

Chemical fingerprints and bioactivities of 12 Anatolian *Achillea L.* species by LC-MS/MS with chemometric approach: novel phytonutrients, natural food preservatives and chlorogenic acid sources

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Abstract: The objective of this study was to evaluate the biological activities and chemical fingerprint profiles of the extracts obtained from twelve *Achillea L.* species (*A. lycaonica*, *A. biebersteinii* (syn: *A. arabica*), *A. kotschy* subsp. *kotschy*, *A. schischkinii*, *A. millefolium* subsp. *millefolium*, *A. sintenisii*, *A. setacea*, *A. teretifolia*, *A. wilhelmsii* subsp. *wilhelmsii* (syn: *A. santolinoidea* subsp. *wilhelmsii*), *A. nobilis*, *A. goniocephala*, *A. spinulifolia*). The antioxidant, enzyme inhibitory and cytotoxic effects were evaluated to investigate their bioactivity profiles. Furthermore, the total flavonoid and phenolic contents were determined and LC-MS/MS analysis was performed to reveal the phytochemical profile of the investigated extracts. *A. kotschy* and *A. nobilis* species were detected to have very high antioxidant potential as well as high total phenolic content (260.00 ± 3.38 and 282.97 ± 3.14 µg of PEs mg extract⁻¹, respectively). According to the LC-MS/MS results, *A. kotschy* and *A. nobilis* species were found to contain very high concentrations of chlorogenic acid (55812.20 and 46407 µg analyte g extract⁻¹). Besides, the bioactivities and phenolic composition of these species were chemometrically analyzed using principal component analysis (PCA) and hierarchical clustering analysis (HCA) techniques. It has also been determined that *Achillea* species generally exhibit quite high cytotoxic activity against the HeLa cell line. The studied species showed high urease enzyme activities.

Key words: *Achillea*, antioxidant, cytotoxicity, urease and tyrosinase, LC-MS/MS, chemometric analysis

1. Introduction

Being found in the Anthemideae tribus of the Asteraceae (Compositae) family, *Achillea L.* genus is known to contain 140 species which spread naturally in some parts of the world, mostly in Eurasia, North America, and North Africa (Bremer and Humphries, 1993; Guo et al., 2004; Arabacı, 2006). *Achillea* genus grown in Turkey, gathered under 6 sections (*Ptarmica*, *Anthemideae*, *Arthrolepis*, *Babounya*, *Santolinoidea*, *Achillea*), is represented by a total of 52 taxa, 46 of which are species. There are 21 endemic species (28 taxa) belonging to this genus (Davis, 1975).

In Anatolia, different local names of *Achillea* species are known. Beside the fact that *Achillea* is commonly known as “yarrow” among the people, many other names

in Turkish could be listed as follows: akbaşlı, civanperçemi beyazı, çetuğçe, yılan çiçeği, yavşan otu, mayasıl otu, kurpotu, sarı civanperçemi (Baytop, 1999; Fırat, 2013).

Achillea L. is a genus widely distributed around the world and its species have been utilized since ancient times. It has been revealed from the phytochemical studies carried on *Achillea* species that quite a few bioactive compounds have been obtained from this genus. Most of the *Achillea* species not only have therapeutic applications but also have economic importance in Anatolia (Aytac et al., 2016). Besides, several *Achillea* species have been ethnopharmacologically prescribed due to their use as folk medicine for variety of purposes. Some herbal teas prepared from *Achillea* species have been reported to be

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commonly used for menstrual regulation, abdominal pain, diuretic, stomach gas, rheumatism, hepatitis, diarrhea, and wound healing purposes. Additionally, various extracts of many *Achillea* species are utilized for their analgesic, antiinflammatory, antimicrobial, spasmolytic, human erythrocyte and leukocyte protective, antioxidant, digestive, collagogenic, and hemostatic effects (Akkol et al., 2009; Polat et al., 2012; Ceylan et al., 2016; Yener et al., 2020a; Yilmaz, 2020). Innumerable scientific studies based on the phytochemical profiling of *Achillea* species revealed that they are rich in terms of diterpenes, sesquiterpene lactones, triterpenes, phenolic acids, flavonoids, volatile oils, and lignans as well as some other groups of molecules such as alkanes, fatty acids, amino acids, and inulin (Verma et al., 2017; Hichri et al., 2018; Yener et al., 2020b).

In determining the phytochemical constituents of medicinal plant species chromatography with suitable detectors as well as proper extraction techniques are quite effective (Ersoy et al., 2019). H-1 qNMR, HPLC-UV, HPLC-DAD, CE-UV, GC-MS, LC-MS/MS, LC-Q-TOF-MS, LCMS-IT-TOF are some of the analytical techniques that have been used in screening bioactive phytochemicals in medicinal and aromatic plants so far (Hamad et al., 2016; Çölgeçen et al., 2018; Yener et al., 2018; Yilmaz et al., 2018; Zengin et al., 2018; Bakır et al., 2020; Zourgui et al., 2020; Aşkun et al., 2021; Khiari et al., 2021; Rifna and Dwivedi, 2021; Takim et al., 2021). Being one of the most sophisticated techniques in investigating the phytochemical profile of the plant extracts, liquid chromatography tandem mass spectrometry has been widely utilized among the scientists owing to its high sensitivity, selectivity, and signal to noise ratio.

The therapeutic efficacy of aromatic and medicinal plants is mostly associated with the presence of various secondary metabolites found in these plants. Because of their numerous pharmacological and biological properties associated with their antioxidant capacity, flavonoid and phenolic acids found in plant species are considered to be the most promising secondary metabolite compounds (Boğa et al., 2016). By examining phenolic compounds in food and medicinal plants, it has been proven that these compounds have beneficial properties in terms of health and diet. Being bioactive compounds, phenolic acid and flavonoids are powerful scavengers of reactive oxygen species (ROS) that cause many human ailments. Moreover, antioxidants of natural origin are highly preferred over synthetic antioxidants such as BHT (Butylated hydroxytoluene) and BHA (Butylated hydroxyanisole) (Zengin et al., 2017b). Nonetheless, phenolic compounds obtained from plants have not been fully evaluated due to their complex chemical nature. With their ancient utilization in medicine and food, phenolic compounds are also known to modulate transcriptional regulation,

membrane permeability, and signal transduction (Güzel et al., 2021).

Taking into account that *Achillea* species are rich in biological activities and valuable phytochemicals, it was aimed to carry out comprehensive chemical and biological screening of 12 *Achillea* species, which are medicinally important and some species are consumed as tea. The ethanol extracts of the root and aerial parts of these 12 species have been prepared. In order to reveal the antioxidant potential of the ethanol extracts, DPPH free radical scavenging, β -carotene-linoleic acid, cupric reducing antioxidant capacity (CUPRAC), and ABTS cation radical scavenging methods were used in addition to total phenolic and flavonoid content analysis. Subsequently, their cholinesterase, urease and tyrosinase enzyme inhibitory activities were identified. Moreover, extracts were examined for their cytotoxic effects on HeLa cells. In addition to these biological parameters, the chemical content of all extracts was qualitatively and quantitatively screened by a comprehensive LC-MS/MS method (Yilmaz et al., 2018). Biological and chemical analysis results were also analyzed by chemometric methods such as hierarchical cluster analysis (HCA) and principal component analysis (PCA).

2. Materials and methods

2.1. Supporting information file

The chemometric evaluation of antioxidant activities and phenolic composition of the samples (S1), abbreviations used in the manuscript (S2), methods of the biological activity studies including total flavonoid and phenolic contents (S3) (Slinkard and Singleton, 1977; Moreno et al., 2000), antioxidant activities by CUPRAC, ABTS, and DPPH methods (S4) (Miller, 1971; Blois, 1988; Re et al., 1999; Apak et al., 2004; Ertas et al., 2015), cytotoxic activities against HeLa cell lines (S5) (Urcan et al., 2010; Işık et al., 2012), cholinesterase (S6) (Ellman et al., 1961), urease (S7) (Zahid et al., 2015), tyrosinase (S8) (Hearing and Jiménez, 1987; Khan et al., 2006) inhibitory activities of the studied samples and the LC-MS/MS TIC chromatograms of the extracts except from ABiAP and AbiR (Figure S1) were given in the supporting information file.

2.2. Supplement of plant material and preparation of the extracts for LC-MS/MS and biological activities

Voucher specimens have been stored in the Herbarium of Hacettepe University Faculty of Pharmacy (HUEF) and Herbarium of Hacettepe University Faculty of Education (HEF) (Table 1). The collected species were separated into root and aerial parts and air-dried in shade. Then, plant materials were powdered and macerated three times with ethyl alcohol (each for 8 h) at room temperature. Afterwards, dry extracts were obtained by evaporating the solvent at 35 °C using a rotary evaporator. Sample concentrations with 1000 mg L⁻¹ were prepared from dry

Table 1. Information on studied *Achillea* species.

Plant name	Abbreviations	Collection place	Collection time	Herbarium number
<i>A. biebersteinii</i> Afan. (syn: <i>A. arabica</i> Kotschy)	ABiAP and ABiR	A4 Ankara: 39°52'40"N, 32°43'49"E. 992 m	June 2014	HUEF 14053
<i>A. kotschyi</i> Boiss. subsp. <i>kotschyi</i> Boiss.	AKoAP and AKoR	B6 Yozgat: 39°31' 14"N, 36°01'20"E. 2000 m	July 2014	HUEF 14044
<i>A. lycanica</i> Boiss. & Heldr.	ALyAP and ALyR	B6 Sivas: 39°32'44"N, 37°02'11 "E. 1450 m	July 2014	HEF 15095
<i>A. schischkinii</i> Sosn.	AScAP and AScR	B6 Sivas: 40°09'50"N, 37°50'53"E. 1925 m	July 2014	HEF 15097
<i>A. setacea</i> Waldst. & Kit.	ASeAP. and ASeR	B6 Sivas: 39°58'48"N, 37°45'09"E. 1300 m	June 2014	HUEF 14046
<i>A. sintenisii</i> Hub. Mor.	ASiAP and ASiR	B6 Sivas: 39°33' 41"N, 37°00'52"E. 1430 m	July 2014	HUEF 14056
<i>A. millefolium</i> L. subsp. <i>millefolium</i>	AMfiAP and AMfiR	B6 Yozgat: 39° 2'27"N, 36°06'60"E. 1880 m	July 2014	HUEF 14047
<i>A. wilhelmsii</i> C. Koch. subsp. <i>wilhelmsii</i> (syn: <i>A. santolinoides</i> subsp. <i>wilhelmsii</i> (K.Koch) Greuter)	AWiAP and AWiR	C5 Niğde: 38°06'07"K. 34° 49' 17"D. 1350 m	July 2014	HUEF 14050
<i>A. teretifolia</i> Willd.	ATeAP and ATeR	B3 Afyon: Şuhut. Around Tekke Village. 1340 m	July 2014	HUEF 14055
<i>A. goniocephala</i> Boiss. & Balansa	AGoAP and AGoR	C5 Niğde: Çamardı. Yelatan Village exit Slopes under the base station. 1410 m	June 2014	HUEF 09889
<i>A. nobilis</i> L.	ANoAP and ANoR	A3 Bilecik: Pazaryeri. Küçükelmali Nature Park. On the way to Gölet. Roadside. 850 m	June 2014	HUEF 09865
<i>A. spinulifolia</i> Fenzl ex Boiss.	ASpAP and ASpR	C5 Niğde: Ankara: 37°30'41 "N, 34°44' 22"E. 1019 m	June 2014	HUEF 09887

Abbreviations: Aerial parts: AP; Root: R.

extracts and filtrated through syringe filter (0.2 μm) prior to LC-MS/MS analysis (Yilmaz et al., 2018).

