin and structural properties, into six subgroups. These are: flavones (eg apigenin), flavanones (eg naringenin), flavonols (eg quercetin), flavanols - flavan-3-ols (eg. catechin), isoflavones (eg genistein) and anthocyanidins (eg cyanidin) (Ghasemzadeh and Ghasemzadeh, 2011).

Free radicals and oxidative processes in the cell

Free radicals are atoms, molecules or ions with usually one unpaired electron in the last orbital. The basic characteristic of free radicals is that they are very unstable, reactive and non-selective molecules. They are formed by homolytic breaking of covalent bonds by redox reactions, thermolysis, electromagnetic radiation, as well as various chemical and enzymatic processes in the body (Acworth et al., 2003).

The formation of free radicals in the cells of organisms is related to the influence of various factors from the external environment (ultraviolet, infrared and ionizing radiation), but also to the processes that take place in the cell itself (oxidative phosphorylation in mitochondria, the process of lipid peroxidation, phagocytosis, oxidoreduction in the presence of metals), catalytic reactions of some enzymes, etc.) (Koraćević, 1996).

Free radicals and their metabolites, involved in a complex network of different signaling pathways, most likely function as intracellular and intercellular mediators, transforming the initial signal (receptor stimulation) into a biochemical response of the cell. Their basic characteristics (very small, highly reactive and diffusible molecules) support the assumption that free radicals and redox stress actively participate in cell signaling as secondary messengers in the activation of transcription factors and induction of gene expression (Pavlović et al., 2002).

The division of free radicals can be performed on the basis of a chemical element that is highly reactive in the body. Therefore, free radicals can be divided into: reactive oxygen species (ROS), reactive chlorine species (RCS), reactive nitrogen species (RNS), reactive bromine species (RBS) and reactive sulfur species (RSS) (Halliwell and Gutteridge, 2015). The most reactive and abundant free radicals are reactive oxygen species that are represented in cells as superoxide anion radicals, hydroxyl radicals, peroxy radicals, alloxyl radicals (Pham - Huyet al., 2008). A special feature of free radicals, due to their reactivity, they easily bind to biomolecules such as nucleic acids, lipids and proteins, which leads to the initiation of many complex and uncontrolled reactions that lead to damage to cell walls and other parts of cells. Therefore, cells are



subject to various degenerative processes, the aging process of cells, and therefore of the whole organism, is accelerated (Mc Cord, 2000).

Antioxidants are special substances that have the ability to to a certain extent inhibit or completely prevent the action of free radicals, i.e. the oxidation of substrates subject to oxidation (lipids, proteins, nucleic acids, carbohydrates). The mechanism of action of antioxidants is based on their ability to act as electron or hydrogen atom donors, then on their ability to chelate metal ions, prevent the action of singlet forms of oxygen, reduce certain compounds, inhibit some enzymes, etc. (Aruoma, 1996).

In the plants themselves, flavonoids have antioxidant and antimicrobial effects. The protective role of flavonoids in biological compositions is attributed to their ability to couple, that is, capture free radical electrons, chelate ion binding of transitional molecules (Fe^{2+} , Cu^{2+} , Zn^{2+} and Mg^{2+}), activate antioxidant enzymes and inhibit oxidases. That multiple action is probably responsible for the overall positive effect in protection against the influence of free radicals on the body's cells (Kazazić, 2004).

Flavonoids may act as antioxidants in several possible ways. The most important is when they act as scavengers of free radicals and thus interrupt the free radical chain reaction. A flavonoid as an antioxidant must satisfy two conditions (Kazazić, 2004):

- when it is present in a small concentration in relation to the molecule subject to oxidation, it must significantly slow down or prevent the oxidation reaction;
- the resulting radical must be stable in order not to cause a chain reaction.

Depending on the place of origin, antioxidants can be endogenous - which are created inside the body and exogenous - which are introduced into the body with food or drugs. According to the nature and mode of action, they can be divided into enzymatic and non-enzymatic antioxidants (Pham-Huy et al., 2008).

