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Characterization of phenolic compounds, total phenolic content and antioxidant activity of three *Achillea* species

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ABSTRACT: Medicinal plants used in traditional medicine have been increasingly noticed within recent years in pharmaceutical, cosmetic and nutraceutical industry. Herein, methanolic extracts of *Achillea biebersteinii* Afan., *A. setacea* Waldst. Et Kit. and *A. wilhelmsii* C. Koch were investigated for their total phenolic content and antioxidant activity with the main focus on phenolics. The total phenolic content varied widely in different parts of the three tested *Achillea* species, ranging from 113.2±1.9 to 178.4±9.5 mg GAE/g extract using Folin Ciocalteu method. DPPH and ABTS antioxidant activity tests were used to evaluate and compare the antioxidant activity of the species. Both the DPPH and ABTS assay results revealed that the radical scavenging activity of *A. biebersteinii* leaf extract is remarkably high with IC_{50} values of 0.377±0.011 and 0.016±0.002 mg/mL, respectively. Furthermore, a newly validated RP-HPLC-DAD method was developed and used to determine the phenolic compound profile of the methanol extracts. Phenolic components, such as gallic acid, chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, rutin, quercetin, luteolin, apigenin and kaempferol were analyzed by HPLC-DAD. The significant antioxidant properties of the extracts could be attributed to the phenolics of the *Achillea* species.

KEYWORDS: ABTS; *Achillea*; DPPH; HPLC-DAD; phenolic compounds.

1. INTRODUCTION

Latin name of *Achillea* comes from the name of Achilles, who healed the wounds by means of *Achillea* herb at the time of the Trojan War. The genus *Achillea* (Asteraceae) comprises more than 100 species widespread in Northern hemisphere. *Achillea* species have been characterized by a high content and diversity of terpenes, flavonoids, coumarins, phenolic acids, lignans and essential oil some of which are responsible for the antioxidant, estrogenic, antispermatogenic, antiulcerogenic, antimicrobial, antiviral, antispasmodic, immunosuppressive, antitumor and antidiabetic activities [1, 2]. Various species of the genus are traditionally used in Turkey for wound healing, against diarrhea and flatulence, as a diuretic, as emmenagogue agents, and for abdominal pain [3, 4].

The chemical activity of phenolic compounds in terms of their reducing properties as hydrogen or electron-supplying agents displays their potential for action as free-radical scavengers. The commercial development of plants as sources of antioxidants which can be used to enhance the properties of foods, cosmetics and pharmaceuticals for both nutritional purposes and for prevention of diseases, is currently of high interest. Various epidemiological investigations have shown an inverse relationship between the intake of natural antioxidants and the frequency of chronic diseases such as coronary heart disease and certain cancers [5-7].

The need for such a study is especially for quantifying the antioxidant constituents of different parts of three *Achillea* taxa which should contribute positively to the above mentioned activities together with evaluating the antioxidant potential. Therefore, the aim of this study is to evaluate the total phenolic content and antioxidant activity of flowers, leaves and roots of three *Achillea* species, as well as to determine the phenolic components such as gallic acid, chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, rutin, quercetin, luteolin, apigenin and kaempferol qualitatively and quantitatively using a new developed and validated HPLC-DAD method.

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2. RESULTS

2.1. Total phenolic content

Total phenolic content of the *Achillea* species estimated using Folin Ciocalteu reagent was expressed as milligrams of gallic acid equivalents (GAE). Table 1 summarizes that total phenolic content of the different parts of the species varied widely, ranging from 113.2±1.9 and 178.4±9.5 mg GAE/g extract. *A. biebersteinii* leaf extract exhibited the highest total phenolic content together with *A. setacea* leaf and root extract and *A. wilhelmsii* leaf extract.

2.2. DPPH radical scavenging activity

The IC_{50} values of scavenging DPPH radicals for all the *Achillea* extracts were varying in the range of 377-1500 µg/mL. Although the antioxidant potential of the extracts was found to be lower than Trolox (a water soluble vitamin E analogue), *A. biebersteinii* leaf extract showed apparent DPPH radical scavenging activity *A. setacea* leaf and root extracts exhibited prominent radical scavenging activity subsequent to *A. biebersteinii* leaf extract.

2.3. ABTS radical scavenging activity

All the *Achillea* ectracts scavenged ABTS radical in a concentration-dependent way (IC_{50}:16-432 µg/mL) and the results were given in Table 1. Leaf extract of *A. biebersteinii* exhibited prominent ABTS radical scavenging activity with an IC_{50} of 16 µg/mL which was found to be lower than Trolox (IC_{50}:43 µg/mL). In parallel, the highest total phenolic content was determined for *A. biebersteinii* leaf extract, for which the lowest IC_{50} value was obtained. Though the total phenolic content of *A. biebersteinii* leaf extract was found so high among the investigated species, this significant radical scavenging activity should be attributed to phenolics. On the other hand, the least ABTS radical scavenging activity was obtained from the flower extract of *A. setacea* with an IC_{50} of 432 µg/mL.

