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# New data on the *Austropotamobius torrentium* (Schrank, 1803) from the tributaries of the Bosna River

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## Abstract

The crayfish *Austropotamobius torrentium* is a dominant species of the family Astacidae in the rivers of the Black Sea and Adriatic basins of Bosnia and Herzegovina. Molecular phylogenetic studies in Europe revealed the presence of eight phylogroups. No morphometric and meristic characteristics have been determined that would clearly separate them. The paper presents new data on the morphometric parameters species Austropotamobius torrentium with watercourses Presjenica, Jošanica and Crna Rijeka. This analysis is based on 22 parameters that were measured on 38 crayfish individuals, whose size was larger than 5 cm. Using Anova test, Bonfferoni test and discrimination analysis, it was determined that females differ significantly in a small number of parameters, while males showed greater morphological divergence. The great diversity of habitats of Austropotamobius torrentium in Bosnia and Herzegovina obliges the inclusion of the Dinarides region in molecular phylogenetic studies. This will result in more precise and complete data on Austropotamobius torrentium populations.

Keywords: stone crayfish, protect, morphometrics, distribution, divergence

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## 1. Introduction

The crayfish *Austropotamobius torrentium* is the smallest species of the Astacidae family that is still not well known. It is widely distributed in Bosnia and Herzegovina, and its area ranges vertically from 175 to 1100 m (Karaman, 1963; Trožić-Borovac, 2011; Roljić et al., 2021, Trožić-Borovac et al., 2022). As in other European countries, it dominates in hilly and mountainous areas, in sheltered and preserved habitats, with well-developed climatogenic coastal vegetation and lower water temperatures. It is believed that the origin of this species

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is from Central and Southeast Europe (Manfrin et al., 2022). It is listed as a data deficient species (DD) on the IUCN Red List of Threatened Species Füreder et al. (2010). It is internationally protected by the Bern Convention (Appendix III, protected animal species), and by the EU Habitats Directive 92/43/EEC, where it is listed in Appendix II (the central areas of the species' habitat are designated as Areas of Community Importance (SCI) and included in the Natura 2000 network) and in Appendix V (member states can decide how to manage the population, but must ensure that their exploitation and collection from the wild is compatible with maintaining a favorable conservation status). This species is protected by national legislation in most of its range (Füreder et al., 2010, Vigneucs et al., 2002), and in Bosnia and Herzegovina it is included in the Red List of Threatened Species (Škrijelj et al, 2013). The results of previous research (morphology, molecular-genetic, evolutionary) of the species indicate a high genetic diversity represented by eight different mtDNA lineages/phylogroups that have been discovered so far (Klobučar et al., 2013; Petrušek et al, 2017; Lovrenčić et al., 2020b).

## 2. Materials and Methods

Sampling was carried out in Presjenica, Crna rijeka and Jošanica streams in the period from May 2016 to july 2022. The researched watercourses are tributaries of the Željeznica River (Crna Rijeka), the Vogošće River (Jošanica) and the Bijela River (Presjenica). The distance between Crna Rijeka and Presjenica is 1.5 km, and Jošanica is 25 km from the Presjenica River. The riverbanks in the researched sections have partially preserved climatogenic vegetation, without visible sources of degradation, but with the presence of anthropogenic influence (roads, tree felling, livestock, agriculture, cottages, settlements, etc.). All three investigated watercourse sections are in the hilly-mountainous zone with oak-hornbeam forests (table 1). The largest number of microhabitats was registered in the river Presjenica, while Crna rijeka and Jošanica have a smaller number of microhabitats. For water temperature and oxygen concentration, the average measurements were taken during the research (Oxi 3205 Set 3 2BA103 WTW), and the depth was measured several times during the research. Individuals were caught by hand, with a benthos net or hand-made traps that were left overnight. A large number of smaller individuals were observed on all three streams, which were only recorded. Only specimens larger than 50 mm were used for analysis.

### *2.1. Morphometric analysis*

A total of 38 individuals (18 males and 19 females) were analyzed. Morphometric measurements were carried out according to the work of Sint.all. (2005) and all measured morphometric characteristics were normalized for size by dividing them by the corresponding postorbital length (POL = HEL + ARL). A total of 22 morphometric characters were used for analysis: claw length (CLL), claw width (CLW), claw height (CLH), length of the claw palm (CPL), length of the claw finger (CFL), rostrum length (ROL ), rostrum width (ROW), head length (HEL), head width (HEW), areolar length (ARL), areolar width (ARW), abdomen length (ABL), abdomen width (ABW), abdomen height (ABH), telson length (TEL), telson width (TEW), carapace width (CPW), width at the cervical groove (CGW), width of the carapace at the hind edges (CEW), carapace height (CPH), and total length (TL ). All the characteristics were measured with a digital calliper with a precision of 0.01 mm. One-way ANOVA (Post-Hoc-test: Bonferroni) was applied to select morphometric parameters for each group (sex) that differed significantly from other populations. Males and females were analyzed separately because crayfish show sexual dimorphism after reaching sexual maturity (Grandjean et al., 1997, Vlach & Valdmanová, 2015). Multivariate discriminant analysis was used to reveal the morphometric parameters that best characterize different crayfish populations. IBM SPSS Statistics 25 and Excel 2016 software were used for statistical analysis.

## 3. Results

The average total body length (TL) of males from the Crna River was 74.79±1.16 mm, males from the Presjenica River are slightly longer 74.33 ±14.74 mm, and the smallest are from Jošanica 60.21± 12.55 mm. Using the Bonferoni test, a statistically significant difference was found in the width of the rostrum ($p=0.002$), head length HEL ($p=0.002$), (ARL) areolar length ($p=0.000$), carapace width CPW ($p=0.001$), width of the carapace at the hind edges (CEW) ($p=0.006$), abdominal length (ABL) and total body length TL ($p<0.05$). The results of the discriminant analysis determined that males are discriminated the most with the highest loadings in discriminant functions for body length (TL), head length (HEL), cheliped length (CLL) and rostrum width (ROW). Whereas if crayfish individuals have a longer head, a larger width of chelipeds (CLW), a smaller width of the rostrum, it is more likely that they are from the Presjenica River (Table 1-2, Figure 1).

*Tabela 1. Values of measured abiotic parameters on the investigated watercourses*

| river | Altitude | depth | T °C | $O_2$ mg/l | Bottom substratet | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Pebbles | Sand | Stone | fital |
| Crna rijeka | 610 | 450 | 10 | 10,05 | 25 | 40 | 30 | 5 |
| Presjenica | 620 | 370 | 11 | 9,85 | 20 | 60 | 10 | 10 |
| Jošanica | 629 | 200 | 12 | 9,45 | 70 | 25 | 5 | 0 |

*Table 2. Standardized canonical discriminant function coefficients for morphometric characteristics of Austropotamobius torrentium males for each discriminant function; TL total length; W weight, CEW carapace width at the hind edges; ROL rostrum length; ARW areolar width; ROW rostrum width; ARL areolar length; ABL abdomen length, HEL head lenght; CGW width at the cervical groove, CLL claw lenght, CPW carapace width; % expl.var. percentage of explained variance; cum. Prop., cumulative proportions; canonical r, canonical correlations*

| Characteristic | Function 1 | Function 2 |
|---|---|---|
| ROL | -3.903 | -2.173 |
| ROW | 5.503 | -0.545 |
| HEL | -4.435 | 6.220 |
| CGW | 3.206 | -3.682 |
| CLL | 1.769 | -2.399 |
| ARL | -6.295 | 3.189 |
| CLW | -3.463 | 2.348 |
| CPW | 3.328 | 7.696 |
| ARW | .379 | -.132 |
| CEW | 5.415 | 3.937 |
| ABL | -8.883 | -8.804 |
| TL | 24.015 | -8.611 |
| W | -15.949 | 2.980 |
| Eigenvalue | 288.460 | 108.218 |
| % of Variance | 72.7 | 27.3 |
| Cumulative % | 72.7 | 100.0 |
| Canonical Correlation | 0.998 | 0.995 |

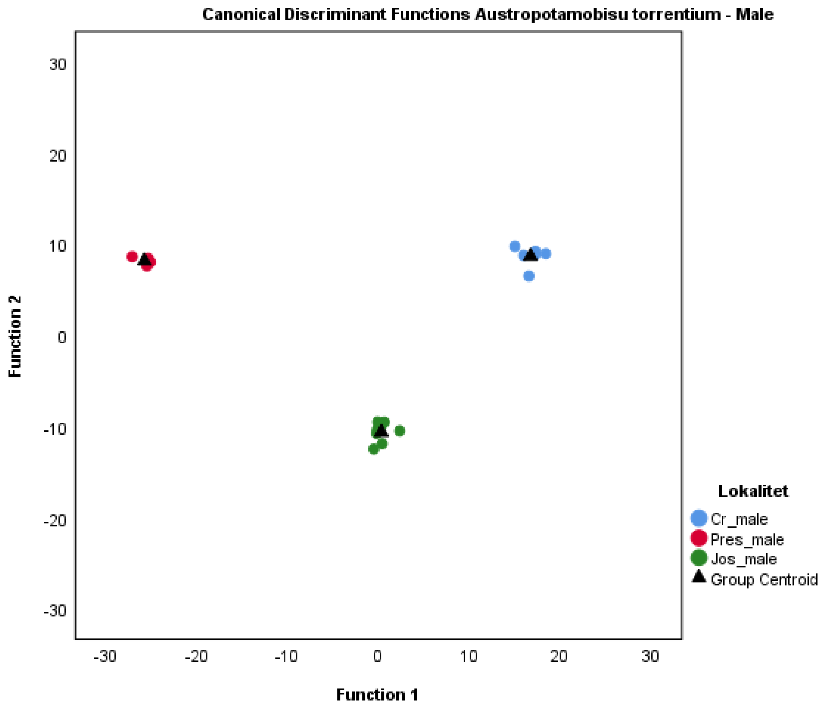


*Figure 1: Discrimination of the different populations of males of austropotamobius torrentium by the first two discriminant functions*

Anova test in females revealed a significant difference in the characters head lenght HEL (p=0.006), ARL (p=0.001), CEW (p=0.01). The Bonferroni test showed that there is a significant difference between the individuals of Presjenica and Crna Rijeka (p=0.002) and Presjenica and Jošanica 0.006 in the ARL parameter, in CEW Presjenica and Crna Rijeka (p=0.038) and Presjenica and Jošanica (p=0.034), and in HEL Jošanica and Presjenica (p=0.006). Discriminative analysis of females revealed that individuals are most discriminated by the parameters CLW, ARW and width at the hind edges of the carapace (CEW), and (ABL) abdomen lenght (Table 3, Figure 2).

*Table 3. Standardized canonical discriminant function coefficients for morphometric characteristics of Austropotamobius torrentium females for each discriminant function; ROL, rostrum length; ROW, rostrum widt; CEW, carapace width at the hind edges; ARW, areolar width; ARL, areolar length; ABL, abdomen length; HEL, head lenght; CGW width at the cervical groove, CLL claw lenght, CPW, carapace width; % expl.var., percentage of explained variance; cum. Prop., cumulative proportions; canonical r, canonical correlations*

| Characteristic | Function 1 | Function 2 |
|---|---|---|
| ROL | 0.173 | 0.454 |
| ROW | -1.247 | -1.354 |
| HEL | -1.698 | -1.491 |
| CGW | 10.154 | 11.430 |
| CLL | -1.830 | 5.021 |
| ARL | 2.783 | 2.529 |
| CLW | 5.014 | -2.746 |
| CPW | -12.955 | -16.889 |
| ARW | -1.332 | 3.662 |
| CEW | 4.212 | 1.366 |
| ABL | -2.299 | -.563 |
| Eigenvalue | 11.110 | 62.2 |
| % of Variance | 62.2 | 37.8 |
| Cumulative % | 62.2 | 100.0 |
| Canonical Correlation | 0.958 | 0.933 |

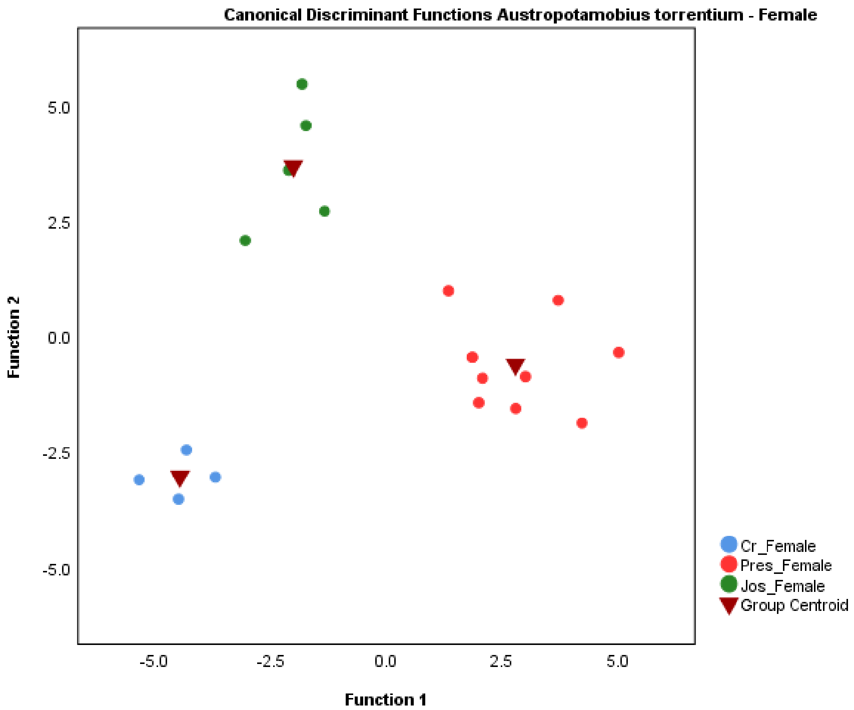


*Figure 2: Discrimination of the different populations of females of Austropotamobius torrentium by the first two discriminant functions*

## 4. Discusion

Based on the analysis, relatively stable crayfish populations were determined in Jošanica, Presjenica and Crna Rijeka. Morphometric measurements show statistically significant differences in females in CEW, ARL and HEL. In males, statistically significant differences ($p>0.05$) were observed for head length, TL - total body length and cheliped length (CLL). According to research (Maguire et al. 2017, Trožić-Borovac et al. 2022), male crayfish individuals show a statistically significant difference in body length and pincer length, as an adaptation to habitat conditions. Discriminative analysis confirmed that populations from geographically close areas show differences in morphometric parameters, as a response to ecological conditions and the degree of anthropogenic pressures. Research on males from the populations of Sopotski Slap, Zeleni Vir and Blato (Maguire et al., 2017) found that males differ significantly in rostrum length, CPH and telzone width (TEW). Unnamed stream, Preodac and the Korana river in the locality of Bosansko Grahovo (Lovrenčić et al, 2020b), and research area of this paper, Crna rijeka, Presjenica and Jošanica are inhabited by representatives of the phylogroup central and south-eastern Europe (CSE). Morphometric analysis within the same phylogroup showed diversity, which was stated for the area of Croatia (Maguire et. al. 2017, Lovrenčić et al. 2020a) and Romania (Pârvulescu et al., 2019). In similar studies, males from three different populations were discriminated based on the shape of the chelipeds (CLW, CLL, CFL, CPL, CPH) and rostrum length (ROL ), rostrum width (ROW). (Maguire et. al., 2017). Males and females show different adaptations to environmental conditions, and on the other hand, ecological conditions result in different adaptations. Species delimitation requires integrative taxonomy, and an approach that combines molecular, morphological, ecological, and geographic data to build species hypotheses. Pârvulescu et al. (2019), have identified the existence of a new phylogroup endemic to the Romanian Apuseni Mountain Region (APU). The molecular studies of this species included morphological data that resulted in the description of a new species of the genus *Austropotamobius*. It is necessary to implement the provisions for the protection of habitats of this species, which are also under significant anthropogenic pressure in the analyzed watercourses. The intensity of these impacts is particularly pronounced on the Jošanica watercourse.