General characteristics of the serpentine geological substratum

Serpentine is a massive metamorphic rock formed by autometamorphism of ultrabasic rocks. The word serpentine is of Latin origin - "serpentinus" and translates as "snake". The characteristic color of these rocks is greenish-yellow, interwoven with winding patterns (Best, 2003).

Serpentine rocks are also called "ultramafic" and "ultramafic", depending on the percentage of a specific mineral from which the rock itself is built. "Ultramafic" rock is a term that refers to rocks that have more than 70% ferromagnesian minerals in their composition, while the term "ultrabasic" refers to rocks that contain up to 45% silicon. Regardless of the aforementioned, for a long period of time most scientists have classified all ultramafic rocks under the name "serpentine" (Brooks, 1987).

Ultrabasic rocks occur within the ophiolitic zone of the Dinarides, where they occupy large areas in this magmatic complex better known as the serpentine zone with a serpentine geological base (Kapović- Solomunet al., 2015).

Serpentinities are made up of one or more serpentine minerals of the same composition, but different structures. The minerals that make up serpentine rocks are: serpentine (more or less fibrous and pale green in color), antigorite (laminar structure and dark green in color), chrysolite (fibrous and paler than serpentine) and



serpophyllite (without pronounced structure and the palest of all minerals). Depending on the combination of minerals, the color of the rock also depends. The parent rock (peridotite) is a rock of volcanic origin, and goes through several stages during its transformation to serpentine. Characteristic levels in the transformation of this bedrock are serpentine, chlorite, asbestos, clay and loess (Knežević-Djordjević and Djordjević, 1976).

It should be emphasized that the surface decay of serpentine rocks is present even today, but on a much smaller scale due to the current atmospheric characteristics. The influence of surface agents leads to the complete destruction of primary ingredients and the creation of dark-yellow to reddish soil. The process of transformation/disintegration of rocks first involves the olivine grains in the cracks of which antigorite appears, which acquires a dark-reddish color. Yellow-brown to reddish soil appears as the end product of decomposition on peridotite terrains, which is largely washed off the surface and is found in deeper parts of the soil profile. Due to the effect of atmospheric precipitation, the ultrabasic environment of the soil is neutralized, because it washes out Mg, as well as oxidizes Fe (Maksimović, 1957).

A specific chemical composition is characteristic of the serpentine geological base. The concentration of heavy metals is greatly increased here, of which the following stand out: iron, nickel, cobalt and chromium, a disturbed ratio of calcium and magnesium, a basic and highly basic pH environment, etc. The specific edaphic characteristics of the habitat on the serpentine substrate are reflected in the floristic and vegetation composition of the plant world, as well as in the anatomical, morphological and physiological characteristics of the plant species. Specific ecological groups of plants - serpentinophytes - live on these geological substrates. They are characterized by special adaptations to this type of habitat, both morpho-anatomical and physiological (Stanković, 2015).

Land on a serpentine geological base

Serpentinite soils are formed on metamorphic, serpentinite rock and occur in various parts of the world. They are formed most often in places above old rocks or where there was once increased magmatic activity. Metamorphic serpentinite rock is formed by the transformation of peridotite (igneous rock) and is rich in magnesium, iron, chromium and nickel. Therefore, in serpentinite soil, the amount of magnesium exceeds the amount of calcium; also basic mineral elements - macroelements, are present in small amounts (nitrogen, potassium, phosphorus), while for most plants toxic heavy metals (chromium and nickel) are present in large concentrations (Stanković, 2015). Since high concentrations of magnesium are present, the availability of calcium to plants is reduced, and the negative effects of high levels of magnesium are most pronounced when the level of calcium in the soil is reduced (Alexander et al., 2007; Vicić et al., 2014).

Such edaphic habitat conditions impose and encourage the development of structural-functional plant adaptations, primarily in connection with the insufficient amount of calcium and its importance for the ionic regulation of biomembranes. In serpentinite



soil there is an extremely unfavorable Sa:Mg ratio, especially in the root zone where, due to the process of respiration and bacterial decomposition, the concentration of carbon dioxide increases, which in the soil solution accelerates the dissolution of magnesium from the serpentinite rock. Serpentinic soils are, by the way, ultrabasic rN reactions (Stanković, 2015).

