Chemical constituent and radical scavenging antioxidant activity of *Anthemis kotschyana* Boiss.

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Abstract

Phenolic content and antioxidant activity of *Anthemis kotschyana* Boiss. var. *discoidea* (*A. kotschyana*) were reported in this study. The ethanol extract of *Anthemis kotschyana* (EEA) and the water extract of *Anthemis kotschyana* (WEA) were prepared and used for biochemical analyses. Radical scavenging antioxidant capacities of EEA and WEA were evaluated by DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging method. Another goal of the study was to evaluate the phenolic compositions of *A. kotschyana* by liquid chromatography and tandem mass spectrometry (LC-MS/MS). Rhamnetin (5.484±0.020 ppm; µg/g extract) and quinic acid (2.251±0.012 ppm; µg/g extract) were identified as major two compounds in the plant sample. This study will be a scientific base for further studies about *A. kotschyana* for plant biochemistry and plant-based pharmacological industry.

Keywords: *Anthemis kotschyana*; antioxidant activity; phenolic content; LC-MS/MS

Experimental

Chemicals

BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), DPPH (1,1-diphenyl-2-picryl-hydrazyl), neocuproine (2,9-dimethyl-1,10-phenanthroline), α-tocopherol, and trolox were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). The standard phenolic compounds of LC-MS/MS; Apigenin (95%), caffeic acid (98%), chlorogenic acid (95%), fumaric acid (99%), kaempferol (96%), pyrogallol (98%), rosmarinic acid (96%), rutin (94%), syringic acid (95%), t-ferulic acid (99%), quercitrin (97%), salicylic acid (99%), p-coumaric acid (98%), and pyrogallol (98%) were obtained from Sigma-Aldrich. Rhamnetin (98%) from ExtraSynthese, gallic acid (99%,) from Merck, and luteolin (99%) from AppliChem were used. Methanol obtained from Merck and all other reagents were purchased from Sigma-Aldrich or Fluka.

Identification and collection of the plant material

The aerial parts of samples were collected from their natural habitats. *Anthemis kotschyana* var. *discoidea*, was collected from Bingol, a Southeast city of Turkey, on July 2013 by Dr. Ömer Kılıç. The habitats were on the road edges at 1350-1400 m altitude. The taxonomic description of the plant sample was made according to the "The Flora of Turkey and East Aegean Islands, Volume 5 (Davis 1975) by taxonomist Ö. Kılıç from Adiyaman University. The voucher specimen was deposited in the Bingöl University, Department of Park and Garden Plant Herbarium with 5232 herbarium number.

Preparation of water and ethanol extracts for antioxidant methods

The ethanol and water extractions of *A. kotschyana* were carried out according to a previous study (Bingol and Bursal 2018). The leaves of *A. kotschyana* were dried at room condition (the room temperature was 25 ^{o}C). For the preparation of WEA and EEA, 20 g of leaves were powdered and mixed with 200 mL distilled water or ethanol (1/10:w/v). The mixtures were homogenized by a magnetic mixer about 12 h, at room conditions. The homogeneous mixtures were filtered with filter papers. The filtrate sample from water solvent was lyophilized in a lyophilizator (Labconco, Freezone 1L) at 5 mm Hg at -50 °C for preparing water extract (WEA). The filtrate sample from ethanol solvent was evaporated with

a rotary evaporator at 40 °C (Heidolph 94200, Bioblock Scientific) for preparing ethanol extract (EEA). The lyophilized and evaporated samples were stored at -30 °C until used.

Determination of phenolic compounds by LC-MS/MS analysis

The phenolic compounds of *A. kotschyana* were determined by LC-MS/MS technique. The compounds were quantified by comparison to twenty-seven different organic compounds. Initially, the extraction for LC-MS/MS system was prepared. For this aim, the aerial parts of the plant were air-dried and powdered. The powder (100 g) was extracted three times with 300 mL of ethanol for 24 h, at room conditions. The solvent was evaporated by using vacuum at 30 °C with a rotovap. Dry filtrate sample was diluted to 1.0 mg/mL and filtrated with 0.2 µm microfiber filter before LC-MS/MS assay.

LC−MS/MS measurements were carried out with a 8040 model LC-MS triple quadrupole mass spectrometer (Shimadzu) integrated with an ESI source. ESI was used for detection of positive and negative ionization. The LC device was integrated with binary pumps (LC-30AD), degasser (DGU-20A3R), column oven (CTO-10ASvp) and auto sampler (SIL-30AC). A reversed-phase C18 analytical column (Inertsil ODS-49) was used for chromatographic identification of compounds. A sample volume of 4 µL was injected during analysis. HPLC was runned at 0.5 mL/min flow rate. The solvent A was water containing ammonium formate (5 mM) with formic acid (0.1%) and the solvent B was methanol containing ammonium formate (5 mM) with formic acid (0.1%). The following flow gradient was used for solvent B; 40% for 0-20 min, 90% for 20-24 min, and 40% for 24-30 min. The analyses of samples were carried out after two or three transitions for per sample. First transition was for quantitative aim and the other transition was made up for verification. Electrospray ionization (ESI) circumstances were set at 300 °C, 3 L/min nebulizing gas flow and 15 L/min drying gas flow. The temperature was adjusted at 40 °C for column (Aras et al. 2017, Ertaş et al. 2014).

