

Chemical profiles and biological properties of methanol extracts of *Allium pallens* L. from different localities in Turkey

Ahmet Emir* and Ceren Emir

Department of Pharmacognosy, Faculty of Pharmacy, Ege University, 35100, Bornova, İzmir, Turkey

*Corresponding author: ahmet.emir@ege.edu.tr

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Abstract: Many species of the *Allium* genus, principally the cultivated forms, are widely used as vegetables, spices and natural therapeutics due to their beneficial health properties. This study aimed to identify the phenolic composition and biological activities of the bulb, stem and flower parts of *Allium pallens* L., collected from two different localities. A total of 28 phenolic compounds were investigated by LC-ESI-MS/MS, and gallic acid, 4-hydroxybenzoic acid, and benzoic acid were found to be the major phenolic compounds in the plants from both locations. Total phenolic- and flavonoid-content analyses of samples were carried out using spectrophotometry, and the stem extracts were found to be rich in phenolics. DPPH, ABTS, FRAP and CUPRAC assays were used to determine the antioxidant capacities of the extracts. A linear relation was observed between the phenolic contents of the extracts and their antioxidant activities, and the stem extracts of plants from both locations were found to have potent antioxidant capacity. The inhibitory activities of the extracts against acetylcholinesterase, butyrylcholinesterase and tyrosinase were determined using a 96-well microplate reader. The anti-butyrylcholinesterase activity of the extracts was found to be the highest. The outcomes of these investigations were further explored, and the underlying structure of multivariate data was revealed using principal component analysis. This study presents the distribution of chemical constituents and biological activities of the different parts of *A. pallens*, and also contributes to further investigations of *Allium* species.

Keywords: *Allium*; phenolics; antioxidant; anticholinesterase; antityrosinase

INTRODUCTION

Secondary metabolites are chemically quite different from each other and synthesized in different pathways in plants. These metabolites are not directly necessary for the basic functions of plants and are thought to be synthesized for protection from insects, oxidizing agents and ultraviolet radiation, as well as for pollination [1]. The characterization of plant secondary metabolites using modern chromatographic methods is a significant subject both in plant physiology and phytochemistry. Plant phenolics are one of the largest groups of secondary metabolites. Phenolics are formed through either the shikimic acid or the malonate/acetate pathways, and are chemically divided into sixteen subgroups according to their basic structure. A large volume of research has focused on phenolic compounds due to their wide range of pharmacological and biological effects [2].

Plant phenolics have considerable efficacy as modulators of cellular biomarkers relating to oxidative stress; they can thus lower the risk for various chronic diseases [3]. The phenyl rings and hydroxyl groups in their chemical structures cause these molecules to exhibit strong antioxidant activity. The redox characteristics of phenolics provide them with the ability to scavenge reactive oxygen species (ROS), reducing agents and metal chelating [4]. Additionally, increasing numbers of studies have focused on the inhibitory properties of phenolic compounds against enzymes such as α -amylase, α -glucosidase, lipase, cholinesterases and tyrosinase [5]. Enzymes are essential molecules owing to their catalytic roles in different life processes and the pathophysiology of numerous ailments and are thus attractive molecular targets in human diseases. In fact, 47% of all marketed small molecule drugs act by inhibiting various enzymes [6]. However, such drugs

can cause numerous side effects, including nausea, vomiting, diarrhea, dizziness, abdominal pain and headache, all of which negatively affect the quality of life of patients [7]. Therefore, studies focused on finding natural enzyme inhibitors with minimal side effects are of great interest to many researchers.

