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Contributed Paper

Evaluation of Antioxidant Capacity and Analysis of Major Phenolic Compounds in *Achillea grandifolia* by HPLC-DAD with Q-TOF LC/MS Confirmation

Duygu Taşkın*[a], Dilek B. Alkaya [b] and Emre Dölen [b]

[a] Department of Analytical Chemistry, Faculty of Pharmacy, Istanbul Yeni Yuzyil University, Istanbul-Turkey.
[b] Department of Analytical Chemistry, Faculty of Pharmacy, Marmara University, Istanbul-Turkey.
*Author for correspondence; e-mail: duygu.susuz@yeniyuzyil.edu.tr

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Abstract

The aim of this study was to examine for the first time antioxidant properties from ethanol extracts of *Achillea grandifolia* flowers, leaves and stems using various antioxidant capacity methods. Subsequently, phenolic compounds in the extracts were identified for the first time by ESI-Q-TOF LC/MS and HPLC-DAD methods. The phenolic compounds in *A. grandifolia* was identified as quercetagetin 3,6-dimethyl ether, quercetin, luteolin-7-*O* glucoside, rutin, luteolin, caffeic acid, chlorogenic acid, salicylic acid and dicaffeoylquinic acids. Additionally, HPLC-DAD quantification of major phenolic acids and flavonoids was carried out to all of extracts. Compherensive HPLC-DAD-MS analysis showed that flower extract represent the most abundant phenolic compounds. Also, our study clearly demonstrated that the flower showed better antioxidant effects, total phenolic and flavonoid contents than leaf and stem. Therefore, the flower extract could be a rich source of natural antioxidants and is worthy of further studies that may lead to the discovery of new natural antioxidants.

Keywords: phenolic compounds, antioxidant capacity, HPLC-DAD/Q-TOF-MS, *A. grandifolia*

1. Introduction

Natural polyphenols are commonly found in plant and are major antioxidants present in the diet. There is increasing interest in polyphenols' antioxidant properties and their possible role in the prevention of various diseases associated with oxidative stress including cancer, cardiovascular and neurodegenerative diseases [1]. Synthetic antioxidants have been used to preserve of foods and animal feeds as well as to improve the stability of pharmaceuticals and cosmetics. But, currently used synthetic antioxidants have been suspected to cause negative health effects because of their toxicity and other side-effects [2]. Hence, there is a trend to substitute them with naturally occurring antioxidants. Besides the well-known and traditionally used natural antioxidants from tea, fruits, vegetables and spices, many other plant species have been investigated in the search for novel antioxidant [3, 4].

Asteraceae (Compositae) family is the richest vascular plant in the world, with about 1509

genera and 20.000 species [5]. *Achillea* species is from Asteraceae family and there are about 115 species in the genus *Achillea* distributed in the central southwest Asia and south-eastern Europe and North America extends across Eurasia. In section 52 taxa collected in Turkey Flora 5 represented by 46 species of which 23 are endemic to Anatolia [6, 7]. *Achillea* L. are widely used in folk medicine due to pharmacological properties, such as antimicrobial, antispasmodic, antidiabetic agents, cytotoxic, estrogenic, immunosuppressive, anti-tumor, antihyperlipidemic, antihypertensive, antifertility [8], analgesic, anti-inflammatory, antipyretic and especially antioxidant effects [9].

Achillea grandifolia is a flowering plant in the family Asteraceae, into distributed in Balkan, Peninsula and Turkey [10]. We focused on this plant as there have been no antioxidant (excluding essential oil of this plant) and chromatographic studies to the best of our team's knowledge. Therefore, the aim of this study was to identify and quantify for the first time the phenolic compounds and antioxidant capacity of flower, leaf and stem in ethanol extracts, which showed the highest total amount of phenolics, of *A. grandifolia*. The major phenolic composition of each parts of the plant was quantified by HPLC-DAD. The antioxidant capacity of the extracts was analysed by five spectrophotometric methods (e.g cupric reducing antioxidant capacity (CUPRAC), ferric ion reducing antioxidant power (FRAP), DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, trolox equivalent antioxidant capacity (TEAC) and metal chelating capacity methods). Additionally, total phenolic and total flavonoid contents of ethanolic extracts were determined as gallic acid and quercetin equivalents, respectively. Lastly, the correlation between the extracts phenolic and flavonoid contents and the antioxidant capacities were also determined in this study.

2. MATERIALS AND METHODS

2.1 Chemicals

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) and butylated hydroxytoluene was sourced from Fluka. 2,4,6-tripyridyl-s-triazine, 2,2-diphenyl-1-picryl-hydrazyl (DPPH•), gallic acid, quercetin ethylenediaminetetraacetic acid disodium salt dihydrate, Folin Ciocalteu's phenol reagent, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid sodium salt, rutin, chlorogenic acid, caffeic acid, luteolin-7-*O* glucoside and luteolin were obtained from Sigma Chemical Co. Quercetagetin 3,6-dimethyl eter were kindly donated by Prof. Dr. Ufuk Kolak. All the other reagents were of analytical grade. High purity water was obtained by passing water though a Milli-Q treatment system (Millipore, Bedford, MA, USA).