2.3. Chemicals and reagents

Reference standards of phytochemical compounds (coumarin, hesperidin, *p*-coumaric acid, *o*-coumaric acid, gallic acid, caffeic acid, vanillic acid, salicylic acid, quinic acid, 4-OH-benzoic acid, ferulic acid, chlorogenic acid, rosmarinic acid, protocatechuic acid, cinnamic acid, sinapinic acid, fumaric acid, vanillin, pyrocatechol, malic acid, syringic acid, hesperetin, naringenin, rutin, quercetin, quercitrin, apigenin, chrysin, liquiritigenin, isoquercitrin, apigenin, rhoifolin, nicotiflorin, fisetin, luteolin, myricetin, kaempferol) were obtained from Sigma-Aldrich (Steinheim, Germany). Formic acid, ammonium formate, chloroform, methanol and HPLC grade acetonitrile were purchased from Merck (Darmstadt, Germany). β -carotene, linoleic acid, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitro blue tetrazolium chloride, acetylcholinesterase (AChE), butyrylcholinesterase (BChE), α -tocopherol, potassium peroxodisulfate ($\text{K}_2\text{S}_2\text{O}_8$), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), copper (II) chloride dihydrate ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$), Tween 40, dichloromethane, ethanol, aluminium nitrate ($\text{Al}(\text{NO}_3)_3$), potassium acetate (KCH_3COO), galanthamine hydrobromide, neocuproine (2,9-dimethyl-1,10-phenanthroline), trypsin EDTA (%25), penicillin streptomycin solution, fetal bovine serum (heat inactivated) and Dulbecco's modified Eagle's medium-high glucose (DMEM) were obtained from Sigma-Aldrich (Steinheim, Germany). Acetylthiocholine iodide and Folin-Ciocalteu phenol reagent was from Applichem (Steinheim, Germany). Butyrylcholine iodide (Fluka), sodium carbonate, ammonium acetate, sodium hydrogen phosphate and sodium dihydrogen phosphate were purchased from Riedel-de-Haen (Germany). Urease from *Canavalia ensiformis* (Jack bean) Type III, powder, 15,000–50,000 units/g solid), phenol, urea and kojic acid (analytical standard) were from Sigma (Germany). Tyrosinase (from mushroom lyophilized powder, ≥ 1000 unit/mg solid) and 3,4-Dihydroxy-L-phenylalanine were from Sigma (Germany). Thiourea was from Merck (Germany). HeLa cells were kindly obtained from Prof. Dr. İbrahim Demirtaş (Çankırı Karatekin University). Ultrapure water was obtained from Sartorius (Goettingen, Germany) Arium Pro Ultrapure Water System.

2.4. Quantitation of phytochemicals by LC-MS/MS and method validation

The previously described LC-MS/MS technique was applied to evaluate the quantitative content of twelve

Achillea species phenolics (Yilmaz et al., 2018). The performance characteristics of the method, namely, accuracy (recovery), linearity, detection and quantification limits (LOD/LOQ), intraday and interday precision (repeatability), and relative standard uncertainty (U% at 95% confidence level ($k = 2$)) were given previously. Details of the procedure explaining the evaluation of uncertainty have been previously reported (Yilmaz et al., 2018).

2.5. Statistical analysis

All statistical calculations were performed by Minitab Statistical Software v. 16.2.1. The results were evaluated using an unpaired *t*-test and one-way analysis of variance ANOVA. The differences were regarded as statistically significant at $p < 0.05$. Phenolic components, antioxidant activities, total phenolic contents, total flavonoid contents and enzyme activity data of *Achillea* species collected in six regions (Ankara, Yozgat, Sivas, Niğde, Afyon, and Bilecik) and divided parts of the species (root, aerial) were evaluated chemometrically by using PCA and HCA techniques.

3. Results and discussion

3.1. Quantitative analysis of phenolic compounds by LC-MS/MS

Ethanol extracts of 12 *Achillea* species were screened using a previously developed and validated LC-MS/MS method (Yilmaz et al., 2018). According to the results of LC-MS/MS, extracts of the both aerial and root parts were determined to be rich in flavonoid and phenolic acid content (Figure 1, Table 2).

It has been found that there are considerable amounts of quinic (10,354.27 and 8024.03 $\mu\text{g g extract}^{-1}$, respectively), malic (3613.71 and 6984.96 $\mu\text{g g extract}^{-1}$, respectively) and chlorogenic acids (21,303.1 and 46,407 $\mu\text{g g extract}^{-1}$, respectively) in the aerial and root parts of *A. nobilis*. Additionally, the amount of the flavonoids; rutin (2440 $\mu\text{g g extract}^{-1}$), hesperidin (511.66 $\mu\text{g g extract}^{-1}$), isoquercitrin (2234.09 $\mu\text{g g extract}^{-1}$), quercetin (621.45 $\mu\text{g g extract}^{-1}$) and luteolin (563.37 $\mu\text{g g extract}^{-1}$) draw attention in the aerial and root parts of *A. nobilis*. Also, significant amounts of quinic (8945.28 and 4749.99 $\mu\text{g g extract}^{-1}$, respectively), malic (3268.45 and 1025.9 $\mu\text{g g extract}^{-1}$, respectively), chlorogenic (13,663.8 and 5440.9 $\mu\text{g g extract}^{-1}$, respectively) and vanillic acids (1321.29 and 5376.57 $\mu\text{g g extract}^{-1}$, respectively) were present in the aerial and root parts of *A. gonioccephala* species. In addition, there are considerable amounts of rutin (5961.2 $\mu\text{g g extract}^{-1}$), hesperidin (1859.84 $\mu\text{g g extract}^{-1}$), isoquercitrin (2053.51 $\mu\text{g g extract}^{-1}$) and luteolin (830.08 $\mu\text{g g extract}^{-1}$) in the aerial part of this species. Phenolic compounds found in significant amounts in the aerial and root parts of *A. sintenisii* are: quinic acid (2689.41 and 6335.38 $\mu\text{g g extract}^{-1}$, respectively) and chlorogenic acid (3085.73 and

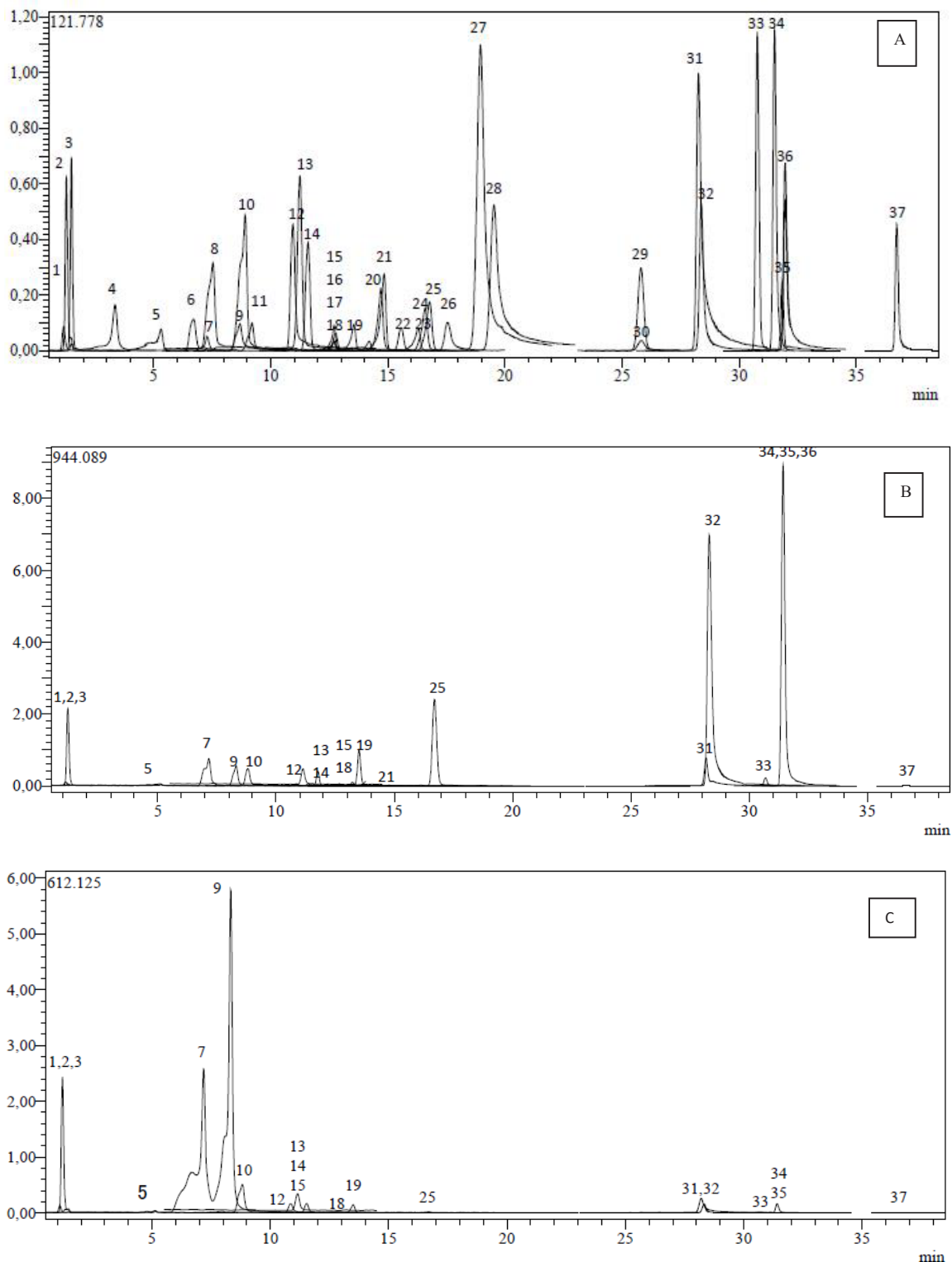


Figure 1. A: LC-MS/MS chromatograms of 37 standard mix. 1: Quinic acid, 2: Malic acid, 3: Fumaric acid, 4: Gallic acid, 5: Protocatechuic acid, 6: Pyrocatechol, 7: Chlorogenic acid, 8: 4-OH-benzoic acid, 9: Vanillic acid, 10: Caffeic acid, 11: Syringic acid, 12: Vanillin, 13: Salicylic acid, 14: p-Coumaric acid, 15: Rutin, 16: Ferulic acid, 17: Sinapinic acid, 18: Hesperidin, 19: Isoquercitrin, 20: Rosmarinic acid, 21: Nicotiflorin, 22: o-Coumaric acid, 23: Rhoifolin, 24: Quercitrin, 25: Apigetrin, 26: Coumarin, 27: Myricetin, 28: Fisetin, 29: Cinnamic acid, 30: Liquiritigenin, 31: Quercetin, 32: Luteolin, 33: Naringenin, 34: Apigenin, 35: Hesperetin, 36: Kaempferol, 37: Chrysin. B: ABiAP. C: ABiR.

Table 2. Identification and quantification of phenolic compounds in ethanol extracts of *Achillea* species by LC-MS/MS^b.