## 5. Conclusion

Morphometric measurements of *Austropotamobius torrentium* populations, in two geographically close watercourses and one distant one, which include a large number of parameters, proved to be a powerful tool in the analysis of the similarity/difference of the conditions in which they exist. A lower degree of diversity, especially in female individuals, confirms their belonging to the same phylogroup but also to similar ecological conditions in which they exist. For more complete data, it is necessary to include new meristic parameters and intensive molecular phylogenetic research.

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# Diversity and distribution of genus *Amegilla* Friese, 1897 in Bosnia and Herzegovina (Hymenoptera: Apoidea)

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## Abstract

In Bosnia and Herzegovina, the genus *Amegilla* Friese, 1897 is represented by four species. The known diversity of species of the genus *Amegilla* in Bosnia and Herzegovina was compared with current data on the diversity of bees in the Balkan Peninsula and Europe as a whole. Through extensive field research carried out in Bosnia and Herzegovina, the presence of the following *Amegilla* species was established: *Amegilla albigena* (Lepeletier, 1841), *Amegilla garrula* (Rossi, 1790), and *Amegilla quadrifasciata* (de Villers, 1789). Additionally, the species *Amegilla magnilabris* (Fedtschenko, 1875) was documented based on specimens stored in the Zoological Collection of the National Museum in Bosnia and Herzegovina. The *Amegilla* species are distributed in the Mediterranean and Pannonian regions. It's significant to mention that the distribution of *Amegilla* species in Bosnia and Herzegovina appears to be influenced by specific climatic and geographic factors. These species tend to be absent in the Dinaric region characterized by a continental climate, suggesting a preference for other ecological niches. Analyzing the distribution data further reveals the thermophilic nature of *Amegilla* species, primarily favoring open-type habitats. The research results highlight the significance of certain climatic variables in shaping their habitat suitability. For instance, the variable Bio 9, representing the mean temperature of the quarter with the least rainfall (the driest period), significantly influences the suitability of the habitat for *Amegilla albigena* (38.0%) and *Amegilla quadrifasciata* (16.1%).

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Meanwhile, for *Amegilla garrula*, the variable Bio 1, representing the mean annual temperature, plays a pivotal role, accounting for 36.2% of its habitat suitability. These findings underscore the importance of understanding the intricate relationships between climatic factors and species distribution, shedding light on the ecological intricacies of *Amegilla* species in Bosnia and Herzegovina.

**Keywords:** Solitary bees, biogeography, biodiversity, digger-bees, blue-banded bees.

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## 1. Introduction

The genus *Amegilla* Friese, 1897 belongs to the tribe Anthophorini, a group within the family Apidae that encompasses a vast diversity of bee species, totaling over 750 worldwide. Within this extensive genus, *Amegilla* comprises approximately 260 species distributed across various regions globally, with 36 of these species found in the West-Palaearctic region. *Amegilla* species, like their fellow Anthophorini members, are known as "digger bees" due to their distinctive nesting behavior, which involves excavating simple nests in dry ground. They are solitary bees, meaning they do not form colonies, and they are particularly well adapted to arid or sub-arid ecosystems, including matorrals, steppes, sub-deserts, and deserts. Only a small number of *Amegilla* species venture north of the 45th parallel where they are restricted to thermophilic habitats (Brooks, 1988).

In terms of their foraging habits, *Amegilla* bees primarily feed on flowers with long corollae, such as those from the Boraginaceae, Lamiaceae, Leguminosae, Scrophulariaceae, and Compositae families. They are also known for their impressive flying abilities, which enable them to move at high speeds. This agility makes them efficient pollinators, and it's important to note that their abundance and distribution may sometimes be underestimated due to their quick and agile nature. Due to their agility, their abundance and distribution may be underestimated. In Europe, the genus *Amegilla* is represented by 10 species, of which seven species are recorded in the Balkan Peninsula (Rasmont, 2014). In the bee fauna of Greece, the genus *Amegilla* is represented with a total of seven distinct species recorded. As we move northward, however, the number of *Amegilla* species gradually declines. In the neighboring faunas of Serbia and Croatia, the genus *Amegilla* is represented by five species each, indicating a slightly lower diversity compared to Greece. Further to the west in Bosnia and Herzegovina, four species of *Amegilla* have been recorded, while in Slovenia, three species have been documented.

Within the western Balkan Peninsula, the genus *Amegilla* is notably represented by five species, all of which have been included in the bee checklists of Croatia (as recorded by Vogrin in 1918), Slovenia (as documented by Gogala in 2023), and Serbia (as noted by Mudri-Stojnić in 2021). These records serve as a valuable resource for understanding the distribution and diversity of *Amegilla* species in the region, contributing to our broader knowledge of bee populations and their ecological roles within these ecosystems. The primary aim of this paper is to offer a overview of the species belonging to the genus *Amegilla* found in Bosnia and Herzegovina. Additionally, the paper aims to conduct a detailed analysis of the distribution patterns of these species concerning the biogeographical regions within Bosnia and Herzegovina, as well as in neighboring countries. Data on the distribution of species of the genus *Amegilla* from western Balkan were used for ecological niche modeling. Using bioclimatic variables in raster form and the distribution of known geocoordinates of the site, a map of the predictive model was created to predict the distribution of species from the genus *Amegilla* within the raster GIS environment in Bosnia and Herzegovina. In essence, this research represents a systematic effort to compile and analyze data on Amegilla species, both within Bosnia and Herzegovina and in its neighboring regions. The utilization of predictive modeling techniques, coupled with bioclimatic variables, enables a deeper understanding of the distribution dynamics of these species, contributing valuable insights to the field of biodiversity and ecological research.

## 2. Material and Methods

The ecological niche modeling of the genus *Amegilla*, a diverse dataset was assembled to analyze their distribution in the western Balkan region. This dataset included a total of 40 GPS points from the western Balkan area, with 27 of them representing records from Bosnia and Herzegovina. To build this dataset, a combination of literature data and various sources of distribution data were utilized. Literature review provided an overview of published information on bee diversity in the Balkan Peninsula. These sources covered several countries, including Bosnia and Herzegovina (Apfelbeck, 1896), Croatia (Vogrin, 1918), Serbia (Mudri-Stojnić, 2012, 2018, 2021, Nieto et al., 2014, Kuhlmann et al., 2020, Rafajlović and Seleši, 1958, Vogrin, 1955, 1918, Mocsáry, 1897, Markov 2017, Markov et al., 2016, Živojinović, 1950, Petrik, 1958, Lebedev, 1931), Slovenia (Gogala, 2023) and Montenegro (Apfelbeck, 1896). Additional distribution data for

*Amegilla* species were collected from various online platforms, including the Atlas Hymenoptera, Global Biodiversity Information Facility (GBIF), and iNaturalist. These sources contributed to creating a more diverse dataset for the western Balkan region. The additional data source for the distribution of genus *Amegilla* in Bosnia and Herzegovina was the entomological collection of the National History Museum in Bosnia and Herzegovina and the bee collection created by field research of bees in the period 2018-2023.

The analysis of the distribution of *Amegilla* species was conducted using the Maxent 3.4.4 (Steven et al. 2023), a widely used tool for ecological niche modeling. Climate data layers with a spatial resolution of 1 km² (Fick and Hijmans, 2018) were obtained from the WorldClim database and processed to ensure consistency in terms of resolution and coordinate system (WGS 84 CRS) for the entire western Balkan Peninsula. Out of the 19 available bioclimatic variables, 12 were selected for use in the modeling process, while the remaining seven were excluded due to their lack of significance or high correlation with other variables (Table 1). Discontinuity in used bioclimatic variables was tested according to Booth (2022). In the modeling process, raster layers for the western Balkans were cut "as an extent" to cover the entire pixel and aligned in the QGIS program. The pixel values for each climate variable were then assigned to each GPS point representing *Amegilla* species' presence using QGIS 3.10. The ecological niche model was developed using the maximum entropy method implemented in Maxent 3.3 (Phillips et al. 2006), and the resulting model was projected onto geographic space, creating a predictive map. Model testing was conducted through 10 repetitions using the Bootstrap method, with 40 GPS points of *Amegilla* species included in the analysis. Twenty-five percent of the points were reserved for testing the model.

*Table 1. Climate variables used in ecological niche modeling*

| **Variable acronym** | **Description of the bioclimatic variable** | **Variable acronym** | **Description of the bioclimatic variable** |
|---|---|---|---|
| Bio1 | Annual Mean Temperature | Bio10 | Mean Temperature of Warmest Quarter |
| Bio4 | Temperature Seasonality (standard deviation ×100) | Bio11 | Mean Temperature of Coldest Quarter |
| Bio5 | Max Temperature of Warmest Month | Bio12 | Annual Precipitation |
| Bio6 | Min Temperature of Coldest Month | Bio15 | Precipitation Seasonality (Coefficient of Variation) |
| Bio7 | Temperature Annual Range (BIO5-BIO6) | Bio18 | Precipitation of Warmest Quarter |
| Bio8 | Mean Temperature of Wettest Quarter | Bio19 | Precipitation of Coldest Quarter |
| Bio9 | Mean Temperature of Driest Quarter | | |

## 3. Results

As previously mentioned, both Croatia and Serbia have documented the presence of five *Amegilla* species: *Amegilla albigena*, *Amegilla garrula*, *Amegilla quadrifasciata*, *Amegilla magnilabris*, and *Amegilla salviae*. While *Amegilla salviae* is one of the five species recorded in Serbia and Croatia, it has yet to be officially documented in Bosnia and Herzegovina. However, the possibility of the presence of *Amegilla salviae* in Bosnia and Herzegovina cannot be ruled out entirely. This is particularly plausible considering the species' Mediterranean distribution and the fact that it has been observed in neighboring regions. In terms of distribution patterns, the data reveals an equal representation among the species *Amegilla albigena*, *Amegilla garrula*, and *Amegilla quadrifasciata*, each with a range of 8 to 11 records (Table 3, Figure 1.). On the other hand, *Amegilla magnilabris* has only been identified in Sarajevo, standing as the sole locality within Bosnia and Herzegovina where this species has been observed.

*Table 2. Overview of species from the genus Amegilla for Bosnia and Herzegovina and neighboring countries on the Western Balkan Peninsula: BiH - Bosnia and Herzegovina, Srb - Serbia, Croatia - Croatia, Slo - Slovenia*

| Species | BiH | Srb | Cro | Slo |
|---|---|---|---|---|
| 1. *Amegilla (Zebramegilla) albigena* (Lepeletier, 1841) | + | + | + | + |
| 2. *Amegilla (Amegilla) garrula* (Rossi, 1790) | + | + | + | + |
| 3. *Amegilla (Zebramegilla) magnilabris* (Fedtschenko, 1875) | + | + | + | |
| 4. *Amegilla (Amegilla) quadrifasciata* (de Villers, 1789) | + | + | + | + |
| 5. *Amegilla (Zebramegilla) salviae* (Morawitz, 1876) | | + | + | |

The vertical distribution profile of *Amegilla* species in Bosnia and Herzegovina shows distinctive patterns. Approximately 75% of the recorded findings of *Amegilla* species fall within the altitude range of 0 to 300 meters above sea level. However, all the findings of *Amegilla* species at altitudes exceeding 300 meters above sea level exclusively belong to the species *Amegilla garrula*. *Amegilla garrula* is known to be associated with the Mediterranean and sub-Mediterranean regions of Bosnia and Herzegovina, particularly within the altitude range of 0 to 600 meters above sea level. Importantly, during field research conducted in both the Pannonian and Dinaric climate regions of Bosnia and Herzegovina, no *Amegilla* species were recorded. However, discoveries of *Amegilla* species in the northern regions of Pannonia in neighboring Croatia, along with data from Central Europe, indicate the potential extension of their habitat further north. This, in turn, raises the possibility of their presence in the Semberia and Pannonian regions of Bosnia and Herzegovina. This anticipation is supported by the broader geographic distribution of these species, as highlighted by Rasmont, 2014.

Species belonging to the genus *Amegilla* are predominantly found in xerothermic habitats within the Mediterranean and sub-Mediterranean regions. However, in the Dinaric region of Bosnia and Herzegovina, these species have been documented in the canyon part of the Neretva River, characterized by a significant sub-Mediterranean influence and xerothermic environments. The *Amegilla* species recorded in Bosnia and Herzegovina exhibit a distribution pattern that extends into neighboring European countries such as Serbia, Croatia, and Slovenia. Interestingly, while *Amegilla salviae* and *Amegilla magnilabris* have not been recorded in Slovenia, *Amegilla albigena*, *A*. *magnilabris*, A. *quadrifasciata*, and *A*. *garrula* have all been documented in these adjacent regions. This suggests that *Amegilla* species can migrate northward, with records extending as far as Germany (Rasmont, 2014). The multiple records of *Amegilla salviae* and *A*. *magnilabris* in the Western Balkan region indicate that these species are primarily distributed in Mediterranean areas but are also capable of inhabiting xerothermophilic habitats within continental regions. The discovery of *Amegilla* (*Zebramegilla*) *magnilabris* in the Sarajevo area further suggests the potential presence of this species in the Dinaric region, especially within thermophilic habitats that exhibit a strong Mediterranean influence.

The distribution and habitat preferences of *Amegilla* species in Bosnia and Herzegovina and the broader Western Balkan region are characterized by a blend of Mediterranean and continental influences, underscoring the adaptability and ecological versatility of these bee species.