2.2 Plant Material

A. grandifolia was collected from Kocaeli, Kartepe (altitude of 1520-1625 m) province of Turkey in May, 2011. The plant was authenticated by Prof. Ertan Tuzlacı. A voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy (MARE 14583), Marmara University, Istanbul, Turkey. The fresh plant materials were separated into flower, leaf and stem parts.

2.3 Preparation of the Extracts

The plant material was dried in shadow and then ground to small pieces using a grinder (Renas, RBT1250). Powdered dried leaves, flowers and stems (20 g) were macerated with ethanol (100 mL each) for 7 days with occasional shaking at room temperature. The extracts were filtered and evaporated in vacuum rotary evaporator at 45 ^{o}C and kept in a refrigerator at 4-5 °C.

2.4 HPLC–DAD Analyses

A Schimadzu high-performance liquid chromatography (HPLC) system (Japan) equipped with a photodiode array detector (DAD) was carried out for chromatographic

analyses. The separation was performed using a Nova-Pak C18 analytical column (3.9 x 150 mm, 5 μm, Part No WAT 086344, Waters), studied at a flow rate of 0.5 mL/min during a gradient. The HPLC gradient elution was performed water with trifluoroacetic acid (TFA) (0.1%) (phase A) and acetonitrile with trifluoroacetic acid (0.1%) (phase B) as mobile phase [11] and the solvent gradient changed according to the following conditions: 0 min, 5% B; 1 min, 5% B; 20 min, 30% B; 25 min, 60% B; 28 min, 60% B; 33 min, 95% B, 35 min, 95% B, 40 min, 5% B. All the extracts and standards were dissolved with methanol/water (2:1; v/v) solution, then filtered through 0.22 μm membranes and the mobile phases were degassed before injection on to HPLC. The injection volume was 20 μL and the DAD acquisition range was 200-500 nm. 350 nm was chosen for the identification of phenolic compounds and different wavelengths were utilized for their quantification as described in Section 3.3.

2.5 ESI-Q-TOF LC/MS Analyses

Electrospray ionization-quadrupole-time of flight liquid chromatography mass spectrometry (ESI-Q-TOF LC/MS) (Agilent 6530, CA, United States) instrument, in the negative ion mode, (model G6530B, Agilent Technologies, USA), $[M-H]^-$ ions, was operated. The same gradient elution method was used for Q-TOF-MS analyses as used in HPLC**.** The mass range scanned was m/z 100 to 1000. According to this, the optimum values of ESI-Q-TOF-LC/MS parameters in negative mode were: capillary voltage, 3500 V; drying gas temperature, 350 °C; drying gas flow, 8 L/min; nebulizing gas pressure, 2 bar and end-plate offset, −500 V. The accurate mass data for the molecular ions were processed using the Mass Hunter Workstation Software Version (B.O600 Build 6.0.633.10).

2.6 Determination of Total Phenolic and Flavonoid Contents

The amount of total phenolic contents (TPC) in *A. grandifolia* ethanol extracts of flower, leaf and stem were determined according to the procedure described before [12]. A volume of 0.1 mL extract solution (with a concentration range from 50 to 200 μg/mL) was diluted with distilled water (4.6 mL) and 0.1 mL of Folin-Ciocalteu reagent (diluted 1:3, v/v) was added. Then, 3 mL of Na_2CO_3 (2% w/v) were added and the mixture was left standing at ambient temperature for 2 hours. The absorbance value was measured at 760 nm using a Shimadzu UVmini-1240 spectrophotometer. All experiments were performed triplicate. A calibration curve was prepared using gallic acid. The gallic acid stock solution (1000 μg/mL) was diluted to produce five concentrations in the range of 50 to 500 μg/mL and were measured spectrophotometrically in triplicate. The results were given as μg gallic acid equivalents per 100 μg material (μg GAE/100 μg sample).

Total flavonoid content (TFC) was determined using aluminium chloride ($AlCl_3$) according to a known method, using quercetin as a standard [13]. The plant extracts (0.5; 1.0; 2.0 mg/mL) (0.5 mL) were added to 2 mL distilled water followed by 5% $NaNO_2$, w/v (0.15 mL). After 6 min at 25 °C, $AlCl_3$ (0.15 mL, 10%) was added. After further 6 min, the reaction mixture was treated with 2 mL of 4% NaOH, w/v and volume was made up to the 5 mL with distilled water. After 15 min of incubation the mixture turns to pink whose absorbance was measured at 510 nm, distilled water was used as blank. A calibration curve was prepared using quercetin and it proved to be linear over the concentration range from 0.2 to 2.0 mg/mL. The results were expressed as μg quercetin (QUE)/100 μg extract. All experiments were performed in triplicate (n = 3).

