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Non-enzymatic antioxidant capacity of *Carex acuta* L. (Cyperaceae)

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Abstract

Carex acuta leaves were sampled on the edge of the glacial lake Gornje bare (National Park "Sutjeska", Zelengora mountain, Bosnia and Herzegovina). Two types of samples were taken: plants that grew on moist soil at the time of sampling, designated as CAm, and plants that grew partially submerged at the time of sampling were designated as CAs. Our work aimed to compare the content of photosynthetic pigments, concentration of total phenolic compounds and flavonoids as well as antioxidant capacity between two CAm and CAs samples. Our results showed that the concentration of total chlorophyll and carotenoids were higher in the CAm sample. Higher concentrations of phenolic compounds (28.987± 0.502 mg/g DW) and flavonoids (22.079 ± 1.471 mg/g DW) were measured in CAs samples. The antioxidant capacity measured by the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) method and phosphomolybdenum method were higher in CAm extract. The abilities to reduce Fe3+ ions and to chelate Fe were higher for CAs samples. Obtained results indicate that the content of phenolic compounds and the antioxidant capacity of plants are significantly influenced by environmental conditions even for the same species. The present study provides the first insight into the antioxidant characteristics of *C. acuta* from the western Balkans.

Keywords: phenolic compounds, flavonoids, submerged, ABTS, Fe chelating ability, Balkans.

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1. Introduction

In natural habitats, plants are exposed to many environmental factors that affect their growth, development, and survival (Kröel-Dulay et al., 2004). The two most important factors that influence plant metabolism are light and water. Sunlight is the basic regulator of the intensity of photosynthesis and therefore the growth and development of plants. In addition to the amount of light, the metabolic processes of plants are significantly affected by the quality, periodicity, and direction of light (Vidović et al., 2017). The visible part of the spectrum is in the range between 400 and 700 nm, i.e., between ultraviolet and infrared,

and is essential for the process of photosynthesis. Light intensity that exceeds the optimum limits for a particular plant species can have an inhibitory effect on the growth and development of plants. The harmful effects of sunlight on biological systems are caused by the ultraviolet part of the spectrum (Diffey, 1991). In the hierarchy of plant needs, water is in second place, right behind the need for energy from sunlight (Lestari et al., 2011). Changes in the amount of available water in the soil have a direct impact on the plant's metabolic processes, causing stress. Water stress can occur due to an excessive supply of water to plants or a water deficit. Plants that are in a water surplus try to survive these conditions through biochemical and physiological adaptations that include changes in the level of hormones, carbohydrate metabolism, energy metabolism, changes in photosynthesis, and protein synthesis (Sairam et al., 2008; Ashraf, 2012) as well as anatomical and morphological adaptations (changes at the level of lenticels, aerenchyma, adventitious roots) (Pedersen et al., 2021).

