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Phytochemical Traits and Biological Activity of *Eryngium amethystinum* and *E. alpinum* (Apiaceae)

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Abstract: *Eryngium amethystinum* L. is a wild vegetable used in Croatia. Both *E. amethystinum* and *E. alpinum* L. are decorative plants that can also be used in horticulture. To find out the hidden qualities of these two species, the following biological activities were studied: essential oil (EO) composition, analyzed by gas chromatography and gas chromatography with mass spectrometry (GC, GC–MS); phenolic compound content (PC), analyzed by high performance liquid chromatography (HPLC); total phenols as well as total flavonoids, analyzed by ultraviolet–visible spectrophotometry (UV/Vis); antioxidants, analyzed by 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity (DPPH), β-carotene-linoleic acid assay, chelating activity and reducing power of the extracts; and antimicrobial evaluation by micro-dilution assay and inhibition of *Candida albicans* blastospore germination. The major constituents of EO were *β*-caryophyllene (15.2%), *α*-pinene (10.2%) and 2,3,6-trimethylbenzaldehyde (9.3%) for *E. amethystinum* and caryophyllene oxide (27.9%), bicyclogermacrene (13.2%) and germacrene D (8.2%) for *E. alpinum*. The methanol extracts of both species showed a broad spectrum of antibacterial and antifungal activity with minimum inhibitory concentrations (MIC) less than or equal to 1.944 and 1.11 mg/mL, respectively.

Keywords: antifungal activity; antimicrobial activity; *Candida*; essential oils; germ-tube inhibition; phenolic compounds

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1. Introduction

The genus *Eryngium* L. (family Apiacea) includes 230–250 herbs widespread in America, Eurasia, Africa, Malesia and Australia [1]. Twenty-six species of *Eryngium* have been recorded in Europe [2] and five in Croatia [3]. Some *Eryngium* species have been used as remedies, especially in folk medicine, and as horticultural plants. *Eryngium creticum* Lam. has been used in folk medicine in Palestine as a diuretic and emmenagogue, and for the treatment of kidney stones and infections, skin diseases and tumours [4]; *E. foetidum* L. for the treatment of respiratory diseases (cold, cough, asthma, sinusitis) and for diarrhoea and rheumatism in traditional medicine of the Democratic Republic of São Tomé and Principe [5]; *E. billardieri* Delar. for the healing of inflammation in Turkey [6]; *E. campestre* L. as a diuretic and emmenagogue, in respiratory system and gastric diseases, skin diseases and periodontosis [7]; and *E. planum* L. for cough [7]. In the folk medicine of Balkan peoples, *E. campestre* L. is used against hepatitis [8]. In addition, the root, young shoots and leaves of *E. amethystinum* L., *E. campestre* and *E. maritimum* are wild vegetables that are consumed in northern Dalmatia, Croatia [9]. People cook the young shoots and prepare them as asparagus. The young leaves are picked and cooked together with other wild

vegetables [9]. Chemical research on the genus *Eryngium* has detected the presence of essential oils (EO) [5,10–12], saponins [6,13] and phenolic compounds [13].

Oxidative stress is a condition included in the pathogenesis of various illnesses such as neurodegenerative diseases, cancer, diabetes mellitus, rheumatoid arthritis, and cardiovascular diseases [14]. Plant life produces many secondary metabolites with antioxidant properties that are considered to be protective against oxidative damage. Among these plant secondary metabolites, phenolic compounds have a particularly prominent position, as they are believed to be responsible for the lower frequency of coronary diseases in communities that consume polyphenol-rich foods [15]. In addition, constant intake of polyphenols has been shown to be associated with a lower frequency of stomach, pancreatic, lung, and possibly breast cancer [16]. In addition to their health-promoting effects, polyphenols also have the ability to protect fatty acids from oxidative decay.

E. amethystinum is the most widely distributed *Eryngium* species in Croatia, and grows on dry, skeletal, limestone soils. Due to its decorative appearance and resistance to drought, it can also be used as an ornamental species in areas with a dry climate. Considering its use as food and in view of its possible use as an ornamental plant, it is useful to know what this species contains. In order to compare the chemical composition of *E. amethystinum* with another *Eryngium* species, we chose the beautiful mountain plant *E. alpinum* L., which is much less common in Croatia and grows on fresh, moist, mostly calcareous soils in mountainous areas. Thus, the goal of this investigation is to analyze the content of essential oil and phenolic compounds, as well as the antioxidant and antimicrobial activity of both *Eryngium amethystinum* and *E. alpinum*.

2. Materials and Methods

2.1. Herbal Material

Plant cuttings from ten randomly selected wild plants from one locality per species of *Eryngium amethystinum* and *E. alpinum* (Apiaceae) were collected during their flowering season in June and August of 2019 in Croatia. The GPS coordinates and altitude were 44°32′21″ N, 15°09′51″ E, 970 m a.s.l. (*E. amethystinum*) and 44°45′56″ N, 14°59′15″ E, 1560 m a.s.l. (*E. alpinum*). Voucher specimens of *E. amethystinum* (voucher specimen No. HFK-HR-11-2019) and *E. alpinum* (voucher specimen No. HFK-HR-34-2019) from plant materials were deposited at the "Fran Kušan" Herbarium, University of Zagreb, Faculty of Pharmacy and Biochemistry, Zagreb, Croatia.

The plant samples were protected from direct sunlight and air-dried at 22 °C at a humidity of 60% for 15 days.

2.2. Microorganisms

Microbial strains: *Staphylococcus aureus* ATCC (American Type Culture Collections; Rockville, USA) 6538, *Escherichia coli* ATCC 10535, *Candida albicans* ATCC 10231, *C. albicans* MFBF (Collection of the Department of Microbiology Faculty of Pharmacy and Biochemistry University of Zagreb, Croatia) 40630/2, *C. parapsilosis* MFBF 4800, *C. krusei* MFBF 429, *C. glabrata* MFBF 3309 and *Microsporum gypseum* MFBF S3) from the collection of microorganisms of the University of Zagreb (Faculty of Pharmacy and Biochemistry, Department of Microbiology) were used. Sabouraud 2% (*w*/*v*)-glucose agar, Müller–Hinton agar and broth were purchased from Merck (Germany). The RPMI 1640 broth was purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.3. Gas Chromatography and Mass Spectrometry (GC and GC–MS)

2.3.1. Sample Extract Preparation

Dried flowering above ground plant parts (100 g) were subjected to hydrodistillation in Clevenger apparatus for 3 h. After that, the obtained EO was immediately dried with anhydrous sodium sulphate.