2.7 Antioxidant Capacity Assessment

2.7.1 Cupric reducing antioxidant capacity (CUPRAC) assay

Cupric reducing antioxidant capacity (CUPRAC) method were used for evaluate the antioxidant capacity of plant extracts [14]. 1 mL each of Cu(II) (1.10^{-2} M), neocuproine ethanolic solution ($7.3.10^{-3}$ M) and 1 M NH_4Ac buffer solution were mixed in a test tube. 1 mL of the extracts (0.05-0.25 mg/mL) and 0.1 mL pure EtOH were added to the initial mixture so as to make the final volume: 4.1 mL. The mixture was vortexed for 10 s and absorbance measurement was performed exactly after 30 min at 450 nm against a reagent blank. The CUPRAC values of plant extracts were reported as trolox equivalents (μM trolox/mg extract). A standard curve was prepared using trolox with a concentration range from 0.025 to 0.250 μM. All experiments were performed in triplicate.

2.7.2 Determination of ferric reducing antioxidant power (FRAP) assay

The FRAP assay was done following the procedure previously described [15]. FRAP reagent included 25 mL 300 mM acetate buffer (pH 3.6), 2.5 mL 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl and 2.5 mL 20 mM $FeCl_3.6H_2O$ solution. The working solution was prepared by mixing them and then warmed at 37 °C for 30 min before using. 0.2 mL (with a concentration range from 0.5 to 2.0 mg/mL) plant extracts and FRAP reagent (3.8 mL) were mixed. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min; the assay was carried out in triplicates. The standard curve was constructed using $FeSO_4$ solution (50-1000 μM). The results were expressed as μM Fe (II)/mg dry weight of plant material. L-ascorbic acid was also used as positive reference with a concentration range from 0.1 to 1.0 mg/mL.

2.7.3 DPPH free radical scavenging activity assay

The antioxidant activity of plant was tested by the DPPH (2,2'-diphenyl-1-picrylhydrazyl) free radical scavenging method [16]. Stock solution (10 mg/mL) of flowers, stems and leaves extracts from *A. grandifolia* was prepared in pure ethanol from which serial dilutions were carried out to obtain the concentrations 0.1-4.0 mg/mL. In this assay, 4 mL of 0.1 mM ethanolic DPPH solution was added to 1 mL of extract solution. The mixtures were shaken in a vortex for a 10 s and then placed in a dark place. The decrease in absorbance at 517 nm was determined using spectrophotometer after 30 min for all samples. Also, butylated hydroxytoluene (BHT) and L-ascorbic acid were used as the reference antioxidants with a concentration range from 25 to 500 μg/mL. The percentage of DPPH free radical-scavenging activity of plant extract was calculated as:

$$\text{DPPH free-radical scavenging activity (I \%)} = [(A_o - A) / A_o] \times 100$$

Where, A_o is the absorbance of the control solution (containing all reagents except plant extract); A is the absorbance of the DPPH solution containing plant extract. All experiments were performed in triplicate. The IC_{50} (extract concentrations required to scavenge radical by 50%) value is inversely correlated to antioxidant ability of extracts. A lower IC_{50} value reveals higher antioxidant capacity.

2.7.4 Determination of trolox equivalent antioxidant capacity (TEAC) assay

The 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) free radical cation ($ABTS^{\bullet+}$) assay was performed as reported by Wojdylo et al. [17]. This assay is based on the formation of $ABTS^{\bullet+}$ by reaction of ABTS aqueous solution

(7 mM) with potassium persulfate ($K_2S_2O_8$) (2.45 mM) at room temperature, under darkness, for 14 hours. This stock solution was diluted with ethanol to an absorbance of 0.702 at 734 nm. The reaction mixture comprised 4 mL of $ABTS^{\bullet+}$ solution and 40 μL of the extracts at a 0.25-2.00 mg/mL concentrations. After six minutes, the absorbance value was read off at 734 nm against of a reagent blank. Besides a standard curve was obtained using trolox standard solution. 10 mM stock trolox solution was diluted with 75 mM phosphate buffer (pH 7.4). A calibration curve was prepared using different concentrations of trolox (200, 400, 600, 800, 1000 μM). TEAC values were expressed as mM trolox equivalent per mg extract. All the tests were performed in triplicate. The results were compared with the standard curve for calculation of TEAC.

