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Antioxidant Activity, Amino Acid Composition and Trace Element Levels of *Verbascum lasianthum* Boiss. ex Bentham from Erzincan, Türkiye

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Verbascum lasianthum, Trace elements, Amino acids, Antioxidant activity, Medicinal plant

Abstract: This study aims to investigate trace elements and amino acids in the root and stem of *Verbascum lasianthum* Boiss. ex Bentham (VL) from Erzincan and to understand the role of these compounds in adaptation to environmental stress conditions. In the study, ICP-MS and LC-MS/MS methods are used for analyses. In addition, the antioxidant activities of methanol: water extracts (70:30, v:v) of these parts were investigated. The results showed that VL in Erzincan accumulates elements such as sodium and silicium to adapt to arid and stressful environmental conditions and supports these processes by synthesizing amino acids such as L-proline, L-asparagine, and L-glutamine. The high amount of iron indicates that VL can be a potential iron source in the treatment of anemia. However, aluminum accumulation in plants is extremely risky due to its possible neurotoxic effect on humans and plant toxicity. Although these amino acids and trace elements contribute to the antioxidant potential of the plant, VL showed a low antioxidant activity when compared to VL species in other regions. In conclusion, this study emphasizes the importance of comprehensively analyzing phytochemical profiles such as trace elements, amino acids, and phenolic compounds to evaluate the efficacy and safety of medicinal plants.

Türkiye, Erzincan'dan *Verbascum lasianthum* Boiss. ex Bentham'ın Antioksidan Aktivitesi, Amino Asit Bileşimi ve Eser Element Seviyeleri

Anahtar Kelimeler
Verbascum lasianthum, Eser elementler, Amino asitler, Antioksidan aktivite, Tıbbi bitki

Öz: Bu çalışmada Erzincan'da yetişen *Verbascum lasianthum* Boiss. ex Bentham (VL) bitkisinin kök ve gövdesinde bulunan eser elementler ve aminoasitlerin araştırılması ve bu bileşiklerin çevresel stres koşullarına uyumda oynadığı rolün anlaşılması amaçlanmıştır. Çalışmada, analizler için ICP-MS ve LC-MS/MS yöntemleri kullanılmıştır. Ayrıca, bu kısımların metanol:su (70:30, v:v) ekstraklarının antioksidan aktiviteleri araştırılmıştır. Sonuçlar, Erzincan'daki VL'nin kurak ve stresli çevre koşullarına uyum sağlamak için sodyum ve silisyum gibi elementleri biriktirdiğini ve L-prolin, L-asparagin ve L-glutamin gibi amino asitleri sentezleyerek bu süreçleri desteklediğini göstermiştir. Yüksek demir miktarı, VL'nin anemi tedavisinde potansiyel bir demir kaynağı olabileceğini göstermektedir. Ancak alüminyumun bitkilerde birikmesi, insanlarda nörotoksik etki yaratması ve bitki toksisitesi nedeniyle son derece risklidir. Bu amino asitler ve eser elementler bu bitkinin antioksidan potansiyeline katkıda bulunsa da, diğer bölgelerdeki VL türleriyle karşılaştırıldığında düşük bir antioksidan aktivite gösterdi. Sonuç olarak, bu çalışma tıbbi bitkilerin tıbbi etkinliğini ve güvenliğini değerlendirmek için eser elementler ve amino asitler gibi fitokimyasal profillerin kapsamlı bir şekilde analiz edilmesinin önemini vurgulamaktadır.

1. INTRODUCTION

Medicinal plants have a long history as an important aspect of traditional medicine in many cultures throughout the world, and the World Health Organization (WHO) considers herbal medicines, in particular, to be a crucial component of healthcare for millions of people [1]. The demand for these herbal treatment methods is constantly increasing in both developing countries and industrialized regions. Along with this interest, a global awareness is emerging on issues such as effectiveness, safety, quality, and regulation. WHO recommends the establishment of quality standards in herbal treatment. Botanical descriptions of plants, active compounds

analyses and detection of contaminants stand out as important elements [2].

Medicinal plants are frequently used both in the treatment of diseases and are also preferred as nutritional supplements due to the beneficial substances in their structure. These substances also include trace elements (TE) and amino acids (AA). These contents in plants can vary depending on the structure of the soil in which they grow, accumulation from the air, and the ability of the plant to collect elements [3,4]. Along with TE and AA, medicinal plants can also accumulate harmful heavy metals due to growth and environmental factors. Some elements that are necessary for living things can have toxic effects when found in excess. Therefore, the detection of elements in plants and the examination of their effects on human health and plant development have become more important with the increase in environmental pollution [5,6].

The biosynthesis of the AA in plants plays a vital role in plant development and resistance to environmental stresses. Some the AA form chelate complexes with micro and macro elements, facilitating the absorption of these elements by plant roots. Chelate complexes increase the solubility of metal ions, allowing them to be transported more effectively into plant tissues. In addition, the AA activate metabolic pathways and enzymatic processes that support the absorption of elements [7,8].

Turkey hosts a wide variety of plants thanks to the rich ecosystems provided by its various geographical regions. Within this variety, the Verbascum genus is included in the *Scrophulariaceae* family with 245 species and stands out with a high endemism rate of 80%. Plants in the Verbascum genus are used in folk medicine in the treatment of many diseases such as asthma, skin disorders, hemorrhoids, fungal infections, and diarrhea. Studies have reported that these species have anticarcinogenic, wound healing, anti-inflammatory, antifungal, and antioxidant properties [9,10].

