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An evaluation of volatiles and phenolic compounds in conjunction with the antioxidant capacity of endemic endangered species of *Erodium hendrikii* Alpınar

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ABSTRACT

This study considers the chemical composition of an endemic endangered *Erodium* species (*E. hendrikii*), which grows naturally in the Turkish flora. The non-polar compounds and fatty acids from air-dried above-ground parts of the plant were analyzed using GC–MS, while phenolic acids and flavonoids were evaluated using UHPLC-MS/MS. Palmitic acid (5.23 mg/g dw) followed by linoleic acid (3.81 mg/g dw) were the major fatty acids, and *n*-hexadecanal (60.75%, dw) followed by *n*-tetradecanal (12.99%, dw) were the main non-polar compounds identified in the plant. The major phenolic acids (nmol/g dw) in the plant were protocatechuic acid (90.07) in free, ferulic acid (430.90) in ester, gallic acid (2450.51) in glycoside, and ester-bound (527.05) forms. The crude extract was represented by the highest caffeic acid (3189.56) content. In addition, hesperidin (259.56 nmol/g dw) was determined as the major flavonoid in the crude extract. This crude extract was further fractioned via solid-phase extraction and its three further partial fractions were obtained using water, ethyl acetate, and methanol. The methanol fraction yielded the highest antioxidant capacity among the fractions. A strong correlation and linear regression were determined between the phenolic contents and antioxidant capacity values of the crude extract and the three further partial fractions of the plant. These findings represent the first data concerning the phytochemical composition of *E. hendrikii* Alpinar and may be useful in the future development of antioxidant and antimicrobial agents for food preservatives against problems of food deterioration.

1. Introduction

The key agricultural and trade significance of plants is no longer limited to traditional food, forage, and fiber crops (Yadav et al., 2014). Plant-derived secondary metabolites are also of great interest due to their aromatic and/or therapeutic attributes, thus leading to their inclusion in healthcare systems, and use in the perfume and chemical industries (Bouaziz et al., 2009; Yadav et al., 2014). The use and efficiency of plants depend on their secondary metabolites (Yadav et al., 2014), which exhibit considerable diversity, such as terpenoids from essential oils, and phenolic acids and flavonoids, representing important compounds with profound implications for both the plant itself and human uses. Due to their high secondary metabolite contents, a great majority of the world population uses medicinal and aromatic plants to treat diseases and ailments in traditional medicine (UICN et al., 1993).

The genus *Erodium* LHérit (Geraniaceae) is composed of a group of more than 70 species worldwide, although it originates from Asia (Munekata et al., 2019). The majority of the species (> 60) are widespread across the Mediterranean region (Radulović et al., 2009; Munekata et al., 2019). In the Flora of Turkey, the genus is represented by 26 taxa, of which 16 are endemic (Güner et al., 2012). The wide traditional use of *Erodium* spp. in natural medicine for health purposes has attracted recent interest among researchers (Munekata et al., 2019). The data obtained from these studies have revealed uses of several *Erodium* spp. in the treatment of disorders

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including indigestion, urinary tract inflammations, diabetes, constipation, and eczemas, as a carminative agent, in gastrointestinal diseases, hemorrhage, and anasarca (generalized edema), wounds, and burns, and as a hair tonic, for circulatory system diseases, skin problems, hair loss, enteritis, diarrhea, and influenza, for heart problems, abdominal pain, cough, pneumonia, menstruaal pain, and low and high blood pressure (Munekata et al., 2019; Bilić et al., 2020). These uses have also been confirmed with experimental studies reporting results supporting antioxidant, antimicrobial, spasmogenic, interferonogenic, antiviral, and antiproliferative activities in terms of the known phytochemical composition of many *Erodium* species as indicated by Bilić et al. (2020). Therefore, the phytochemical composition of *Erodium* species has also been the subject of recent interest in terms of investigating substantial natural potential bioactive compounds in medical applications in the traditional uses described above and of finding naturally occurring antioxidants for use in foodstuffs to replace synthetic antioxidants and antimicrobial agents due to their carcinogenicity. In this context, essential oil compositions (Nikitina et al., 2007; Radulović et al., 2009; Stojanović-Radić et al., 2010; Munekata et al., 2019; Bilić et al., 2020) and diverse phenolic compounds including some flavonoid glycosides such as rutin, hyperoside, isoquercitrin, condensed tannin derivatives (e.g. catechin, gallic acid/gallotannins, ellagic acid/ellagitannins, geraniin, corilagin, brevifolin, etc.), flavonols (e.g. quercetin, kaempferol, myricetin, etc.), hydroxybenzoic (salicylic acid, *m*– and *p*–hydroxybenzoic acid, 2,3–dihydroxybenzoic acid, gentisic, and vanillic acid) and hydroxycinnamic acids (caffeic acid, *p*–coumaric, and ferulic acid) have bee the subject of investigation in a number of *Erodium* species (Sroka,1994; Lis-Balchin et al., 1993; Fecka et al., 1997; 2001; Fecka and Cisowski, 2005; Nikitina et al., 2007; Munekata et al., 2019; Bilić et al., 2020).

Apart from the reports concerning the uses and chemical compositions of *Erodium* species described above, a literature survey revealed no other reports regarding the phytochemical profile of the endemic *Erodium hendrikii* Alpınar known as "Güdük iğnelik" (Turkish), that grows only in the northeast Anatolian region of Turkey. The present study deals with the determination and quantification of wax volatiles and fatty acids, as well as the phenolic acids and flavonoids of this endemic endangered species. Additionally, the ethyl acetate and *n*-butanol extracts of *E. hendrikii* were tested for comparison with their possible 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) scavenging activities. The aim of the present study was thus to provide the first data concerning the chemical composition of *E. hendrikii* in order to contribute to the challenges of an efficient plant conservation strategy for biological diversity for endemic species.

2. Materials and methods

2.1. Plant material

The *E. hendrikii* specimens were collected from an indigenous natural population (A7 Gümüşhane: Yağmurdere, 40°35′09″N, 39° 52′51″E; 1740 m, Fig. 1) between June and July in 2016 and 2017. Herbarium specimens were dried according to standard herbarium techniques and deposited in the Karadeniz Technical University Faculty of Science, Biology Department, Turkey (KTUB; İmamoğlu & Cüce 03). Fresh *E. hendrikii* material (combined above-ground parts including stem, leaves, and flowers) was treated with liquid nitrogen and dried in a lyophilizer (Christ, Alpha 1–2LD plus, Germany). The dried samples were stored at −80 °C until analysis.

