

Phytochemical Characterization of *Phagnalon graecum* Boiss. by HPLC and GC-MS with its Enzyme Inhibitory and Antioxidant Activity Profiling by Spectrophotometric Methods

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Received: 27 December 2011 / Accepted: 15 February 2012 / Published online: 13 April 2012
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Abstract In the current study, the *n*-hexane, chloroform, ethyl acetate, methanol, and water extracts of *Phagnalon graecum* Boiss. (Asteraceae), a spice plant, were investigated for their enzyme inhibitory activity against acetylcholinesterase, butyrylcholinesterase, lipoxygenase, tyrosinase, and antioxidant activities. Antioxidant activity of the extracts was determined by 2,2-diphenyl-1-picrylhydrazyl radical scavenging, ferric ion-chelation activity, and ferric-reducing antioxidant power tests. Total phenol (gallic acid equivalent) and flavonoid (quercetin equivalent) contents were calculated. Essential oil of the plant was analyzed by GC-MS, while the selected phenolic acids were analyzed by HPLC. The extracts had insignificant inhibition against the

tested enzymes, whereas they displayed a remarkable antioxidant activity. Therefore, to the best of our knowledge, this study presents the first data on biological activity and phytochemical content of *P. graecum* and this species could be utilized as potential antioxidant applicable for food preservation.

Keywords Antioxidant activity · Asteraceae · Enzyme inhibition · Essential oil · *Phagnalon graecum* · Phenolics

Introduction

The genus *Phagnalon* Cass. (Asteraceae) is represented by three species in the Flora of Turkey; namely *Phagnalon graecum* Boiss., *Phagnalon kotschyi* Schultz Bip. & Boiss., and *Phagnalon rupestre* (L.) DC (Davis 1988). Some *Phagnalon* species are used as spice in Italy (Lentini and Venza 2007) or herbal medicine in some folkloric systems such as Israel (Friedman et al. 1986), Spain (Pardo De Santayana et al. 2005), Palestine (Abu-Rabia 2005), and Jordan (Hudaib et al. 2008). A limited number of phytochemical studies performed in some *Phagnalon* species revealed existence of quinine and some phenolic acid conjugates (Hausen and Schultz 1977; Góngora et al. 2001, 2002a, b, c, 2003, Olmos et al. 2005), some terpenes and thymol derivatives (El-Dahmy et al. 1994), and some flavonoids (Góngora et al. 2001, 2002a, b).

However, there has been no report up to date on the phytochemistry or biological activity of *P. graecum*. Only a recent study describing anticholinesterase and antioxidant activities of *Phagnalon saxatile*, a Mediterranean species

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growing in Italy (Conforti et al. 2010), prompted us to investigate similar enzyme inhibitory and antioxidant activities of *P. graecum*, which is another species of this genus growing in the Mediterranean region. Consequently, the *n*-hexane, chloroform, ethyl acetate, methanol, and water extracts prepared from the aerial parts of *P. graecum* were tested for their enzyme inhibitory activity against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), lipoxygenase (LOX), and tyrosinase (TYRO) using ELISA microplate reader at 50, 100, and 200 µg/mL concentrations. Antioxidant activity was determined using three *in vitro* test systems; 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, ferric ion-chelating activity, and ferric-reducing antioxidant power (FRAP) at 125, 250, 500, 1,000, and 2,000 µg/mL concentrations. For phytochemical characterization of the extracts, total phenol (gallic acid equivalent) and flavonoid (quercetin equivalent) contents were established using Folin–Ciocalteu's and aluminum chloride reagents, respectively. Additionally, high-performance liquid chromatography (HPLC) analysis for quantitative determination of phenolic acids and gas chromatography–mass spectrometry (GC-MS) analysis to define essential oil composition were applied in the plant.

Materials and Methods

Plant Material

The plant sample was collected in February, 2009 in the vicinity of Belek town of Antalya province, Turkey and identified by Prof. Dr. Hayri Duman of Department of Biology, Faculty of Science and Art, Gazi University, Ankara, Turkey. The voucher specimen (GUE 2788) is preserved in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Turkey.