The genus *Allium* L. is represented by about 900 species [8] and is a taxonomically problematic and highly variable genus distributed across the northern hemisphere [9]. Although the genus *Allium* was formerly included in the Liliaceae family, the Angiosperm Phylogeny Group (APG) has reevaluated the taxonomic position of this genus using molecular studies, and has finally transferred *Allium* to the Amaryllidaceae family, subfamily Allioideae, tribe Allieae [10]. Many species of the *Allium* genus, principally cultivated forms such as *A. sativum* (garlic), *A. cepa* (onion), *A. porrum* (leeks) and *A. ascalonicum* (shallots), are widely used as vegetables, spices and natural therapeutics due to their beneficial health properties. They are known to have significant beneficial effects on the cardiovascular system due to their hypotensive, hypolipidemic, platelet-preventing and hypocholesterolemic effects [11,12]. Additionally, several studies have reported that many *Allium* species have potent inhibitory activities against cholinesterases and tyrosinase, which are both key enzymes linked to neurodegenerative diseases and skin disorders. Twenty-three different *Allium* species in terms of their respective inhibitory activities against acetylcholinesterase (AChE) were investigated, and all species were reported as active, with *A. obliquum* being the most potent [13]. The anticholinesterase and antityrosinase activities of bulb, stem and flower parts of *A. scorodoprasum* subsp. *rotundum* were examined [14], and all three parts of the plant demonstrated potent inhibitory activity against these enzymes. A previous study by our group investigated the cholinesterase and tyrosinase inhibitory potentials of different parts of *A. nigrum* and *A. subhirsutum* and observed enzyme inhibitory activity in all samples, with the bulb parts of both plants showing the highest activity [15].

Allium pallens L. is a synanthropic species distributed in the Mediterranean region [16], and was collected from Ovacık, Ödemiş, and Bozdağ, Ödemiş, in Turkey for chemical and biological investigations. To the best of our knowledge, this is the first report of the phenolic profile *A. pallens*, along with its inhibitory

activity against AChE, butyrylcholinesterase (BuChE) and tyrosinase as well the antioxidant potentials of its bulb, stem and flower.

MATERIALS AND METHODS

Chemicals

The reference standards used in the LC-MS/MS analysis, AChE (from *Electrophorus electricus*), BuChE (from equine serum), acetylthiocholine/butyrylthiocholine iodide, DTNB (Ellman's reagent) [5,5'-dithio-bis-(2-nitrobenzoic acid)], tyrosinase (from mushroom), galanthamine, L-dopa, kojic acid (used for enzyme inhibitory activities), 1,1-Diphenyl-2-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), potassium persulfate, trolox, copper(II) chloride, neocuproine, 2,4,6-tris(2-pyridyl)-s-triazine and ferric chloride (used for antioxidant activities) were all purchased from Sigma-Aldrich, USA.

Plant materials

Allium pallens L. was collected in July 2018 from Ovacık, Ödemiş (No: 1610), and Bozdağ, Ödemiş (No: 1614), (İzmir, Turkey). The plants were identified by Dr. Hasan Yıldırım. The morphological characteristics of the *Allium* specimens were examined using a binocular stereo microscope (Olympus SZ60 Trinocular Stereo-zoom Microscope) and the identification of *A. pallens* was performed according to the relevant taxonomic literature [17]. Voucher specimens were deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

Sample preparation

For all assays, the bulb, stem and flower parts of the plants were extracted using methanol. Two grams of air-dried, powdered sample and 15 mL of methanol were added to falcon tubes. The extraction was carried out three times under continuous stirring in a rotator (ISOLAB Laborgeräte GmbH). The samples were then centrifuged at 2900 xg for 10 min in order to obtain the supernatants. The liquid phase was then evaporated using a rotary evaporator (Buchi) under reduced pressure and the extracts were kept at 4°C until use.

Total bioactive compounds

A modified Folin-Ciocalteu method [18] was performed to determine the total phenolic content (TPC) of the samples. One mL of ten-fold-diluted reagent plus the samples was added to falcon tubes and neutralized with 4 mL sodium carbonate solution (7.5%). The mixture was incubated at room temperature for 20 min and the absorbance was measured at 765 nm. Gallic acid was used as the standard and the results were expressed as gallic acid equivalents (mg GAE/g extract) based on the standard curve created.

The total flavonoid content (TFC) of extracts was evaluated using the AlCl₃ method with slight modifications, as described [19]. Two mL of extract at a concentration of 1 mg/mL were mixed with 0.1 mL of a 10% AlCl₃ solution. Then 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water were added to the mixture. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm. The results are given as quercetin equivalents (mg QE/g extract) based on a standard curve created with quercetin.