2.2. Extraction

Approximately 10 g of pulverized dry above-ground parts (combined stem, leaves, and flowers) of *E. hendrikii* sample was first extracted twice with 80% methanol. The combined homogenates were then centrifuged at 7 000 g for 15 min at room temperature. The supernatants were concentrated via a rotary evaporator (Heidolph Laborota-4000, Germany) under reduced pressure at 35 °C, and the slurry was dried in a lyophilizer. The dried sample prepared as crude extract was dissolved in 5 mL of deionized water (DIW). The crude extract was further fractioned via solid-phase extraction (SPE)

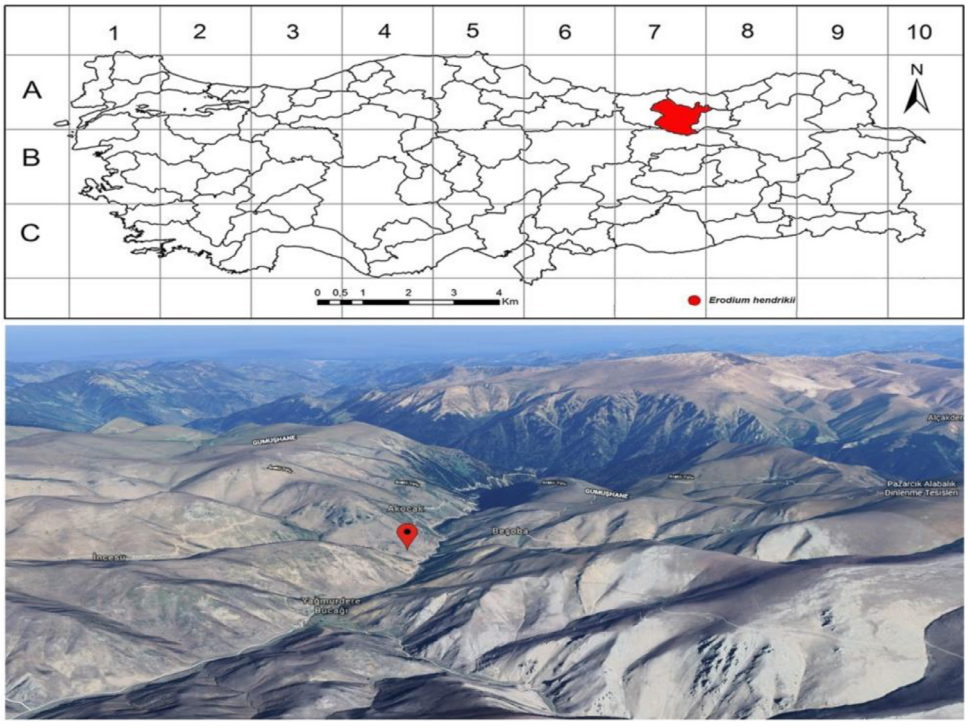

Fig. 1. Distribution and habitat of *Erodium hendrikii* in Yağmurdere village of Gümüşhane, Turkey.

on a Grace Pure C-18 column (max. 500 mg packed bed, 3 mL, Deerfield, IL, USA) according to the slightly modified method described by Rodriguez-Saona and Wrolstad (2001).

2.3. Determination of total phenolic compounds (TPC) content

In order to determine the TPC content of the plant methanolic extract, Folic-Ciocalteu (FC) reagent (Merck, Darmstadt) was used as described by Slinkard and Singleton (1977). The reaction mixture containing a 500 μL diluted sample, 25 μL 2 N FC reagent, and 975 μL 2% Na_2CO_3 (totally 1.5 mL) was measured against the standard curves (Fig. 2A) at 750 nm in a UV–VIS spectrophotometer (Thermo, Evolution 201, England). The results were expressed as gallic acid equivalents per gram dry weight (mg GAE/g dw).

2.4. Determination of total flavonoid (TF) content

In order to evaluate the TF content of methanolic extract of aboveground samples, the colorimetric assay was used as described by Huang et al. (2004). The reaction mixture containing 2% $AlCl_3$ and 500 μL diluted sample was measured against calibration curves (Fig. 2B) at 510 nm in a UV–VIS spectrophotometer, and the values were expressed as mg quercetin equivalent per g dry weight (mg QE/g dw).

2.5. Measurement of antioxidant capacity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity. The scavenging activity of the extract was evaluated using the free radical, following the method described by Blois (1958). Briefly, a diluted sample (100 μL) in 1 mL of freshly prepared methanolic DPPH solution was measured against calibration curves (Fig. 2C) at 520 nm in a UV–VIS spectrophotometer, and the values were expressed as μmol Trolox equivalent (μmol TE/g dw).

FRAP (ferric reducing antioxidant power). The FRAP assay was performed using the method described by Benzie and Strain (1999) with some modifications. The stock solution contained 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine), and 20 mmol/L $FeCl_3$ x $6H_2O$ in proportions of 10:1:1 (v/v/v). Freshly prepared FRAP reagent (2900 μL) was added to 100 μL of each sample, and the mixture was measured (Fig. 2D) at 593 nm in a UV–VIS