Preparation of the Extracts

The aerial parts of the plant were dried in shade at room temperature and ground in a mechanic grinder to fine powder and weighed accurately (218.54 g). The powdered plant material was firstly macerated with 500 mL of *n*-hexane at room temperature for 2 days and then successively with chloroform, ethyl acetate, methanol, and distilled water in the same manner. Then, the organic phases obtained in each extraction were filtered through filter paper and evaporated in reduced pressure until dryness to give the crude extracts, which were kept at +4 °C until the time bioactivity and phytochemical studies were performed. Yields (*w/w*) of the prepared extracts were obtained as follows; *n*-hexane extract: 9.38 g; chloroform extract: 13.65 g; ethyl acetate extract: 12.23 g; methanol extract: 18.30 g; water extract: 16.79 g.

Isolation of the Essential Oil

The air-dried aerial parts of the plant (38.0 g) were hydro-distilled for 3 h using a Clevenger-type apparatus to produce a trace amount of essential oil which was trapped into *n*-hexane.

Enzyme Inhibitory Activity Assays

AChE and BChE Inhibitory Activity

AChE and BChE inhibitory activity was measured by slightly modifying the spectrophotometric method developed by Ellman et al. (1961). Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) and horse serum BChE (EC 3.1.1.8, Sigma) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as substrates of the reaction. 5,5'-Dithio-bis(2-nitrobenzoic)acid (DTNB, Sigma, St. Louis, MO, USA) was used for the measurement of the cholinesterase activity. All the other reagents and conditions were the same as described in our recent publication (Senol et al. 2010). Briefly, in this method, 140 µL of sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of test solution and 20 µL of AChE/BChE solution were added by multichannel automatic pipette (Eppendorf, Germany) in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of 10 µL of acetylthiocholine iodide/butyrylthiocholine chloride. The hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm utilizing a 96-well microplate reader (VersaMax Molecular Devices, CA, USA). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software. Percentage of inhibition of AChE/BChE was determined by comparison of rates of reaction of samples relative to blank sample (ethanol in phosphate buffer pH=8) using the formula $(E - S)/E \times 100$, where *E* is the activity of enzyme without test sample and *S* is the activity of enzyme with test sample. The experiments were done in triplicate. Galanthamine, the anticholinesterase alkaloid-type of drug isolated from the bulbs of snowdrop (*Galanthus* sp.), was purchased from Sigma (St. Louis, MO, USA) and used as reference.

LOX Inhibitory Activity

Inhibitory activity of the extracts against LOX was measured by modifying the spectrophotometric method developed by Tappel (1962). LOX (EC 1.13.11.12, type I-B), baicalein (reference drug), and linoleic acid (substrate) were purchased from Sigma (St. Louis, MO, USA). The reaction

mixture of total volume of 190 μL contained sodium phosphate buffer (pH 8.0), sample and enzyme solutions, which were later incubated for 10 min at 25°C. The reaction was initiated by addition of 10 μL of linoleic acid solution. Due to the formation of (9Z, 11E)-(13S)-13-hydroperoxyoctadeca-9,11-dienoate, the change of absorbance at 234 nm was followed for 6 min. All of the reactions were performed in 96-well microplates using ELISA microplate reader (Spectramax Plus₃₈₄ Molecular Devices, CA, USA). The percentage (%) inhibition was calculated as $(E - S)/E \times 100$, where E is the activity of the enzyme without test compound and S is the activity of enzyme with test compound. The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software.

