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Characterization of the Chemical Profile of *Euphorbia* Species from Turkey by Gas Chromatography–Mass Spectrometry (GC-MS), Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS), and Liquid Chromatography–Ion Trap–Time-of-Flight–Mass Spectrometry (LC-IT-TOF-MS) and Chemometric Analysis

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LIQUID CHROMATOGRAPHY

Characterization of the Chemical Profile of *Euphorbia* Species from Turkey by Gas Chromatography–Mass Spectrometry (GC-MS), Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS), and Liquid Chromatography–Ion Trap–Time-of-Flight–Mass Spectrometry (LC-IT-TOF-MS) and Chemometric Analysis

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ABSTRACT

The *Euphorbiaceae* family comprises of about 300 genera and 5000 species primarily distributed in America and tropical Africa. The *Euphorbia* genus is represented by 105 species and locally named as "Sütleğen" and "Xaşîl" in Turkey. The present study aimed to determine the chemical constituents of *E. aleppica*, *E. eriophora*, *E. macroclada*, *E. grisophylla*, *E. seguieriana* subsp. *seguieriana*, *E. craspedia*, *E. denticulata, E. falcata*, and *E. fistulosa*, and classify them by utilizing the chemometric techniques of principal component analysis (PCA) and hierarchical cluster analysis (HCA). Linoleic acid, 17-tetratriacontane, palmitic acid, and hexatriacontane were the major fatty acids from the gas chromatography–mass spectrometry (GC/MS) analyses. Characterization of 268 constituents of the studied species was achieved by liquid chromatography–ion trap–time-of-flight–mass spectrometry (LC-IT-TOF-MS). Furthermore, a new liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was developed and validated for the simultaneous quantitative determination of 11 compounds (quinic acid, protocatechuic acid, rutin, hesperidin, eugenol, *p*-coumaric acid, piceatannol, scopoletin, DL-kavain, chrysophanic acid, and resiniferatoxin) in these species. The developed method was validated for the linearity, limit of detection, limit of quantification, repeatability, and recovery.

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Chemometric approach; *Euphorbia*; gas chromatography–mass spectrometry (GC-MS); hierarchical cluster analysis (HCA); liquid chromatography–ion trap–time-of-flight-mass spectrometry (LC-IT-TOF-MS); liquid chromatography–tandem mass spectrometry (LC-MS/MS); method validation; principal component analysis (PCA)

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Introduction

The *Euphorbiaceae* family is one of the largest families including approximately 300 genera and 5,000 species (Brummitt 1992; Fırat 2013). The genus *Euphorbia* L. belonging to this family comprises about 2,000 species (Willis 1996). There are 105 species of this genus in Turkey and 14 are endemic.

A literature survey revealed that the major secondary metabolites of this genus were terpenoids, coumarins, steroids, and aromatic compounds. Several *Euphorbia* species have been used as folk medicine. Some species have antiviral, anti-inflammatory, antipyretic, and analgesic activities (Lanhers et al. 1990) and are effective against skin cancer and warts (Evans and Taylor 1983). Furthermore, they exhibit antitumor, antifungal, antibacterial, and cytotoxic effects (Lanhers et al. 1991). It was proposed that the biological activities of the species could be due to their terpenoid constituents (Hamburger et al. 1989). The characteristic milky sap of *Euphorbia* species is highly toxic and irritant. Macrocyclic diterpenes were thought to be the reason for the irritation (Jeske, Jakupovic, and Berendsohn 1995).

The determination of the chemical constituents of herbal samples is an essential issue since plant materials have a variety of compounds with different chemical structures and complex matrices. Nowadays liquid chromatography–mass spectrometry (LC-MS) is the most widely used technique to characterize the secondary metabolites of plants (Sun et al. 2018; Selvi et al. 2018; Wang and Wang 2018).

One of the crucial advantages of time-of-flight–mass spectrometry (TOF-MS) instruments is the accurate mass determination up to 1/10,000 sensitivity. They also provide the elemental compositions of molecular ion and fragments used in the analysis of unknown matter (Li et al. 2017). The TOF-MS instruments have the properties of fast scanning and high mass resolution, but they do not possess the capability of sequential (multiple stages) mass spectrometry (MS^n).

Ion trap–mass spectrometry (IT-MS) instruments, in particular, play a crucial role in the structure elucidation of molecules by MS^n, but they have low resolution (generally 1 Da). Nowadays, liquid chromatography–ion trap–time-of-flight–mass spectrometry (LC-IT-TOF-MS) is one of the most sophisticated LC-MS instrument designs. The IT-TOF-MS has much higher sensitivity and accuracy than both TOF-MS and IT-MS. The IT-TOF-MS has the capability to scan natural compounds in MS^n mode by IT and perform accurate mass determination by TOF spontaneously (Liang et al. 2010; Liu et al. 2011; Rui et al. 2018; Taşkın et al. 2018).

The present study aimed to evaluate fatty acid profiles and chemical constituents of *E. aleppica*, *E. eriophora*, *E. grisophylla*, *E. seguieriana* subsp. *seguieriana*, *E. craspedia*, *E. denticulata, E. falcate*, and *E. fistulosa* using GC-MS and LC IT-TOF-MS, respectively. Furthermore, a new LC-MS/MS method was developed for the quantification of 11 compounds (Figure S1). The developed method was validated concerning linearity, limit of detection, limit of quantification, repeatability, and recovery. Moreover, chemometric techniques, namely, principal component analysis (PCA) and hierarchical cluster analysis (HCA), were applied to the chemical constituent data for the classification of the analyzed *Euphorbia* species.

Experimental

Plant material

Whole plants of *Euphorbia* species were collected from the southeastern part of Turkey on July 2015 by Dr A. Ertaş (Department of Pharmacognosy, Faculty of Pharmacy, Dicle University), M. Fırat (Department of Biology, Faculty of Education, Yüzüncü Yıl University), and Dr Y. Yeşil (Department of Pharmaceutical Botany, Faculty of Pharmacy, Istanbul University) and were identified by M. Fırat and Y. Yeşil. Voucher specimens were kept in the Herbarium of Yüzüncü Yıl University (Table 1).

Preparation of plant extracts for GC-MS, LC-MS/MS, and LC-IT-TOF-MS

Roots and aerial parts (branches, leaves, flowers, seeds) of the plant materials were air dried. Individual methanol extracts of roots, branches, leaves, flowers, and seeds were prepared by maceration (3 times for 24 h) at 25 °C. In addition, methanol and petroleum ether extracts of whole-plant materials (roots and aerial parts were mixed) were prepared in the same manner. After filtration, the solvent was removed under reduced pressure. The extraction yields were given in Table 1. The residues were diluted to 250 mg/L with methanol and passed through a 0.2-µm microfiber filter before LC-MS/MS and LC-IT-TOF-MS analyses.

Esterification of total fatty acids for GC-MS analysis

One hundred milligrams of the petroleum ether extract was refluxed with 2 mL of 0.1 M NaOH solution in methanol for 1 h. The solution was cooled and 5 mL of water was added. The aqueous mixture was neutralized with 0.5 mL of HCl and was extracted with diethyl ether:hexane (3.5:1, mL). The separated organic phase was washed with 10 mL of water and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure, and fatty acid methyl esters were obtained (Ertaş et al. 2014). The analyses were performed using an ion trap Varian Saturn 2100 T GC-MS coupled with DB-5 nonpolar column (length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 µm) (Ertaş et al. 2014).

LC-IT-TOF-MS conditions

The phytochemical constituents of methanol extracts of nine *Euphorbia* species were qualitatively identified using a Shimadzu LC-IT-TOF-MS. This hybrid instrument is an integration of ultra-high-performance liquid chromatography (UHPLC) with IT-TOF-MS, a high-resolution mass spectrometer. The UHPLC system (Shimadzu) consisted of a gradient pump (LC-20AD), an autosampler (SIL-20AC), a degasser (DGU-20A3), a communication bus module (CBM-20A), and a column oven (CTO-20AC).

