



In vitro antioxidant capacity and phytochemical characterization of *Eryngium kotschy* Boiss.

[Capacidad antioxidante *in vitro* y caracterización fitoquímica de *Eryngium kotschy* Boiss.]

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Abstract

Context: *Eryngium kotschy* is one of the species of *Eryngium* genus and was endemic to Turkey. It was known, that traditionally uses of this plant in the South-Western part of Turkey for the treatment of various diseases.

Aims: To evaluate the antioxidant capacities of methanol extract and ethyl acetate, *n*-butanol and water sub-extracts as well as to determine the phytochemical composition and to quantify the major antioxidant compounds in the active sub-extract.

Methods: Besides the total phenolic compounds and total flavonoid compounds, the antioxidant capacity of *E. kotschy* was evaluated by DPPH•, ABTS•• and FRAP methods and qualitative and quantitative determination of phytochemical constituents in active sub-extract by LC-MS/MS.

Results: The highest total phenolic (173.710 ± 1.088 mg gallic acid equivalent/ g_{extract}) and total flavonoid content (86.978 ± 0.650 mg catechin equivalent/ g_{extract}) was found in ethyl acetate sub-extract of *E. kotschy* (EKE) and this sub-extract showed the highest antioxidant capacity on DPPH• ($IC_{50} = 0.264 \pm 0.040$ mg/mL), ABTS•• (at a concentration of 0.125 mg/mL equivalent to 0.497 μ M Trolox) and FRAP (at a concentration of 1 mg/mL equivalent to 1476 ± 5.292 mmol Fe^{2+}) tests. Preliminary examination of the mass spectrums revealed the presence of 24 phytochemical compounds in this sub-extract and 7 of them was quantified. According to quantitative analyses the main compounds of EKE sub-extract were rosmarinic acid (490.820 ± 0.703 μ g/ mg_{extract}), chlorogenic acid (80.405 ± 0.170 μ g/ mg_{extract}), isorhamnetin 3-*O*-rutinoside (72.280 ± 0.33 μ g/ mg_{extract}), rutin (63.020 ± 0.052 μ g/ mg_{extract}) and caffeic acid (55.153 ± 0.523 μ g/ mg_{extract}).

Conclusions: The data suggest that EKE possess utilizable antioxidant properties *in vitro*. The antioxidant capacity of this sub-extract could be due to high content of phenolic and flavonoid contents.

Keywords: ABTS••; antioxidant DPPH•; *Eryngium kotschy*; FRAP; LC-MS/MS.

Resumen

Contexto: *Eryngium kotschy* es una de las especies del género *Eryngium* y era endémica de Turquía. Se sabía que tradicionalmente utiliza esta planta en la parte suroeste de Turquía para el tratamiento de diversas enfermedades.

Objetivos: Evaluar la capacidad antioxidante de extracto de metanol y los sub-extractos en acetato de etilo, *n*-butanol y agua, así como determinar la composición fitoquímica y cuantificar los principales compuestos antioxidantes en el sub-extracto activo.

Métodos: Además de los compuestos fenólicos totales y los compuestos flavonoides totales, la capacidad antioxidante de *E. kotschy* se evaluó mediante los métodos DPPH•, ABTS•• y FRAP y la determinación cualitativa y cuantitativa de los componentes fitoquímicos en el sub-extracto activo por LC-MS / MS.

Resultados: Los contenidos más altos de fenoles totales (173.710 ± 1.088 mg equivalente de ácido gálico/g de extracto) y flavonoides totales (86.978 ± 0.650 mg equivalente de catequina/g de extracto) se encontraron en el sub-extracto de acetato de etilo de *E. kotschy* (EKE) y este sub-extracto mostró la mayor capacidad antioxidante en las pruebas de DPPH• ($IC_{50} = 0,264 \pm 0,040$ mg/mL), ABTS•• (a una concentración de 0,125 mg/mL equivalente a 0,497 μ M Trolox) y FRAP (a una concentración de 1 mg/mL equivalente a 1476 ± 5.292 mmol Fe^{2+}). El examen preliminar de los espectros de masas reveló la presencia de 24 compuestos fitoquímicos en este sub-extracto y 7 de ellos se cuantificaron. De acuerdo con los análisis cuantitativos, los compuestos principales del sub-extracto EKE fueron ácido rosmarínico ($490,820 \pm 0,703$ μ g/mg de extracto), ácido clorogénico ($80,405 \pm 0,170$ μ g/mg de extracto), isorhamnetin 3-*O*-rutinoside ($72,280 \pm 0,33$ μ g/mg de extracto), rutina ($63,020 \pm 0,052$ μ g/mg de extracto) y ácido cafeico ($55,153 \pm 0,523$ μ g/mg de extracto).

Conclusiones: Los datos sugieren que EKE posee propiedades antioxidantes utilizables *in vitro*. La capacidad antioxidante de este sub-extracto podría deberse al alto contenido de contenido fenólico y flavonoide.

Palabras Clave: ABTS••; antioxidante DPPH•; *Eryngium kotschy*; FRAP; LC-MS/MS.

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INTRODUCTION

Free radicals are linked to pathology of various diseases such as diabetes, cancer and cirrhosis. Reactive oxygen species (ROS) can react with fatty acids in the cell membrane and with sulfhydryl bonds in nucleotides and proteins, leading to cell damage. Natural antioxidants can scavenge these free radicals that are responsible for the pathology of ROS-related diseases. Because of their natural antioxidant compositions, the plants are rich sources and are being widely investigated in such diseases (Roopan et al., 2009).

The genus of *Eryngium* L. (*Apiaceae*, *Saniculoideae*) is widely distributed in the world and used in traditional medicine for different therapeutic purposes. In Turkish folk medicine, various species of the genus are used for a wide range of ailments; particularly, roots are used against various inflammatory disorders, edema, sinusitis, urinary infections or inflammations and snake or scorpion bites or goiter; roots and leaves for infertility and herbs for wound healing (Küpeli et al., 2006).

