Cent. Eur. J. Biol. • 9(2) • 2014 • 149-155
DOI: 10.2478/s11535-013-0247-0

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Central European Journal of **Biology**

Composition, antimicrobial and antioxidant activity of the extracts of *Eryngium palmatum* Pančić and Vis. (Apiaceae)

Research Article

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Received 18 April 2013; Accepted 05 August 2013

Abstract: The chemical composition, antimicrobial and antioxidant activity of *Eryngium palmatum*, an endemic plant species from the Balkan Peninsula, were investigated. The flavonoids apigenin (9.5±0.3 mg g^{-1}) and apigenin 7-O-glucoside (2.4±0.1 mg g^{-1}) were determined in a methanol extract of aerial parts using HPLC analysis. The methanol extract of roots contained catechin (5.0±0.1 mg g^{-1}), epicatechin (2.9±0.1 mg g^{-1}), chlorogenic acid (1.6±0.0 mg g^{-1}), gallic acid (0.9±0.0 mg g^{-1}) and rosmarinic acid (0.9±0.2 mg g^{-1}). GC-FID and GC-MS analysis of a chloroform extract of aerial parts showed that the main volatile constituents were falcarinol, linoleic acid, hexadecanoic acid and methyl linoleate (comprising 32.6%; 24.4%; 19.9; 13.2% of the volatile fraction, respectively), while octanoic acid, tetradecanol and dodecanol dominated in the chloroform extract of the roots (34.9%; 25.8%; 22.2% of the volatile fraction, respectively). Investigation of antimicrobial activity by broth microdilution showed that the methanol and chloroform extracts of aerial parts and roots exerted a significant effect (MIC 3.5-15.6 μg mL^{-1}) against tested Gram-positive and Gram-negative bacteria. The methanol extracts of aerial parts or roots exerted moderate ferric reducing antioxidant power, DPPH radical scavenging activity and hydroxyl radical scavenging activity.

Keywords: *Eryngium palmatum* • *Extracts* • *Composition* • *Antimicrobial activity* • *Antioxidant activity*

1. Introduction

The genus *Eryngium* L. (Apiaceae) comprises about 250 species distributed in temperate regions [1]. Some *Eryngium* species are cultivated as vegetables, ornamental or medicinal plants. In traditional medicine they are used to treat various inflammatory disorders, fever, diarrhea, hypertension, oedema, sinusitis and snake or scorpion bite [2,3]. Previous investigations show that species of this genus contain flavonoids, triterpene saponins, coumarins, rosmarinic acid derivatives, polyacetylenes and essential oils [4-6]. Aqueous extracts of leaves and flowers of *E. bourgatii* Gouan exhibit antioxidant and anti-inflammatory activity [6], and ethanol extracts of different *Eryngium* species have anti-inflammmatory and antinociceptive activity [2]. The essential oil of aerial parts of *E. duriaei* J. Gay has demonstrated antifungal activity [7], while the essential oils from *E. campestre* L., *E. thorifolium* Boiss. and *E. creticum* Lam. inhibit the growth of methicillin-resistant *Staphylococcus aureus* strains [8].

Eryngium palmatum Pančić and Vis. is an endemic species of the Balkan Peninsula [9]. The aim of this work was to investigate the composition and antimicrobial and antioxidant activity of extracts of the aerial parts and roots of this plant. Increasing bacterial resistance to commonly used chemotherapeutics requires a constant search for new compounds with new mechanisms of action [10]. Antioxidant compounds may be also valuable since oxidative

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stress presents a risk to human health and can lead to the development of conditions such as cardiovascular diseases and cancer [11]. Plants are one of the most promising sources for discovering novel antimicrobial and antioxidant agents [11,12]. Beside potential therapeutic values, investigations of *E. palmatum* provide valuable information about these vulnerable endemic species, helping to contribute to their future protection and conservation. The composition and antimicrobial and antioxidant activity of *E. palmatum* extracts have not previously been investigated.