2.3.2. GC, GC–MS Conditions

The chromatographic conditions during analysis were as follows: carrier gas helium at 1 mL·min^{-1}, temperature of injector of 250 °C, while the *flame ionization* detector temperature was 300 °C. The temperature programme of the VF-5MS column was: isothermal at 60 °C for 3 min, then up to 246 °C at a rate of 3 °C·min^{-1} and finally remaining isothermal for 25 min. The injection quantity was set at 1 µL while the split ratio was set up to 1:20. The mass spectrometry conditions were: ion temperature 200 °C; mass scan range: 40–350 mass units; ionisation voltage 70 eV. Analyses were performed in duplicate. Each peak was determined by comparing of their retention indices (for VF-5MS based on C8-C40 n-alkanes) with those of the homemade library, original samples, and by comparison of their mass spectra from the literature [17,18], NIST02 (Gaithersburg, MD, USA), and Wiley 9 mass spectrometry (Wiley, New York, NY, USA) mass spectral database. A home library was constructed from original commercially available chemicals and from the major compounds of several EOs from our previous investigations. The percentage of each component was obtained as averages of peaks area from GC and GC–MS using the normalisation method but without correction factors. Finally, the percentage of each compound was calculated as mean from three both GC and GC–MS analyses.

2.4. HPLC Analysis

2.4.1. Sample Extract Preparation

Ultrasonic extraction was performed at 25 °C for 60 min with 500 mg of powdered plant material and 20 mL of 80% ethanol. The each obtained extract was filtered and the filtrate was diluted to a volume of 25.0 mL using 80% ethanol. After that, extracts were filtered using a 0.45 µm PTFE 25 mm filter (Restek, Bad Homburg, Germany). Finally, 5 µL of each prepared extract was inserted into the HPLC instrument for analysis.

2.4.2. Preparation of Standard Solutions

The solutions of all tested standard compounds were obtained according to Kremer et al. [19]. Briefly, stock solutions of each standard were separately diluted in a solution of methanol and water (1:1, *v*/*v*) to obtain a concentration of exactly 1.0 mg/mL. After that, the working solution was diluted with a mixture of methanol and water (1:1, *v*/*v*) to obtain a concentration of 0.01 mg/mL. The standard mixture was obtained by the diluting all stock solution separately to a final concentration of 0.01 mg/mL using the same mixture of solvents.

2.4.3. HPLC Conditions

HPLC analysis of investigated samples was done using an HPLC system Agilent 1100 Series (Agilent, Santa Clara, CA, USA) and the procedure described by Kremer et al. [19] and Čeh et al. [20]. The Agilent Zorbax Eclipse XDB-C_{18} reversed-phase packed column (5 µm,150 mm × 4.6 mm) was utilized for separation. The separation was carried out at a temperature of exactly 30 °C. A gradient elution was applied on the chromatographic system. The gradient elution mobile phase consisted of water with acetic acid (pH value of 2.50; solvent A) and acetonitrile (solvent B). The mobile phase used with the program was as follows: starting with 85% solvent A and 15% solvent B, then from 15% solvent B to 22.5% B over a period of 15 min, followed by 40% solvent B for a duration of 10 min and then staying consistent for another period of 5 min. Initial conditions were adjusted after 5 min. The volume of injection for all investigated plant extracts and standards was 5 µL, while the flow rate was set to 1.0 mL/min. A diode array detector was used for identification of compounds. A wavelength of 280 nm was used for the detection of chrysin, naringenin, vanillic, syringic, gallic and protocatehuic acid. On the other hand, a wavelength of 320 nm was applied for detection of ferulic, *p*-coumaric, sinapic, rosmarinic and caffeic acid, while a wavelength of 370 nm was used for the detection of quercetin and rutin.

Determination of specific compounds was done by comparison of unknown peaks in the plant extracts with the retention times of the standards. The method of standard addition was used to avoid errors in the interpretation of results. External standards were used for quantification.

2.5. Total Phenol and Total Flavonoid Content

For determination of total flavonoid and polyphenol content, antimicrobial as well as antioxidant activity, extracts of flowers, stems and leaves of both species were prepared. Extract preparation was performed by ultrasonication of 2.5 g of powdered material with 8 mL of 80% ethanol at 30 °C for 30 min. The obtained samples were filtered using Whatman paper No. 542. All plant samples were re-extracted two times. After that, each combined filtrate was evaporated to dryness under diminished pressure and stored in the dark in a fridge at +4 °C until use. The total polyphenol content in the obtained extracts was detected with the use of the Folin–Ciocalteu colorimetric method described by Singleton et al. [21]. The calibration curve of gallic acid was constructed and the results were expressed as gallic acid equivalents (mg/g). On the other hand, the determination of flavonoid content was made using the method of Kumazawa et al. [22]. The obtained results were calculated and expressed as quercetin equivalents in mg/g.

2.6. Antioxidant Capacity

2.6.1. DPPH Radical-Scavenging Activity

The method described by Zovko Končić et al. [23], with some minor modifications, was performed to investigate DPPH radical-scavenging activity. In short, 1 mL of each extract was mixed with 1 mL of 0.16 mM methanolic DPPH solution. The reaction solution was incubated over a period of 30 min at room temperature. After incubation, the degree of absorbance was determined at a wavelength of 517 nm using methanol as a blank.

RSA (radical scavenging activity) was obtained according to the equation $RSA = (1 - A_{sample}/A_{control}) \times 100$. In this equation, $A_{control}$ denotes the absorbance degree of the methanol (which is used as a control), while A_{sample} denotes the absorbance degree of the examined extract. Butylated hydroxyanisole (BHA) was taken as a radical scavenging standard. The radical-scavenging activity of DPPH was expressed as EC_{50}, denoting the concentration at which 50 percent of free radicals were scavenged from DPPH.