Table 1. Total phenol content and IC_{50} values of methanol extracts of three *Achillea* species according to DPPH and ABTS assays (Trolox IC_{50} (mg/mL): 0.042 for DPPH and 0.043 for ABTS methods).

	Species	Folin Ciocalteu (mgGAE/g extract)	DPPHIC_{50} (mg/mL)	ABTSIC_{50} (mg/mL)
Flower	***A.biebersteinii***	131.4 ± 2.5	0.670 ± 0.044	0.138 ± 0.024
	A. setacea	121.2 ± 4.4	0.947 ± 0.023	0.432 ± 0.003
	A. wilhelmsii	119.4 ± 1.4	1.500 ± 0.024	0.329 ± 0.009
Leaf	***A. biebersteinii***	178.4 ± 9.5	0.377 ± 0.011	0.016 ± 0.002
	A. setacea	167.9 ± 3.5	0.470 ± 0.002	0.144 ± 0.005
	A. wilhelmsii	155.5 ± 8.7	0.812 ± 0.013	0.214 ± 0.001
Root	***A. biebersteinii***	113.2 ± 1.9	0.773 ± 0.053	0.279 ± 0.007
	A. setacea	154.1 ± 5.6	0.497 ± 0.013	0.143 ± 0.004
	A. wilhelmsii	136.4 ± 8.8	0.991 ± 0.017	0.184 ±0.011

2.4. RP-HPLC-DAD Analysis

To precisely and exactly analyze six phenolic compounds in three *Achillea* species, a suitable HPLC method was established. In general, reverse phase columns were used to assay phenolic compounds in natural products. So that, we selected ACE 5 µ C18 (150 X 4.60 mm) column which is an efficient preference for separation of polar compounds. The mobile phase consisting of water (% 0.2 trifloroacetic acid) pH: 2.4 A, Acetonitrile : Methanol (80:20 v/v) B was tested among various gradient systems and an adequate gradient ratio was selected. The composition of the gradient was (A:B), 95:5 at 0 min, 85:15 at 5 min, 85:15 at 15 min, 80:20 at 20 min, 70:30 at 25 min, 65:35 at 30, 50:50 at 35 min and 95:5 at 39 min. The UV wavelength of the DAD detector was set at 230, 330, 360, 340and 280 nm. Most of the absorbed UV wavelength of each compound was selected in the UV spectrum. Chlorogenic and caffeic acids were analyzed at 330 nm, luteolin was measured at 340 nm, rutin, quercetin and apigenin were analyzed at 360 nm. The peak of each compound was confirmed by comparing the retention time and UV spectrum of each analyzed compound with authentic ones. Moreover, authentic compounds were added to the extracts and the increase in the peaks were observed.

Retention times for peaks of chlorogenic acid, caffeic acid, rutin, quercetin, luteolin and apigenin were 8.0, 8.9, 20.3, 29.2, 29.48, 32.66 min, respectively (Figure 1). HPLC chromatograms of authentic compounds and extracts were given in Figure 2-4.