The distribution of serpentine geological substrata in Serbia

The largest serpentine areas in Europe are located on the Balkan Peninsula (peripheries of Epirus, Thessaly and Sterea Elás in Greece, northern, north-central and southeastern Albania, central and eastern Bosnia, western, southwestern and central Serbia, eastern and central Rhodope Mountains in Bulgaria and gorges of the Pčinja River, southeastern slopes of Šar Mountain in the northeastern part of the Republic of Macedonia) (Jakovljević et al., 2011).

In Serbia, there are several areas with a serpentine geological base, which are located primarily in the central, western and southwestern parts of Serbia, as well as in Kosovo. Some of the characteristic areas with a serpentine geological base stand out: Brđanska klisura, Suvobor, Maljen, Tara, Zlatibor, Mokra Gora, Goč, Stolovi, Kopaonik, the Ibra valley, Peć and its surroundings, Orahovac and Koznik, Ostrovica mountain, etc. (Jakovljević et al., 2011)

The largest serpentine massif in Serbia is Zlatibor, which forms the central part of the spread of this bedrock. From there, the serpentine bed spreads out in several different directions. The northwestern part extends to Bosnia and Croatia, and the southern part is represented along the river Ibar. One part extends along the western slopes of Kopaonik via Studena Planina to Albania, while the other part extends across Kosovo and Metohija, as well as across Macedonia to Greece (Vasić and Diklić, 2001).

Specific adaptations of plants from the serpentine geological substrate

The specific edaphic conditions of the habitat impose and encourage the development of structural and functional plant adaptations, which are primarily related to the insufficient amount of calcium and its importance for the ionic regulation of biomembranes. In serpentinite soil, there is an extremely unfavorable Sa:Mg ratio, especially in the zone of the root system (Stanković, 2015). Thus, serpentine soils are characterized by low calcium values. However, the low concentration of calcium is not the primary cause of the infertility of these soils, but the condition is worsened by an unfavorable Ca/Mg ratio. Calcium has the ability to neutralize harmful effects if it is present in sufficient concentrations of ions (Brooks, 1987). Adaptation to low calcium values occurs through selective uptake, whereby plants retain high levels of calcium in their leaves regardless of low calcium levels in the soil or through the ability of plants to grow despite low calcium levels in the plant (Alexander et al., 2007).

Serpentinic soils are inhabited by a special group of plants - serpentrophytes, which include ecophysiologicaly specifically adapted plant species or subspecies, as well as



special ecological races - i.e. ecotypes of those plant species, which normally grow on some different substrates and soils (especially limestone terrain). Plants from serpentinite soils are adapted to absorb calcium even in conditions where it is present in small amounts (Stanković, 2015).

Serpentinophytes tolerate, excrete or exclude, or immobilize in certain places in tissues and cells, large amounts of magnesium by binding it to oxalates), as well as toxic metals such as nickel, chromium or cobalt. (Stanković, 2015).

The adaptation of plant species to the conditions of serpentine soils is reflected in certain morpho-anatomical specificities that distinguish them from related species that are not adapted to such environmental conditions (Bradu et al., 2005). Already at first glance, the morphological characteristics of serpentinite plants and the physiognomy of serpentinite communities are noticeable, which are characterized by a poorer general appearance of the habitus compared to the vegetation from the surrounding, non-serpentinite habitats. Serpentinophytes have a looser appearance, weak branching, non-specific hairiness, with often a whitish-smooth-shiny surface of leaves that are smaller or narrower than in related species or subspecies from non-serpentinic soils (Stanković, 2015). Due to the specific water, temperature and mineral regime, serpentine habitats are mostly characterized by barren soils. Vegetation on such lands is sparse, uniform, while the plants are characterized by low growth and specific functional and structural adaptive characteristics and do not form a dense assembly (Bradu et al., 2005).

