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Original article

Phytochemical compositions, antioxidant properties, enzyme inhibitory effects of extracts of four endemic *Lathyrus* L. taxa from Türkiye and a taxonomic approach

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ABSTRACT

Lathyrus is an economically important genus, with different parts of some species used as foodstuff or animal feed. In this study, phytochemical compositions and bioactivities of *Lathyrus brachypterus* var. *brachypterus*, *L. brachypterus* var. *haussknechtii*, *L. nivalis* subsp. *sahinii* and *L. tefennicus* taxa which are endemic to Türkiye were investigated. Total phenolic and flavonoid contents (TPC, TFC) of methanolic extracts were detected. Then, phytochemical compositions, antioxidant features (radical scavenging (DPPH: 1,1-diphenyl-2-picrylhydrazyl, ABTS: 2,2'-azino-bis(3 ethylbenzothiazoline) 6 sulfonic acid), reducing power (FRAP:Ferric ion reducing antioxidant power, CUPRAC:Cupric ion reducing antioxidant capacity), metal chelating activity (MCA), and the phosphomolybdenum assays (PDA)) and enzyme inhibitory properties of the extracts were also determined. The highest values were found at *L. brachypterus* var. *brachypterus* for TPC, *L. brachypterus* var. *haussknechtii* for TFC. The highest antioxidant properties were seen in extracts of *L. brachypterus* var. *brachypterus* in DPPH, ABTS, FRAP, CUPRAC and PDA assays, while in extract of *L. nivalis* subsp. *sahinii* in MCA. The highest enzyme inhibitory activity was found in extract of *L. brachypterus* var. *brachypterus* in tyrosinase and glucosidase assays, while in extracts of *L. nivalis* subsp. *sahinii* in AChE (acetylcholinesterase), BChE (butyrylcholinesterase) and amylase. Finally, a taxonomic evaluation was made by considering the phytochemicals.

Keywords: antioxidant properties, enzyme inhibitory, *Lathyrus*, phenolic compounds, phytochemical composition, taxonomy.

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Introduction

Free radicals can be described as molecular entities or molecular fragments that capable of independent existence (hence 'free') and have unpaired electron(s) in outer atomic orbits or molecular orbits (hence 'radical') (Martemucci *et al.* 2022). Provenance of these radicals may be endogenous (as products of normal metabolisms of the cell organelles such as peroxisomes, mitochondria and endoplasmic reticulum, enzyme activities or phagocytosis, etc.) or exogenous (pollution, tobacco smoke, heavy or transition metals, alcohol, pesticides, etc.) (Phaniendra *et al.* 2015; Martemucci *et al.* 2022).

Many radicals are unstable and show a highly reactive property that tends to accept or donate an electron. Because of these properties, they act as oxidant or reductant (Mohammed *et al.* 2015). The presence of unpaired electron(s) in these radicals causes oxidative stress that can brings about the damages of the proteins, carbohydrates, enzymes, lipids and DNA and even lead to cell death through DNA fragmentation and lipid peroxidation. These outcomes of oxidative stress constitute the molecular basis of the diabetes, cancer, autoimmune diseases, cardiovascular diseases and neurodegenerative disorders (Ratnam *et al.* 2006).

Antioxidants, which have the potential as prophylactic and therapeutic agents in many diseases, have acquired great significance in the recent times due to the understanding of the role of free radicals in diseases and disorders mentioned above and aging (Ratnam *et al.* 2006). Several antioxidant-based mechanisms are used by human body to ward off effects of the oxidative stress. Antioxidants, which may be endogenous or exogenous origin, act as "free radical scavengers" in these mechanisms (Pham-Huy *et al.* 2008).

Since endogenously produced antioxidants are inadequate to prevent oxidative damage caused by free radicals, the antioxidants got exogenously except hige doses are beneficial in preserving against free radicals (Koçyiğit & Selek 2016).

Many plants and their derivatives have been considerably used to prohibit oxidative stress because they contain important natural antioxidants (Akbari *et al.* 2022). Recently, researches on the extraction methods, antioxidant and enzyme inhibitory features of the bioactive compounds, which have great importance for human health, have also increased.

Lathyrus L., which is an economically important plant genus belongs to *Fabaceae* family, is one of the studied plant genus on these subjects. The genus *Lathyrus*, which has more than 200 species naturally distributed in the world, is represented by 79 taxa at the species, subspecies and variety level in Türkiye and 25 of these taxa are endemic (Genc *et al.* 2022). Some species of the genus are cultivated for different purposes in different parts of the world. The seeds of some species are used as human food, while the aerial parts of some species are used as animal feed (Yildirim *et al.* 2023). It has been stated that *L. tuberosus* and *L. undulatus* Boiss. species have positive effects on health (Baytop 1984; Sakinoglu-Oruc *et al.* 2021). Within the genus, the main phenolic compounds were reported as chlorogenic acid, epicatechin, and benzoic acid for *L. czeczottianus* Bässler (Ceylan *et al.* 2021), quercetin and kaempferol for both *L. cicera* L. and *L. digitatus* (Bieb.) Fiori (Llorent-Martinez *et al.* 2017a)

There are a number of studies testing the bioactive components, antioxidant properties and enzyme inhibitory properties of extracts obtained using the aerial parts or seeds of some taxa of the genus (Pastor-Cavada *et al.* 2009; Fratianni *et al.* 2014; Heydari *et al.* 2015; Llorent-Martinez *et al.* 2016; 2017a; b; Ozbek-Yazici *et al.* 2020; Ceylan *et al.* 2021; Eyiiş & Karadeniz-Pekgöz 2021). In these studies, there are differences in the used parts of the plant, the used tests, and the way the results are expressed. In Pastor-Cavada *et al.* (2009), it has been stated that there are few studies on *Lathyrus* species, although they are seen as a source of functional compounds such as antioxidant phenolics.