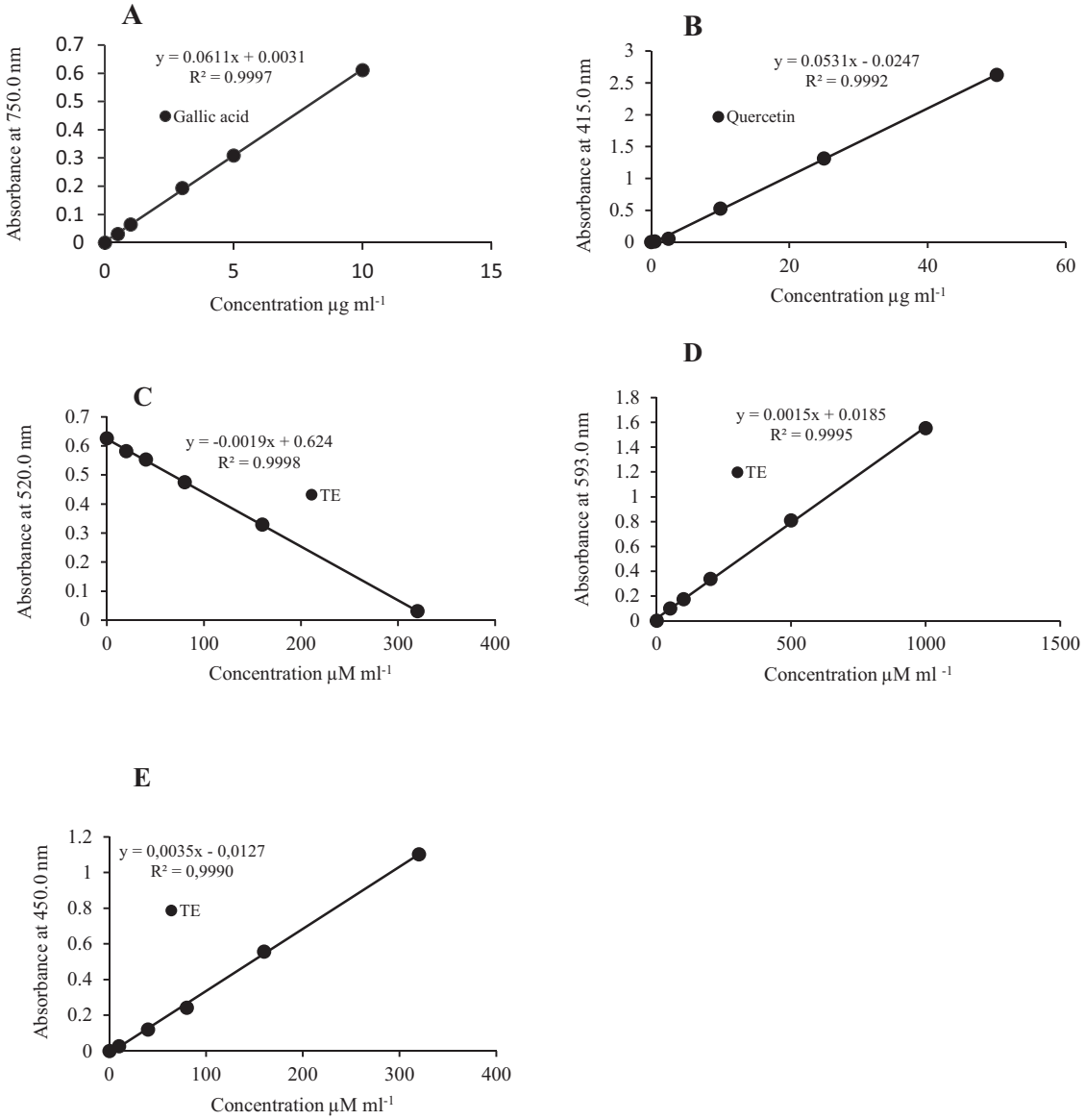

Fig. 2. Standard calibration curve of (A) gallic acid, (B) quercetin, (C) DPPH (TE), (D) FRAP (TE) and (E) CUPRAC (TE).

spectrophotometer, the values being expressed as μmol of Trolox/g (μmol TE/g dw).

CUPRAC (cupric ion reducing antioxidant capacity). For the CUPRAC assay, the procedure followed the slightly modified method described by Apak et al. (2004). The reaction mixture containing 100 μL of the diluted sample redistilled water (900 μL), acetate buffer solution (1 mL, 1 mmol/L, pH: 7.0), $CuCl_2$ (1 mL, 10 mmol/L), and neocuproine (1 mL 7.5 mmol/L) was measured (Fig. 2E) at 450 nm in a UV–VIS spectrophotometer, and the results were expressed as μmol Trolox equivalent per g (μmol TE/g dw).

2.6. UHPLC-MS/MS determination of phenolic acids and flavonoids

Ten grams of pulverized dry above-ground plant parts (combined stem, leaves, and flowers) of *E. hendrikii* sample were extracted thrice with aqueous methanol (80:20, v/v, 3 × 40 mL) including DBC [(2,6-ditertbutyl-*p*-cresol, 6 mg/100 mL) until the solution became colourless. The combined homogenates were centrifuged at 7000 g for 15 min at room temperature. The supernatants were concentrated in a rotary evaporator (Heidolph Laborota-4000, Germany) under reduced pressure at 35 °C, and the slurry was dried in a lyophilizer (Christ, Alpha 1–2 LD). The dried sample prepared as crude extract was dissolved in 30 mL of deionized water (DIW), and phenolic acids were further fractioned using a previously described method by Ayaz et al. (2005) to yield liberated phenolic acids in free, ester, glycoside, and ester-bound forms. All forms of phenolic acids were identified and quantified according to the method described by Gruz et al. (2008). An ACQUITY Ultra Performance LC™ system (Waters, Milford, MA, USA) linked to a Micromass Quattro micro™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) was used in the analysis. Phenolic acids purified with further fraction methods were injected into the BEH C8 reversed-phase column (1.7 μm, 2.1 × 150 mm, Waters, Milford, MA). 2,6-di-tertbutyl-*p*-Cresol (DBC, 6 mg/100 mL) was used during the extraction stage to avoid any oxidation problems. Deuterium-labeled 4-hydroxybenzoic (2,3,5,6-D_4) and salicylic (3,4,5,6-D_4) acids were used as the internal standard to quantify phenolic acid samples.

The phenolic acid and flavonoid contents in crude extracts were determined via UHPLC-MS/MS analysis on an UltiMate™ 3000 liquid chromatographic system consisting of binary pumps, an autosampler, and a column thermostat coupled to a TSQ Quantum Access Max triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed on an Acquity BEHC18 (150 × 3.0 mm; 1.7 μm particle size) UHPLC column (Waters Corp., Milford, MA, USA) kept at 40 °C. The mobile phase consisted of 10 mM formic acid in water (component A) and acetonitrile (component B). Twenty-nine compounds (15 phenolic acids and 14 flavonoids) were separated using a binary gradient starting at 5% B for 0.8 min, increasing to 10% B in 0.4 min with an isocratic run for 0.7 min, then rising to 15% B for 0.5 min and isocratic run for 1.3 min, subsequently increasing to 20% B for 0.3 min and isocratic for 1.2 min, then rising to 25% B for 0.5 min with next increase to 35% B within 2.3 min, next increasing to 70% B for 2.5 min. This was followed by a further increase to 100% B for 1 min, with an isocratic run for 1 min, and then returned to 5% B for 0.5 min. Finally, the equilibration to the initial conditions took 3.3 min, with a total chromatographic run of 16 min. The flow rate was 0.4 mL/min and the injection volume was 10 μL. All analytes were detected in negative ionization mode ESI- and multiple reaction monitoring (MRM) mode was used for quantification. The spray voltage was 3 kV, and the vaporizer and ion transfer tube temperatures were 320 °C.

2.7. GC–MS analysis of non-polar compounds and fatty acid methyl esters

The pulverized plant samples (10 g) were extracted with *n*-hexane by Soxhlet extraction (150 mL) for 2 h. The homogenates were combined, filtered, and concentrated on a rotary evaporator at 40 °C to yield the crude hexane extract for GC–MS analyses for the non-polar compounds and fatty acid content. The crude *n*-hexane extract was first re-dissolved in *n*-hexane containing 0.1% n-undecane as an internal standard and analyzed directly on an Agilent GC–MS system (GC 7890 A; MSD 5975C series II). Analytes were separated on a fused silica HP-5MS UI column (30 m x 0.25 mm x 0.25 mm), with He as carrier gas (1.1 mL/min), with a temperature program of 60 °C for 5 min, folowed by 5 °C/min from 60 °C to 200 °C, then 10 °C/min from 200 °C to 310 °C, and next by 310 °C for 6 min. The temperature of the injection port was 250 °C, that of the MS source 230 °C, and that of the quadrupole 150 °C. Ionization was performed in the EI mode (70 eV). The linear retention indices (RI) were determined by injection of C_8–C_{26} n-alkanes (Van Den Dool and Kratz, 1963). Compound identification was performed by comparing RIs and mass spectra with data from the literature (Adams, 2007) by means of a computer library search (HP Chemstation computer library NBS75K. L, NIST/EPA/NIH Mass Spectral Library 2.0 and Mass Finder 4 Computer Software and Terpenoids Library), and in the laboratory's own database.