The analytes were separated using an Agilent Eclipse XDB column (150 × 4.6 mm, 3.5 µm) at 35 °C using a flow rate of 0.35 mL/min. The injection volume was 4 µL. The mobile phase consisted of aqueous 5 mM ammonium formate (A) and acetonitrile (B) with a gradient program that began at 7.5% of eluent B flow from 0 to 5 min. A linear

Table 1. Yields of the petroleum ether and methanol extracts and species abbreviations.

Sample	Abbreviation	Methanol yield (%)	Petroleum ether yield (%)	Collection site	Collection time	Herbarium number
E. craspedia seed	ECMS	9.46				
E. craspedia root	ECMR	2.17				
E. craspedia branch	ECMB	9.95				
E. craspedia leaf	ECML	8.8				
E. craspedia flower	ECMF	8.1				
E. craspedia mixed	ECMM	13.98		Mardin	June 2015	M. Fırat 31625 (VANF)
E. craspedia mixed	ECMMP	–	4.40			
E. denticulata seed	EDKS	9.28				
E. denticulata root	EDKR	11.86				
E. denticulata branch	EDKB	8.76				
E. denticulata leaf	EDKL	10.93				
E. denticulata flower	EDKF	12.49				
E. denticulata mixed	EDKM	11.8		Kayseri	June 2015	M. Fırat 31630 (VANF)
E. denticulata mixed	EDKMP	–	4.03			
E. aleppica root	EADR	5.28				
E. aleppica branch	EADB	8.9				
E. aleppica leaf	EADL	19.62				
E. aleppica mixed	EADM	10.85		Diyarbakir		M. Fırat 31626 (VANF)
E. aleppica mixed	EADMP	–	1.67			
E. eriophora root	EEDR	3.92				
E. eriophora branch	EEDB	4.62				
E. eriophora leaf	EEDL	10.94				
E. eriophora mixed	EEDM	8.14		Diyarbakir	June 2015	M. Fırat 31627 (VANF)
E. eriophora mixed	EEDMP	–	1.52			
E. falcata mixed	EFDM1	14.51		Diyarbakir	June 2015	M. Fırat 31629 (VANF)
E. grisophylla seed	EGVS	13.92				
E. grisophylla root	EGVR	6.31				
E. grisophylla branch	EGVB	14.4				
E. grisophylla leaf	EGVL	12.54				
E. grisophylla mixed	EGVM	13.03		Van	June 2015	M. Fırat 30910 (VANF)
E. grisophylla mixed	EGVMP	–	1.44			
E. seguieriana subsp. *seguieriana* seed	ESDS	5.31				
E. seguieriana subsp. *seguieriana* root	ESDR	4.58				
E. seguieriana subsp. *seguieriana* branch	ESDB	5.43				
E. seguieriana subsp. *seguieriana* leaf	ESDL	5.9				
E. seguieriana subsp. *seguieriana* flower	ESDF	13.13				
E. seguieriana subsp. *seguieriana* mixed	ESDM	5.13		Diyarbakır	June 2015	M. Fırat 30905 (VANF)
E. seguieriana subsp. *seguieriana* mixed	ESDMP	–	2.09			
E. fistulosa root	EFDR	2.96				
E. fistulosa branch	EFDB	3.1				
E. fistulosa leaf	EFDL	8.26				
E. fistulosa flower	EFDF	12.04				
E. fistulosa mixed	EFDM	5.66		Diyarbakir	June 2015	M. Fırat 31628 (VANF)
E. fistulosa mixed	EFDMP	–	2.84			
E. macroclada root	EMMR	13.58				
E. macroclada branch	EMMB	7.71				
E. macroclada leaf	EMML	8.43				
E. macroclada flower	EMMF	15.59				
E. macroclada mixed	EMMM	7.08		Malatya	June 2015	M. Fırat 30906 (VANF)
E. macroclada mixed	EMMMP		3.43			
E. macroclada seed	EMDS	3.42				
E. macroclada root	EMDR	9.81				
E. macroclada branch	EMDB	5.93				

(*continued*)

Table 1. Continued.

Sample	Abbreviation	Methanol yield (%)	Petroleum ether yield (%)	Collection site	Collection time	Herbarium number
E. macroclada leaf	EMDL	14.47				
E. macroclada mixed	EMDM	6.57		Diyarbakir	June 2015	M. Fırat 30906 (VANF)
E. macroclada mixed	EMDMP	–	1.42			
E. macroclada seed	EMVS	2.93				
E. macroclada root	EMVR	5.69				
E. macroclada branch	EMVB	6.05				
E. macroclada leaf	EMVL	12.71				
E. macroclada flower	EMVF	4.4				
E. macroclada mixed	EMVM	10.51		Van	June 2015	M. Fırat 30906 (VANF)
E. macroclada mixed	EMVMP	–	2.70			
E. macroclada seed	EMTS	8.91				
E. macroclada root	EMTR	14.79				
E. macroclada branch	EMTB	12.94				
E. macroclada leaf	EMTL	13.58				
E. macroclada flower	EMTF	14.23				
E. macroclada mixed	EMTM	10.38		Trabzon	June 2015	M. Fırat 30906 (VANF)
E. macroclada mixed	EMTMP	–	1.54			

gradient was applied from 5 to 45 min from 7.5% to 95% of eluent B flow that was maintained for 7 min. The eluent B flow was then set to its original percent at 13 min.

The hybrid IT-TOF-MS high-resolution spectrometer had an electrospray ionization (ESI) source that operated in both positive and negative ionization modes. The optimized mass spectrometry (MS) conditions were as follows: positive and negative ionization modes; nebulizer gas (N_2) flow rate, 1.5 L/min; drying gas (N_2) pressure, 100 kPa; curved desolvation line temperature, 200 °C; block heater temperature, 200 °C; detector voltage, 1.63 kV; electrospray voltage, −4.5 kV; mass range, *m/z* 100–1000 (MS1); TOF pressure, 1.4×10^{-4} Pa; IT pressure, 1.8×10^{-4} Pa; ion accumulation time, 30 msec. The data obtained were analyzed by the software LC-MS Solution Version 3.4.1 (Shimadzu, Japan).

LC-MS/MS method development and validation

LC-MS/MS conditions. The LC-MS/MS analyses of the phenolic compounds were performed by using a Nexera model Shimadzu UHPLC coupled to a tandem MS instrument. The liquid chromatograph was equipped with LC-30AD binary pumps, a DGU-20A3R degasser, a CTO-10ASvp column oven, and a SIL-30AC autosampler. The chromatographic separation was performed on a C18 reversed-phase Inertsil ODS-4 (150 mm ×4.6 mm, 3 µm) analytical column. The column temperature was fixed at 40 °C. The elution gradient consisted of mobile phase A (aqueous 5 mM ammonium formate and 0.1% formic acid) and mobile phase B (5 mM ammonium formate and 0.1% formic acid in methanol). The gradient program with the following proportions of solvent B was applied; time (min), B %: (0, 35), (5, 50), (10, 90), (12, 90), (13, 35). The flow rate was 0.5 mL/min, and the injection volume was 2 µL.

MS detection was performed using a Shimadzu LCMS 8040 triple quadrupole mass spectrometer equipped with an ESI source operating in both positive and negative ionization modes. The LC-MS/MS data were collected and processed by LabSolutions software (Shimadzu, Kyoto, Japan). The multiple reaction monitoring (MRM) mode was used to quantify the analytes. The analysis of the investigated compounds was

Table 2. Analytical parameters of the LC-MS/MS method for the phytochemicals.