DPPH radical scavenging method

The DPPH$^{\bullet}$ free radical scavenging antioxidant effects of EEA and WEA were determined according to the previously described study (Gülçin et al. 2019). DPPH free radical molecules shows maximum absorbance at 517 nm. Thus, antioxidant molecules can reduce the absorbance. For this aim, DPPH radicalic solution in ethanol (0.5 mL, 0.1 mM) was transferred to the different concentrations (10-30 µg/mL) of the sample solutions in ethanol (1.5 mL) and incubated in dark for 30 min. Finally, the absorbance were recorded at 517 nm.

The analyses were achieved in triplicate. A declining absorbance indicates radical scavenging potential. The radical scavenging percentages of the sample and standards were measured from the following equation.

$$Radical\ scavenging\ (\%) = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) x\ 100$$

Statistical analysis

The experimental results were performed in triplicate. The data were assessed using the Microsoft Office Excel program. In our study, the values are presented as a mean ± standard deviation.

Supplementary References

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Table S1. LC-MS/MS parameters and amounts of standard compounds and *A. kotschyana*

No	Analytes	[a]RT	[b]Parent ion (m/z)	Ionization Mode	[c]R^2	[d]RSD%	Linearity Range (µg/L)	[e]LOD/ LOQ (µg/L)	Recovery (%)	[f]U	[g]Amount
1	Quinic acid	3.32	190.95	Neg	0.9927	0.0388	250-10000	22.3 / 74.5	103.3	4.8	2.251±0.012
2	Malic acid	3.54	133.05	Neg	0.9975	0.1214	250-10000	19.2 / 64.1	101.4	5.3	0.497±0.005
3	tr-Aconitic acid	4.13	172.85	Neg	0.9933	0.3908	250-10000	15.6 / 51.9	102.8	4.9	
4	Gallic acid	4.29	169.05	Neg	0.9901	0.4734	25-1000	4.8 / 15.9	102.3	5.1	
5	Chlorogenic acid	5.43	353	Neg	0.9932	0.1882	250-10000	7.3 / 24.3	99.7	4.9	0.314±0.009
6	Protocatechuic acid	5.63	152.95	Neg	0.9991	0.5958	100-4000	25.8 / 85.9	100.2	5.1	0.131±0.006
7	Tannic acid	6.46	182.95	Neg	0.9955	0.9075	100-4000	10.2 / 34.2	97.8	5.1	
8	tr- caffeic acid	7.37	178.95	Neg	0.9942	1.0080	25-1000	4.4 / 14.7	98.6	5.2	0.117±0.002
9	Vanillin	8.77	151.05	Neg	0.9995	0.4094	250-10000	10.1 / 33.7	99.2	4.9	0.037±0.002
10	p-Coumaric acid	9.53	162.95	Neg	0.9909	1.1358	100-4000	15.2 / 50.8	98.4	5.1	0.112±0.006
11	Rosmarinic acid	9.57	358.9	Neg	0.9992	0.5220	250-10000	10.4 / 34.8	101.7	4.9	
12	Rutin	10.18	609.1	Neg	0.9971	0.8146	250-10000	17.0 / 56.6	102.2	5.0	
13	Hesperidin	9.69	611.1	Poz	0.9973	0.1363	250-10000	21.6 / 71.9	100.2	4.9	
14	Hyperoside	10.43	463.1	Neg	0.9549	0.2135	100-4000	12.4 / 41.4	98.5	4.9	
15	4-OH Benzoic acid	11.72	136.95	Neg	0.9925	1.4013	25-1000	3.0 / 10.0	106.2	5.2	0.057±0.001
16	Salicylic acid	11.72	136.95	Neg	0.9904	0.6619	25-1000	4 / 13.3	106.2	5.0	0.051±0.001
17	Myricetin	11.94	317	Neg	0.9991	2.8247	100-4000	9.9 / 32.9	106.0	5.9	
18	Fisetin	12.61	284.95	Neg	0.9988	2.4262	100-4000	10.7 / 35.6	96.9	5.5	
19	Coumarin	12.52	146.95	Poz	0.9924	0.4203	100-4000	9.1 / 30.4	104.4	4.9	
20	Quercetin	14.48	300.9	Neg	0.9995	4.3149	25-1000	2.0 / 6.8	98.9	7.1	0.230±0.003
21	Naringenin	14.66	270.95	Neg	0.9956	2.0200	25-1000	2.6 / 8.8	97.0	5.5	0.004±0.001
22	Hesperetin	15.29	300.95	Neg	0.9961	1.0164	25-1000	3.3/ 11.0	102.4	5.3	0.104±0.002
23	Luteolin	15.43	284.95	Neg	0.9992	3.9487	25-1000	5.8 / 19.4	105.4	6.9	0.039±0.001
24	Kaempferol	15.43	284.95	Neg	0.9917	0.5885	25-1000	2.0 / 6.6	99.1	5.2	0.168±0.003
25	Apigenin	17.31	268.95	Neg	0.9954	0.6782	25-1000	0.1 / 0.3	98.9	5.3	0.008±0.002
26	Rhamnetin	18.94	314.95	Neg	0.9994	2.5678	25-1000	0.2 / 0.7	100.8	6.1	5.484±0.020
27	Chrysin	21.18	253	Neg	0.9965	1.5530	25-1000	0.05 / 0.17	102.2	5.3	