Verbascum lasianthum Boiss. ex Bentham (VL), known as " Yünlü sığırkuyruğu", is a biennial plant that grows widely throughout Turkey and is preferred in the treatment of hemorrhoids, especially in Southwestern Anatolia. (Figure 1) Previous studies have reported that VL has wound healing, antioxidant, cytotoxic, antibacterial, antinociceptive, and anti-inflammatory properties [11–13]. Although the phenolic compounds and biological activities of the plant have been investigated in these studies, the AA content has not yet been reported. In a single study on the TE profile, it was reported that Ca and Mg were found in high concentrations, but Se and Co could not be detected [13].

In this study, TE analysis of root and stem parts of VL collected from Erzincan and its surroundings was carried out by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) method. In addition, the AA profile of methanol-water extracts of root and stem parts was evaluated by liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique. In addition, the antioxidant activities of these extracts were also studied.

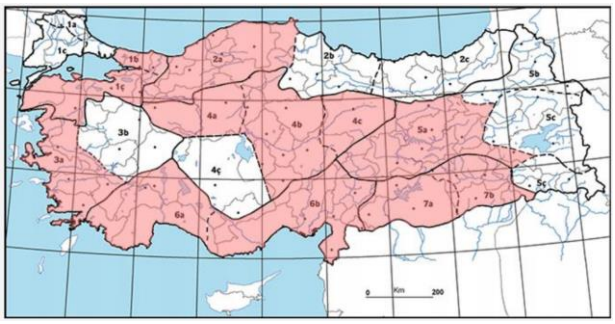

Figure 1. Distribution areas of VL species in Turkey

2. MATERIAL AND METHOD

2.1. Plant Collection and Extraction Protocol for Analytical Procedures

Plant samples were collected from the Kemah and İliç countryside of Erzincan in June 2023. One of these samples was identified by senior taxonomist Prof. Dr. Ali Kandemir and preserved in the herbarium of the Faculty of Arts and Sciences, Erzincan Binali Yıldırım University (EBYU), Turkey (Herbarium ID number: EBYU-000009).

The samples were separated into roots and stems to make them suitable for analysis, and both parts were dried under natural conditions at the room temperature. These dried parts were pulverized with a blender for ICP-MS analysis. On the other hand, hydromethanolic extracts were prepared for antioxidant capacity and AA analyses. In the extraction process, the root and stem parts of the plants were dried at the room temperature and then pulverized into fine powder. 10 grams of plant samples were macerated overnight in 500 mL of methanol and water mixture (7:3, v:v). This process was repeated three times in total. After removing the plant residues, the solvents were combined, and methanol was evaporated. Finally, the water phase was lyophilized to obtain an amorphous solid (1.2 g for root and 1.7 g for stem). The prepared extracts were stored in dark bottles at +4 °C until use.

2.2. Reagents

All aqueous solutions used in ICP-MS analysis were prepared with pure water obtained with Milli-Q Advanced A 10 purification system (Millipore, USA). A mixed solution containing 1% HNO_3 (Merck, USA) and 1% acetonitrile (Sigma Aldrich, Germany) was used for preperation of plant samples, internal standards, and standard solutions. All equipment used in the study, such as tubes, glass vials, and micropipette tips, were cleaned with 10% HNO_3 solution and rinsed with deionized water to prevent contamination during sample preparation and analysis. Agilent® Trace Elements solution contained the following TEs at a concentration of 100 mg L^{-1}: Boron (B), sodium (Na), aluminum (Al), silicium (Si), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), selenium (Se), molibdant (Mo), and cadmium (Cd). This solution was diluted to 1000 µg

dL^{-1} to establish a calibration curve for the TE. The HNO_3 solution used had 99.99% trace metal purity.

2.3. Sample Preperation for ICP-MS Analysis

Milestone Connect ETHOS UP microwave system and Direct-Q 8 UV Ultrapure Water system were used to prevent contamination of atmospheric pollutants during sample preparation for ICP-MS analyses. The microwave degredation method was applied to remove organic compounds in plant samples and to bring inorganic components into a soluble form. Root and stem samples were weighed 200 mg each and 1.8 mL of ultrapure water was added. A vortex device was used to ensure homogeneous dissolution of the mixture. 0.5 mL samples from the prepared mixtures were taken into Teflon containers, then 8 mL of HNO_3 and 2 mL of H_2O_2 solution were added and subjected to a microwave-assisted digestion process. Ramp parameters used in the microwave program are presented in detail in Table 1.

Table 1. The ramping conditions of the microwave program.

Step	Time	T1	T2	Pressure	Power
1	00:10:00	200 °C	100 °C	45 bar	Max power*
2	00:15:00	200 °C	100 °C	45 bar	Max power*

*Max power: 1500W for Ethos and 1200W for Start units.

After the degredation process was completed, ultrapure water was added to complete the sample volume to 15 mL. The mixtures were purified by passing through 0.45 µm syringe filters. Final samples were analyzed three times on the ICP-MS instrument to ensure the reliability of the analytical process and the average values were reported. Dilution factors were calculated using the formula "(final volume or weight / initial sample amount) * dilution coefficient" to verify the accuracy of the results.