Identification of phenolic compounds

Twenty-eight phenolic compounds were discovered in the different parts of the plants using a TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer (Thermo Scientific™, USA). Chromatographic separation of phenolics was performed using a GL Sciences (Japan) ODS C18 column (150 mm × 4.6 mm × 5 µm) with a gradient mobile phase that consisted of water (A) and methanol (B), both of which were acidified with 0.1% formic acid. Gradient elution was carried out as follows: 5-20% B (0-1.5 min), 20-30% B (1.5-3 min), 30-50% B (3-4.75 min), 50-70% B (4.75-6.25 min), 70-80% B (6.25-7.5 min), 80-90% B (7.5-10 min), 90-95% B (10-12.5 min), 95-5% B (12.5-15 min). The flow rate and the injection volume were 1.0 mL/min and 5 µL, respectively. ESI parameters were as follows: capillary temperature: 400°C; vaporizer temperature: 500°C; the flow rate of the sheath gas, aux gas, and sweep gas were kept at 75 arb, 20 arb and 0 arb, respectively. The identity of the phenolic compounds in the samples was determined by comparing their retention times and MS/MS fragments with reference standards. An external standard method was performed, and the results are expressed as µg per gram of extracts.

Enzyme inhibitory activities

The anticholinesterase activity of the extracts was determined according to Ellman's method [20], which is based on the spectrophotometric measurement of the yellow color formed as a result of released thiocholines through the reaction with Ellman's reagent, with previously described modifications [21], using a microplate reader (Varioskan Flash Multimode Reader, Thermo Scientific, USA).

The antityrosinase activity of the samples was determined using the dopachrome method with a 96-well microplate reader [22]. The procedures of this experiment have been defined in our previous study [15]. The IC₅₀ values of the samples were determined using GraphPad Prism V5.0 software (GraphPad Software, San Diego, CA, USA) and the results given represent the averages from three independent experiments.

Estimation of radical scavenging activities

DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay

The DPPH assay was performed as detailed [23], with slight modifications. Specifically, 0.1 mM DPPH was mixed with the methanol extract of the sample and incubated for 30 min at room temperature. The absorbance was measured at 517 nm and Trolox, a hydrophilic analog of vitamin E, was used as a positive control. The results were given in milligrams of Trolox equivalents/g of extract (mg TE/g extract).

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay

The ABTS⁺ radical cation was obtained by mixing an equal proportion of 7.4 mM ABTS and 2.45 mM potassium persulfate solutions and allowing the mixture to stand at room temperature in the dark. This solution was then diluted with methanol until the absorbance reached 0.700±0.02 at 734 nm. Then, 0.2 mL of methanol extract and 2.80 mL of ABTS⁺ solution were mixed together and incubated for 2 h at room temperature, after which the absorbance was measured at 734 nm [24]. Trolox was used as the positive control and the results were given in milligrams of Trolox equivalents/g of extract (mg TE/g extract).

Estimation of reducing power activities

Cupric ion reducing (CUPRAC) assay

The CUPRAC assay was performed as described [25], with slight modifications. Ten mM CuCl₂, 7.5 mM neocuproine and the sample solution were mixed at pH 7.0. After incubation in the dark for 30 min, the absorbance values were measured at 450 nm. The results are given in mg TE/g extract.

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP reagent was prepared by mixing 25 mL of 0.3 M acetate buffer (pH 3.6) and 2.5 mL of 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl and 2.5 mL of 20 mM ferric chloride. Methanol extracts (0.1 mL) from the samples were added to 2 mL of this solution and the mixture was incubated for 30 min at room temperature, after which the absorbance was recorded at 593 nm [26]. Trolox was used as the positive control and the results were given in mg TE/g extract.

Statistical analysis

The results of all analyses were subjected to principal component analysis (PCA) using SPSS.25 software. The number of variables was reduced and the most discriminating phenolics and biological activities of the different parts of plants from both localities were determined for the purpose of assigning resemblances and distinctions. Additionally, a one-way ANOVA test (Tukey's test) was used to determine differences among means and the differences were considered as significant with $P<0.05$.

RESULTS

Phenolic profile

Bulb, stem and flower parts of *A. pallens* were collected from different localities and their compounds were extracted using a quick, efficient and straightforward method. The extraction yields, identification (ID), TPC and TFC of the samples are given in Table 1. In each case, the highest yield was obtained from the bulb parts, followed by the flower parts and then the stem.

Table 1. Sample ID and extraction yield of extracts.