[a] RT: Retention time
[b] Parent ion (*m/z):* Molecular ions of the standard compounds (mass to charge ratio)
[c] R^2: coefficient of determination
[d] RSD: relative standard deviation
[e] LOD/LOQ (µg/L): Limit of detection/Limit of quantification
[f] U (%): Percent relative uncertainty at 95% confidence level (k=2).
[g] Amount: Quantitative phenolic acid composition of *A. kotschyana* (ppm; µg analyte/g extract)

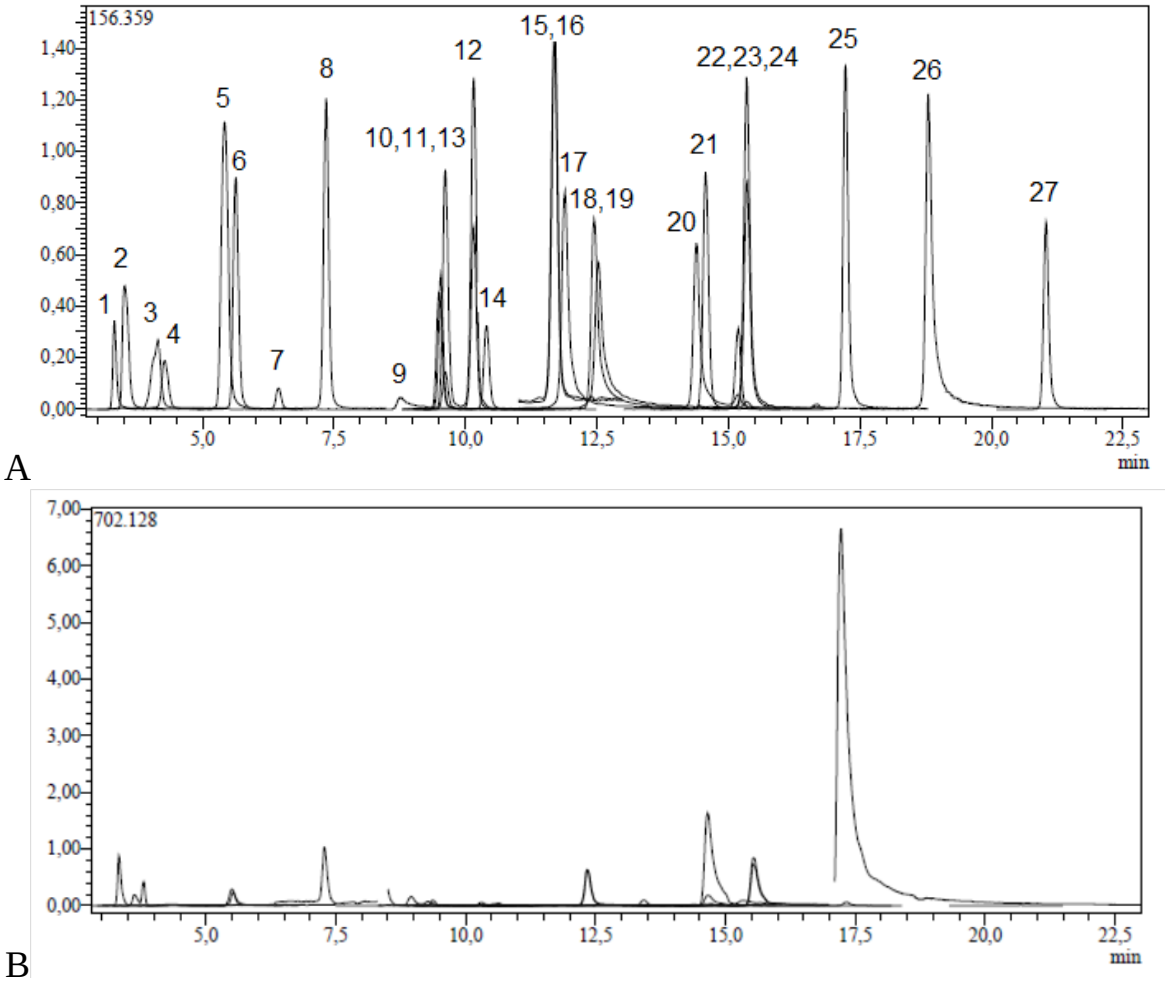

Figure S1. UHPLC-ESI-MS/MS chromatograms of standard compounds (**A**) and *A. kotschyana* (**B**)

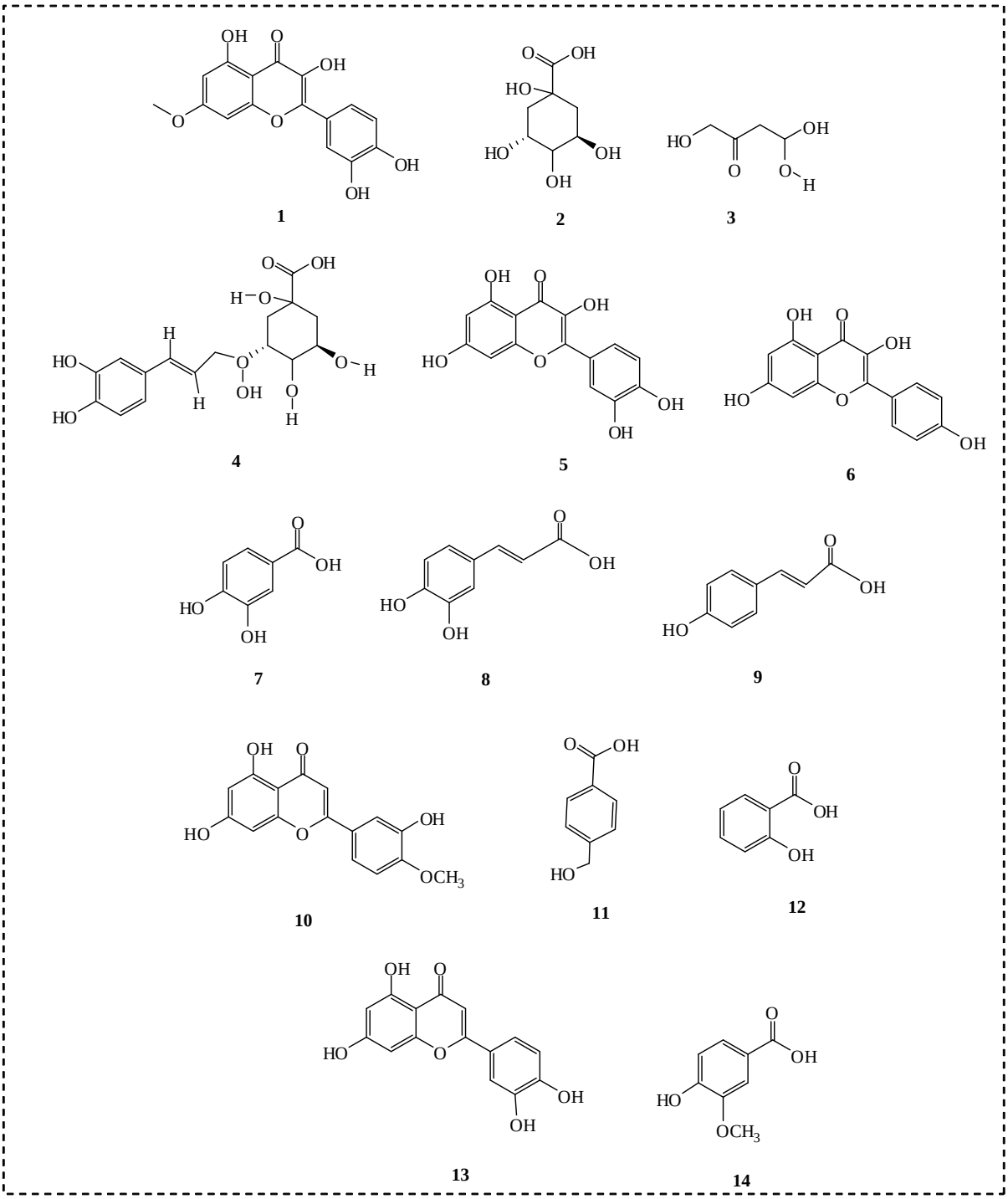

Figure S2. Chemical structures of compounds identified from *A. kotschyana*