2.4. ICP-MS Conditions

For the TE analysis, Agilent 7800 Quadrupole ICP-MS (Agilent Technologies, Japan) was used by operating it with a rotary pump. During the sample injection process, the Integrated Sample Input System (ISIS 3) and Agilent ASX-500 Series ICP-MS Autosampler (Agilent Technologies, Japan) were preferred. Instrument control and data analysis were performed by Mass Hunter 4.2 Workstation Software 7800 ICP-MS Top C.01.02. Measurements were made using a nickel sampler, MicroMist glass concentric nebulizer, and quartz Scott-type spray chamber in quantitative ICP-MS analysis mode. After activating the instrument, basic calibration procedures such as torch alignment, resolution calibration, standard lens settings, plasma optimization, spectrum analysis, and performance reports were applied. Instrument calibration was performed with an Agilent calibration solution containing cerium, cobalt, lithium, magnesium, thallium, and yttrium at a concentration of 1 $\mu g\ mL^{-1}$. Helium collision mode was used for sensitive determination of the TE, and argon was preferred as carrier gas. Before the measurement, the purification process was performed with helium gas for 45 minutes to ensure the accuracy of the ICP-MS system. Device settings and process parameters are presented in detail in Table 2. In the sample preparation phase, the tube and probe of the autosampler were cleaned with 2% HNO_3 and 1% HCl solutions, then rinsed with ultrapure water and made ready for use.

Table 2. Agilent 7800 Quadrupole ICP-MS device parameters

Parameters	Value
Plasma conditions	Forward power 1200W
Plasma gas flow	15.0 L min^{-1}
Carrier gas flow	1 L min^{-1}
Carrier gas pressure	1.45 kPa
Dilution gas flow	1 L min^{-1}
He gas flow	4.5 mL min^{-1}
QP bias	-15 V
Oct bias	-18 V
Cell entrance	-40 V
Cell exit	60 V
Deflect	-0.8 V
Plate bias	-60 V
Nebulizer pump speed	0.30 rps
Sample uptake rate	1.5 mL min^{-1}

Before starting the analysis of plant samples, the instrument was calibrated and validated to ensure the reliability of the method. These operations were performed using Agilent certified reference materials accompanied by daily quality control tests in accordance with the instructions provided by the manufacturer. The calculation of measurements and data analysis process were completed by means of the software 'Mass Hunter 4.2 Workstation Software 7800 ICP-MS Top C.01.02'.

2.5. Chromatographic and Mass Spectroscopic Conditions for AA Analysis

The LC-MS/MS method described in previous studies was used for the separation of the AA [14]. Experiments were carried out using an Agilent 6470 triple quadrupole LC-MS/MS system (Agilent Technologies, Santa Clara, CA, USA). This system includes a 1290 fast pump (G7120A), a 1290 automatic sampler (G7129A), and a 1260 multi-column thermostat (G7116A). Electrospray ionization (ESI) source was preferred for the ionization process. Jasem Quantitative Amino Acids LC-MS/MS Analysis Kit (Altium International Lab. Cih. A.Ş., Istanbul, Turkey) was used to determine the AA concentrations in root and stem extracts. The kit has a CE-IVD certificate, and its validation has been completed. This analysis kit; Calibration curves consisting of 7 different standards include a mixture of 27 isotope-labeled amino acids used as internal standards (IS), two-level quality control samples (QC) and a dilution solution (reagent-1).

Standards, quality control, and extract samples were prepared in accordance with the sample preparation protocol of the kit. For calibration standards, 50 µL of standard solution was taken into a vial. 50 µL of IS and 700 µL of reagent-1 were added. The vial was vortexed for 5 seconds before being placed in the autosampler before being prepared for LC-MS/MS analysis. For extract samples, 10 mg of extract was dissolved in the Jasem's mobile phase (A: 3% formic acid–5% methanol–30 mM ammonium formate, B: Acetonitrile; 1:1, v:v). After filtration, 50 µL of extract sample was mixed with 50 µL of IS and 700 µL of reagent-1. The mixture was

vortexed for 5 seconds and centrifuged at 3600 *g* for 5 minutes at room temperature to precipitate insoluble particles. The supernatant was transferred to a vial for LC-MS/MS analysis.

Chromatographic separation of the AA was performed using the analytical column, gradient elution program, and mobile phases specified in the kit protocol. The analytical column was kept at 30 °C and 3 µL of standard, control, and sample solutions were injected into the HPLC system. Jasem's A and B mobile phases were applied at a flow rate of 0.7 mL min^{-1} with the gradient elution program specified in Table 3. The entire analysis took 7.5 minutes.

Table 3. The mobile phase composition during gradient elution

Time	Change Solvent Composition	
	A	B
1.00 min	22.00 %	78.00 %
4.00 min	70.00 %	30.00 %
5.00 min	70.00 %	30.00 %
5.10 min	22.00 %	78.00 %
9.00 min	22.00 %	78.00 %

*A: %3 formic acid–%5 methanol–30 mM ammonium formate, B: Acetonitrile

The mass spectrometer was operated in positive ion multiple reaction monitoring (MRM) mode to perform mass determination of the AA. The analysis method included the following mass parameters: capillary voltage of 2000 V, drying gas temperature of 150 °C, drying gas flow rate of 10 L min^{-1}, 40 psi nebulizer pressure, 400 °C sheath gas temperature, and 10 L min^{-1} sheath gas flow rate. MS/MS determinations were performed using the collision-induced dissociation (CID) process, with the dissociation of precursor ions into product ions. For each ion transition, the MRM of the the AA to monitor the transitions and determine the IS, appropriate breakdown voltage (FV), and collision energy (CE) values were set. Table 4 summarizes the mass spectrometry parameters and precursor/product ion mass transitions of each analyte.