2.7.5 Determination of ferrous ion-chelating capacity

The chelating effect on ferrous ion of the prepared extracts was estimated by the method of Bakir and Ozmen [18]. 200 μL of each extract (5.0 mg/mL) and 1.0 mg/mL positive control ethylenediaminetetraacetic acid (EDTA) solution were mixed with 50 μL of $FeCl_2$ (2 mM) solution, 350 μL distilled water. Then ethanol was added to this mixture until a final volume of 4 mL was achieved. The reaction was initiated by the addition 200 μL of ferrozine (5 mM), stirred with vortex 10 s, left standing at ambient temperature for ten minutes. Then, the absorbance was measured at 562 nm against blank (without extract). Distilled water instead of the extracts was used as a control against ethanol. EDTA was used as a positive reference compound. The activity was calculated by using the same formula which was used for DPPH radical scavenging activity. Results were given the percentages of ferrous ions (Fe^{2+}) chelating capacity of the extracts and EDTA. All experiments were performed in triplicate.

3. RESULTS

3.1 Total Phenolic and Total Flavonoid Contents of *A. grandifolia* Flowers, Leaves and Stems

The total phenolic contents (TPC) in the extracts were determined from the regression equation of calibration curve (Y =18.78x–0.0031 (R^2 = 0.9855) and expressed in gallic acid equivalents (GAE). TPC of the extracts showed the following order: flowers > leaves> stems. The total flavonoid contents (TFC) in the extracts were determined from the regression equation of calibration curve (Y =1.5460x + 0.2210 (R^2 = 0.9904) and expressed in quercetin equivalents (QUE). The TFC in the flower were higher than leaf and stem. The results are shown in Table 1.

Table 1. The TPC and TFC of extracts from *A. grandifolia* flowers, leaves and stems.

	Sample(μg/mL)	Flowers	Leaves	Stems
TPC (μg GAE)	200	9.22 ± 0.01	7.28 ± 0.01	4.97 ± 0.04
	100	5.18 ± 0.01	4.19 ± 0.04	2.92 ± 0.01
	50	2.68 ± 0.03	2.16 ± 0.01	1.44 ± 0.02
TFC(μg QUE)	100	15.90 ± 0.01	15.6 ± 0.02	9.90 ± 0.02
	50	8.90 ± 0.04	0.04 ± 0.02	0.03 ± 0.02
	25	5.90 ± 0.01	0.014 ± 0.01	0.006 ± 0.01

3.2 HPLC-DAD and ESI-Q-TOF LC/MS-MS Analysis of Phenolic Compounds

Phenolic compounds in all of extracts were identified by HPLC-DAD based on comparing on-line ultraviolet absorption spectrum data and retention times acquired with authentic standards. After that, they were confirmed by ESI-Q-TOF LC/MS-MS in negative ionization by comparison of MS spectral data of identified peaks from HPLC-DAD with standards. A total of eleven phenolic compounds were identified, as summarised in Table 2, including the retention time, molecular weight, formula and fragments. Fragmentation patterns of phenolic acids and flavonoids of *A. grandifolia* are shown in Figure 1.

The fragmentation of the $[M-H]^-$ molecular ion at m/z 353.0878 gave product ions at m/z 191 and 179 with respect to quinic through the loss of a caffeoyl and through the loss of a quinic respectively, was identified as chlorogenic acid [19]. Luteolin-7-*O* glucoside showed $[M-H]^-$ value at m/z 447.0933 and the product ion at m/z 285 representing luteolin aglycone through the absence of a glucoside moiety (447-162) [20]. Luteolin gave $[M-H]^-$ value at m/z 285.0405 and the product ions at m/z 175 [M-H-110], 151 [M-H-134] and 133 [M-H-152]. The fragmentation of the $[M-H]^-$ molecular ion at m/z 515.1277 with fragment ions 353 (chlorogenic acid), 191, 179 was identified as dicaffeoylquinic acid [21]. Trifluoroacetic acid (TFA) was used as the mobile phase adducted to dicaffeoylquinic acid. Because the $[M-H]^-$ value was observed to be m/z 629.0977 (515+114). The spectra generated in negative ion detection gave the deprotonated molecule $[M-H]^-$ (m/z 137.0244) for salicylic acid. Quercetagetin 3,6-dimethyl ether showed $[M-H]^-$ value at m/z 345.0616 and gave product ions at m/z 330 [M-H-CH_3], 315 [M-H-CH_3-CH_3] and 287 [M-H-CH_3-CH_3-CO]. Caffeic acid showed $[M-H]^-$ value at m/z 179.0329. It was found that the fragment ions at m/z 161 and 135 by

Table 2. List of the phenolic compounds identified in ethanol extracts of *A. grandifolia* flower, leaf and stem by LC/ESI-MS/MS.