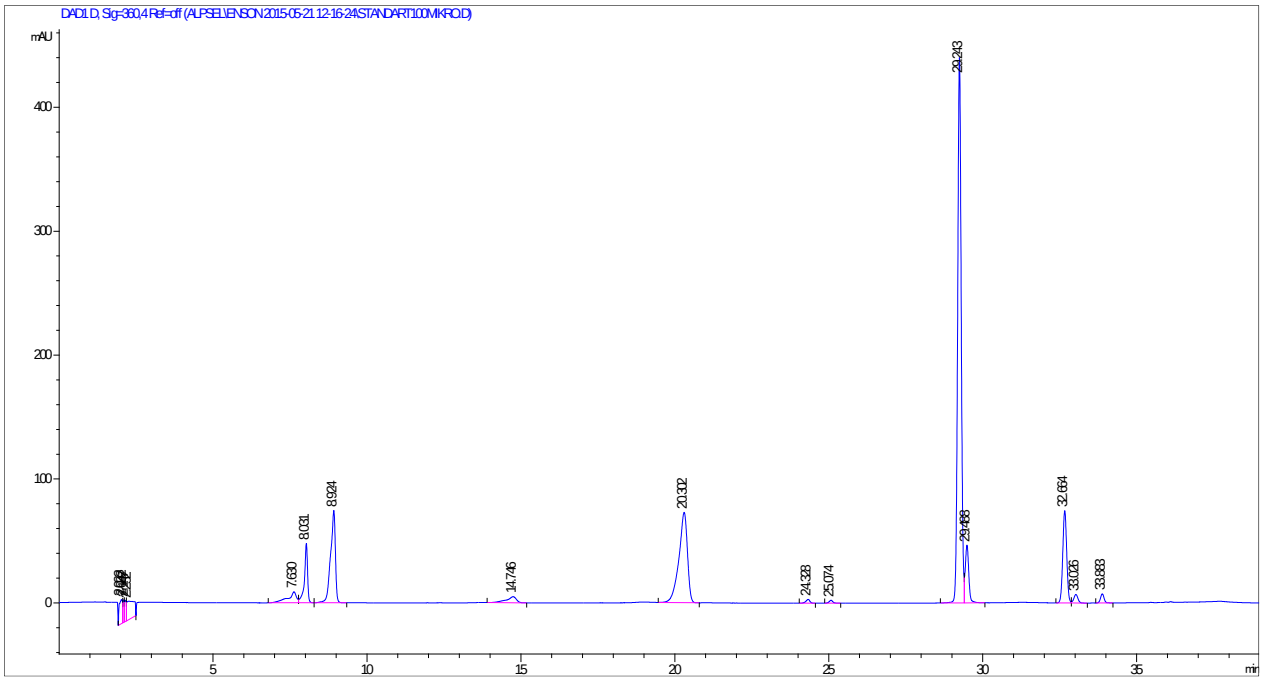

Figure 1. HPLC chromatogram of authentic phenolic compounds: Chlorogenic acid 8.0 min, caffeic acid 8.9 min, rutin 20.3 min, quercetin 29.24 min, luteolin 29.48 min, apigenin 32.66 min.

This new method was validated and fine results were obtained. For the method validation, linearity was confirmed by the correlation coefficient (R^2). To calculate the regression equation, five different concentrations of the standard solutions were used to establish calibration curves. The regression equation formed was Y = ax + b (a - slope of the calibration curve, b - intercept of the calibration curve); Y axis was the value of peak area and X axis was the concentration of phenolic compounds. The correlation coefficient of six phenolic compounds showed good linearity (R^2>0.9977). The RSD values for intra-day variations (repeatability) for chlorogenic acid, caffeic acid, rutin, quercetin, luteolin and apigenin were 2.54%, 1.40%, 0.93%, 2.23%, 3.25%, 2.29%, respectively. The RSD values for inter-day variations (intermediate precision) for chlorogenic acid, caffeic acid, rutin, quercetin, luteolin and apigenin were 5.98%, 6.16%, 2.53%, 4.67%, 6.13%, 3.72%, respectively. The recovery values were given in paranthesis for phenolic compounds: chlorogenic acid (88.9-98.8%), caffeic acid (93.3-112%), rutin (82.6-99.9%), quercetin (93.4-111.8%), luteolin (84.8-98.8%) and apigenin (94.6-97%). Retention times, linear relationships between peak areas and concentrations, test ranges, LOD and LOQ values were given in Table 2. The content of phenolic compounds in different parts of *Achillea* species were given in Table 3.

Table 2. Retention times, linear relationships between peak areas and concentrations, test ranges, LOD and LOQ.

Analyte	Retention time (min)	Standard curve	R^2	Test range (µg/mL)	LOD[a] (µg/mL)	LOQ[b] (µg/mL)
Chlorogenic acid	8.0	y=19008x+5.2795	0.9997	0.06-333	0.0198	0.066
Caffeic acid	8.9	y=62629x+0.565	0.9977	0.03-166	0.0111	0.037
Rutin	20.3	y=15129x-39.937	0.9991	6.6-333	0.0066	0.022
Quercetin	29.24	y=41336x-6.5413	0.9993	0.052-333	0.0156	0.052
Luteolin	29.48	y=40892x-16.331	0.9998	0.8-40	0.0195	0.065
Apigenin	32.66	y=41303x-25.856	0.9995	0.04-66	0.0132	0.044

y, peak area; x, concentration of analyte (mg/mL); [a]LOD= limit of detection S/N:3 (n=9); [b]LOQ= limit of quantification S/N:10 (n=9)

Table 3. The content of phenolic compounds in *Achillea* species.

		Content (g/100g dw)					
	Species	**Chlorogenic acid**	**Caffeic acid**	**Rutin**	**Quercetin**	**Luteolin**	**Apigenin**
Flower	***A.biebersteinii***	0.0519±0.0012	0.0008±0.0004	0.0205±0.0024	0.0035±0.0001	0.0240±0.0002	0.0044±0.0001
	A.setacea	0.0707±0.0039	Nd	0.0169±0.0004	Nd	0.0293±0.0004	0.0044±0.0001
	A.willhelmsii	0.0696±0.0009	0.0021±0.0001	0.0307±0.0015	0.0036±0.0001	0.0076±0.0001	0.0034±0.0001
Leaf	***A.biebersteinii***	0.1094±0.0048	0.0006±0.0001	0.0527±0.0058	0.0017±0.0001	0.0042±0.0001	Nd
	A.setacea	0.1937±0.0003	0.0007±0.0001	0.1358±0.0033	0.0018±0.0001	0.0053±0.0001	0.0041±0.0001
	A.willhelmsii	0.2745±0.0116	0.0013±0.0001	0.0202±0.0011	Nd	Nd	Nd
Root	***A.biebersteinii***	0.1139±0.0004	0.0018±0.0004	Nd	Nd	Nd	Nd
	A.setacea	0.4778±0.0213	0.0083±0.0010	Nd	Nd	Nd	Nd
	A.willhelmsii	0.1328±0.0125	0.0007±0.0001	Nd	Nd	Nd	Nd