2.6.2. β-Carotene-Linoleic Acid Assay

The β-carotene-linoleic acid test described by Amarowicz et al. [24] was applied to investigate the antioxidant activity of prepared plant extracts. An emulsion of β-carotene-linoleic acid mixture was prepared in a few steps. At first, 0.2 mg of β-carotene was dissolved in 10 mL of chloroform. After that, 1 mL of the solution was added to a mixture made from 200 mg of Tween 40 and 20 mg of linoleic acid. Chloroform as an undesirable component was eliminated using reduced pressure. Then, distilled water saturated with oxygen (50 mL) was added with energetic shaking. The 5 mL of the prepared emulsion was transferred into tubes with 2 mg of extract or 0.5 mg of BHA. An emulsion without added antioxidant was used as a control. After that, each tube was put into a water bath for 2 h at a temperature of 50 °C. During the next 2 h, the absorbance degree of each plant extract and control was measured at a wavelength of 470 nm at intervals of 15 min. The measurement was begun immediately after plant sample preparation (t = 0 min) and finished after 2 h (t = 120 min). The first-order kinetics were used to calculate R (rate of β-carotene bleaching) for the extracts, BHA and water. The antioxidant activity degree or ANT was expressed as a percentage this was calculated according to the equation $ANT = (R_{control} - R_{sample}/R_{control}) \times 100$. In this equation, $R_{control}$ and R_{sample} denote the average bleaching rates of control (water) and antioxidant (extract made from herbal material and BHA), respectively.

2.6.3. Fe^{2+} Chelating Activity (ChA)

The method of Decker and Welch [25] was used to estimate the chelation of iron ions (II). At first, the methanol extract (1.3 mL) was mixed with 2 mM $FeCl_2$ (100 µL). Following this, the reaction was initiated after 5 min by addition of 5 mM ferrozine (200 µL). The obtained solution was left at room temperature for a period of 10 min. Methanol (1.3 mL) was used as a control. The absorbance degree of the mixture and control was estimated at a wavelength of 562 nm. The ChA was calculated using the equation: $ChA = (1 - A_{sample}/A_{control}) \times 100$. In this equation, A_{sample} is the absorbance value of the extract, while $A_{control}$ is the absorbance value of the control. The $ChEC_{50}$ (the concentration which chelates 50 percent of the Fe^{2+} ions) was calculated at the end. Quercetin and EDTA (ethylenediaminetetraacetic acid) were used for comparison.

2.6.4. The Reducing Power of the Extracts

The method described by Yen et al. [26], with some minor variations, was used for estimating the reducing power of the extracts. At first, extracts in concentrations from 0.1 to −0.5 mg/mL were dissolved in 0.5 mL of distilled water. Following this, each solution was mixed with 200 mM of sodium phosphate buffer with a pH value of 6.6 (1.25 mL) and 1% (*m*/*v*) of potassium ferricyanide (1.25 mL). The prepared mixture was left to stand at 50 °C for a period of 20 min. After incubation, 10% (*m*/*v*) trichloroacetic acid (1.25 mL) was added to each sample. The obtained mixture was then centrifuged at 2795× *g*; only the 1.25 mL of the upper layer was removed. The obtained solution was mixed with 0.1% (*m*/*v*) ferric chloride (0.25 mL) and deionized water (1.25 mL). At the end of the procedure, the absorbance value of the mixture solution was measured at a wavelength of 700 nm against water as a blank. For the comparison, ascorbic acid was used.

2.7. Antimicrobial Susceptibility Assay

2.7.1. Micro-Dilution Assay

Prior to analysis, plant extracts were diluted. The dilution was made with 70% (*v*/*v*) ethanol and a final concentration of 20 mg/mL was obtained. A series of two-fold diluted extracts ranged from 10 mg/mL to 4.89 µg/mL was used for implementation of the micro-dilution assay. Fresh cultures of microbial strains cultured on tryptic-soy agar at a temperature of 37 °C for a period of 18 h (for bacteria) and of 48 h (for fungi) were used for preparation of inoculums. Yeasts and dermatophyte were cultivated with the addition of 50 mg/L of chloramphenicol on Sabouraud 2% (*w*/*v*) glucose agar at a temperature of 37 °C for 48 h. A nephelometer was used to set the density of the inoculums to 0.5 McFarland units. The final concentrations were 1.5×10^8 CFU (colony forming units) per mL for tested bacteria and 5×10^5 CFU/mL for tested fungi. The MIC, that is, the minimal inhibitory concentration, was estimated using the twofold micro-dilution method. The assay was conducted using Müller–Hinton broth for bacteria and RPMI (Roswell Park Memorial Institute) 1640 medium (pH value of 7.0) for yeasts, adhering to the Clinical and Laboratory Standards Institute (formerly known as NCCLS) recommendation M07-A8 and recommendation M-27A [27,28]. The MIC value was determined as the lowest concentration of the extract which allowed microbial growth up to 20 percent. This is observed as a reduced number of microbial colonies after removal of a loop with 10 µL of each rarefaction on a substrate made from tryptic-soy agar or Sabouraud agar, with addition of 2% glucose agar and incubation for 18–48 h at a temperature of 37 °C. The growth of microbes was determined by the use of a microbial culture medium. All analyses were performed in triplicate. The obtained values were calculated as mean ± SD (standard deviation).

2.7.2. Inhibition of Germination of Candida Albicans Blastospores

Inhibition of creation of germ-tube as a virulence factor of *C. albicans* was done using the method of Ishida et al. [29]. Briefly, the suspensions of *C. albicans* blastospores were cultivated in Sabouraud 2% (*w*/*v*) glucose broth at a temperature of 37 °C for 24 h. After

that, suspensions were centrifuged at 2500× *g* and washed twice using phosphate buffer (pH 7.4). Approximately 1–5 × 10^4 of blastospores were treated with 100 mg and 300 mg of both *Eryngium* L. spp. in fetal bovine serum (FBS; Sigma-Aldrich, USA) for 3 h at 37 °C. Blastospores in FBS without extracts were served as a negative control in this investigation. After the incubation period, the ratios between germinated and non-germinated blastospores were calculated using a haemocytometer under 1000× magnification by phase-contrast microscopy. Sixty corner squares were observed for each test tube. During observation and counting, the tubes were kept at +4 °C.