Due to specific chemical, physical and biotic factors that act on serpentinite habitats and cause various morpho-anatomical and physiological adaptations, these adaptations are also called the general term "serpentinic syndrome". In serpentinite syndrome, there is a whole series of solutions that are often observed in serpentinite plants in one and the same habitat, for each species special and specific adaptive responses. The basic morphological characteristics of serpentinite plants are (Stanković, 2015):

- reduction of the size of the aerial part, so that instead of trees, bushy forms develop, and in the case of herbaceous plants, a dwarf and prostrate habit;
- strongly developed and branched root system;
- scleromorphic, small leaves that are smooth or covered with different types of hairs, or are distinct gray-green in color (glaucous) due to a specific wax coating on them.

Aforementioned features are manifested in different ways and to varying degrees in serpentinophytes. For example, on serpentinite habitats in our country, species with smooth leaves, such as *Haplophyllum boissierianum*, and species with hairy leaves, such as *Potentilla visiani* and *Centaurea melanocephala*, can be found next to each other, and next to them, plants with smooth and grayish-green leaves, as in the species *Saponaria intermedia*, or smooth, but distinctly green and finely divided leaves, as in the species *Centaurea koshaninii*. Some of the serpentinophytes are accumulators or hyperaccumulators of certain elements from the serpentinite substrate (Stanković, 2015).



Plant species that accumulate certain elements from the serpentinite substrate have the ability to transfer and accumulate heavy metals in the aerial parts, regardless of the amount of metals in the soil (Baker and Brooks, 1989).

Hyperaccumulators represent plant representatives that accumulate metals in concentrations up to a hundred times higher than those normally present in the plant. Hyperaccumulators can be presented as accumulators that exhibit extreme adaptation in the transfer and accumulation of heavy metals that are present in high concentration in serpentine soils (Brooks et al., 1977; Chaney et al., 2007).

Serpentinophytes can be divided into two groups (Stevanović et al., 2003):

- facultative serpentinophytes that, in addition to serpentine ones, also inhabit habitats on other types of geological substrates;
- obligate serpentinophytes, species that grow only in serpentine habitats.

Between these two groups of plants there are certain differences between structural and functional properties that allow them to adapt to the conditions of the habitat they live in (Stevanović et al., 2003).

Therefore, some serpentinophytes can be marked as distinct indicator species of such habitats - obligate serpentinophytes. On the Balkan Peninsula, such species are: *Cheilanthes marantae*, *Asplenium adulterinum*, *A. cuneifolium*, *Silene paradoxa*, *Echium rubrum*, *Alyssum murale* and others. However, a greater number of species are facultative serpentinophytes, which means that they are able to adapt to serpentinite soils, although their populations are more numerous and lush in limestone habitats. Such species are: *Asplenium ruta-muraria*, *A. ceterach*, *Ramonda nathaliae*, *Teucrium montanum*, *Seseli rigidum*, *Scabiosa fumarioides*, *Potentilla arenaria*, *Dorycnium germanicum*, *Chrysopogon gryllus*, *Cotynus coggigia*, *Pinus mugo*, *Syringa vulgaris*, *Daphne blagayana*, *Erica carnea* and others. (Stanković, 2015).

Also, serpentinite habitats are characterized by the presence of a large number of endemic species, which over a long time have adapted to the special mineral conditions of the soil and have found their refuges and safety in these places, primarily due to less pronounced competitive relations. About 110 endemic species of the Balkan Peninsula are strictly related to the serpentinite substrate, so endemic serpentinophytes make up about 5% of the total endemic flora of the Balkan Peninsula. Balkan endemic serpentinophytes include: *Aster albanicus*, *Potentilla mollis*, *P. visiani*, *Saponaria intermedia*, *Bornmullera dieckii*, *B. tymphaea*, *Leptoplax emarginata*, *Haplophyllum boissierianum*, *Centaurea koshaninii*, *Aristolochia merxmulleri*, *Silene schwarzenbergiana*, *Cerastium smolikanum*, *Gypsophila spergulifolia*, *Alyssum markgrafii*, *Thlaspi epirotum*, *Genista hassertiana*, *Euphorbia serpentini*, *Viola dukadjinica*, *Fumana bonapartei*, *Halacsya sendtneri*, *Onosma stridii*, *Fritillaria epirotica*, *Festucopsis serpentini* and others (Stanković, 2015).