Samples	AB/AP	AB/R	AKoAP	AKoR	ALyAP	ALyR	AScAP	AScR	AScAP	AScR	ASiAP	ASiR	AMiAP	AMiR	AWiAP	AWiR	AtEAP	AtER	AGoAP	AGoR	ANoAP	ANoR	AsPAP	AsPR	
1	1373.68	1794.30	15784.55	22706.73	2509.4	7695.40	38293.02	24116.77	10067.71	8023.43	26889.41	6335.38	8960.77	8287.72	16163.94	15776.34	6194.91	7643.08	8945.28	4749.99	10354.27	8024.03	6720.17	17631.42	
2	3040.6	3536.30	5343.31	3474.18	7056.43	1730.38	9990.55	3007.55	5812.82	6192.07	694.04	494.55	10067.45	5514.18	11038.45	3722.71	3405.83	4000.83	3268.45	1025.9	3613.71	6984.96	2799.63	9711.22	
3	954.07	1488.68	1097.5	1016.95	803.66	859.52	1443.39	1121.66	1333	1412.62	747.29	1395.23	1216.86	1444.08	2011.01	702.62	1442.57	1100.88	1240.63	1293	1097.8	897.76	1348.42	1727.79	
4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
5	451.93	213.47	343.84	116.90	86.75	92.22	412.13	214.30	129.66	93.59	73.12	78.07	144.03	55.73	213.91	67.91	326.67	361.12	204.23	ND	178.77	101.4	228.39	254.85	
6	1874.46	7533.31	24257.9	55812.20	4978.32	9030.99	17894.4	21860.60	7435.26	15923.70	3085.73	2474.77	11249.7	11406.9	18354.7	15483.6	5646.06	15820.3	13663.8	5440.9	21303.1	46407	10670.5	28277.8	
7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
9	648.84	1622.97	599.11	489.77	429.91	581.47	476.06	1981.17	948.37	1802.89	812.19	1982.04	348.86	1188.17	438.23	443.61	1278.64	2035.01	1321.29	5376.57	703.49	1401.27	1609	2481.97	
10	129.7	179.23	307.11	237.59	105.31	1396.22	345.6	2414.94	232.09	341.94	165.22	637.12	231.22	301.18	202.82	105.02	265.26	599.27	309.86	511.03	160.83	189.1	302.89	1305.97	
11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
12	132.27	459.70	81.37	160.26	61.15	207.01	110.24	904.02	184.19	603.78	237.22	1707.28	67.19	333.23	70.84	204.9	156.5	411.97	376.14	1573.02	121.05	377.04	239.41	1278.91	
13	78.95	63.56	140.89	48.99	41.33	7.56	149.32	34.85	83.16	105.80	29.62	30.89	80.75	42.35	240.36	14.57	152.64	140.82	209.88	ND	164.24	58.62	96.83	175.5	
14	17.86	57.32	20.45	55.32	14.78	134.79	63.74	297.71	12.2	40.26	101.32	197.77	17.09	33.85	15.74	83.81	12.7	46.87	35.95	222.83	ND	35.85	21.41	134.08	
15	61.09	ND	4018.88	115.36	39.22	5.80	1108.34	173.82	7461.23	87.36	650.31	152.32	7696.17	115.18	359.92	32.38	672.56	672.12	5961.2	135.74	2440	50.44	3383.97	310.4	
16	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
17	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
18	28.42	ND	1210.77	73.81	14.35	6.89	441.85	101.15	2562.7	49.06	275.1	104.91	2245.19	89.77	106.59	21.42	236.06	292.88	1859.84	112.92	511.66	21.91	1309.23	205.2	
19	918.84	151.37	3535.16	90.11	48.55	42.06	1167.57	50.60	1162.85	42.91	114.27	38.71	1328.87	62.08	727.4	23.53	773.75	635.42	2053.51	53.96	2234.09	106.99	2241.42	149.5	
20	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
21	27.64	ND	176.95	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
22	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
23	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
24	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
25	1599.57	13.35	5338.4	57.06	15.58	ND	44.24	ND	1098.25	12.13	160.86	9.50	1464.77	29.74	19.37	ND	19.11	ND	123.16	ND	42.74	ND	19.89	ND	
26	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
27	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
28	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
29	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
30	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
31	662.56	285.05	591.84	75.44	26.08	9.84	515.66	27.14	153.39	14.44	52.36	40.72	307.29	18.34	36.48	ND	4432.36	ND	302.98	ND	621.45	ND	229.67	ND	
32	2535.41	46.75	1518.62	19.90	480.52	55.38	1930.59	88.07	1824.69	63.88	1395.23	275.17	1497.72	64.33	359.44	ND	2613.32	9085.56	830.08	ND	563.37	31.75	727.23	41.84	
33	26.8	ND	27.78	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	69.14	190.56	35.34	39.31	ND	23.13	328.63	92.2
34	1496.74	25.71	2167.55	26.76	ND	33.42	85.67	ND	533.16	10.78	608.18	42.56	419.53	12.43	20.05	ND	33.92	121.98	67.58	11.78	26.05	ND	19.53	ND	
35	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	37.7	14.99	47.74	ND	8.09	ND	90.3	ND
36	40.47	ND	25.07	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
37	7.45	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

a: Phytochemical compounds are numbered. 1: Quinic acid, 2: Malic acid, 3: Fumaric acid, 4: Gallic acid, 5: Protocatechuic acid, 6: Pyrocatechol, 7: Chlorogenic acid, 8: 4-OH-benzoic acid, 9: Vanillic acid, 10: Caffeic acid, 11: Syringic acid, 12: Vanillin, 13: Salicylic acid, 14: *p*-Coumaric acid, 15: Rutin, 16: Ferulic acid, 17: Sinapinic acid, 18: Hesperidin, 19: Isoquercitrin, 20: Rosmarinic acid, 21: Nicotiflorin, 22: *o*-Coumaric acid, 23: Rhoifolin, 24: Quercitrin, 25: Apigetrin, 26: Coumarin, 27: Myricetrin, 28: Fisetin, 29: Cinnamic acid, 30: Liquiritigenin, 31: Quercetin, 32: Luteolin, 33: Naringenin, 34: Apigenin, 35: Hesperetin, 36: Kaempferol, 37: Chrysin, b: µg analyt g extract⁻¹, c: Not detected.

2474.77 $\mu\text{g g extract}^{-1}$, respectively). Vanillic acid (1982.04 $\mu\text{g g extract}^{-1}$) and vanillin (1707.28 $\mu\text{g g extract}^{-1}$) were the phenolic compounds present in significant quantities in the root of this extract. Moreover, aerial parts of *A. sintenisii* contain notable amounts of rutin (650.31 $\mu\text{g g extract}^{-1}$), luteolin (1395.23 $\mu\text{g g extract}^{-1}$) and apigenin (608.18 $\mu\text{g g extract}^{-1}$) flavonoids.

When the aerial and root parts of *A. kotschyi* were examined, quinic (15,784.55 and 22,706.73 $\mu\text{g g extract}^{-1}$, respectively), malic (5343.31 and 3474.18 $\mu\text{g g extract}^{-1}$, respectively) and chlorogenic acids (24,257.9 and 55,812.2 $\mu\text{g g extract}^{-1}$, respectively) were determined to be in significant amounts. However, in the aerial part of this species, there are significant amounts of rutin (4018.88 $\mu\text{g g extract}^{-1}$), hesperidin (1210.77 $\mu\text{g g extract}^{-1}$), isoquercitrin (3535.16 $\mu\text{g g extract}^{-1}$), apigenin (5358.4 $\mu\text{g g extract}^{-1}$), quercetin (591.84 $\mu\text{g g extract}^{-1}$), luteolin (1518.62 $\mu\text{g g extract}^{-1}$) and apigenin (2167.55 $\mu\text{g g extract}^{-1}$) flavonoids.

It was detected that, there are considerable amounts of quinic (8960.77 and 8287.72 $\mu\text{g g extract}^{-1}$, respectively), malic (10,067.45 and 5514.18 $\mu\text{g g extract}^{-1}$, respectively) and chlorogenic acids (11,249.7 and 11,406.9 $\mu\text{g g extract}^{-1}$, respectively) in the aerial and root parts of *A. millefolium*. Besides, the aerial parts of this species contain considerable amounts of rutin (7696.17 $\mu\text{g g extract}^{-1}$), hesperidin (2245.19 $\mu\text{g g extract}^{-1}$), isoquercitrin (1328.87 $\mu\text{g g extract}^{-1}$), apigenin (1464.77 $\mu\text{g g extract}^{-1}$), luteolin (1497.72 $\mu\text{g g extract}^{-1}$) and apigenin (419.53 $\mu\text{g g extract}^{-1}$). When it comes to the species *A. lycaonica*, it contains remarkable amounts of quinic (root; 7695.4 $\mu\text{g g extract}^{-1}$), malic (aerial; 7056.43, root; 1730.38 $\mu\text{g g extract}^{-1}$), chlorogenic (root; 9030.99, aerial; 4978.32 $\mu\text{g g extract}^{-1}$), caffeic acids (root; 1396.22 $\mu\text{g g extract}^{-1}$) and luteolin (aerial; 480.52 $\mu\text{g g extract}^{-1}$). If we look at the root and aerial parts of *A. wilhelmsii*, quinic (16,163.94 and 15,776.34 $\mu\text{g g extract}^{-1}$, respectively), malic (11,038.45 and 3722.71 $\mu\text{g g extract}^{-1}$, respectively) and chlorogenic acids (18,354.7 and 15,483.6 $\mu\text{g g extract}^{-1}$, respectively) appear to be important. When the aerial and root parts of *A. spinulifolia* were examined, quinic acid (6720.17 and 17,631.42 $\mu\text{g g extract}^{-1}$, respectively), chlorogenic acid (10,670.5 and 28,277.8 $\mu\text{g g extract}^{-1}$, respectively), vanillic acid (1609 and 2481.97 $\mu\text{g g extract}^{-1}$, respectively) and in the root part vanillin (1278.91 $\mu\text{g g extract}^{-1}$) were detected to be in significant amounts. Nonetheless, rutin (3383.97 $\mu\text{g g extract}^{-1}$), hesperidin (1309.23 $\mu\text{g g extract}^{-1}$), isoquercitrin (2241.42 $\mu\text{g g extract}^{-1}$), luteolin (727.23 $\mu\text{g g extract}^{-1}$) and naringenin (328.63 $\mu\text{g g extract}^{-1}$) flavonoids were present in notable amounts in the aerial parts of this species. However, in the aerial and root parts of *A. teretifolia*, there are significant amounts quinic (6194.91 and 7643.08 $\mu\text{g g extract}^{-1}$, respectively), malic (3405.83 and 4000.83 $\mu\text{g g extract}^{-1}$, respectively), chlorogenic

(5646.06 and 15820.3 $\mu\text{g g extract}^{-1}$, respectively) and vanillic acids (2035.01 $\mu\text{g g extract}^{-1}$ in root part). Besides, in its aerial and root parts, rutin (672.56 and 672.12 $\mu\text{g g extract}^{-1}$, respectively), isoquercitrin (635.42 and 672.12 $\mu\text{g g extract}^{-1}$, respectively), quercetin (4432.36 and 9085.56 $\mu\text{g g extract}^{-1}$, respectively) and in the aerial parts luteolin (2613.32 $\mu\text{g g extract}^{-1}$) were present in fair amounts.

If we examine the aerial and root parts of *A. biebersteinii*, malic (3040.6 and 3536.3 $\mu\text{g g extract}^{-1}$, respectively) and chlorogenic (1874.46 and 7533.31 $\mu\text{g g extract}^{-1}$, respectively) acids draw attention. Besides, isoquercitrin (918.84 $\mu\text{g g extract}^{-1}$), apigenin (1599.57 $\mu\text{g g extract}^{-1}$), quercetin (662.56 $\mu\text{g g extract}^{-1}$), luteolin (2535.41 $\mu\text{g g extract}^{-1}$) and apigenin (1496.74 $\mu\text{g g extract}^{-1}$) flavonoids were found to be in remarkable amounts in the aerial parts of this species. When it comes to the aerial and root parts of *A. setacea*, quinic (10,067.71 and 8023.43 $\mu\text{g g extract}^{-1}$, respectively), malic (5812.82 and 6192.07 $\mu\text{g g extract}^{-1}$, respectively), chlorogenic acids (7435.26 and 15,923.7 $\mu\text{g g extract}^{-1}$, respectively) were found to be important. In addition, rutin (7461.23 $\mu\text{g g extract}^{-1}$), hesperidin (2562.7 $\mu\text{g g extract}^{-1}$), isoquercitrin (1162.85 $\mu\text{g g extract}^{-1}$), apigenin (1098.25 $\mu\text{g g extract}^{-1}$), luteolin (1824.69 $\mu\text{g g extract}^{-1}$) and apigenin (523.16 $\mu\text{g g extract}^{-1}$) were found to be significant in the aerial parts of this species. In the aerial and root parts of *A. schischkinii*, quinic (38,293.02 and 24,116.77 $\mu\text{g g extract}^{-1}$, respectively), malic (9990.55 and 3007.55 $\mu\text{g g extract}^{-1}$, respectively) and chlorogenic (17,894.4 and 21,860.6 $\mu\text{g g extract}^{-1}$, respectively) acids were in significant amounts. Furthermore, rutin (1108.34 $\mu\text{g g extract}^{-1}$), hesperidin (441.85 $\mu\text{g g extract}^{-1}$), isoquercitrin (1167.57 $\mu\text{g g extract}^{-1}$), quercetin (515.66 $\mu\text{g g extract}^{-1}$) and luteolin (1930.59 $\mu\text{g g extract}^{-1}$) were found to be the most abundant flavonoids in the aerial parts of this species.