Compounds	t_R(min.)	λmax (nm)	$[M-H]^-$ (m/z)	Molecular formula	Fragment ions	Ref./Std.
Chlorogenic acid	10.99	218, 235, 325	353.0913	$C_{16}H_{18}O_9$	191, 179	Std.
Caffeic acid	11.66	217, 234, 323	179.0350	$C_9H_8O_4$	135	Std.
Rutin	16.46	255, 353	609.1875	$C_{27}H_{30}O_{16}$	301	Std.
Luteolin-7-*O* glucoside	17.02	255, 354	447.0876	$C_{21}H_{20}O_{11}$	285	Std.
Dicaffeoylquinic acid I	18.19	216, 234, 327	515.1113	$C_{25}H_{24}O_{12}$	353,191, 179	Ref.
Dicaffeoylquinic acid II	18.71	216, 234, 327	515.1042	$C_{25}H_{24}O_{12}$	353,191, 179	Ref.
Dicaffeoylquinic acid III	19.10	216, 234, 327	629.1022	$C_{25}H_{24}O_{12}$	515, 353, 179	Ref.
Salicylic acid	22.12	237, 303	137.0284	$C_7H_6O_3$	137	Std.
Luteolin	23.60	252, 347	285.0368	$C_{15}H_{10}O_6$	133, 151, 175, 199, 217	Std.
Quercetin	24.02	254, 370	300.9021	$C_{15}H_{10}O_7$	151, 121	Std.
Quercetagetin 3,6-dimethyl ether	24.67	258, 350	345.0565	$C_{17}H_{14}O_8$	330, 315, 287	Std.