The consequence of high light intensity or water surplus is an increased concentration of reactive oxygen species (ROS: superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$)). An increase in ROS concentration leads to oxidative stress in the cell (Halliwell and Gutteridge, 2015). In conditions of oxidative stress, damage occurs to essential cellular macromolecules: proteins, nucleic acids, and lipids. By damaging the structure of macromolecules, they change their function and eventually lead to cell death. In response to increased ROS concentrations, cells synthesize or activate enzymatic and/or non-enzymatic antioxidants (Karuppanapandian et al., 2011). The term "antioxidant" refers to any substance that can inhibit or slow down the oxidation of the substrate by interrupting the chain of oxidative reactions or preventing the formation of reactive species (Somogyi et al., 2007). Enzymatic antioxidants include superoxide dismutase, catalase, and peroxidase class III, while the most important non-enzymatic antioxidants are ascorbate, glutathione, and phenolic compounds. Phenolic compounds are ubiquitous in plants, characterized by the abundance of different structural classes and subclasses and a large number of representatives within them. Among the most abundant phenolic compounds are flavonoids and phenolic acids. Flavonoids have a common C6–C3–C6 structure (diphenylpropane skeleton). Their C skeleton consists of two aromatic rings (A and B) connected by a three-carbon chain, often organized as an oxygenated heterocycle (ring C) (Heim et al., 2002). According to their structural characteristics, flavonoids are divided into flavones, flavonols, flavanones, flavanonols, flavanol, anthocyanins, and chalcones. Phenolic acids have one carboxyl functional group, with two basic C skeletons: hydroxycinnamic and hydroxybenzoic structures. Hydroxybenzoic acids most often have a C6-C1 structure (p-hydroxybenzoic, gallic, protocatechuic, vanillic, and syringic acids), while hydroxycinnamic acids have a C6-C3 structure (caffeic, ferulic, p-coumaric) (Cirillo et al., 2012). Antioxidative activities of phenolic compounds are related to their molecular structure: the presence, orientation, and number of hydroxyl groups and double bond conjugation (Rice-Evans et al., 1996). The antioxidant activities of phenolic compounds include various mechanisms: removal of free radicals in direct reactions, metal chelation, and removal of free radicals as substrates for POX (Michalak, 2006; Ferreres et al., 2011; Vidović et al., 2017; Jovanović et al., 2018). In wet ecosystems (swamps, lakes), changes in hydrological conditions are one of the key factors that influence plant growth and development (Zhang et al., 2019). Plants possess various biochemical mechanisms to cope with environmental pressures, and one of them is the activation of non-enzymatic antioxidant metabolism.

The genus *Carex* L. belongs to the family Cyperaceae Juss., which is the third-largest family of monocotyledons. That genus occurs worldwide, includes 1500 to 2000 species, and inhabits moist to wet habitats (POWO, 2023). *Carex acuta* L. is a perennial, caespitose geophyte or hydrophyte with a long, stout rhizome (Chater, 1980). Its native range comprises Azores, Europe to Mongolia, and Western Iran (POWO, 2023). *C. acuta* often occurs as a marginal fringe along rivers, streams, marshes, and hollows (Chater, 1980; Lansdown, 2014). At the global level, *C. acuta* has been assessed as the Last Concern for The IUCN Red List of Threatened Species. Populations of this species are stable in the northern part of its distribution range. However, in much of southern Europe occurrences of *C. acuta* are scattered with locally decreasing populations due to changes in hydrological regimes (Lansdown, 2014).

During field research in the National Park "Sutjeska" (Zelengora mountain, Bosnia and Herzegovina), we found a dense stand of *C. acuta* that formed the marginal fringe of the glacial lake Gornje bare. Individuals of *C. acuta* in their natural habitat (National Park "Sutjeska", Zelengora, lake Gornje Bare) at high altitudes where they are exposed to increased intensity of sunlight, and strong changes in hydrological regimes. In the zone around the lake, some of the individuals were partially submerged, while others grew on moist soil.

Our work aimed to examine the non-enzymatic antioxidant capacity of *C. acuta* and the differences in antioxidant capacity between plants that were on moist soil at the time of sampling and plants that were partially submerged.

2. Material and Methods

2.1. Plant material

Leaves of *C. acuta* were sampled on the edge of the lake Gornje bare (National Park "Sutjeska", Zelengora mountain, Bosnia and Herzegovina) (Figure 1).

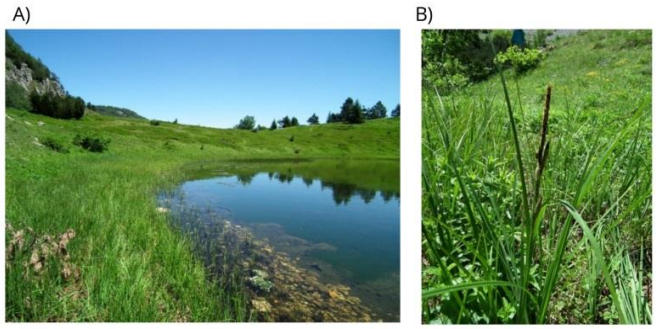

Figure 1. Collection site (A); Carex acuta in natural habitat (B) (Photos by S. Škondrić)

The collection site is located at 1520 m a.s.l. (43.32049˚ N, 18.60683˚ E). Identification of plant material was carried out according to Chater (1980). Voucher specimens are deposited in the Herbarium of the Department of Botany, Faculty of Natural Sciences and Mathematics, University of Banja Luka. Nomenclature and systematics were aligned according to Euro+Med (2023). Individuals were in the anthesis (June). During the collection, care was taken not to harvest individuals and habitat. Two types of leaf samples were taken: plants that were on moist soil at the time of sampling were designated as CAm, while plants that were partially flooded at the time of sampling were designated as CAs.