Eryngium genus comprises about 250 species, growing in Eurasia, North and South America, North Africa, and Australia. It is the most species-rich genus of the *Apiaceae* (Pimenov and Leonov, 1993). The most recent monograph of *Eryngium* is now over 90 years old (Wolff, 1913) and outdated. Many regional treatments in "Floras" were subsequently published, among them Davis (1972) for Turkey, Pimenov and Tamamschian (1987) for the Flora Iranica area and Mathias and Constance (1941) for North America (Wörz and Duman, 2004). There are 23 taxa in Turkey according to Turkey Plant List (Güner and Aslan, 2012).

It was reported that some species of *Eryngium* have different biological activities such as cytotoxic (Kartal et al., 2005; Bogucka-Kocka et al., 2008; Zhang et al., 2008; Vukic et al., 2018), anti-inflammatory and anti-nociceptive (Küpeli et al., 2006), anti-amebicide (Derda et al., 2013), anti-snake and scorpion venom (Alkofahi et al., 1997), anti-leishmania (Rojas-Silva et al., 2014), anti-malarial (Fokialakis et al., 2007), antioxidant

(Thomas et al., 2017), antibacterial (Çelik et al., 2011), antifungal (Cavaleiro et al., 2011) and anti-diabetic (Pereira et al., 2019). These pharmacological effects are mainly related to the terpenoids, triterpenoid saponins, flavonoids, coumarins, polyacetylenes and steroids (Küpeli et al., 2006; Çelik et al., 2011; Wang et al., 2012).

There are few studies concerning the antioxidant activity and chemical composition of *E. kotschy*. In light of this, the present study is mainly designed to evaluate the total phenolic and flavonoid content, and antioxidant capacity by 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]) scavenging activity, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS^{•+}) scavenging activity and ferric-reducing ability power (FRAP) methods of *E. kotschy* methanol, ethyl acetate, *n*-butanol and water extract. Further the phytochemical profile of active sub-extract was evaluated, and quantitative analyses was carried out on this sub-extract using LC-MS/MS.

MATERIAL AND METHODS

Chemicals

All chemicals and reagents were analytical or HPLC grade and purchased from Sigma-Aldrich.

Plant material

E. kotschy Boiss. was harvested from Konya; South Hadim at 1600 m altitude of steppe areas on 2015 year (GPS coordinates: 36°58'32.1"N 32°23'13.6"E). The plant was identified and collected by Osman Tugay (Selçuk University). Plant samples was deposited in the Herbarium of Science Faculty at Selcuk University (Herbarium No: KNYA 26907). In this study flowering aerial parts were air dried and cut into small pieces and grounded to coarse powder using a blender (IKA MF 10 Basic, 1000 W, Grinder).

Preparation of extracts

Air dried parts of *E. kotschy* (500 g) were powdered and extracted three times (during 24 h, at room temperature) with 600 mL 70% methanol by

maceration. Combined macerates filtered and evaporated to dryness under reduced pressure at 37°C using a rotary evaporator. *E. kotschy* methanol extract (EKM) dispersed with water and partitioned with ethyl acetate (EKE) and *n*-butanol (EKB) sequentially. The crude extracts were lyophilized and stored in dark at -20°C. A total 3 sub-extracts were obtained from EKM extract. Yields of extract and sub-extracts are given in Table 1.

Table 1. Yield of extracts and sub-extracts.

Extracts	Code	Yields (%)
Methanol	EKM	10
Ethyl acetate	EKE	25
<i>n</i> -butanol	EKB	30
Water	EKW	30

EKM: *E. kotschy* methanol extract; EKE: *E. kotschy* ethyl acetate sub-extract; EKB: *E. kotschy* *n*-butanol sub-extract; EKW: *E. kotschy* water sub-extract.

Determination of Total Phenolic Content (TPC)

The TPC was estimated using method of Saeed et al. (2012). Briefly, 100 µL of the sample solution or standard was mixed with 100 µL of Folin-Ciocalteu (FC) reagent and 900 µL of ultrapure water and after 5 min 1 mL of 7% sodium carbonate added to start the reaction. The absorbance was measured at 765 nm after 90 min of reaction in dark at ambient temperature. The TPC was reported as gallic acid equivalents (GAE) per gram of dry extract. Response function of the gallic acid calibration curve was $y = 4.5906x + 0.0129$ and the correlation coefficient (r^2) of the calibration curve was 0.999.

Determination of Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was carried out according to the Marinova et al. (2015) with some modifications. A volume of 250 µL of a sample was mixed with 1 mL ultrapure water and 75 µL of NaNO₂ solution (5%, w/v) and after 5 min 75 µL of AlCl₃ solution (10%, w/v) was added. A sample was mixed and six minutes later was neutralized with 0.5 mL of 1 mol/L NaOH solution. The mixture was left for 10 min at room temperature and then absorbance was measured at 510 nm. Cate-

chin was used for the construction of a standard curve. All tests were carried out in triplicate. The TFC was reported as catechin equivalents (CA) per gram of dry extract. Response function of the catechin calibration curve was $y = 3.0525x - 0.0085$ and the correlation coefficient (r^2) of the calibration curve was 0.999.