For the fatty acids composition, 30 mg of crude n-hexane extract was redissolved into 0.5 mL of 0.5 M NaOMe in methanol and vortexed. The reaction mixture was left at room temperature for 5 min. Fatty acid methyl esters (FAMEs) were then extracted with *n*-hexane (2 × 0.5 mL), and the solvent was evaporated under reduced pressure at 35 °C. The dry sample was re-dissolved in 1 mL of n-hexane containing 0.1% *n*-undecane, and the sample was injected into the Agilent GC–MS system described above. FAME separation was conducted on the same capillary column and with the carrier gas as described above. The temperature program was 40 °C for 2 min, followed by 10 °C/min from 40 °C to 200 °C, then 2 °C/min from 200 °C to 250 °C, and finally 250 °C for 2 min. The temperature of the injection port and MS source was 230 °C, and that of the quadrupole was 150 °C. Ionization was performed in the EI mode (70 eV). Identification and quantification of FAMEs were performed by comparison of retention times and mass spectra with authentic standards. Quantification was performed in SIM mode, both for the standards (Supelco® 37 Component FAME Mix, Merck, Germany) and the samples (Fig. 4).

2.8. Statistical analysis

All extractions and analyses were performed in triplicate (n = 3). Data were expressed as mean ± pooled standard deviation and were compared within each column or row of the data. For comparisons among mean values, analysis of variance (one-way ANOVA) and the Multiple Range Test or Pearson correlation (r) analysis were performed on IBM SPSS Statistics V22.0 software. Linear regression (R) and correlation (r) analyses were carried out on Microsoft Office Excel 2010. Differences at $P < 0.05$ were considered significant.

3. Result and discussion

3.1. Total phenolic contents and antioxidant capacity of E. hendrikii extract

The TPC and TF contents in the crude extract and the three fractions (aqueous, ethyl acetate, and methanolic fractions) of *E. hendrikii* are shown in Table 1. The TPC content (mg GaE/g dw) was 87.95 ± 6.47 in the crude extract, 9.34 ± 1.49 in the aqueous fraction, 20.78 ± 1.28 in the ethyl acetate, and 65.28 ± 3.22 in the methanolic. Variations in the TPC content of this genus are reported to be species-

Table 1
Total phenolic contents and antioxidant capacity values in *Erodium hendrikii*.

	Fractions			
	Crude phenolic extract	Aqueous	Polyphenolic	Methanolic
TPC$^{\alpha}$	87.95 ± 6.47^{d}	9.34 ± 1.49^{a}	20.78 ± 1.28^{b}	65.28 ± 3.22^{c}
TF$^{\beta}$	12.14 ± 0.08^{d}	1.81 ± 0.02^{a}	5.49 ± 0.55^{b}	7.26 ± 0.05^{c}
DPPH$^{\gamma}$	605.06 ± 34.36^{d}	1.41 ± 0.02^{a}	46.27 ± 2.41^{b}	560.35 ± 21.68^{c}
FRAP$^{\gamma}$	842.16 ± 23.66^{d}	8.18 ± 2.87^{a}	225.27 ± 13.34^{b}	675.85 ± 26.26^{c}
CUPRAC$^{\gamma}$	1205.44 ± 12.05^{d}	160.59 ± 1.65^{a}	350.17 ± 7.25^{b}	913.96 ± 26.04^{c}

$^{\chi}$ Values are expressed as mean ± SE (n = 3). In the same row, values marked with different small letter at superscript indicate significant differences ($P < 0.05$).
$^{\alpha}$ TPC; total phenolic compounds, mg GAE/g DW.
$^{\beta}$ TF; total flavonoid, mg QE/100 g DW.
$^{\gamma}$ DPPH; 2,2-diphenyl-1-picrylhydrazyl, μmol Trolox/g DW $^{\gamma}$FRAP; ferric reducing antioxidant power, μmol Trolox/g DW. $^{\gamma}$CUPRAC; cupric ion reducing antioxidant capacity, μmol Trolox/g DW.

dependent (Munekata et al., 2019). The reported TPC content in several *Erodium* species ranged from 10.3 to 364 mg GaE/g dw (Alali et al., 2007; Jaradat et al., 2017; Sarikurkcu et al., 2017; Bakari et al., 2018; Hamza et al., 2018; Barba et al., 2020). The TPC content determined in the present *Erodium* species (87.95 mg GaE/g dw) was within the ranges reported in the literature. The TF content (mg QE /g dw) in *E. hendrikii* was 12.14 ± 0.08 in the crude extract, 1.81 ± 0.02 in the aqueous fraction, 5.49 ± 0.55 in the ethyl acetate, and 7.26 ± 0.05 in the methanolic. Findings concerning the TF contents in the literature showed wide variations among *Erodium* species, ranging between 30.5 and 91.97 mg QE /g dw (Hamza et al., 2018; Bakari et al., 2018). However, in the present study the content was relatively lower (12.14 ± 0.08 mg) than those reported in the literature.

Table 1 shows the antioxidant capacity (AC) values (μmol TE/g dw) of the extracts measured using three different assays (DPPH, FRAP, and CUPRAC). Following the TPC and TF contents, the results indicated an increasing trend in the AC values within the tested assays (CUPRAC>FRAP>DPPH) and among the fractions in the order methanol>ethyl acetate>aqueous fractions. In principle, the crude extract had the highest AC values as well as phenolic compound contents (TPC and TF). Among the fractions, depending on the polarity, the methanol fraction gave the highest AC values (560.35 ± 21.68, 675.85 ± 26.26, and 913.96 ± 26.04, respectively) in the extracts of *E. hendrikii* (Table 1). Measured antioxidant capacity values from *E. cicutarium* showed good agreement with the results of Bilić et al. (2020). Those authors used FRAP, DPPH, ABTS, and CUPRAC assays to compare water and methanolic extracts of the plant, and three of the four assays (DPPH, ABTS, and CUPRAC) demonstrated higher antioxidant capacity in methanolic extraction than in water extract.