2.8. Statistical Analysis

Comparison of the content of investigated phenolic constituents in different plant parts, as well as antioxidant activity among plant parts were estimated using ANOVA (one-way analysis of variance) and a Dunnett's post-hoc test. Student's t-test was used to compare different extracts while Welch's t-test was used for comparisons between species. p values < 0.05 were taken as statistically significant. These analyses were carried out using SAS software (JMP V6 procedure) [30]. Statistical comparisons of antimicrobial activity among species were performed using a one-way ANOVA and a Scheffe's post-hoc test. p values < 0.05 were again taken as significant. Analysis was carried out with help of Statistica 7 software package [31]. Logarithmic transformation was used for data transformation before the analysis.

3. Results and Discussion

3.1. Gas Chromatography and Mass Spectrometry (GC and GC–MS)

Thirty-nine (*E. amethystinum*) and thirty-six components (*E. alpinum*) were determined in the EO, accounting for 91.3% and 90.4% of the total oil composition, respectively (see Table 1). The oil of *E. amethystinum* was mainly composed of sesquiterpenes (54.9%), especially sesquiterpene hydrocarbons (35.2%). Eleven sesquiterpene hydrocarbons were determined in this oil, and among them β-caryophyllene (15.2%) and germacrene D (5.9%) predominated. Dunkić et al. [32] also found that β-caryophyllene (19.7%) was the major compound in *E. amethystinum* from Croatia [32], while germacrene D (31.3%) was identified as the main compound in *E. amethystinum* growing in Italy [12,33]. Germacrene D was also the main compound (19.7%) in *E. serbicum* Pančić from Serbia [11] and one of the main compounds (0.4–53.4%) in *Eryngium campestre* from Western Algeria [34]. Oxygenated sesquiterpenes (athulenol, ledol and α-bisabolol) were also the dominant compound in *E. dichotomum* C. Presl, *E. ilicifolium* Lam. and *E. triquetrum* Vahl growing in Tunisia [35]; a higher concentration of carbonylic compound 2,3,6-trimethylbenzaldehyde (9.3%) was also determined, which was also identified in high concentrations in the oils of Italian *E. amethystinum* [12] and in oils of *E. foetidum* from São Tomé and Principe [5]. However, in the oil of *E. triquetrum* Vahl [36], the isomers of germacrene D and trimethylbenzaldehyde are completely absent.

Table 1. Composition (expressed in %) of the essential oils of both *E. amethystinum* and *E. alpinum*.

Component	R	*E. amethystinum*	*E. alpinum*	Identification	CAS No.
Monoterpene hydrocarbons		14.5	12.0		
α-Pinene	938	10.2 ± 0.01	1.9 ± 0.01	RI, MS, Co-GC	80-56-8
Camphene	962	–	0.8 ± 0.01	RI, MS	79-92-5
β-Pinene	982	0.3 ± 0.1	3.3 ± 0.07	RI, MS, Co-GC	127-91-3
Myrcene	992	2.3 ± 0.01	5.1 ± 0.01	RI, MS	123-35-3.
Limonene	1032	0.9 ± 0.05	–	RI, MS, Co-GC	5989-27-5
(*Z*)-β-Ocimene	1052	–	0.6 ± 0.07	RI, MS	3338-55-4
Terpinolene	1089	0.8 ± 0.07	0.3 ± 0.02	RI, MS	586-62-9
Oxygenated monoterpenes		8.9	4.7		