2. Experimental Procedures

2.1 Chemicals

Folin-Ciocalteu reagent, 2,4,6-tripyridyl-*s*-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), polyvinylpyrrolidone (PVP), 2-deoxyribose and analytical standards gallic acid, (+)-catechin, (-)-epicatechin, chlorogenic acid, rosmarinic acid, apigenin and apigenin 7-glucoside were obtained from Sigma-Aldrich, St. Louis, MO, USA. Thiobarbituric (TBA) and phosphoric acid were purchased from Merck, Darmstadt, Germany; trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA) and L-ascorbic acid from Lachema, Neratovice, Czech Republic; 2-deoxyribose from Acros Organics, New Jersey, USA. Acetonitrile used for HPLC analysis was obtained from J. T. Baker, Phillipsburg, NJ, USA, HPLC grade. All other solvents and chemicals were of analytical grade. Ampicillin, amikacin and nystatin were purchased from Galenika, Belgrade, Serbia; Müeller-Hinton broth and Sabouraud dextrose broth from Institute of Immunology and Virology Torlak, Belgrade, Serbia.

2.2 Plant material and extraction procedure

The aerial parts and roots of *E. palmatum* were collected in Sićevačka gorge, in Eastern Serbia in April 2007. Identification was confirmed by curator botanist Dr. Marjan Niketić from the Natural History Museum, Belgrade, Serbia and a herbarium specimen was deposited there (2007040201 BEO). The herbal material was air dried, ground to a coarse powder and extracted with chloroform at room temperature. After chloroform extraction, the material was air-dried and extracted with methanol. Solvents were evaporated under reduced pressure at less than 40°C. Aerial parts yielded 1.02% of the chloroform extract and 1.35% of the methanol extract, and roots yielded 6.33% and 11.22% respectively. All results are calculated per g of dry weight of plant extracts (DW).

2.3 Total phenolic and tannin contents

The total phenolic and tannin contents in the methanol extracts of *E. palmatum* were analyzed spectrophotometrically by the Folin-Ciocalteu assay. The removal of tannin was achieved with insoluble polyvinylpyrrolidone (PVP) which binds tannins [13]. Total phenolic and tannin contents were expressed as mg of gallic acid equivalents per g of dry weight of extract (mg g^{-1} DW; GAE).

2.4 HPLC analysis of methanol extracts

HPLC analysis was performed on Agilent 1100, using a Zorbax Eclipse XDB-C18 analytical column (4.6 × 250 mm with 5 µm particle size), with photodiode array detector. Samples (10 mg mL^{-1}) were gradiently eluted with a two phase system, phase A-water/phosphoric acid (99.97:0.03), pH 2.75 and phase B-10% A in acetonitrile, flow rate of 0.8 mL min^{-1}, at 25ºC. Gradient profile was: 0 min 90% A, 10% B; 5-15 min 75% A, 25% B; 20 min 70% A, 30% B; 25 min 50% A, 50% B; 30 min 30% A, 70% B and 35 min 90% A, 10% B. Identification of components was performed by comparison of retention time and spectra with representative standards. Quantification of compounds was achieved by construction of the calibration curves of gallic acid, (+)-catechin, (-)-epicatechin, chlorogenic acid, rosmarinic acid, apigenin and apigenin 7-glucoside.

2.5 GC-FID and GC-MS analysis of chloroform extracts

The volatile constituents were determined by GC-FID and GC-MS. The GC analysis was performed on Agilent 6890N GC system equipped with 5975 MSD and FID, using a HP-5 MS column (30 m × 0.25 mm, 0.25 µm film thickness). The injection volume was 2 µL and the injector temperature was 200°C with a 10:1 split ratio. Helium was the carrier gas and its flow rate was 1.0 mL min^{-1} (constant flow mode). The column temperature was linearly programmed in the range 60-280°C at a rate of 3°C min^{-1} and held at 280°C for 5 min. The transfer line was heated at 250°C. The FID detector temperature was 300°C. EI mass spectra (70 eV) were acquired in the *m z-1* range 35-550. The retention indices were experimentally determined using *n*-alkanes (C_8-C_{20} and C_{21}-C_{40}), under the same chromatographic conditions. The identification of the compounds was based on the comparison of their retention indices (RI), their retention times (t_R) and mass spectra with those obtained from authentic samples and/or the NIST AMDIS (Automated Mass Spectral Deconvolution and Identification System) software, Wiley libraries, Adams data base and literature [14]. The relative percentages of the identified compounds were computed from the GC-FID peak area.