Peak number	Analyte	Retention time (min)	Parent ion (*m/z*)[a]	MS[2b]	Ion mode	Calibration equation	Coefficient of determination	Linear range (μg/L)	Limits of detection/ quantification (μg/L)	Relative standard deviation %		Recovery %		Relative uncertainty %[c]
										Intraday	Interday	Intraday	Interday	
1	Quinic acid	0.944	190.95	85.25–93.15	Neg	$y = 34.5791x + 65729.5$	0.9951	500–20,000	20.88/69.61	0.0011	0.0011	1.0067	1.0035	0.0041
2	Protocatechuic acid	1.397	153	109.10–108.10	Neg	$y = 1101.00x + 59048.1$	0.9967	50–2000	2.48/8.28	0.0038	0.0100	0.9959	0.9993	0.0172
3	Rutin	1.399	609.05	271.1	Neg	$y = 193.476x - 32739.8$	0.9994	250–10,000	19.63/65.45	0.0076	0.0107	0.9974	1.0029	0.0279
4	Hesperidin	1.773	610.95	303.00–449.00	Poz	$y = 364.317x - 65780.1$	0.9999	250–1000	10.91/36.37	0.0046	0.0036	0.9962	0.9973	0.0132
5	Eugenol	2.056	162.6	119.30–62.15	Neg	$y = 2330.77x + 136202.0$	0.9943	25–1000	3.01/10.04	0.0142	0.0148	0.9995	0.9999	0.0270
6	*p*-Coumaric acid	2.072	162.75	119.30–93.20	Neg	$y = 3061.45x + 767001.0$	0.9947	100–4000	6.62/22.07	0.0069	0.0112	1.0045	0.9995	0.0281
7	Piceatannol	2.564	242.95	159.20–01.20	Neg	$y = 896.611x + 135143.0$	0.9956	100–4000	8.23/27.43	0.0234	0.0230	1.0102	1.0099	0.0422
8	Scopoletin	2.649	190.95	176.05–04.25	Neg	$y = 2838.63x + 64443.1$	0.9995	25–1000	3.81/12.71	0.0191	0.0153	1.0092	1.0050	0.0422
9	DL-Kavain	7.877	230.9	115.10–53.10	Poz	$y = 14905.7x + 5453.0$	0.9999	5–100	0.41/1.36	0.0145	0.0177	1.0065	1.0053	0.0306
10	Chrysophanic acid	11.12	252.95	225.10–82.20	Neg	$y = 617.568x - 12169.4$	0.9988	25–1000	0.90/2.98	0.0201	0.0233	1.0006	1.0078	0.0404
11	Resiniferatoxin	11.16	629.05	311.00–93.00	Poz	$y = 8815.06x + 11367.2$	0.9999	5–100	0.34/1.14	0.0181	0.0162	0.9996	1.0004	0.0292

[a]Molecular ions of the standard compounds (*m/z* ratio).
[b]Fragment ions.
[c]95% confidence level ($k = 2$).

Table 3. Fatty acid constituents of the *Euphorbia* species[a].

Retention time (min)	Compound[b]	Composition (%)[c]										
		ECMMP	EDKMP	EADMP	EEDMP	EGVMP	ESDMP	EFDMP	EMMMP	EMDMP	EMVMP	EMTMP
19.03	Lauric acid	–	–	–	1.28 ± 0.01	1.26 ± 0.02	0.8 ± 0.01	0.94 ± 0.01	–	–	–	0.53 ± 0.00
19.24	Azelaic acid	–	–	–	4.08 ± 0.06	1.19 ± 0.01	0.98 ± 0.01	1.18 ± 0.01	–	–	–	2.76 ± 0.07
26.83	Myristic acid	–	–	–	11.4 ± 0.24	0.79 ± 0.01	4.8 ± 0.10	5.81 ± 0.12	–	10.25 ± 0.25	1.23 ± 0.03	0.71 ± 0.01
30.50	Pentadecanoic acid	–	–	–	0.87 ± 0.02	0.23 ± 0.00	–	0.34 ± 0.00	–	–	0.15 ± 0.00	–
34.04	Palmitic acid	19.64 ± 0.39	1.85 ± 0.02	13.51 ± 0.20	43.83 ± 0.94	12.85 ± 0.25	14.62 ± 0.28	18.72 ± 0.36	1.54 ± 0.04	39.48 ± 0.88	9.3 ± 0.22	11.3 ± 0.27
37.36	Heptadecanoic acid	–	–	–	0.82 ± 0.01	–	0.37 ± 0.00	–	–	–	0.15 ± 0.00	0.3 ± 0.00
39.30	9.12-Octadecadienoic acid	8.63 ± 0.18	–	–	–	0.64 ± 0.01	4.54 ± 0.09	3.92 ± 0.08	–	–	–	7.53 ± 0.14
39.42	Linoleic acid	40.52 ± 1.09	–	–	3.35 ± 0.04	0.48 ± 0.00	3.15 ± 0.06	3.8 ± 0.08	0.2 ± 0.00	–	53.45 ± 1.19	10.34 ± 0.20
39.61	Oleic acid	12.12 ± 0.22	–	–	4.21 ± 0.10	8.93 ± 0.17	4.2 ± 0.09	1.77 ± 0.04	0.35 ± 0.00	–	10.79 ± 0.21	15.82 ± 0.30
39.79	Elaidic acid	–	–	–	–	–	0.35 ± 0.00	–	–	–	1.01 ± 0.02	2.1 ± 0.05
40.61	Stearic acid	4.16 ± 0.06	–	1.77 ± 0.02	10.43 ± 0.20	3.89 ± 0.09	3.55 ± 0.07	3.37 ± 0.07	0.25 ± 0.00	5.44 ± 0.13	2.77 ± 0.06	7.79 ± 0.19
45.70	Eicosenoic acid	–	–	–	–	0.2 ± 0.00	0.43 ± 0.00	0.42 ± 0.00	–	–	1.76 ± 0.04	1.95 ± 0.05
46.62	Arachidic acid	–	–	–	5.54 ± 0.13	1.7 ± 0.03	4.39 ± 0.08	4.07 ± 0.08	–	3.45 ± 0.07	0.73 ± 0.01	1.39 ± 0.03
51.90	Erucic acid	–	–	2.45 ± 0.03	–	0.26 ± 0.00	3.3 ± 0.07	2.19 ± 0.04	–	2.34 ± 0.06	0.48 ± 0.00	0.9 ± 0.01
52.25	Behenic acid	–	–	–	1.62 ± 0.04	1.38 ± 0.03	2.76 ± 0.06	3.43 ± 0.07	–	6.02 ± 0.15	0.95 ± 0.01	3.4 ± 0.08
52.67	17-Tetratriacontane	–	64.75 ± 1.44	31.59 ± 0.39	12.56 ± 0.24	3.99 ± 0.08	19.86 ± 0.38	1.58 ± 0.03	41.03 ± 0.91	9.52 ± 0.23	9.85 ± 0.24	12.22 ± 0.29
54.02	Hexatriacontane	–	2.31 ± 0.04	8.50 ± 0.20	–	52.32 ± 1.16	18.78 ± 0.36	38.13 ± 0.85	33.03 ± 0.73	16.23 ± 0.39	0.05 ± 0.00	9.68 ± 0.23
55.25	Lanosterol	–	1.07 ± 0.02	3.22 ± 0.03	–	0.62 ± 0.01	0.39 ± 0.00	0.88 ± 0.01	0.71 ± 0.01	1.06 ± 0.03	1.38 ± 0.03	2.11 ± 0.05
55.99	β-Sitosterol	–	14.69 ± 0.28	20.13 ± 0.38	–	0.85 ± 0.01	1.19 ± 0.01	0.68 ± 0.01	11.54 ± 0.28	3.51 ± 0.08	0.66 ± 0.01	0.53 ± 0.00
56.48	17-Pentatriacontene	–	12.33 ± 0.24	18.72 ± 0.46	–	8.33 ± 0.17	6.93 ± 0.14	4.82 ± 0.10	9.76 ± 0.24	–	3.79 ± 0.08	6.44 ± 0.13
59.62	Tetracontane	11.28 ± 0.19	0.78 ± 0.01	0.11 ± 0.00	–	0.09 ± 0.00	4.6 ± 0.09	39.6 ± 0.88	0.99 ± 0.01	2.71 ± 0.06	1.42 ± 0.03	2.22 ± 0.05

[a]Results are presented as the mean ± standard deviation of three parallel measurements.
[b]Nonpolar fused silica column.
[c]Relative weight percent.

performed following two or three transitions per compound, the first for quantitative purposes and the second and/or the third for confirmation.