Table 1. *Cont.*

Component	R	*E. amethystinum*	*E. alpinum*	Identification	CAS No.
β-Thujone	1121	0.3 ± 0.01	-	RI, MS	1125-12-8
trans-Pinocarveol	1147	0.7 ± 0.01	–	RI, MS	547-61-5
Camphor	1151	4.1 ± 0.03	2.8 ± 0.01	RI, MS, Co-GC	76-22-2
Borneol	1176	–	0.1 ± 0.02	RI, MS	507-70-0
Terpinen-4-ol	1184	0.7 ± 0.01	0.3 ± 0.01	RI, MS	562-74-3
β-Thujone	1121	0.3 ± 0.01	-	RI, MS	471-15-8
Myrtenol	1197	0.9 ± 0.01	0.6 ± 0.01	RI, MS	515-00-4
Linalyl acetate	1252	0.8 ± 0.02	0.3 ± 0.02	RI, MS	115-95-7
Bornyl acetate	1285	0.4 ± 0.07	–	RI, MS	76-49-3
α-Terpenyl acetate	1349	0.1 ± 0.02	0.4 ± 0.03	RI, MS	80-26-2
Sesquiterpene hydrocarbons		35.2	28.9		
α-Copaene	1377	0.9 ± 0.01	–	RI, MS	3856-25-5
β-Bourbonene	1383	1.7 ± 0.01	0.6 ± 0.07	RI, MS	5208-59-3
α-Gurjunene	1407	3.6 ± 0.01	tr	RI, MS	489-40-7
β-Caryophyllene	1424	15.2 ± 0.01	2.1 ± 0.01	RI, MS, Co-GC	87-44-5
β -Copaene	1429	0.8 ± 0.02	0.5 ± 0.07	RI, MS	18252-44-3
trans-α-Bergamotene	1433	0.4 ± 0.01	–	RI, MS	13474-59-4
(*Z*)-β-Farnesene	1454	0.3 ± 0.01	0.2 ± 0.03	RI, MS	28973-97-9
α-Humulene	1456	1.1 ± 0.01	–	RI, MS	6753-98-6
allo-Aromadendrene	1465	0.6 ± 0.01	–	RI, MS	25246-27-9
Germacrene D	1481	5.9 ± 0.01	8.2 ± 0.01	RI, MS	23986-74-5
β-Bisabolene	1494	–	1.3 ± 0.01	RI, MS	495-61-4
Bicyclogermacrene	1500	4.7 ± 0.01	13.2 ± 0.01	RI, MS	24703-35-3
δ-Cadinene	1517	–	2.8 ± 0.01	RI, MS	483-76-1
Oxygenated sesquiterpenes		19.7	41.4		
Spathulenol	1577	0.3 ± 0.02	–	RI, MS	6750-60-3
Caryophyllene oxide	1581	4.2 ± 0.01	27.9 ± 0.01	RI, MS, Co-GC	1139-30-6
γ-Eudesmol	1632	6.4 ± 0.01	5.7 ± 0.01	RI, MS	1209-71-8
α-Cadinol	1655	0.5 ± 0.01	–	RI, MS	481-34-5
α-Bisabolol	1688	8.3 ± 0.01	7.8 ± 0.01	RI, MS	515-69-5
Phenolic compounds		2.0	1.7		
Thymol	1290	1.2 ± 0.01	0.9 ± 0.01	RI, MS, Co-GC	89-83-8
Carvacrol	1299	0.5 ± 0.01	0.6 ± 0.01	RI, MS, Co-GC	499-75-2
Eugenol	1370	0.3 ± 0.07	0.2 ± 0.01	RI, MS, Co-GC	97-53-0
Carbonylic compounds		10.0	0.2		
3-Octanol acetate	1125	0.4 ± 0.02	0.2 ± 0.01	RI, MS	4864-61-3
Butylhexanoate	1193	0.3 ± 0.01	–	RI, MS	626-82-4
2,3,6-Trimethylbenzaldehyde	1340	9.3 ± 0.01	–	RI, MS	34341-29-2
Hydrocarbons		1.0	1.5		
Eicosane	2000	–	0.2 ± 0.03	RI, MS, Co-GC	112-95-8
Docosane	2200	0.5 ± 0.01	0.3 ± 0.01	RI, MS, Co-GC	629-97-0
Tricosane	2300	–	0.2 ± 0.01	RI, MS, Co-GC	638-67-5
Tetracosane	2400	0.2 ± 0.01	–	RI, MS, Co-GC	646-31-1
Pentacosane	2500	–	0.3 ± 0.01	RI, MS, Co-GC	629-99-2
Hexacosane	2600	–	0.1 ± 0.02	RI, MS, Co-GC	630-01-3
Octacosane	2800	–	0.3 ± 0.02	RI, MS, Co-GC	630-02-4
Nonacosane	2900	0.3 ± 0.02	0.1 ± 0.1	RI, MS, Co-GC	630-03-5
Total identified (%)		91.3	90.4		
Yield (%)		0.1	0.1		

Note: R = retention indices were defined relative to a series of n-alkanes (C8–C40) on capillary column VF5-MS; RI = identification made using the literature [17]; MS = identification made with help of database NIST02, Wiley 7 and homemade library; Co-GC = identification using reference compounds; – = component is not determined; tr = traces (mean value below 0.1%); SD = standard deviation (N = 3); CAS No. = CAS Registry Number [18].

In EOs of *E. alpinum*, the major fraction consisted of sesquiterpenes (70.3%). Among these, oxygenated sesquiterpenes (41.4%) prevailed on sesquiterpene hydrocarbons (28.9%). The main components were caryophyllene oxide (27.9%) and bicyclogermacrene (13.2%). Caryophyllene oxide was also found in *E. alpinum* from Croatia (21.6%) [32] and in *E. palmatum* Pančić et Vis. (16.0%) from Serbia [11]. On the other hand, bicyclogermacrene (12.5%) was the main compound in *E. rosulatum* P. W. Michael from Australia [10]. Kikowska et al. [37] found that the main compounds in leaves of ground (intact) plants of *E. alpinum* growing in a botanical garden in Poznań (Poland) were elemenone (10.3%), selina-4(15),7(11)-diene (7.1%), selina-3,7(11)-diene (6.7%) germacrone (5.8%) and 1,8-cineole (5.3).

3.2. HPLC Analysis

The concentrations of the phenolic compounds are shown in Table 2. Quercitrin, protocatehuic and rosmarinic acid were identified in *E. amethystinum*, while coumaric acid was identified in *E. alpinum*. Only rutin was identified in both species. In both the shoots of the ground plant of *E. alpinum* and shoots cultured in vitro (developed from axillary buds and regenerated from callus tissue) Kikowska et al. [38] found chlorogenic, isochlorogenic, rosmarinic and 3,4-dihydroxyphenylacetic acid. On the other hand, caftaric, neochlorogenic and caffeic acid were found only in shoots cultured in vitro. Moreover, isoquercitin was identified only in shoots of ground plants, while quercitrin was found only in shoots cultured in vitro [38].

Table 2. Contents of investigated phenolic compounds (%) determined in methanolic solvent.

Compound	*E. amethystinum*	*E. alpinum*
Chrysin	–	–
Rutin	0.002 ± 0.000	0.001 ± 0.000
Quercetin	–	–
Quercitrin	0.026 ± 0.005	–
Cichoric acid	–	–
Coumaric acid	–	tr
Ferulic acid	–	–
Protocatehuic acid	0.015 ± 0.003	–
Rosmarinic acid	0.005 ± 0.001	–
Syringic acid	–	–
Tannic acid	–	–

Extracts of *E. amethystinum* and *E. alpinum*. Tr = traces (mean < 0.001%); N = 3.

Rutin was also identified as one of the flavonoids in *E. campestre* and *E. octophyllum* Korovin, while quercitrin was found in *E. campestre* [39,40] and *E. creticum* [41]. In addition, isoquercitrin was found in *E. campestre*, *E. maritimum* L. and *E. giganteum* M. Bieb. [39,42,43]. According to Vukic et al. [44], the most abundant phenolic compounds in *E. serbicum* were chlorogenic and rosmarinic acid. Le Claire et al. [45] identified rosmarinic acid in *E. alpinum*, *E. amethystinum*, *E. maritimum* and *E. campestre*. However, rosmarinic acid was not found in our analysed sample of *E. amethystinum*. This difference could be ascribed to some ecological conditions and genetic variations within the species.