Nd: Not dedected; dw:dry weight

3. DISCUSSION

A rich complex of biologically active compounds is responsible for the wide spectrum of biological activities of *Achillea* species [8]. In the last decades, interest in the antioxidant properties of *Achillea* genus is increasing, so that the assessment of total phenolic content and radical scavenging activity of these plants are of high importance.

Several *in-vitro* methods have been used to present the antioxidant activity of plant extracts and pure compounds in order to set light and plan *in-vivo* experiments [6]. In our study, DPPH and ABTS radical scavenging assays were preferred to determine the antioxidant activity of *Achillea* species. The radical scavenging potential of the extracts can be measured by evaluating 2,2'-diphenyl-1- picrylhydrazyl radical (purple-coloured) bleaching. The degree of bleeching is directly proportional to the potency and concentration of the antioxidants. Herein, among all the investigated species, *A. biebersteinii* leaf extract showed apparent DPPH radical scavenging activity together with *A. setacea* leaf and root extracts. In ABTS radical scavenging assay, a blue/green $ABTS^{+}$ chromophore occurs via the reaction of ABTS and $K_2S_2O_8$. The reduction of the ABTS radical cation in the presence of hydrogen-donating antioxidants is measured spectrophotometrically at 734 nm. In this study, leaf extract of *A. biebersteinii* exhibited prominent ABTS radical scavenging activity with an IC_{50} of 16 µg/mL. *A. biebersteinii* leaf extract was found to possess notable radical scavenging activity with both methods, and total phenolic content of the *A. biebersteinii* leaf extract was found very high among all the investigated extracts supporting our results that this activity could be due to the phenolics.

Phenolic compounds can be classified in several categories such as phenolic acids, flavonoids etc. Phenolic acids and flavonoids are naturally occuring secondary metabolites of the plants and most of the studies have shown their positive effects on public health. Phenolic compounds are supposed to be highly effective scavengers of most oxidizing molecules [9, 10]. Our results suggested that phenolic acids and flavonoids may be the major contributors for the radical scavenging activity of the *Achillea* extracts.

To present the active principles responsible for the antioxidant activity, reverse phase HPLC-DAD method was developed and validated. For the best separation, different combinations of solvent systems consisting of water, methanol and acetonitrile were tried using various flow rates. For method validation, linearity, precision, test range, detection and quantification limits and recovery values were calculated. In approximetaly 35 minutes, two phenolic acids and 4 flavonoids were well separated and quantified by this newly developed method. Other compounds such as gallic acid, *p*-coumaric acid, ferulic acid and kaempferol were not detected in any of the investigated species. According to our HPLC results, rutin was determined in significant amount in *A. setacea* leaves while chlorogenic acid was one of the most abundant compound in the roots. Flavonoids were not detected in any of the investigated root samples.

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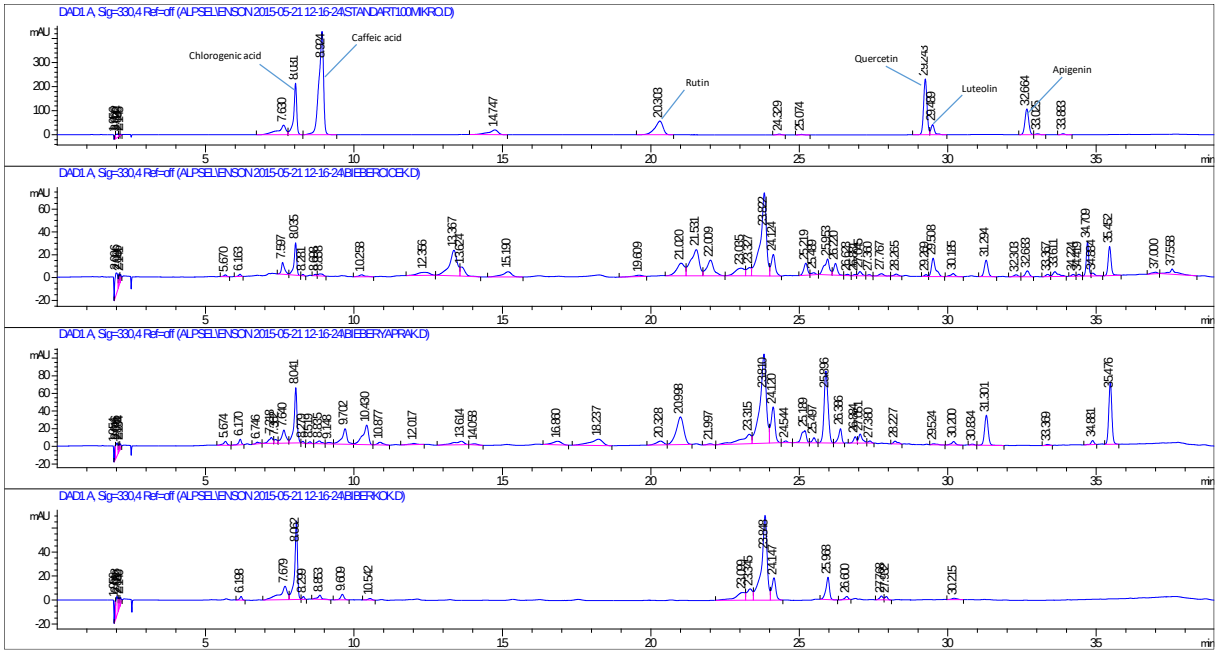