The purposes of this investigation are to exhibit the phytochemical composition, total bioactive components, antioxidant capacity and enzyme inhibitory potential of the aerial parts of *Lathyrus brachypterus* var. *brachypterus, L. brachypterus* var. *haussknechtii*, *L. nivalis* subsp. *sahinii* and *L. tefennicus* taxa which are endemic for Türkiye. According to our knowledge, there is no report on these properties of these taxa.

Material and methods

Plant materials and preparation of extracts

Plant samples were collected from the natural distribution areas at the flowering period. Plant samples were identified by YILDIRIM and GENC. Locality informations are given below.

- *L. brachypterus* Čel. var. *brachypterus*: Foothills of Erciyes mountain, 1730 m, Kayseri, Türkiye.
- *L. brachypterus* var. *haussknechtii* (Širj.) P.H.Davis: Karadağ mountain, 1975 m, Karaman, Türkiye.
- *L. nivalis* Hand.-Mazz. subsp. *sahinii* H.Genç: Karadağ mountain, 1680 m, Karaman, Türkiye.
- *L. tefennicus* H.Genç&A.Şahin: Bezirgan plateau road, 1300 m, Tefenni, Burdur, Türkiye.

From this point of the manuscript, the abbreviations *L. b.* var. *brachypterus* for *L. brachypterus* var. *brachypterus*, *L. b.* var. *haussknechtii* for *L. brachypterus* var. *haussknechtii* and *L. n.* subsp. *sahinii* for *L. nivalis* subsp. *sahinii* will be used.

The aerial parts (as mix) of these plants were carefully separated and they were dried at the dark conditions for ten days. The dried plant materials were powdered by using a laboratory mill. The extracts were prepared using methanol through maceration. Overnight, the air-dried powdered

samples (10 g) were macerated at room temperature with 200 mL of methanol. Finally, the solvents were evaporated from the mixtures. The extracts were stored at 4 °C until further analysis was required.

Phytochemical composition

In the quantitative screening of fifty-three compounds (phenolics, flavonoids, organic acid, etc.), Shimadzu brand LCMS-8040 tandem mass spectrometer and a Nexera model ultra-high performance liquid chromatograph (U-HPLC) were used. The separation system consisted of binary pumps (LC 30AD), a column oven (CTO 10 ASvp), an autosampler (SIL 30 AC), and a degasser (DGU 20 A3R) (the chromatograph). The previously developed and validated liquid chromatography-mass spectrometry/mass spectrometry method was used in the analyses (Yilmaz 2020). The analytical column used for the chromatographic seperation was a reversed phase Agilent brand Poroshell 120 EC-C18 model (150 mm×2.1 mm, 2.7 µm) column. In addition, the temperature of the column was arranged as 40°C. The mobile phases used for the gradient elution was; mobile phase A (water, 5 mM ammonium formate, 0.1% formic acid) and mobile phase B (methanol, 5 mM ammonium formate, 0.1% formic acid). Moreover, the gradient program started with 20% mobile phase B, followed by a ramp from 20% to 100% for 25 minutes. Then the mobile phase system remained constant at 100% B for 10 minutes. Finally, the initial mobile phase system (20% B) was followed for 10 minutes. Furthermore, the injection volume and the mobile phase flow rate was set to 5 µL and 0.5 mL/min, respectively.

Bioactive compounds

The total phenolic content (TPC) and total flavonoid content (TFC) were determined using previously published methods (Zengin & Aktumsek 2014). TPC and TFC values were expressed as mg gallic acid equivalents (GAE)/g extract and mg rutin equivalents (RE)/g extract, respectively.

Antioxidant and enzyme inhibitory assays

In this study, various techniques were used to evaluate the antioxidant properties of the examined extracts (Grochowski *et al.* 2017). These tests included the radical scavenging (DPPH: 1,1-diphenyl-2-picrylhydrazyl and ABTS: 2,2'-azino-bis(3 ethylbenzothiazoline) 6 sulfonic acid), ferric and cupric ion reducing ability (FRAP and CUPRAC), metal chelating ability (MCA), and the phosphomolybdenum (PDA) assay. The results were expressed as mg Trolox equivalent (TE)/g extract (for DPPH, ABTS, CUPRAC, and FRAP tests), mg EDTA equivalent (EDTAE)/g extract (for MCA test) and mmol Trolox equivalent (TE)/g extract (for PDA test).

The effects of the extracts on the activity of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase, amylase, and glucosidase enzymes were also tested. In AChE and BChE assays used galanthamine as positive control, results were expressed as mg galanthamine equivalents (GALAE)/g extract. Kojic acid, a standard tyrosinase enzyme inhibitor, was used in the tyrosinase tests and the results were given as mg kojic acid equivalents (KAE)/g extract (Uysal *et al.* 2017). Amylase and glucosidase enzyme inhibition test results of the extracts were calculated as mmol acarbose equivalents (ACAE)/g extract.

Data analysis

All data were given as mean ± standard deviation. Statistical analysis was performed by analysis of variance (ANOVA). A post hoc test (Tukey) was done when the differences shown by data were significant ($p < 0.05$). Also, hierarchical clustered analysis (HCA) was achieved to assess the (dis)similarity between samples in terms of their molecules. SIMCA 14.0 statistical program was used for all analysis.

Results and discussion

Phytochemical composition

The phytochemical structures of four taxa were revealed by LC-MS/MS analysis and the amounts of their some major compounds were calculated. The findings are given in Tab. 1.