Antioxidant capacity

1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging capacity

1,1-diphenyl-2-picrylhydrazyl radical (DPPH•) scavenging abilities of samples were determined using the method of Gyamfi and Aniya (2002). Stock solutions of samples were prepared in methanol at 4 mg/mL concentrations. Then the stock solutions were diluted to obtain working concentrations (0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1 and 2 mg/mL). A volume of 50 µL of a sample concentration was mixed with 950 µL 0.05 M Tris-HCl buffer and 1 mL of DPPH solution. The mixture was left for 30 min at room temperature and then absorbance was measured (Shimadzu UV-1800 UV-Vis Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) at 517 nm. In this study, butylated hydroxyanisole (BHA) was the reference standard and all tests carried out in triplicate. The % inhibition was calculated using Equation [1]. The IC₅₀ (concentration providing 50% inhibition) values were calculated use the dose inhibition curve in nonlinear regression mentioned of plots of the percentage of antiradical capacity against the concentration of the samples.

$$\% \text{ inhibition} = \frac{[(\text{Abs control} - \text{Abs sample}) / \text{Abs control}] \times 100}{[1]}$$

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS•+) radical scavenging capacity

In this study, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) radical (ABTS•+) model was used as an alternative radical scavenging capacity test and was estimated using method of Thaipong et. al. (2006) with some modifications. The ABTS•+ stock solution was prepared by reacting 7.4 mM ABTS with 2.6 mM K₂S₂O₈ in equal quantities and allowing the mixture to stand in the dark for 12 h before use. The ABTS+ working solution was di-

luted with a methanol to an absorbance of 1.1 ± 0.02 (734 nm). A volume of 150 μ L of sample were mixed with fresh 2850 μ L of ABTS⁺ reagent. The absorbance reading was taken after 30 min of incubation at room temperature in the dark. Trolox was chosen as a reference compound. Absorbance was measured on a UV spectrophotometer (Shimadzu UV-1800 UV-Vis Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) at 734 nm and results are given in terms of TEAC (Trolox equivalent antioxidant capacity). The assay was carried out in triplicate.

Ferric-reducing ability power (FRAP)

The FRAP assay was carried out with method described by Guo et al. (2003) with some modifications. Briefly, 40 μ L of the extract or standard were mixed with 200 μ L ultrapure water and 1800 μ L of freshly prepared FRAP reagent. The FRAP reagent contained 2.5 mL of a 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mmol/L HCl adding 2.5 mL of 20 mmol/L FeCl₃ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6 and was prepared freshly and warmed at 37°C. Samples were incubated for 10 min at 37°C, and then, absorbance was recorded at 593 nm. FRAP values were calculated using FeSO₄·7H₂O as standard ferric reducing activity. Trolox was used as a reference compound in this assay. The results were expressed as mmol of Fe²⁺ equivalents per g of extract weight (mmol Fe²⁺/g). The assay was carried out in triplicate.

LC-MS/MS instrumentation

Compounds in active sub-extract were determined by using liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry (LC-ESI-MS/MS, Shimadzu 8040, Shimadzu Corporation, Kyoto, Japan). Mass spectrometry was conducted using a Shimadzu LC-MS/MS-8040 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface in the negative ion mode.

The samples were prepared in methanol. The following instrument settings were used for analysis: column Restek C18 (150 × 4.6 mm × 3 μ m); column heat, 40°C; heat block temperature, 400°C; DL

temperature, 250°C; nebulizing gas (N₂), 3 L/min; drying gas (N₂), 15 L/min; collision energy, 25.0, 12.0, 9; dwell time, 100 msec. A mixture of methanol (A) and water: formic acid (99:1, v/v) (B) was selected as the mobile phase. The mobile phase consisted of 50% solvent A and 50% solvent B at a flow rate of 0.4 mL/min, and injection volume was 1 μ L.

Statistical analysis

Statistical analysis was performed GraphPad Prism Software Version 8.0 (La Jolla, CA, USA) using to compare differences in values between the standard and experimental group. The results are expressed as the mean \pm standard deviation (SD). Statistically significant values were compared using two-way ANOVA with Tukey Multiple Comparison Test and p-values of less than 0.05 were considered statistically significant.

RESULTS

Determination of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The results for TPC and TFC of the extracts are shown in Table 2. The TPC and TFC of methanol extract were 58.329 ± 0.804 mg GAE/g dry_{extract} and 31.941 ± 1.083 mgCA /g_{extract}, respectively. Moreover, it was found that the EKE sub-extract contains higher (**p<0.001) TPC (173.710 ± 1.088 mg GAE/g_{extract}) as well as TFC (**p<0.001) (86.978 ± 0.650 mgCA/g_{extract}) than other extracts. Furthermore, lower results (*p<0.05) for TPC (and TFC) were detected in water extract (39.737 ± 0.902 mg GAE/g_{extract} and 12.503 ± 1.250 mgCA /g_{extract}, respectively).

Antioxidant capacity

1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging capacity

The DPPH• assay determines the ability of antioxidants to eliminate DPPH radicals by an electron transfer reaction. The results for DPPH• antioxidant capacity of EK extract and sub-extracts are presented in Table 2. According to results EKM

extract showed moderate antioxidant capacity with 0.470 ± 0.023 mg/mL IC_{50} values. In the present study, the higher DPPH radical scavenging capacity was determined in EKE sub-extract ($IC_{50} = 0.264 \pm 0.040$ mg/mL) and was followed by EKB, EKM and EKS extracts ($IC_{50} = 0.520 \pm 0.020$ mg/mL, 0.470 ± 0.023 mg/mL and 1.061 ± 0.006 mg/mL, respectively). The EKE sub-extract showed statistically similar capacity ($p < 0.05$) with BHA ($IC_{50} = 0.056 \pm 0.004$ mg/mL), but none of the extracts showed high capacity than BHA.