TYRO Inhibitory Activity

Inhibition of tyrosinase (EC 1.14.1.8.1, 30 U, mushroom tyrosinase, Sigma) was determined using the modified dopachrome method with L-DOPA as substrate (Masuda et al. 2005). Assays were conducted in a 96-well microplate and an ELISA microplate reader (VersaMax Molecular Devices, CA, USA) was used to measure absorbance at 475 nm. The extracts dissolved in DMSO (50%) with 80 μL of phosphate buffer (pH 6.8), 40 μL of tyrosinase, and 40 μL of L-DOPA were put in each well. Each sample was accompanied by a blank that had all the components except for L-DOPA. Results were compared with a control consisting of 50% DMSO as well as the reference (α -kojic acid). Percentage inhibition of tyrosinase was calculated according to the formula given below:

$$\% \text{ Inhibition} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}}} \times 100 \quad (1)$$

Antioxidant Activity Assays

DPPH Free Radical-Scavenging Assay

The stable DPPH radical-scavenging activity was determined by Hatano's method (Hatano 1995). The samples dissolved in ethanol (75%) were mixed with DPPH solution (1.5×10^{-4} M) and left for 20 min incubation in dark at room temperature. Remaining DPPH amount was measured at 520 nm using a Unico 4802 UV-visible double beam spectrophotometer (USA). Gallic acid was employed as the reference and the experiments were performed in triplicate. Inhibition of DPPH in percent (I%) was calculated as given below: $I\% = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts/reference.

Fe⁺²-Ferrozine Test System for Ferric Ion-Chelating Activity

The ferric ion-chelating effect of the extracts by Fe⁺²-ferrozine test system was estimated by the method of Chua et al. (2008). Briefly, 740 μL of methanol and the samples were incubated with 2 mM FeCl₂ solution. The reaction was initiated by the addition of 40 μL of ferrozine solution into the mixture and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. The ratio of inhibition of ferrozine-Fe²⁺ complex formation was calculated as follows:

$\% \text{ Inhibition} = [(\text{Absorbance of control} - \text{Absorbance of test sample})/\text{Absorbance of control}] \times 100$. The control contained only FeCl₂ and ferrozine. Analyses were run in triplicate and expressed as average values with S.E.M. (standard error of the mean). The reference used in this method was ethylenediamine tetraacetic acid.

Ferric-Reducing Antioxidant Power Assay (FRAP)

The ferric-reducing power was tested using the assay of Oyaizu (1986). According to the method; the samples were added to phosphate buffer (pH 6.6) and potassium ferricyanide. Later, the mixture was incubated at 50 °C for 20 min, which was followed by addition of trichloroacetic acid. After the mixture was shaken vigorously, the solution was mixed with distilled water and FeCl₃ (0.1%, w/v). After 30 min incubation, absorbance was read at 700 nm using a Unico 4802 UV-visible double beam spectrophotometer (USA). Analyses were achieved in triplicate. Increasing absorbance of the reaction indicated increase in reducing power. The reference used in this method was chlorogenic acid.

Phytochemical analyses

Total Phenol and Flavonoid Contents

Phenolic content of the extracts was determined in accordance with Folin-Ciocalteu's method (Singleton and Rossi 1965). In brief, the samples dissolved in ethanol (75%) were mixed with 750 μL of Folin-Ciocalteu's reagent and 600 μL of sodium carbonate in test tubes. The tubes were then vortexed and incubated at 40 °C for 30 min. A number of dilutions of gallic acid were obtained to prepare a calibration curve. Afterward absorption was measured at 760 nm at a Unico 4802 UV-visible double beam spectrophotometer (USA). Total flavonoid content was calculated by aluminum chloride colorimetric method (Woisky and Salatino 1998). A number of dilutions of quercetin were obtained to prepare a calibration curve. Then, the extracts and quercetin were separately mixed with 75% ethanol, 10%

aluminum chloride, 1 M sodium acetate. Following incubation for 30 min at room temperature, absorbance of the reaction mixture was measured at 415 nm with a Unico 4802 UV–visible double beam spectrophotometer (USA). The total phenolic and flavonoid contents of the extracts were expressed as gallic acid and quercetin equivalents (in milligrams per gram extract), respectively.