Figure 2. HPLC chromatograms of authentic phenolic compounds, and flower, leaf and root of *A. Biebersteinii.*

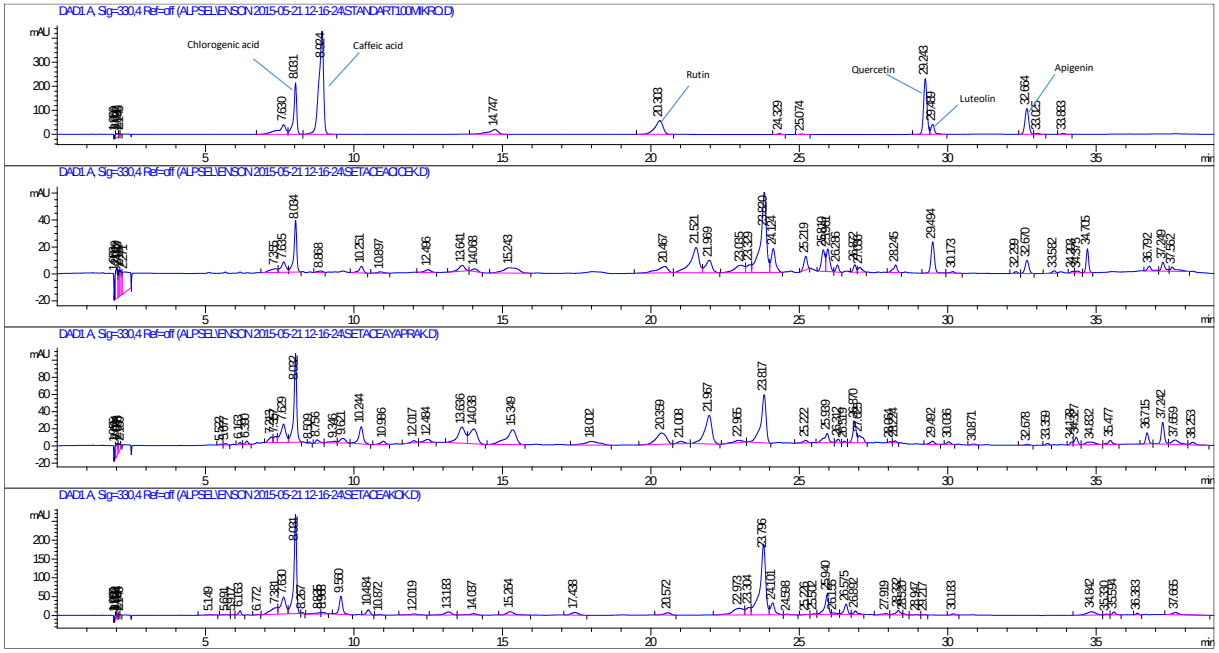

Figure 3. HPLC chromatograms of authentic phenolic compounds, and flower, leaf and root of *A. setacea.*