The AC values exhibited significantly high and strong linear relationships and correlations with the TPC (range; r = 0.957 – 0.999, $P <$ 0.01; 0.05) and TF (range; r = 0.735 – 0.948, $P < 0.01$; 0.05) contents (Fig. 3). Treatment and solvent type, depending on polarity, has a significant impact on TPC content as well on AC values in *Erodium* extracts (Munekata et al., 2019; Barba et al., 2020). Similar findings were also reported by Wie et al. (2010) after using solvents of increasing polarities for the characterization of medicinal plants and by Carbonell-Capella et al. (2017) using combinations of ethanol: water vs. water to extract TPC from *Stevia rebaudiana* Bertoni leaves. The results of the present study showed that methanolic and ethyl acetate fractions yielded higher AC values compared to the aqueous extract of *E. hendrikii*. The higher the extracts' TPC or AC values, the higher their enzyme inhibitory effect (Aghraz et al., 2018). Moreover, Owczarek et al. (2019) reported significant inhibitory effects of polyphenol-rich extract on the insulin-like growth factors that participate in several cellular processes (e.g. proliferation, differentiation, and apoptosis).

The present and the earlier reports confirmed the presence of high variation in TPC and TF or AC values between the present and referred species *Erodium* species (Munekata et al., 2019). Factors such as species, variety, soil composition, rainfall, temperature, and humidity, can all affect the levels of phenolic compounds in plants (Fratianni et al., 2007; Colak et al., 2017; Munekata et al., 2019). In addition, collection, handling, and drying conditions, plant development (vegetative or flowering stage), and isolation procedures (extraction solvent content such as more polar or non-polar solvent, extraction time, and repeated number of the extractions) are other factors affecting the composition and properties of phenolics (Fratianni et al., 2007). For the reasons discussed, it is difficult to entirely compare the results of the present study with those in the previous literature. Comparison with the available literature shows that antioxidant activity and total phenolic results for *E. hendrikii* extracts in the described assays represent the first such reports. Considering the ethnopharmacological use of *E. hendrikii*, we think that further research targeting the particularly diverse activity of further water extracts together with ethyl acetate or methanol extracts will be of particular interest.

3.2. Phenolic acid and flavonoid contents in E. hendrikii

UHPLC-MS/MS analysis revealed five phenolic acids in the crude extract and 13 phenolic acids in four forms in the extract liberated after acid and alkaline hydrolysis (free, ester, glycoside, and ester-bounded) in the aerial parts of *E. hendrikii*. These are shown in Table 2. Caffeic acid (CaA), with a concentration of 3189.56 nmol/g dw, was the most abundant phenolic acid in the crude extract, followed by ferulic acid (FeA, 357.47 nmol/g dw) and *p*-coumaric acid (*p*-CoA, 335.25 nmol/g dw). Protocatechuic acid (PA) in free (90.07 nmol/g dw), ferulic acid (FeA) in ester (430.90 nmol/g dw), gallic acid (GaA) in glycoside (2450.51 nmol/g dw), and ester-bound (527.05 nmol/g dw) forms were the major phenolic acids identified and quantified. Intraspecies variation of phenolic acids among *Erodium* species was previously reported by Fecka et al. (2001). Those authors observed considerable variation in gallic acid content, ranging from 0.1 to 12.40 mg/g, for *E. gruinum* and *E. cicutarium*, respectively. The presence of the major phenolic acids in the genus was reported by Al-Snafi (2017) and Bilić et al. (2020) for *E. cicutarium* and Bakari et al. (2018) for *E. glaucophyllumin* (avg. of flower and leaves; 6224.6 mg/kg dw). Consistent with these findings, Bilić et al. (2020) recently reported that the largest group of identified compounds consisted of gallic acid derivatives (24 out of 85). The results of the present study are in agreement with previously published data (Bilić et al., 2020) with the majority of gallic acid in *E. hendrikii* being determined in the glycoside and ester-bound forms. Concentrations of this acid generally range from 0.679 to 2.310 mg/g dw in *Erodium* species (Bilić et al. (2020). In comparison to the polyphenolic profile of

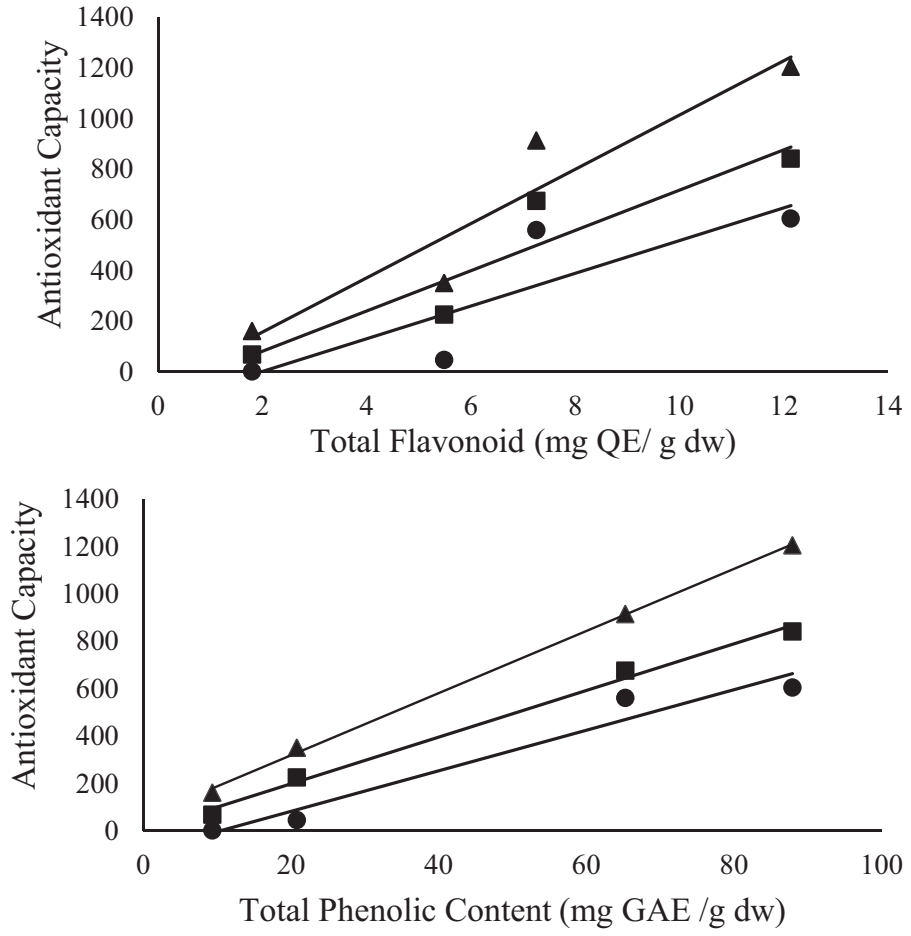

Fig. 3. Correlation between total phenolic compounds (TPC) and total flavonoids (TF) contents with further fractionated plant extract (*Erodium hendrikii*) by SPE (aqueous, polyphenolic and methanol fractions) in conjunction with antioxidant capacity values (μmol TE/g dw). DPPH: 2,2-diphenyl-1-picrylhydrazyl, FRAP: ferric reducing antioxidant power; CUPRAC: cupric ion reducing antioxidant capacity.