Calibration curves were used to quantify analytes, considering matrix effects and procedural losses. During LC-MS/MS analysis, data collection, characterization, and quantification were performed with the help of Agilent MassHunter software (versions 10.1 and 10.0).

2.6. Antioxidant activity assays

2.6.1. Determination of total phenolic content (TPC)

The TPC of the extracts was measured by a spectrophotometric method using the Folin-Ciocalteu reagent [15]. In this analysis, first, each extract and standard solutions (1 mg mL^{-1}) were diluted with 4.5 mL of distilled water. Then, 100 µL of Folin-Ciocalteu reagent was added to the mixture, and the mixture was incubated at room temperature for 120 minutes. After the incubation period, 300 µL of 2% Na_2CO_3 solution was added to each sample. The samples were vortexed to make them homogeneous and the absorbance values were measured at a wavelength of 760 nm. All analyses were performed in triplicate, and the calibration curve was obtained with the linear equation y = 0.117x - 0.011 using various gallic acid concentrations (1–1000 µg mL^{-1}). The results obtained were expressed in mg gallic acid equivalent phenolic compound per extract (mg GAE g $extract^{-1}$).

2.6.2. Determination of total flavonoid content (TFC)

The TFC of the extracts was analyzed according to the method described by Varol et al [16]. In this process, firstly, 100 µL of extract and standard solutions (1 mg mL^{-1}) were mixed with 4.7 mL of methanol. Then, 100 µL of ammonium acetate (NH_4CH_3COO, 1 M) and 100 µL of 10% aluminum chloride ($AlCl_3$) solution were added to the mixture. The resulting mixture was vortexed and incubated at room temperature for 45 minutes. At the end of the incubation period, absorbance measurements were made at 415 nm wavelength. All tests were performed in triplicate and the results were reported as mg quercetin equivalent/g extract based on the calibration curve (y = 0.117x - 0.011) obtained using various quercetin concentrations (1–1000 µg mL^{-1}).

2.6.3. DPPH• Radical scavenging activity

DPPH• radical scavenging capacities of the extracts were evaluated using the methods specified in the literature [17]. Samples prepared at different concentrations (5–100 µg mL^{-1}) were mixed with 1 mL of DPPH• (0.26 mM) solution and vortexed for homogenization. The mixtures were incubated at room temperature in the dark for 30 minutes. After incubation, absorbance measurements were performed at 517 nm wavelength. DPPH• activity was calculated with Equation 1.

$$\text{Activity (\%)} = [(A_0-A_1)/A_0]x100 \quad (1)$$

In this equation, A_0 is the absorbance of the control sample and A_1 is the absorbance of the test sample. Analyses were performed with triplicates and the results were given as IC_{50} (µg mL^{-1}) value, mean ± standard deviation. The DPPH• scavenging activities of the extracts were compared with standard antioxidants such as Trolox, butylated hydroxytoluene (BHT), butylated hydroxy anisole (BHA), and ascorbic acid.

2.6.4. Ferric reducing antioxidant power (FRAP)

The ferric-reducing antioxidant power (FRAP) analysis of the extracts was performed according to the method described by Akman et al [18]. In this method, 250 µL of extracts or standard solutions (1 mg mL^{-1}) were mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of 1% [$K_3Fe(CN)_6$] solution. The mixtures were incubated in a water bath at 50°C for 20 min. After incubation, 0.25 mL of 0.1% $FeCl_3$ and 1.25 mL of 10% trichloroacetic acid were added and the absorbance of the mixture was measured at 700 nm. All tests were performed in triplicate and the results were expressed as mg Trolox equivalent (TE) activity g $extract^{-1}$ according to the calibration curve obtained with various concentrations of Trolox (10–100 µg mL^{-1}). FRAP values

of the extracts were compared with standard antioxidants BHT, BHA, and ascorbic acid.

Table 4. MRM conditions of the AA.

Amino Acid	Precursor Ion (*m/z*)	Product Ion (*m/z*)	FV* (V)	CE** (V)	Polarity	Retention time (min)
1-Methyl-l-Histidine	170.1	124.1	100	10	Positive	4.354
3-Methyl-l-Histidine	170.1	126.2	120	10	Positive	4.334
Argininosuccinic acid	291.0	70.2	140	30	Positive	3.982
Beta-Alanine	90.1	72.1	80	2	Positive	2.537
DL- 5-Hydroxylysine	163.1	128.1	90	6	Positive	4.083
DL-homocystine	269.0	136.0	90	8	Positive	3.789
Ethanolamine	62.1	44.2	80	4	Positive	2.518
Gamma-aminobutyric acid	104.0	87.1	100	6	Positive	2.188
Glycine	76.2	30.1	80	1	Positive	3.129
L-2-Aminoadipic acid	162.0	98.0	90	10	Positive	2.619
L-2-Aminobutyric acid	104.2	58.3	80	4	Positive	2.759
L-alanine	90.2	44.2	80	4	Positive	2.954
L-anserine	241.1	170.0	80	10	Positive	4.507
L-arginine	175.2	70.2	110	20	Positive	3.995
L-asparagine	133.1	74.2	70	10	Positive	3.184
L-aspartic acid	134.1	74.1	80	8	Positive	2.767
L-carnosine	227.1	110.1	110	22	Positive	4.255
L-citrulline	176.2	159.3	80	3	Positive	3.520
L-cystathionine	223.0	134.0	100	8	Positive	4.032
L-cystine	241.1	74.2	100	24	Positive	3.974
L-glutamic acid	148.1	84.2	80	12	Positive	2.494
L-glutamine	147.1	84.2	80	12	Positive	3.289
L-histidine	156.1	110.1	100	8	Positive	4.006
L-homocitrulline	190.1	173	80	12	Positive	3.461
L-isoleucine	132.2	69.2	100	14	Positive	2.147
L-leucine	132.2	43.3	100	24	Positive	2.060
L-lysine	147.1	84.2	80	12	Positive	4.113
L-methionine	150.1	104.1	80	4	Positive	2.174
L-norvaline	186.0	140.0	100	8	Positive	2.478
L-ornithine	133.2	70.3	80	14	Positive	4.075
L-phenylalanine	166.1	120.1	80	6	Positive	1.921
L-proline	116.2	70.2	90	12	Positive	3.273
L-serine	106.2	60.2	80	4	Positive	2.924
L-threonine	120.2	74.2	80	4	Positive	2.807
L-tryptophan	205.1	188.1	80	1	Positive	1.764
L-tyrosine	182.1	165	80	1	Positive	1.968
L-valine	118.2	72.2	80	4	Positive	2.478
o-phospo-L-serine	186.0	88.1	90	8	Positive	3.655
o-phosphoryl Ethanolamine	142.0	44.2	80	4	Positive	3.735
Sarcosine	90.1	44.2	90	8	Positive	3.468
Taurine	126.1	44.3	110	14	Positive	1.730
Trans-4-hydroxy-l-proline	132.2	68.2	90	20	Positive	3.165