In particular, it has been determined that some of the species studied can be a source for certain phenolic compounds as they are present in high levels. These components and species might be listed as chlorogenic acid (55,812.20 and 46,407.00 $\mu\text{g g extract}^{-1}$, respectively) in the root extracts of *A. kotschyi* and *A. nobilis*, rutin (7696.17 and 7461.23 $\mu\text{g g extract}^{-1}$, respectively) and hesperidin (2245.19 and 2562.70 $\mu\text{g g extract}^{-1}$, respectively) in the aerial extracts of *A. millefolium* and *A. setacea*, quercetin (9085.56, 4432.36 $\mu\text{g g extract}^{-1}$, respectively) in the aerial and root extracts of *A. teretifolia*, vanillic acid (5376.57 $\mu\text{g g extract}^{-1}$) in the root extract of *A. goniocephala*, apigenin (2167.55 $\mu\text{g g extract}^{-1}$) in the aerial extract of *A. kotschyi*, isoquercitrin (3535.16, 2241.42, 2234.09 and 2053.51 $\mu\text{g g extract}^{-1}$, respectively) in the aerial extracts of *A. kotschyi*, *A. spinulifolia*, *A. nobilis* and *A. goniocephala*, apigenin (5358.40 $\mu\text{g g extract}^{-1}$) in the aerial extract of *A. kotschyi* and luteolin (2613.32, 2535.41 $\mu\text{g g extract}^{-1}$, respectively) in the aerial extract of *A. teretifolia* and *A. biebersteinii*.

In literature, there are few studies on the LC-MS/MS based phytochemical screening of some *Achillea* species (Vitalini et al., 2011; Agar et al., 2015; Becker et al., 2016; Zengin et al., 2017a). Methanol extract of *A. Phrygia* was analyzed by LC-MS/MS and chlorogenic and sinapinic acids (3524 ± 102 and $5072 \pm 22 \mu\text{g g extract}^{-1}$, respectively) were found to be the major components (Zengin et al., 2017a). Luteolin was detected to be the major compound of *A. distans* in the study of Benedec et al. They also found that chlorogenic acid was in trace amount (Benedec et al., 2013). Moreover, in the study of Vitalini et al. the phenolic screening of *A. millefolium* was mainly identified by the existence of chlorogenic acid and its caffeoylquinic derivatives, apart from apigenin, luteolin, and rutin flavonoid glycosides (Vitalini et al., 2011). In another study conducted by Agar et al. (2015), chlorogenic acid (511.9 ± 25.1 , 2890.6 ± 141.6 , and $778.0 \pm 38.1 \mu\text{g g extract}^{-1}$, respectively) was found to be the major component of *A. lycaonica*, *A. kotschyi* subsp. *kotschyi*, and *A. coarctata*. In the study of Taşkın et al., the phenolic compounds identified were chlorogenic acid, caffeic acid, rutin, dicaffeoylquinic acid, salicylic acid, luteolin, quercetin, naringenin, apigenin, and 8-hydroxy-salvigenin (Taşkın et al., 2018). There is not any study related to the screening phenolic compounds in *A. gonocephala* in literature. As a consequence, it might be said that the results of the present study are consistent with the literature in terms of the major compounds. Besides, flavonoids and phenolic acids were quite rich in the studied extracts.

3.2. Total flavonoid-phenolic content and antioxidant activities

Cupric reducing antioxidant capacity (CUPRAC), ABTS cation radical scavenging, β -carotene-linoleic acid test system, and DPPH free radical methods were used to uncover the antioxidant activities of the 24 extracts of twelve *Achillea* species (Table 3). α -TOC and BHT were used as standard compounds in the applied antioxidant activity test methods. Total flavonoid and phenolic amounts of the crude extracts were determined by expressing as quercetin and pyrocatechol equivalents, respectively ($y = 0.039 + 0.0041x$, quercetin (μg), $r^2 = 0.9959$ and $y = 0.0185 + 0.0283x$, pyrocatechol (μg), $r^2 = 0.9906$).

Within the extracts, it was determined that the total phenolic content ($282.97 \pm 3.14 \mu\text{g pyrocatechol equivalent mg extract}^{-1}$) of *A. nobilis* was the highest, while the total phenolic content ($36.30 \pm 1.0 \mu\text{g pyrocatechol equivalent mg extract}^{-1}$) of *A. biebersteinii* was the lowest. Moreover, the total flavonoid content ($35.36 \pm 0.54 \mu\text{g quercetin equivalent mg extract}^{-1}$) of the root extract of *A. schischkinii* was determined to be the highest, while the total flavonoid content ($11.15 \pm 0.47 \mu\text{g quercetin equivalent mg extract}^{-1}$) of the root extract of *A. wilhelmsii* was the least. The total phenolic contents of the aerial parts of *A. biebersteinii*, *A.*

setacea and *A. schischkinii* species were higher than those of the root parts whereas the total phenolic contents of the root parts of other species were higher than those of the aerial parts.

The extracts of the studied *Achillea* species were determined to have a moderate-high activity in the β -carotene-linoleic acid test system. In this method, aerial parts of *A. gonocephala* ($\text{IC}_{50}: 48.42 \pm 0.25$), *A. millefolium* (53.59 ± 0.29), *A. setacea* (54.70 ± 0.65) and root of *A. wilhelmsii* (55.19 ± 0.86) were detected to be the most active extracts.

In general, it has been determined that the *Achillea* species studied exhibit DPPH free radical scavenging activity at very good level. According to the DPPH free radical scavenging activity method, *A. nobilis*-root ($\text{IC}_{50}: 12.23 \pm 0.24$), *A. kotschyi*-root (12.88 ± 0.45) and aerial (27.58 ± 0.15) extracts showed the highest activities. Besides, it has been determined that the studied *Achillea* species generally exhibits very good activity also in the ABTS cation radical scavenging method. Roots and aerial parts of *A. kotschyi* (4.74 ± 0.07 and 13.19 ± 0.97 , respectively) and *A. nobilis* (3.38 ± 0.06 and 22.16 ± 0.01 , respectively) showed the highest activities in the ABTS cation radical scavenging method. These extracts showed better activities than α -tocopherol and BHT which were used as standard compounds. It was determined that root parts of the species being active both in ABTS and DPPH methods were more active than their aerial parts.

Four different concentrations (10, 25, 50, 100 $\mu\text{g mL}^{-1}$) were studied in the CUPRAC antioxidant assay and the corresponding $A_{0.5}$ values were calculated. Root extract of *A. nobilis* ($A_{0.5}: 7.97 \pm 0.01$) and root and aerial parts extracts of *A. kotschyi* showed remarkable activities in CUPRAC antioxidant activity assay. Furthermore, root extracts showed better activity than the aerial parts for all species in the assay. These extracts detected to show better activities than α -tocopherol and BHT which were used as standard compounds.

It is noteworthy that, same species showed the best activity in both DPPH and ABTS activity assays. The high activities of these species may be attributed to their high total phenolic and flavonoid contents. When the results of the LC-MS/MS data were thoroughly evaluated, it might be said that the high activity of the studied extracts could be due to the flavonoids such as luteolin, apigenin, quercetin, and rutin, as well as phenolic acids such as protocatechic, *p*-coumaric, chlorogenic, and caffeic acids which have antioxidant potential.

According to the literature, there are numerous studies on the total phenolic-flavonoid contents and antioxidant activities of *Achillea* species (Agar et al., 2015; Tušek et al., 2016; Zengin et al., 2017a). In a previous study, total phenolic contents of the methanol extracts of *A. lycaonica*,

Table 3. Antioxidant and anticholinesterase, antiurease, antityrosinase activities and total phenolic-flavonoid contents of the *Achillea* species^a.