3.3. Total Phenol and Total Flavonoid Content

The results indicated that the quantity of total polyphenols in *E. alpinum* was almost twice as high as the content of phenols in *E. amethystinum* (Table 3). On the other hand, the extracts from the leaves and stems of *E. amethystinum* contained significantly greater amounts of total flavonoids than corresponding extracts of *E. alpinum*. The total phenolic contents in aqueous, ethyl acetate and *n*-hexan extracts of *E. caucasicum* Trautv were 214.18, 140.57, and 29.06 mg of gallic acid equivalent per gram of investigated extract, respectively. On the other hand, the total flavonoid contents in *E. caucasicum* were 75.36, 31.51, and 97.37 mg of quercetin equivalent per gram of extract powder, respectively [46]. According

to Daneshzadeh et al. [47] the total phenolic content in *E. billardieri* F. Delaroche ranged from 10.71 to 33.38 mg gallic acid equivalent per gram of dry extract, while the total flavonoids ranged between 15.04 and 27.13 mg quercetin equivalent per gram of dry extract. Finally, the content of total phenols in the methanolic extract of *E. pyramidale* Boiss. & Hausskn. was 0.36 ± 0.01 mg of gallic acid equivalent per gram of dry plant material, while the content of total flavonoid in the methanolic extract was 0.90 ± 0.05 mg of quercetin equivalent per gram of dry plant material [48].

Table 3. Content of total phenols (TP) and flavonoids (TF), radical scavenging (EC_{50}), and antioxidant activity obtained in β-carotene-linoleate assay (ANT), metal chelating activity ($ChEC_{50}$) and slope of trendline in a reducing power assay (SRP) of *E. amethystinum* and *E. alpinum* extracts. Values are calculated as means ± SD. N = 3.

Species	Plant Part	TP (mg/g)	TF (mg/g)	EC_{50} (μg/mL)	ANT (%)	$ChEC_{50}$ (μg/mL)	SRP (mg^{-1})
E. amethystinum	leaf	34.48 ± 0.33 ^{A}	17.24 ± 1.16 ^{A}	30.73 ± 0.29 ^{A}	90.94 ± 1.58 ^{A}	682.58 ± 4.56 ^{A}	1.05 ± 0.02 ^{A}
	flower	11.77 ± 0.45 ^{B}	9.65 ± 0.52 ^{B}	169.78 ± 2.86 ^{B}	84.71 ± 0.19 ^{B}	484.12 ± 17.44 ^{B}	0.17 ± 0.01 ^{B}
	stem	23.8 ± 0.71 ^{C}	16.21 ± 0.97 ^{A}	100.25 ± 2.24 ^{C}	85.26 ± 1.42 ^{B}	684.14 ± 39.95 ^{A}	0.38 ± 0.00 ^{C}
E. alpinum	leaf	73.42 ± 1.05 ^{D}	1.78 ± 0.24 ^{C}	268.47 ± 17.19 ^{D}	48.81 ± 5.24 ^{C}	1049.68 ± 21.59 ^{C}	1.12 ± 0.08 ^{A}
	flower	29.39 ± 2.92 ^{E}	11.34 ± 0.30 ^{D}	1510.17 ± 29.74 ^{E}	75.89 ± 3.09 ^{D}	331.2 ± 6.88 ^{D}	0.33 ± 0.04 ^{C}
	stem	33.77 ± 1.93 ^{A}	13.50 ± 0.27 ^{E}	241.08 ± 4.66 ^{F}	71.59 ± 1.45 ^{D}	951.14 ± 6.20 ^{E}	0.67 ± 0.06 ^{D}
Standard		-	-	^{a} 2.83 ± 0.02 ^{A}	^{a} 95.39 ± 0.21 ^{A}	^{b} 219.16 ± 4.70 ^{Fc} 5.49 ± 0.25 ^{G}	^{d} 7.59 ± 0.08 ^{E}

Note: samples connected by the same capital letter (A–F) are statistically different at $p < 0.05$. Inside column ^{a} = BHA; ^{b} = quercetin; ^{c} = EDTA; ^{d} = ascorbic acid.

3.4. Antioxidant Capacity

3.4.1. DPPH Radical-Scavenging Activity

The radical-scavenging activity of the extracts varied between 30.73 μg/mL (*E. amethystinum* leaf) and 1510.17 μg/mL (*E. alpinum* flower; Table 3). In addition, the activity of the *E. amethystinum* leaf was statistically equal to the activity of BHA (butylated hydroxyanisol). In general, the extracts of *E. amethystinum* were more potent radical scavengers than the extracts of *E. alpinum*. For comparison, the radical-scavenging activity (DPPH IC_{50}) of methanol, acetone, ethyl acetate, and butanol extracts of *E. maritimum* root were 0.2350, 0.0818, 0.1200, and 0.0104 mg/mL [49]. According to Meot-Duros et al. [50], the ABTS IC_{50} for *E. maritimum* was 0.28 mg/mL. Additionally, the concentration of the investigated extracts that reduced 50 percent of DPPH radicals was 0.6 ± 0.0 mg/mL for the methanol extract prepared from the above-ground parts of *E. palmatum* Pančić and Vis. and 0.7 ± 0.0 mg/mL for the methanol extract obtained from the root [51].

3.4.2. β-Carotene-Linoleic Acid Assay

The extracts of *E. amethystinum* were found to be more efficient antioxidants than the extracts of *E. alpinum* in this test (Table 3; Figure 1). The extract which showed the most antioxidant activity in this assay was extract made from *E. amethystinum* leaves. Its activity was statistically the same as the activity of synthetic antioxidant BHA.

3.4.3. Chelating Activity

The extract with the highest activity in this assay was extract made from *E. alpinum* flower (Table 3). However, the chelating properties of all the extracts were somewhat lower than the activity of quercetin and EDTA. It was also noticed that the chelating effect of the extracts did not differ between species.