Ref: Literature reference; Std: Standards

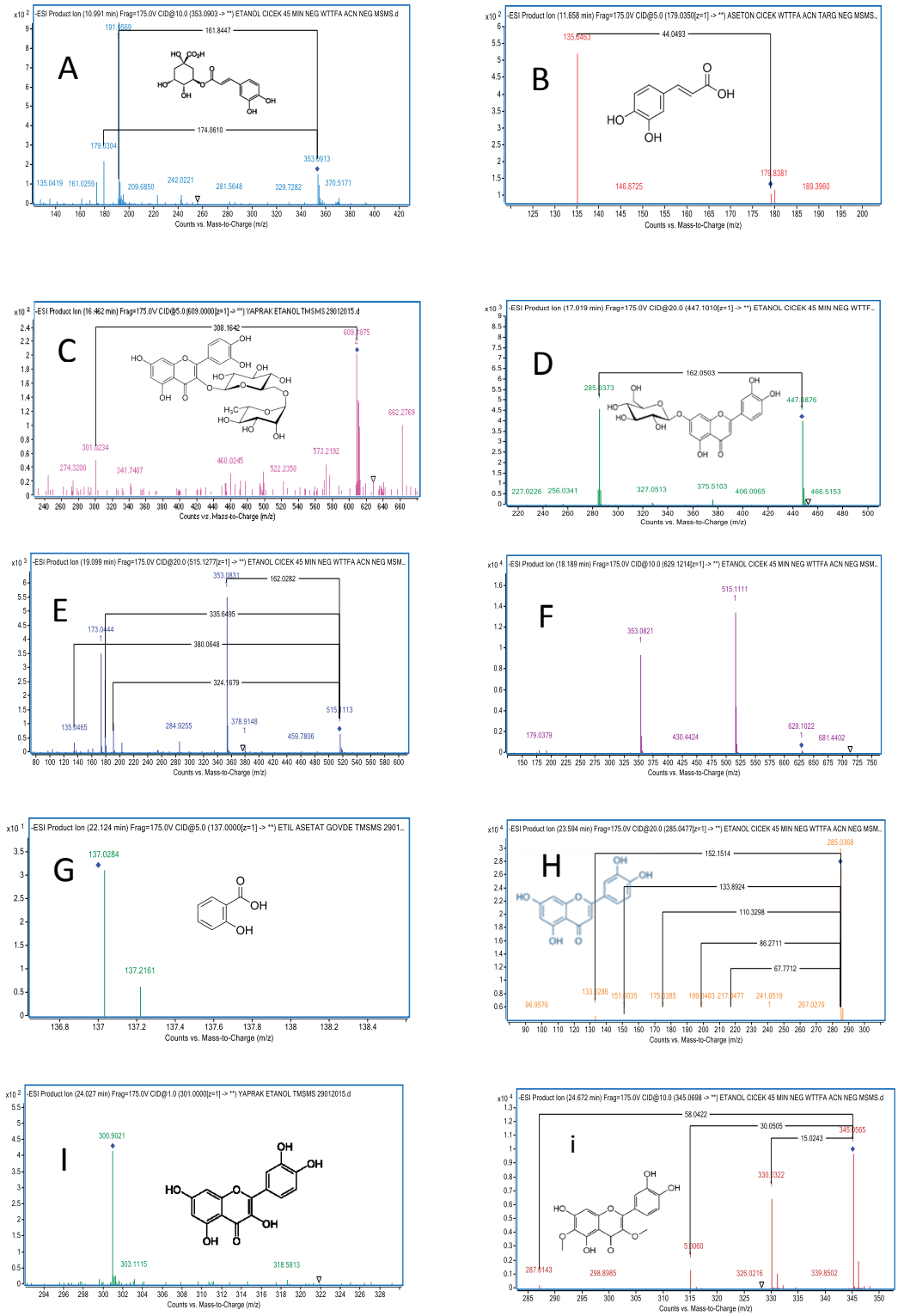

Figure 1. MS/MS spectra and fragmentation patterns of chlorogenic acid (A), caffeic acid (B), rutin (C), luteolin-7-*O* glucoside (D), dicaffeoylquinic acid (E), Isomer of dicaffeoylquinic acid (F), salicylic acid (G), luteolin (H), quercetin (I), quercetagetin 3,6-dimethyl ether (i).

losses of a H_2O molecule and a CO_2 molecule, respectively. Rutin showed $[M-H]^-$ value at m/z 609.2080 gave product ions at m/z 301 by losses rutinose. The fragmentation of the $[M-H]^-$ molecular ion at m/z 300.9021 gave product ions 151, 121 which was attributed to the aglycone quercetin.

3.3 Quantitative Analysis of *A. grandifolia* Extracts

The standard phenolic compounds were prepared in triplicate and they were chromatographed individually by injection 20 µL of 500 µg/mL then detecting the corresponding peak at their maximum wavelength. The calibration curves were constructed by plotting the peak area against the known concentration of each phenolic compound. Chromatogram peak areas on 354 nm for luteolin-7-*O* glucoside; 325 nm for chlorogenic acid; 353 nm for rutin; 370 nm for quercetin; 347 nm for luteolin were plotted against the known concentrations of the standard solutions to establish calibration equations. The calibration curves were obtained on five levels of concentration of standards with three injections per level. A linear regression equation was calculated by the least squares method. The limit of detection (LOD) and limit of quantification (LOQ) were calculated from standard deviation of the regression (σ) line and the slope (S) as follows: LOD = $3.3\sigma/S$; LOQ = $10\sigma/S$. The linear range, regression equation and determination coefficient of each standard, LOD and LOQ values were shown in Table 3.

Data of quantitative analyses are expressed as mean ± standard deviation and are listed in Table 4. The results were expressed in µg

Table 3. Quantitative determination of five phenolic compounds using HPLC-DAD.

Compounds	Regression equation	R^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Chlorogenic acid	y= 406496x+279519	0.9998	5.0-30	0.46	1.38
Rutin	y = 64641x - 5024.6	0.9966	5.0-30	2.15	6.53
Luteolin	y= 203849x+ 2013.9	0.9986	5.0-50	2.22	6.75
Quercetin	y= 163014x+205756	0.9959	5.0-50	3.83	11.62
Luteolin7-*O* glu	y = 17067x + 26827	0.9983	10-75	3.45	10.46

Table 4. The polyphenol contents in *A. grandifolia* flower, leaf and stem extracts presented as average values ± standard deviation of three measurements (µg/mg extracts).

Compounds	Average amount ± SD (Flowers)	Average amount ± SD (Leaves)	Average amount ± SD (Stems)
Chlorogenic acid	0.70 ± 0.01	0.68 ± 0.04	0.48 ± 0.01
Rutin	4.56 ± 0.05		
Luteolin-7-*O* glucoside	38.65±1.52		1.19 ± 0.03
Luteolin	1.71 ± 0.37		
Quercetin		1.23 ± 0.03	

per mg of ethanolic extracts. The content of dicaffeoylquinic acid and its isomers were not quantified due to the lack of standards. So, their structure was identified by MS/MS.

3.4 Antioxidant Capacity of *A. grandifolia* Flowers, Leaves and Stems

3.4.1 Cupric reducing antioxidant capacity (CUPRAC) assay

The cupric reducing antioxidant capacity of ethanol extracts from *A. grandifolia* flowers, leaves and stems were expressed as μM trolox and are shown as Table 5. CUPRAC values of the extracts are exhibited in the following order: flowers > leaves > stems extract.

(The calibration equation for trolox was Absorbance = 3.5858 trolox (μM)–0.0072 (R^2 = 0.9994)).

3.4.2 DPPH free radical scavenging activity assay

The free radical scavenging capacity of the extracts was measured by DPPH assay. Butylated hydroxytoluene and ascorbic acid were used as standard antioxidants for comparison of antiradical capacity. The DPPH radical scavenging capacity of the extracts and standards showed the following order: ascorbic acid > BHT > flowers > leaves > stems extract. The results showed in Table 6 are represented in IC_{50} values.