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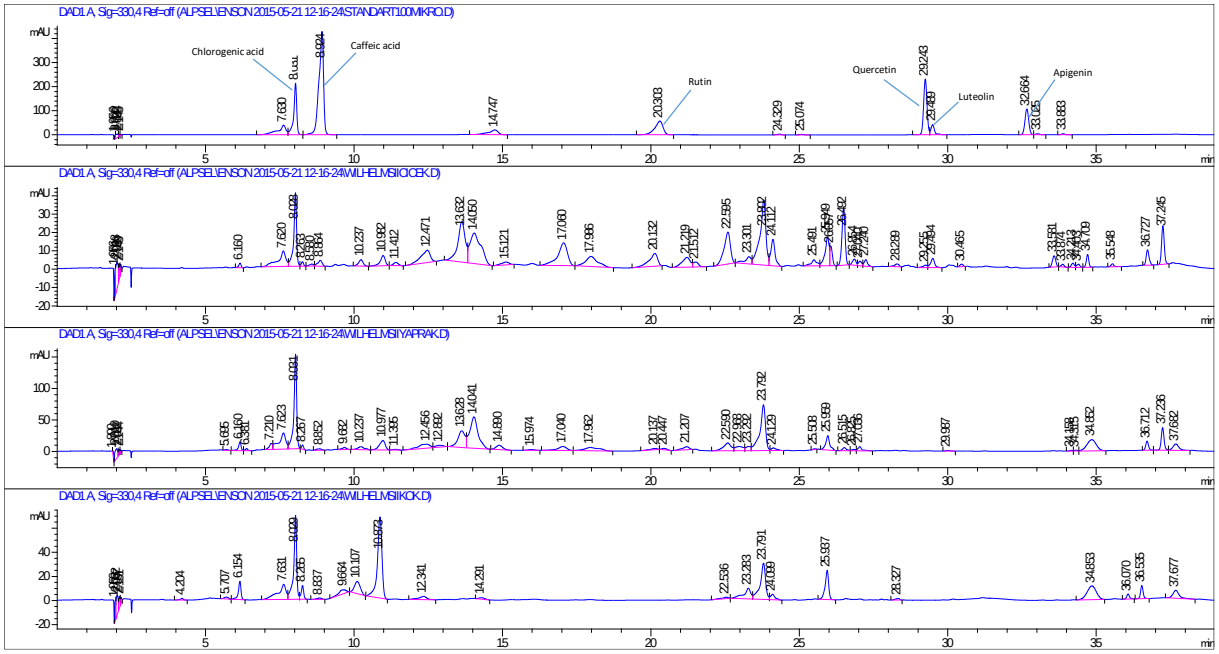

Figure 4. HPLC chromatograms of authentic phenolic compounds, and flower, leaf and root of *A. wilhelmsii*.

Researchers are mostly focused on *A. millefolium* and some other taxa of *Achillea* genus, so that some of the antioxidant activity assay results and phenolic composition of the species were summarized to see the distinction among the *Achillea* plants. Investigations on *A. millefolium* were summarized: Benetis et al. [8] quantified some phenolic compounds in *A. millefolium* and the results revealed that chlorogenic acid amount was 2.06 g/100g, rutin was 0.27 g/100g, luteolin was 0.015 g/100g and apigenin was 0.008 g/100g in the leaves of yarrow. Bobis et al. [11] determined total phenolic content and phenolic composition of *A. millefolium* and the results exhibited that chlorogenic acid amount was 0.001 g/100g, rutin was 0.14 g/100g and luteolin was 0.09 g/100g. The total phenolic content was determined as 134.65 ± 9.52 mg GAE/g dry weight of plant. Trumbeckaite et al. [12] analyzed the phenolic components of *A. millefolium*, and the results showed that chlorogenic acid (1.93 ± 0.13 g/100g) predominated in the mixture of identified secondary metabolites. Regarding the composition of the flavonoid complex, the pattern of distribution in *A. millefolium* was characterised by the dominance of apigenin (1.42 ± 0.04 g/100g) and luteolin (1.02 ± 0.03 g/100g), whereas their glucosides were determined in considerably lower quantities. Furthermore, rutin (0.32 ± 0.01 g/100g) was also found to be minor components amongst the identified flavonoids. Raudonis et al. [13] investigated some compounds in the extracts of *A. millefolium* that possessed radical-scavenging properties, and reported these compounds as chlorogenic acid, luteolin-7-O-glucoside, rutin, and luteolin. Vitalini et al. [14] investigated the phenolic composition and antioxidant activity of the methanol extract of *A. millefolium*, and determined the antioxidant compounds as chlorogenic acid, rutin, luteolin and apigenin glycosides. Investigations on *A. biebersteinii* and *A. wilhelmsii* were summarized and compared to our results: Fathi et al. [2] investigated antioxidant activity and phenolic content of the methanol extracts of *A. wilhelmsii* aerial parts and IC_{50} for DPPH radical-scavenging activity was revealed as 58.9 ± 2.7 µg/mL and the total phenolic content was determined as 37.4 ± 0.3 mg GAE/g of extract. These findings are too low when compared to our results. Bashi et al. [15] investigated antioxidant activity and total phenolic contents of *A. biebersteinii* and *A. wilhelmsii* methanol extracts with different extraction methods and reported the total phenolic contents of the species in the ranges of 20.16-108.54 and 17.18-59.61 mg GAE/g extract, respectively. Ultrasonic extraction results are close to our findings. Baris et al. [16] investigated the antioxidant activity and total phenolic content of the ethanol extract of *A. biebersteinii* aerial parts and indicated that the IC_{50} for DPPH radical-scavenging activity was 33 µg/mL, which was too low compared to our flower, leaf and root extract results. Total phenolic content was determined as 134 mg GAE/g extract, which was close to our results. Ashgar et al. [17] examined the antioxidant activity of methanol extracts of various parts of *A. wilhelmsii* and their fractions. The methanol extract and different fractions of various parts of *A. wilhelmsii* were found to contain appreciable levels of total phenolic contents. The results of the study have shown significant variations in the antioxidant activities of various parts of *A. wilhelmsii* and their fractions which the results were in parallel to our findings.