2.2. Extraction and concentration determination of phenolic compounds

Dry *C. acuta* leaves were used for the work. The leaves were ground to a powder in an electric mill. For the extraction of phenolic compounds, 5 g of ground plant material and 70

mL of 80% ethanol were measured. The samples were sonicated for 5 minutes in an ultrasonic bath. After that, the samples were mixed for 30 minutes on a magnetic stirrer at a speed of 750 rpm. The samples were then filtered using filter paper and the filtrate was centrifuged for 10 min at 10,000 rpm. The supernatant was used for further analyses. The content of total phenolic compounds was determined according to the method given by Singleton and Rossi (1965). Quantification of the content of phenolic compounds was performed based on the calibration curve for gallic acid. The content of total flavonoids was determined according to Chang et al. (2002). The concentration of flavonoids was determined based on the equation of the standard curve for quercentin.

2.3. Determination of the concentration of photosynthetic pigments

The concentration of photosynthetic pigments in the ethanol extract was determined spectrophotometrically by measuring the absorbance at: 664 nm, 649 nm and 470 nm.

2.4. Determination of total antioxidant capacity with the phosphomolybdenum method

Determination of the total antioxidant capacity with the phosphomolybdenum method was determined by the method given by Prieto et al. (1999). The method is based on the reduction of Mo (VI) to Mo (V) by the action of ethanol extracts of plants, whereby the green Mo (V) complex is formed. The antioxidant capacity measured by the phosphomolybdenum method was expressed as the equivalent of vitamin E and vitamin C through standard curve equations.

2.5. Determination of ability to remove ABTS radicals

The ability to remove ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radicals was determined by the method according to Re et al. (1999). The antioxidant capacity is expressed as an IC_{50} value. The IC50 value represents the concentration of phenolic compounds for which achieves 50% inhibition of the formation of ABTS radicals.

2.6. Determination of the ability of the extract to reduce Cu

The method for determining the ability of a plant extract to reduce Cu^{2+} was described in the paper by Apak et al. (2007). As a standard, Trolox was used in a concentration of 20 to 300 µg/mL, and the ability of the plant extract to reduce copper was calculated based on the equation of the standard curve for Trolox.

2.7. Determination of the ability of the extract to reduce Fe

The ability of the extract to reduce Fe, the FRAP (ferric reducing antioxidant power) method, was done according to Benzie and Sreain (1996). The method is based on the reduction of the yellow-colored complex TPTZ (ferric 2,4,6-tripyridyl-s-triazine, which forms a blue-colored complex. The calibration curve was constructed based on the measured absorbance values of known concentrations of the standard $Fe_SO_4x7H_2O$.

2.8. Determination of the ability of the plant extract to chelate Fe

The ability of the plant extract to chelate Fe was determined according to the method of Carter (1971). After measuring the absorbance at 562 nm, the ability to chelate Fe is expressed as the concentration of phenolic compounds that removes 50% of Fe.

3. Results

The concentration of total Chl (a+b) was higher in the sample CAm compared to CAs ($p<0.05$) (Figure 2A). In addition, Car concentration was also higher in the sample CAm ($p<0.05$) (Figure 2B). The ratio of Chl a/Chl b, as well as the Chl (a+b)/Car, were not significantly different between the samples (Figure 2C, D).