Table 2
Phenolic compounds (nmol/g dw) in *Erodium hendrikii*.

Compound*	Crude extract	Phenolic acids Free	Ester	Glycoside	Ester-bound
GaA	n.d.**	$22.81 \pm 1.19^{a, x}$	89.25 ± 1.93^{b}	2450.51 ± 43.09^{d}	527.05 ± 4.77^{c}
PA	n.d.	90.07 ± 4.83^{c}	192.48 ± 2.12^{d}	53.96 ± 0.34^{a}	72.70 ± 1.87^{b}
GA	n.d.	4.39 ± 0.64^{a}	n.d.	513.39 ± 24.74^{b}	n.d.
p-HBA	124.02 ± 12.07	9.93 ± 0.18^{a}	88.96 ± 4.63^{d}	60.62 ± 2.43^{c}	45.92 ± 0.22^{b}
VaA	tr***	2.67 ± 0.92^{a}	51.08 ± 1.38^{d}	32.07 ± 0.44^{c}	14.29 ± 2.03^{b}
SA	176.13 ± 23.85	27.31 ± 1.56^{b}	38.71 ± 0.85^{c}	185.23 ± 1.78^{d}	11.05 ± 0.09^{a}
CaA	3189.56 ± 283.92	1.50 ± 0.06^{a}	5.12 ± 0.02^{c}	12.47 ± 0.77^{d}	2.81 ± 0.19^{b}
SyA	n.d.	0.25 ± 0.09^{a}	1.53 ± 0.01^{b}	2.86 ± 0.44^{c}	0.36 ± 0.04^{a}
p-CoA	335.25 ± 18.18	31.61 ± 1.88^{a}	178.25 ± 1.66^{d}	143.78 ± 1.81^{c}	74.37 ± 0.91^{b}
m-CoA	n.d.	n.d.	n.d.	n.d.	0.13 ± 0.01
o-CoA	n.d.	0.40 ± 0.03^{a}	n.d.	0.45 ± 0.01^{b}	n.d.
FeA	357.47 ± 27.27	13.92 ± 1.52^{a}	430.90 ± 1.18^{d}	86.64 ± 1.75^{c}	70.43 ± 4.05^{b}
t-CA	n.d.	3.14 ± 0.42^{b}	5.60 ± 0.60^{c}	6.03 ± 0.86^{c}	0.85 ± 0.00^{a}
ChlA	11.46 ± 1.26				
Catechin	tr				
Hesperidin	259.56 ± 29.86				
Quercetin	tr				
Morin	tr				
Apigenin	tr				
Naringenin	tr				
Kaempferol	tr				

[x] Values are expressed as mean ± SE ($n = 3$). In the same row, values marked with different small letter at superscript indicate significant differences ($P < 0.05$). Comparison is four forms of phenolic acids.

* GaA: Gallic acid, PA: Protocatechuic acid, GA: Gentisic acid, *p*-HBA: *p*-hydroxybenzoic acid, VaA: Vanillic acid, SA: Salicylic acid, CaA: Caffeic acid, SyA: Syringic acid, *p*-CoA: p-coumaric acid, *m*-CoA: m-coumaric acid, *o*-CoA: o-coumaric acid, FeA: Ferulic acid, *t*-CA: Caffeolquinic acid, ChlA: Chlorogenic acid.

** n.d.; not detected,.

*** tr; trace amount.

E. cicutarium previously published by Bilić et al. (2020), the presence of several hydroxycinnamic acid derivatives such as *p*-coumaric, ferulic, and caffeic acid, followed by *p*-hydroxybenzoic acid and vanillic acid confirm the present results in *E. hendrikii*. Several beneficial effects have been reported for gallic acid, including antioxidant, anticarcinogenic, antimutagenic, anti-inflammatory, and antineoplastic properties. It has also been shown to be an efficient apoptosis-inducing agent, to exhibit cardioprotective effects, and to protect the cells against oxidative stress (Kahkeshani et al., 2019; Bilić et al., 2020). The present study was not consistent with these reports, wince caffeic acid emerged as the major phenolic acid in extract. Our results revealed considerable amounts of protocatechuic, ferulic, and gentisic acids in the above described phenolic acid forms in *E. hendrikii* extract. Protocatechuic acid has been reported to exhibit antioxidant, antibacterial, anticancer, antiulcer, antidiabetic, antiageing, antifibrotic, antiviral, anti-inflammatory, analgesic, antiatherosclerotic, and cardio-, hepato-, neuro-, and nephroprotective activities (Kakkar and Bais, 2014; Bilić et al., 2020). As a superior antioxidant with low toxicity, ferulic acid is more easily absorbed into the body and stays in the blood longer than any other phenolic acid. It is involved in many physiological functions, exhibiting anti-inflammatory, antimicrobial, anticancer, anti-arrhythmic, antithrombotic, and antidiabetic activities, ameliorates nerve cell damage, and may help repair damaged cells (Zduńska et al., 2018). Gentisic acid also exhibits beneficial effects on human health such as anti-inflammatory, antigenotoxic, hepatoprotective, neuroprotective, antimicrobial, and especially antioxidant activities (Abedi et al., 2020). The majority of gentisic acid in *E. hendrikii* in the present study was detected in glycoside form (513.39 nmol/g dw), and ferulic acid in ester form (430.90 nmol/g dw).

A recent literature survey concluded that flavonoid/flavonols also exhibit species variation in *Erodium* (Munekata et al., 2019). We identified and quantified only hesperidin in the extract as the major flavonoid compound, while the levels of the remaining compounds were much lower, being determined in trace amounts or alse not detected at all. Naringin, at 855.6 mg/kg in flower and 755.7 mg/kg in leaves, was reported as the major flavonoid in *E. glaucophyllum* (Bakari et al., 2018). However, naringin was not detected at all in the present study. Bakari et al. (2018) determined some quantities of quercetin (14.4 and 6.9 mg/kg) and naringenin (0.2 and 0.1 mg/kg) in the flowers and leaves of *E. glaucophyllum*, respectively, while we detected only trace amounts. Very recently, Bilić et al. (2020) reported rutin (range; 0.11 – 0.67 mg/g dw) and narcissin (range; 0.10 to 0.45 mg/g dw) as the major flavonoid compounds in *E. cicutarium* sampled from four locations in Croatia. Rutin is a well-known flavonoid, which has attracted considerable attention in the literature, mainly for its broad pharmacological properties, including antimicrobial, anti-inflammatory, anticancer, antiallergic, antithrombogenic, and antidiabetic activity (Munekata et al., 2019; Bilic et al., 2020). Despite its disadvantages, including restricted bioavailability, low water solubility, poor stability, and limited membrane permeability, a large number of products containing rutin are currently available on the global market (Bilić et al., 2020).