Investigations on some other *Achillea* species were displayed: Gharibi et al. [18] investigated total phenolic content and antioxidant activity of the methanolic extracts of the leaf samples of three Iranian endemic *Achillea* species. In DPPH assay, *A. aucherii* showed the highest IC_{50} (0.844 mg/mL), while *A. kellalensis* and *A. pachycephalla* possessed 0.518 mg/mL and 0.248 mg/mL, respectively. Benedec et al. [19] quantified some phenolic compounds and determined antioxidant activity of two *A. distans* subspecies. Chlorogenic acid content was found too low (<0.0002 g/100g) in both ssp. In the ethanolic extract of *A. distans* subsp. *distans* flowers, luteolin was the compound found in the largest amount (0.763±0.001 g/100 g) followed by apigenin (0.264 ± 0.001 g/100 g). They detected quercetin at lower levels than major flavonoides (0.0014±0.0001 g/100 g). In the ethanolic extract of *A. distans* subsp. *alpina* luteolin amount was determined as 0.052±0.0001 g/100g while apigenin was found as 0.013±0.0001 g/100g. The highest amount of the total polyphenols was determined in the extract of *A. distans* subsp. *alpina* flowers (174.75 ± 1.47 mg GAE/g extract) followed by *A.distans* subsp. *distans* extract (101.61 ± 1.24 mg GAE/g extract).

There are only a few studies on the phenolic composition of *A. setacea*. One of them, capillary electrophoretic seperation and quantification of flavone-O and C-glycosides in *A. setacea* were carried out, previously [20]. To the best of our knowledge, HPLC analysis of *A. setacea* on phenolic acids and flavonoids were performed first time in this study.

According to the literature given above, antioxidant activity and phenolic content of *Achillea* species are of great interest especially to the researchers who are trying to set light to the traditional usage of these plants against chronic diseases and to present the active principles responsible for the wide range of pharmacological activities.

4. CONCLUSION

The screening of antioxidant properties of three *Achillea* species by DPPH and ABTS assays revealed that they possessed significant antiradical activity, which was due to the presence of radical scavenging components that were quantified using a newly validated HPLC-DAD method. In other words, the phenolic compound profile of the MeOH extracts of *Achillea* species contributed to the definition of antioxidant activity of the plants. To the best of our knowledge, qualitative and quantitative analysis of phenolic compounds of *A. setacea* is reported here for the first time, as well as the results of radical scavenging activity tests and total phenolic content assay. The antioxidant activity values (IC_{50}) obtained from *A. biebersteinii* and *A. wilhelmsii* extracts were found notably high compared to the results obtained from the species of different countries. On the other hand, herein, different parts of the plants such as flowers, leaves and roots were studied separately in all experiment models to set light to the other researchers for efficient usage and evaluation of the plants. Consequently, due to the high content of phenolics, *Achillea* species could be evaluated as natural antioxidants in pharmaceutical, cosmetic and nutraceutical industry.

5. MATERIALS AND METHODS

5.1. Chemicals

Chromatographic grade double-distilled water, HPLC grade methanol, acetonitrile and analytical grade trifluoroacetic acid were used for HPLC analysis. Folin Ciocalteu reagent (F9252) and the following phenolic compounds were purchased from Sigma (Germany): gallic acid (G7384), chlorogenic acid (C3878), caffeic acid (C0625), ferulic acid (46278), *p*-coumaric acid (C9008), rutin (R5143), quercetin (Q4951), luteolin (L9283), apigenin (10798) and kaempferol (K0133). Also, DPPH (D9132) and ABTS (A1888) were purchased from Sigma (Germany). All other chemicals were analytical grade and obtained from either Sigma or Merck.

5.2. Plant material

Achillea biebersteinii Afan. (AEF 26686), *A. setacea* Waldst. Et Kit. (AEF 26688) *and A. wilhelmsii* C. Koch (AEF 26687) were collected near Yahyalı-Kayseri in their flowering stages (2014). Voucher specimens are deposited in the herbarium of Ankara University Faculty of Pharmacy (AEF).

5.3. Extraction procedure

For antioxidant activity tests, 5 g of dried and milled flowers, leaves and roots of the plants were extracted with methanol (100 mL) by magnetic stirrer for 6 h (room temperature, 250 rpm) [21]. After filtration, the organic phases were evaporated completely in a rotary evaporator (Buchi-R200). The crude extracts were used for antioxidant activity and total phenolic content assays.

For HPLC analysis, 200 mg of dried and milled flowers, leaves and roots were extracted with methanol, using a magnetic stirrer, for 6 h (room temperature, 250 rpm). The extracts were then filtered, made up to 10.0 mL in a volumetric flask with methanol, passed through a 0.45 µm filter, and injected into the HPLC system.