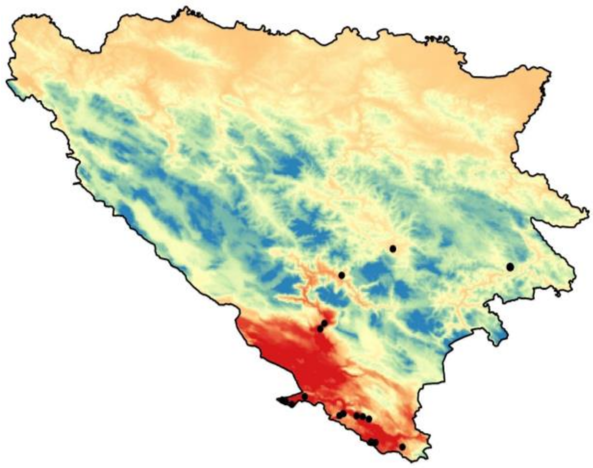

*Figure 1. Distribution of finds of species from the genus Amegilla in Bosnia and Herzegovina shown by black dots on the Bio1-Annual Mean Temperature background*

*Table. 3. Overview of species from the genus Amegilla with localities in Bosnia and Herzegovina and the source of data, which were used for species modeling, given in format yyyymmdd, ECNHMBIH-entomological collection Natural History Museum Bosnia and Herzegovina, DD-data deficient*

| Name | Locality | Date | Data source |
|---|---|---|---|
| *1. Amegilla albigena* | Bijeljina | 20190702 | Field data |
| *2. Amegilla albigena* | Klek_Neum | 20190702 | Field data |
| *3. Amegilla albigena* | Mostar | 20190722 | Field data |
| *4. Amegilla albigena* | Zavala | 20180714 | Field data |
| *5. Amegilla albigena* | Herceg Novi | 192107?? | ECNHMBiH |
| *6. Amegilla garula* | Krmeci | 20180617 | Field data |
| *7. Amegilla garula* | Krmeci | 20180617 | Field data |
| *8. Amegilla garula* | Klek_Neum | 20180617 | Field data |
| *9. Amegilla garula* | Klek_Neum | 20180617 | Field data |
| *10. Amegilla garula* | Fortica | 20190722 | Field data |
| *11. Amegilla garula* | Ovčari | 20200615 | Field data |
| *12. Amegilla garula* | Zavala | 20200612 | Field data |
| *13. Amegilla garula* | Ustiprača | 1882???? | ECNHMBiH |
| *14. Amegilla magnilabris* | Sarajevo | DD | ECNHMBiH |
| *15. Amegilla magnilabris* | Herceg Novi | 192107?? | ECNHMBiH |
| *16. Amegilla quadrifasciata* | Krmeci | 20180616 | Field data |
| *17. Amegilla quadrifasciata* | Krmeci | 20180616 | Field data |
| *18. Amegilla quadrifasciata* | Klek_Neum | 20180617 | Field data |
| *19. Amegilla quadrifasciata* | Češljari | 20200612 | Field data |
| *20. Amegilla quadrifasciata* | Dobromani | 20200612 | Field data |
| *21. Amegilla quadrifasciata* | Batković | 20210724 | Field data |
| *22. Amegilla quadrifasciata* | Bosanski Brod | 1875???? | ECNHMBiH |
| *23. Amegilla quadrifasciata* | Ustiprača | 1882???? | ECNHMBiH |
| *24. Amegilla quadrifasciata* | Jablanica | 1882???? | ECNHMBiH |
| *25. Amegilla magnilabris* | Sarajevo | DD | ECNHMBiH |
| *26. Amegilla quadrifasciata* | Bosanski Brod | 1875/ inv. Num. | ECNHMBiH |
| *27. Amegilla garrula* | Ustiprača | 1882/46 | ECNHMBiH |
| *28. Amegilla sp?* | Ustiprača | 1882/46 | ECNHMBiH |
| *29. Amegilla sp?* | Jablanica | 1882/46 | ECNHMBiH |

The predictive model depicting habitat suitability for species within the *Amegilla* genus across Bosnia and Herzegovina and the broader Western Balkan Peninsula is presented in (Figures 2-4.). This model offers a detailed resolution of 1x1 km. To gain insights into the factors shaping habitat suitability, the influence of climate variables is delineated in (Table 2.). Among these variables, two standout factors with the most significant statistical influence have been identified and highlighted for emphasis. Specifically, in the ecological niche model for *Amegilla albigena*, the variable Bio9 (Mean Temperature of Driest Quarter) exhibited the most pronounced impact on habitat suitability. For *Amegilla garrula*, the Bio1 (Annual Mean Temperature) variable played a pivotal role in shaping its ecological niche model. Meanwhile, in the case of *Amegilla quadrifasciata*, the ecological niche model was primarily influenced by the Bio11 (Mean Temperature of Coldest Quarter) variable (Table 2.).

*Table. 2. Analysis of variable contributions with estimates of relative contribution of the environmental variables to the Maxent model Percent contribution%/Permutation importance; the model is reevaluated on the permuted data, and the resulting drop in the training AUC is normalized to percentages*

| | bio_1 | bio_5 | bio_6 | bio_9 | bio_10 | bio_11 | bio_12 | |
|---|---|---|---|---|---|---|---|---|
| *Amegilla albigena* | 28.4%/0 | | 6.8%/9.8 | 38%/0 | 10.2%/37.1 | 12.2%/0 | | AUC T = 0.888<br>AUC P=0.5 |
| *Amegilla garula* | 36.2% | 2.8%/9.6 | | 0.2% | 1.9%/57.8 | 7.7% | 7.5%/25 | AUC T = 0.915<br>AUC P=0.5 |
| *Amegilla quadrifasciata* | 12.3%/91.3 | 3.7% | | 16.1% | 18.5% | 47.5% | | AUC T = 0.856<br>AUC P=0.5 |

The ecological niche model for species within the genus *Amegilla* is visually represented with a color gradient, ranging from colder to warmer colors, illustrating the suitability of different habitats. Habitats within the Mediterranean and sub-Mediterranean regions exhibit a high level of ecological suitability for *Amegilla* species. This underscores the significance of temperature-related variables in shaping the ecological niche models for these species, indicating that temperature plays a critical role in determining their potential distribution.

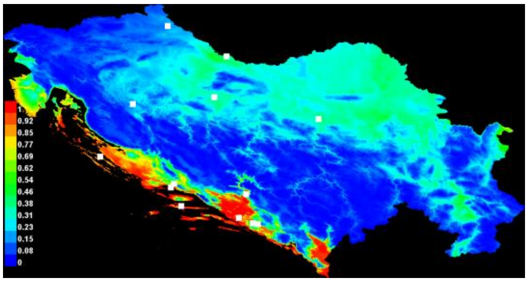

*Figure 2. The representation of the Maxent model for Amegilla albigena*

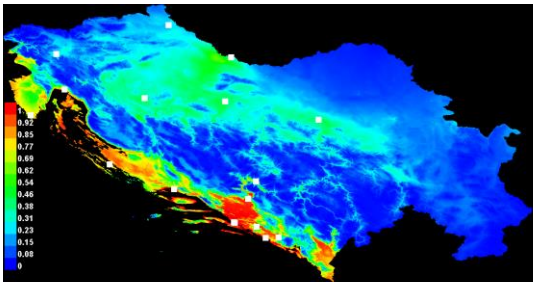

*Figure 3. The representation of the Maxent model for Amegilla garula*

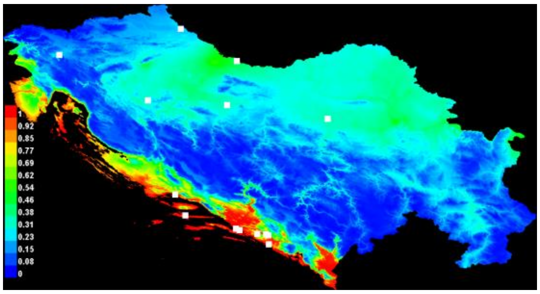

*Figure 4. The representation of the Maxent model for Amegilla quadrifasciata*

**Discussion**

Species from the genus *Amegilla* are widespread in the Mediterranean area of Herzegovina and spread from the south to the north. Climatic conditions change significantly as the altitude increases, the conditions on the horizontal profile of Bosnia and Herzegovina change and cause the absence of species in hilly, mountainous and alpine areas. *Amegilla* species pass through the canyon of the Neretva River to Jablanica, which is one of the northernmost localities in the Dinarides in Bosnia and Herzegovina. Members of the genus are recorded in the Pannonian region where they are strongly influenced by the warm climate of the Pannonian and Peripannonian areas along the Sava and Drina rivers. The ecological niche of the species will be considered through three dimensions: climatic conditions, nutrition and nesting places. The area with a continental climate includes the temperature valence of *Amegilla* species. In the Pannonian area, the nesting site is most likely the limiting factor. In the area of the vegetation where the groundwater level and humidity are high, which is not conducive to the species that nest in the soil. Finding microhabitats with dry vertical cliffs and embankments is a limiting factor for species in the Pannonian area. In the foothills and mountainous area of the Dinarides, temperature is a limiting factor for species.

It was not possible to obtain information about the habitats where the individuals were collected from literature data and museum collections. Field data provides conclusive evidence that species predominantly reside within the Mediterranean and sub-Mediterranean regions, specifically favoring rocky terrain. However, it is noteworthy that the majority of these specimens were procured from locations characterized by pre-existing maquis vegetation and deposols. Remarkably, every locality in which these specimens were collected exhibited a common feature - the predominance of skeletal soils, accounting for a substantial 80% of the soil composition. One exception to this trend pertains to the species *Amegilla garrula*, which exhibits a distinct ecological niche within the sub-Mediterranean area. Here, it diverges from the rocky habitat preference exhibited by other species from the genus and instead favors meadow communities with a pronounced thermophilic character, comprising approximately 20% of its observed habitat range. This species primarily feeds on plant species belonging to the Lamiaceae family, including *Salvia officinalis* and *Salvia pratensis*, as well as those from the Boraginaceae family, such as *Echium vulgare* and *E. italicum*. These specific plant preferences provide valuable insights into the ecological interactions and dependencies of *Amegilla garrula* within its chosen habitat, offering a glimpse into its foraging behavior and the intricate relationships it forms with its plant hosts.

## 4. Conclusions

Field research conducted in Bosnia and Herzegovina has provided valuable insights into the diversity of bee species within the *Amegilla* genus. Specifically, three species have been definitively confirmed through field research: *Amegilla albigena*, *A*. *quadrifasciata*, and *A*. *garrula*, all of which represent significant findings for Bosnia and Herzegovina.

Furthermore, the species *Amegilla magnilabris* has been recorded, with records based on collections stored in the National History Museum of Bosnia and Herzegovina. This underscores the importance of museum collections in expanding our knowledge of the distribution of bee species.

Notably, although the species *Amegilla salviae* is known to exist in the broader region of the western Balkan peninsula, it was not conclusively confirmed during the fieldwork conducted in Bosnia and Herzegovina. This absence in the current research is interesting, given that neighboring countries have recorded the presence of this species. Therefore, it is reasonable to anticipate that future research efforts in Bosnia and Herzegovina may eventually establish the presence of *Amegilla salviae*. This expectation is supported by the fact that this species is distributed in neighboring countries such as Croatia and Serbia, suggesting that it may indeed be part of the local bee fauna but has yet to be documented in Bosnia and Herzegovina. Consequently, the continued exploration and study of bee populations in Bosnia and Herzegovina are likely to yield additional insights into the distribution and diversity of *Amegilla* species in this area.

While it's worth noting that in the Peripanonian region there are no records on the presence of species from this genus, it is reasonable to anticipate that members of the genus may potentially inhabit this area, particularly within open stony dry habitats. This expectation is based on the genus's general ecological preferences, which tend to favor such habitats. These species are predominantly distributed in Mediterranean and sub-Mediterranean regions, typically up to elevations of 600 meters above sea level. Their favored habitats include open xerothermophilic environments characterized by arid and warm open habitats.

Interestingly, modeling efforts aimed at predicting the distribution of species from this genus have shown a strong correlation with Mediterranean and thermophilic habitats, aligning with their known ecological preferences. The model also demonstrates a notably high degree of correlation with the canyon of the Neretva river in the Dinaric area, indicating that this specific locality might be particularly of value for the presence of these species. This finding underscores the significance of understanding and identifying suitable habitats for these species, as it can assist in conservation efforts and enhance our knowledge of their distribution patterns.

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# Allelopathic Effect of *Lavandula angustifolia* Mill. and *Thymus serpyllum* L. Essential Oils on Five Selected Plant Species

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## Abstract

In this study allelopathic effects of essential oils from English lavender (*Lavandula angustifolia*) and creeping thyme (*Thymus serpyllum*) on seed germination and seedling growth of mint (*Mentha spicata*), lemon balm (*Melissa officinalis*), tomato (*Lycopersicon esculentum*), cucumber (*Cucumis sativus*), and black locust seeds (*Robinia pseudoacacia*) was evaluated. The assessment of the allelopathic effect of essential oils was examined in a laboratory biological test. Working solutions of essential oils were prepared in three concentrations (10, 20 and 30 μg/mL). Both oils exhibited allelopathic effects, however, the concentrations that exhibited suppressing effects were different among the plants. It was noticed that English lavender and creeping thyme oils reduced the germination and growth of mint and black locust seeds and significantly suppressed tomato seed germination at higher concentrations (20 and 30 μg/mL), while it increased the lemon balm seed germination.

Keywords: alleopathy, creeping thyme, English lavender, essential oils, *Lavandula angustifolia*, *Thymus serpyllum*.

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### 1. Introduction

The allelopathic effect of essential oils and their chemical components is very well known. Their herbicidal, insecticidal, nematicidal, fungicidal, and bactericidal effects have been described in previous works (Isman et al., 2011; Pavela and Banelli, 2016; Martinez et al., 2017). On the other hand, they have low toxicity for mammals and they decompose faster, and therefore represent a "green" alternative to the synthetic agents that are used in agricultural production today.

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The Lamiaceae family contains highly active components such as α-pinene, limonene, camphor, carvacrol, thymol, etc. Citronella oil and D-limonene are components of some registered herbicides such as Barrier H®, Avenger Organic Weed Killer®, and Organic Interceptor® (Giepen et al., 2014). The Lamiaceae family also contains thymol and carvacrol, which, in addition to having a strong antimicrobial, antiviral, antifungal, and antioxidant effect, have also been shown to have a strong effect on the inhibition of plant germination (Edris, 2007; Synowiec et al., 2017; Synowiec et al., 2019), for controlling plant diseases, and life extending of fruits and vegetables (Solgi and Ghorbanpour, 2014).

Essential oils of *Thymus vulgaris* L. and *Lavandula angustifolia* Mill. (Lamiaceae) have long been known for their medicinal properties, i.e. antibacterial, antimicrobial, antifungal, and insecticidal effects (Verma et al., 2014). Previous research has indicated the promising potential of these essential oils for aphid control (Yousefzadi et al., 2009). There are also reports on the allelopathic effect of extracts and essential oils of these plants on various other species (Cavalieri and Caporali, 2010; Zheljazkov et al., 2021; Zhou et al., 2021).