2.6 Antimicrobial activity

The antimicrobial activity of *E. palmatum* methanol and chloroform extracts was examined by the broth microdilution method [15] against the eight different laboratory control strains of bacteria and one strain of yeast. Minimal inhibitory concentrations (MICs) were determined according to the Clinical and Laboratory Standards Institute [15]. All tests were performed in Müller-Hinton broth for bacterial strains and in Sabouraud dextrose broth for *Candida albicans*. Overnight broth cultures of each strain were prepared at a final concentration of 2×10^6 CFU mL^{-1} for bacteria, and at 2×10^5 CFU mL^{-1} for yeast, in a 96-well microtitre plate. The extracts were investigated in serial doubling dilutions from 1.8 to 100.0 μg mL^{-1}. Triphenyl tetrazolium chloride (TTC) was added to the culture medium as a growth indicator (0.05%). Microbial growth was determined after incubation at 37°C for 24 h for bacteria, and after incubation for 48 h at 26°C for yeast. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate visible growth. The MIC of ampicillin, amikacin and nystatin were determined in parallel experiments. All determinations were performed in duplicate; two positive growth controls were included.

2.7 Ferric reducing antioxidant power

The total antioxidant potentials of methanol extracts were determined using a ferric reducing antioxidant power (FRAP) assay based on the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) and formation of a blue complex (Fe^{2+}- 2,4,6-tripyridyl-*s*-triazine), which increases absorption at 593 nm [16,17]. The amount of reduced Fe^{2+}- TPTZ complex was calculated from an equation of the regression line constructed from the absorbance of water solutions of $FeSO_4 \times 7\ H_2O$ (0.1-1.0 mmol L^{-1}) under experimental conditions. Results are expressed as mmol Fe^{2+} g^{-1} of dry weight of extracts.

2.8 DPPH radical scavenging activity

DPPH radical scavenging activity is based on the reduction of the free radical DPPH▪ (2,2-diphenyl-1-picrylhydrazyl) and decrease of absorbance at 517 nm [18]. The SC_{50} value, which is the concentration of the tested extract that reduces 50% of the free-radical concentration, was calculated as mg mL^{-1} from the plot of logarithms of concentration *vs*. probits of scavenging effect.

2.9 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined in a non-site-specific deoxyribose assay. The extract (20 μL of different concentrations) was mixed with 100 μL of 4 mmol L^{-1} $FeCl_3$, 100 μL of 4 mmol L^{-1} EDTA, 200 μL of 0.05 mol L^{-1} 2-deoxyribose, 20 μL of 1.5% H_2O_2 and 100 μL of 4 mmol L^{-1} L-ascorbic acid in phosphate buffer, pH=7.4 (4 mL final solution). The mixtures were then incubated at 37°C for 60 min. After addition of 1 mL of 1% solution of thiobarbituric acid (TBA) in 0.05 mol L^{-1} NaOH and 1 mL of 2.8% solution of trichloroacetic acid (TCA), the mixtures were heated at 100°C for 15 min, and then cooled on ice. Absorbance of the samples was measured at 532 nm [19]. Rutin was used as the control.