Optimization of the LC-MS/MS method. Trials of different combinations were performed to have rich ionization and a good chromatographic separation. Gradient elution was achieved using two solvents as (A) water (5 mM ammonium formate and 0.1% formic acid) and (B) methanol (5 mM ammonium formate and 0.1% formic acid). Among the most commonly used atmospheric pressure ionization sources including ESI, atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI), ESI was selected because the phenolics were small and relatively polar. Furthermore, LC-MS/MS was used for the current study due to its ion fragmentation stability (Ertaş et al. 2014; Ertaş, Yilmaz, and Firat 2015). The optimum ESI conditions were determined to be: an interface temperature 350 °C, desolvation line temperature 250 °C, heat block temperature 400 °C, nebulizing gas flow (nitrogen) 3 L/min, and drying gas flow (nitrogen) 15 L/min.

Validation of the developed LC-MS/MS method. In this study, 10 phenolic compounds and 1 nonphenolic organic acid were qualified and quantified in 9 *Euphorbia* species. The developed method was validated in terms of linearity, limit of detection, limit of quantification, precision, and accuracy. The quantification was performed by the standard external method.

Calibration curves were plotted from six replicate analyses using the linear regression model of least squares. The linearity was examined using the coefficient of determination (R^2) values. Ten independent solutions at lowest acceptable concentration were analyzed, and the standard deviations were determined. Limits of detection and quantification were determined to be the mean concentration +3 standard deviations and mean concentration +10 standard deviations, respectively.

The precision and the accuracy of the developed method were determined using samples spiked at low, middle, and high concentrations (25.00, 100.00, and 500.00 µg/L). The precision was examined as the repeatability (intraday) and intermediate precision (interday). The accuracy was characterized as the recovery given by the percentage of the ratio of observed concentration to the nominal concentration of the spiked sample.

Rectilinear regression equations and the linear ranges of the studied standard compounds are provided in Table 2. The correlation coefficients were higher than 0.99. The limits of detection and quantification of the reported analytical method are shown in Table 2. For the studied compounds, limits of detection ranged between 0.34 and 20.88 µg/L, and the limits of quantification were between 1.14 and 69.61 µg/L (Table 2 and Table S1). Moreover, the recoveries of the phenolic compounds ranged from 99.59% to 101.02%.

Relative standard uncertainty (U^{95}). The standard uncertainties of the analytes were determined by the accuracy (recovery) and precision (repeatability) studies according to the EURACHEM Guide. The calculated uncertainties are provided in Table 2. The intra- and interday precision, accuracy, and uncertainty studies were performed individually for all of the compounds. The required measurements for eugenol are given in Tables S2–S4 as examples (Ellison and Williams 2012; Ertaş et al. 2014; Yilmaz et al. 2018).

Chemometric analyses

The chemometric analyses of fatty acid contents of *Euphorbia* species were carried out using PCA and HCA, which are multivariate data analysis methods. Both methods for

clustering and classification are mainly based upon the PCA. The PCA reduces multiple variables into a set of fewer components created by their linear combinations by hindering correlations between those examined variables. The PCA-based methods can classify the samples by clustering into various groups.

The HCA classifies samples in a given data set and defines those data according to their similarities. The HCA can be applied directly to the original variables or to the results obtained from PCA in case of existing too many variables. Herein, HCA applied to data of the chemical constituents. The measurement is based on the Euclidean distance. The Ward's method was used as the clustering method.

Statistical analysis

All statistical calculations for chemometric analysis were performed using the Minitab 16.2.1 statistical software (Minitab Inc. 2010). The sections of the *Euphorbia* species were classified regarding fatty acid components using PCA and HCA techniques.

Results and discussion

Fatty acid composition by GC-MS

The fatty acid compositions of the petroleum ether extracts of 11 *Euphorbia* species were analyzed by GC-MS (Table 3). Six components were identified, constituting 100% of the petroleum ether extract of *E. craspedia* (Table 3). The major constituents of the fatty acids obtained from the petroleum ether extract were identified to be linoleic acid (C18:2 omega-6) (40.52%), palmitic acid (C16:0) (19.64%), oleic acid (C18:1 omega-9) (12.12%), and tetracosanoic acid (C24:0 lignoceric acid) (11.28%). Seven components were identified, including 100% of the petroleum ether extract of *E. denticulata*.

The main constituents of the fatty acid composition of the petroleum ether extract were identified to be 17-tetratriacontane (64.75%), β-sitosterol (14.69%), and 17-pentatriacontene (12.33%). Nine components were identified, including 100% of the petroleum ether extract of *E. aleppica*. The main constituents of the petroleum ether extract were identified to be 17-tetratriacontane (31.59%), β-sitosterol (20.13%), 17-pentatriacontene (18.72%), and palmitic acid (13.51%). Twelve components were identified, including 100% of the petroleum ether extract of *E. eriophora* with the main fatty acids as palmitic acid (43.83%), 17-tetratriacontane (12.56%), myristic acid (11.40%), and stearic acid (10.43%).

Nineteen components were identified, comprising 100% of the petroleum ether extract of *E. grisophylla*. The main constituents were identified as hexatriacontane (52.32%), palmitic acid (12.85%), oleic acid (8.93%), and 17-pentatriacontene (8.33%). Twenty components were identified, including 100% of the petroleum ether extract of *E. seguieriana* subsp. *seguieriana*. The main constituents of the fatty acid composition of the petroleum ether extract were identified to be 17-tetratriacontane (19.86%), hexatriacontane (18.78%), and palmitic acid (14.62%). Nineteen components were identified, including 100% of the petroleum ether extract of *E. fistulosa*. The main constituents were identified as tetracosanoic acid (39.60%), hexatriacontane (38.13%), and palmitic acid (18.72%).

Table 4. Identification and quantification of phenolic compounds of methanol extracts of *Euphorbia* species by LC-MS/MS[a].