Fifty-three compounds were considered and twenty-five of them were detected in at least one of the studied taxa. The first three compounds detected with the highest amount are Isoquercitrin, Quinic acid and Chlorogenic acid (for *L. b.* var. *brachypterus*), Quinic acid, Isoquercitrin and Chlorogenic acid (for *L. b.* var. *haussknechtii*), Quinic acid, Salicylic acid and Protocatechuic acid (for *L. n.* subsp. *sahinii*), Quinic acid, Fumaric acid and Hesperidin (for *L. tefennicus*).

It was stated that three of the mentioned compounds with the highest rate have favourable effects as given below: isoquercitrin against oxidative stress, cancer, cardiovascular disorders, diabetes and allergic reactions (Valentová *et al.* 2014), quinic acid against prostate cancer (Inbathamizh & Padmini 2013), chlorogenic acid which also has hepatoprotective and renoprotective features, against oxidative stress, inflammatory stress, cardiovascular disorders and diabetes (Maalik *et al.* 2016).

Total bioactive components

Phenolics, including flavonoids, have been reported to be effective on various pharmacological activities (Mondal & Rahaman 2020). There are many studies showing that flavonoids have benefical effects in many different clinical areas such as cardiovascular diseases, neurology, urology, immunology and gastroenterology (Hoensch & Oertel 2015).

Extraction yields, Total phenolic content (TPC) and Total flavonoid content (TFC) values of the extracts of the examined *Lathyrus* taxa were presented in Tab. 2.

TPC values vary in the range of 24.91-44.31 mg GAE/g in the current study. *L. b.* var. *brachypterus* possessed the highest TPC (44.31±0.48 mg GAE/g), followed by *L. b.* var. *haussknechtii* (34.99±1.03 mg GAE/g) and *L. n.* subsp. *sahinii* (27.25±0.62 mg GAE/g). The lowest TPC was found in *L. tefennicus* (24.91±0.06 mg GAE/g).

TFC values vary in the range of 25.78-42.98 mg RE/g. TFC contents of *L. b.* var. *haussknechtii* and *L. b.* var. *brachypterus* are very close to each other, 42.98 mg RE/g and 41.84 mg RE/g, respectively. TFC content of *L. tefennicus* is 37.14 mg RE/g and of *L. n.* subsp. *sahinii* is 25.78 mg RE/g. Assays carried out on four *Lathyrus* taxa mentioned above have revealed different results on the amount of bioactive components.

Various studies have been conducted to investigate the TPC and TFC values of the extracts of different *Lathyrus* taxa obtained by using different solvents, such as ethyl acetate, methanol, water, etc. Aerial parts of plants have been used in some of these studies (Heydari *et al.* 2015; Llorent-Martinez *et al.* 2016; 2017a; b; Ceylan *et al.* 2021; Eyiiş & Karadeniz-Pekgöz 2021) and seeds have been used in others (Pastor-Cavada *et al.* 2009; Marathe *et al.* 2011; Fratianni *et al.* 2014; Ozbek-Yazici *et al.* 2020; Eyiiş & Karadeniz-Pekgöz 2021). It is not possible to make healthy comparisons with some of the studies mentioned above, as there are differences in solvents, used plant parts or the ways the results are expressed.

TPC values of methanolic extracts (as mg GAE/g) have been found as 150.63, 179.69, 390.94, 397.00, 452.19 mg GAE/g for *L. armenus* (Boiss. & Huet) Širj., *L. cilicicus* Hayek & Siehe, *L. pratensis* L., *L. laxiflorus* (Desf.) O. Kuntze and *L. aureus* (Stev.) Brandza (Heydari *et al.* 2015), 22.74

Table 1. Phytochemical compositions (mg/g extract).

Analyte	*L. b.* var. *brachypterus*	*L. b.* var. *haussknechtii*	*L. n.* subsp. *sahinii*	*L. tefennicus*
Isoquercitrin	16.037	3.304	0.017	0.061
Quinic acid	4.623	3.998	1.274	1.648
Chlorogenic acid	3.582	2.408	0.015	0.027
Luteolin	0.412	0.032	0.011	0.039
p-Coumaric acid	0.121	0.084	0.028	0.030
Salicylic acid	0.175	0.102	0.089	0.222
Acacetin	0.183	0.283	0.073	0.053
Astragalin	1.196	0.921	-	0.018
Quercetin	1.158	0.230	-	0.008
Fumaric acid	0.146	0.156	-	0.262
Hesperidin	0.139	0.323	-	0.259
Rutin	0.109	0.282	-	0.240
Protocatechuic acid	0.283	0.093	0.079	-
Hesperetin	0.089	0.014	-	-
Naringenin	0.005	0.006	-	0.005
Kaempferol	0.094	0.067	-	0.033
Apigenin	0.030	0.012	-	0.040
Nicotiflorin	0.013	0.107	-	0.029
Cosmosiin	0.034	0.011	-	0.007
Vanillin	-	0.075	-	0.080
Coumarin	-	0.015	-	0.014
Genistein	0.017	-	-	0.004
Cynaroside	0.301	0.023	-	-
Gallic acid	0.010	-	-	-
Caffeic acid	0.027	-	-	-

The areas indicated by "-" in the table could not be detected. The following compounds were not detected in the extracts of any of the studied taxa: Daidzin, Gentisic acid, Tannic acid, Vanilic acid, Epigallocatechin, Catechin, 4-OH Benzoic acid, Epigallocatechin gallate, Cynarin, Epicatechin, Syringic acid, Epicatechin gallate, Sinapic acid, Ferulic acid, Genistin, Ellagic acid, Rosmarinic acid, Fisetin, Quercitrin, Daidzein, Amentoflavone, Chrysin, Miquelianin, Aconitic acid, O-Coumaric acid, Piceid, Protocatechuic aldehyde, Syringic aldehyde).