3. Results and Discussion

3.1 Total phenolic and tannin contents

The composition of methanol and chloroform extracts of *E. palmatum* aerial parts and roots was investigated. The total phenolic content in the methanol extract of aerial parts (29.0±2.0 mg GA/g) was higher than in the methanol extract of roots (13.9±1.0 mg GA g^{-1} (Table 1). The total phenolic content of the methanol extracts of *E. palmatum* were higher than in the previously researched ethanol extracts of *E. bornmuelleri* Nábělek (25.8 mg GA g^{-1} leaf; 8.9 mg GA g^{-1} stem and 3.6 mg GA g^{-1} root) [20]. Tannin content was similar in the methanol extract of aerial parts and in the methanol extract of roots (4.5±1.0 and 4.7±1.0 mg GA g^{-1}, respectively).

3.2 Composition of methanol and chloroform extracts

The methanol extract of aerial parts contained flavonoids: apigenin (9.5±0.3 mg g^{-1}), apigenin 7-O-glucoside

		Phenolic (mg GA g^{-1})	Tannin (mg GA g^{-1})	FRAP (mmol Fe^{2+} g^{-1})	DPPH (mg mL^{-1})	Hydroxyl radical (mg mL^{-1})
Methanol extract	aerial parts	29.0±2.0	4.5±1.0	0.2±0.0	0.6±0.0	0.5±0.0
	roots	13.9±1.0	4.7±1.0	0.1±0.0	0.7±0.0	0.3±0.0
Rutin		-	-	5.9±0.0	0.006±0.0	0.002±0.0

Table 1. Total phenolic and tannin contents as mg GA g^{-1}, ferric reducing ability (FRAP) as mmol Fe^{2+} g^{-1}, DPPH radical and hydroxyl radical scavenging activity as SC_{50} (mg ml^{-1}) of the methanol extracts of aerial parts and roots of *E. palmatum*.

DPPH- 2,2-diphenyl-1-picrylhydrazyl

(2.4±0.1 mg g^{-1}), another apigenin glycoside and also one kaempferol diglucoside (Table 2). Flavonoids were not detected in the methanol extract of roots, which did contain catechin (5.0±0.1 mg g^{-1}), epicatechin (2.9±0.1 mg g^{-1}), chlorogenic acid (1.6±0.0 mg g^{-1}), gallic acid (0.9±0.0 mg g^{-1}) and rosmarinic acid (0.9±0.2 mg g^{-1}). The presence of chlorogenic acid was previously reported in the leaves and flowers of *E. bourgatii* [6], leaves of *E. foetidum* L. [3] and leaves and roots of *E. planum* L. [21]. The content of chlorogenic acid in the roots of *E. palmatum* (1.6±0.0 mg g^{-1}) was higher than the previously reported content of chlorogenic acid in the roots of *E. planum* (0.19±0.00 mg g^{-1}) [21]. The roots of different species of the genus *Eryngium* contain rosmarinic acid in the range 0.4-272.0 mg g^{-1} [5,21]. In the methanol extract of roots of *E. palmatum* a moderate quantity of rosmarinic acid was determined.

The main compounds of the volatile part of the chloroform extract of aerial parts (Table 3) were polyacetylene falcarinol (32.6%), linoleic acid (24.4%), hexadecanoic acid (palmitic acid) (19.9%) and methyl linoleate (13.2%). The volatile fraction of the chloroform extract of roots contained octanoic acid (caprylic acid) (34.9%), tetradecanol (myristyl alcohol) (25.8%) and dodecanol (lauryl alcohol) (22.2%).

3.3 Antimicrobial activity

The antimicrobial activity of extracts of *E. palmatum* was investigated against eight bacterial strains and yeast *C. albicans* (Table 4). The methanol and chloroform extracts showed high antimicrobial activity with MIC values in the range 3.5-15.6 μg mL^{-1}. The investigated extracts were active against both Gram-positive and Gram-negative bacteria. Compared to Gram-positive bacteria, Gram-negative bacteria are usually more resistant to commonly-used antibiotics as well as to plant secondary metabolites, having a complex cell wall, which acts as an effective permeability barrier to restrict the penetration of compounds. Furthermore a set of multidrug resistance pumps in their cell wall extrudes toxins across the outer membrane [11,22]. The highest antimicrobial activity was obtained with the methanol extract of aerial parts against *M. luteus* (MIC 3.5 μg mL^{-1}). This effect may be due to its flavonoid content. The methanol extract of aerial parts contained apigenin and its glycosides as well as kaempferol glycoside. Apigenin and kaempferol exert antimicrobial activity [23] and probably have multiple cellular targets. Phenolic compounds inhibit microbial adhesions and inactivate enzymes and cell envelope transport proteins [24]. Apigenin inhibits *E. coli* DNA gyrase and it has been suggested that the B ring of the flavonoids plays a