Sample	1	2	3	4	5	6	7	8	9	10	11
ECMS	11500 ± 47.15	151 ± 2.60	N.D.	N.D.	14 ± 0.38	51 ± 1.43	N.D.	7.8 ± 0.33	N.D.	N.D.	N.D.
ECMR	6324 ± 25.93	N.D.	N.D.	N.D.	5.6 ± 0.15	N.D.	N.D.	47.8 ± 2.02	N.D.	N.D.	N.D.
ECMB	844 ± 3.46	N.D.	N.D.	N.D.	11.5 ± 0.31	20.48 ± 0.58	N.D.	67 ± 2.83	N.D.	N.D.	N.D.
ECML	13287 ± 54.48	28.98 ± 0.50	N.D.	N.D.	4.4 ± 0.12	65 ± 1.83	N.D.	32.9 ± 1.39	N.D.	N.D.	N.D.
ECMF	5999 ± 24.60	113 ± 1.94	N.D.	27.6 ± 0.36	N.D.	20.38 ± 0.57	N.D.	N.D.	N.D.	N.D.	N.D.
ECMM	12701 ± 52.07	60 ± 1.03	N.D.	N.D.	4.9 ± 0.13	49.09 ± 1.38	N.D.	20.3 ± 0.86	N.D.	N.D.	N.D.
EDKS	15348 ± 62.93	30.15 ± 0.52	607 ± 16.94	1407 ± 18.57	N.D.	74 ± 2.08	N.D.	N.D.	N.D.	N.D.	N.D.
EDKR	110 ± 0.45	N.D.	3.09 ± 0.09	N.D.	11.7 ± 0.32	50.87 ± 1.43	N.D.	136 ± 5.74	N.D.	N.D.	N.D.
EDKB	60 ± 0.25	N.D.	61 ± 1.70	207 ± 2.73	8.4 ± 0.23	31.51 ± 0.89	N.D.	283 ± 11.94	N.D.	N.D.	N.D.
EDKL	1410 ±	79 ±	2303 ±	3205 ±	21.3 ±	112 ±	N.D.	121 ±	N.D.	N.D.	N.D.
EDKF	14967 ± 61.36	42.3 ± 0.73	509 ± 14.20	1206 ± 15.92	7.6 ± 0.21	100 ± 2.81	N.D.	73 ± 3.08	N.D.	N.D.	N.D.
EDKM	935 ± 3.83	N.D.	224 ± 6.25	609 ± 8.04	8.8 ± 0.24	60 ± 1.69	N.D.	61 ± 2.57	N.D.	N.D.	N.D.
EADR	80 ± 0.33	N.D.	N.D.	N.D.	13.8 ± 0.37	37.07 ± 1.04	N.D.	682 ± 28.78	N.D.	N.D.	N.D.
EADB	50.2 ± 0.21	N.D.	N.D.	N.D.	5.7 ± 0.15	35.02 ± 0.98	N.D.	386 ± 16.29	N.D.	N.D.	N.D.
EADL	901 ± 3.69	N.D.	N.D.	N.D.	22.4 ± 0.60	126 ± 3.54	N.D.	117 ± 4.94	N.D.	N.D.	N.D.
EADM	N.D.	N.D.	N.D.	N.D.	9.6 ± 0.26	40.22 ± 1.13	N.D.	277 ± 11.69	N.D.	N.D.	N.D.
EEDR	323 ± 1.32	N.D.	N.D.	N.D.	28.7 ± 0.77	120 ± 3.37	N.D.	266 ± 11.23	N.D.	N.D.	N.D.
EEDB	582 ± 2.39	40.89 ± 0.70	N.D.	N.D.	35.7 ± 0.96	154 ± 4.33	N.D.	174 ± 7.34	N.D.	N.D.	N.D.
EEDF	1076 ± 4.41	30.01 ± 0.52	N.D.	N.D.	15.6 ± 0.42	64 ± 1.80	N.D.	37.9 ± 1.60	N.D.	N.D.	N.D.
EEDM	81 ± 0.33	N.D.	N.D.	N.D.	26.6 ± 0.72	27.46 ± 0.77	N.D.	74 ± 3.12	N.D.	N.D.	N.D.
EFDM1	968 ± 3.97	N.D.	241 ± 6.72	696 ± 9.19	22.9 ± 0.62	70 ± 1.97	N.D.	145 ± 6.12	N.D.	N.D.	N.D.
EGVS	825 ± 3.38	250 ± 4.30	256 ± 7.14	481 ± 6.35	8.7 ± 0.23	129 ± 3.62	N.D.	11.9 ± 0.50	N.D.	N.D.	N.D.
EGVR	N.D.	50.16 ± 0.86	N.D.	N.D.	6.3 ± 0.17	47.9 ± 1.35	N.D.	400 ± 16.88	N.D.	N.D.	N.D.
EGVB	60.09 ± 0.25	136 ± 2.34	64 ± 1.79	172 ± 2.27	8.3 ± 0.22	71 ± 2.00	N.D.	69 ± 2.91	N.D.	N.D.	N.D.
EGVL	8080 ± 33.13	120 ± 2.06	295 ± 8.23	503 ± 6.64	8.7 ± 0.23	49.22 ± 1.38	N.D.	8.9 ± 0.38	N.D.	N.D.	N.D.
EGVM	2908 ± 11.92	160 ± 2.75	167 ± 4.66	362 ± 4.78	8.1 ± 0.22	61 ± 1.71	N.D.	43.8 ± 1.85	N.D.	N.D.	N.D.
ESDS	11986 ± 49.14	33.43 ± 0.57	22.56 ± 0.63	58 ± 0.77	10.8 ± 0.29	34.93 ± 0.98	N.D.	64 ± 2.70	N.D.	N.D.	N.D.
ESDR	55 ± 0.23	N.D.	N.D.	39.02 ± 0.52	17.5 ± 0.47	50.36 ± 1.42	N.D.	495 ± 20.89	N.D.	N.D.	N.D.
ESDB	139 ± 0.57	26.03 ± 0.45	33.92 ± 0.95	91 ± 1.20	10.1 ± 0.27	30.88 ± 0.87	N.D.	181 ± 7.64	N.D.	N.D.	N.D.
ESDL	130 ± 0.53	N.D.	24.05 ± 0.67	66 ± 0.87	N.D.	29.29 ± 0.82	N.D.	29.4 ± 1.24	N.D.	N.D.	N.D.
ESDF	5674 ± 23.26	45.3 ± 0.78	37.15 ± 1.04	120 ± 1.58	13.7 ± 0.37	80 ± 2.25	N.D.	40.1 ± 1.69	N.D.	N.D.	N.D.
ESDM	705 ± 2.89	N.D.	56 ± 1.56	139 ± 1.83	14.3 ± 0.39	63 ± 1.77	N.D.	189 ± 7.98	N.D.	N.D.	N.D.
EFDR	1325 ± 5.43	26.59 ± 0.46	N.D.	N.D.	7.6 ± 0.21	29.4 ± 0.83	N.D.	47.7 ± 2.01	N.D.	1.19 ± 0.05	N.D.
EFDB	2037 ± 8.35	27.03 ± 0.46	N.D.	N.D.	14.5 ± 0.39	29.83 ± 0.84	N.D.	26.7 ± 1.13	N.D.	N.D.	N.D.
EFDL	13883 ± 56.92	235 ± 4.04	40.43 ± 1.13	55 ± 0.73	10.3 ± 0.28	29.02 ± 0.82	N.D.	22.2 ± 0.94	N.D.	N.D.	N.D.
EFDF	27074 ± 111.00	361 ± 6.21	N.D.	N.D.	14.3 ± 0.280.39	29.46 ± 0.83	N.D.	314 ± 13.25	N.D.	N.D.	N.D.
EFDM	8753 ± 35.89	168 ± 2.89	42.33 ± 1.18	45.02 ± 0.59	8.8 ± 0.24	43.78 ± 1.23	N.D.	97 ± 4.09	N.D.	N.D.	N.D.
EMMR	N.D	N.D	N.D	N.D	N.D.	N.D	N.D	48.6 ± 2.05	N.D	N.D	N.D
EMMB	N.D	27.13 ± 0.47	N.D	N.D	5.5 ± 0.15	N.D	N.D	37.9 ± 1.60	N.D	N.D	N.D

Table 4. Continued.