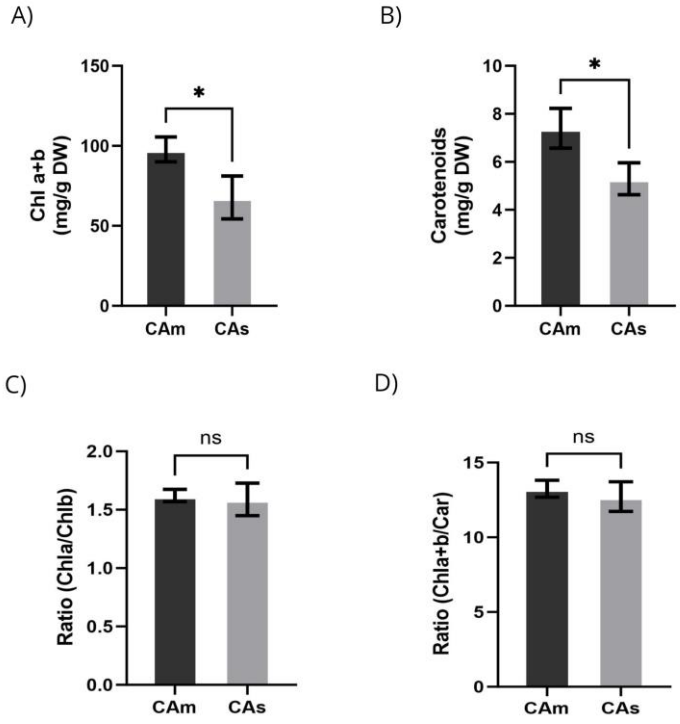

Figure 2. Concentration of total chlorophyll (Chl (a+b)) (A); Concentration of carotenoids (Car) (B); Ratio Chl(a+b)/Car (C); Ratio Chl a/b (D). Asterisk indicate statistically significant differences between samples (* $p<0.05$). ns – no statistical significance

In contrast to the Chl content, higher concentrations of phenolic compounds were measured in CAs (28,987± 0,502 mg/g DW) ($p<0.0001$) samples (Figure 3A). Also, a higher concentration of flavonoids was measured in CAs samples (22.079 ± 1.471 mg/gDW) ($p<0.01$) (Figure 3B).

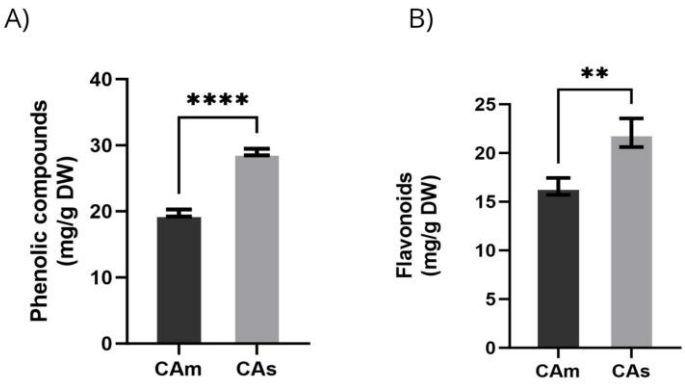

Figure 3. Concentration of total phenolic compounds (A); Flavonoid concentration (B). Asterisks indicate statistically significant differences between samples (** $p<0.01$; **** $p<0.0001$).

The antioxidant activity of the extract measured by the ABTS method was higher in CAm ($p<0.01$) sample (Figure 4A). Also, the total antioxidant activity measured by the phosphomolybdenum method was higher in the CAm sample expressed according to

equivalents of Vitamin E and Vitamin C ($p<0.05$) (Figure 4B, C). The total antioxidant capacity for both samples, CAm and CAs, was higher expressed as vitamin E equivalents.