Samples	IC ₅₀ (µg mL ⁻¹)			µg PEs mg extract ^{-1 b}	µg QEs mg extract ^{-1 c}	A _{0.5}	Inhibition (%) ^d			
	Lipid peroxidation	DPPH free radical	ABTS cation radical	Phenolic content	Flavonoid content	CUPRAC	Anti-AChE	Anti-BChE	Antityrosinase	Antiurease
ABiAP	84.55 ± 1.69	100.93 ± 2.70	44.45 ± 0.75	111.35 ± 1.91	16.43 ± 0.32	55.07 ± 1.77	NA ^e	26.84 ± 0.01	9.52 ± 0.39	24.75 ± 1.71
ABiR	116.11 ± 1.71	80.83 ± 0.24	30.11 ± 0.01	36.30 ± 1.03	21.26 ± 0.28	42.42 ± 0.91	NA	23.94 ± 0.87	NA	52.47 ± 3.09
AKoAP	113.43 ± 1.79	27.58 ± 0.15	13.19 ± 0.97	180.27 ± 1.28	25.87 ± 0.67	18.59 ± 0.09	NA	NA	NA	27.21 ± 1.04
AKoR	113.00 ± 2.13	12.88 ± 0.45	4.74 ± 0.07	260.00 ± 3.38	20.22 ± 0.79	9.17 ± 0.02	NA	14.69 ± 0.91	22.58 ± 0.27	83.30 ± 3.09
ALyAP	68.30 ± 0.93	254.41 ± 3.60	112.16 ± 1.02	80.27 ± 1.38	13.42 ± 0.25	95.81 ± 2.34	NA	1.95 ± 0.08	NA	NA
ALyR	92.54 ± 1.14	71.96 ± 0.22	28.32 ± 0.92	105.95 ± 0.91	12.08 ± 0.77	48.26 ± 1.08	11.19 ± 0.18	14.54 ± 1.97	21.61 ± 2.04	26.17 ± 0.33
AScAP	71.93 ± 0.78	68.31 ± 0.55	31.63 ± 0.67	110.00 ± 1.-10	14.89 ± 0.29	34.96 ± 0.47	NA	NA	6.87 ± 0.20	NA
AScR	100.75 ± 1.02	37.21 ± 0.72	17.38 ± 0.44	95.34 ± 1.37	35.36 ± 0.54	20.49 ± 0.27	NA	2.25 ± 0.07	28.58 ± 1.58	83.92 ± 2.88
ASeAP	54.70 ± 0.65	70.50 ± 0.22	54.13 ± 0.56	134.32 ± 1.83	18.39 ± 0.42	37.52 ± 0.31	NA	NA	27.09 ± 0.88	NA
ASeR	192.09 ± 3.51	44.91 ± 1.44	30.26 ± 0.69	42.13 ± 0.13	25.10 ± 0.12	29.76 ± 0.07	NA	NA	28.21 ± 1.26	85.54 ± 3.03
ASiAP	69.63 ± 0.63	101.32 ± 1.47	56.36 ± 1.07	97.39 ± 1.69	13.18 ± 0.25	62.57 ± 1.09	7.36 ± 0.21	30.88 ± 1.91	NA	26.28 ± 0.56
ASiR	210.99 ± 1.14	109.54 ± 0.32	39.51 ± 0.22	108.65 ± 0.73	14.33 ± 0.15	44.08 ± 0.56	NA	17.54 ± 0.66	25.48 ± 0.71	53.13 ± 1.31
AMiAP	53.59 ± 0.29	64.61 ± 0.70	31.61 ± 0.38	123.51 ± 0.24	18.27 ± 0.75	37.24 ± 0.33	NA	9.30 ± 0.18	11.73 ± 0.35	32.61 ± 1.49
AMiR	185.56 ± 3.27	62.22 ± 0.40	31.35 ± 0.97	127.57 ± 0.76	12.70 ± 0.34	32.31 ± 0.43	NA	NA	23.34 ± 0.54	67.37 ± 0.15
AWiAP	55.19 ± 0.86	89.07 ± 2.39	40.99 ± 0.61	110.00 ± 2.38	15.70 ± 0.20	51.03 ± 0.87	NA	NA	8.02 ± 0.04	NA
AWiR	144.78 ± 4.10	57.25 ± 0.63	26.51 ± 0.62	135.68 ± 1.36	11.15 ± 0.47	33.77 ± 0.65	NA	NA	28.80 ± 1.96	79.70 ± 0.46
ATeAP	95.73 ± 1.01	59.19 ± 1.28	33.52 ± 0.11	146.49 ± 1.37	18.35 ± 0.33	31.16 ± 0.35	NA	11.90 ± 0.81	16.91 ± 0.28	NA
ATeR	126.63 ± 2.27	57.92 ± 0.88	26.02 ± 1.07	184.32 ± 2.27	14.15 ± 0.65	29.02 ± 0.26	NA	NA	11.08 ± 0.26	NA
AGoAP	141.42 ± 0.09	58.05 ± 0.81	34.78 ± 0.47	123.51 ± 1.82	16.95 ± 0.55	43.76 ± 0.21	NA	13.14 ± 0.96	NA	27.65 ± 0.51
AGoR	48.42 ± 0.25	76.23 ± 0.49	31.93 ± 0.05	132.97 ± 1.91	11.99 ± 0.32	36.77 ± 0.13	NA	6.15 ± 0.05	14.39 ± 0.03	65.42 ± 2.50
ANoAP	64.51 ± 3.94	32.24 ± 1.44	22.16 ± 0.01	151.89 ± 1.91	24.77 ± 0.71	24.24 ± 0.06	NA	23.64 ± 0.26	8.82 ± 0.44	84.78 ± 2.70
ANoR	109.53 ± 0.55	12.23 ± 0.24	3.38 ± 0.06	282.97 ± 3.14	22.23 ± 0.11	7.97 ± 0.01	NA	NA	28.18 ± 1.07	80.89 ± 1.07
ASpAP	239.62 ± 3.13	67.86 ± 2.84	29.64 ± 0.52	124.86 ± 1.73	16.65 ± 0.97	35.94 ± 0.12	NA	NA	NA	36.43 ± 1.16
ASpR	84.88 ± 1.32	53.56 ± 0.51	19.35 ± 0.44	154.59 ± 3.54	14.14 ± 0.35	23.44 ± 0.05	NA	4.15 ± 0.07	20.26 ± 1.05	77.68 ± 1.04
α-TOC	15.62 ± 0.12	16.30 ± 0.79	9.88 ± 0.23	-	-	19.05 ± 0.02	-	-	-	-
BHT	8.38 ± 0.08	58.86 ± 0.50	12.29 ± 0.67	-	-	14.80 ± 0.01	-	-	-	-
Galantamine	-	-	-	-	-	-	76.08 ± 0.39	76.52 ± 0.41	-	-
Kojic acid	-	-	-	-	-	-	-	-	89.00 ± 0.12	-
Thiourea	-	-	-	-	-	-	-	-	-	95.89 ± 6.36

^aValues expressed are means ± SD of three parallel measurements and values were calculated according to negative control. Values with different letters in the same column were significantly different ($p < 0.05$), ^bPyrocatechol equivalent phenolic content ($y = 0.0185x + 0.0283$ $R^2 = 0.9906$), ^cQuercetin equivalent flavonoid content ($y = 0.039x + 0.0041$ $R^2 = 0.9959$), ^d200 µg mL⁻¹, ^eNA: Not active.

A. kotschyi subsp. *kotschyi*, and *A. coarctata* were found to be 76.49 ± 1.67 , 148.00 ± 0.92 , and 55.16 ± 0.96 mg GAE g extract⁻¹, respectively (Agar et al., 2015). In another study conducted by Zengin et al. (2017a), total phenolic and flavonoid contents of methanol extract of *A. phrygia* were detected to be 41.13 ± 0.74 mg GAE g extract⁻¹ and 21.73 ± 1.45 mg RE g extract⁻¹, respectively. A previous report showed that, total phenolic and flavonoid contents of *A. millefolium* were determined to be 135.26 ± 1.72 mg GAE g extract⁻¹ and 30.82 ± 2.35 mg QE g⁻¹, respectively (Milutinović et al., 2015). Generally speaking, our results are consistent with the literature. However, in parallel with the results of LC-MS/MS, it appears that the species we studied were richer in total phenolic content.

3.3. Cytotoxic activity

Figure 2 demonstrates the xCELLigence real-time monitoring results of the proliferation of HeLa cells treated with methanol-chloroform extracts of aerial and root parts of eleven *Achillea* species. The cytotoxic effect of *A. biebersteinii* extract could not be determined due to some problems arised in the analysis. Differences in cytotoxic activities between the extracts might be due to their phytochemical content. The cell index measurements indicate that each extract showed notable cytotoxic activity (Figure 2 and Table 4).

HeLa cells were highly inactivated by AKoAP, ASiAP and ASiR extracts at concentrations of 250 and 100 µg mL⁻¹ (dark blue and pink lines), while lower activities

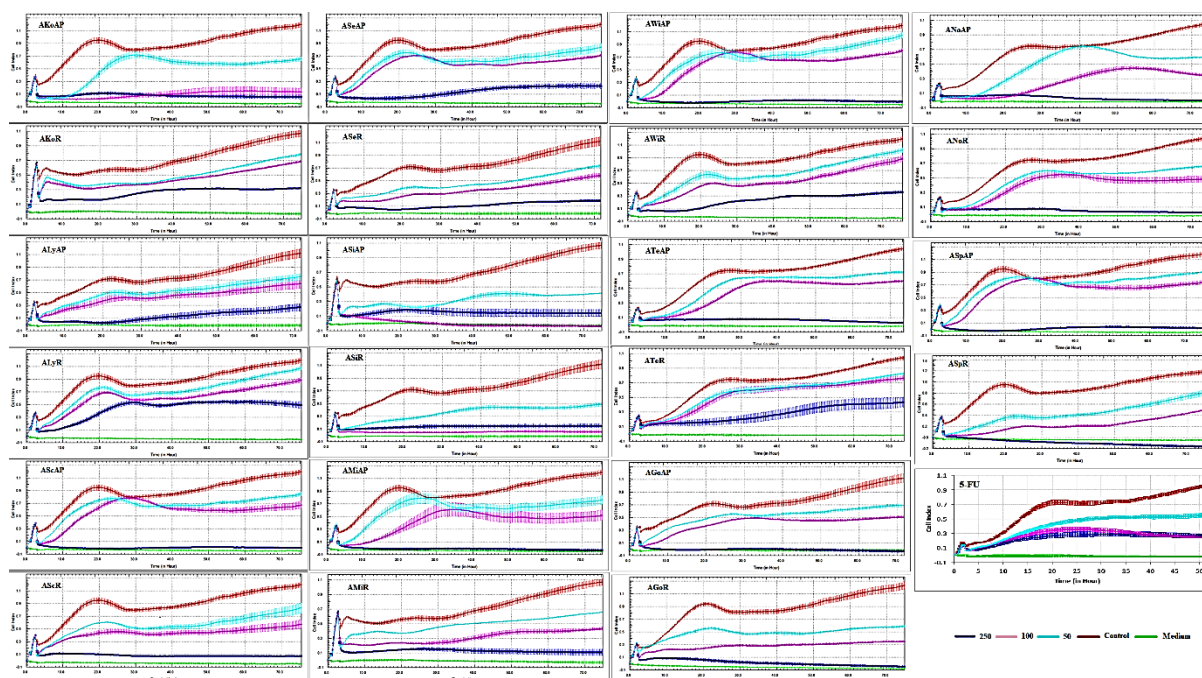


Figure 2. Cytotoxicity assay graphs of studied *Achillea* species.

were obtained at lower concentration of $50 \mu\text{g mL}^{-1}$ (light blue line) in a dose-dependent manner. On the other hand, HeLa cells were highly inhibited by ASaAP, ASaR, ASiAP, ASiR, AWiAP, AWiR, ATeAP, AGoAP, AGoR, ANoAP, ANoR, ASpAP, and ASpR extracts at $250 \mu\text{g mL}^{-1}$ concentration. Nevertheless, lower inhibition was obtained at low concentrations (100 and $50 \mu\text{g mL}^{-1}$) in a dose dependent manner. Moreover, ALyAP and AWiR extracts highly inhibited the HeLa cells at $250 \mu\text{g mL}^{-1}$ concentration (for 20 and 15 h, respectively) then the inhibition decreased slowly in a time dependent manner. Moreover, AKoR and ATeR extracts showed moderate inhibition against the HeLa cells at $250 \mu\text{g mL}^{-1}$ concentration for 20 h, then the inhibition decreased slowly with time. Nevertheless, ALyR extract showed little inhibition at all concentration levels (Figure 2 and Table 4). It is noteworthy to say that, while the aerial parts of *A. kotschyi*, *A. lycaonica*, *A. wilhelmsii*, *A. teretifolia*, *A. nobilis* species showed better inhibitory activity than their root parts against HeLa cells, the opposite is true for *A. schischkinii*, *A. setacea*, *A. millefolium*, *A. gonioccephala*, and *A. spinulifolia* species. Nevertheless, both the aerial and root parts of *A. sintenisii* species showed almost same inhibitory activity against HeLa cells. Interestingly, inhibitory activity of $100 \mu\text{g mL}^{-1}$ ASiAP extract at was better than $250 \mu\text{g mL}^{-1}$ extract and worse than $50 \mu\text{g mL}^{-1}$.

Being used as positive control, 5-FU suppressed the HeLa cell proliferation once the cells were added to the wells (Figure 2, Table 4). This happened only at lower

values than the CI values obtained from the control (red) and proceeded until the end of the experiment. The CI values (red) gathered from the wells of normal growing cells at the end of the 48 h reached to the level 0.9 and the CI values obtained from 5-FU added to the wells at 250 and $100 \mu\text{g/mL}$ concentrations were close to each other during the process progressed and remained below 0.4.

Though the effect of all doses was similar up to 20 h after adding 5-FU, the effect of 5-FU progressively decreased in the wells where the effects of $50 \mu\text{g/mL}$ concentration were investigated and the CI values increased steadily till the end of the experiment. 5-FU was quite active against HeLa cells at 250 and $100 \mu\text{g/mL}$; however, the cell proliferation was partially inhibited and did not stop completely. On the other hand, at $50 \mu\text{g/mL}$ concentration, the HeLa cells were freed from the antiproliferative effect of 5-FU as time proceeded, resulting in higher CI values.

A literature review demonstrated the in vitro anticancer potential of some *Achillea* species and their metabolites using various tumor cell lines. When these reports are examined, it is seen that *Achillea* species have high cytotoxic potential, as in our results (Taşkın et al., 2016; Awad et al., 2017). Awad et al. (2017) have investigated the cytotoxic effects of some components they isolated from *A. fragrantissima* Sch. Bip. species on 5 different human cell line (MCF7, HepG2, A549, PC3, and HeLa) and found them to be quite active in general. Additionally, Taşkın et al. (2016) reported that the chloroform extract of *A. multifida* species was highly active against the HeLa

Table 4. Cell index values from cytotoxicity tests of *Achillea* methanol-chloroform extracts on HeLa cells.