and 40.54 for *L. aureus* and *L. pratensis* (Llorent-Martinez *et al.* 2016), 24.09 and 33.18 for *L. cicera* and *L. digitatus* (Llorent-Martinez *et al.* 2017a), 25.47 and 63.16 for *L. nissolia* L. and *L. czeczottianus* (Llorent-Martinez *et al.* 2017b), 13.18, 13.85, 22.36, 32.84, 67.60 and 273.16 for *L. setifolius* L., *L. cicera*, *L. aphaca* L. var. *pseudoaphaca* (Boiss.) Davis, *L. digitatus*, *L. aureus* and *L. sphaericus* Retz. (Eyiiş & Karadeniz-Pekgöz 2021), respectively. According to the results of the current study, TPC values were found to be relatively compatible with the literature data except Heydari *et al.* (2015). In particular, it has been seen that *L. b.* var. *haussknechtii* has closer results to *L. digitatus*, *L. n.* subsp. *sahinii* to *L. nissolia*, and *L. tefennicus* to *L. cicera* and *L. nissolia*. The examined taxa in Heydari *et al.* (2015) have very high TPC values compared to other literature data and the results of our study.

TFC values of the methanolic extracts (as mg RE/g) have been found as 5.31 and 26.16 for *L. aureus* and *L. pratensis* (Llorent-Martinez *et al.* 2016), 3.75 and 16.10 for *L. cicera* and *L. digitatus* (Llorent-Martinez *et al.* 2017a), 14.16 and 20.94 for *L. czeczottianus* and *L. nissolia* (Llorent-Martinez *et al.* 2017b), respectively. According to the findings of our work, TFC values of all taxa except *L. n.* subsp. *sahinii* were higher than the literature data. Although *L. n.* subsp. *sahinii* gave close results with *L. pratensis*, it was determined that it had higher TFC content than the other taxa given in the literature.

Even in the extracts of the same species using different solvents, the total content of bioactive components can be different. Comparisons become difficult due to differences in the methods of obtaining the extracts, the used parts of the plant, and the presentation of the results (Llorent-Martinez *et al.* 2016). It has been stated that differences in bioactive components are related to genetic variations, climatic conditions, soil structure, region where the plants grow, habitat properties and the other ecological factors (Llorent-Martinez *et al.* 2016; Ozbek-Yazici *et al.* 2020; Zengin *et al.* 2020; Ceylan *et al.* 2021). The total bioactive components can be different even in the extracts obtained from the same species according to differences in the solvent, the plant parts used, or region which the plants were collected. In general, the total bioactive components are closely related to the solvent polarity (Fratianni *et al.* 2014; Llorent-Martinez *et al.* 2016; Ceylan *et al.* 2021; Eyiiş & Karadeniz-Pekgöz 2021).

Antioxidant properties

Some commonly performed in vitro methods were used in this study: DPPH, ABTS, FRAP, CUPRAC, MCA and PDA. Tab. 3 summarizes antioxidant properties of the investigated taxa.

As seen in the Tab. 3, rankings of the taxa according to the antioxidant properties are the same considering the DPPH, ABTS, CUPRAC and FRAP tests. When the MCA and PDA tests are taken into account, the rankings change.

Radical scavenging assays (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$)

The free radical scavenging abilities of antioxidants are used to measure their antioxidant powers. DPPH and ABTS+ are free radicals used for this purpose (Pisoschi & Negulescu 2012; Sadeer *et al.* 2020; Flieger *et al.* 2021; Muntenau & Apetrei 2021). DPPH$^{\bullet}$ discovered by Goldschmidth and Renn in 1922, is one of the most commonly used stable free radicals. The DPPH scavenging test serves to reduce this radical by an antioxidant. The DPPH solution is deep violet in colour with a maximum absorbance at a wavelength of 517 nm. When it interacts with a substance (such as an

Table 2. Extraction yields, total phenolic and flavonoid contents.

Taxon	Extraction yields (%)	TPC (mg GAE/g)	TFC (mg RE/g)
L. b. var. *brachypterus*	10.61	44.31±0.48^{a}	41.84±0.20^{b}
L. b. var. *haussknechtii*	11.74	34.99±1.03^{b}	42.98±0.08^{a}
L. n. subsp. *sahinii*	6.54	27.25±0.62^{c}	25.78±0.28^{d}
L. tefennicus	8.85	24.91±0.06^{d}	37.14±0.14^{c}

Values are reported as mean±S.D of three paralel experiments. Different superscripts indicate significant differences between the tested extracts. ($p<0.05$)

Table 3. Antioxidant properties.