Conditions of HPLC Analysis for Phenolic Acid Determination

Chemicals used for HPLC (methanol and formic acid) were of chromatographic grade (Sigma-Aldrich, St. Louis, MO, USA). Standards of the phenolic acids used in this study; gallic acid (GA), protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, chlorogenic acid, syringic acid, ferulic acid, and propylparaben as internal standard (IS) were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck (GmbH, Darmstadt, Germany). Analysis of phenolic acids in the extracts of *P. graecum* was carried out with Agilent 1100 series auto sampler system from Agilent, GL Sciences Inc (Waldbronn, Germany) equipped with a system controller, DAD detector (G 1315B, 280 nm), an quaternary LC pump (G1311A). The separation was carried out with a Zorbax Eclipse XDB-C18 column (150 mm, 4.6 mm i.d. and 5 µm particle size) (Agilent, Waldbronn,

Germany) with the column temperature set at 25°C. Chromatographic separation was carried out using two solvent systems: (A) methanol/water/formic acid (10:88:2, v/v/v); (B) methanol/water/formic acid (90:8:2, v/v/v), as reported elsewhere (Ozturk et al., 2007). Ultrapure water was supplied from Human UP 9000 System (18 mW) water purification system. The analyses were performed by using a linear gradient program. A gradient elution program was applied as follows: the pumping solution was 100% A between 0 and 15 min; it decreased to 85% A from 15 to 20 min, further decreasing to 50% A from 20 to 30 min, to 0% A from 30 to 35 min and at 100% A between 35–42 min. The flow-rate was adjusted as 1 mL/min and the injection volume was 10 µL. Signals were detected at 280 nm. Besides, the IS (propylparaben) technique was applied to the analysis in order to increase the repeatability. The extracts were dissolved in a mixture of methanol and water (1:1, v/v) and injected into the HPLC. Identification of the peaks was achieved as follows: All of the phenolic acid standards were dissolved individually. Propylparaben was used as an IS to increase the repeatability of the method. The integrated peak areas and their retention times were computed to get the rate of peak normalization (peak area/peak retention time) of the relevant phenolic acids, and their amounts were calculated in the related extracts *via* their calibration curves.

Table 1 Enzyme inhibitory activities (percentage±S.E.M.) of *P. graecum* extracts

Enzymes	Concentrations (µg/mL)	Enzyme inhibitory activity (percentage±S.E.M. ^a)				
		<i>n</i> -Hexane	Chloroform	Ethyl acetate	Methanol	Water
AChE	50	– ^b	–	–	–	–
	100	–	–	–	–	20.89±3.24
	200	–	–	–	–	41.26±0.07
BChE	50	–	–	–	–	–
	100	–	–	–	–	–
	200	–	–	22.35±3.29	–	9.14±0.25
LOX	50	–	–	–	–	–
	100	–	–	–	–	–
	200	19.29±1.21	13.06±2.15	–	–	–
TYRO	50	–	–	–	–	–
	100	–	–	–	5.08±2.24	–
	200	–	–	3.95±0.01	8.82±1.22	–
Galanthamine ^c	100	98.97±0.24 for AChE and 89.95±0.87 for BChE at 100 µg/mL				
Baicalein ^d	100	77.94±1.03 at 100 µg/mL				
α-Kojic acid ^e	100	78.89±0.09 at 100 µg/mL				

^a Standard error of the mean.

^b No activity.

^c Reference for AChE and BChE

^d Reference for LOX

^e Reference for TYRO

Table 2 DPPH radical-scavenging activity (inhibition %±S.E.M.) and ferrous ion-chelating capacity (inhibition % ± S.E.M.) and total phenol±S.E.M. and total flavonoid ± S.E.M. contents of *P. gracuum* extracts