Samples	250 µg mL ⁻¹				100 µg mL ⁻¹				50 µg mL ⁻¹			
	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h
AKoAP	0.0965	0.1070	0.0786	0.0680	0.0250	0.063	0.1054	0.1473	0.1749	0.6906	0.6219	0.5800
AKoR	0.1609	0.2163	0.2952	0.3111	0.3142	0.3596	0.4152	0.4837	0.3469	0.3760	0.4388	0.5408
ALyAP	0.0040	0.0578	0.1289	0.1887	0.2926	0.4257	0.4635	0.5076	0.3674	0.4850	0.528	0.5918
ALyR	0.2266	0.5196	0.4938	0.5393	0.5022	0.6035	0.5976	0.6732	0.6059	0.6534	0.6788	0.7861
AScAP	-0.0034	-0.0084	0.0065	0.0134	0.4734	0.7858	0.6279	0.5953	0.6330	0.7416	0.6645	0.6938
AScR	0.1094	0.0833	0.0734	0.0768	0.3982	0.4562	0.4310	0.4537	0.5026	0.5425	0.5301	0.5880
ASeAP	0.0355	0.0685	0.1513	0.2113	0.5166	0.6985	0.5671	0.5729	0.6074	0.6788	0.6217	0.6584
ASeR	0.0535	0.0768	0.1057	0.1579	0.1922	0.2932	0.3521	0.4078	0.2930	0.3883	0.4383	0.5162
ASiAP	0.1559	0.1728	0.1470	0.1429	0.0540	0.0065	-0.025	-0.026	0.2618	0.2281	0.3522	0.3971
ASiR	0.1179	0.1425	0.1472	0.1487	0.0509	0.0514	0.0564	0.0656	0.2249	0.3108	0.4281	0.4350
AMiAP	-0.0059	-0.0059	-0.0049	-0.0119	0.1727	0.5114	0.5874	0.4921	0.5380	0.7822	0.6349	0.6335
AMiR	0.1274	0.1523	0.1299	0.1186	0.2269	0.2297	0.2996	0.3867	0.3814	0.4190	0.4926	0.5327
AWiAP	-0.0146	-0.0029	0.0019	0.0213	0.4080	0.7631	0.6620	0.6472	0.5665	0.7548	0.7193	0.7726
AWiR	0.0674	0.1986	0.2593	0.3013	0.3191	0.4743	0.5014	0.5882	0.4729	0.5753	0.6073	0.7260
ATeAP	0.0709	0.0653	0.0302	0.0159	0.1567	0.1318	0.3113	0.4367	0.2357	0.4708	0.7215	0.6311
ATeR	0.1487	0.0745	0.0506	0.0374	0.2524	0.4794	0.5311	0.4645	0.3003	0.5581	0.5530	0.5546
AGoAP	-0.009	0.0061	0.0045	-0.0064	0.3069	0.4666	0.4739	0.4556	0.4055	0.5533	0.5454	0.5865
AGoR	0.0813	0.0356	0.0086	-0.0119	0.2277	0.2858	0.2880	0.3084	0.4591	0.5193	0.4812	0.4819
ANoAP	0.0678	0.0755	0.0783	0.0633	0.0342	0.4492	0.5917	0.5663	0.1271	0.6027	0.6370	0.6362
ANoR	0.0709	0.1842	0.2573	0.3619	0.1654	0.5588	0.6160	0.6480	0.2397	0.5702	0.6219	0.6635
ASpAP	-0.0200	-0.0025	0.0330	0.0415	0.5259	0.8055	0.6785	0.6537	0.6630	0.8001	0.7342	0.7731
ASpR	-0.0300	-0.0707	0.0994	-0.1174	0.0831	0.2052	0.2104	0.2491	0.2465	0.3619	0.4022	0.5048
5-FU	0.2281	0.2892	0.2859	0.2623	0.2808	0.3553	0.2910	0.2535	0.3197	0.4934	0.5263	0.5516

cell line. Consequently, in our study, most of the studied species, especially *A. gonioccephala*, *A. millefolium*, *A. wilhelmsii* and *A. spinulifolia*, determined to show high cytotoxic activity against HeLa cell line.

3.4. Anticholinesterase, antiurease and antityrosinase inhibition activities

Generally speaking, *Achillea* species determined to show low anticholinesterase activity (Table 3). The highest activity among the species studied was found to be *A. sintensii* (inhibition %: 30.88 ± 1.91) in the inhibition of butyrylcholinesterase enzyme and *A. lycanica* (inhibition %: 11.19 ± 0.18) in the inhibition of the acetylcholinesterase enzyme. In addition, it has been determined that almost all extracts exhibit low and moderate activity in tyrosinase enzyme activity assay. However, it has been found that the extracts studied in urease enzyme activity were highly active. Moreover, AKoR, AScR, ASeR, AWiR, ASpR, ANoR, and ANoAP extracts detected to show 83.30 ± 3.09 , 83.92 ± 2.88 , 85.54 ± 3.03 , 79.70 ± 0.46 , 84.78 ± 2.70 ,

80.89 ± 1.07 and 77.68 ± 1.04 percent inhibition values, respectively. Remarkably, the species studied are generally found to be active in urease enzyme activity assay.

3.5. Chemometric analysis

The chemometric properties of the phenolic components of aerial and root parts of 12 *Achillea* species were determined using PCA and HCA techniques. To better understand the bioactivity interactions of the phenolic compounds, the total phenolic contents (TPh), total flavonoid contents (TFI), antioxidant and enzyme inhibitory activities of these 12 species were also taken into account. β -carotene-linoleic acid test, ABTS cation radical and DPPH free radical scavenging activities and cupric reducing antioxidant capacity (CUPRAC) methods were used to uncover the antioxidant activities of the 24 extracts of twelve *Achillea* species, and the corresponding IC₅₀ values were used in chemometry studies. Enzyme inhibition activities were determined using butyrylcholinesterase inhibition (BIA), tyrosinase inhibition (TIA) and urease inhibition (UIA)

tests, and inhibition values at 200 $\mu\text{g mL}^{-1}$ were used in chemometry analyses.

As a result of PCA analysis of 12 different *Achillea* species with 29 variables; the first two components explain 33.6% of variance, while the first 10 components describe 87.2% of the variance. With the PCA analysis 29 variables were reduced to 10 variables. Figure 3 shows the biplot graphs of PC1 and PC2. As shown in Figure 3, root samples of the species, apart from *A. teretifolia*, *A. lycaonica* and *A. biebersteinii*, form a group. As it can be seen from the biplot graph where the scor and loading graphs were given together, *A. lycaonica* (ALyR and ALyAP) and *A. biebersteinii* (ABiR) samples showed weak ABTS, DPPH and CUPRAC activities and the same samples showed higher BIA. Interestingly, in the biplot graph, it is also seen that quantities of quercetin, salicylic acid, routine and hesperidin were dominant in the mentioned samples. In addition, ASiR, AGoR, AWiR, AMiR, ASeR, ASpR, AScR, AKoR, and ANoR samples belonging only to the root parts formed a group. According to the biplot graph, LPIA and BIA activities of these stem samples were low but ABTS, DPPH, CUPRAC, TIA, UIA activities were high (Figure 3). The same examples were rich in vanillin, caffeic acid, vanillic acid, chlorogenic acid and apigenin. Especially, ANoR, AKoR and AScR extracts that belong to the same group were rich in chlorogenic acid, TPh and TFl contents. Aerial samples were located at the bottom of biplot graph and they formed three different groups as shown in Figure 3. However, The ALyAP sample is outside these three groups. Moreover, naringenin, hesperetin, nicotiflorin, *p*-coumaric acid and luteolin phenolics were more dominant in ASiAP and ABiAP samples. Besides, the LPIA activities of these two samples were higher than the other examples. On the contrary, DPPH, ABTS and CUPRAC activities were lower in these samples.

ANoAP and AKoAP extracts belonged to another group. Apigenin, fumaric acid, quinic acid, and chlorogenic acid were predominant and TPh and TFl contents were high in these samples. Additionally, ASeAP, ATeAP, AGoAP, AMiAP, AWiAP, ASpAP, AScAP samples belonged to another aerial group. Malic acid, isoquercitrin, protocatechuic acid and luteolin were dominant in these samples. Although the ATeR extract is a root sample belonging to *A. teretifolia*, it is present in this last group.

Classification of *Achillea* samples with 24×29 variables was also performed using 3D PC score graphs. Figure 4 shows the 3D scatterplot that belong to PC1, PC2 and PC3. PC1-PC2-PC3 describes the 45.4% of total variance. As shown in Figure 4, ALyR and ALyAP samples behaved differently from other samples. The behavior of the AScR, ANoR and AKoR samples against these variables was very similar. These samples were collected from Sivas, Bilecik,

and Yozgat, respectively. Likewise, the characteristics of ASeR, AGoR, and ASiR samples were similar and they were collected from Sivas, Niğde, and Sivas, respectively.

Figure 5 shows the results of clustering analysis (HCA) of samples of *Achillea* species divided into two parts, root and aerial. Phenolic compounds, total phenolic content, total flavonoid content and various activities were analyzed by clustering to determine similarities between the aerial and root parts of 12 *Achillea* species. Clustering analysis was applied to the analysis results of 29 variables in *Achillea* species. The measurements were based on squared Euclidean distance. Ward method was used as the classification method. Dendrogram obtained by Ward method is given in Figure 5.

Examining Figure 5, it is seen that there are 6 clusters (Similarity 21).

Cluster 1: AKoR and ANoR

Cluster 2: AGoR, ASpR, AScR, AWiR, AMiR, ASeR, ASiR and ABiR

Cluster 3: ATeAP, AMiAP, ASeAP, AWiAP, AScAP, ANoAP, ATeR, AGoAP, ASpAP and AKoAP

Cluster 4: ALyR

Cluster 5: ALyAP

Cluster 6: ABiAP and ASiAP

The HCA analysis results are consistent with the results of the PCA analysis and support each other.

4. Conclusion

Natural bioactive molecules act as a notable role in the management of major health problems such as cancer and Alzheimer's disease. Modern scientific studies on those natural agents have acquired great interest recently (Zengin et al., 2017a). In this regard, the present study was planned to investigate the chemical and biological properties of *Achillea* species. Within this context, 24 ethanol extracts from the root and aerial parts of 12 *Achillea* species (*A. kotschyi*, *A. biebersteinii*, *A. lycaonica*, *A. millefolium*, *A. setacea*, *A. schischkinii*, *A. sintenisii*, *A. wilhelmsii*, *A. teretifolia*, *A. gonioccephala*, *A. nobilis*, *A. spinulifolia*) were screened by LC-MS/MS for phytochemical content and a number of biological parameters.

In terms of biological activity and chemical content, various parts of the species have been found to have quite distinct effects and contents. For example, in this study it was determined that the extracts prepared from root portions of *Achillea* species were biologically more active. Furthermore, according to the results of LC-MS/MS, it appears that there were differences in chemical composition. Thus, different utilization of extraction methods and solvents ought to be tested on the species to be studied and different parts of the species should be separately investigated in phytochemistry studies.