*FV: Fragmentor voltage, **CE: Collision energy.

3. RESULTS

3.1. ICP-MS Analysis

In this study, the TE amounts in the root and stem of VL were determined using a validated ICP-MS method [19]. According to the analysis results, it was determined that the TE levels in the root and stem parts showed similar distributions. (Table 5) Na stood out as the most abundant TE in both parts. Especially high amount of Na accumulation was observed in the root and found remarkable (6226.7 µg g dry weight (dw)$^{-1}$). After Na, Si was detected in high amounts at 387.0 µg g dw^{-1}for root and 593.5 µg g dw^{-1} for stem and Fe was detected in high amounts at 208.8 µg g dw^{-1} for root and 216.3 µg g dw^{-1} for stem. B, Al, Mn, Co, Ni, Cu, Zn, and Se were determined in both parts but in very low concentrations. Mo was only detected in root with low concentration, while Cd was not detected in both parts.

Table 5 The TE concentrations in root and stem parts of VL. (µg g dw^{-1})

Parts	B	Na	Al	Si	Mn	Fe	Co	Ni	Cu	Zn	Se	Mo	Cd
Root	18.2	6226.7	126.6	387.0	8.8	208.8	0.1	8.5	9.8	63.2	0.1	0.1	<0.000
Stem	23.7	644.4	195.9	593.5	10.2	216.3	0.1	9.7	8.0	6.9	0.1	<0.000	<0.000

3.2. LC-MS/MS Analysis for AA

An LC-MS/MS analysis method with high sensitivity, accuracy, and precision was used to evaluate the AA profiles in the root and stem parts of VL. A total of 43 AA were quantitatively analyzed in these parts by LC-MS/MS. Figure 2 shows the MRM chromatograms obtained from the LC-MS/MS analysis of the root and stem extracts.