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS^{•+}) radical scavenging capacity

The ABTS radical scavenging capacity of EKM extract and EKB, EKW sub-extracts were determined at the concentrations of 0.50 and 1 mg/mL and EKE 0.125, 0.25 and 0.5 mg/mL. The concentrations of BHA as a standard were 0.125, 0.25 and 0.5 mg/mL. The methanol extract and EKB, EKW sub-extract revealed the highest capacity at 1 mg/mL, but EKE sub-extract showed it at 0.125 mg/mL. The ABTS value of EKM extract was determined with 0.490 ± 0.050 μ M Trolox/ g_{extract} . EKE

sub-extract was found more active at 0.125 mg/mL concentration than water and n-butanol sub-extracts (0.497 ± 0.065 μ M Trolox/ g_{extract}) (Table 2). Except of EKE sub-extract none of extract and sub-extracts surpassed the activity of BHA. The activity of the EKE at a concentration of 0.125 mg/mL was found statistically higher from the capacity at a concentration of 0.125 mg/mL of BHA ($p < 0.05$) (0.126 ± 0.078 μ M Trolox/ g_{BHA}).

Ferric-reducing ability power (FRAP)

To determine the antioxidant activity of *E. kotschyi* extract and sub-extracts FRAP assay was applied, which represented single electron mechanism. Table 2 shows FRAP values obtained from EKM extract and sub-extracts. According to this results ethyl acetate sub-extract from EKM extract was the most active (1476 ± 5.292 mmol $Fe^{2+}/g_{\text{extract}}$) and was followed by EKW, EKM and EKB (866 ± 10.000 , 375 ± 10.000 and 284 ± 6.028 mmol $Fe^{2+}/g_{\text{extract}}$, respectively). Trolox was used as a standard and EKE sub-extract was not significantly different from Trolox ($p < 0.05$).

Table 2. Total phenolic and flavonoid content and antioxidant activity results of *Eryngium kotschyi* extract and sub-extracts.

Samples	TPC (mg GAE/ g_{extract})	TFC (mg CA/ g_{extract})	DPPH (IC_{50}) (mg/mL)	FRAP (mmol $Fe^{2+}/g_{\text{extract}}$) (mmol $Fe^{2+}/g_{\text{Trolox}}$)	ABTS (μ M Trolox/ g_{extract}) (μ M Trolox/ g_{BHA})
EKM	$58.329 \pm 0.804^*$	$31.941 \pm 1.083^*$	$0.470 \pm 0.023^*$	375 ± 10.000	0.490^{a*} 0.356^{b*}
EKE	$173.710 \pm 1.088^{**}$	$86.978 \pm 0.650^{**}$	$0.264 \pm 0.040^*$	$1476 \pm 5.292^*$	0.603^{c**} 0.497^{d**}
EKB	$83.816 \pm 0.105^*$	38.821 ± 1.665	$0.520 \pm 0.020^*$	284 ± 6.028	0.563^{a*} 0.428^b
EKW	$39.737 \pm 0.902^*$	$12.503 \pm 1.250^*$	1.061 ± 0.006	$866 \pm 10.000^*$	0.324^a
Trolox	-	-	-	2516 ± 15.275	-
BHA	-	-	0.056 ± 0.004	-	0.608^{b**} 0.304^{c*} 0.126^{d*}

Values are mean \pm SD, n = 3. Different letters for the same column indicate significant differences at $*p < 0.05$, $**p < 0.001$ and $***p < 0.0001$.

^aConcentration at 1 mg/mL; ^bconcentration at 0.5 mg/mL; ^cconcentration at 0.25 mg/mL; ^dconcentration at 0.125 mg/mL. EKM: *E. kotschyi* methanol extract, EKE: *E. kotschyi* ethyl acetate sub-extract, EKB: *E. kotschyi* n-butanol sub-extract, EKW: *E. kotschyi* water sub-extract.

Qualitative analyses of compounds

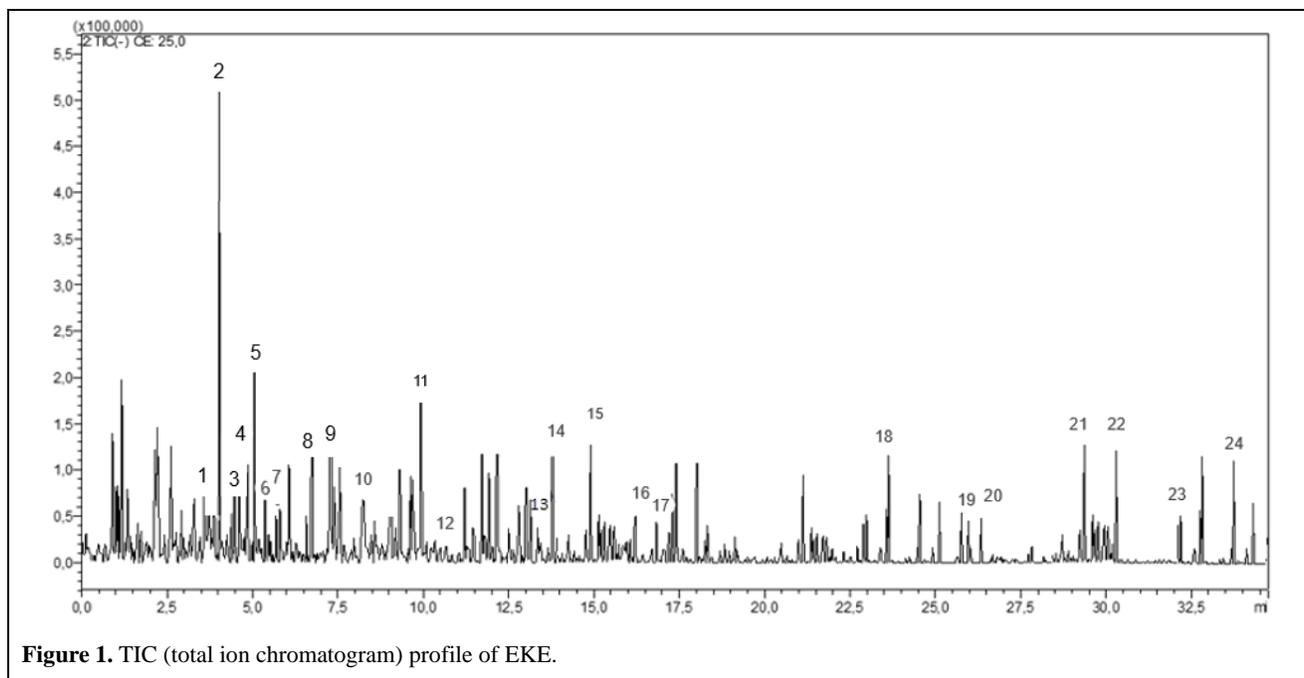
The structural characterizations of compounds in active sub-extract were evaluated based on the registered mass spectra fragmentation patterns, NIST (National Institute of Standards and Technology) mass spectral database (version 2.3, USA) and literature data. The mass spectrometric behavior of compounds was studied using both positive-ion and negative-ion mode. But negative-ion mode provided a better sensitivity for these compounds due to more efficient ionization, simpler fragmentation, and lower baseline noise.