Extracts	DPPH radical-scavenging activity (inhibition %±S.E.M. ^a)			Total phenol ^b content±S.E.M.		Total flavonoid ^c content±S.E.M.	
	125 µg/mL	250 µg/mL	500 µg/mL	1,000 µg/mL	2,000 µg/mL	1,000 µg/mL	2,000 µg/mL
<i>n</i> -Hexane	10.87±0.09	10.80±0.01	11.0±0.10	11.61±0.01	12.67±0.03	37.69±4.85	149.03±1.11
Chloroform	10.26±0.01	9.85±0.76	11.68±0.29	14.84±0.38	22.0±0.57	47.07±4.33	711.46±8.71
Ethyl acetate	23.62±0.38	32.32±1.05	49.19±0.86	78.61±0.66	88.33±0.47	219.08±6.96	380.45±6.14
Methanol	22.07±0.47	33.94±0.09	55.20±2.10	80.91±0.67	83.81±0.38	150.74±4.95	285.61±6.25
Water	10.26±0.57	10.19±0.48	9.32±0.19	13.90±0.01	81.65±0.01	39.86±1.45	31.12±3.86
Gallic acid (Reference for DPPH scavenging activity)							
Extracts							
Ferric ion-chelating capacity (Inhibition %±S.E.M.)							
	125 µg/mL	250 µg/mL	500 µg/mL	1,000 µg/mL	2,000 µg/mL	Phenolic acid amounts found by HPLC analysis (mg/g)	
<i>n</i> -Hexane	2.64±0.74	38.16±3.58	48.69±3.29	45.0±3.35	66.32±4.38	Ferulic acid	– ^d
Chloroform	15.79±3.72	27.29±2.12	51.32±3.34	57.90±0.74	41.32±1.86	<i>o</i> -Coumaric acid	–
Ethyl acetate	– ^e	–	–	–	–		0.71
Methanol	–	–	–	–	18.69±4.33		5.03
Water	47.10±2.60	63.95±1.12	70.0±4.20	75.26±0.01	75.0±0.37		–
Ethylenediamine tetraacetic acid (EDTA) (Reference for ferric ion-chelating capacity)			35.40±3.21	57.08±2.88	80.13±2.49		–

^a Standard error mean.^b Data expressed in mg equivalent of gallic acid (GAE) to 1 g of extract.^c Data expressed in mg equivalent of quercetin to 1 g of extract.^d Not found.^e No activity.

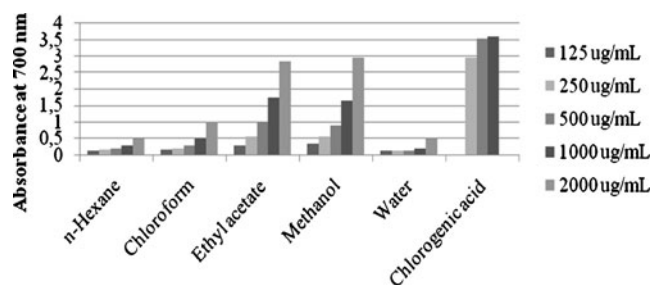


Fig. 1 FRAP results of the *P. graecum* extracts and the reference (chlorogenic acid)

Conditions of GC-MS Analysis of the Essential Oil

The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. Innovax FSC column (60 m×0.25 mm, 0.25 μm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. Mass range was from m/z 35 to 450. The GC analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300°C. To obtain

the same elution order with GC-MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from the FID chromatograms.