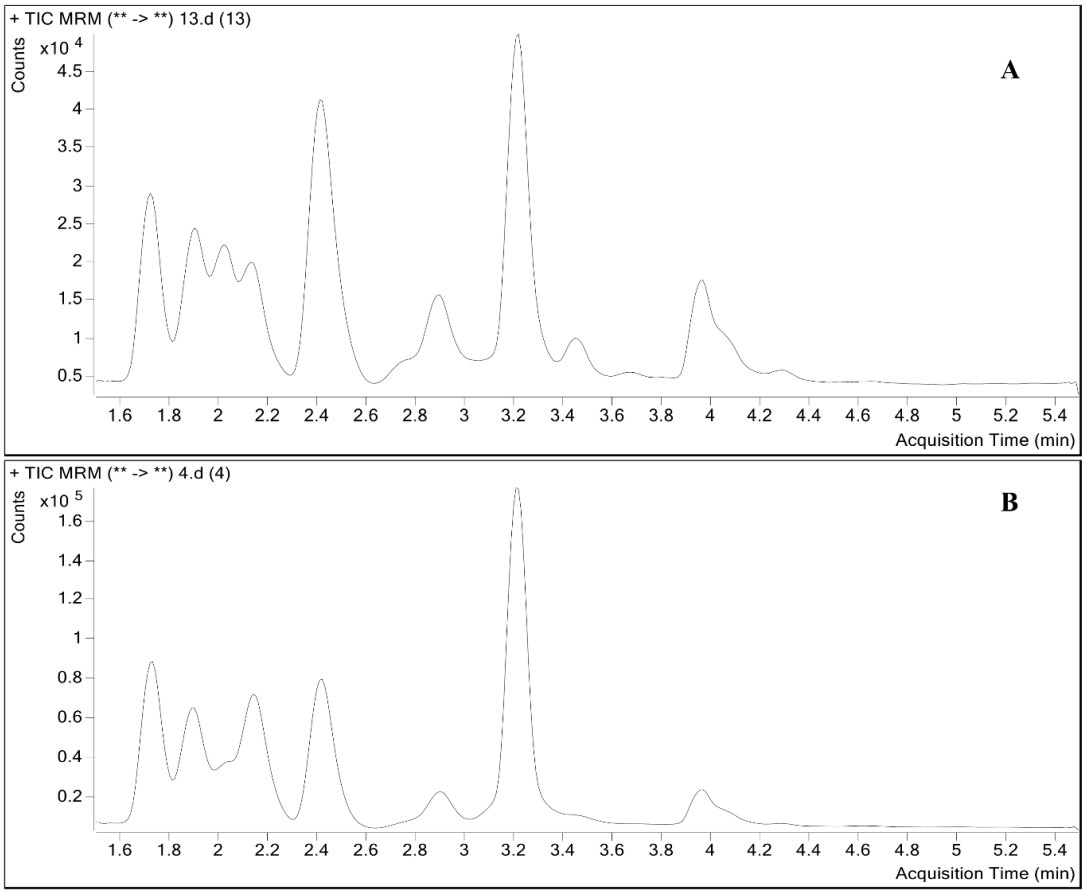

Figure 2. The MRM chromatograms of the A) root and B) stem extracts

The AA levels in these extracts were determined in nmol mL^{-1}. The linear regression equations, correlation coefficients (R^2), and quantification limit values (LOQ) of the AA are presented in Table 6.

Table 6. Calibration curve equations, correlation coefficients, and LOQ values of the AA in the LC-MS/MS method

Amino Acids	Calibration curve equations	R^2	LOQ (nmol L^{-1})
1-methylhistidine	y= 0.134550x + 0.030534	0.9963	1.0
3-methylhistidine	y= 0.007255x + 0.009899	0.9970	1.0
2-aminoadipic acid	y= 0.012476x - 0.005961	0.9924	0.5
2-aminobutyric acid	y= 0.164021x - 0.304197	0.9969	2.5
3-aminoisobutyric acid	y= 0.005320x + 0.006929	0.9979	1.0
5-hydroxylysine	y= 0.003114x + 0.003675	0.9971	1.0
Argininosuccinic acid	y= 0.013154x - 7.593827E-004	0.9992	1.0
Beta-alanine	y= 0.006016x + 0.002325	0.9970	0.2
Ethanolamine	y= 0.386430x + 1.384151	0.9961	2.0
Gamma-aminobutyric acid	y = 0.039074x + 0.058776	0.9962	1.0
Glycine	y= 0.001120x - 0.008773	0.9955	5.0
Homocitrulline	y= 0.004492x - 0.007928	0.9986	2.0
Homocystine	y= 0.039396x + 0.012211	0.9998	0.25
L-alanine	y= 0.002234x - 0.003392	0.9991	1.0
L-anserine	y= 0.020276x + 0.003041	0.9994	0.4
L-arginine	y= 0.012304x + 7.375877E-004	0.9994	2.0
L-asparagine	y= 0.012480x - 0.008593	0.9962	2.0
L-aspartic acid	y = 0.020336x – 0.028037	0.9995	1.0
L-carnosine	y= 0.025459x - 0.002913	0.9989	1.0
L-citrulline	y= 0.018775x + 0.039661	0.9986	0.5
L-cystathionine	y= 0.101093x + 0.001578	0.9998	0.1
L-cystine	y= 0.010862x - 0.007446	0.9987	2.0
L-glutamic acid	y= 0.017219x - 0.038986	0.9994	5.0
L-glutamine	y= -1.686812E-006x^2 + 0.006569x + 0.054237	0.9994	5.0
L-histidine	y= 0.010063x + 0.072616	0.9984	5.0
L-isoleucine	y= 0.002582x - 0.007951	0.9976	1.0
L-leucine	y= 0.001315x - 7.336834E-004	0.9980	5.0
L-lysine	y= 0.025117x + 0.130604	0.9984	1.0
L-methionine	y= 0.021032x + 0.025020	0.9966	1.0
L-norvaline	y= 0.002458x^2 + 00.042305x + 0.014883	0.9975	0.1
L-ornithine	y= 0.013625x + 0.526848	0.9993	2.0
L-phenylalanine	y= 0.020012x + 0.067728	0.9996	1.0

L-proline	y= 0.005376x + 0.024883	0.9979	5.0
L-serine	y= 0.013133x + 0.214409	0.9962	5.0
L-threonine	y= 0.010757x + 0.078723	0.9953	2.5
L-tryptophan	y= 0.028557x - 0.053366	0.9990	1.0
L-tyrosine	y= 0.013461x - 0.019332	0.9995	1.0
L-valine	y= 0.002968x + 0.014195	0.9989	5.0
o-phosporhyl ethanolamine	y= 9.059945E-004x - 1.944540E-004	0.9997	0.25
o-phosphoserine	y= 5.074562E-004x - 7.213929E-006	0.9999	0.25
Sarcosine	y= 0.050509x + 0.228475	0.9991	5.0
Taurine	y= 0.001442x – 0.001064	0.9979	5.0
Trans-4-hydroxy L-proline	y= 0.005091x - 0.003910	0.9993	1.0

*R^2: Correlation coefficient

In this study, it was determined that the root and stem parts of the plant have different AA profiles. It was observed that the concentrations of the AA generally found in the stem were higher than in the root. (Table 7)

Table 7. AA levels in the root and stem extracts of VL (nmol mL^{-1})