Taxon	DPPH (mg TE/g)	ABTS (mg TE/g)	FRAP (mg TE/g)	CUPRAC (mg TE/g)	MCA (mg EDTAE/g)	PDA (mmol TE/g)
L. b. var. *brachypterus*	41.74±0.04^{a}	75.87±3.43^{a}	77.32±0.23^{a}	114.92±2.00^{a}	25.28±0.36^{b}	1.21±0.07^{a}
L. b. var. *haussknechtii*	36.59±0.35^{b}	67.61±4.37^{b}	56.67±1.48^{b}	85.52±1.71^{b}	25.29±0.16^{b}	1.08±0.04^{a}
L. n. subsp. *sahinii*	19.43±0.31^{c}	65.23±0.98^{b}	45.37±0.30^{c}	62.32±0.23^{c}	30.81±0.31^{a}	1.20±0.09^{a}
L. tefennicus	18.90±0.57^{c}	57.73±0.09^{c}	42.33±0.25^{d}	51.88±1.09^{d}	23.25±0.28^{c}	1.06±0.07^{a}

Values are reported as mean±S.D. of three paralel experiments. Different superscripts indicate significant differences between the tested extracts. ($p<0.05$)

antioxidant) that can donate hydrogen, the reduced form of DPPH is formed and the colour of the solution becomes pale yellow and the colour change serves as an indicator of antioxidant activity. The decrease in absorbance is linearly related to antioxidant concentration. The degree of colour change in the solution also indicates the level of antioxidant capacity. In other words, substances with strong antioxidant properties cause more lightening of the solution colour (Pisoschi & Negulescu 2012; Sadeer *et al.* 2020; Flieger *et al.* 2021). $ABTS^{\bullet+}$ assay, which was first reported in Miller *et al.* (1993), is also known as TEAC (Trolox Equivalent Antioxidant Capacity) (Sadeer *et al.* 2020; Muntenau & Apetrei 2021). $ABTS^{\bullet+}$, a bluish-green chromophore of maximum absorption at 734 nm, is another radical used in scavenging assays, and it is formed as a result of losing an electron by the nitrogen atom of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (Pisoschi & Negulescu 2012). When $ABTS^{\bullet+}$ receives an electron from electron donors such as antioxidants, it forms the stable form of ABTS and changes in colour to pale blue or colourless (Pisoschi & Negulescu 2012; Sadeer *et al.* 2020; Flieger *et al.* 2021; Muntenau & Apetrei 2021).

Findings of the radical scavenging assays are presented in Tab. 3. As a result of the $DPPH^{\bullet}$ scavenging assay in the current study, the ordering of the taxa according to their antioxidant capacities (as trolox equivalent) is *L. b.* var. *brachypterus* > *L. b.* var. *haussknechtii* > *L. n.* subsp. *sahinii* > *L. tefennicus*. The antioxidant capacities of the varieties of the *L. brachypterus* are higher than the others. In particular, $DPPH^{\bullet}$ scavenging capacity of *L. b.* var. *brachypterus* (41.74 mg TE/g) is more than twice that of *L. n.* subsp. *sahinii* and *L. tefennicus*. In the same assay, capacity of *L. b.* var. *haussknechtii* (36.59 mg TE/g) is also close to twice that of the mentioned taxa.

Considering the $ABTS^{\bullet+}$ scavenging assay result, the ordering of the taxa in terms of their antioxidant capacities is the same as the ordering made according to the $DPPH^{\bullet}$ scavenging assay result. $ABTS^{\bullet+}$ scavenging capacities of the taxa are close to each other and vary in the range of 57.73-75.87 mg TE/g. $ABTS^{\bullet+}$ scavenging capacities are approximately twice the $DPPH^{\bullet}$ scavenging capacities for *L. b.* var. *brachypterus* and *L. b.* var. *haussknechtii*, and approximately three times for *L. n.* subsp. *sahinii* and *L. tefennicus*.

DPPH and ABTS radical scavenging tests were performed on *Lathyrus* species (Llorent-Martinez *et al.* 2016; 2017a; b; Heydari *et al.* 2015; Ceylan *et al.* 2021; Ozbek-Yazici *et al.* 2020). Radical scavenging abilities were measured as 126.68 and 4.09 mg TE/g extract (for DPPH) and 67.85 and 34.37 mg TE/g extract (for ABTS) for the methanolic extracts of *L. pratensis* and *L. aureus*, respectively (Llorent-Martinez *et al.* 2016). For methanolic extracts of the *L. digitatus* and *L. cicera*, DPPH radical scavenging abilities were 40.28 and 25.23 mg TE/g DE, while ABTS radical scavenging abilities were 147.83 and 65.18 mg TE/g DE, respectively (Llorent-Martinez *et al.* 2017a).

Relatively, *L. b.* var. *brachypterus* and *L. b.* var. *haussknechtii* are closer to *L. digitatus; L. n.* subsp. *sahinii* and *L. tefennicus* are closer to *L. cicera* in terms of DPPH tests. Similarly, *L. b.* var. *brachypterus* and *L. b.* var. *haussknechtii* are closer to *L. pratensis*, and *L. n.* subsp. *sahinii* and *L. tefennicus* closer to *L. cicera* in terms of ABTS tests.

Reducing antioxidant abilities (FRAP and CUPRAC)

The Ferric Reducing Antioxidant Power (FRAP) and Cupric Reducing Antioxidant Capacity (CUPRAC) are important tests applied to determine antioxidant properties. Iron and copper ions are reduced by taking advantage of the electron-donating ability of antioxidants. The reducing power is also a measure of the antioxidant ability (Llorent-Martinez *et al.* 2017a). The FRAP assay is based on the reduction of the Fe(TPTZ)3+ (iron trotripyridyltriazine) complex to a strongly blue coloured Fe(TPTZ)2+ complex by antioxidants under acidic (pH 3.6) conditions. Results occur in terms of absorbance increase at 593 nm and are expressed as micromolar Fe2+ equivalent or relative to an antioxidant standard. Nevertheless, it was also found that the measured reduction capacity does not necessarily reflect antioxidant activity (Benzie & Strain 1996; Antolovich *et al.* 2002). In the CUPRAC test, reducing capability of the Cu(II)-neocuproine complex formed by Cu(II) and neocuproine (2,9-Dimethyl-1,10-phenanthroline) to Cu(I)-neocuproine, which gives maximum absorbance at 450 nm, by the presence of the antioxidants is utilized (Apak *et al.* 2004; Büyüktuncel 2013).