Sample	1	2	3	4	5	6	7	8	9	10	11
EMML	4460 ± 18.29	N.D	95 ± 2.65	214 ± 2.82	8.6 ± 0.23	33.7 ± 0.95	N.D	44.8 ± 1.89	N.D	N.D	N.D
EMMF	110 ± 0.45	160 ± 2.70	N.D.	N.D.	25.5 ± 0.69	205 ± 5.76	N.D	473 ± 19.96	N.D	N.D	N.D
EMMM	110 ± 0.45	99 ± 1.70	34.9 ± 0.97	123 ± 1.62	10.2 ± 0.28	35.65 ± 1.00	N.D	112 ± 4.73	N.D	N.D	N.D
EMDS	26467 ± 108.51	139 ± 2.39	1113 ± 31.05	1736 ±	28.6 ± 0.77	331 ± 9.30	N.D	45.1 ± 1.90	N.D	N.D	N.D
EMDR	N.D.	46.04 ± 0.79	N.D	34.37 ± 0.45	5.5 ± 0.15	43.83 ± 1.23	N.D	342 ± 14.43	N.D	N.D	N.D
EMDB	451 ± 1.85	27.07 ± 0.47	40.29 ± 1.12	97 ± 1.28	15.5 ± 0.42	117 ± 3.29	N.D	291 ± 12.28	N.D	N.D	N.D
EMDL	1801 ± 7.38	N.D	412 ± 1.49	887 ± 11.71	10.5 ± 0.28	63 ± 1.77	N.D	11.9 ± 0.50	N.D	N.D	N.D
EMDM	3830 ± 15.70	31.87 ± 0.55	400 ± 11.16	840 ± 11.09	6.7 ± 0.18	134 ± 3.77	N.D	39.4 ± 1.66	N.D	N.D	N.D
EMVS	1665 ± 6.83	N.D	N.D	N.D	13.8 ± 0.37	50.72 ± 1.43	N.D	121 ± 5.11	N.D	N.D	N.D
EMVR	29.04 ± 0.12	N.D	N.D	N.D	3.7 ± 0.10	N.D	N.D	444 ± 18.74	N.D	N.D	N.D
EMVB	71 ± 0.29	N.D	N.D	N.D	20.1 ± 0.54	63 ± 1.77	N.D	256 ± 10.80	N.D	N.D	N.D
EMVL	32.07 ± 0.13	39.2 ± 0.67	N.D	N.D	49.4 ± 1.33	275 ± 7.73	N.D	113 ± 4.77	N.D	N.D	N.D
EMVF	2700 ± 11.07	128 ± 2.20	N.D	N.D	15.9 ± 0.43	106 ± 2.98	N.D	154 ± 6.50	N.D	N.D	N.D
EMVM	58 ± 0.24	66 ± 1.14	N.D	N.D	24.4 ± 0.66	84 ± 2.36	N.D	183 ± 7.72	N.D	N.D	N.D
EMTS	N.D	N.D	N.D	N.D	N.D.	52 ± 1.46	N.D	12.3 ± 0.52	N.D	N.D	N.D
EMTR	N.D	N.D	N.D	N.D	3.3 ± 0.09	58 ± 1.63	N.D	338 ± 14.26	N.D	N.D	N.D
EMTB	20.5 ± 0.08	28.89 ± 0.50	N.D	N.D	4.9 ± 0.13	121 ± 3.40	N.D	86 ± 3.63	N.D	N.D	N.D
EMTL	985 ± 4.04	86 ± 1.48	N.D	N.D	N.D.	415 ± 11.66	N.D	30.4 ± 1.28	N.D	N.D	N.D
EMTF	30.2 ± 0.12	N.D	N.D	N.D	4.7 ± 0.13	90 ± 2.53	N.D	39.6 ± 1.67	N.D	N.D	N.D
EMTM	8735 ± 35.81	62 ± 1.07	N.D	N.D	4.2 ± 0.11	50.98 ± 1.43	N.D	59 ± 2.49	N.D	N.D	N.D

Compound identification: (1) quinic acid, (2) protocatechuic acid, (3) rutin, (4) hesperidin, (5) eugenol, (6) p-coumaric acid, (7) piceatannol, (8) scopoletin, (9) DL-kavain, (10) chrysophanic acid, and (11) resiniferatoxin.

Values provided as μg/g (w/w) of plant methanol extract.

N.D: not detected.

[a]μg analyte/g extract.

Table 5. Loading, eigenvalue, variance, and cumulative variance values of *Euphorbia* samples by principal component analysis.

Fatty acid	Principal component 1	Principal component 2	Principal component 3	Principal component 4
Palmitic acid	−0.304	0.247	**0.513**	−0.068
Linoleic acid	−0.211	−0.536	−0.275	0.235
Oleic acid	−0.278	−0.444	−0.258	−0.297
Stearic acid	−0.364	0.025	**0.397**	−0.342
17-Tetratriacontane	**0.406**	−0.070	0.191	0.359
Hexatriacontane	0.046	**0.512**	−0.424	−0.262
Lanosterol	0.270	−0.239	0.058	−0.671
β-Sitosterol	**0.451**	−0.042	0.177	0.002
17-Pentatriacontene	**0.443**	−0.050	−0.075	−0.295
Tetracontane	−0.119	**0.354**	−0.425	0.009
Eigenvalue	4.3303	1.8333	1.5310	0.9864
Variance (%)	43.3	18.3	15.3	0.099
Cumulative (%)	43.3	61.6	76.9	86.8

Larger number indicates a significant contribution of that fatty acids to the separation along the principal component (PC) axes.
The values in bold were the major contributors to each principal component.

Furthermore, the fatty acid components constituting 100% of *E. macroclada* samples collected from four regions were determined. The sample from Diyarbakır contained palmitic acid (39.48%), whereas linoleic acid (53.45%), oleic acid (15.82%), and 17-tetratriacontane (41.03%) were identified in samples from Van, Trabzon, and Malatya, respectively. Factors such as climate conditions and soil characteristics were significantly influential on the fatty acid profiles of *Euphorbia* species.

Ertaş et al. (2015) collected *E. macroclada* from Diyarbakır in 2013 and determined the palmitic acid concentration to be 33.3%. It was observed that the samples, which were collected at a time interval of approximately 2 years from the same region, contained similar fatty acid constituents (Ellison and Williams 2012). Considering these results, it could be concluded that factors other than climate and soil structure had no significant effect on the fatty acid profile of this species.

Generally, the fatty acid profile of the 11 studied *Euphorbi*a species was different from each other. Besides, it was observed that the saturated fatty acid amount of these species was much higher than their unsaturated fatty acid amount except for *E. craspedia*. To our knowledge, this is the first report on the fatty acid compositions of *E. aleppica*, *E. eriophora*, *E. grisophylla*, *E. seguieriana* subsp. *seguieriana*, *E. craspedia*, *E. denticulata, E. falcate*, and *E. fistulosa* species.

There are very few studies on the fatty acid contents of *Euphorbia* species. Carriere et al. (1992) reported linoleic acid to be the major fatty acid of *E. characias*. In another study, the main fatty acid component of *E. acanthothamnos* extracts was determined to be palmitic acid (Meletiou-Christou, Rhizopoulou, and Diamantoglou 1992). Consequently, when the results of the present and the previous studies in literature were examined together, it could be said that the saturated fatty acid content of *Euphorbia* species was higher than their unsaturated fatty acid content.

Elucidation of phytochemical profiles by LC-IT-TOF-MS

The *Euphorbiaceae* family represents 300 genera and 5,000 species in the world. These species are quite rich in terms of phenolics, aromatic esters, steroids, terpenoids,