Collection site	Part used	Sample ID	Yield (%)	TPC (mg GAEx/g extract)	TFC (mg QEY/g extract)
Ovacık/ Ödemiş	Bulb	AP-1B	16.91	12.52±0.95a	2.01±0.22a
	Flower	AP-1F	11.65	11.28±0.63a	1.84±0.43b
	Stem	AP-1S	9.27	30.74±0.97b	2.15±0.34a
Bozdağ/ Ödemiş	Bulb	AP-2B	18.63	15.85±0.85b	1.77±0.31b
	Flower	AP-2F	14.75	12.64±0.76a	1.14±0.26c
	Stem	AP-2S	10.52	39.32±0.69b	3.29±0.12d

Values are means±SD of three parallel measurements. ^aGallic acid equivalent, ^bQuercetin equivalent. Superscripts indicate significant differences in the studied extracts ($P<0.05$).

The TPCs and TFCs of all the samples were screened spectrophotometrically. The TPC of the samples was determined using the Folin-Ciocalteu method, which is based on a redox reaction, and the results are stated in gallic acid equivalents. The results indicate that AP-2S had the highest TPC level at 39.32 mg/g. The extracts from the other samples demonstrated TPCs that varied from 11.28 mg/g for AP-1B to 30.74 mg/g for AP-1S. Additionally, the TFCs of the samples were determined using the AlCl₃ assay, which is based on the generation of a complex between aluminum ions, the C-4 keto group and either the C-3 or C-5 hydroxyl groups of flavonoids. The results of this assay are stated in quercetin equivalents and they showed similarity to the TPC assay. Specifically, the sample extracts demonstrated TFCs varying from 1.14 mg/g for AP-2F to 3.29 mg/g for AP-2S.

The aim of liquid chromatography tandem-mass spectrometry analysis was to simultaneously determine and quantify the different classes of phenolic compounds, which vary widely in their concentrations in the three parts of both plant samples. Also, this type of analysis provides information that is useful in predicting the structures of phenolics responsible for biological activities. Twenty-eight phenolic compounds, including flavonoids and phenolic acids in methanol extracts, were chosen for the LC-ESI-MS/MS analysis and the results are detailed in Table 2. Among the compounds identified, luteolin, daidzein, chrysins, myricetin and hesperidin were found as trace amounts in all samples, while vitexin and rutin were not detected in any of the samples. Twenty-one compounds were quantified from the plant extracts and these results are expressed as µg/g extract. The results indicated that gallic acid, 4-hydroxybenzoic acid and benzoic

Table 2. Concentrations of phenolic compounds ($\mu\text{g g}^{-1}$ of extract) of different parts of *A. pallens*.