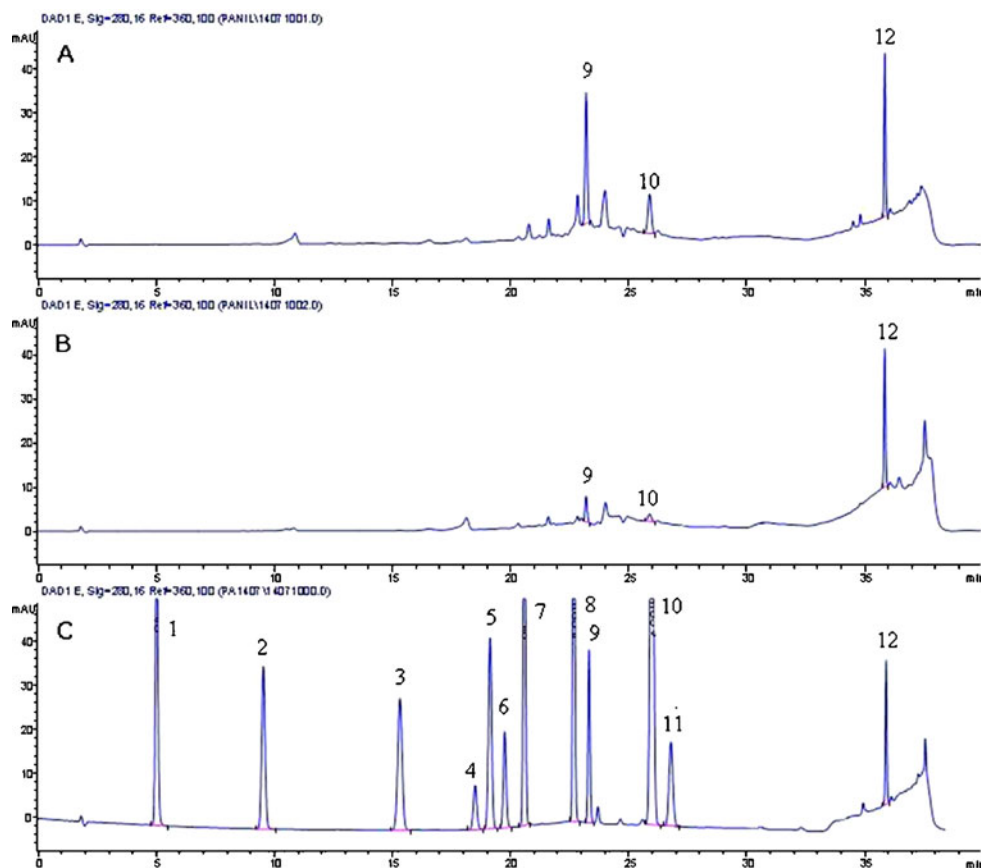
Identification of the essential oil components was carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index to a series of *n*-alkanes. Computer matching against commercial (Wiley GC-MS Library, Adams Library, MassFinder 3 Library; McLafferty and Stauffer 1989; Koenig et al 2004) and in-house “Başer Library of Essential Oil Constituents” built up by genuine compounds and components of known oils, as well as MS literature data (Jennings and Shibamoto 1980; Joulain and Koenig 1998; ESO 1999) was used for the identification.

Results and Discussion

Enzyme Inhibitory and Antioxidant Effects

The results of enzyme inhibitory activity of the extracts of *P. graecum* against AChE and BChE (in Alzheimer’s disease), LOX (in inflammation), and TYRO (in

Fig. 2 The representative chromatograms of **a** methanol and **b** ethyl acetate extracts prepared from *P. graecum*, and **c** mixture of standard phenolic acids: The phenolic acids are symbolized as 1 (gallic acid), 2 (protocatechuic acid), 3 (*p*-hydroxybenzoic acid), 4 (vanillic acid), 5 (caffeic acid), 6 (chlorogenic acid), 7 (syringic acid), 8 (*p*-coumaric acid), 9 (ferulic acid), 10 (*o*-coumaric acid), 11 (*trans*-cinnamic acid) and 12 (propylparaben as internal standard) on the chromatograms



hyperpigmentation and melasma) are shown in Table 1. Accordingly, among the extracts, only the water extract showed a moderate inhibition against AChE having 20.89 ± 3.24 and $41.26 \pm 0.07\%$ at 100 and 200 $\mu\text{g/mL}$, respectively. However, the extracts displayed either no or very low inhibition against the tested enzymes. The extracts were tested for their antioxidant activity at 125, 250, 500, 1,000, and 2,000 $\mu\text{g/mL}$ concentrations using three *in vitro* test systems. As shown in Table 2, the ethyl acetate, methanol, and water extracts exerted a high scavenging activity against DPPH radical. The most effective extract in this test was found to be the ethyl acetate extract ($88.33 \pm 0.47\%$) at 2,000 $\mu\text{g/mL}$. On the other hand, the water extract had the highest ferric ion-chelating capacity ($75.0 \pm 0.37\%$), followed by the *n*-hexane extract ($66.32 \pm 4.38\%$; Table 2). In the FRAP assay, the ethyl acetate and methanol extracts showed a remarkable activity at 2,000 $\mu\text{g/mL}$, which was similar to that of reference (chlorogenic acid) at 1,000 $\mu\text{g/mL}$ (Fig. 1).