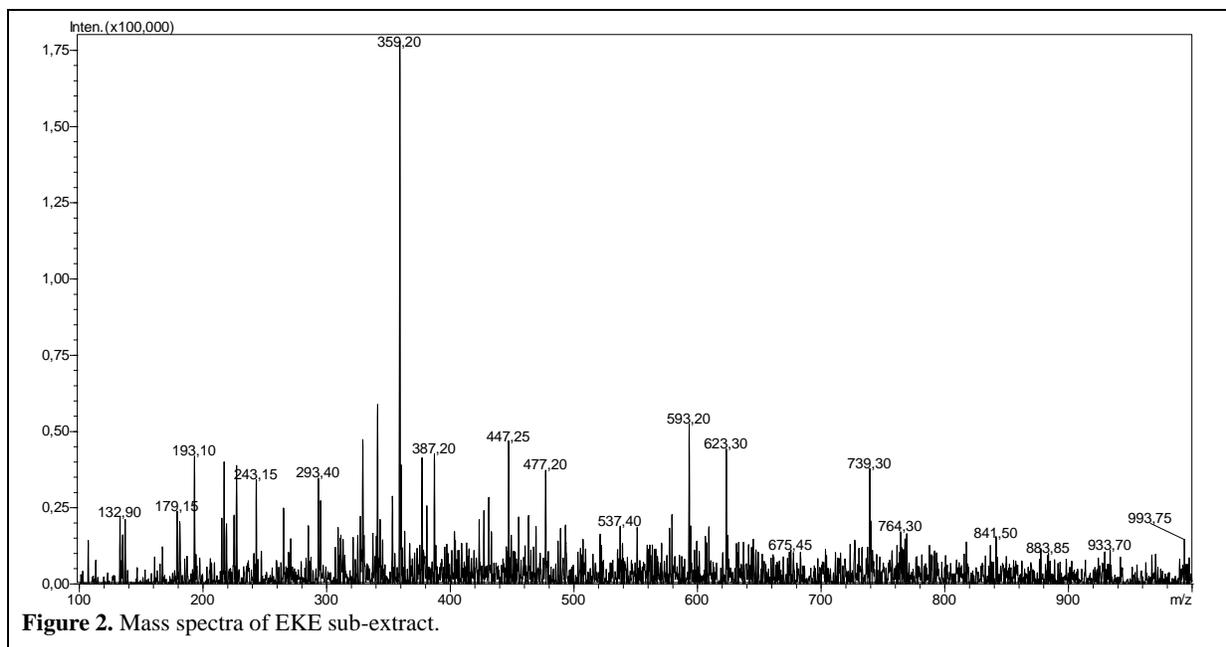
Preliminary examination of the mass spectrums revealed the presence of apigenin-7-*O*-rutinoside (Lin et al., 2000), caffeic acid-3-glucoside (Sadeghi et al., 2018), caffeic acid derivative-I (Riethmüller et al., 2013), chlorogenic acid (Lin et al., 2000), epicatechin-3-*O*-(4-*O*-metil) gallate (Kelebek, 2016), ferulic acid dimer (Bravo et al., 2007), isorhamnetine 3-*O*-rutinoside (Lin et al., 2000), kaempferol cumaroil hexoside (Simirgiotis and Schmeda-Hirschmann, 2010), kaempferol-3-*O*-

glucoside (Ribeiro et al., 2008), malic acid (González et al., 2011), quinic acid (Clifford et al., 2003) rosmarinic acid (Tang et al., 2016), rutin (Karaçelik et al., 2015), 5-cynapoil quinic acid (Lin et al., 2000) and others. The total ion chromatogram and mass spectra of sub-extract are shown in Figs. 1 and 2. Molecular ion, retention time (RT), MS/MS data of identified compounds are given in Table 3. Preliminary examination of the mass spectrums revealed the presence of flavonoid and flavonoid glycosides, phenolic acids and derivatives, organic acids, pentacyclic triterpene and hydroquinone glycoside in EKE sub-extract.

Quantitative analyses of compounds

Compounds were subsequently analyzed in Q1Scan (Product Ion Scan) mode, using [M-H]⁻ ions as precursors. Obtained MS2 spectra were used to select the optimal product ions. The MRM parameters, such as the precursor ion *m/z*, collision energy, and product ion *m/z* for compounds were optimized by an automatic MRM optimization function.