Compound	AP-1B	AP-1F	AP-1S	AP-2B	AP-2F	AP-2S
Gallic acid	1054.3 \pm 2.18 ^a	1181.6 \pm 3.52 ^a	1269.2 \pm 4.15 ^b	958.4 \pm 2.98 ^a	998.8 \pm 1.75 ^a	1264.4 \pm 3.72 ^c
<i>p</i> -Coumaric acid	202.0 \pm 1.67 ^a	293.9 \pm 2.89 ^b	197.1 \pm 1.22 ^c	239.9 \pm 1.65 ^b	208.2 \pm 1.93 ^b	270.9 \pm 1.22 ^b
Benzoic acid	441.6 \pm 1.83 ^a	424.7 \pm 2.61 ^a	461.6 \pm 3.05 ^a	391.6 \pm 3.17 ^a	353.4 \pm 2.16 ^a	412.5 \pm 4.63 ^a
4-Hydroxybenzoic acid	727.5 \pm 3.05 ^a	711.1 \pm 1.83 ^a	824.7 \pm 4.38 ^a	570.6 \pm 1.09 ^a	495.8 \pm 3.09 ^a	539.0 \pm 1.38 ^a
Vanillic acid	7.1 \pm 1.26	4.0 \pm 1.24	10.8 \pm 0.51	6.2 \pm 0.39	5.5 \pm 2.78	4.7 \pm 1.26
Ferulic acid	14.8 \pm 1.53 ^a	8.5 \pm 1.63 ^b	17.8 \pm 2.33 ^c	3.4 \pm 0.55 ^d	4.0 \pm 1.21 ^e	10.2 \pm 1.53 ^f
Syringic acid	8.4 \pm 0.95 ^a	12.3 \pm 0.88 ^b	49.5 \pm 1.78 ^c	10.4 \pm 1.73 ^d	13.8 \pm 0.52 ^e	6.5 \pm 0.87 ^f
Kaempferol	33.6 \pm 2.64 ^a	22.9 \pm 1.71 ^b	65.6 \pm 1.49 ^c	60.0 \pm 2.81 ^d	32.8 \pm 1.64 ^e	109.6 \pm 2.54 ^b
Luteolin	T	T	T	T	T	T
Fisetin	9.3 \pm 0.88 ^a	14.5 \pm 2.76 ^b	4.5 \pm 0.72 ^c	8.7 \pm 1.64 ^d	16.8 \pm 0.74 ^e	6.7 \pm 0.55 ^f
Morin	1.2 \pm 0.56 ^a	2.8 \pm 0.44 ^b	1.2 \pm 0.16 ^a	T	1.3 \pm 0.28 ^c	T
Quercetin	5.4 \pm 0.72	3.2 \pm 0.79	T	3.5 \pm 1.26	2.2 \pm 0.53	T
3-O-methylquercetin	66.2 \pm 2.18 ^a	87.3 \pm 4.09 ^b	91.3 \pm 2.35 ^c	67.0 \pm 2.61 ^d	76.9 \pm 2.95 ^e	83.4 \pm 1.97 ^f
Daidzein	T	T	T	T	T	T
Chrysin	T	T	T	T	T	T
Isorhamnetin	6.9 \pm 1.74	6.4 \pm 1.28	4.1 \pm 0.72	4.2 \pm 0.97	4.4 \pm 1.88	6.8 \pm 0.61
Galangin	43.9 \pm 3.09	54.6 \pm 2.16	58.3 \pm 1.48	49.9 \pm 1.25	51.5 \pm 2.69	53.8 \pm 1.73
Myricetin	T	T	T	T	T	T
Vitexin	ND	ND	ND	ND	ND	ND
Hesperidin	T	1.9 \pm 0.23	T	T	T	1.5 \pm 0.24
3-Hydroxyflavone	4.8 \pm 1.55	5.8 \pm 1.42	5.8 \pm 0.41	2.9 \pm 0.52	4.2 \pm 0.98	4.5 \pm 0.31
Naringenin	11.2 \pm 0.91 ^a	12.5 \pm 0.65 ^b	20.2 \pm 1.49 ^c	14.9 \pm 1.77 ^d	7.9 \pm 1.82 ^e	11.1 \pm 0.89 ^f
Genistein	27.2 \pm 4.07 ^a	33.4 \pm 2.96 ^b	37.2 \pm 2.53 ^c	31.5 \pm 1.83 ^b	13.2 \pm 3.03 ^d	31.7 \pm 1.64 ^b
Rutin	ND	ND	ND	ND	ND	ND
Catechol	143.3 \pm 3.65 ^a	100.0 \pm 4.12 ^b	190.0 \pm 3.74 ^b	146.7 \pm 3.79 ^c	6.7 \pm 0.21 ^b	286.7 \pm 3.49 ^b
(+)-Catechin	1.3 \pm 0.78	T	T	T	T	T
(-)-Epicatechin	5.1 \pm 0.91	4.6 \pm 0.67	4.3 \pm 0.22	2.0 \pm 0.83	7.1 \pm 1.53	2.7 \pm 1.03
(-)-Epigallocatechin gallate	10.2 \pm 2.16 ^a	8.3 \pm 0.44 ^b	23.9 \pm 0.54 ^c	11.3 \pm 1.63 ^d	2.8 \pm 0.95 ^e	14.9 \pm 1.28 ^e

Values are means \pm SD of three parallel measurements. ND – not detected, T – trace amounts. Superscripts indicate significant differences in the studied extracts ($P<0.05$).

acid were the major phenolic acids in all samples. In terms of flavonoids, kaempferol (109.6 \pm 2.54 $\mu\text{g/g}$) in sample AP-2S and 3-O-methylquercetin (91.3 \pm 2.35 $\mu\text{g/g}$) and galangin (58.3 \pm 1.48 $\mu\text{g/g}$) in sample AP-1S were the most abundant compounds.

Enzyme inhibitory activities

Cholinesterase inhibitors are important drugs used in the symptomatic treatment of Alzheimer's disease [7]. The AChE enzyme localized in neurons is thought to be responsible for 80% of the cholinesterase activity in glial cells, while the remaining 20% is provided by the BuChE enzyme [27]. Methanol extracts from the bulb, stem and flower parts of the plants were tested for their AChE and BuChE inhibitory activities (Table 3)

Table 3. Enzyme inhibitory properties of the samples.