Total Phenol and Flavonoid Contents

Calibration equations were calculated as $y = 2.8034x + 0.009$ ($r^2 = 0.9796$) and $y = 2.7319x - 0.0308$ ($r^2 = 0.9934$) for total phenol and total flavonoid contents, respectively. According to the results of total phenol and flavonoid amounts of the extracts listed in Table 2, the ethyl acetate extract possessed the highest total phenol content (219.08 ± 6.96 mg/g extract as gallic acid equivalent). On the other hand, the chloroform extract had the richest total flavonoid amount as quercetin equivalent (711.46 ± 8.71 mg/g extract), followed by the ethyl acetate extract (380.45 ± 6.14 mg/g extract).

Analysis of Phenolic Acids in the Methanol and Ethyl Acetate Extracts

Determination of phenolic acids was realized by injecting the methanol and ethyl acetate extracts prepared from *P. graecum* into the column after addition of IS. The related chromatograms are demonstrated in Fig. 2. The amounts of the relevant phenolic acids were calculated by using their calibration equations as reported previously by one of us (Ozturk et al 2007). HPLC analysis of the extracts revealed presence of mainly two phenolic acids; ferulic and *o*-coumaric acids, which were only found in the ethyl acetate and methanol extracts (Table 2). According to our results, the methanol extract was rather richer in these phenolic acids than the ethyl acetate extract. Rest of the extracts did not contain any of the phenolic acids analyzed herein.

Essential oil composition

Composition of the essential oil of *P. graecum* was established by GC-MS and 38 components were identified representing 86.0% of the oil. Table 3 shows the identified

Table 3 Composition of the essential oil of *P. graecum*

RRI ^a	Compounds	% ^b
1032	α -Pinene	0.2
1118	β -Pinene	0.3
1400	Nonanal	0.1
1497	α -Copaene	0.7
1535	β -Bourbonene	0.6
1597	β -Copaene	0.2
1612	β -Caryophyllene	9.4
1661	Alloaromadendrene	0.4
1670	<i>trans</i> -Pinocarveol	0.1
1687	α -Humulene	1.6
1704	γ -Muurolene	2.0
1726	Germacrene D	21.3
1740	α -Muurolene	1.0
1773	δ -Cadinene	3.2
1776	γ -Cadinene	1.4
1784	(<i>E</i>)- α -Bisabolene	0.9
1827	(<i>E,E</i>)-2,4-Decadienal	tr ^c
1849	Calamenene	tr
1868	(<i>E</i>)-Geranyl acetone	0.8
1900	<i>epi</i> -Cubebol	0.2
1958	(<i>E</i>)- β -Ionone	2.0
1973	Dodecanol	1.5
1992	2-Phenylethyl isovalerate	0.6
2008	Caryophyllene oxide	6.0
2037	Salvial-4(14)-en-1-one	0.7
2069	Germacrene D-4 β -ol	1.1
2071	Humulene epoxide-II	0.9
2130	Salviadienol	1.1
2131	Hexahydrofarnesyl acetone	9.6
2144	Spathulenol	0.6
2187	<i>trans</i> -Cadinol	1.8
2209	<i>trans</i> -Muurolol	2.1
2255	α -Cadinol	2.7
2278	Torilenol	1.1
2369	Eudesma-4(15),7-dien-4 β -ol	1.7
2700	Heptacosane	1.1
2900	Nonacosane	0.9
2931	Hexadecanoic acid	6.1
	Total	86.0

^a Relative retention indices calculated against *n*-alkanes

^b % calculated from FID data

^c Trace (<0.1%)

compounds and their percentages obtained by GC-MS as well as the retention indices listed in order of their elution from the HP Innowax capillary column. According to GC-MS data, germacrene D (21.3%) was the dominant compound in the oil, followed by hexahydrofarnesyl acetone (9.6%), β -caryophyllene (9.4%), hexadecanoic acid (6.1%), caryophyllene oxide (6.0%), and δ -cadinene (3.2%).