The aim of the research

Based on the fact that the quantitative-qualitative composition and intensity of secondary metabolites play a key role in the adaptation of plants to environmental stress factors, the aim of this work is based on determining the differences in the amount and activity of secondary metabolites of the species *Teucrium montanum* from a habitat with a serpentine geological substrate. The research was carried out in order to determine the variability of the amount and activity of secondary metabolites in the plants living in the investigated localities through a comparative analysis of samples of the investigated species from five different localities with serpentine substrate (Kamenica, Maglič, Orovica, Zlatibor, Divčibare). Variability of the amount and activity of secondary metabolites it was established by analyzing whole plants, shoots, flowers, trees and leaves from the aspect of variability and their adaptive significance. Based on the previously stated facts, **the objectives of the research** are:

- obtaining methanolic extracts from the aerial parts of the plant *Teucrium montanum* from the test sites;
- comparative quantitative analysis of total phenolic compounds in methanolic extracts of *Teucrium montanum* species;
- comparative quantitative analysis of total flavonoids in methanolic extracts of *Teucrium montanum* species;
- comparative quantitative analysis of antioxidant activity of methanolic extracts of *Teucrium montanum* species.

Research results and discussions

Amount of total phenolic compounds

In plant extracts of shoots of the investigated species, the amount of total phenolic compounds is in the range from 11.77 to 145.32 mg GA/g extract, in flower extracts from 100.83 to 138.25 mg GA/g extract, in tree extracts it is in the range from 83.26 to 152.12 mg GA/g extract, while in leaf extracts it is from 98.66 to 129.45 mg GA/g extract.

The highest concentration of phenolic compounds (152.12 mg GA/g extract) was measured in the extract of trees from the Kamenica location, and the lowest concentration (11.77 mg GA/g extract) was measured in the shoot extract from the Orovica location.

The studied localities of Maglič (305 m above sea level) and Orovica (538 m above sea level) represent serpentine rock fields, while Kamenica (447 m above sea level) and Divčibare (873 m above sea level) are localities with coniferous forests. The basic difference between the mentioned localities is reflected in relation to the type of habitat, but also to the existing ecological factors that rule in those habitats.

There is a certain difference if we compare the localities with the highest (Kamenica) and the lowest content of phenolic compounds (Zlatibor). The difference is reflected



in the fact that these two localities differ in habitat type and altitude. Kamenica penetrated conifer forests located on a serpentine geological bed at an altitude of 447 m above sea level. On the other hand, the locality Zlatibor is a pasture with a serpentine substrate and is located at an altitude of 1044 m. Therefore, the differences in the amount of phenolic compounds are reflected in the differences between the investigated localities.

According to Bakhshi and Arakawa, 2006, the amount of secondary metabolites increases with altitude, however, in our study, the opposite results were obtained. Hamid et al., 2010, indicate that environmental factors act simultaneously so that it is not possible to determine the relationship between the amount of secondary metabolites and environmental factors based on only one factor. On the other hand, more thermophilic environmental conditions prevail at lower altitudes, so there is a possibility that for this reason the amount of phenolic compounds is higher in locations with a lower altitude. In addition, due to the increased concentration of heavy metals on the serpentine substrate, the amount of total phenolic compounds is increased.

According to the average values obtained, the highest amount of total phenolic compounds is found in the shoot, then in the stem and flower, and the lowest amount of total phenolic compounds is represented in the leaves.

In the research Stanković et al. (2011), in methanolic extracts of different parts of the plant species *Teucrium montanum* somewhat different results were obtained. The highest value of the amount of total phenolic compounds was found in the shoot extracts, then in the leaf and flower, and the lowest value was found in the tree extract.

Amount of flavonoids

The amount of flavonoids in the plant extracts of the shoots of the investigated species is in the range of 29.87 to 39.71 mg Ru/g extract, in the flower extracts from 23.61 to 36.27 mg Ru/g extract, in the tree extracts it is in the range of 19.93 to 29.77 mg Ru/g extract, while in leaf extracts it is from 30.43 to 45.98 mg Ru/g extract.