Sample	AChE Inhibition (IC_{50} $\mu\text{g/mL}$)	BuChE Inhibition (IC_{50} $\mu\text{g/mL}$)	Tyrs Inhibition (IC_{50} $\mu\text{g/mL}$)
AP-1B	63.89 \pm 0.23 ^a	54.16 \pm 1.08 ^b	327.78 \pm 1.22 ^c
AP-1F	102.95 \pm 1.48 ^a	99.84 \pm 1.73 ^b	147.21 \pm 1.35 ^c
AP-1S	33.76 \pm 1.82 ^a	20.75 \pm 1.64 ^b	104.38 \pm 0.79 ^c
AP-2B	38.48 \pm 1.33 ^a	34.63 \pm 1.98 ^b	96.65 \pm 0.85 ^c
AP-2F	124.61 \pm 2.04 ^a	42.55 \pm 0.86 ^b	138.43 \pm 1.93 ^c
AP-2S	24.29 \pm 0.57 ^a	10.82 \pm 0.21 ^b	54.58 \pm 0.38 ^c
Galanthamine	0.106 \pm 0.01 ^a	1.04 \pm 0.01	-
Kojic acid	-	-	7.9 \pm 0.02

Values expressed are means \pm SD of three parallel measurements. AChE – acetylcholinesterase; BuChE – butyrylcholinesterase; Tyrs – Tyrosinase. Superscripts indicate significant differences in the studied extracts ($P<0.05$).

according to Ellman's method, with galanthamine used as a positive standard, and the IC_{50} values of galanthamine for AChE and BuChE were calculated as 0.106 μ g/mL and 1.04 μ g/mL, respectively. All samples displayed inhibitory activities against AChE (IC_{50} 24.29–124.61 μ g/mL) and BuChE (IC_{50} 10.82–99.84 μ g/mL), with the most potent sample being AP-2S (IC_{50} 24.29 and 10.82 μ g/mL for AChE and BuChE, respectively).

Tyrosinase is the central enzyme in melanin synthesis in the human body, and it also causes the enzymatic browning of fruits and vegetables [28,29]. Moreover, tyrosinase oxidizes dopamine and levodopa to first form dopaquinone and then neuromelanin in the brain. Dopaquinone is known to be a neurotoxic metabolite that leads to the loss of dopaminergic neurons. Neuromelanin interacts with the α -synuclein protein, which is thought to be responsible for familial Parkinson's disease, and it also renders neurons in the substantia nigra pars compacta more susceptible to toxic effects [30,31]. The tyrosinase inhibitory potentials of the methanol extracts are shown in Table 3, with kojic acid used as the positive control (IC_{50} 7.9 μ g/mL). The IC_{50} values of the samples for tyrosinase were found to be higher than those for the cholinesterase enzymes. The extracts of parts from both plant samples demonstrated antityrosinase activity ranging from 54.58 to 327.78 μ g/mL. Similar to the results of the anti-cholinesterase activity assay, the highest tyrosinase inhibitory activity was observed in sample AP-2S.

Antioxidant activities

Methanol extracts of the bulb, stem and flower parts of both plant samples were investigated in terms of their radical scavenging and reducing power activities in order to deduce their respective antioxidant capacities, with the results expressed as equivalents of Trolox (Table 4). The radical scavenging activity of the samples was evaluated using the DPPH and ABTS assays. Sample AP-2S exhibited the most potent DPPH (38.19 mg/g) and ABTS (95.68 mg/g) scavenging activities. The reducing powers of the extracts were determined by the CUPRAC and FRAP assays, which measure the reduction of copper(II) and iron(III), respectively. Similar to the results of the radical scavenging activity assays, the highest activities for both reducing assays were observed in the stem extract of AP-2 (119.3 and 70.12 mg/g, respectively).

Table 4. Antioxidant capacities of the samples.