Amino Acids	**Root**	**Stem**	**Amino Acids**	**Root**	**Stem**
1-methylhistidine	0.0000	0.0000	L-glutamic acid	8.9035	28.4213
3-methylhistidine	0.0000	0.0000	L-glutamine	14.9213	379.9220
2-aminoadipic acid	2.4261	5.6595	L-histidine	0.0000	4.6044
2-aminobutyric acid	2.2302	3.2828	L-isoleucine	5.6796	34.5097
3-aminoisobutyric acid	21.0044	66.0686	L-leucine	6.2863	32.7559
5-hydroxylysine	0.0000	0.0000	L-lysine	1.2469	11.0819
Argininosuccinic acid	0.3042	1.4487	L-methionine	0.0000	0.0000
Beta-alanine	0.8958	0.7660	L-norvaline	0.2209	3.6957
Ethanolamine	2.3116	103.5776	L-ornithine	24.7736	0.0000
Gamma-aminobutyric acid	14.5879	53.9212	L-phenylalanine	0.0000	20.4949
Glycine	10.6834	38.2563	L-proline	0.0000	470.0972
Homocitrulline	1.7979	1.8431	L-serine	0.0000	83.7654
Homocystine	0.0000	0.0000	L-threonine	0.0000	31.4394
L-alanine	30.4803	93.4081	L-tryptophan	2.4485	23.8490
L-anserine	0.0000	0.0000	L-tyrosine	3.1378	11.5716
L-arginine	2.0282	53.5069	L-valine	3.0923	68.5982
L-asparagine	2.0480	403.7208	*o*-phosporhyl ethanolamine	0.8348	0.2532
L-aspartic acid	7.9867	36.4775	*o*-phosphoserine	0.1288	0.0609
L-carnosine	0.2780	0.3002	Sarcosine	0.0000	0.2969
L-citrulline	0.0000	5.3701	Taurine	1.1490	0.9507
L-cystathionine	0.3446	0.1189	Trans-4-hydroxy L-proline	1.5211	1.7520
L-cystine	1.7094	1.4065			

As a result of the analyses, a total of 30 AA were determined in the root part, and the ones with the highest concentration were L-alanine (30.4803 nmol mL^{-1}), L-ornithine (24.7736 nmol mL^{-1}) and 3-aminoisobutyric acid (21.0044 nmol mL^{-1}), respectively. L-citrulline, L-histidine, L-phenylalanine, L-proline, L-serine, L-threonine, and sarcosine were detected in the stem, but not in the root. Among the 36 AA detected in the stem, the ones with the highest concentrations were determined as L-proline (470.0972 nmol mL^{-1}), L-asparagine (403.7208 nmol mL^{-1}), and L-glutamine (24.7736 nmol mL^{-1}), respectively. (Figure 3) The concentrations of 3-aminoisobutyric acid, ethanolamine, gamma-aminobutyric acid, L-alanine, L-arginine, L-aspartic acid, L-isoleucine, L-leucine, L-serine and L-valine, which were found in very low amounts in the root, were detected at quite high levels in the stem (32.7559-103.5776 nmol mL^{-1}). However, L-ornithine, one of the most abundant AA in the root, was not detected in the stem. The AA such as 2-aminoadipic acid, 2-aminobutyric acid, argininosuccinic acid, beta-alanine, homocitrulline, L-carnosine, L-cystationine, L-cystine, L-glutamic acid, L-lysine, L-norvaline, L-tryptophan, L-tyrosine, *o*-phosphorylethanolamine, *o*-phosphoserine, taurine and trans-4-hydroxy L-proline were found in very low concentrations (0.1288-23.8490 nmol mL^{-1}) both in root and stem. Additionally, 1-methylhistidine, 3-methylhistidine, 5-hydroxylysine, homocystine, L-anserine, and L-methionine were not observed in any part of the plant.