The highest concentration of flavonoids (45.98 mg Ru/g extract) was measured in the leaf extract from the Zlatibor site, while the lowest concentration (19.93 mg Ru/g extract) was measured in the tree extract from the Maglič site.

The locations where the highest amounts of flavonoids were measured are located at different altitudes: the Zlatibor location at 1044 m, the Divčibare location at 873 m, while the Maglič location, where the lowest amount of flavonoids was measured, is located at 305 m above sea level, which also represents and the lowest altitude from which the plant material was sampled. This tells us that with increasing altitude, the amount of flavonoids in plants increases.

The highest measured concentration of flavonoids is in Zlatibor, which is located at an altitude of 1044 m. It can be concluded that the altitude has an influence on the total amount of secondary metabolites in the plant, but it is also not a crucial factor for the increased synthesis of secondary metabolites.



The mean values of the amount of flavonoids in extracts of different plant parts show that the highest concentration of flavonoids is found in leaves (38.85), followed by shoots (33.89) and flowers (27.96), while the lowest concentration is found in trees (24.69).

According to Stanković et al. (2011) slightly different values of flavonoid content were obtained. The highest amount of flavonoids in the methanolic extracts of different parts of the plant *Teucrium montanum* was obtained in the shoot extract, somewhat lower values were measured in the leaf and flower extracts, while the lowest flavonoid values were obtained in the stem extract.

The role of flavonoids is reflected in the protective functions of the organism, which is primarily reflected in the antioxidant and antimicrobial effect. The protective role of flavonoids in biological compositions is attributed to their ability to couple, that is, capture electrons of free radicals, chelate ions of transition molecules, activate antioxidant enzymes and inhibit oxidases (Kazazić, 2004). In addition, flavonoids can build complexes with heavy metals, on the basis of which they are the main factors in the adaptation of plants to stressful environmental conditions caused by elevated concentrations of heavy metals (Michalak, 2006).

The different concentration of secondary metabolites in plants can be attributed to the different type of habitat as well as to the abiotic factors on which the populations are represented. Increased synthesis of flavonoids in plants occurs as a response to stress caused by heavy metals in the substrate and plant tissues (Michalak, 2006).

Antioxidant activity

Antioxidant activity in plant extracts of *Teucrium montanum* shoots is in the range from 73.84 to 81.92 $\mu\text{g/mL}$, in flower extracts from 76.21 to 84.21 $\mu\text{g/mL}$, in tree extracts from 77.26 to 81.13 $\mu\text{g/mL}$, in leaf extracts from 72.35 to 84.45 $\mu\text{g/mL}$.

The highest intensity of antioxidant activity (84.45 $\mu\text{g/mL}$) was measured in the leaf extract from the Divčibare site, while the lowest intensity (72.35 $\mu\text{g/mL}$) was measured in the leaf extract from the Orovica site.

The mean values of antioxidant activity range from 78.64 $\mu\text{g/mL}$ to 79.72 $\mu\text{g/mL}$. The highest intensity of antioxidant activity is found in flower extracts, followed by stems and shoots, while the lowest intensity of antioxidant activity is represented by leaf extracts.

Stanković et al. (2011) compared methanolic extracts of different plant parts of the species *Teucrium montanum*, and obtained slightly different results. The highest intensity of antioxidant activity was present in flower extracts, followed by shoot and leaf extracts, while the lowest intensity of antioxidant activity was present in the stem extract.

Based on the results obtained for the amount of phenolic compounds, the amount of flavonoids and antioxidant activity, it can be observed that the plant samples differ in the amount of these parameters. This difference is reflected regardless of the fact that the same geological substrate - serpentine - is represented in all the examined localities. Due to different temperature differences, differences in the water regime,



different amounts of heavy metals and different altitudes, different results were obtained from the localities where the examined plants are located. During growth, plants are exposed to various types of stress factors such as weather, drought, heavy rainfall, air pollution, various pathogens and herbivores. Therefore, the increased concentration of secondary metabolites in plants is a consequence of the physiological adaptation of plants to unfavorable environmental conditions.