	Rt[a]	Methanol extract	
		aerial parts	roots
Gallic acid	4.1	0.9±0.0[b]	0.9±0.0
(+)-Catechin	7.6	-	5.0±0.1
Chlorogenic acid	8.4	-	1.6±0.0
(-)-Epicatechin	9.1	t	2.9±0.1
Kaempferol diglucoside	9.3	+[c]	-[d]
Apigenin glycoside	10.5	+	-
Apigenin 7-O-glucoside	16.2	2.4±0.1	-
Rosmarinic acid	18.7	-	0.9±0.2
Apigenin	27.4	9.5±0.3	-

Table 2. HPLC analysis of the methanol extracts of aerial parts and roots of *E. palmatum*.

[a] retention time on Zorbax Eclipse XDB-C18 column; [b] mg mL^{-1} of dry extract; [c] present in investigated methanol extracts; [d] not detected

RI[a]	Compounds	Volatile part of chloroform extract (%)	
		aerial parts	roots
1004	Octanal	-[b]	2.0
1177	Octanoic acid (caprylic acid)	-	34.9
1474	Dodecanol (lauryl alcohol)	4.5	22.2
1693	Tetradecanol (myristyl alcohol)	1.3	25.8
1790	Pentadecanol	-	3.0
1963	Hexadecanoic acid (palmitic acid)	19.9	-
2035	Falcarinol	32.6	4.8
2096	Methyl linoleate	13.2	-
2130	Linoleic acid	24.4	t[c]

Table 3. GC-FID and GC-MS analysis of the volatile part of the chloroform extracts of aerial parts and roots of *E. palmatum*.

[a] Retention indices on HP-5 MS column; [b] not detected; [c] trace (<0.1%)

Microorganism	MIC (μg mL^{-1})				
	Methanol extract		Chloroform extract		
	aerial parts	roots	aerial parts	roots	
Staphylococcus aureus ATCC 25923	15.6	15.6	7.8	15.6	0.5^{Amp}
Staphylococcus epidermidis ATCC 12228	7.8	7.8	15.6	7.8	1.0^{Amp}
Micrococcus luteus ATCC 9341	3.5	15.6	7.8	7.8	0.5^{Amp}
Enterococcus faecalis ATCC 29212	15.6	15.6	15.6	15.6	1.0^{Amp}
Bacillus subtilis ATCC 6633	15.6	15.6	15.6	7.8	2.0^{Amp}
Pseudomonas aeruginosa ATCC 27853	15.6	15.6	7.8	15.6	4.0^{Ami}
Escherichia coli ATCC 25922	15.6	15.6	15.6	15.6	2.0^{Ami}
Klebsiella pneumoniae NCIMB 9111	15.6	15.6	15.6	15.6	2.0^{Ami}
Candida albicans ATCC 10259	7.8	7.8	7.8	7.8	3.1^{Nys}

Table 4. Antimicrobial activity of the methanol and chloroform extracts of aerial parts and roots of *E. palmatum*.

^{Amp} *Ampicillin;* ^{Ami} *Amikacin;* ^{Nys} *Nystatin*

role in inhibiting DNA or RNA synthesis [25]. The methanol extract of roots, which contained catechins, also inhibited the growth of tested microorganisms (MIC 7.8-15.6 μg mL^{-1}). Catechins damage bacterial membranes and induce leakage of small molecules. Although it was reported that catechins have greater activity against Gram-positive than Gram-negative bacteria [25], in our study the methanol extract of roots restrained the growth of both bacterial groups. This activity is probably based on a synergistic activity of phenolic compounds.