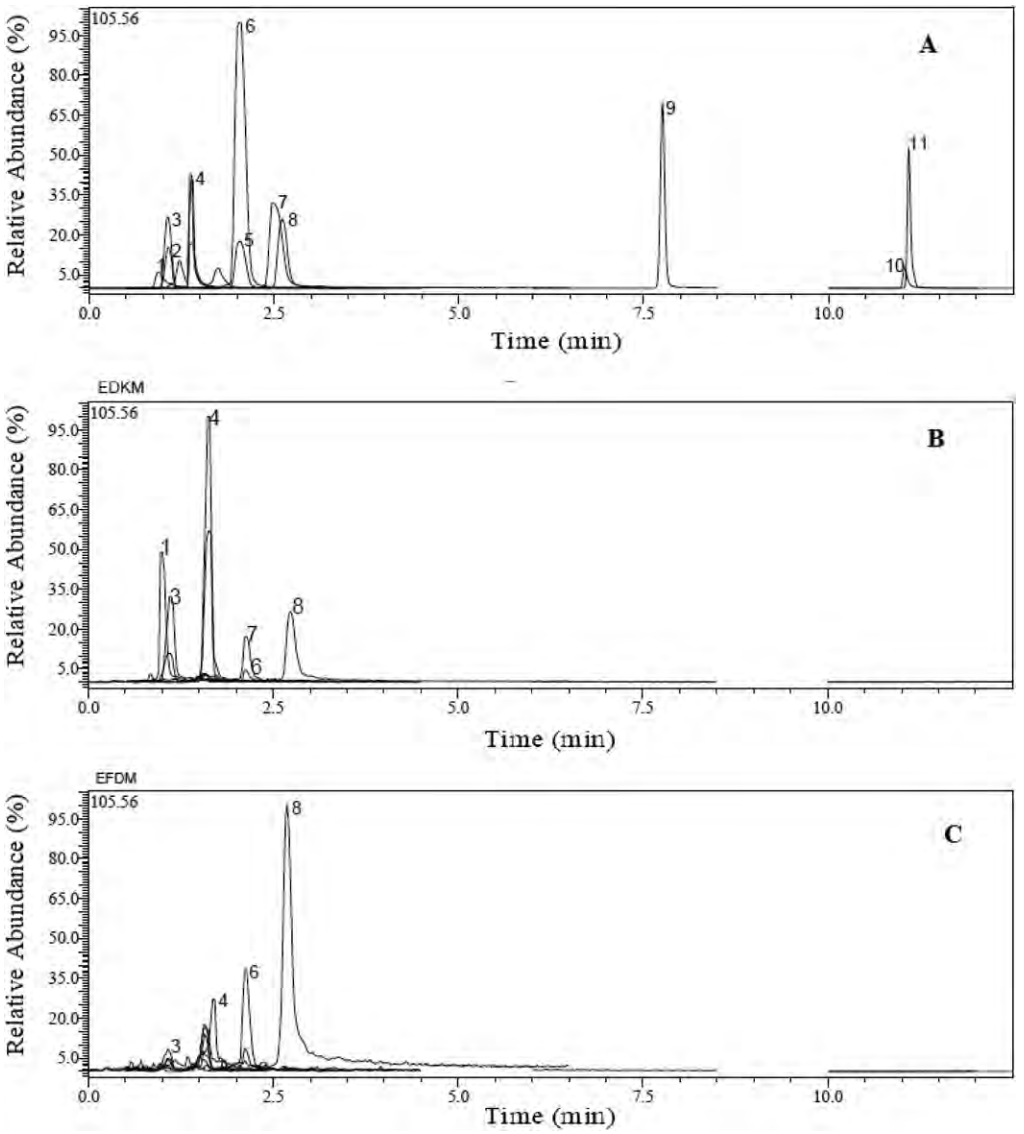

Figure 1. LC-MS/MS chromatogram of (A) 250 ppb standard mixture, (B) *E. denticulata* and (C) *E. fistulosa*. Peak identification: (1) quinic acid, (2) protocatechuic acid, (3) rutin, (4) hesperidin, (5) eugenol, (6) *p*-coumaric acid, (7) piceatannol, (8) scopoletin, (9) DL-kavain, (10) chrysophanic acid, and (11) resiniferatoxin. LC-MS/MS conditions: C18 reversed-phase Inertsil ODS-4 (150 mm ×4.6 mm, 3 µm) analytical column using (A) aqueous 5 mM ammonium formate with 0.1% formic acid and (B) 5 mM ammonium formate with 0.1% formic acid in methanol as the mobile phase. Gradient program: time (min), B %: (0, 35), (5, 50), (10, 90), (12, 90), (13, 35). The flow rate was 0.5 mL/min at a column temperature of 40 °C. ESI conditions: interface temperature; 350 °C, desolvation line temperature; 250 °C, heat block temperature; 400 °C, nebulizing gas flow (nitrogen); 3 L/min and drying gas flow (nitrogen), 15 L/min.

essential oils, and other bioactive constituents. As it is well-known, the isolation of chemicals responsible for biological activities from natural products involves a series of very challenging steps.

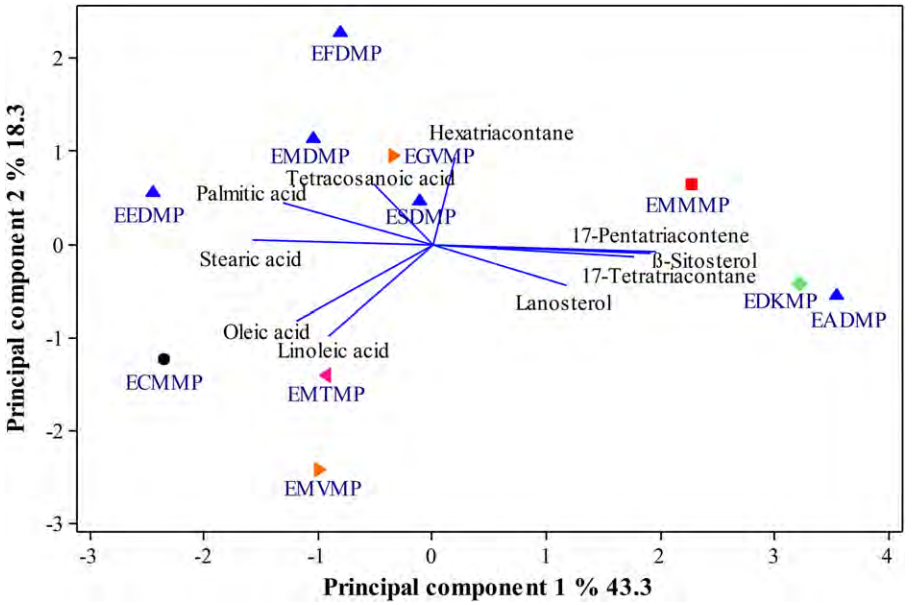

Figure 2. Biplot for principal components 1 and 2 in the *Euphorbia* samples: ▲Diyarbakır, ■ Malatya, ◆ Kayseri, ● Mardin, ▶ Van, and ◀Trabzon. The sample codes are defined in Table 1.

The screening of plant chemicals (terpenes, alkaloids, anthocyanins, saponins, and so on) before isolation studies is of great importance for the efficient production of biologically active compounds. Therefore, LC-MS-IT-TOF results are very important. However, screening of the studied species with the time-of-flight mass spectrometry is insufficient, and for proper identification, isolation and NMR studies are necessary for exact structural identification of the major compounds in the future. Nevertheless, prescreening studies on some species of a family with so many species and chemical variability sheds light on isolation studies of these species.

The methanol extracts of the *Euphorbia* species were examined by an optimized LC-IT-TOF-MS method (75 min), and their phytochemical profiles were determined. The analyte peaks having intensities higher than 2,500,000 were evaluated. A total of 268 secondary metabolites belonging to different classes were identified in the analyzed species. Retention times, forms of detected ions, measured and expected *m/z* values (with errors), and exact masses of the tentatively identified phytochemicals are given in Table S5. The LC-IT-TOF-MS chromatograms are shown in Figure S4.

LC-MS/MS method development – validation and quantification results

Euphorbia species from around the world have been considered to select the compounds for method validation studies. Therefore, the developed method has the distinction of being a valid method, not only for *Euphorbia* species in Turkey, but also *Euphorbia* species worldwide.

A new LC-MS/MS method was developed to quantify 11 phytochemicals (quinic acid, protocatechuic acid, rutin, hesperidin, eugenol, *p*-coumaric acid, piceatannol, scopoletin, DL-kavain, chrysophanic acid, and resiniferatoxin) of the analyzed *Euphorbia* species (Table 4

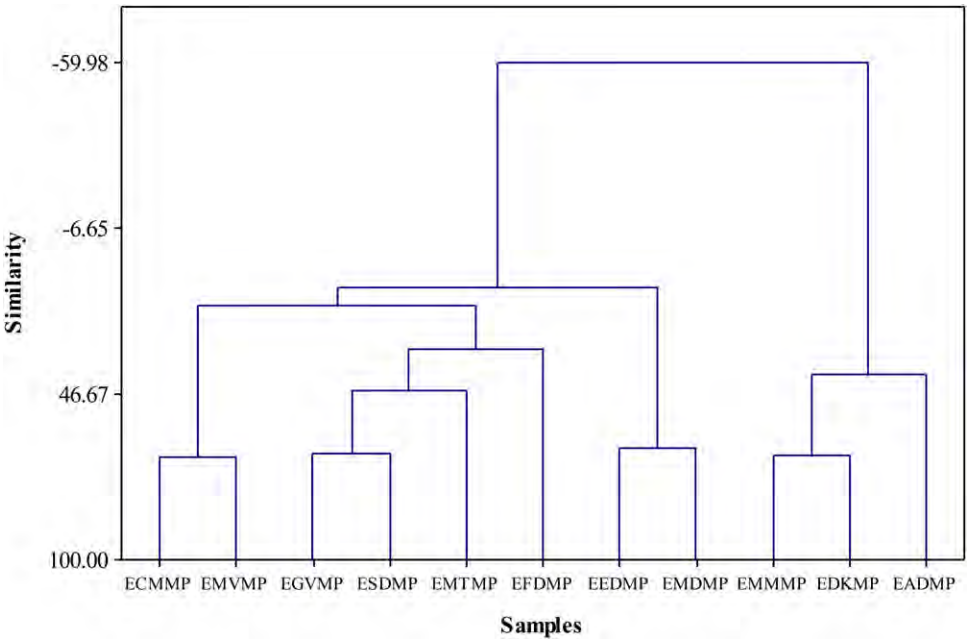

Figure 3. Dendrogram results obtained by the Euclidean distance and Ward linkage methods. The sample codes are defined in Table 1.

and Figure 1, Figure S2, and Figure S3). The linearity was determined by six-point calibration curves with 6 replicates. The R^2 values were higher than 0.99 (Table 2). The limits of detection and quantification of the compounds were in the range of 0.34–20.88 µg/L and 1.14–69.61 µg/L, respectively (Table 2). Retention times, linear equations, linear ranges, coefficients of determination, limits of detection, and quantification are given in Table 2.