3.3. *Fatty acid and non-polar compounds in* E. hendrikii

GC–MS analysis of hexane extract revealed 34 fatty acids ranging in carbon chain lengths from C4 to C24 (Table 3, Fig. 4). Palmitic acid (C16:0, 5.23 mg/g dw) was the major fatty acid, followed by stearic acid (C18:0, 4.27 mg/g dw) and linoleic acid (C18:2, 3.81 mg/g dw). Approximately, 44.04% of the total fatty acid constituted saturated fatty acids and 55.95% unsaturated fatty acids. These results are in agreement with previous findings regarding *E. cicutarium* reported by Sarikurkcu et al. (2017). Those authors confirmed that linolenic acid (35.61%) was the most abundant fatty acid in *E. cicutarium* total oil, followed by linoleic acid (25.94%) and saturated palmitic acid

Table 3
Fatty acid profile of *Erodium hendrikii* extract.

Peak No	CHL*	Fatty acids	(mg/g dw)
1	C4:0	Butanoic	0.28 ± 0.00
4	C8:0	Octanoic	0.44 ± 0.02
5	C10:0	Decanoic	0.59 ± 0.00
7	C12:0	Dodecanoic	0.90 ± 0.09
8	C13:0	Tridecanoic	0.38 ± 0.00
9	C14:1 (D9)	Myristoleic	0.45 ± 0.39
10	C14:0	Tetradecanoic	1.66 ± 0.38
11	C15:1 (D10)	*cis*-10-Pentodecanoic	3.17 ± 0.97
12	C15:0	Pentadecanoic	0.45 ± 0.00
13	C16:1 (D9)	Palmitoleic	0.83 ± 0.04
14	C16:0	Hexadecanoic	5.23 ± 1.46
15	C17:1 (D10)	*cis*-10-Heptadecenoic	0.53 ± 0.00
16	C17:0	Heptadecanoic	0.53 ± 0.04
17	C18:3 (D9.12.15)	Linolenic	0.76 ± 0.09
18	C18:2 (D9.12)	9,12-Octadecadienoic	3.81 ± 1.34
19	C18:1 (D9)	*cis*-Oleic	2.59 ± 0.62
20	C18:1 (D9)	trans-Elaidic	0.76 ± 0.09
21	C18:0	Stearic	4.27 ± 1.24
22	C20:4 (D5.8.11.14)	*cis*-5,8,11,14-Eicosatetranoic	1.15 ± 0.22
23	C20:5 (D5.8.11.14.17)	*cis*-5,8,11,14,17-Eicosapentanoic	1.22 ± 0.26
24	C20:3 (D8.11.14)	*cis*-8,11,14-Eicosatrienoic	1.38 ± 0.33
25	C20:2 (D11.14)	*cis*-11,14-Eicosadienoic	3.29 ± 1.11
26	C20:1 (D11)	*cis*-11-Eicosenoic	0.46 ± 0.00
27	C20:0	Eicosanoic	1.07 ± 0.09
28	C21:0	Heneicosanoic	0.54 ± 0.00
29	C22:6 (D4.7.10.13.16.19)	*cis*-4,7,10,13,16,19-Docosahexaenoic	1.38 ± 0.04
30	C22:2 (D13.16)	*cis*-13,16-Docasadienoic	0.61 ± 0.00
31	C22:1 (D9)	Erucic	0.54 ± 0.00
32	C22:0	Docosanoic	1.08 ± 0.00
33	C23:0	Tricosanoic	0.54 ± 0.00
34	C24:1 (D9)	*cis*-15-Tetracosenoic	1.31 ± 0.48
35	C24:0	Tetracosanoic	1.08 ± 0.09

* CHL; carbon chain length.