As a result of LC-MS/MS analysis for malic acid the loss of water $[M-H-H_2O]^-$ provided an ion at m/z 115 and with the loss of CO_2 an intense ion at m/z 71 (Fernández-Fernández et al., 2010). The peak identified as a chlorogenic acid (m/z 353), produced to the loss of one of the caffeoyl moieties $[M-H-caffeoyl]^-$, and subsequent fragmentation of ion yielded the fragments at m/z 191 (deprotonated quinic acid), 179 [caffeic acid- H] $^-$, 135 and the peak of the ion at m/z 173 (the absence of a C4 substituent) (Barros et al., 2013). Fragmentation of $[M-H]^-$ ion (m/z 609) of rutin resulted in two major ions at m/z 300 and 301, showing the loss of rhamnose-glucose unit. The other flavonol diglycoside isorhamnetin 3-*O*-rutinoside is a 3'-methoxylated derivative of rutin. Fragmentation of this molecule $[M-H]^-$ ion (m/z 623) resulted ions m/z 285, 300 and 315. Isorhamnetin represents specific fragmentation with the loss of CH_3 radical from the deprotonated aglycone, thus giving m/z 315 \rightarrow m/z 300 and the m/z 285 pattern as a result of fragmentation in C-ring (Martucci et al., 2014). The tentative mass spectrum for rosmarinic acid ($[M-H]^-$ ion at m/z 359.08) showed the caffeic acid at m/z 179.0 and m/z 161.0, m/z 135.0 corresponding to loss of water and carbon dioxide molecules respectively from the precursor ion (Hossain et al., 2010). The obtained LC-MS/MS chromatogram and mass spectrum of compounds are presented in Fig. 3.

Preparation of standard and sample solutions

Stock solutions of compounds were prepared in methanol at 8 $\mu\text{g}/\text{mL}$ concentrations. The extract and sub-extracts solutions were prepared in methanol at 10 $\mu\text{g}/\text{mL}$.

Calibration curve

Linearity of the methods was established by triplicate injections of each concentration (0.01-8 $\mu\text{g}/\text{mL}$) of standard solutions. Response function of the standards calibration curve was $y = 2842x + 54.151$ for rosmarinic acid, $y = 10074x + 994.36$ for malic acid, $y = 33716x - 2152.2$ for chlorogenic acid, $y = 16535x + 275.47$ for quinic acid, $y = 181197x + 9999$ for caffeic acid, $y = 511143x - 4056$ for rutin and $y = 18006x + 928.47$ for isorhamnetin 3-*O*-rutinoside. The correlation coefficient (r^2) of the calibration curves was 0.9989, 0.9988, 0.9995, 0.9994, 0.9991, 0.9997 and 0.9996, respectively.

The quantitative results of compounds are given in Table 4. As seen in the table, rosmarinic acid ($490.820 \pm 0.703 \mu\text{g}/\text{mg}_{\text{extract}}$), chlorogenic acid ($80.405 \pm 0.170 \mu\text{g}/\text{mg}_{\text{extract}}$), isorhamnetin-3-*O*-rutinoside ($72.280 \pm 0.336 \mu\text{g}/\text{mg}_{\text{extract}}$), rutin ($63.020 \pm 0.052 \mu\text{g}/\text{mg}_{\text{extract}}$) and caffeic acid ($55.153 \pm 0.523 \mu\text{g}/\text{mg}_{\text{extract}}$) were the main constituents of EKE sub-extract.

Table 3. Mass spectral characteristics and identify of compounds in EKE sub-extract.

Pick No	RT (min)	[M-H] ⁻ (m/z)	MS/MS (m/z)	Compounds	References
1	3.60	455	189, 207, 248, 456	Betulinic acid	NIST MS database
2	4.31	359	133,161,179	Rosmarinic acid	(Tang et al., 2016)
3	4.42	377	341, 215, 179, 161,119	Caffeic acid derivative-I	(Riethmüller et al., 2013)
4	5.15	191	191, 93, 85	Quinic acid	(Clifford et al., 2003)
5	5.16	353	191	Chlorogenic acid	(Lin et al., 2000)
6	5.21	341	179,135	Caffeic acid-3-glucoside	(Gardana et al., 2007)
7	5.61	179	135, 87	Caffeic acid	(Horai et al., 2010)
8	6.82	477	477, 315, 287, 271, 187	Nepetin 7-glucoside	(Zou et al., 2015)
9	7.23	133	115	Malic acid	(González et al., 2011)
10	8.10	609	301	Rutin	(Karaçelik et al., 2015)
11	10.22	623	315	İsorhamnetin 3-O-rutinoside	(Lin et al., 2000)
12	11.83	537	284, 537	Hinokiflavone	(Yin et al., 2017)
13	13.81	271	108	Arbutin	(de la Luz Cádiz-Gurrea et al., 2013)
14	13.91	593	285, 255	Kaempferol cumaroil hexoside	(Simirgiotis and Schmeda-Hirschmann, 2010)
15	14.22	387	223,191,179	5-cynapoil quinic acid	(Lin et al., 2000)
16	16.31	447	273, 285, 257, 151	Kaempferol-3-O-glucoside	(Ribeiro et al., 2008)
17	16.93	331	168	Glycogallin	(de la Luz Cádiz-Gurrea et al., 2013)
18	23.62	739	285, 593	Robinin	NIST MS database
19	26.21	521	359, 161, 197	Glucopyranosyl rosmarinic acid	(de la Luz Cádiz-Gurrea et al., 2013)
20	26.73	463	300	Isoquercitrin	(de la Luz Cádiz-Gurrea et al., 2013)
21	29.32	577	269	Apigenin-7-O-rutinoside	(Lin et al., 2000)
22	30.41	579	285	Naringenin-7-O-rhamnoside-O-β-glucoside	(de la Luz Cádiz-Gurrea et al., 2013)
23	32.12	455	289,183	Epicatechin-3-O-(4-O-methyl) gallate	(Kelebek, 2016)
24	34.79	735	191,193,367	Ferulic acid dimer	(Bravo et al., 2007)