5.4. Determination of total phenolic content

The total phenolic content of the extracts was determined spectrophotometrically using a modified Folin Ciocalteu method [22]. The reduction of the reagent, which resulted in the formation of a blue colour, was recorded at 765 nm. One hundred µL of the methanol extract of each plant part (2 mg/mL) was mixed with 7.9 mL of distilled water. Folin Ciocalteu reagent (500 µL) was added and the contents of the flask shaken vigorously. After 8 min, 1.5 mL of 20% Na_2CO_3 was added. After 2 h incubation at room temperature, the absorbance was measured at 765 nm with a Shimadzu spectrometer. Gallic acid was used as standard. All measurements were performed in triplicate, and the average values were used to express the mg of gallic acid equivalents (GAE)/g dry extract.

5.5. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity

The capacity to scavenge the stable free radical DPPH wasmonitored according to the modified method of Barros et al. [23]. Various concentrations of extracts (0.25 mL) were mixed with 2.75mL of methanolic solution containing DPPH radical. The mixture was shaken vigorously and left to stand for 10 min in the dark (until stable absorption values were obtained). The reduction of the DPPH radical was determined by measuring the absorption at 517 nm. The radical scavenging activity (Inh%) was calculated as a percentage of DPPH discoloration using the equation: Inh% = [(A_{DPPH}-A_s) /A_{DPPH}] X 100, where A_S is the absorbance of the solution when the sample extract was added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage against extract concentration. Trolox (Sigma, Germany) was used as standard.

5.6. ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] assay

ABTS radical scavenging activity was measured using a modification of the method of Re et al. [24]. ABTS was dissolved in methanol to a concentration of 7 mM. ABTS radical cation was produced by reaction of ABTS stock solution with 2.45 mM $K_2S_2O_8$ (as an oxidant for conversion of ABTS into a radical cation). The color of the resulting solution was blue-green. This radical solution was kept in the dark at room temperature for 12-16 h before use in precise measurements. The ABTS radical cation solution was diluted with 96% ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. An aliquot of each extract (0.25 mL) was mixed with 2.75 mL of diluted ABTS radical cation solution. After reaction at room temperature for 6 min, the reduction in absorbance at 734 nm was measured. The radical scavenging activity (Inh%) was calculated as a percentage of ABTS inhibition using the equation: Inh% = [(A_{ABTS}-A_s) /A_{ABTS}] X 100. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage against extract concentration. Trolox (Sigma, Germany) was used as standard.

5.7. RP-HPLC-DAD analysis

5.7.1. HPLC conditions

RP-HPLC systems equipped with DAD are frequently used for qualitative and quantitative analysis of phenolic compounds, and one of our previous studies should be given as an instance for such studies [21]. In this study, analysis was performed on Agilent 1260 Series HPLC system which was equipped with a quaternary pump, an auto-sampler, a column oven, and a diode-array UV/VIS detector. Data analysis was performed using Agilent Chemstation software. The separation was executed on ACE 5 µ C18 (150 X 4.60 mm id) column. The mobile phase was composed of A: Water (%0.2 trifloro acetic acid) pH: 2.4, B: Acetonitrile:Methanol (80:20 v/v) with the gradient elution system at a flow rate of 0.9 mL/min. The composition of the gradient was (A:B), 95:5 at 0 min, 85:15 at 5 min, 85:15 at 15 min, 80:20 at 20 min, 70:30 at 25 min, 65:35 at 30, 50:50 at 35 min and 95:5 at 39 min. The injection volume was 10 µl. The detection UV wavelength was set at 230, 330, 360, 340, and 280 nm. The column temperature was set to 25°C. Method validation was performed according to the ICH guideline [25].

5.7.2. Calibration

Five different concentrations of chlorogenic acid, caffeic acid, rutin, quercetin, luteolin and apigenin were prepared in methanol ranging between 0.066-333 µg/mL, 0.037-166 µg/mL, 6.6-333 µg/mL, 0.052-333

µg/mL, 0.8-40 µg/mL and 0.044-66µg/mL, respectively. Triplicate 10 µL injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak areas obtained from injections were plotted against the concentrations to establish the calibration graph.

5.7.3. Limits of detection and quantification

Limits of detection (LOD) were established at a signal to noise ratio (S/N) of 3. Limits of quantification (LOQ) were established at a signal to noise ratio (S/N) of 10. LOD and LOQ were experimentally verified by the nine injections of reference compounds in LOQ concentrations.

5.7.4. Precision

The precision of the method (intra-day and inter-day variations of replicate determinations) was checked by injecting nine times of reference compounds at the LOQ levels in the same day and in two different days. The area values were recorded and RSD% values were calculated.

5.7.5. Recovery

The spike recovery was carried out by the standard addition method. For the determination of the recovery from the methanol extract, three different concentrations of reference compounds were added prior to the extraction. In each additional level, six determinations were carried out and the mean value of recovery percentage was calculated.

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