Sample	DPPH scavenging capacity (mg TE/g extract)	ABTS scavenging capacity (mg TE/g extract)	CUPRAC (mg TE/g extract)	FRAP (mg TE/g extract)
AP-1B	24.18 \pm 0.62 ^a	61.09 \pm 1.03 ^a	93.55 \pm 0.89 ^a	41.93 \pm 0.39 ^a
AP-1F	9.44 \pm 0.81 ^a	41.34 \pm 0.95 ^b	62.85 \pm 1.22 ^a	21.43 \pm 0.42 ^b
AP-1S	35.42 \pm 0.59 ^a	76.45 \pm 0.83 ^b	101.78 \pm 1.33 ^a	55.86 \pm 0.83 ^b
AP-2B	15.73 \pm 0.44 ^a	55.29 \pm 0.26 ^b	71.26 \pm 0.84 ^a	41.68 \pm 1.07 ^c
AP-2F	18.93 \pm 0.71 ^a	58.96 \pm 0.99 ^c	79.64 \pm 0.54 ^a	45.71 \pm 0.52 ^b
AP-2S	38.19 \pm 0.26 ^a	95.68 \pm 0.17 ^b	119.63 \pm 1.08 ^a	70.12 \pm 0.16 ^b

Values are means \pm SD of three measurements; TE – Trolox equivalents. Superscripts indicate significant differences in the studied extracts ($P<0.05$).

Statistical analysis

To gain insight into the relationship between the results of the various analyses, PCA was applied and a two-dimensional PCA scatter plot (based on the first two principal components (PCs)) was created (Fig. 1). The two principal components (PC1 and PC2) were found to provide for 91.34% of the total variance in the data, with PC1 and PC2 individually explaining 67.56% and 23.78% of the variability, respectively. It should be noted that the IC_{50} values of the samples were found to be inversely proportional to their activity potency. Therefore, the negative correlation between the samples with the data of enzyme inhibitory activity should be taken into consideration. As shown in Fig. 1, AP-2S had the most potent total bioactive components. The most distinguishing compounds were catechol and kaempferol for AP-2S and the phenolic acids, except for *p*-coumaric acid for AP-1S. In addition, the Tukey test was applied to the data. Significant differences were observed among all groups except vanillic acid, quercetin, isorhamnetin, galangin, 3-hydroxyflavone and epicatechin (Table 2).

DISCUSSION

High yields were obtained by an effective extraction method; however, no correlation was expected between the yields and the biological activities. Because methanol is the general solvent for primary and secondary metabolites in plants, these extracts contain not only the phenolic compounds but also substances from other parts of the plant. Therefore, the Folin-Ciocalteu and AlCl₃ methods were used to determine TPC and TFC in the plants. Compared with the AP-1 samples,

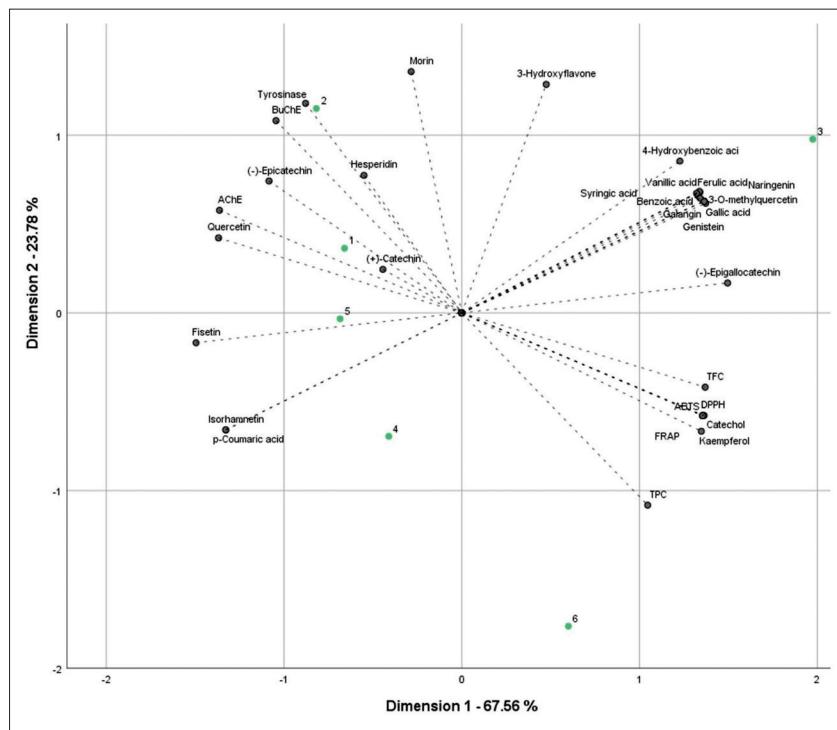


Fig. 1. Principal component analysis of the results of investigations. 1 – AP-1B; 2 – AP-1F; 3 – AP-1S; 4 – AP-2B; 5 – AP-2F; 6 – AP-2S.