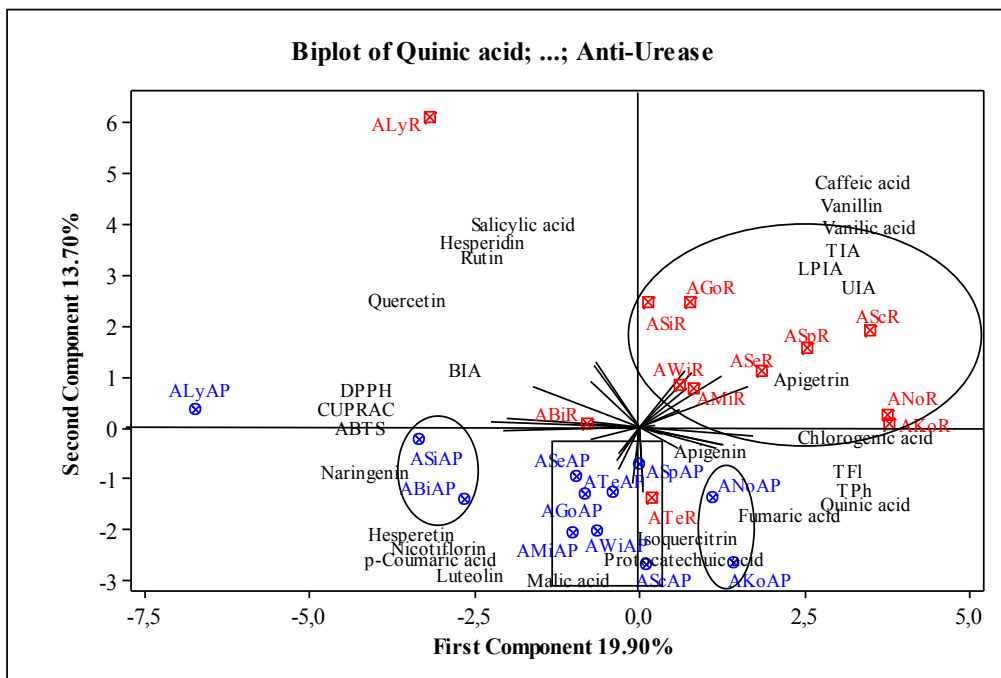


Figure 3. PC1 and PC2 biplot graphs of *Achillea* samples, ⊗ Aerial, ⊠ Root.

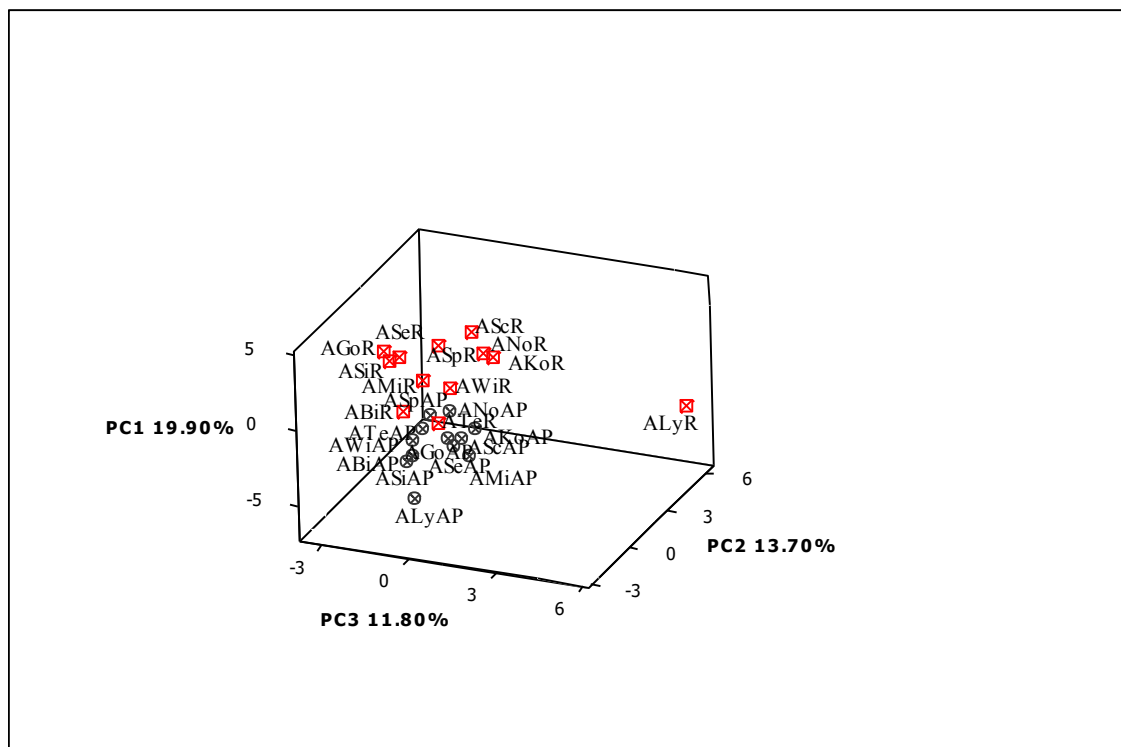


Figure 4. 3D scatterplot that belongs to PC1, PC2, and PC3 for *Achillea* samples, ⊗ Aerial, ⊠ Root.

The species studied have been found to possess a particularly high antioxidant and cytotoxic potential. It has also been determined that these species are rich in fumaric, chlorogenic, vanillic acids and flavonoids such as rutin,

hesperidin, isoquercitrin, apigenin, luteolin and apigenin. It can even be said that some species have the potential to be a source for some components. In this context, from the obtained results, *Achillea* species, which are natural sources

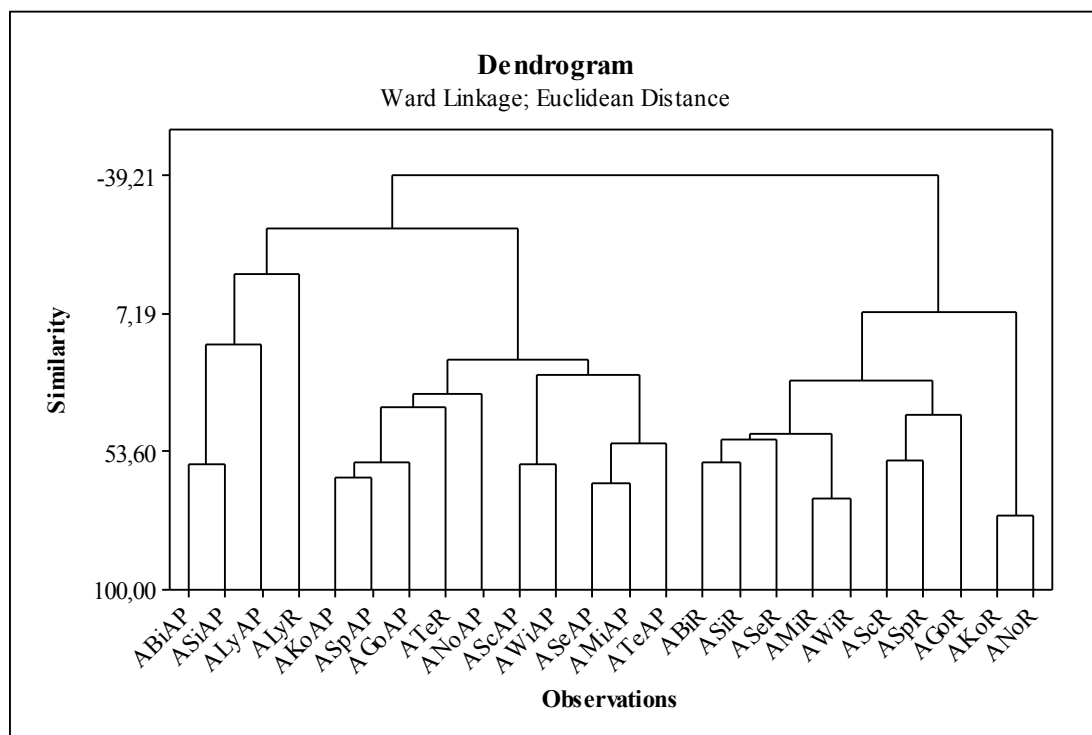


Figure 5. Dendrogram results obtained from Euclidean distance and Ward linkage method.

of bioactive agents, have a high potential to develop novel nutraceutical and functional products. In this context, the current study might be considered as a starting point for these species. Nevertheless, additional research is required to evaluate the toxic effects of those species.

Supporting information file: The chemometric evaluation of antioxidant activities and phenolic composition of the samples (S1), abbreviations used in the manuscript (S2), methods of the biological activity studies including total flavonoid and phenolic contents (S3), antioxidant activities by CUPRAC, ABTS, and DPPH methods (S4), cytotoxic activities against HeLa cell lines (S5), cholinesterase (S6), urease (S7), tyrosinase (S8) inhibitory activities of the studied samples and the LC-MS/MS TIC chromatograms of the extracts except from

ABiAP and AbiR (Figure S1) were given in the supporting information file.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting information

†Supporting information: The chemometric evaluation of antioxidant activities and phenolic composition of the samples (S1), abbreviations used in the manuscript (S2), methods of the biological activity studies including total flavonoid and phenolic contents (S3), antioxidant activities by CUPRAC, ABTS, and DPPH methods (S4), cytotoxic activities against HELA cell lines (S5), cholinesterase (S6), urease (S7), tyrosinase (S8) inhibitory activities of the studied samples and the LC-MS/MS TIC chromatograms of the extracts except from ABiAP and ABiR (Figure S1) were given in the supporting information file.

S.1. The chemometric analysis

The chemometric analyses of antioxidant and phenolic contents of roots and aerial parts of *Achillea* species were carried out using principal component analysis (PCA) and hierarchical clustering analysis (HCA), which are multivariate data analysis methods. Both methods for clustering and classification are mainly based upon the principal component analysis. PCA reduces multiple variables into a set of fewer components created by their linear combinations by hindering correlations between those examined variables. PCA-based methods can classify the samples by clustering into various groups. Hierarchical clustering analysis (HCA) classifies samples in a given data set and defines those data according to their similarities. HCA can be applied directly to the original variables, as well as possible to be applied to the results obtained from PCA, in case of existing too many variables. In this study, HCA applied to the analysis of LC-MS/MS, antioxidant (β -carotene, ABTS, DPPH and CUPRAC), and enzyme inhibition (butyrylcholinesterase, urease and tyrosinase) results. The measurement is based on the Euclidean distance. The Ward's method was used as a clustering method. In this context, all classification and clustering analyses for *Achillea* species were carried out using MINITAB Statistical Software.

The total phenolic-flavonoid contents, antioxidant, cytotoxic, urease inhibition, tyrosinase inhibition and anticholinesterase activity assay results were shown as means \pm standard deviation. The results were evaluated using an unpaired *t*-test and one way analysis of variance ANOVA. The differences were regarded as statistically significant at $p < 0.05$.