The reducing abilities of the investigated taxa are given in Tab. 3. According to the FRAP assay results in this study, the ferric reducing abilities of the taxa are in the form of *L. b.* var. *brachypterus* > *L. b.* var. *haussknechtii* > *L. n.* subsp. *sahinii* > *L. tefennicus* from strong to weak. Power of *L. b.* var. *brachypterus* to reduce Fe^{3+} to Fe^{2+} close to two times that of *L. tefennicus*.

The CUPRAC assay also gave similar results with the FRAP test. The same ranking was seen in terms of copper reduction capabilities of taxa. Nevertheless, copper reduction ability of *L. b.* var. *brachypterus* is more than two times that of *L. tefennicus*, and close to two times that of *L. n.* subsp. *sahinii*.

According to literature, FRAP and CUPRAC assays were performed on some *Lathyrus* species (Llorent-Martinez *et al.* 2016; 2017a; b; Ceylan *et al.* 2021). Taking into account parameters such as solvent, plant parts used and ways of expressing the results, Llorent-Martinez *et al.* (2016; 2017a) can be compared with current study. For methanolic extracts of the *L. aureus* and *L. pratensis*, results of the reducing power tests were 31.98 and 13.32 mg TE/g extract (FRAP), 71.22 and 35.33 mg TE/g extract (CUPRAC), respectively (Llorent-Martinez *et al.* 2016). FRAP values were determined with 55.84 and 40.99 mg TE/g DE, CUPRAC values were determined as 54.39 and 53.96 mg TE/g DE for methanolic

extracts of *L. digitatus* and *L. cicera*, respectively (Llorent-Martinez *et al.* 2017a).

According to the FRAP test results, *L. tefennicus* and *L. n.* subsp. *sahinii* showed closeness to *L. cicera*, and *L. b.* var. *haussknechtii* to *L. digitatus*. Extract of the *L. b.* var. *brachypterus* has the highest FRAP value. CUPRAC test results show that *L. tefennicus* is closer to *L. cicera* and *L. digitatus*, while *L. b.* var. *brachypterus* and *L. b.* var. *haussknechtii* have the highest values.

Metal chelating activity (MCA)

Chelating agents are organic compounds that bind metal ions to form a structure called a chelate. Chelators form a complex together with toxic ions. These complexes, which have lower toxicity, are more easily removed from the body by the excretory system. Metal chelation therapy is commonly used to treat metal poisoning (Flora *et al.* 2007). Iron and copper, which are transition metals, play an important role in the Fenton and Haber-Weiss reactions that lead to the formation of free radicals, which are very reactive and harmful in the body. Substances that can chelate these metals are important in terms of antioxidants (Llorent-Martinez *et al.* 2017a; b).

The metal chelating activities of the extracts obtained from the taxa (displayed in Tab. 3) are generally close to each other and vary in the range of 23.25-30.81 mg EDTAE/g. It was observed that extract of the *L. nivalis* subsp. *sahinii* was more effective than the others.

The metal chelating activities of the methanolic extracts were found as 44.48, 23.78, 9.39 and 0.62 mg EDTAE/g for *L. digitatus*, *L. cicera*, *L. aureus* and *L. pratensis*, respectively (Llorent-Martinez *et al.* 2016; 2017a). Different studies (Llorent-Martinez *et al.* 2017b; Ceylan *et al.* 2021) on the chelating abilities of extracts obtained from *Lathyrus* species were also carried out. However, these studies do not make it possible to compare in terms of the used solvents and the ways the results were expressed.

The results of the MCA tests showed that the taxa we investigated generally had better chelating ability than the taxa given in the literature, except *L. digitatus*.

Phosphomolybdenum assay (PDA)

The phosphomolybdenum assay based on the reduction of Mo(VI) to Mo(V) is a method used for the quantitative determination of antioxidant capacity. At the end of the reduction reactions, a green coloured phosphate-Mo(V) complex is formed at acidic pH. This complex has a maximum absorbance value at 695 nm (Prieto *et al.* 1999).

Phosphomolybdenum tests results are shown in Tab. 3. As a result of PDA, the antioxidant abilities of the taxa were found to be close to each other, as in the MCA test. *L. b.* var. *brachypterus* and *L. n.* subsp. *sahinii* have the higher values (1.21 and 1.20, respectively) while *L. b.* var. *haussknechtii* and *L. tefennicus* have the lower (1.08 and 1.06, respectively).

Ability of the methanolic extracts to reduction Mo(VI) to Mo(V) were specified as 2.40 (*L. cicera*), 1.47 (*L. digitatus*), 1.41 (*L. aureus*) and 1.40 mmol TE/g (*L. pratensis*) (Llorent-Martinez *et al.* 2016; 2017a).

All of the taxa we studied showed less efficiency in terms of phosphomolybdate assays than the taxa given in the literature.

Enzyme inhibitory properties

AChE (acetylcholinesterase) and BChE (butyrylcholinesterase) are enzymes that act on Alzheimer's and learning, and amylase and glucosidase on diabetes. Excess of these enzymes in the body can lead to the mentioned health problems. The excess of the tyrosinase enzyme, which catalyzes the production of melanin pigment, which helps to prevent UV light, also can casuses hyperpigmentation and neurodegenerative diseases like Parkinson's (Yırtıcı 2019). Enzyme inhibitors are secondary metabolites that bind to enzymes and reduce their activity (Rauf & Jehan 2017). Enzyme inhibition, which is an important pharmacological research area today, is popular in treatment strategies of global health problems. One of the most important characteristics of enzyme inhibitors is that they are used as drugs in many physiological conditions (Çakmak *et al.* 2017). For this reasons, it is necessary to search for natural compounds that do not have unfavorable effects from plants in order to gain new approaches in the treatment of the aforementioned diseases (Llorent-Martinez *et al.* 2016).