RT: Retention time

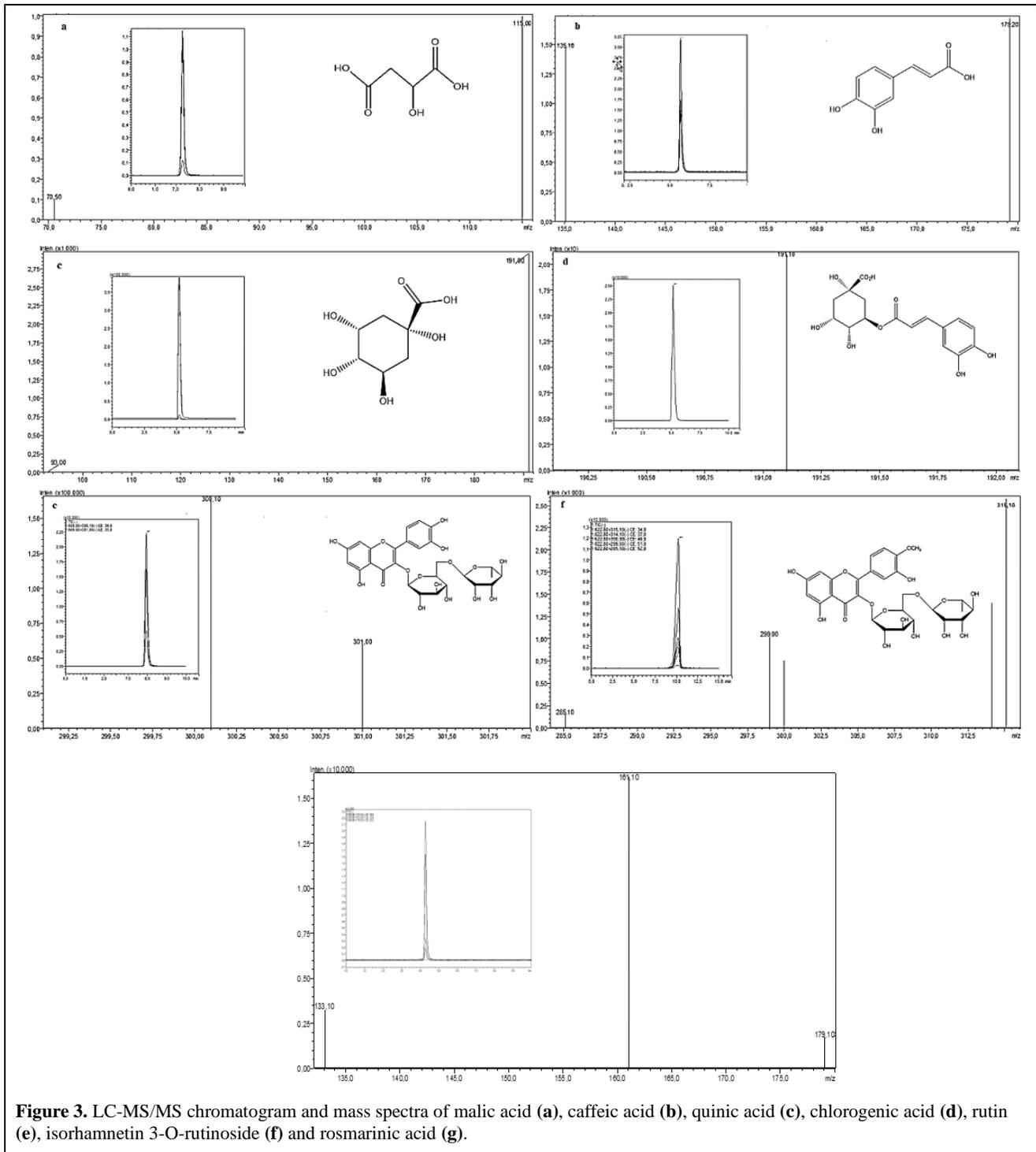


Figure 3. LC-MS/MS chromatogram and mass spectra of malic acid (a), caffeic acid (b), quinic acid (c), chlorogenic acid (d), rutin (e), isorhamnetin 3-O-rutinoside (f) and rosmarinic acid (g).

DISCUSSION

Many studies have shown that, natural antioxidants are capable of preventing oxidative stress related diseases such as cancer, rheumatoid arthritis, cardiovascular diseases, and neurodegenera-

tive diseases. On the other hand, because of their wide application, therapeutic efficacy and low toxicity, increasing attention has been paid to natural products for evaluation of their antioxidant activities. In this direction, flavonoids and phenolic acids are known as potential natural antioxidant

compounds and have been related to the capacity to scavenge free radicals and to reduce Fe³⁺ to Fe²⁺ (Pham-Huy et al., 2008; Fadus et al., 2017).

Table 4. Contents of compounds in EKE sub-extract.

Constituent	Content (µg/mg _{extract})
Malic acid	N.D.
Caffeic acid	55.153 ± 0.523
Quinic acid	19.161 ± 0.238
Chlorogenic acid	80.405 ± 0.170
Rutin	63.020 ± 0.052
Isorhamnetin 3- <i>O</i> -rutinoside	72.280 ± 0.336
Rosmarinic acid	490.820 ± 0.703

Results were given as means ± SD. ND: Not determined.

According to previous studies revealed that different *Eryngium* species have demonstrated various biological activities including cytotoxic, antioxidant, apoptotic, antifungal, antimicrobial and anti-inflammatory effect (Yurdakök et al., 2013; Toktas et al., 2017; Roshanravan et al., 2018).