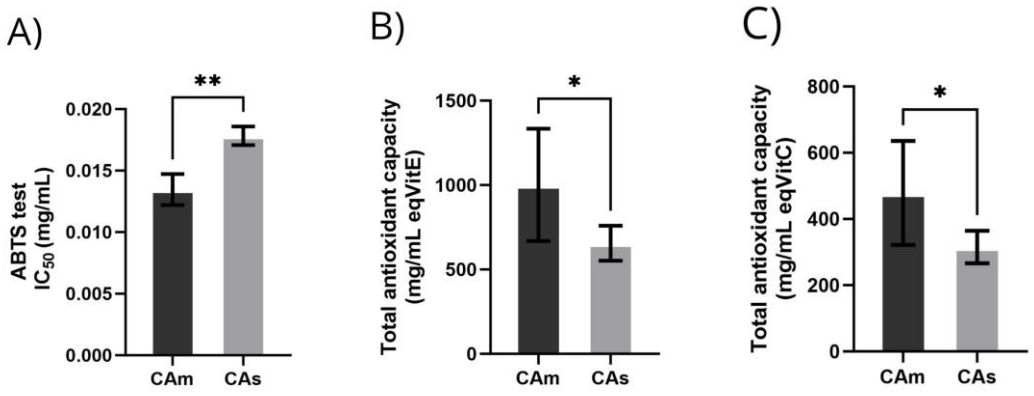

Figure 4. Antioxidant capacity of extracts measured by different methods: ABTS test (A); by the phosphomolybdenum method (B and C). Asterisks indicate statistically significant differences between samples (* $p<0.05$;** $p<0.01$).

Both extracts have a similar ability to reduce Cu^{2+} ions (Figure 5A).

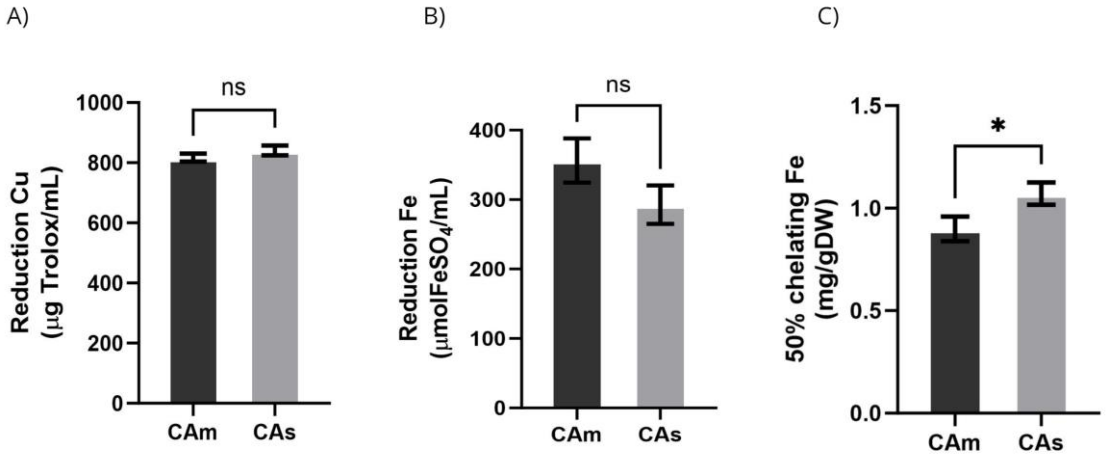

Figure 5. Antioxidant capacity of extracts measured by different methods: reduction of Cu (A); reduction of Fe (B); chelation of Fe (C). Asterisks indicate statistically significant differences between samples (* $p<0.05$). ns – no statistical significance

The ability to reduce Fe^{3+} ions was higher for CAm samples, but without statistical significance (Figure 5B). The CAs sample showed a higher ability to chelate Fe ($p<0.05$) (Figure 5C).

4. Discussion

Photosynthetic pigments determine the photosynthetic capacity, and thus the growth of plants. In natural habitats, plants to some extent could optimize light absorption and photosynthesis by adjusting the content and ratio of photosynthetic pigments (Li et al., 2018). Temperature and water significantly affect photosynthesis. Nagata et al. (2005)