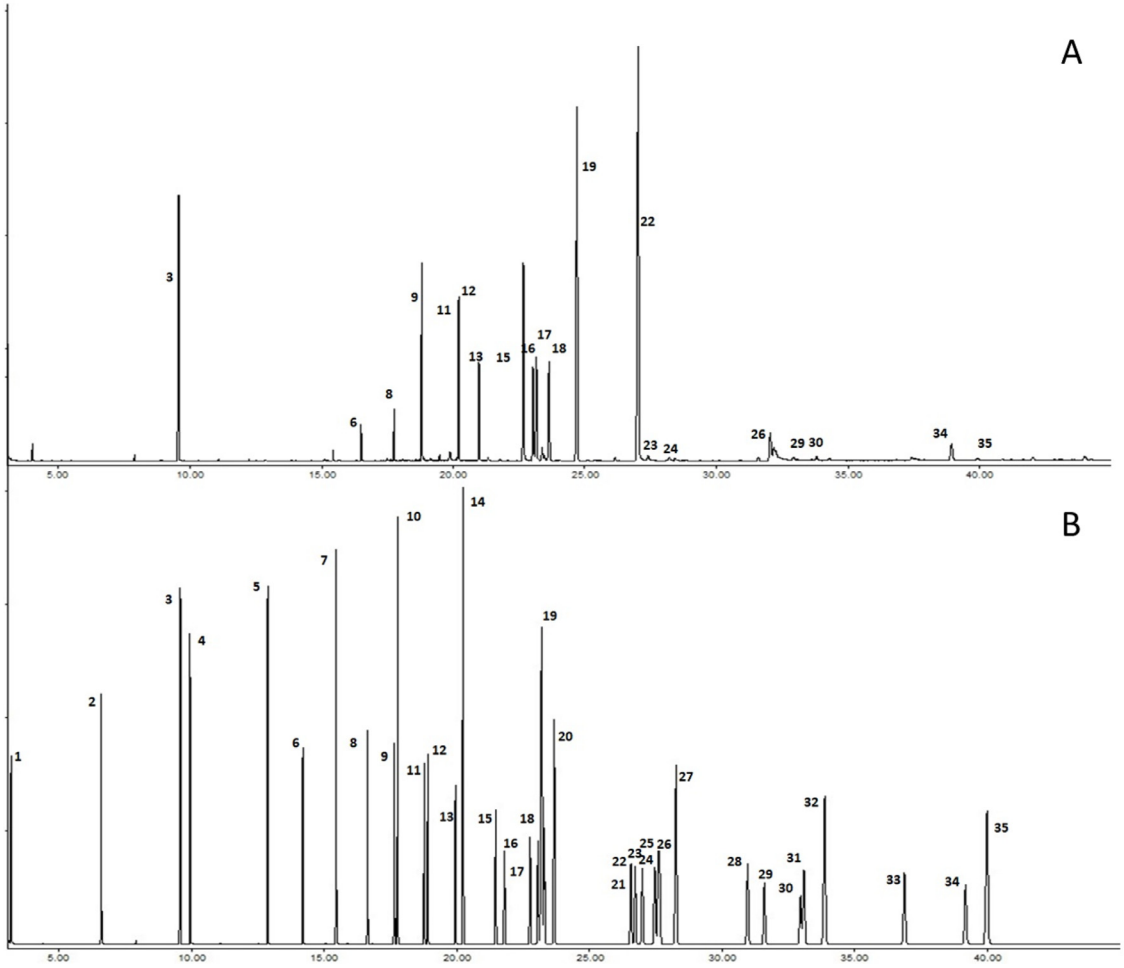

Fig. 4. GC–MS chromatograms of fatty acid methyl esters (FAME) of *Erodium hendrikii* (A) and FAME standards mixture (B). Peak numbers are given in Table 3, except with peaks 2 (C6:0), 3 (*n*-undecane as internal standard) and 6 (C11:0) in the FAME standard chromatogram (lower).

(20.90%). The difference between these findings may be due to our performing hexane extraction, while Sarikurkcu et al. used total lipid extraction for fatty acid analysis.

Twelve non-polar compounds in the hexane fraction were identified and quantified (Table 4) from the aerial parts of *E. hendrikii*. In the hexane extract, the major compound was the long-chained *n*-hexadecanal, known as palmitaldehyde, followed by *n*-tetradecanal (myristaldehyde, 12.99%). The concentrations of the remaining 10 non-polar compounds in the extract ranged from 0.37% to 2.48%. Volatiles of the entire plant, in the form of aerial parts including stem, leaf, and inflorescence, but not the root, were analyzed in the present study. Radulović et al. (2009) reported that the roots of *E. cicutarium* did not contain the same quantities of volatiles as the aerial parts of the plant. They also found that the inflorescences of the plant had very few or non-volatile constituents, especially because the flowers represent only a minute part of the overall plant mass (Radulović et al., 2009). In agreement with the present findings, *n*-hexadecanoic acid (35.9%) was the most abundant fatty acid in *E. cicutarium* collected from Serbia. Hexadecanal represented the highest amount of non-polar compound in *E. hendrikii* in the present study, while this substance was present at very low levels in other *Erodium* species, and was even not detected in some taxa (e.g. *E. absinthioides*) (M. Lis-Balchin 1993; Radulović et al., 2009; Stojanović-Radić et al., 2010). Stojanović-Radić et al. (2010) later compared the essential oil composition of three *Erodium* taxa (*E. ciconium* L. and *E. cicutarium* L. from Serbia, and *E. absinthioides* from Macedonia) and found that hexadecanoic acid levels varied significantly among the species, at 38%, 8.7%, and 5.4%, respectively, depending on the geography. Al-Snafi (2017) also identified hexadecanoic acid as the major constituent in the essential oil extract of *E. cicutarium* obtained by hydrodistillation. However, M. Lis-Balchin (1993) analyzed leaf essential oil (more specifically the hexane extract of the leaves) of *E. cicutarium* and found that the leaf was rich in geraniol (16.7%), citronellol (15.4%), isomenthone (11.2%), and methyl eugenol (10.6%). One of the other major compounds in the essential oil (hydrodistillation or hexane extraction) in *Erodium* is hexahydrofarnesyl acetone (6,10,14-trimethylpentadecan-2-one), an auto-oxidation compound derived from chlorophyll phytyl side-chain (Rontani et al., 1996; Radulović et al., 2009). This compound, like hexadecanoic acid, has been reported to exhibit an intraspecies variation ranging from 10.8% - 15.5% in *E. cicutarium*, 9.9% in *E. ciconium*, and 8.3% in *E. absinthoides*. The studies cited in the present study reported that the main constituent in the essential oil or non-polar fraction of *Erodium* species comprises fatty acids and fatty acid-derived compounds at levels between approximately 26% and 73% (M. Lis-Balchin 1993; Radulović et al., 2009; Stojanović-Radić et al. (2010). The difference between Radulović et al.'s findings and those of the present study may be due to either chemotypification or an ecological effect, or may simply the consequence of the different isolation procedures.

Table 4
GC–MS profile of the non-polar compounds in *Erodium hendrikii*.

Rt (min)	RI	SI (%)	Non-polar compounds	(%)
8.84	1057	92	Artemisia ketone	0.65 ± 0.04
11.92	1141	96	Camphor	0.64 ± 0.07
30.84	1611	89	Tetradecanal	12.99 ± 0.62
33.99	1697	87	2-Pentadecanone	1.62 ± 0.25
38.12	1815	92	Hexadecanal	60.75 ± 1.28
41.01	1901	93	2-Heptadecanone	2.48 ± 0.39
41.74	1924	98	Methyl hexadecanoate	0.58 ± 0.01
46.91	2090	98	Methyl 9,12-Octadecadienoate	0.37 ± 0.03
47.13	2097	95	Methyl *cis*-Oleate	1.01 ± 0.03
47.49	2110	89	Phytol	1.88 ± 0.33
50.44	2211	96	trans-Elaidic acid	0.82 ± 0.12
58.30	2400	89	2-Monopalitin	0.73 ± 0.00

RT: Retention time, RI: Retention index, SI: Similarity index according to the NBS75K.L, NIST/EPA/NIH Mass Spectral Library 2.0 database .

Conclusion

The present study represents the first report concerning different phytochemicals extracted from endemic, endangered *E. hendrikii* and compares the phenolics and the antioxidant capacity values obtained from its crude extract and its three further partial fractions. A strong correlation and linear regression were determined between the phenolic content and antioxidant capacity values of the crude extract and the three further partial fractions of the plant. Significant variation in the antioxidant capacity values was generally dependent on the type of fraction as well as the TPC content. The crude extract mainly contains caffeic acid, free and ester forms of protocatechuic acid, glycoside, and ester-bound forms of gallic acid. Palmitic acid as a fatty acid and n-hexadecanal as a non-polar compound were the major components in the hexane extract of the plant. Our results showed that the methanolic and crude extracts contained the most active phenolic compounds and exhibited high antioxidant capacity values. Overall, *E. hendrikii* may be considered a potential candidate for use as a natural food preservative and in medicinal plant use in primary health care systems. Further research into the extracts, including detailed identification and characterization, together with qualitative standardizations, as well as safety issues and toxicity studies aimed at food technologies or security are now needed.

No potential conflict of interest was reported by the authors.

Declaration of Competing Interest

None.

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