The precision was determined to be the repeatability and the intermediate precision concerning relative standard deviation (Table 2). All of the relative standard deviation values were lower than 0.03%. The accuracy of the developed method was evaluated using the recovery. The recovery values of the spiked samples (25.00, 100.00, and 500.00 µg/L) were between 0.9956–1.0102% (intraday) and 0.9973–1.0078% (interday). The uncertainty range was 0.0041–0.0422.

Quinic acid, hesperidin, eugenol, *p*-coumaric acid, and scopoletin were detected in most of the samples, whereas DL-kavain, chrysophanic acid, and resiniferatoxin were not detected above the limit of quantification values in any (Table 4). The studied species were generally rich in quinic acid, and the EFDF extract of *E. fistulosa* species had the highest concentration (27074 µg/g extract). Protocatechuic acid was determined in all species, except for *E. Aleppica*. The EFDF extract was found to be the richest in terms of protocatechuic acid (361 µg/g extract).

The rutin content of EDKL extract of *E. denticulata* species was particularly high (2,303 µg/g extract). For the *E. macroclada* samples, the EMDS extract was the richest in terms of phenolic constituents (1113 µg/g extract) of the samples collected from Diyarbakır. The EDKL (3,205 µg/g extract) and EMDS (1736 µg/g extract) contained the highest concentration of hesperidin. Hesperidin was not detected in *E. macroclada* samples collected from Van and Trabzon, like rutin.

Eugenol was detected in all samples in low concentrations. *p*-Coumaric acid was also determined in all samples The plant tissue with the highest concentration (415 µg/g extract) in the EMTL extract of *E. macroclada* was collected from Trabzon. Similar to *p*-coumaric acid, scopoletin was also found in all samples and all plant tissues. The EADR extract of *E. denticulata* had the highest scopoletin content (682 µg/g extract).

There are few studies on phenolic and flavonoid compounds of *Euphorbia* species determined by high-performance liquid chromatography (HPLC) and GC-MS. Pintus et al. (2013) characterized different phenolic compounds like quercetin, *p*-coumaric acid, and caffeic acid in the *E. characias* latex qualitatively by GC-MS. Liu et al. (2011) developed a quantitative method for quercetin in *E. helioscopia* L. and reported the content as 1.42 mg/g dry weight (Liu et al. 2011). Moreover, Jahan, Khalil-Ur-Rahman, and Asi (2013) quantified phenolic compounds such as chlorogenic, *p*-coumaric, caffeic, and ferulic acids and quercetin in *E. tirucalli* by reversed-phase HPLC (Jahan et al. 2013). Flavonoids were present in plants as their glycosides. Parallel to this study, being a glycoside of quercetin, rutin was rich in the EDKL extract.

Chemometric analysis results

The PCA and HCA of 10 fatty acids (common in studied *Euphorbia* samples) were carried out and the results are shown in Table S6. The PCA results of *Euphorbia* samples collected from Mardin, Malatya, Kayseri, Diyarbakır, Van, and Trabzon are given in Table 5. According to the results obtained from the determination of the 10 fatty acids, the first three principal components explained 76.9% of the variation, with the first two contributing 61.6%. The results with bold character were more useful to define the principal components than the others in Table 5. Considering the data set, 17-tetratriacontane, *β*-sitosterol, and 17-pentatriacontene were determined to be the dominant fatty acids with high positive loadings on the first principal component. Hexatriacontane and tetracosanoic acid had a high score on the second principal component, whereas palmitic acid and stearic acid were dominant on the third principal component.

The scores of the first three principal components of *Euphorbia* species collected from various regions are given in Table S7. In extracts of EMMMP, EDKMP, and EADMP, 17-tetratriacontane, *β*-sitosterol, and 17-pentatriacontene, which explained the first principal component, were present at higher concentrations. Hexatriacontane and tetracosanoic acid were the dominant fatty acids in EFDMP extract, defining the second principal component. In EEDMP extract, palmitic acid and stearic acid (the third principal component) were determined at the highest concentrations.

The biplot of the first and the second principal components of *Euphorbia* samples is provided in Figure 2. The distribution of the samples according to the fatty acids present and the regions are defined in different colors in Figures S5–S7. Three groups were formed as the result of the PCA analysis. The first group involved all of the Diyarbakır samples except for the EADMP sample. The EGVMP sample from Van was also included in this group.

Palmitic and stearic acids were at higher concentrations in the EEDMP sample, whereas EMDMP and ESDMP samples contained higher tetracosanoic acid and hexatriacontane contents. The Mardin ECMMP sample was rich in oleic acid and linoleic acid. Trabzon EMTMP and Van EMVMP samples were in the second group, whereas

Malatya EMMMP, Diyarbakır EADMP, and Kayseri EDKMP samples were present in the third group. The samples of the third group were differentiated from the others in terms of 17-tetratriacontane, β-sitosterol, 17-pentatriacontene, and lanosterol.

HCA was applied to the results obtained from the determination of the 10 fatty acids in the *Euphorbia* species. The measurements were dependent on the squared Euclidean distance. Ward's method was used as the classification method. The dendrogram obtained using Ward's method is given in Figure 3. Three groups formed as the result of HCA where the first group included the EMMMP, EDKMP, and EADMP samples, and the second group contained EEDMP and EMDMP. The third group, however, contained the EFDMP, EMTMP, ESDMP, EGVMP, EMVMP, and ECMMP samples.

Conclusions

To the best of our knowledge, the present study may be the first report on the chemical profiles of methanol extracts of *Euphorbia* species collected from different regions of Turkey. The chemical compositions of petroleum ether extracts of nine *Euphorbia* species were determined by GC-MS. The chemometric analyses of the results were carried out by PCA and HCA. The methanol extracts of the species were scanned by LC-IT-TOF-MS, and molecular formulas of 268 compounds were tentatively identified by exact mass and molecular formulas prediction. Further studies are needed for structural elucidation of the identified compounds. Also, an LC-MS/MS method with high accuracy and precision was developed and validated for the quantification of 11 compounds present in the studied species.

The PCA results allowed the studied samples to be evaluated in terms of region and species. Especially, EFDMP, EDMP, ESDMP, EEDMP samples (except EADMP) collected from Diyarbakır were similar in terms of fatty acid contents (palmitic acid, tetracosanoic acid, stearic acid, hexatriacontane) and grouped. Herein different species grouped. It means that the regional characteristics affected the fatty acid contents. The aspects of this group were also seen in the EGVMP sample collected from Van.

The different species gathered from Diyarbakır had similar features indicated the importance of the regional effects. On the other hand, the EMVMP and EGVMP samples collected from Van were included in different groups. Another evaluation was made by comparing four *E. macroclada* collected from different regions. The EMTMP (Trabzon), EMVMP (Van), EMMMP (Malatya), and EMDMP (Diyarbakir) samples belong to this species exhibited different characteristics. Herein, it may be concluded that the local properties affected the fatty acid content of the samples. The samples belonging to the same species were differentiated due to environmental factors. The HCA showed that the EEDMP and EMDMP samples collected from Diyarbakır were the most similar.

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