showed that the optimal temperature for chlorophyll synthesis is 30°C. Water is the medium that plants use to transport nutrients, so the amount and frequency of rainfall can affect the content of photosynthetic pigments (Zhou et al., 2003). Changing the water regime in terms of flooding - droughts affect the metabolism and physiological reactions of *Carex schmidtii* Meinsh. (Zhang et al., 2019). The authors showed that in the earlier stages of development, *C. schmidtii* has a better ability to adapt to changes in hydrological conditions. Chlorophyll content in *C. schmidtii* plants exposed to flooding was higher in May, while in June there was a decrease in chlorophyll content (Zhang et al., 2019). Our results show that the content of total Chl a+b was lower in CAs plants, and the reason may be the sampling season (they were sampled in June) (Figure 2A). Carotenoids have several functions in plant cells. In addition to participating in light absorption, they are involved in the photoprotection of cells and have an antioxidant effect. An important antioxidant role has β-Car because it plays a key role in the removal of triplet Chl and singlet oxygen (Esteban et al., 2015). According to our results, the Car concentration was higher in CAm plants (Figure 2B), while the Chl a+b/Car ratio was similar between the two groups of plants (Figure 2D). Other studies also showed a decrease in the concentration of carotenoids in the leaves of terrestrial plants Vigna radiata (L.) R. Wilczek (Kumar et al., 2013), *Triticum aestivum* L. (Collaku and Harrison, 2002), *Allium fistulosum* L. (Yiu et al., 2008) and *Zea mays* L. (Lukić et al., 2021) when exposed to a water surplus.

Phenolic compounds are involved in numerous metabolic processes in plant cells and have many functions: in the processes of growth and reproduction, they provide morphological and sensory properties to plants (pigmentation, aroma), they have an important defensive role against herbivores, pathogens, and an antioxidant role in conditions of exposure of plants to various types of stress, a protective role against UV radiation (Turunen et al., 1999; Vidović et al., 2017; Březinová and Vymazal, 2018; Veljović Jovanović et al., 2018). The concentration of phenolic compounds is influenced by genetic predispositions and environmental factors. The qualitative and quantitative composition of phenolic compounds differs between species, and differences can also exist between genotypes of the same species, which is significantly influenced by exposure to environmental factors (Smolders et al., 2000; Březinová and Vymazal, 2018). Phenolic compounds (localized in vacuoles and cell walls of epidermal cells, and very often in hairs on the leaf surface, play an important role in the absorption of UV rays in superficial plant tissues (Landry et al., 1995). With increasing intensity of UV-B radiation, the synthesis of phenolic compounds (e.g., tannins) is induced in leaves (Turunen et al., 1999).

As shown in our results, the measured concentration of phenolic compounds was higher in partially submerged samples (Figure 3A). Phenolic concentration in CAs sample was very similar to the concentration of total phenolic compounds in the aerial part of *Carex nigra* (L.) Reichard (27 mg/g DW) (Březinová and Vymazal, 2017). Based on the obtained results, Březinová and Vymazal (2017) concluded that the content of phenolic compounds is affected by the season, as well as the environmental conditions. In our experiment, the content of flavonoids also differed between the samples even though it was the same species which could be attributed to the surplus of water to which CAs plants were exposed at the time of sampling. An increase in the total concentration of polyphenols, such as flavonoids, is a response to various abiotic stress factors (water deficit, UV radiation, low temperatures, etc.) (Chalker-Scott, 1999; Sakihama et al., 2002; Michalak, 2006).

Our results showed that CAs leaves have a significantly higher content of phenolic compounds, and flavonoids compared to CAm plant (Figure 3). Such results may indicate that the conditions of the habitat significantly influence the synthesis of phenolic compounds because they are the same plant species. It is possible that exposure to partial submergence induced oxidative stress and an increase in phenolic compounds synthesis as important antioxidants. On the other hand, *C. acuta* plants in moist soil are possibly more exposed to herbivores and birds because they are an important source of food (Huang et al., 2022), so the response to biotic stress can lead to increased consumption of phenolic compounds. The analysis of 18 species of the genus Carex showed that they differ based on phenolic acid content (Bogucka-Kocka et al., 2011). Differences in the composition of phenolic acids can be caused by habitat and ecological conditions. Carex spp. are plants that inhabit very different habitats: wet and moist (peatlands, swamps, meadows) and dry and extremely dry habitats (Bogucka-Kocka et al., 2011). One of the possible reasons for their plasticity and settlement of different habitats may be the possibility of rapid changes in the metabolism of phenolic compounds. Antioxidant activity is a critical indicator of the bioactivity of plant extract. Plant extracts are complex mixtures of chemical compounds that differ in terms of functional groups, polarity and chemical properties, and whose qualitative and quantitative proportion significantly affects the biological activities of the extract. The ABTS method is often used to estimate the antioxidant capacity of plant extracts.