The main objective of this study was to determine the allelopathic effects of essential oils of English lavender (*Lavandula angustifolia*) and creeping thyme (*Thymus serpyllum*) on seed germination and growth of mint (*Mentha spicata* L.), lemon balm (*Melissa officinalis* L.), tomato (*Lycopersicon esculentum* Mill.), cucumber (*Cucumis sativus* L.), and black locust seeds (*Robinia pseudoacacia* L.).

## 2. Material and Methods

### *2.1. Plant material and essential oils*

Commercially produced seeds [S.i.p.a.s, Via Emilia 1810/A, Longiano (FC), Italia] of mint (*Mentha spicata* L.), lemon balm (*Melissa officinalis* L.), tomato (*Lycopersicon esculentum* Mill.) and cucumber (*Cucumis sativus* L.) were used. Black locust seeds (*Robinia pseudoacacia* L.), in their reproductive phase, were collected in Dariva areas of Sarajevo (Bosnia and Herzegovina; 43°51'25'' N, 18°26'42'' E). The collected seeds were cleaned and stored at 4°C until use. Essential oils of English lavender (*Lavandula angustifolia* Mill.) and creeping thyme (*Thymus serpyllum* L.), commercially available (Cydonia d.o.o., Lipa 73, Gračanica, BiH), were used. Stock solutions of essential oils were prepared using DMSO (dimethyl sulfoxide; Sigma-Aldrich, Deinheim, Germany) and serial dilutions were prepared with distilled water to give the concentrations of 10, 20 and 30 μg/mL.

### *2.2. Cultivation of the seeds*

The effect of the essential oils of *L. angustifolia* and *T. serpyllum* was evaluated by cultivating the seeds in a growth chamber. In Petri dishes, with two layers of filter paper, 25 seeds of each species were placed. Five mL of essential oils at three different concentrations (10, 20 and 30 μg/mL) were added to Petri dishes, and distilled water was used as the control. The Petri dishes were then placed in a growth chamber at 23°C and 16 hours photoperiod. Seeds were considered as germinated when radicles were longer than 1 mm and with a visible root (Kolb et al. 2016). The test was performed in four replications in completely randomized design. After five, seven and ten days, the results were recorded. We analyzed index of germination (AOSA, 1983), germinationpercentage (Scott et al., 1984); germination vigor index (Ranal and Santana, 2006), percentage of germination inhibition (Ali et al., 2015), and phytotoxicity index (Mekki et al., 2007). Phytotoxicity index ranges between (0) and (1), where a higher value indicates a negative (toxic) effect and a lower value a positive (stimulating) effect.

***2.3. Statistical analysis***

All results were expressed as the mean of four replicates (± standard deviation). The one-way ANOVA, followed by Duncan’s multiple range test and Pearson’s correlation coefficient were applied. The significance level of $p<0.01$ is considered as very significant.

**3. Results**

Essential oils of English lavender and creeping thyme significantlly decreased germinatin index of *M. spicata*, *L. esculentum* and *C. sativum*, especially at higher concentrations (Table 1). Seeds of *M. officinalis* were not affected by English lavender and creeping thyme oils while *R. pseudoacacia* seeds were not affected only by English lavender oil. Germination index in all cases decreased with the increasing oils concentrations

***Table 1.*** *Effect of different concentrations of* L. angustifolia *and* T. serpyllum *essential oils on germination index (GI), percentage of germination (G%), germination vigor index (GVI), percentage of germination inhibition (GI%), and index of phytotoxicity (IPH)*

| | | GI | | G% | | GVI | | GI% | | IPH | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **English lavander** | **Creeping thyme** | **English lavander** | **Creeping thyme** | **English lavander** | **Creeping thyme** | **English lavander** | **Creeping thyme** | **English avander** | **Creeping thyme** |
| *Mentha spicata* | Control | 18.74±2.84$^{c}$ | 18.74±2.84$^{c}$ | 87.00±6.00$^{a}$ | 87.00±6.00$^{a}$ | 11.92±2.28$^{c}$ | 11.92±2.28$^{c}$ | - | - | - | - |
| | 10 µg/mL | 18.36±0.64$^{c}$ | 15.08±1.63$^{b}$ | 86.00±9.52$^{a}$ | 86.00±2.31$^{a}$ | 11.72±1.38$^{c}$ | 10.21±1.06$^{b.c}$ | 6.07 | 6.79 | -0.10 | 0.07 |
| | 20 µg/mL | 13.75±1.49$^{b}$ | 13.12±1.37$^{b}$ | 81.00±3.83$^{a}$ | 88.00±7.30$^{a}$ | 8.48±1.73$^{b}$ | 8.19±1.29$^{b}$ | 6.73 | 5.81 | 0.21 | 0.25 |
| | 30 µg/mL | 10.22±1.65$^{a}$ | 10.00±1.09$^{a}$ | 73.00±12.38$^{a}$ | 82.00±2.31$^{a}$ | 4.38±1.31$^{a}$ | 5.41±0.41$^{a}$ | 15.5 | 13.21 | 0.54 | 0.50 |
| *Melissa officinalis* | Control | 21.56±3.49$^{a}$ | 21.56±3.49$^{a}$ | 75.00±8.25$^{a}$ | 75.00±8.25$^{a}$ | 29.47±7.60$^{b}$ | 29.47±7.60$^{b}$ | - | - | - | - |
| | 10 µg/mL | 21.19±3.18$^{a}$ | 20.45±4.15$^{a}$ | 75.00±6.83$^{a}$ | 78.00±15.49$^{a}$ | 26.17±4.55$^{b}$ | 33.03±6.51$^{b}$ | 2.75 | 22.25 | 0.10 | -0.13 |
| | 20 µg/mL | 20.46±3.53$^{a}$ | 19.59±2.24$^{a}$ | 83.00±11.49$^{a}$ | 83.00±7.57$^{a}$ | 22.81±5.96$^{a.b}$ | 24.80±3.03$^{a.b}$ | 10.50 | 14.25 | 0.32 | 0.22 |
| | 30 µg/mL | 17.52±2.86$^{a}$ | 17.65±0.64$^{a}$ | 81.00±8.87$^{a}$ | 85.00±3.83$^{a}$ | 16.90±2.40$^{a}$ | 17.90±1.56$^{a}$ | 11.25 | 14.50 | 0.50 | 0.48 |
| *Lycopersicon esculantum* | Control | 26.05±2.50$^{c}$ | 26.05±2.50$^{c}$ | 84.00±8.64$^{c}$ | 84.00±8.64$^{c}$ | 80.83±15.10$^{b}$ | 80.83±15.10$^{c}$ | - | - | - | - |
| | 10 µg/mL | 14.61±1.44$^{b}$ | 16.45±1.48$^{b}$ | 72.00±5.66$^{c}$ | 81.00±5.03$^{c}$ | 57.75±3.49$^{b}$ | 73.87±2.39$^{c}$ | 16.00 | 13.50 | 0.20 | 0.00 |
| | 20 µg/mL | 3.07±2.39$^{a}$ | 4.43±1.32$^{a}$ | 25.00±13.22$^{b}$ | 38.00±9.52$^{b}$ | 8.27±6.68$^{a}$ | 17.83±5.37$^{b}$ | 70.50 | 53.75 | 0.73 | 0.51 |
| | 30 µg/mL | 0.61±0.60$^{a}$ | 2.41±1.11$^{a}$ | 8.00±7.90$^{a}$ | 17.00±5.03$^{a}$ | 0.79±0.77$^{a}$ | 3.29±1.03$^{a}$ | 89.25 | 79.00 | 0.92 | 0.81 |
| *Cucumis sativus* | Control | 44.07±4.21$^{c}$ | 44.07±4.21$^{c}$ | 98.00±2.31$^{a}$ | 98.00±2.31$^{a}$ | 38.88±3.85$^{b}$ | 38.88±3.85$^{b}$ | - | - | - | - |
| | 10 µg/mL | 33.12±0.82$^{b}$ | 44.64±4.82$^{c}$ | 100.00±0.00$^{a}$ | 99.00±2.00$^{a}$ | 48.16±3.43$^{c}$ | 42.50±9.49$^{b}$ | 2.00 | 1.00 | -0.27 | -0.15 |
| | 20 µg/mL | 31.37±0.58$^{b}$ | 30.62±1.19$^{b}$ | 100.00±0.00$^{a}$ | 97.00±3.83$^{a}$ | 43.73±2.31$^{c}$ | 45.75±4.50$^{b}$ | 2.00 | 3.00 | -0.18 | -0.33 |
| | 30 µg/mL | 20.39±1.01$^{a}$ | 20.23±1.79$^{a}$ | 92.00±5.66$^{a}$ | 95.00±5.03$^{a}$ | 23.28±1.08$^{a}$ | 18.51±2.91$^{a}$ | 8.00 | 3.00 | 0.30 | 0.48 |
| *Robinia pseudoacacia* | Control | 29.68±7.87$^{a}$ | 29.68±7.87$^{b}$ | 87.00±18.29$^{a}$ | 87.00±18.29$^{b}$ | 41.87±5.83$^{c}$ | 41.87±5.83$^{c}$ | - | - | - | - |
| | 10 µg/mL | 26.33±5.81$^{a}$ | 29.39±4.96$^{b}$ | 85.00±11.94$^{a}$ | 90.00±7.66$^{b}$ | 29.51±8.61$^{b}$ | 36.41±9.34$^{b.c}$ | 28.75 | 21.00 | 0.26 | 0.21 |
| | 20 µg/mL | 23.90±2.92$^{a}$ | 21.55±3.19$^{a}$ | 83.00±5.03$^{a}$ | 77.00±5.03$^{a.b}$ | 30.54±3.34$^{b}$ | 30.02±5.18$^{b}$ | 20.25 | 18.50 | 0.20 | 0.22 |
| | 30 µg/mL | 21.48±7.51$^{a}$ | 14.81±1.60$^{a}$ | 76.00±4.62$^{a}$ | 68.00±6.53$^{a}$ | 20.58±1.55$^{a}$ | 18.52±2.74$^{a}$ | 24.75 | 24.75 | 0.40 | 0.41 |

**Note**: Values are expressed as means ± standard deviations of four replications; Means in the same column with different letters in superscript are significantly different at $p<0.01$

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*Lavandula angustifolia* and *T. serpyllum* essential oils exhibited a dose-dependent phytotoxic activity against the seed germination *of L. esculentum*, with significantly decreased germination at the lower dose (20 µg/mL) tested (25%), and almost total inhibition at the highest dose (30 µg/mL) applied (8% with English lavender and 17% with creeping thyme oil) (Table 1). Germination percentage of *R. pseudoacacia* was slightly affected only by creeping thyme essential oils at higher concentrations (20 and 30 µg/mL). All other species did not show significantly changes in germination percentages after application these two essential oils (Table 1). The percentage of inhibition of germination follows these results with the greatest effect on tomatoes, where the percentage of inhibition of germination was 89.25 (at 30 μg/mL of English lavender oil, and 79% at the same concentration of creeping thyme oil).
Furthermore, both oils at the highest concentration (Table 1) completely suppressed vigor index in tomato (0.79 and 3.29 μg/mL respectively), moderate suppressed in mint (4.38 and 5.41 μg/mL respectively), and slightly in lemon balm (16.90 and 17.90 μg/mL respectively), black locust (20.58 and 18.52 μg/mL respectively), and cucumber (23.28 and 18.51 μg/mL respectively). The opposite effect was noticed only in cucumber at 10 and 20 µg/mL concentrations of both oils (Table 1).
Negative phytotoxic index was noticed at the highest concentration of both oils in mint, lemon balm and tomato (Table 1), and at 20 μg/mL concentration in tomato. In all other cases the phytotoxic indexes were positive.

### 3. Discussion

The essential oils of English lavender and creeping thyme showed different effects on seed germination and early growth of the investigated plant species (Table 1). The percentage of germination decreased with the increase in the concentration of essential oils with the exception of *M. officinalis*. Higher concentrations (20 and 30 μg/mL) of both essential oils showed a high percentage of inhibition in tomato seeds germination, with almost complete inhibition of seed germination at a concentration of 30 μg/mL. A similar observation of the inhibitory effect of *L. angustifolia* essential oil on the percentage of tomato germination was observed by Ibáñez and Blázquez (2019). It is well documented that some allelochemicals suppress the mitotic activity of initially developed cells, which leads to inhibition of seed germination (Rice, 1987).
In most cases, growth parameters varied due to different percentage concentrations of essential oils with increasing concentrations, the inhibitory effects progressively increased. The root length of the germinated seeds was more sensitive to allelochemicals compared to the shoot, which was reflected in the percentage of inhibition of germination and the phytotoxicity index. Such effect could be expected, considering that roots first absorb allelochemicals and then transfer them to shoots that is confirmed in previous studies (Turk and Tawaha, 2003; Sarkar et al., 2012).
Seed vigor is an important parameter for testing seed quality. Essential oils of English lavender and creeping thyme at application rates of 20 and 30 µg/mL completely suppressed germination vigor index (Table 1). In addition, when applied at 10 µL both essential oils slightly decreased the vigor index compared to the control or, in the case of the creeping thyme essential oil, increased it in *M. officinalis*. The results were similar to

those of Zheljazkov et al. (2021) in vigor index of wheat and barley seeds, especially at higher concentrations of *L. angustifolia* and *T. vulgaris* essential oils.

The percentage of inhibition of germination is dose-dependent and varies in different species. Both essential oils have an inhibitory effect on tomato seedlings with relatively high rates of inhibition, which was not observed in the other tested species. The inhibition of germination under the influence of essential oils, mainly terponoids, monoterpenes and sesquiterpenes, has already been recorded in different crops and weeds (Batis et al., 2004; Uremis et al., 2009; Gitsopoulus et al., 2013). Thus, it was observed that carvacrol and thymol showed complete inhibition of seed germination and seedling growth of numerous plant species (Kordali et al., 2008; de Almeida et al., 2010; Ibáñez and Blázquez, 2017). Ibáñez and Blázquez (2017) observed that oregano essential oil with 60.42% carvacrol exhibited complete inhibition of *Portulaca oleracea* L., *Lolium multiflorum* Lam., and *Echinochloa crus-galli* (L.) P. Beauv., while de Almeida et al. (2010) observed a complete inhibition of *Lepidium sativum* L., *Raphanus sativus* L., and *Lactuca sativa* L. under the influence of, among others, oils of oregano and thyme. These results show that the applied oils could be both effective and an alternative to synthetic herbicides and cause less problems for the environment and human health.