S.2. Abbreviations used

DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), CUPRAC: Cupric reducing antioxidant capacity, LC-MS/MS: Liquid chromatography-tandem mass spectrometry, PCA: Principal component analysis, HCA: Hierarchical clustering analysis, HPLC: High performance liquid chromatography, BHT: Butylated hydroxytoluene, BHA:

Butylated hydroxyanisole, DTNB: 5,5'-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent), NADH: Nicotinamide adenine dinucleotide, PMS: Phenazine methosulfate, AChE: Acetylcholinesterase, BChE: Butyrylcholinesterase, EDTA: Ethylenediaminetetraacetic acid, DMEM: Dulbecco's modified Eagle's medium, LOD: Limit of detection, LOQ: Limit of quantitation, HeLa: The first continuously cultured human malignant cell line, derived from the cervical carcinoma of Henrietta Lacks, SP: Single plate, RTCA: Real-time cell analyzer, L-DOPA: L-3,4-dihydroxyphenylalanine, OD: Optical density, SEM: Standard error of the mean, ANOVA: Analysis of variance, α -TOC: α -tocopherol, GAE: Gallic acid equivalent, QE: Quercetin equivalent, μ g: microgram, mg: milligram, IC_{50} : The half maximal inhibitory concentration, MCF7: Breast cancer cell line isolated in 1970 from a 69-year-old Caucasian woman, HepG2: Perpetual cell line derived from the liver tissue of a Caucasian American male, A549: Adenocarcinomic human alveolar basal epithelial cells, PC1: First principal direction, PC2: Second most important direction, PC3: Third most important direction, TPh: Total phenolic content, TFl: Total flavonoid content, BIA: Butyrylcholinesterase inhibition analysis, TIA: Tyrosinase inhibition analysis, UIA: Urease inhibition analysis, LPIA: Latent pathway identification analysis, ABiAP: *Achillea biebersteinii* aerial parts, ABiR: *Achillea biebersteinii* root, AKoAP: *Achillea kotschyi* aerial parts, AKoR: *Achillea kotschyi* root, ALyAP: *Achillea lycaonica* aerial parts, ALyR: *Achillea lycaonica* root, AScAP: *Achillea schischkinii* aerial parts, AScR: *Achillea schischkinii* root, ASeAP: *Achillea setacea* aerial parts ASeR: *Achillea setacea* root, ASiAP: *Achillea sintenisii* aerial parts, ASiR: *Achillea sintenisii* root, AMiAP: *Achillea millefolium* aerial parts, AMiR: *Achillea millefolium* root, AWiAP: *Achillea wilhelmsii* aerial parts, AWiR: *Achillea wilhelmsii* root, ATeAP: *Achillea teretifolia* aerial parts, ATeR: *Achillea teretifolia* root, AGoAP: *Achillea goniocephala* aerial parts, AGoR: *Achillea goniocephala* root, ANoAP: *Achillea nobilis* aerial parts, ANoR: *Achillea nobilis* root, ASpAP: *Achillea spinulifolia* aerial parts, ASpR: *Achillea spinulifolia* root, 5-FU: 5-fluorouracil.

S.3 Determination of total phenolic and flavonoid contents

Phenolic and flavonoid contents expressed as pyrocatechol and quercetin equivalents, respectively, were determined as reported in the literature (Moreno et al., 2000; Slinkard and Singleton, 1977). The following equations were used to calculate total phenolic and flavonoid contents of the extracts:

$$\text{Absorbance} = 0.0498 + 0.0434 \text{ pyrocatechol } (\mu\text{g}) \quad (r^2 = 0.9918)$$

$$\text{Absorbance} = 0.0535 + 0.0748 \text{ quercetin } (\mu\text{g}) \quad (r^2 = 0.9960)$$

S.4 Antioxidant activity

β -carotene-linoleic acid test system, DPPH free radical and ABTS cation radical scavenging activities and cupric reducing antioxidant capacity (CUPRAC) methods were used to determine the antioxidant activity of the *Achillea* extracts (Apak et al., 2004; Blois, 1958; Miller, 1971; Re et al., 1999; Yilmaz, 2018).

S.5 Cytotoxic activity

The xCELLigence real-time cell analyzer-single plate (RTCA-SP) instrument (Roche Applied Science, Basel, Switzerland) was used to visualize the cytotoxic effects of the *Achillea* extracts on human cervical cancer (HeLa) cells. The instrument is a combination of four parts: an E-Plate 96, a single plate (SP) station that is kept in an incubator and holds the E-Plate 96, an analyzer and a computer with RTCA software. The wells of the E-Plate 96 have an inner volume of $243 \pm 5 \mu\text{L}$ and their bottoms are coated with golden electrodes (Urcan et al., 2010). The system measures impedance differences in order to derive cell index values at time points whose intervals can be set by the operator. These impedance differences and thus the cell index values depend on the cell activity at the bottom of the wells. The higher the cell population growing at the bottom and the greater the spreading of the cells, the higher is the cell index value. This system allows the user to analyze cell behavior in a label-free environment and produces a real-time profile of the cells (Işık et al., 2012).

DMEM containing 10% fetal bovine serum and 2% streptomycin-penicillin was used as the medium and incubated at 37 °C in an incubator (5% CO₂). real-time cell analyzer-single plate was added to each of the 96 wells of the E-plate to form an incubation chamber. The E-plate 96 plates were then placed in the xCELLigence station and background impedance measurements were taken for 1 min. Then, 50 μL of each cell suspension was placed in wells containing medium and adjusted to 20,000 HeLa cells mL⁻¹. For attachment of the cells to E-plate 96 wells, plates were left in sterile cabinet for 30 min at room temperature. Finally, HeLa cells were observed every 10 min for 3 h with the internal sensor electrode sequences of E-Plate 96 for binding, growth and proliferation. The extracts dissolved in DMSO were added to the plate wells at concentrations of 50, 100 and 250 $\mu\text{g mL}^{-1}$ and the final volume was adjusted to 200 μL . Plates were then immediately incubated and observed every 10 min for 48 h. All the measurements were performed in triplicates. 5-FU (5-fluorouracil) was used as a positive control in this assay against HeLa cells. The 5-FU solution prepared at 4 different concentrations (250, 100 and 50 $\mu\text{g/mL}$) was added to the wells as three replicates diluted (1:20) with DMEM. The antiproliferative

effect of 5-FU and the studied extracts against HeLa cells were examined for 48 h and the results obtained were given in Figure 2.

S.6 Anticholinesterase activity

A spectrophotometric method developed by Ellman et al. (1961) was used to indicate the acetyl- and butyrylcholinesterase inhibitory activities (Ellman et al., 1961).

S.7 Urease inhibitory activity

Urease inhibitory activity of the extracts of *Achillea* species was determined according to the reported protocol (Zahid et al., 2015). Final volume of reaction mixture was 200 mL at pH 8.2. 25 mL of urease (Jack bean) solution was mixed with 10 mL of each extract (4000 mg mL⁻¹) and incubated at 30 °C for 15 min. Aliquots were taken and immediately transferred to assay mixtures containing urea (100 mM) in buffer (50 mL) and reincubated for 30 min in 96-well plates. 45 mL each of phenol reagent (1% w v⁻¹ phenol and 0.005% w v⁻¹ sodium nitroprusside) and 70 mL of alkali reagent (0.5% w v⁻¹ sodium hydroxide NaOH and 0.1% sodium hypochlorite NaOCl) were added to wells. Increase in absorbance value was measured after 50 min at 630 nm against blank. All reactions were performed in triplicates. Thiourea was used as positive control. The percentage inhibition was determined by using the following equation:

$$\text{Urease inhibition (\%)} = 100 - (\text{OD test well OD}^{-1} \text{ control}) \times 100 .$$

S.8 Tyrosinase inhibitory activity

Tyrosinase inhibition assays were performed according to the method Hearing's protocol (Hearing and Jimenez, 1987). Briefly, the extracts were screened for the *o*-diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. All the extracts were dissolved in methanol to reach to a concentration of 4000 mg mL⁻¹. 150 μL of phosphate buffer (pH = 6.8), 10 μL of the ethanol extracts of the extracts and 20 μL of the enzyme solution were added to the wells in the microplate, and the initial absorbance at 475 nm was read after stirring for 3 min. This solution was then incubated for 10 min at 37 °C, after 10 min 20 μL of L-DOPA was added and incubated again at 37 °C, after 10 min the final absorbance at 475 nm was read in the Microplate ELISA reader. Tyrosinase activity (% inhibition) was calculated using the following equation.

$$\text{Tyrosinase inhibition (\%)} = 100 - (\text{OD test well OD control}^{-1}) \times 100 .$$

All the experiments were carried out at least in triplicate and the results represent means \pm SEM (standard error of the mean). Kojic acid was used as a standard inhibitor for the tyrosinase inhibition (Khan et al., 2006).

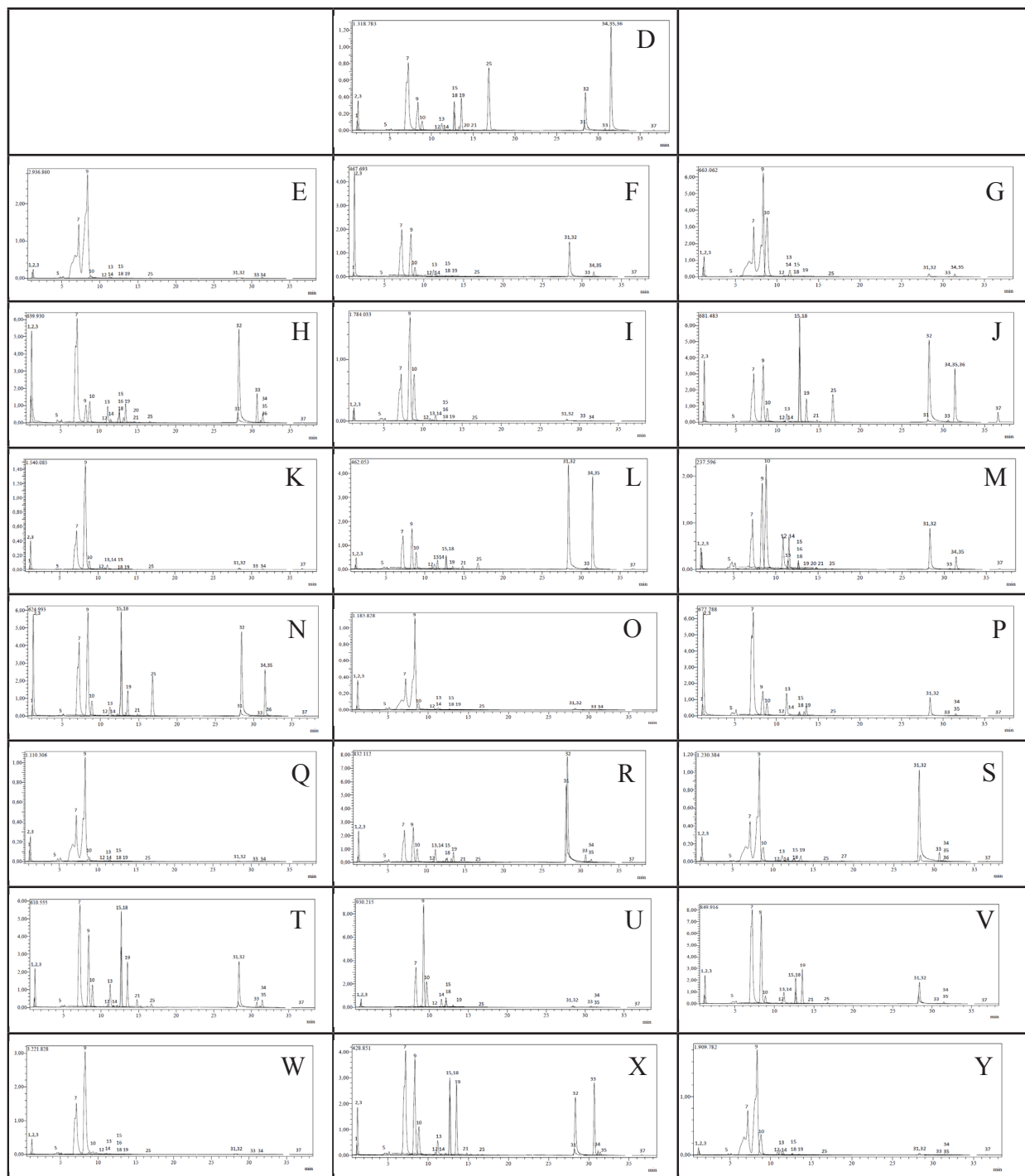


Figure S1. LC-MS/MS TIC chromatograms of B: ABiAP, C: ABiR, D: AKoAP, E: AKoR, F: ALyAP, G: ALyR, H: AScAP, I: AScR, J: ASeAP, K: ASeR, L: ASiAP, M: ASiR, N: AMiAP, O: AMiR, P: AWiAP, Q: AWiR, R: ATeAP, S: ATeR, T: AGoAP, U: AGoR, V: ANoAP, W: ANoR, X: ASpAP, Y: ASpR