Our results show that both extracts, CAm, and CAs, have a low IC50 value for ABTS indicating significant antioxidant activity (Figure 4A). CAm extract had a lower IC50 value and a significantly higher antioxidant capacity (Figure 4B, C), although they have a lower content of phenolic compounds. Such results can indicate that the antioxidant activity of the extract is determined not only by the total concentration of phenolic compounds but also by the presence of specific phenolic compounds. The reason may be the conditions of the habitat in terms of the influence of different types of biotic and abiotic stress on the synthesis of specific phenolic components. The ABTS IC_{50} value for CAm was 13.46 mg/mL (Figure 4A), which indicates a lower antioxidant capacity compared to the essential oil extract of *Carex meyeriana* Kunth (10.79 mg/mL) (Cui et al., 2018). The authors showed that ABTS IC_{50} of *C. meyeriana* was much higher than the IC_{50} for standards: vitamin C (IC_{50} 1.02 µg/mL) and butylated hydroxytoluene (IC_{50} 0.01 µg/mL). Measuring the total antioxidant capacity by the phosphomolybdenum method is important because it shows the antioxidant capacity of the hydrophilic and hydrophobic components of the extract. The obtained results also indicate that the CAm extract has a higher total antioxidant capacity measured by the phosphomolybdenum method (Figures 4B, C). Transition metals (Fe and Cu) are essential elements, however, at increased concentrations, in Fenton-type reactions with H_2O_2, they can lead to the formation of an extremely reactive and dangerous hydroxyl radical that disrupts the structure and function of cellular macromolecules. Due to participation in the Fenton reaction, plants must possess secondary metabolites capable of reducing the flow of transition metals through the Fenton reaction. As our results show the reducing ability of the extracts to reduce Cu and Fe was not significantly different between the two extracts (Figures 5A, B). As for other measured parameters, according to the available literature, we were unable to find data on the reducing ability of *C. acuta*. Data on the reducing ability we found for essential oil of *C. meyeriana* plants of the same genus (Cui et al., 2018). The authors showed that the reducing activity is dependent on the concentration (EC_{50} 12.59 mg/mL) and that it is significantly lower than the reducing ability of the vitamin C as standard (EC_{50} 0.03 µg/mL) and butylated hydroxytoluene (EC_{50} 0.12

μg/mL). In addition to Fe reduction, an important way of regulating elevated Fe concentrations in the cell and preventing participation in the Fenton reaction is chelation. In our research, plant extract CAm showed a slightly higher ability to chelate Fe (Figure 5C).

The methods for determining the antioxidant capacity include different reaction mechanisms. It is important to test the antioxidant activity of the extract using different methods to get a more complete picture of its antioxidant activity. Our results show that the antioxidant capacity is not only influenced by the total concentration of phenolic compounds but also by the specific components of the extract.

5. Conclusions

The paper compared the content of phenolic compounds and antioxidant capacity in *C. acuta* leaves between individuals growing on moist soil and partially submerged individuals. The obtained results showed differences between the two samples that can indicate the influence of environmental conditions (hydrological regime) on the content of phenolic compounds and antioxidant capacity. Also, the differences in antioxidant capacity between the samples, obtained for different methods, may indicate that, in addition to the quantitative the antioxidant capacity is also affected by the qualitative composition of phenolic compounds. According to the available literature, these are the first data on the antioxidant characteristics of *C. acuta* from the western Balkans. This work opens a lot of questions and possibilities for future work that could include examination of the enzymatic antioxidant metabolism and other biochemical and physiological mechanisms that enable *C. acuta* to quickly adapt to significant changes in water level and other ecological factors that life at higher altitudes brings.

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