### 5. Conclusions

Our results show that essential oils of *Lavandula angustifolia* and *Thymus serpyllum* stimulated the germination of *Melissa officinalis* seeds; negatively affected the germination of *Lycopersicon esculentum* and *Robinia pseudoacacia* seeds, and had a moderate inhibitory effect on Mentha spicata seed germination. Both essential oils can be considered as potential allelopathic agents that, due to their organic origin, easy degradability in nature, without known negative effects on humans and nature, could significantly improve agricultural practices.

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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,

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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).

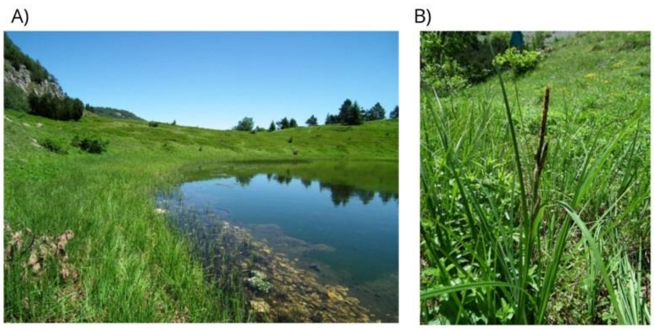


*Figure 1. Collection site (A); Carex acuta in natural habitat (B) (Photos by S. Škondrić)*

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 $Fe_5O_4x7H_2O$.

### *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).

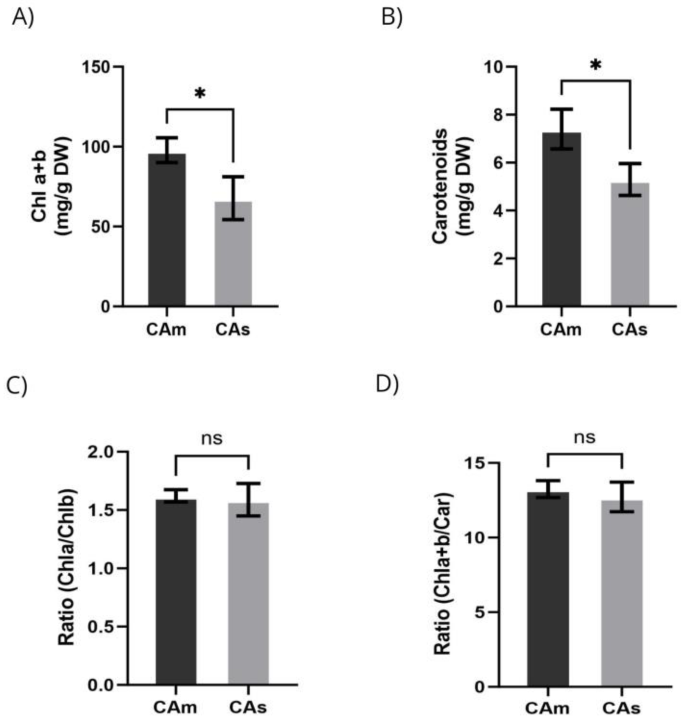


Figure 2. Concentration of total chlorophyll (Chl (a+b)) (A); Concentration of carotenoids (Car) (B); Ratio Chl(a+b)/Car (C); Ratio Chl a/b (D). Asterisk indicate statistically significant differences between samples (* $p<0.05$). ns – no statistical significance

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).

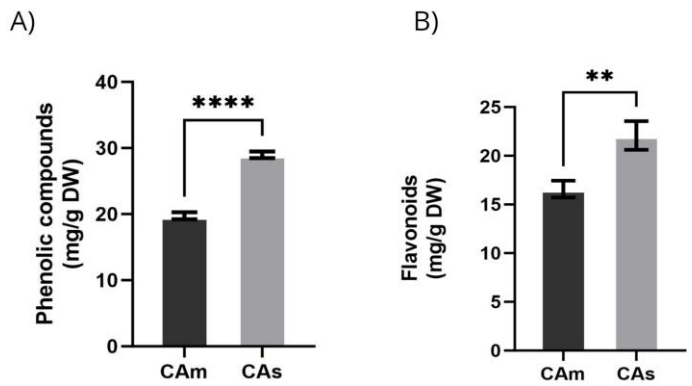


Figure 3. Concentration of total phenolic compounds (A); Flavonoid concentration (B). Asterisks indicate statistically significant differences between samples (** $p<0.01$; **** $p<0.0001$).

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.

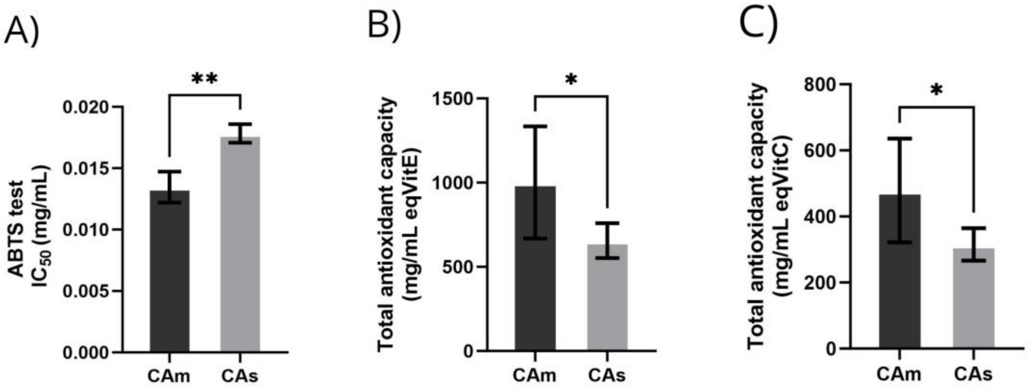


Figure 4. Antioxidant capacity of extracts measured by different methods: ABTS test (A); by the phosphomolybdenum method (B and C). Asterisks indicate statistically significant differences between samples (* $p<0.05$;** $p<0.01$).

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

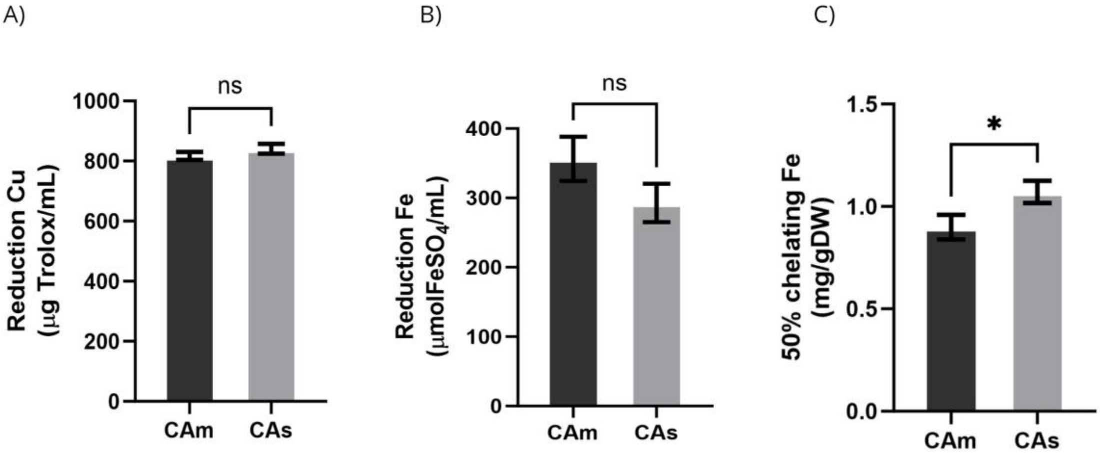


Figure 5. Antioxidant capacity of extracts measured by different methods: reduction of Cu (A); reduction of Fe (B); chelation of Fe (C). Asterisks indicate statistically significant differences between samples (* $p<0.05$). ns – no statistical significance

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 $\beta$-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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# The Review on the Antimicrobial Potential of Maidenhair Fern, *Adiantum capillus-veneris* L.

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## Abstract

*Adiantum capillus-veneris* L. or Maidenhair fern is a perennial fern that belongs to the family Pteridaceae. It is widespread in temperate and tropical regions with high humidity. *A. capillus-veneris* has a long history of medicinal use, and recent investigations suggest the presence of many bioactive compounds in this plant. This review debates the antimicrobial potential of *Adiantum capillus-veneris* in a comprehensive manner that includes data about the antibacterial, antifungal, and antiviral activity. Data regarding the antimicrobial potential of *A. capillus-veneris* were collected from scientific databases Web of Science, Scopus, PubMed, and Google Scholar. The largest number of reviewed studies were related to the antibacterial activity of extracts made from *A. capillus-veneris* plant. Mainly, leaves, stems, roots, and rhizomes were tested, while water, methanol, ethanol, ethyl acetate, and hexane were used as solvents. For the testing of antimicrobial susceptibility, standard tests were implemented, such as the disk-diffusion method, agar dilution method, and determination of MIC and MBC values. There are also studies testing the antibiofilm effects of the investigated plant. Our review showed that *A. capillus-veneris* exhibits large antibacterial potential, with the capacity to inhibit the growth of different bacteria, including multidrug-resistant strains. The antifungal and antiviral activity of the investigated plant was also recorded. This review summarized data regarding the antimicrobial potential of *A. capillus-veneris* and suggests that future phytochemical investigations of ferns may lead to the discovery of new therapeutic agents.

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## 1. Introduction

Ferns are a plant group with worldwide distribution in different habitats, and the floristic wealth of pteridophytes is estimated to be 12.000 species, globally (Johnson et al, 2017). According to Kumar & Kumari (2023) pteridophytes are represented by 48 families with 587 genera in the world. The genus name *Adiantum* has etymological origin in the ancient Greek word "Adiantos", which could be translated as "unwetted" (Kumar & Kumari, 2023). Prado et al (2007) state that around 200 species were reported in the large and diverse genus *Adiantum*.

*Adiantum capillus-veneris* L. or Maidenhair fern is a delicate, perennial fern of ca. 35 centimeters in height, with a creeping rhizome (Sallam et al, 2019). Taxonomically, it belongs to the family Pteridaceae, subfamily Vittarioideae (Singh et al, 2020). The plant is widespread in regions with warm or tropical temperatures with high humidity (Kashkooe et al, 2021). Therefore, it could be found in different areas in southern Europe, the Atlantic coast of Ireland, and northern Africa (Boukada et al, 2022). Typical habitats are limestone cliffs away from direct insolation (Al-Snafi, 2015). Furthermore, the plant prefers well-drained, neutral, and alkaline soils, and it can grow in the semi-shade (Ansari & Ekhlasi-Kazaj, 2012).

Pteridophytes are indeed one of the oldest and most primitive vascular plant groups on earth, possessing leaves, roots, and erect stems (Kumar & Kumari, 2023). Interestingly, there are observations that pteridophytes are not infected with microbial pathogens, which could be related to their evolutionary success and the fact that they survived more than 350 million years (Johnson et al, 2017).

Historically, ferns have been known in folk medicine for more than 2000 years, and they are an indispensable part of many traditional medical practices and remedies due to the exploration and utilization of different species because of their beneficial properties (Baskaran et al, 2018). Numerous species of the genus

*Adiantum* are used in traditional medicine, such as *Adiantum philippense* L. (syn. *A. lunulatum* Burm), *A. caudatum* Klotzsch, *A. flabellulatum* L., *A. pedatum* L., *A. venustum* D. Don, *A. aethiopicum* L., *A. tenerum* Sw. (Brahmachari et al, 2003).

*A. capillus-veneris* is characterized by a long history of medicinal use. The medicinal parts of the plant are the leafy fronds, rhizomes, and roots (Boukada et al, 2022). The fresh and dried leaves are used as antidandruff, antitussive, astringent, demulcent, depurative, diuretic, emetic, emmenagogue, emollient, expectorant, galactagogue, laxative, pectoral, refrigerant, stimulant, sudorific and tonic. A tea or syrup of this plant is used to treat cough and bronchitis (Ansari & Ekhlasi-Kazaj, 2012), as well as for fever, pneumonia, mucous formation, diabetes, erysipelas, urinary insufficiency, and hepatitis (Yumkham et al, 2018; Kumar & Kumari, 2023). In general, the fresh plant is more effective, but it could be collected in the summer and dried for later use (Nazim et al, 2018).

### *1.1. Antimicrobial resistance (AMR)*

According to Hernando-Amado et al (2019), antimicrobial resistance (AMR) is one of the major challenges to global health. It is estimated that around 750.000 annual deaths worldwide are caused by AMR (Chassagne et al, 2021) and this number is likely to reach 10 million annually by the year 2050 (O'Neill, 2016). AMR is a consequence of the microorganisms' ability to survive in the presence of drugs that would usually inhibit their growth (Founou et al, 2017).

The development of multidrug-resistant (MDR) microbial strains is related to the accumulation of various resistance mechanisms inside the same strain (Harbottle et al, 2006), and despite the production of new antibiotics, resistance to drugs increased (Hussain et al, 2014). Therefore, new anti-infective agents are needed to overcome the abovementioned issues (Thabit et al, 2015).

The acronym ESKAPE refers to bacteria recognized by The Infectious Disease Society of America and the American Society of Microbiology as very compelling in terms of the resistance exhibiting, and defined as a high priority for drug discovery. ESKAPE stands for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, and *Enterobacter* species (Boucher et al, 2009). Besides bacteria, other microbial pathogens such as fungi and viruses exhibit multidrug-resistance patterns and there is an emphasized need for adequate antimicrobial treatment. Due to the described reasons, recent studies are directed toward plant products, in order to identify and develop new antimicrobial agents with antifungal, antibacterial, and antiviral activities (Maiyo et al, 2010).

From ancient times plants and their products have been used in the promotion of human health, and modern findings recognize them as a valuable source of compounds with various biological potentials. There is also evidence that herbal medicines were used throughout history against different infectious diseases, in the form of crude plant extracts as well as pure compounds (Parekh & Chanda, 2007). The World Health Organization (WHO) states that 88% of the global population relies on traditional medicine (WHO, 2023) since that represents an easily available treatment method. The usage of plants in traditional medicinal practices is based on their capacity to inhibit the growth and virulence of numerous microbes (Khan et al, 2018), but at the same time, they have fewer side effects and decreased toxicity (Chai et al, 2013).

Secondary metabolites *per se* are synthesized by plants in order to overcome evolutionary challenges, for self-defense reasons, as well as for communication with other organisms in the ecosystems (Khan et al, 2018). Nevertheless, these compounds are involved in the development of new antimicrobial drugs of natural origin, since they are generally bioactive and even exhibit synergy with other secondary metabolites as a part of the plant's multicomponent defense system (Harvey et al, 2015). Novel studies showed that plants have a wide bioactive potential, particularly antimicrobial (Zhang et al, 2019).

This review debates the antimicrobial potential of *Adiantum capillus-veneris* in a comprehensive manner that includes data regarding antibacterial, antifungal, and antiviral activity, as well as the potential phytochemicals related to described effects.