On the other hand, non-polar chloroform extracts of aerial parts or roots also showed an antimicrobial effect. The chloroform extract of aerial parts significantly inhibited the growth of resistant Gram-negative bacteria *P. aeruginosa* (MIC 7.8 μg mL^{-1}). Falcarinol, linoleic acid and palmitic acid, the main compounds in the chloroform extract of the aerial parts, exert antimicrobial activity [26,27]. Unsaturated fatty acids such as linoleic acid inhibit bacterial fatty acid synthesis by blocking bacterial enoyl-acyl carrier protein reductase (FabI) which is essential for production of lipid-containing components such as cell membranes [28]. It has been previously reported that octanoic acid, one of the main compounds in the chloroform extract of the roots, also restrains bacterial growth [29]. Other components in the root chloroform extract were saturated alcohols, which have an antimicrobial activity that depends on their carbon chain length [30]. The greatest growth inhibition activity is seen in long-chained fatty alcohols with an aliphatic carbon chain of 12 or 13 carbon atoms. The fatty alcohols with aliphatic chains of fewer than 7 carbon atoms have no activity, and alcohols with chains longer than 17 carbon atoms have very low antimicrobial effect [31]. Considering the structure of fatty alcohols identified in the chloroform extracts of *E. palmatum*, it can be presumed that these compounds contribute to the observed antimicrobial activity.

3.4 Antioxidant activity

The antioxidant activity of secondary metabolites, especially phenolic compounds, is based on their ability to donate hydrogen atoms or electrons, chelate metal cations or scavenge free radicals [18,32]. The ferric reducing antioxidant power (FRAP) assay indicates the total antioxidant potential of the investigated extract. The total antioxidant potential of the methanol extract of aerial parts was 0.2±0.0 mmol Fe^{2+} g^{-1} and of the methanol extract of roots was 0.1±0.0 mmol Fe^{2+} g^{-1} (Table 1). The reducing power of the methanol extract of aerial parts found in this study was similar to that of the water extract of leaf of *E. bornmuelleri* (0.25 mmol Fe^{2+} g^{-1}). The reducing power of the methanol extract of roots of *E. palmatum* was higher than the activity of the water extract of roots of *E. bornmuelleri* (0.05 mmol Fe^{2+} g^{-1}) [20]. In the DPPH assay, the reactivity of potential antioxidant with stable, free DPPH radical is measured. The concentration of the extract that reduces 50% of the DPPH radical was 0.6±0.0 mg mL^{-1} for the methanol extract of aerial parts and 0.7±0.0 mg/ml for the methanol extract of roots. Previously reports of different extracts of *E. caucasicum* Trautv. showed analogous DPPH scavenging activity (0.4-0.8 mg mL^{-1}) [33]. The methanol extract of aerial parts inhibited highly reactive and toxic $OH^{\cdot}$ radicals in the concentration of 0.5 mg mL^{-1} and the methanol extract of roots did so in the amount of 0.3 mg mL^{-1}. The antioxidant activity of *E. palmatum* methanol extracts was not significant in

comparison with the control antioxidant rutin, but was comparable to the previously-investigated antioxidant activity of other species of this genus.

4. Conclusions

Our research showed that methanol and chloroform extracts of aerial parts or roots of *E. palmatum* possessed significant antimicrobial activity against Gram-positive and Gram-negative bacteria. The antimicrobial effect of the methanol extract of aerial parts is probably based on the flavonoid content; the activity of the methanol extract of roots is likely to be due to catechins and other phenolic constituents. Non-polar chloroform extracts also inhibited the growth of the tested microorganisms. The chloroform extracts contained polyacetylene, fatty alcohols, saturated and unsaturated fatty acids, all of which have proven antimicrobial activity.

Acknowledgements

This work was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project No. 173021.

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