Extracts of four *Lathyrus* taxa were tested by spectrophotometric methods to determine enzyme inhibitory activities. Findings reached at the end of the tests are summarized in Tab. 4.

Table 4. Enzyme inhibitory activities.

Taxon	AChE (mg GALAE/g)	BChE (mg GALAE/g)	Tyrosinase (mg KAE/g)	Amylase (mmol ACAE/g)	Glucosidase (mmol ACAE/g)
L. b. var. *brachypterus*	1.85±0.05^{b}	0.20±0.01^{c}	49.33±1.47^{a}	0.55±0.01^{b}	0.65±0.02^{a}
L. b. var. *haussknechtii*	1.96±0.08^{ab}	1.53±0.24^{b}	41.67±1.00^{b}	0.53±0.01^{c}	0.51±0.01^{b}
L. n. subsp. *sahinii*	2.06±0.02^{a}	2.68±0.20^{a}	47.79±1.06^{a}	0.57±0.01^{a}	0.39±0.04^{c}
L. tefennicus	2.01±0.03^{a}	1.52±0.43^{b}	48.98±1.68^{a}	0.50±0.01^{d}	0.39±0.05^{c}

Values are reported as mean±S.D. of three paralel experiments. Different superscripts indicate significant differences between the tested extracts. ($p<0.05$)

As seen in the Tab. 4, *L. n.* subsp. *sahinii* extracts show the highest enzyme inhibitory activities in AChE, BChE and amylase tests, while *L. b.* var. *brachypterus* is the best in tyrosinase and glucosidase tests. *L. b.* var. *brachypterus* in the AChE and BChE tests, *L. b.* var. *haussknechtii* in the tyrosinase test, *L. n.* subsp. *sahinii* in the glucosidase test and *L. tefennicus* in the amylase and glucosidase tests have the lowest enzyme inhibitory activities.

The enzyme inhibitory activities for *L. cicera*, *L. digitatus*, *L. pratensis* and *L. aureus* taxa were determined as follows, respectively: not active, 2.06, 1.13, 1.31 mg GALAE/g (for AChE), 2.01, 0.60, not active, 0.14 mg GALAE/g (for BChE), unstudied, unstudied, 35.08, 62,85 mg KAE/g (for Tyrosinase), 0.53, 0.56, 0.37, 0.39 mmol ACAE/g (for Amylase), 30.33, 25. 01, 10.12, 3.18 mmol ACAE/g (for Glucosidase) (Llorent-Martinez *et al.* 2016; 2017a).

In terms of AChE test, value of the AChE inhibition of the *L. n.* subsp. *sahinii* is found as 2.06 mg GALAE/g like *L. digitatus*. On the other hand, the other taxa we investigated showed a stronger effect than the taxa given in the literatüre except *L. digitatus*. For BChE test, *L. n.* subsp. *sahinii* showed the highest inhibition property among both the taxa investigated and the taxa given in the literature. Moreover, *L. b.* var. *haussknechtii* and *L. tefennicus* have the higher inhibitory effect than the other taxa mentioned in the literature. *L. aureus* had a strong tyrosinase inhibitory effect. All of the taxa we investigated have been shown to have stronger tyrosinase inhibitory properties than *L. pratensis* given in the literature. Considering the test results of amylase inhibition test, the taxa we studied gave closer results to *L. digitatus* and *L. cicera* reported in the literature, but showed more potent effects than *L. aureus* and *L. pratensis*. In glucosidase inhibition assays, all of the taxa we studied have the lowest inhibitory properties compared to the data in the literature, in contrast to the other enzyme inhibition assays.

Taxonomic evaluations

In this study, various biochemical properties of the 4 taxa, which have been classified in the Sect. *Platystylis* of the Genus *Lathyrus* (*Fabaceae* family), were examined. All of these taxa are endemic to Türkiye.

Lathyrus brachypterus Čel. and *Lathyrus haussknechtii* Širj. were described as different species in Čelakovský (1888) and Fedde (1934), respectively. Second taxon named by G. Širjaev was transferred to the *L. brachypterus* species and reduced to the variety level in Davis (1970). *L. brachypterus* also decreased to the variety level by itself due to the rules of nomenclature. Based on the current taxonomic situation, *L. brachypterus* has 2 varieties: var. *brachypterus* and var. *haussknechtii*.

In Flora of Turkey (Davis 1970), *Lathyrus* species were classified and prepared an identification key according to the morphological features. The morphological features of taxa given in Davis (1970), Genc (2009) and Genc and Sahin (2011) have been summarized in Tab. 5.

The most important differences that distinguish the studied taxa from each other are flower colours and style shapes. Three different style types as filiform, linear and spathulate are seen in Sect. *Platystylis* according to the Flora of Turkey. In the aforementioned study, varieties of the *L. brachypterus* differ from the other taxa with differences of the sulphur or cream flower colours and filiform style shape. These two varieties are very close to each other taxonomically according to the morphological classification. *L. n.* subsp. *sahinii* differs from varieties of the *L. brachypterus* with its violet to light purple flower colour and linear style shape. *L. tefennicus* is similar to *L. n.* subsp. *sahinii* with its purple (or light purple) flower colour, but differs from both *L. n.* subsp. *sahinii* and varieties of the *L. brachypterus* with its spathulate style shape.