## 2. Material and Methods

### *2.1. Literature search*

The available data regarding the antimicrobial potential of *Adiantum capillus-veneris* L. were collected from scientific databases such as Web of Science, Scopus, PubMed, and Google Scholar. The search terms used for this review included: "*Adiantum capillus-veneris*", "antimicrobial activity", "antimicrobial resistance", "plant antimicrobials", "phytochemical composition", and "traditional use". This article represents an overview of the current state of knowledge about the antimicrobial properties of *Adiantum capillus-veneris* L.

## 3. Results

### *3.1. Antimicrobial potential of Adiantum capillus-veneris*

According to Chassagne et al. (2021) who systematically examined plants with antibacterial activity, only four families of ferns: Polypodiaceae, Pteridaceae, Salviniaceae, and Lygodiaceae were noted with antibacterial effects. Additionally, in each of these families, a single species was studied. Recently, there has been increased interest in the antimicrobial properties of ferns, because available data confirms large potential in that sense, especially when compared to the higher plants, which could be related to the presence of numerous defensive biochemical constituents (Rani et al, 2010). Although the genus *Adiantum* comprises many species, only a few are studied in terms of biological activity, while others are still unexplored (Brahmachari et al, 2003).

### *3.2. Antibacterial activity*

The study of Mahmoud et al (1989) investigated the antibacterial effects of aqueous and methanolic extracts made from *A. capillus-veneris* aerial parts and obtained results showed inhibition of *Bacillus subtilis*, *Proteus vulgaris*, and *Staphylococcus aureus* by crude extracts obtained from organic solvents, while water extract did not cause inhibition. Gram-negative species included in the investigation, *Pseudomonas aeruginosa* and *Escherichia coli* were resistant to the tested substances.

The antibacterial activity of the active oils from the *A. capillus-veneris* leaves is proven against *Klebsiella pneumoniae*, *Pseudomonas* sp., *Salmonella typhi*, *S. aureus*, and *Streptococcus pyogenes* (Victor et al, 2003). There are also data regarding the antibacterial activity of particular *A. capillus-veneris* compounds: adiantone, 22,29ξ-Epoxy-30-norhopane-13β-ol, fern-9(11)-en-28-ol, fern-9(11)-en-25-oic acid, fern-9(11)-en-6α-ol, fern-9(11)-ene, filicenol B, and 6-oxofern-9(11)-ene against Gram-negative bacteria *E. coli*, *P. aeruginosa*, *S. typhi,* and Gram-positive bacteria *Bacillus sphaericus*, B. subtilis, and *S. aureus* (Reddy et al, 2001).

Essential oil of the *A. capillus-veneris* is also investigated by Nasrollahi et al (2022) in terms of antibacterial activity, and inhibition is proven against *S. aureus*, *S. pyogenes*, and Diphteroid. Observed effects are probably related to the detected chemical constituents: carvone, carvacrol, hexadecanoic acid, hexahydrofarnesyl acetone, and n-nonanal. These findings are in accordance with the previous study of Khodaie et al (2015) that identified several phytochemicals in the volatile oil of

*A. capillus-veneris*: carvone, carvacrol, hexadecanoic acid, thymol, hexahydrofarnesyl acetone, and n-nonanal related to its antioxidant capacity.

Guha et al (2004) observed inhibition of *E. coli*, *S. aureus*, *Agrobacterium tumefaciens*, *Salmonella arizonae*, and *S. typhi* by the aqueous and alcoholic leaves extract of *A. capillus-veneris*. Results of Parihar et al (2010) confirmed that the aqueous and alcoholic leaves extract of *A. capillus-veneris* are effective against *A. tumefaciens*, *E. coli*, *S. arizonae*, *S. typhi,* and *S. aureus*.

The broad antibacterial activity of *A. capillus-veneris* methanolic extract is noted by Singh et al (2008), who also determined values of the minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC). Low MIC values are given against *Streptococcus pneumoniae* (7.81 μg/ml), and *E. coli* (0.48 μg/ml). Similar findings are presented in the work of Pan et al (2011), where the MIC of *A. capillus-veneris* sample against *E. coli* was very low, 0.97 μg/ml. Besides the already mentioned bacteria, Shirazi et al (2011) observed the antibacterial activity of *A. capillus-veneris* methanolic extract against *Helicobacter pylori*. Ethanolic extracts of *A. capillus-veneris* were investigated by Nyarko et al (2012) and antibacterial activity is noted against *Proteus mirabilis*, *K. pneumoniae*, and *S. aureus*. Further phytochemical investigation revealed the presence of sugars, flavonoids, triterpenoids, and steroids in the tested sample.

Ishaq et al (2014) presented a study of the antibacterial potential of different *A. capillus-veneris*, namely aqueous, methanolic, ethanolic, ethyl acetate, and hexane extracts derived from leaves, stems, and roots of the plant. The investigation included MDR strain and gave a comparative illustration of the activity of the following commercial antibiotics against tested strains: amoxicillin, ampicillin, cefaclor, ciprofloxacin, cephradine, cefotaxime, cefoperazone-sulbactam, ceftriaxone, gentamicin, moxifloxacin, nalidixic acid, tetracycline, norfloxacin, and trimethoprim-sulfamethoxazole. Bacteria included were: *Citrobacter freundii*, *E. coli*, *Providencia* sp., *K. pneumoniae*, *P. vulgaris*, *S. typhi*, *Shigella* sp., *Vibrio cholerae*, *P. aeruginosa*, and *S. aureus*. Overall results suggested higher growth inhibition of bacteria caused by the leaves extracts, excluding the hexane leaves extract, that achieved inhibition only of *E. coli*, *P. aeruginosa*, and *Shigella* sp. From all stem extracts, methanolic and ethanolic extracts had the broadest activity, while hexane root extract was inactive against tested strains. This study detected the presence of the following phytochemicals: alkaloids, flavonoids, tannins, saponins, terpenoids, steroids, glycosides, and reducing sugar.

In a similar study by Hussain et al (2014), leaves and stem extracts of *A. capillus-veneris* achieved antibacterial activity against the following MDR strains: *C. freundii*, *E. coli*, *Providencia* sp., *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *P. vulgaris*, *S. typhi*, *Shigella* sp., and *V. cholerae*. Medrar et al (2014) demonstrated that the aqueous and methanolic extracts of *A. capillus-veneris* showed comparatively higher competence against *P. aeruginosa* in comparison to the drug amoxicillin.

Antibacterial activity of the methanolic extract of *A. capillus-veneris* is described against clinical pathogens in the investigation of Hussein et al (2016), more precisely against *B. subtilis*, *P. aeruginosa*, *Streptococcus faecalis*, *S. typhi*, and S. *aureus*, with *S. faecalis* being the most sensitive species to the tested substance. Major phytochemical compounds identified in the investigated extract that possess antibacterial activity were: d-Mannose, imidazole-4-carboxylic acid, 2-fluoro-1-methoxymethyl-ethyl ester, D-Carvone, Pyrrolizin-1,7-dione-6-carboxylic acid, methyl (ester), phenol,2-methyl-5-(1-methylethyl)-, tetraacetyl-d-xylonic nitrile, curan-17-oic acid, 2,16-didehydro-20-hydroxy-19-oxo, methyl ester, tributyl acetylcitrate, 10,13-dioxatricyclo[7.3.1.0(4,9)]tridecan-5-ol-2-carboxylic acid, 9-Octadecenamide,(Z)-.

Additionally, other studies confirmed the antibacterial potential of different *A. capillus-veneris* extracts against *B. subtilis*, *P. vulgaris*, *S. aureus*, *A. tumefaciens*, *E. coli*, *S. arizonae*, and *S. typhi*, *S. enterica*, with various patterns of solvent-dependent and species-specific activity (Moradi et al, 2018; Nermin & Sadik, 2018; Parihar et al, 2018; Rautray et al, 2018). Moradi et al (2018) observed the high flavonoid content in the investigated sample (57.2 mg/ml). Additionally, other secondary metabolites are also described such as triterpenes, flavonoids, phenylpropanoids, carotenoids, quercetin, rutin, shikimic acid, violaxanthin, and zeaxanthin (Rautray et al, 2018).

In the comprehensive study of Khan et al (2018) it was shown that ethanolic and aqueous extracts of *A. capillus veneris* exhibited growth inhibition ≥50% ($IC_{50}$) against ESKAPE pathogens *E. faecium* and *S. aureus* when screened at 256 µg/ml of extract. Furthermore, regarding the antibiofilm activity, statistically significant *quorum sensing* inhibition was observed in the case of *S. aureus*.

Boukada et al (2022) investigated the antibacterial properties of A. *capillus-veneris* hydro-methanolic extract and noted growth inhibition of *E. coli*, *S. pneumoniae*, and *S. aureus*. In this case, antibacterial effects are in relation to the presence of phenolic compounds that use different mechanisms to kill bacteria, including the inhibition of nucleic acid synthesis, disruption of the plasma membrane, as well as the enzymatic and energy metabolism of the bacterial cell. In the investigated sample, the most abundant compounds were flavonoids: quercetin 3-O-glucoside and kaempferol 3-O-glucoside; and phenolic acids: 3,5-Di-O-caffeoylquinic acid and hydroxycinnamic derivative. This is in accordance with the previous results (Yuan et al, 2012; Al-Hallaq et al, 2015; Zeb & Ullah, 2017).

Yazdani & Kashi (2021) performed an investigation on the antibacterial potential of different *A. capillus-veneris* extracts, including water, methanolic, and hexane extracts of the leaves. The antibacterial potential is confirmed for *B. subtilis*, *Staphylococcus epidermidis*, *S. aureus*, *E. coli*, *Shigella dysenteriae*, *P. vulgaris*, *P. aeruginosa*, with different diameters of the inhibition zones, and MIC values ranging from 125 to 500 µg/ml of the extract. The high total phenolic content and considerable amounts of phenols and flavonoids are detected in the sample. The plant extracts have contained saponins, triterpenes, alkaloids, glycosides, tannins, and flavonoids.

Since the selection of the active compounds in medicinal plants is of great interest and may serve as a promising source of new prototype antibiotics and have great therapeutic potential against infectious diseases (Boukada et al, 2022), many investigations are performed in order to identify bioactive compounds of *A. capillus veneris*. Al-Snafi (2015) highlighted the importance of flavonoids, triterpenoids, oleananes, phenylpropanoids, carbohydrates, and carotenoids in this plant species. Many triterpenoids were isolated from the leaves, such as 21-hydroxyadiantone, adiantoxide, isoadiantone, isoglaucanone, isoadiantol, hydroxyadiantone, olean-12-en-3-one, olean-18-en-3-one, fern-9(11)-ene, fern-α-7,9(11)-diene, 7-fernene, hop- 22(29)-ene, filic-3-ene, neohop-12-ene, pteron-14-en-7α-ol, fern-9(11)-en-3α-ol, fern-7-en-3α-ol, adian-5(10)-en-3α-ol, adian5-en-3α-ol, fern-9(11)-en-28-O, fern-9(11)-en-12-β-ol, and 4-α-hydroxyfilican-3-one (Berti et al, 1969; Marino et al, 1989; Shiojima et al, 1992; Shiojima et al, 1993; Ageta et al, 1994; Shiojima et al, 1995; Nakane et al, 1999; Abdel-Halim et al, 2002; Nakane et al, 2002; Shinozaki et al, 2008). The leaves also contain flavonoids such as rutin, quercetin, quercetin-3-O-glucuronide, isoquercetin, nicotiflorin, naringin, astragalin, populnin, procyanidin, prodelphinidin, and kaempferol-3-sulfate (Akabori & Hasagava, 1969; Cooper-Driver & Swain, 1977; Imperato, 1982; Ibraheim et al, 2011).

Although the chemical composition of plant extracts could be influenced by the cultivation method, the phenological stage, the nature of the soil, the climate at the time of sampling, as well as the constitution of the sample (Boukada et al, 2022), it is possible to establish major bioactive phytochemical compounds of some species. According to Singh et al (2020) in *A. capillus-veneris*, these are 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester, Docosane, 1,2-Benzenedicarboxylic acid, butyloctyl ester, phthalic acid, butyl octyl ester, Hexadecanoic acid, ethyl ester, 9-Octadecenoic acid, Di-n-octyl phthalate, and Tetracontane.

### *3.3. Antifungal activity*

So far, antifungal activity of *A. capillus-veneris* preparations is confirmed against different fungal species, starting with one of the oldest available records in Mahmoud et al (1989), who described antifungal effects of the methanolic extract against *Candida albicans*. Singh et al (2008) also recorded antifungal activity against *C. albicans*, with a MIC value of 3.90 μg/ml, as well as the activity toward *Cryptococcus albidus*, *Trichophyton rubrum*, *Aspergillus niger*, *A. flavus*, *A. terreus*, *A. spinulosus*, and *A. nidulans*. Preliminary phytochemical screening showed the presence of flavonoids and tannins.

Together with *C. albicans*, *A. flavus*, and *A. niger*, Ishaq et al (2014) presented the study with the described antifungal activity of various *A. capillus-veneris* extracts against *Pythium* sp. and *Trichoderma* sp. Inhibition is achieved with all tested extracts, but nevertheless, hexane extract exhibited very low antifungal action.

Similarly, Yazdani & Kashi (2021) conducted results regarding the antifungal effects of aquatic, methanolic, acetate, and hexane extracts of the leaves and stems of *A. capillus-veneris* against *C. albicans*. Their results suggested broader activity of the leaves extracts in comparison to those made from stems, as well as that hexane extracts performed relatively low activity.

The aqueous extracts and phenols extracted from gametophytes of *A. capillus-veneris* performed antifungal activity against *A. niger* and *Rhizopus stolonifer* (Piyali et al, 2005).

An investigation by Hussein et al (2016) described the antifungal potential of the methanolic extract against the following subjects: *A. niger*, *A. terreus*, *A. flavus*, *A. fumigatus*, *C. albicans*, *Saccharomyces cerevisiae*, *Fusarium* sp., *Microsporum canis*, *Mucor* sp., *Penicillium expansum*, *Trichoderma viride*, *T. horzianum*, and *Trichophyton mentagrophytes*.

Rautray et al (2018) reported antifungal activity against *T. rubrum*, *Scedosporium apiospermum*, *A. fumigatus*, *A. niger*, and *A. flavus*, followed by the observation of Sallam et al (2019) who noted antifungal effects on *S. cerevisiae* and highlighted flavonoids, triterpenoids, phenylpropanoids, carbohydrates, carotenoids, and alicyclics as potential bioactive compounds of *A. capillus-veneris*.