3.4.3 Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC value is assigned by comparing the scavenging capacities of extracts with that of trolox and a high TEAC value indicated a high level of antioxidant activity. The TEAC values were determined from the regression equation of calibration curve (Y=37.22 trolox(mM)+1.66 (R^2= 0.9986) and expressed in trolox equivalents (mM trolox/mg extract). The TEAC values of the extracts are exhibited in the following order: flower > leaf > stem extract. The results are shown in Table 6.

3.4.4 Determination of ferrous ion-chelating capacity

The percentages of ferrous ion chelating capacity of 5.0 mg/mL concentration ethanol extracts from *A. grandifolia* flowers, leaves, stem and EDTA are shown in Table 6. The

Table 5. CUPRAC values (μM trolox) of flowers, leaves and stems extracts.

	CUPRAC values (μM trolox)		
Extracts	0.05 mg/mL	0.1 mg/mL	0.25 mg/mL
Flowers	0.0570 ± 0.0010	0.0929 ± 0.0020	0.2032 ± 0.0040
Leaves	0.0480 ± 0.0006	0.0660 ± 0.0010	0.1570 ± 0.0020
Stems	0.0140 ± 0.0006	0.0297 ± 0.0007	0.0610 ± 0.0020

Table 6. Antioxidant capacity of ethanol extracts from *A. grandifolia* and standard antioxidants.

Extracts/ Standards	TPC (μg CE/100 μg extract)	TFC (μg QUE/ 100 μg extract)	DPPH (IC_{50}:mg/ mL)	TEAC (mM trolox/mg extract)	CUPRAC (μM trolox) (1.0 mg/ mL)	FRAP (μM Fe (II) / mg extract)	Metal chelating (%) (100 μg)
Flowers	5.18± 0.01	15.90±0.01	1.14 ± 0.01	14.25 ± 0.03	0.929 ± 0.002	2.60 ± 0.01	9.58 ± 0.12
Leaves	4.19 ± 0.04	15.60±0.02	1.45 ± 0.02	15.38 ± 0.03	0.660 ± 0.001	1.70 ± 0.01	1.0 ± 0.47
Stem	2.92 ± 0.01	9.90 ± 0.02	4.95 ± 0.03	4.25 ± 0.02	0.297 ± 0.001	1.60 ± 0.01	4.25 ±1.17
Ascorbic acid			0.13 ± 001			21.16 ± 0.02	
BHT			0.37 ± 0.01				
EDTA (20μg)							9.54 ± 0.22

metal chelating activity of the extracts and the EDTA at the 100 μg amounts are exhibited in the following order: EDTA > flower > leaf > stem extract.

3.4.5 Determination of ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant/power (FRAP) activity of ethanol extracts are shown in Table 6. Ascorbic acid was used as a standard. FRAP values (μM$FeSO_4$/mg extract) of the extracts and standard are in the following order: ascorbic acid > flowers > leaves > stem extract.

(The calibration equation for $FeSO_4$ was Absorbance =1.6750 $FeSO_4$(μM)–0.1216 (R^2= 0.9983)).

3.5 Correlation Between Antioxidant Capacity and Total Phenolic and Flavonoid Contents

The correlations between the antioxidant capacity and the phenolic and flavonoid contents were also determined in this study (Table 7). There was a significant correlation between the DPPH, TEAC and CUPRAC methods and total phenolic and flavonoid contents of flowers and stems. However, leaves of *A. grandifolia* show poor linear correlation between FRAP values and total phenolic and flavonoid contents. FRAP assays results were obtained in 4 minutes, but in theoretically some polyphenols react more slowly and require longer reaction times for complete redox reaction. The correlation value of FRAP and leaf indicated that quercetin is mainly responsible for the FRAP assays.

4. DISCUSSION

The antioxidant capacity of *Achillea* species were widely studied by *in vitro – in vivo* methods which are as follows: DPPH, FRAP etc. Phenolic compounds have been reported in *Achillea* species that are antioxidants with the potential to protect the body from some disease [22-24].

To the best of our knowledge, there are only four reports of *Achillea grandifolia*. Both of the reports are about essential oil contents and especially found thujone, camphor (15.6%), ascaridole (15.5%), borneol (5.2%) and 1,8-cineol [25, 26] antioxidant activities of essential oil [27] and the last one is isolation of methyl ethers

Table 7. Correlation between antioxidant capacity and total phenolic and flavonoid contents.