The results of the hierarchical cluster analysis (HCA) made by considering the phytochemical contents of these

Table 5. Morphological features of the investigated *Lathyrus* taxa.

Morphological feature	*L. b.* var. *brachypterus*	*L. b.* var. *haussknechtii*	*L. n.* subsp. *sahinii*	*L. tefennicus*
Life form	Perennial	Perennial	Perennial	Perennial
Stem	Erect	Erect	Ascending-erect	Ascending-erect
Stipules	Lanceolate-subulate	Lanceolate-subulate	Lanceolate to subulate	Lanceolate to subulate
Leaves	Median leaves pinnate	Median leaves subdigitate	Median leaves pinnate	Median leaves subdigitate
Leaflets	2-3 paired	2-3 paired	2-4(-5) paired	1-3 paired
Inflorescence	2-10 flowered	2-10 flowered	2-4(-5) flowered	2-4(-5) flowered
Peduncle	1-2 x leaves	1-2 x leaves	22-52 mm	12-125 mm
Pedicels	n.i.	n.i.	3-4 mm	4-9.5 mm
Flower	Sulphur or cream	Sulphur or cream	Violet to light purple	Purple to light purple
Calyx	(5-)6-9 mm, teeth unequal	(5-)6-9 mm, teeth unequal	5-7 mm, teeth unequal	3.5-5 mm, teeth unequal
Style	Filiform	Filiform	Linear	Spathulate
Legume	Linear	Linear	Oblong-linear	Oblong to linear
Flowering time (months)	5-7	5-7	6-8	4-5

taxa are given in Fig. 1. Considering their morphological similarities, *L. b.* var. *brachypterus* and *L. b.* var. *haussknechtii* can be expected to be very close to each other in HCA. However, the HCA data revealed different results from the affinities determined according to the morphological features.

L. b. var. *brachypterus* and *L. b.* var. *haussknechtii* are separated from each other in HCA, which are taxonomically close to each other according to the morphological classification. *L. b.* var. *brachypterus* quite differs from other taxa in terms of phytochemical content. *L. tefennicus*, which is less morphologically similar to other taxa, appears to be very close to *L. b.* var. *haussknechtii* in HCA.

It is normal for extracts from different species to have different test results. Yet, even varieties of the same species are less similar to each other according to the HCA. It is understood that the morphological similarities of the taxa and their phytochemical compositions are not compatible. If these differences, seen even in varieties of *L. brachypterus* species, are not caused by environmental differences such as geographical and ecological conditions, it can be assumed that genetic factors may be effective.

As a result of the morphological and anatomical studies carried out on *L. b.* var. *haussknechtii*, which was first described as a species and then reduced to the variety level, it was suggested that the taxon be classified as a different species as *L. haussknechtii* (Çildir 2011). In other words, these two varieties (*L. b.* var. *brachypterus* and *L. b.* var. *haussknechtii*) belonging to the same species are thought to be two different species. In Güner *et al.* (2012), this taxon has been evaluated as a separate species as *L. haussknechtii*. The results of the HCA in this study also support this opinion.

Conclusions

The tests applied to determine the amount of bioactive components in plants may give different results depending on the polarity of the solvent. Antioxidant properties and enzyme inhibitory properties of the plant extracts may also vary depending on the ratio of bioactive components. The results of studies carried out on taxa of the Genus *Lathyrus* indicate that variations in bioactive components and their amounts are related with pedo-climatic, geographic and the other ecologic differences. In addition, genetic factors can also affect the mentioned properties of the plants. In addition, the phytochemical profiles of *L. b.* var. *brachypterus* and *L. b.* var. *haussknechtii* are not compatible with current taxonomic affinities based on morphological similarities. It has been concluded that the phytochemical profile data in current study supports the views that accept these varieties as *L. brachypterus* and *L. haussknechtii* as separate species.

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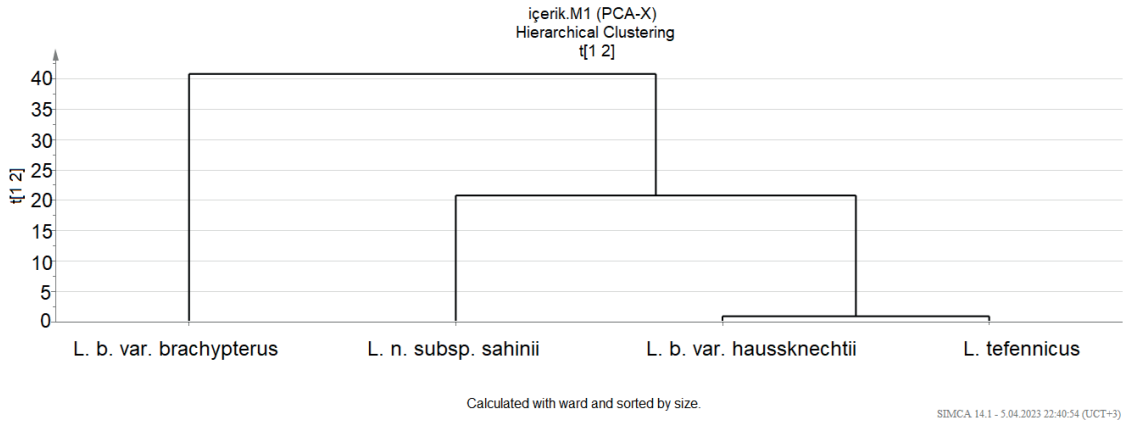

Figure 1. Hierarchical cluster analysis for tested taxa based on their phytochemical composition (by Ward method).

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