### *3.4. Antiviral activity*

Antiviral drugs are a specific class of medicines used for the treatment of viral infections. Since the viruses use the host's cell for replication, the design of a safe and effective antiviral drug is challenging (Kausar et al, 2021). There are some data regarding the antiviral properties of *A. capillus-veneris*. One of them refers to the *in vitro* antiviral activity of ethanolic extract made from a rhizome, that acts against vesicular stomatitis virus (Husson et al, 1986). Ansari & Ekhlasi-Kazaj (2012) noted that this particular activity could be related to the traditional usage of Maidenhair fern preparations in the treatment of respiratory and urinary infections.

Moradi et al (2017; 2018) investigated the antiviral potential of *A. capillus veneris* against Herpes simplex virus-1 and Influenza, but despite other observed bioactive properties, there was no antiviral activity. In contrast, there are recorded antiviral properties of this plant against the Tobacco mosaic virus (Biniaz et al, 2023). Since viruses don't act in a taxonomically limited host range, this observation could be promising in future investigations.

## 4. Conclusions

Our review showed that *A. capillus-veneris* exhibit large antibacterial potential, with the capacity to inhibit the growth of different Gram-positive and Gram-negative bacteria, including multidrug-resistant strains, as well as some species recognized as ESKAPE pathogens. The antifungal and antiviral activity of investigated plant was also recorded. Considering the growing emergence of antimicrobial resistance, the need for the identification and characterization of novel antimicrobial agents couldn't be overemphasized. Due to the fewer side effects, low toxicity, and variable phytochemical profile, plant products are a promising source of new antibacterial, antifungal, and antiviral compounds. Ferns are generally poorly investigated in antimicrobial terms when compared to angiosperms, but data presented in this review suggest that they should be a focus of such investigations. This review summarizes current knowledge on the antimicrobial potential of *A. capillus-veneris* and could be used as the starting point in future studies regarding the antimicrobial, phytochemical, toxicological, and molecular properties of ferns.

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# The Impact of Online Education on Biology Students Motivation and Academic Achievement

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## Abstract

Online teaching has changed the dynamics of students' work and led to a series of changes concerning student engagement, motivation and academic success. The main goal of this research was to analyze the success of students from the Department of Biology at the Faculty of Science in Sarajevo during online classes compared to traditional classes, and to assess their motivation in online classes. Independent samples t-test, descriptive statistics and one-way ANOVA were used for data analysis and coding. The impact of online teaching on student success was assessed by comparing the average grades in subjects from the first and second year of study during online and face-to-face teaching. In the academic year 2018/2019, 79 students attended the first year of study, and 66 students attended in 2020/2021. For the second year of study, 74 students attended in the 2018/2019 academic year, while 90 students attended in 2020/2021. From the third and fourth year of study 64 students took part in in the questionnaire to assess motivation. The results showed that online teaching had a positive impact on the academic success of students with a statistically significant difference in the success achieved during online and face-to-face teaching. The research showed that students have a neutral academic motivation and a high degree of cooperation with professors and colleagues, with a significant difference between years of study.

Keywords: *online teaching, academic success, motivation, biology students*

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### 1. Introduction

During 2020 and 2021, educational systems worldwide faced significant challenges in combating the spread of the virus. To ensure the continuity of schooling and academic progress during the 2019/2020 academic year, educational institutions at all levels swiftly adapted and developed emergency online education models. These models embraced the

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concept of "disrupted classrooms, uninterrupted learning" (Huang et al., 2020), ushering in a new era of online learning (Anwar et al., 2021).

Higher education in Bosnia and Herzegovina (BiH) has not been exempt from the challenge of rapidly transitioning to online teaching. In both entities, due to the interruption of regular classes, online education has been organized through television, the internet, and other means. Given that education in the Federation of BiH is under the jurisdiction of cantons, there have been different approaches to maintaining the teaching process (Dedić, 2020).

### *1.1. Motivation and academic success in online classes*

Motivation plays a significant role in determining learning outcomes, but due to its complexity, it represents one of the most challenging aspects to measure (Graham & Golan, 1991). Motivation in online education is primarily influenced by individual characteristics and specific contexts (Hartnett et al., 2011). According to previous research, a decrease in student motivation for active participation in online education has been observed (Kyewski & Krämer, 2018). In the field of educational research, the most easily measurable behavior is academic achievement, but the relationship between motivation and academic success is complex, particularly due to difficulties in clearly understanding intelligence. As a result, researchers must consider individual differences in motivation when drawing conclusions (Breen & Lindsay, 1999). The results of some previous studies indicate improved learning outcomes in online education (Green et al., 2018), while other studies have not identified significant differences (Pickering & Swinnerton, 2019).

Online education has become inevitable in times of crisis, but it is increasingly gaining durability and institutionalization (Meyer, 2014). Therefore, questions regarding the effectiveness of online teaching and the satisfaction of students and teachers are crucial. Considering the limited research on this issue in Bosnia and Herzegovina, especially at the university level, the main objective of this study is to evaluate the level of motivation and academic achievement among students of the Department of Biology at the Faculty of Natural Sciences and Mathematics in Sarajevo during online learning. Before the research, the following research hypotheses were formulated:

1. There is no statistically significant difference in the achieved success of students in online and traditional classes;
2. Students have no expressed motivation in online classes;
3. There are no differences in intrinsic motivation in online classes with regard to the year of study and major;
4. There are no differences in the satisfaction of students with online teaching with regard to the year of study and major;
5. There are no differences in academic motivation for cooperation with colleagues and professors in online classes with regard to the year of study and major;
6. There are no differences in the overall academic motivation regarding the year of study and major.

## 2. Material and Methods

### *2.1. Student success*

The study examined the performance of students in 15 subjects from the first two years of study at the Department of Biology. Out of these subjects, seven belonged to the first year, while eight were related to the second year of study. To compare the performance of students during online and traditional classes, the academic years 2018/2019 (when face-to-face classes were held) and 2020/2021 (when classes were conducted online) were analyzed. In the academic year 2018/2019, 79 students attended the first year of study, whereas in the 2020/2021 academic year, this number decreased to 66 students. For the second year of study, 74 students attended in the academic year 2018/2019, and in the 2020/2021 academic year, the number increased to 90 students. Table 1 presents the subjects included in this study.

*Table 1. Subjects of the first and second year of study at the Department of Biology used in research*

| | | |
|---|---|---|
| **First year of study** | | |
| **Winter Semester** | | **Summer Semester** |
| 1 | Cell Biology | Plant Morphology |
| 2 | Systematics of Algae and Fungi | Histology and Embryology of Animals and Humans |
| 3 | Systematics of Lower Non-Chordates | Systematics of Higher Non-Chordates |
| 4 | | Organic Chemistry |
| **Second year of study** | | |
| **Winter Semester** | | **Summer Semester** |
| 1 | Systematics of Chordates | Molecular Biology |
| 2 | Comparative Anatomy of Animals and Humans | Systematics of Cormophytes |
| 3 | Biochemistry | General Physiology of Animals and Humans |
| 4 | General Microbiology | General Genetics |

*2.2. Student motivation*

An online survey was used to collect data. The questions, with certain modifications, were taken from studies conducted by Pesidas et al. (2022) and Babakova et al. (2021) among students of culture and arts. The survey consisted of 23 items divided into three categories (satisfaction, intrinsic motivation, and collaboration), and students responded using a Likert scale: 5 - Strongly agree, 4 - Agree, 3 - Neutral, 2 - Disagree, 1 - Strongly disagree.

The research sample consisted of third- and fourth-year students of the Department of Biology at the Faculty of Science and Mathematics in Sarajevo. A total of 64 students responded to the survey, of which 38 (59.4%) were third-year students and 26 (40.6%) were fourth-year students. Of the total number of respondents, 6 were male (9.4%) and 58 were female (90.6%). The research included all five study majors, and Table 2 presents the distribution of participants across these majors.

*Table 2. Distribution of participants' responses according to variable: major*

| | Major | f | % |
|---|---|---|---|
| 1 | Biochemistry and Physiology | 16 | 25% |
| 2 | Microbiology | 14 | 21,9% |
| 3 | Genetics | 10 | 15,6% |
| 4 | Ecology | 14 | 21,9% |
| 5 | Teaching major | 10 | 15,6% |

*2.3. Statistical analysis*

All research results were analyzed using statistical software programs Microsoft Excel and Past4.13 (Microfost Excel, 2019; Hammer, 2001). Alpha Cronbach was used to analyze the reliability of the entire instrument and its subscales. The reliability of the subscale "Satisfaction with online teaching" is 0.81, for the subscale "Intrinsic motivation" is 0.81, and for the subscale "Cooperation with professors and students" is 0.91. Overall, reliability for the entire instrument is 0.77, indicating moderate reliability.

To determine if there was a significant difference in academic motivation levels, when data was grouped by study program, inferential statistics known as one-way ANOVA were applied. Independent t-tests were used to analyze data on students' academic performance, comparing the mean exam grades of the subjects between the group that attended face-to-face classes during the 2018/2019 academic year and the group that underwent online teaching during the 2020/2021 academic year. The significance level was set at 0.05, with a confidence interval of 95%. This test was also used to compare the level of motivation, for each scale individually, between third-year and fourth-year students.

## 3. Results

### *3.1. Academic achievement*

The average grades from the subjects that students took during face-to-face instruction (2018/2019) were compared with the average grades from the subjects that students took online (2020/2021). The compared subjects were from the first two years of study as all majors had the same subjects. Figure 1 shows the average grades from the subjects in the first year of study.

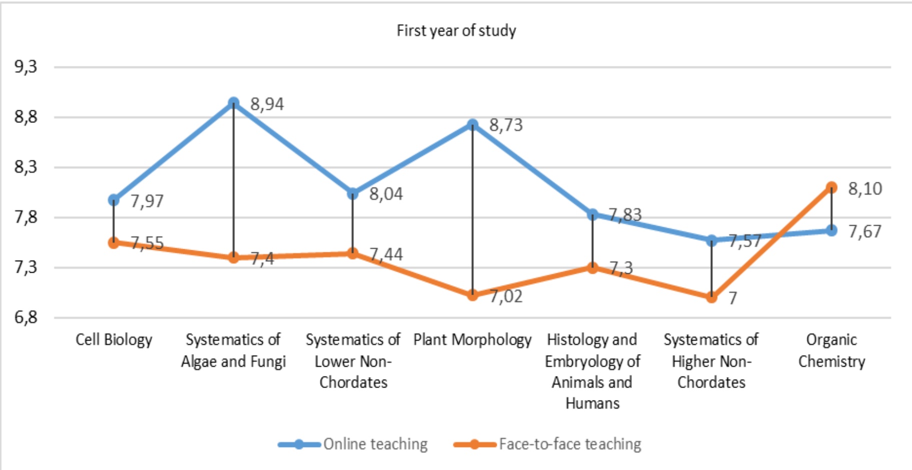


Figure 1. Success of students in the first year of study during online and face-to-face classes

The analysis of the results of students in the first year of study shows that the grades in the subject Organic Chemistry were better during traditional classes, while the results in all other subjects were better in online classes.

The results of descriptive statistics for the first year of studies can be seen in Table 3. The obtained p-value of 0.01 indicates statistically significant differences in favor of online teaching.

Table 3. Results of the analysis of the average grades from the first year of study

| | Online teaching | Face-to-face teaching |
|---|---|---|

| | Online teaching | Face-to-face teaching |
|---|---|---|
| Average | 8,11 | 7,4 |
| Standard deviation | 0,52 | 0,37 |
| Variance | 0,27 | 0,13 |
| Minimum value | 7,57 | 7 |
| Maximum value | 8,94 | 8,1 |
| P-value | 0,01 | |

In addition to the analysis for the first year of study, an analysis of academic success in subjects from the second year of study was also performed, the average grades of which can be seen in Figure 2.

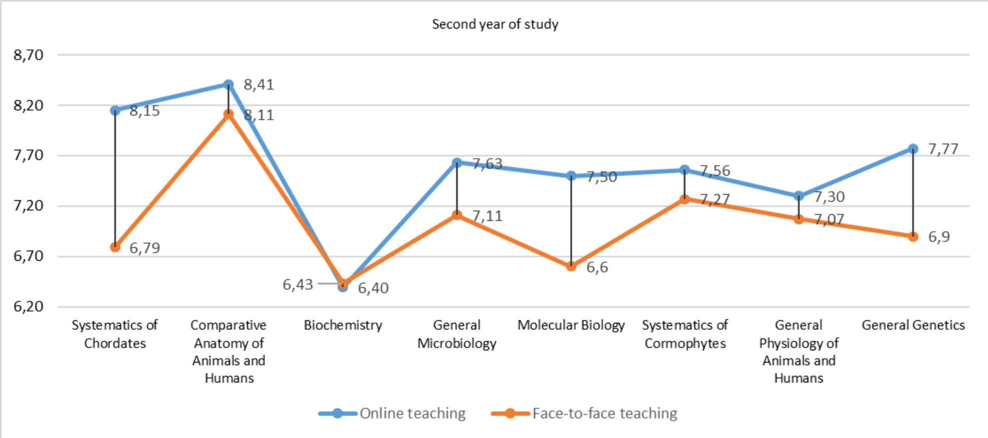


Figure 2. Success of students in the second year of study during online and face-to-face classes

Among the eight courses taken by students in the second year of study, the most significant difference is visible in the course Systematics of chordates, where students achieved significantly better results during online classes. There were no significant deviations in all other courses. The results of descriptive statistics for the second year of study can be seen in Table 4.

*Table 4. Results of the analysis of the average grades from the second year of study*

| | Online teaching | Face-to-face teaching |
|---|---|---|
| Average | 7,58 | 7,03 |
| Standard deviation | 0,6 | 0,5 |
| Variance | 0,36 | 0,26 |
| Minimum value | 6,39 | 6,43 |
| Maximum value | 8,41 | 8,11 |
| P-value | 0,06 | |

The p-value was 0.06, which means that there is no statistically significant difference in the success of students during online and traditional classes when it comes to subjects from the second year of study. Using the t-test for independent samples, the success in all subjects was compared for both years of study, in online and traditional classes, and a p-value of

0.004 was obtained. This means that there is a statistically significant difference in the success achieved in online and face-to-face classes.

*3.2. Student motivation*

*3.2.1. Academic motivation: Satisfaction with online teaching*

Responses to the subscale "Satisfaction with online teaching" were carefully examined and subjected to coding and analysis. The findings are presented in Figure 3, which illustrates the participants' responses to each item on the questionnaire, along with the corresponding mean (M) value. The data were coded based on the following scale: 1.0 – 1.79 (Strongly Disagree), 1.80 – 2.59 (Disagree), 2.60 – 3.39 (Neutral), 3.40 – 4.19 (Agree), and 4.20 – 5.00 (Strongly Agree).