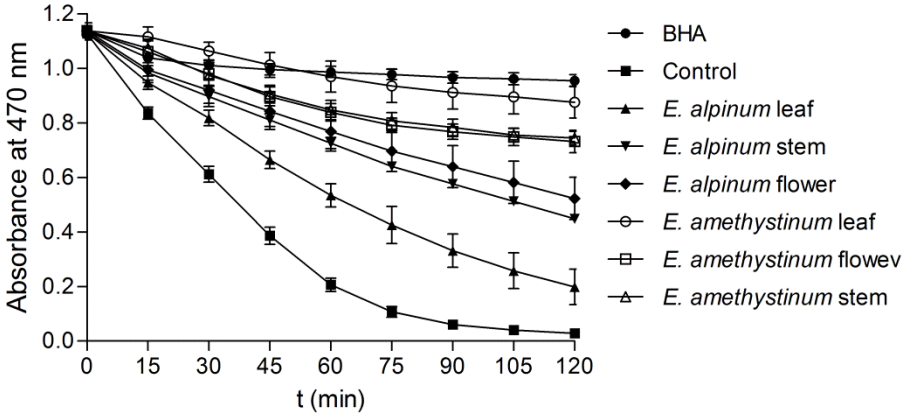

Figure 1. The reduction of absorbance of β-carotene-linoleic acid emulsion caused by *Eryngium* spp. extracts. Each value represents the mean of three independent measurements with SD error bar.

3.4.4. The Reducing Power of the Extracts

All the investigated extracts showed some degree of reducing activity (Figure 2). The reducing power of the extracts was increased linearly with higher concentration ($r^2 \geq 0.98$). On the other hand, the absorbance of the reaction solution with ascorbic acid remains relatively constant at higher concentrations, probably due to the restrictions of the Beer–Lambert law. For comparison, slopes of the trend lines were obtained for the lowest three concentrations where $r^2 > 0.97$ for all investigated samples (Table 3). It was also noticed that the activity of all investigated extracts was somewhat lower in comparison with ascorbic acid activity. However, the extracts also differed in their activity; the most active were the leaf extracts of the two species.

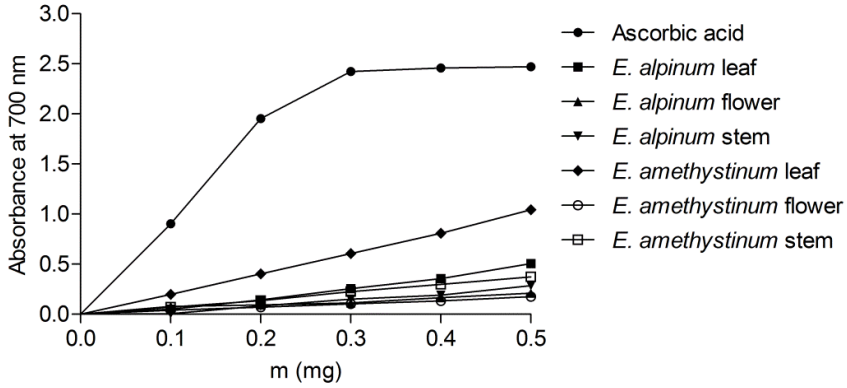

Figure 2. Reductive capacities of the *Eryngium* spp. extracts. Each value shown represents the mean of three independent measurements with SD error bar (SD are not visible due to their small size relative to the mean).

These studies have shown that the extracts prepared from both *Eryngium* species have significant antioxidant activity, especially the leaves of *E. amethystinum*. The antioxidant activity of the leaves of *E. amethystinum* studied via DPPH radical-scavenging activity and the β-carotene-linoleic acid assay was statistically equal to the activity of synthetic antioxidant BHA. This significant antioxidant activity can be attributed primarily to the phenol content of the species studied. Phenolics contain one or more aromatic rings with added hydroxyl groups in their structure and their antioxidant activity is directly related to the phenolic rings and hydroxyl groups [52]. The relationshiop between phenol content and antioxidant activity has been studied by many authors and their results showed a statistically significant relationship between total phenol content and antioxidant activity [53–56]. Moreover, some authors suggested that phenolic compound content could be used as an indicator of antioxidant properties [54]. Additionally, the antioxidant activity

of EO cannot be ignored either. For example, the studies conducted by Guo et al. [55] have shown a synergistic activity between γ-terpinene and polyphenols.

3.5. Antimicrobial Activity

Screening of antimicrobial activity showed that extracts exhibited antimicrobial activity with MIC values below or equal 1.94 ± 0.48 mg/mL (Table 4). For comparison, Thiem et al. [57] found that ethanolic extracts (70%) from *Eryngium planum*, *E. campestre* and *E. maritimum* showed antibacterial activity against *Staphylococcus aureus* with MIC values ranging from 400 (*E. planum* leaves) to 1900 (*E. campestre* leaves) mg/mL. Hołderna-Kędzia and Kędzia [58] reported that ethanolic extract (50%) from the roots of *E. maritimum* showed antibacterial activity against *S. aureus* (MIC = 2500 mg/mL). Chloroformic fractions of the methanolic extract of *E. maritimum* leaf inhibited the growth of several microorganisms, mainly *S. aureus* (MIC = 10 mg/mL) [49]. Moreover, aqueous and chloroformic fractions possess a strong antimicrobial activity against tested *Pseudomonas aeruginosa* and *P. fluorescens* (MIC = 1 and 2 mg/L, respectively) [49]. Ethanolic extract obtained from above-ground parts of *E. caeruleum* M. Bieb. and *E. thyrsoideum* Boiss. showed antimicrobial activity against tested *Staphylococcus epidermidis* with an MIC value of 3.125 mg/mL [59].

Table 4. Antimicrobial activity of methanolic extracts obtained from *Eryngium alpinum* and *E. amethystinum*. Presented values denote means ± SD. N = 3.