the AP-2 samples were observed to contain higher levels of total phenolic contents. The stem extracts of both plants were found to have the highest TPC. Using LC-MS/MS analysis to quantify various phenolic compounds, thirteen of the analyzed compounds (gallic acid, benzoic acid, 4-hydroxybenzoic acid, vanillic acid, ferulic acid, syringic acid, kaempferol, 3-O-methylquercetin, galangin, naringenin, genistein, catechol, epigallocatechin gallate) in AP-1S were found to be major compounds, while AP-1F had four major compounds (*p*-coumaric acid, fisetin, 3-hydroxyflavone, hesperidin), and AP-1B had three (quercetin, isorhamnetin, epicatechin). A similar situation was observed in the extracts of AP-2. Phenolic compounds are thought to serve various functions in plants, including attracting pollinators, providing antioxidant activity and protecting against UV light and pests [2]. Therefore, environmental factors, such as the soil structure, temperature and/or physiological factors of the plants were most likely responsible for these differences in the phenolic contents of the samples. In the literature, bulbs of *A. subhirsutum* [15] and *A. roseum* var. *odoratissimum* [32], leaves of *A. ursinum* [33], aerial parts of *A. nigrum* [15] and *A. orientale*

[34] and flowers of *A. scorodoprasum* [14] have been reported to be richer in phenolics than other parts. Additionally, the phenolic compounds of some *Allium* species have previously been investigated quantitatively with LC. Specifically, eriodictyol in *A. scorodoprasum* [14], 3-hydroxybenzoic acid in *A. nigrum* [15], *p*-coumaric acid in *A. subhirsutum* [15], protocatechuic acid in *A. hookeri* [35], isorhamnetin in *A. flavum* subsp. *flavum* [36] and malic acid in *A. macrochaetum* [37] are the most dominant phenolic compounds in these species.

Phenolic compounds have significant antioxidant activity, therefore a linear relation was observed between TPCs and antioxidant capacities of samples. AP-2 samples showed higher radical scavenging (DPPH, ABTS) and reducing power (CUPRAC, FRAP) activities compared to AP-1 extracts. The flower extract of AP-1, which had a low TPC, had the lowest antioxidant activity. These results are similar to those found for the different parts of *A. ursinum* [32], *A. orientale* [33], *A. roseum* var. *odoratissimum* [31], *A. scorodoprasum* [14] and *A. hookeri* [35], in which the parts with the highest TPC showed the highest antioxidant activity.

Owing to the greater amounts of bioactive compounds, AP-2S was found to be the most potent sample in terms of both antioxidant and enzyme inhibitory activities. Flavonoids, which were determined to be present at higher concentrations in LC-MS/MS analysis, may well have contributed to the cholinesterase inhibitory activity of all samples. Kaempferol, galangin, naringenin, genistein, morin and (-)-epigallocatechin gallate have been reported to possess potent anticholinesterase activities [38-40]. Additionally, phenolic substances such as 4-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, (-)-epigallocatechin gallate, kaempferol and catechol may account for some of the antityrosinase activity of the extracts. In the literature, regarding these phenolics, 4-hydroxybenzoic acid, vanillic acid, (-)-epigallocatechin gallate and kaempferol

have been identified as being strong antityrosinase compounds [41]. Also, catechol, with its dihydroxyphenol structure, and *p*-coumaric acid, with a similar chemical structure to L-tyrosine, serve as substrate molecules for tyrosinase [42,43].

In conclusion, in the present study the phenolic composition and anti-cholinesterase, antityrosinase and antioxidant activities of different parts of *A. pallens* collected from different localities, were analyzed for the first time. The results of our analyses revealed that the concentrations and distributions of phenolic compounds of the various samples were quite different. The extracts had variable biological activities due to their differing contents of bioactive compounds. This study contributes to further investigations of *Allium* species. It shows that *A. pallens* collected from Bozdağ, Ödemiş, Turkey could serve as a natural source for *in vivo* research of diseases related to cholinesterase and tyrosinase inhibition.

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Author contributions: CE examined the biological activities of the samples. AE determined the phenolic profiles of the samples, designed the experiments, analyzed the data, supervised the study and wrote the article.

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