The content of secondary metabolites in plants also depends on altitude. With the increase in altitude, the amount of secondary metabolites in plants also increases, which is related to the fact of protecting plants from the negative effect of radiation (Stanković et al., 2013).

Different amounts of heavy metals are present in the soils from the serpentine geological base. Therefore, there are also differences in the composition of secondary metabolites of the plants that were examined from that soil. Due to heavy metals in the soil, the synthesis of secondary metabolites in plants increased (Stanković, 2011).

Conclusion

Based on the review of the literature and the analysis of the content of secondary metabolites as well as the antioxidant activity in the extracts of the aerial parts of *Teucrium montanum* species from different serpentine localities (Kamenica, Maglič, Orovica, Zlatibor, Divčibare), the following conclusions can be drawn :

- *Teucrium montanum* is a perennial, bushy plant, of low growth, with a developed spindle root. Facultative is a serpentinophyte and the largest number of sites was recorded at an altitude between 500 and 1000 m;
- serpentine soils are inhabited by a special group of plants - serpentinophytes, which include ecophysiologicaly specifically adapted plant species;
- the adaptability of plant species to the conditions of serpentine soils is reflected in certain morpho-anatomical specificities that distinguish them from related species that are not adapted to unfavorable environmental conditions;
- g ratio , also basic mineral elements - macroelements, are present in small amounts (nitrogen, potassium, phosphorus), while for most plants toxic heavy metals (chromium and nickel) are present in large concentrations;
- due to unfavorable environmental conditions, plants produce higher concentrations of secondary metabolites that play a role in the adaptation of plants to unfavorable conditions, but also enable interactions with the external environment, thus they have a protective role;
- based on the obtained results, it was proven that the amount of total phenolic compounds, flavonoids, as well as the antioxidant activity in *Teucrium montanum* extracts varies in the examined localities, which is related to the fact that the same ecological factors are not represented in different localities and do not act with the same intensity;
- the obtained results also show that the content of total phenolic compounds, flavonoids and antioxidant activity vary depending on the examined plant organs,



which is conditioned by the different metabolic activities that take place in these parts;

- the highest concentration of phenolic compounds (152.12 mg GA/g extract) was measured in the extract of trees from the Kamenica location, and the lowest concentration (11.77 mg GA/g extract) was measured in the shoot extract from the Orovica location;
- the highest concentration of flavonoids (45.98 mg Ru/g extract) was measured in the leaf extract from the Zlatibor site, while the lowest concentration (19.93 mg Ru/g extract) was measured in the tree extract from the Maglič site;
- the highest intensity of antioxidant activity (84.45 $\mu\text{g}/\text{mL}$) was measured in the leaf extract from the Divčibare locality, while the lowest intensity (72.35 $\mu\text{g}/\text{mL}$) was measured in the leaf extract from the Orovica locality .

Acknowledgements

The organizer gratefully acknowledges the work done by Scientific Committee of the SETI V 2023 International Scientific Conference for efforts done for the success of this event.

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SETI V 2023

Book of Proceedings



CIP - Каталогизacija у публикацији Народна библиотека Србије, Београд

0/9(082)(0.034.2)

IRASA. International Scientific Conference Science, Education, Technology and Innovation (5 ; 2023 ; Beograd)

Book of Proceedings [Електронски извор] / [IRASA] International Scientific Conference Science, Education, Technology and Innovation SETI V, Belgrade, October 14, 2023 ; [editors Vladica Ristić, Marija Maksin, Jelena Bošković]. - Belgrade : IRASA - International Research Academy of Science and Art, 2023 (Belgrade : Instant System). - 1 elektronski optički disk (CD-ROM) ; 12 cm

Sistemski zahtevi: Nisu navedeni. - Nasl. sa naslovne strane dokumenta. - Tiraž 150. - Napomene i bibliografske reference uz radove. - Bibliografija uz svaki rad. - Abstracts.

ISBN 978-86-81512-11-1

a) Наука -- Зборници b) Технологија -- Зборници v) образовање -- Зборници
g) Животна средина -- Зборници d) Одрживи развој -- Зборници
đ) Национална безбедност -- Зборници

COBISS.SR-ID 128307465