		Determination coefficients			
		DPPH	**TEAC**	**FRAP**	**CUPRAC**
TPC	Flower	y= 10.824x-11.113 (R^2: 0.9987)	y=14.752x- 1.6103 (R^2: 0.9847)	y=0.0695x +0.0883 (R^2: 0.9946)	y =0.0167x +0.0099 (R^2: 0.9985)
	Leaf	y =11.083x-11.103 (R^2: 0.9734)	y=18.519x– 6.5633 (R^2: 0.9991)	y = 0.0486x +0.0261 (**R^2: 0.9110**)	y = 0.0162x + 0.0068 (R^2: 0.9822)
	Stem	y=5.284x-0.4495 (R^2: 0.9800)	y=8.0687 x -1.7941 (R^2: 0.9970)	y=0.0560x+0.0150 (R^2: 0.9880)	y = 0.0098x + 0.0002 (R^2: 0.9999)
TFC	Flower	y=3312.7x-9.5462 (R^2: 0.9966)	y= 3601.7x-2.3123 (R^2: 0.9880)	y=22.658x- 0.2508 (R^2: 0.9958)	y = 4.7017x + 0.0165 (R^2: 0.9996)
	Leaf	y=1442.4x+7.8275 (R^2: 0.9739)	y=3227.7x– 6.0409 (R^2: 0.9822)	y= 8.3224x + 0.2132 (**R^2: 0.8574**)	y = 3.263x + 0.0262 (R^2: 0.9720)
	Stem	y=971.93x+4.9177 (R^2: 0.9852)	y =1575.2x –1.6908 (R^2: 0.9999)	y= 10.356x + 0.1584 (R^2: 0.9914)	y=2.2889x+ 0.0061 (R^2: 0.9999)

of 6-hydroxyflavones and 6-hydroxyflavonols from acetone washes of leaves and stems [28]. The method of Stankovic and co-workers [27] are based on the antioxidant activities of essential oils from *A. grandifolia*, but there have been no studies of the antioxidant activity of ethanol extract from *A. grandifolia* flower, leaf and stem in seperately. Also, there have been no studies about the chromatographic analysis of ethanol extracts from *A. grandifolia*.

In this study, using various *in vitro* assay systems, the antioxidant potential of *A. grandifolia* extract was evaluated based on DPPH, CUPRAC, FRAP, TEAC and metal chelating assays. Our results demonstrated that *A. grandifolia*, rich in respect to flavonoids and phenolic acids, could be a good source of natural antioxidants. There was a significant correlation between the DPPH, TEAC and CUPRAC methods and total phenolic and flavonoid contents of flowers and stems. However, leaves of *A. grandifolia* show poor linear correlation between FRAP values and total phenolic and flavonoid contents. Quercetin was only found in leaves and rutin was only found in flowers of the plant. Pulido et al [29] reported that the absorbance of quercetin didn't stabilize within the measurement period of the FRAP assay and absorbance of quercetin was increasing within several hours, whereas FRAP assays results are obtained in 4 min. Some polyphenols react more slowly and require longer reaction times for complete redox reaction and so 4 min is very short for finish the reaction [30]. Also, formation of Fe^{2+} can be easily interfered by the other reduction agents. Hence, the leaves of *A. grandifolia* did not show significant correlation by FRAP assay, it may be concluded about above reasons. In addition to the study of Apak and co-workers [31] are based on the TEAC coefficients of various phenolics. Their results showed that the TEAC coefficient of quercetin (2.77 mM trolox equivalents of 1 mM quercetin) is higher than of rutin (1.15 mM trolox equivalents of 1mM rutin). Because rutin, having an *O*-rutinose substituent instead of –OH in the 3-position, showed the lower capacity. Hence, the leaves of *A. grandifolia* are richest antioxidant capacity in TEAC assay.

Qualitative and quantitative analysis of major phenolic compounds of *A. grandifolia* could be helpful for clarifying the relationships between the content of total phenolic and flavonoid compounds and its antioxidant capacity. According to the results of antioxidant capacity assays and HPLC-DAD-MS, especially flower of *A. grandifolia* could be used as a source of natural antioxidant after further research on cytotoxic effect. Furthermore, future studies should focus on the assesments of economic benefits and *in vivo* activities of the extracts before their commercial exploitation.

5. Conclusions

Eleven phenolic compounds in the ethanol extracts of *A. grandifolia* were identified for the first time by ESI-Q-TOF LC/MS and HPLC-DAD methods. The results obtained from the HPLC-DAD and LC-MS/MS showed that the flower extract contains the most amount of phenolic compounds. This study clearly exhibited that the flower had the highest antioxidant capacity of extracts. The antioxidant capacity of the flower extract was better correlated with the phenolic and flavonoid contents, which showed that mainly phenolic acids and flavonoids were responsible for the antioxidant capacity. Consequently, the flower extract might be a rich source of natural antioxidant agent in food and pharmaceutical industry.

Statistical analysis

All experiments were carried out in triplicate and expressed as mean ± standard deviation (SD). Statistical analyses were performed by using Graphpad Prism 5 Demo and Microsoft Office Excel 2007.

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