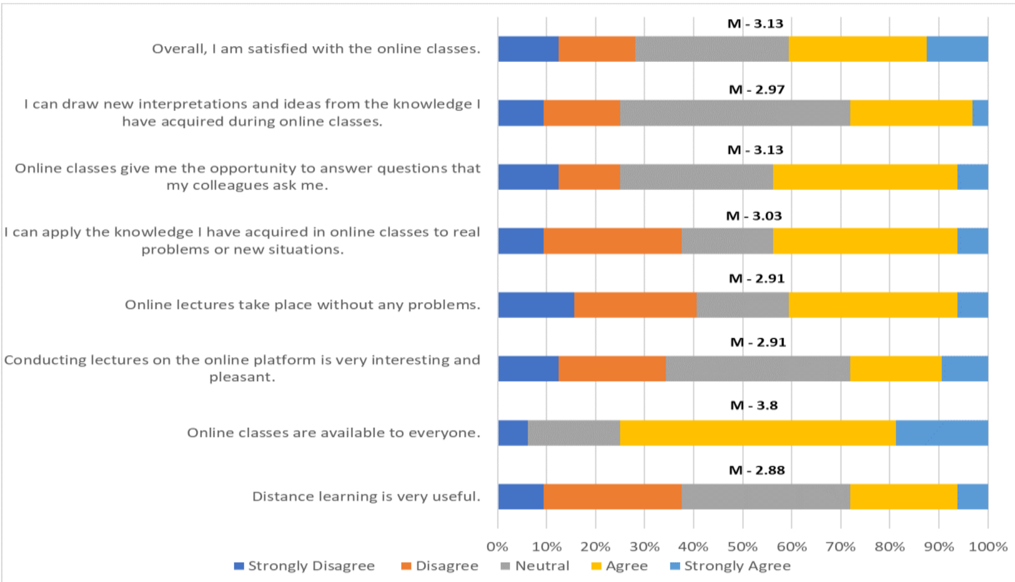


Figure 3. Results of student responses on academic motivation: Satisfaction with online teaching

The results shown in Figure 3 indicate that the respondents expressed a "neutral" attitude in most of the statements, which is further confirmed by the mean value (M) of 3.09 for all analyzed questions. However, the one statement that most respondents agreed with was that online classes are accessible to everyone.

*3.2.2. Academic motivation: Intrinsic motivation*

Responses to the subscale "Intrinsic motivation" were carefully examined and subjected to coding and analysis. The findings are presented in Figure 4, which illustrates the participants' responses to each item on the questionnaire, along with the corresponding mean (M) value. The data were coded based on the following scale: 1.0 – 1.79 (Strongly Disagree), 1.80 – 2.59 (Disagree), 2.60 – 3.39 (Neutral), 3.40 – 4.19 (Agree), and 4.20 – 5.00 (Strongly Agree).

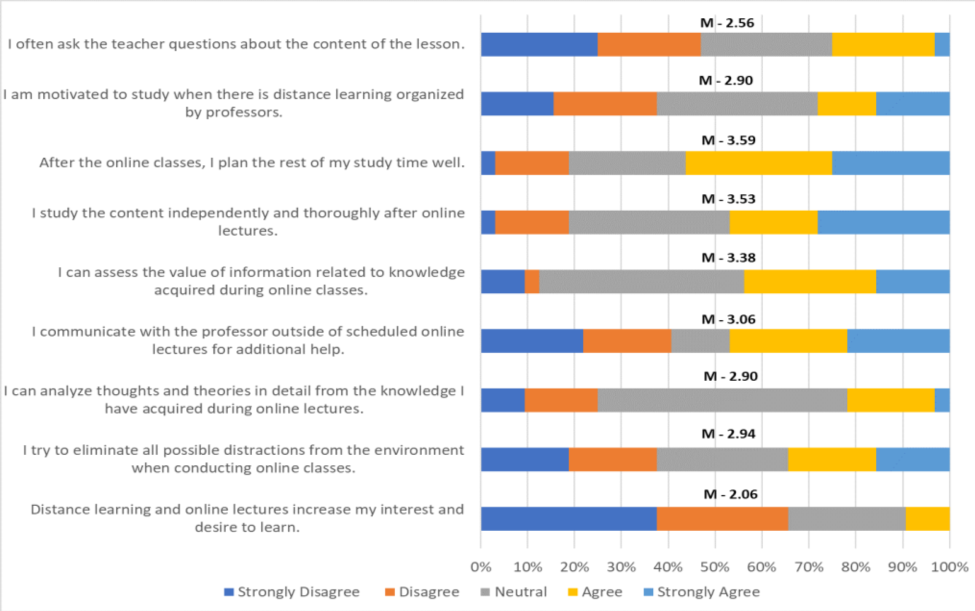


Figure 4. Results of student responses on academic motivation: Intrinsic motivation

The analysis of the data shown in Figure 4 indicates the academic motivation of the students of the Department of Biology in terms of intrinsic motivation (items 1-9). The results show that the respondents are "neutral" in statements 2, 5, 6, 7 and 8. Students agree with statements 3 and 4 and disagree with statements 1 and 9. The mean value for the entire subscale is 2.99 which means that the respondents expressed a neutral attitude towards internal motivation in online classes.

### *3.2.3. Academic motivation: Cooperation with professors and students*

Responses to the subscale "Cooperation with professors and students" were carefully examined and subjected to coding and analysis. The findings are presented in Figure 5, which illustrates the participants' responses to each item on the questionnaire, along with the corresponding mean (M) value. The data were coded based on the following scale: 1.0 – 1.79 (Strongly Disagree), 1.80 – 2.59 (Disagree), 2.60 – 3.39 (Neutral), 3.40 – 4.19 (Agree), and 4.20 – 5.00 (Strongly Agree).

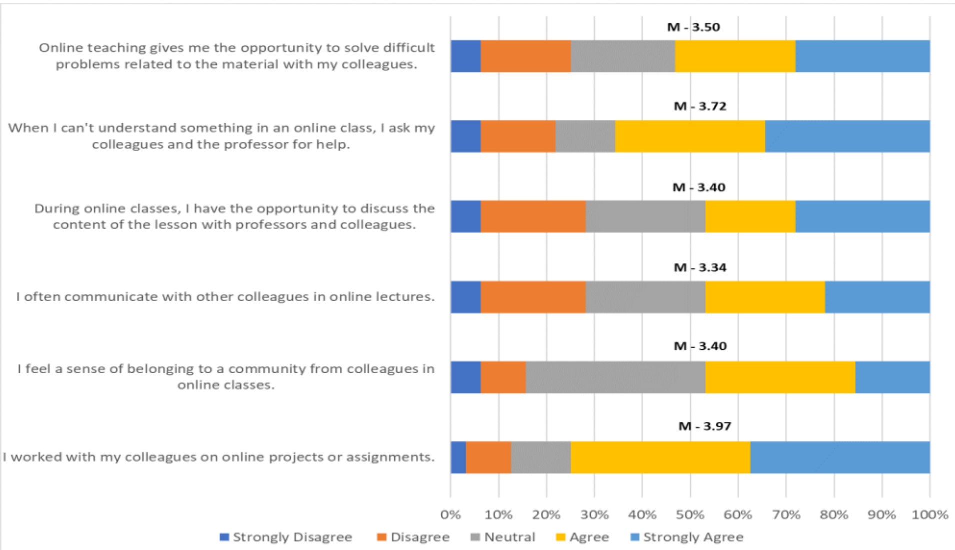


Figure 5. Results of student responses on academic motivation: Cooperation with professors and students

The mean value of the answers for this subscale is 3.56, which shows that the respondents "agree" that there is cooperation between professors and students in online classes. They expressed a neutral attitude only in the claim related to communication with colleagues in online lectures.

#### 3.2.4. *Motivation of students in relation to the year of study*

To determine if there is a significant difference in academic motivation during online classes with regard to year of study, the data set was analyzed using inferential and parametric statistics known as the T-test for independent samples (Table 5). Based on the data presented in table 5, it can be concluded that there are significant differences in overall academic motivation in online classes in relation to the year of study (value sig.= 0.04 < a = 0.05). The year of study has a significant impact on students' motivation.

*Table 5. Academic motivation of students in online classes in relation to the year of study*

| Variables | | | | | |
|---|---|---|---|---|---|
| Dependent | Independent | N | M | SD | Sig. (2-tailed) |
| Satisfaction with online teaching | third year | 38 | 2,96 | 0,74 | 0,24 |
| | fourth year | 26 | 3,26 | 0,64 | |
| Intrinsic motivation | third year | 38 | 2,85 | 0,75 | 0,21 |
| | fourth year | 26 | 3,19 | 0,74 | |
| Cooperation with professors and students | third year | 38 | 3,16 | 1,03 | 0,003 |
| | fourth year | 26 | 4,12 | 0,67 | |
| Overall motivation | third year | 38 | 2,97 | 0,70 | 0,04 |
| | fourth year | 26 | 3,47 | 0,60 | |

#### 3.2.5. *Students' motivation in relation to the major*

To determine whether or not there was a significant difference in the level of academic motivation during online classes on the subscales when the data were grouped by major, the data set was subjected to an inferential statistic known as one-way ANOVA.

Table 6 shows the results of the one-way ANOVA. As shown in the table, there was no statistically significant difference in academic motivation among majors in terms of academic motivation in online classes. P-values are greater than 0.05 implying that academic motivation in online classes is statistically similar among majors. Since p-values are greater than 0.05, performing post hoc tests is no longer necessary.

*Table 6. Academic motivation of students in relation to the major*

| Subscale | | MS | Sig. |
|---|---|---|---|
| Satisfaction with online teaching | Between groups | 0,48 | 0,49 |
| | Within groups | 0,54 | |
| Intrinsic motivation | Between groups | 0,50 | 0,5 |
| | Within groups | 0,58 | |
| Cooperation with professors and students | Between groups | 0,18 | 0,96 |
| | Within groups | 1,15 | |
| Overall motivation | Between groups | 0,20 | 0,82 |
| | Within groups | 0,53 | |

## 4. Discussion

### *4.1. Academic success*

The recent global pandemic of COVID-19 suddenly forced teachers to replace traditional written exams with alternative assessment methods (Borgaonkar et al., 2021; Clark et al., 2021; Sletten, 2021). Thus, at the Department of Biology, which is located at the Faculty of Science, all exams were held online during the pandemic.

The analysis of the results of this research showed significant differences in the academic success of students between face-to-face and online classes. In the first year of study, it was noticed that the results of most subjects were better in online classes. This leads to the conclusion that online teaching was equally and even more effective in most subjects compared to traditional teaching. Although the subject Histology and Embryology of Animals and Humans requires a lot of practical work, the knowledge was transferred well and the students achieved better results in online classes. Similarly, the results of a study conducted by Zheng and colleagues (2021) show that students had an equal or higher percentage of success in obtaining an "A" grade in online classes compared to face-to-face classes in Anatomy and Histology.

The analysis of the results for the second year of study showed that the most significant difference in the success of students is visible in the subject Systematics of chordates. From this, it can be concluded that online teaching methods are potentially useful, especially in areas that focus on theoretical education rather than practical skills. Although there were no significant differences in other subjects, the majority showed slightly better results in online classes. These results are consistent with previous studies in which researchers concluded that online teaching has a positive impact on students' academic achievement (Mahmoodi et al., 2015; Handique, 2017; Gonzalez et al., 2020). In the research conducted by Lestari et al. (2022), students achieved significantly better results in microbiology in online classes, which also coincides with this research. A recent study by Stevens and colleagues (2019) found similar results, showing that online teaching in clinical microbiology was well received by students and had a positive impact on their academic performance.

It is clear, based on this research, that students from subjects from the first year of study had statistically significantly better results on exams during online classes, while there was no statistically significant difference in subjects from the second year of study. Similarly, in the research conducted by Lestari et al. (2022), first- and second-year students had good results on exams during online classes, unlike third-year students, which means that the year of study has a significant impact on the success of students during online classes.

Limitations of this research include the limited sample of students from the same faculty and the specific subjects included in the analysis. Also, the duration of the research was limited to one academic year. Future research could expand the sample and include more faculties in order to gain a more comprehensive view of the impact of online teaching on students' academic success.

### *4.2. Student motivation*

Due to the widespread phenomena brought about by the pandemic, the results show that the respondents do not have a pronounced or positive academic motivation for studying online. These findings support the conclusions of Lee and Choi (2011), who pointed out that the distance between teachers and students hinders full communication and interaction, leading to a decrease in engagement in teaching. Babakova et al. (2021) and Pesidas et al. (2022) also investigated similar topics, but the difference in results is possible due to the different populations in their studies. Also, it is important to note that Babakova et al. (2021) and Pesidas et al. (2022) conducted research with a focus on students from the fields of culture and art.

In this research study, it is observed that there is a neutral degree of satisfaction among students regarding online teaching. It is possible that the root cause of low satisfaction with online teaching lies in the expectations that students have. It is likely that students had high expectations regarding online classes, but these expectations were not met. Some authors emphasize the importance of the psychological contract between students and teachers and claim that this contract plays a key role in achieving satisfaction with online teaching (Dziuban et al., 2015). The results of the intrinsic motivation test showed that students have neutral motivation, which is in line with the research conducted by Pesidas et al. (2022). The respondents expressed a neutral attitude towards most of the statements, but they agree with the statements related to independent study of the content after online lectures and good planning of study time after online lectures. The research on the cooperation of students with professors and colleagues indicates a significant engagement of students in these interactions. Roddy and colleagues (2017) note that teachers play an important role in the motivation and success of students in online classes. In contrast to this research, Bączek et al. (2021) suggest that students are less active during online lectures. Rovai et al. (2007) reveal differences in student motivation according to years of study in online education, which is also confirmed in this study with a statistically significant difference of 0.04 between students in the third and fourth year of study. However, the research results of Osmanagić (2021) show that, despite the existence of differences in the motivation of students of different years of study, these differences are not statistically significant. Regarding the motivation of students in different courses, no statistically significant differences were found, which coincides with the research of Francis et al. (2019), who found that although students in e-learning and traditional face-to-face education may differ in terms of academic achievements, motivation was not significantly different. Overall, these findings highlight the complexity of student motivation and satisfaction in the context of online classes.

## 5. Conclusions

Based on the analysis of student motivation and success in online education, our research gave key insights into the dynamics of contemporary education.

Confirming our first hypothesis, we observed a significant difference in achievement between online and traditional classes. However, the results did not support our expectations from the second hypothesis about expressed motivation in the online environment, which indicates the neutrality of student motivation. Our analysis revealed no significant differences in students' intrinsic motivation and satisfaction, confirming our third and fourth predictions. On the other hand, we observed differences in cooperation with professors depending on the year of study, while differences related to major were less significant, partially supporting our fifth hypothesis. Furthermore, our sixth hypothesis regarding the lack of differences in overall academic motivation by major was also confirmed, while significant differences emerged depending on the year of study, highlighting the need for further research to better understand the specific factors that influence motivation in online environments. These findings emphasize the importance of developing strategies aimed at improving the online education experience for all students.

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