Only some species of the genus *Phagnalon* have been investigated so far in terms of phytochemistry and biological activity. Nevertheless, our literature survey pointed out to absence of phytochemical and bioactivity data on *P. graecum*, which encouraged us to perform the current study. Up to date, there has been only one report on AChE and BChE inhibitory activity of a single *Phagnalon* species; namely *P. saxatile* (Conforti et al. 2010), which also encouraged us to work on *P. graecum* in a similar manner. The methanol extract of *P. saxatile* showed no inhibition against AChE, while it displayed 100% inhibition on BChE at 1,000 $\mu\text{g}/\text{mL}$, which was tested using the same method used in our study. Although the extract per se did not show anti-AChE activity, its main constituents including caffeic acid, apigenin, apigenin-7-*O*- β -glucopyranoside, 3,5-dicaffeoylquinic acid, and luteolin-4'-*O*-glucopyranoside had 100% inhibitory activity against AChE at 100 $\mu\text{g}/\text{mL}$, except for chlorogenic and methylchlorogenic acids. All of the active compounds against AChE were found to have 100% inhibition at 100 $\mu\text{g}/\text{mL}$ towards BChE, whereas only luteolin-4'-*O*-glucopyranoside displayed lower inhibition (50.3%) at the same concentration. It should be noted that cholinesterase inhibitory data of *P. saxatile* seems to differ with our anti-cholinesterase results of *P. graecum*. However, this dissimilarity might be apparently resulting from their different phytochemical contents. Considering the same report on *P. saxatile*, its methanol extract exerted a very high DPPH radical-scavenging activity (over 90% at 100, 250, 500, and 1,000 $\mu\text{g}/\text{mL}$), which is compliant with our results on the methanol extract of *P. graecum*. The extract and its phenolic components also showed remarkable antioxidant activity in β -carotene bleaching assay and inhibition of nitric oxide production in that study (Conforti et al. 2010). Since the phenolic compounds had the high antioxidant activity in *P. saxatile*, the phenolic acids in the methanol extract of *P. graecum* identified in our analysis could be assumed to be responsible for the marked antioxidant activity of this extract.

There has been only one other study on antioxidant activity related to other *Phagnalon* species. In that study, Góngora et al. (2003) described xanthine oxidase inhibitory activity of the caffeoyl conjugates of prenylhydroquinone glucoside and quinic acid isolated from *P. rupestre*. In the conclusion of this study, the authors stated that the caffeoyl moiety is vital for both the antiperoxidative and radical-scavenging activities, and the methylation of the quinic

carboxyl group enhanced the effectiveness on xanthine oxidase inhibitory activity.

Although various derivatives of phenolic compounds were identified in different *Phagnalon* species (Hausen and Schultz 1977; Góngora et al. 2001, 2002a, b, 2003; Olmos et al. 2005), none of the phenolic acids (ferulic and *o*-coumaric acids), that we identified in *P. graecum*, has been reported to exist in other *Phagnalon* species up to date.

Among all species of *Phagnalon*, only the essential oil composition of *Phagnalon sordidum* was elucidated (Salgues 1953), which was dominated by α -pinene and azulogenene, a sesquiterpene derivative commonly found in roman chamomile. Conversely, the essential oil of *P. graecum* examined herein did not contain azulogenene and a quite low amount of α -pinene (0.2%) (Table 3).

Conclusion

Our results on enzyme inhibitory and antioxidant activities of *P. graecum* indicated that the plant has a strong antioxidant activity and quite prosperous in total phenol and flavonoid contents, which can be considered to be a rich source of potential antioxidants. The water extract having moderate AChE inhibitory activity may be considered to be tested for further phytochemical analysis in order to elucidate its possible active constituents. To the best of our knowledge, we herein report the first study describing AChE, BChE, LOX, and TYRO enzyme inhibitory and antioxidant activities of *P. graecum* as well as its phenolic content and essential oil composition.

Acknowledgment F.S. Senol expresses her genuine gratitude to the Scientific and Technological Research Council of Turkey (TUBITAK) for the scholarship provided during her Ph.D. program.

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