Species	Plant	MIC ± SD (mg/mL)			
	Part	***S. aureus* ATCC 6538**	***E. coli* ATCC 10536**	***C. albicans* ATCC 10231**	***M. gypseum* MFBF S2**
E. amethystinum	stem	0.39 ± 0.02	1.94 ± 0.48	0.16 ± 0.12	0.24 ± 0.07
	leaf	0.39 ± 0.020	1.53 ± 0.24	0.06 ± 0.018 *	0.16 ± 0.12
	flower	1.32 ± 0.60	1.53 ± 0.24	0.43 ± 0.334	0.06 ± 0.02 *
E. alpinum	stem	0.24 ± 0.07	1.94 ± 0.48	0.39 ± 0.20	0.21 ± 0.14
	leaf	0.29 ± 0.02	1.94 ± 0.48	0.39 ± 0.20	0.08 ± 0.06 *
	flower	0.24 ± 0.07 *	1.53 ± 0.24	0.32 ± 0.29	0.13 ± 0.13

Note: * = significantly lower MIC value than the MIC of the same organ of another *Eryngium* species tested ($p < 0.05$).

Fungal strains tested for antimicrobial activity were most sensitive to both the extracts with an MIC below 0.39 ± 0.20 mg/mL. Due to the lower MIC values against tested *C. albicans* ATCC 10231, the antifungal testing was expanded to clinical isolates of *Candida* spp.

The results of the determination of MIC values in Table 5 show differences in fungal susceptibility to *E. amethystinum* and *E. alpinum*. *C. albicans*, *C. glabrata*, *C. krusei* were the most sensitive to the extracts of both species. MIC values ranged from 0.12 to 1.11 mg/mL. Below MIC values of 0.37 ± 0.05 mg/mL, the inhibition of germination of clinical isolates of *C. albicans* was determined for both extracts at a concentration of 0.30 mg/mL (Figure 3), but not at a concentration of 0.100 mg/mL (data not shown). This is the first data on antifungal activity and inhibition of germ-tube formation of blastospores of *C. albicans* by extracts of *E. amethystinum* and *E. alpinum*. According to Thiem et al. [57], the ethanolic extracts (70%) of *Eryngium planum*, *E. maritimum* and *E. campestre* exhibited antifungal activity against *Candida albicans* with MIC values from 90 (*E. planum* leaves) to 7500 (*E. campestre* leaves) mg/mL. In addition, antifungal activity against *C. glabrata* ranged from 40 (*E. planum* leaves) to 700 (*E. maritimum* leaves) mg/mL.

Table 5. Antimicrobial activity of prepared methanolic extracts of *Eryngium amethystinum* and *E. alpinum*.

Species	Plant	MIC ± SD (mg/mL)			
	Part	*C. albicans* MFBF 40630/2	*C. parapsilosis* MFBF 4800	*C. krusei* MFBF 429	*C. glabrata* MFBF 3309
E. amethystinum	stem	0.37 ± 0.05	0.37 ± 0.05	0.37 ± 0.05	0.37 ± 0.05
	leaf	0.37 ± 0.05	0.37 ± 0.05	0.37 ± 0.05	0.37 ± 0.05
	flower	0.37 ± 0.05	0.37 ± 0.05	0.37 ± 0.05	0.37 ± 0.05
E. alpinum	stem	0.37 ± 0.05	1.11 ± 0.05	0.37 ± 0.05	0.37 ± 0.05
	leaf	0.37 ± 0.05	1.11 ± 0.05	0.37 ± 0.05	0.37 ± 0.05
	flower	0.37 ± 0.05	1.11 ± 0.05	0.12 ± 0.01	0.37 ± 0.05

On clinical isolates of *Candida* spp. Presented values denote means ± SD. N = 3.

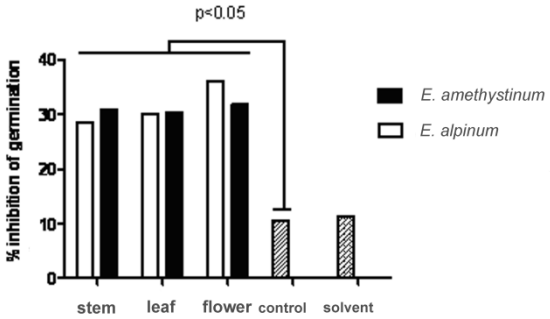

Figure 3. The inhibition of germination of *Candida albicans* due to extracts of *Eryngium* spp. at 300 µg/mL. Control: FBS (foetal bovine serum) without extracts; solvent: 10% (*v*/*v*) 70% ethanol in FBS. N = 3.

Both studied *Eryngium* species showed antimicrobial activity, which can be attributed to the content of phenols and EOs. The antimicrobial activity of polyphenols is explained by changes in the permeability of the microbial cell membrane, changes in cell wall rigidity caused by the interaction of phenols and cell membranes, and some changes in intracellular activity due hydrogen binding of phenols to enzymes [60]. In this study, the antimicrobial activity of *Eryngium* species can be attributed to the effect of phenolic compounds present (Table 2). Rutin, quercitrin and rosmarinic acid have a proven activity against *S. aureus*, *E. coli* as well as against some other microbes [61,62]. The effect of EO on microbes is manifested in several ways. Damage to the cell membrane and degradation of the cell wall, structural changes in the membrane protein, condensation of the cytoplasm, leakage of cytolymph, and alteration of nuclear activity [63–65]. Among the compounds detected in EOs in higher quantities, α- and β-pinene have proven activity against *S. aureus*, *E. coli*, and *C. albicans* [66], α-bisabolol against *S. aureus* and various other microbes [67,68], β-caryophyllene against *S. aureus* [69], and caryophyllene oxide against *S. aureus* and *C. albicans* [70].

In conclusion, the results on the antimicrobial activity of essential oils of *Eryngium campestre, E. thorifolium*, and *E. creticum* [71] against MRSA provide direction for future studies of antimicrobial activity of *E. amethystinum* and *E. alpinum*.

4. Conclusions

E. amethystinum is a wild vegetable consumed in Croatia. Considering its use as food, the chemical composition of *E. amethystinum* was studied and compared with *E. alpinum*. The EOs of *E. amethystinum* and *E. alpinum* show that the oil of both species was characterized by a higher content of sesquiterpenes. Several in vitro assays showed that both species are significant source of polyphenols and other antioxidant substances with chelating and radical-scavenging properties. Screening of antibacterial and antifungal activity revealed that both *E. amethystinum* and *E. alpinum* exhibited antimicrobial activity against tested

microbes. The present results indicate that *E. alpinum* and *E. amethystinum* could be useful as antioxidant and antimicrobial agents.

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