In this study, the antioxidant activity of *E. kotschy* extract and sub-extracts measured with free radical (DPPH, FRAP, ABTS) scavenging capacity tests. Also, TPC and TFC of extract and sub-extracts were investigated. Among the extracts TPC and TFC were found higher in EKE sub-extract than others (TPC: 173.710 ± 1.088 mgGAE/g_{extract}, TFC: 86.978 ± 0.650 mgCA/g_{extract}). Due to the presence of high amount of phenolic acids and flavonoids EKE sub-extract showed higher antioxidant capacity than others (DPPH[•]: IC₅₀ = 0.264 ± 0.040 mg/mL, ABTS^{+•}: at a concentration of 0.125 mg/mL equivalent to 0.497 µM Trolox, FRAP: at a concentration of 1 mg/mL equivalent to 1476 ± 5.292 mmol Fe²⁺). Although TFC and DPPH radical scavenging activity of EKM extract was described before (Yurdakök et al., 2014), there is no information available about ABTS^{+•} and FRAP antioxidant activity and chemical composition of this extract and sub-extract. Unlike this report, to maximize the recovery of phenolic compounds and flavonoids as potential antioxidant agents we applied a sequential extraction procedure using solvents with different polar-

ities and obtained ethyl acetate, *n*-butanol and water sub-extracts. In a result the antioxidant capacity of EKE sub-extract was found significantly high (p<0.05) in DPPH[•] and FRAP tests and higher even BHA as a standard in ABTS^{+•} antioxidant test. Thus, these findings led us to investigate the phytochemical composition of this sub-extract. Hereby, 10 flavonoids and flavonoid glycosides, 12 phenolic acids and derivatives, 1 pentacyclic triterpene and 1 hydroquinone glycoside were determined in EKE sub-extract and major antioxidant compounds, as rosmarinic acid, chlorogenic acid, isorhamnetin-3-rutinoside, rutin and caffeic acid were quantified in this sub-extract. As a result, rosmarinic acid was found higher amount in this extract with 490.820±0.703 µg/mg_{extract} and was followed chlorogenic acid, isorhamnetin-3-rutinoside, rutin and caffeic acid. According to literature rosmarinic acid, isorhamnetin-3-rutinoside, rutin and caffeic acid were played major role in DPPH and ABTS radical scavenging activity and chlorogenic acid in FRAP test (de la Luz Cádiz-Gurrea et al., 2013).

Some *Eryngium* species have previously evaluated for the antioxidant activity such as *E. palmatum* (FRAP value: 0.2 ± 0.0 mmol Fe²⁺/g; DPPH^{+•}: IC₅₀=0.6 ± 0.0 mg/mL), *E. bourgatii* (FRAP value: 59.8 ± 2.4 mmol Fe²⁺/g) (Wang et al., 2012), *E. tricuspidatum* (DPPH^{+•}: IC₅₀ = 180 µg/mL) (Benmerache et al., 2016), *E. caucasicum* (TPC: 105.5 ± 2.8 mgGAE/g_{extract}, TFC: 18.7 ± 0.9 mgCA/g_{extract}, DPPH^{+•}: 83.1 ± 2.1 µg/mL) (Ebrahimzadeh et al., 2009), *E. creticum* (DPPH^{+•}: 89.92%) (Hijazi et al., 2015), *E. bornmuelleri* acetone extract (FRAP: 909.1 ± 37.5 µmol Fe²⁺/g_{DW}) (Dalar et al., 2014), *E. maritimum* (TPC: 8.09 mgGAE/g_{extract}, TFC: 1.03 mgCA/g_{extract}, DPPH^{+•}: IC₅₀ = 47.87 µg/mL) (Rjeibi et al., 2017) and *E. triquetrum* (DPPH^{+•}: IC₅₀ = 136 µg/mL) (Khalfallah et al., 2014). As described in some of these manuscripts, the antioxidant capacity of extract/sub-extract was related to flavonoid and phenolic compounds as similar to our study (Ribeiro et al., 2008; Ebrahimzadeh et al., 2009; Dalar et al., 2014; Benmerache et al., 2016; Rjeibi et al., 2017).

So, the higher antioxidant capacity of EKE sub-extract compared to other extracts may be due to

the higher content of phenolic compounds and flavonoids quantified as well as to the highest content of rosmarinic acid, chlorogenic acid and caffeic acid, which are the most efficient free radical scavengers (Chen and Ho, 1997). According to Rice-Evans et al. (1996) the antioxidant capacity of phenolic acids and their esters related to number of hydroxyl groups in the molecule and the electron-withdrawing properties of the carboxylate group (Rice-Evans et al., 1996). To the best of our knowledge, the higher antioxidant capacity of this sub-extract besides the phenolic acids is correlated to the flavonoids as rutin and isorhamnetin 3-O-rutinoside. It is well known that antioxidant effect of flavonoid compounds is related to structure conformation of these compounds. The free radical scavenging activity of flavonoids is linked presence of -OH groups, 2,3-double bond in conjunction with 4-carbonyl group in ring C, allowing for delocalization of the phenoxyl radical electron to the flavonoid nucleus (Atoui et al., 2005). Antioxidant effect of these compounds have previously been reported (Benavente-Garcia et al., 2000; Heim et al., 2002; Shibano et al., 2008; Salem et al., 2011).

CONCLUSIONS

This is the first report on the antioxidant capacity and phytochemical analyses of *E. kotschyi* species. The results showed that the ethyl acetate sub-extract of this species has highest antioxidant capacity by DPPH•, ABTS•• and FRAP methods. Identification and quantification of the antioxidant constituents of this sub-extract were evaluated in this study and their protective effect with other minor compounds may be investigated. Moreover, because of the limited studies on *E. kotschyi* species and identified compounds this qualitative and quantitative study combined with the antioxidant evaluation will shed new lights to the advanced studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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21404.

AUTHOR CONTRIBUTION:

Contribution	Paşayeva L	Köngül Şafak E	Arğün T	Fatullayev H	Tugay O
Concepts or ideas	x	x		x	
Design	x		x	x	
Definition of intellectual content	x			x	
Literature search			x	x	x
Experimental studies	x	x	x		x
Data acquisition	x	x	x	x	
Data analysis	x			x	
Statistical analysis	x			x	
Manuscript preparation	x	x	x	x	x
Manuscript editing	x	x		x	x
Manuscript review	x	x	x	x	x

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