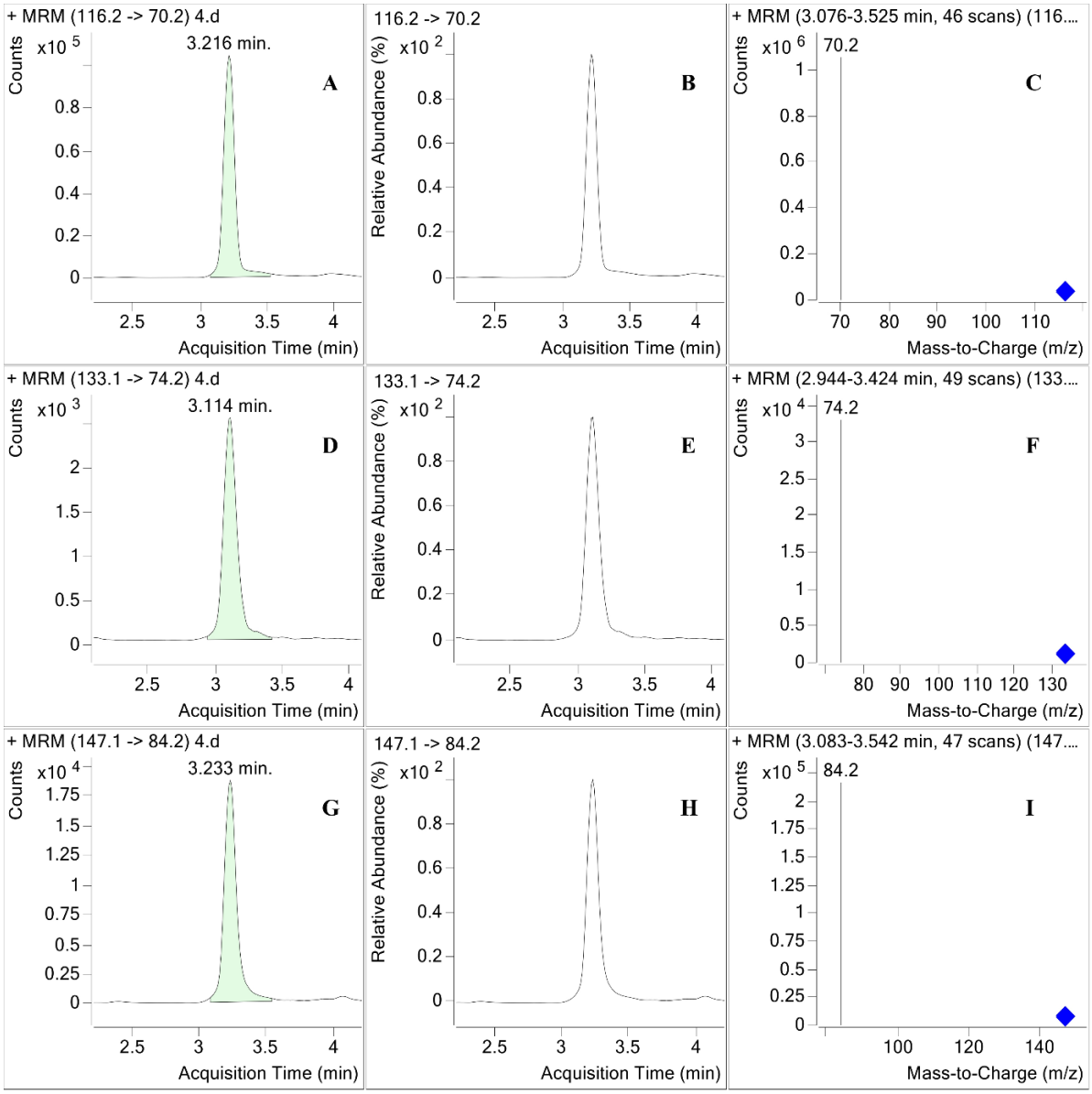

Figure 3. The typical chromatograms showed the peak area and relative abundance respectively of A, B) L-proline, D, E) L-asparagine, and G, H) L-glutamine. The mass spectrums of C) L-proline, F) L-asparagine, and I) L-glutamine.

3.3. Antioxidant Activity Assay

The antioxidant activity and total phenolic and flavonoid contents of root and stem extracts are detailed in Table 8. When DPPH• radical scavenging ability was evaluated in terms of IC_{50} (µg mL^{-1}), it was seen that the IC_{50} values of the root and stem extracts were 141.35±2.03 and 54.97±1.78 µg mL^{-1}, respectively. However, these values indicate lower activity when compared to standard antioxidants such as Trolox, BHA, and BHT. In addition, the TPC value of the stem extract was measured as 62.28±0.28 mg GAE g $extract^{-1}$ and the TFC value was measured as 15.71±0.75 mg QE g $extract^{-1}$, which were significantly higher than the TPC (35.80±1.31 mg GAE g $extract^{-1}$) and TFC (10.52±0.56 mg QE g $extract^{-1}$) values of the root extract. The ferric ion-reducing capacity of root and stem extracts of VL was also investigated in the study. The results showed that the ferric reducing capacity of the stem extract was higher as 122.53±0.58 mg TE g $extract^{-1}$ compared to the root extract (75.83±2.97 mg TE g $extract^{-1}$); however, this capacity was lower than the activity of ascorbic acid at 394.17±0.98 mg TE g $extract^{-1}$.

Table 8. Antioxidant activity, TPC and TFC of VL

Plant Species	DPPH IC_{50} (µg mL^{-1})	Total phenolics (mg GAE g $Extract^{-1}$)	Total flavonoids (mg QE g $Extract^{-1}$)	Reducing power (mg TE g $Extract^{-1}$)
Verbascum lasianthum (root)	141.35±2.03	35.80±1.31	10.52±0.56	75.83±2.97
Verbascum lasianthum (stem)	54.97±1.78	62.28±0.28	15.71±0.75	122.53±0.58
Trolox	11.95±0.15	-	-	-
BHA	8.04±0.69	-	-	338.57± 0.31
BHT	10.70±0.73	-	-	257.80± 1.24
Ascorbic acid	9.91±0.87	-	-	394.17±0.98

4. DISCUSSION AND CONCLUSION

Medicinal plants have various pharmacological and biological effects thanks to the primary and secondary metabolites, phytochemical compounds, and the TE in their structure. Therefore, their use in traditional medicine has become widespread in recent years as an alternative to both nutritional and pharmaceutical treatments. However, human-induced activities such as industrialization, mining, use of chemical fertilizers and pesticides, fossil fuel consumption, and accumulation of domestic waste have led to heavy metal accumulation and related toxicity in medicinal plants. This situation limits the safe use of medicinal plants. Medicinal plants can cause serious side effects when used above the recommended safe consumption limits [20]. Therefore, for the evaluation of the efficacy, safety, and toxicity of medicinal plants, their chemical compositions, especially the TE, AA, and heavy metals, should be examined by analytical methods.

In this study, the TE, heavy metal, and AA levels in the root and stem of VL collected from Erzincan were analyzed. In addition, the antioxidant activities of hydromethanolic extracts obtained from these parts were evaluated. In a previous study, the TPC, antioxidant activity, and TE compositions of methanol and acetone extracts of VL grown in Western Anatolia were reported. In this study, methanol (IC_{50}: 6.44 mg mL^{-1}) and acetone (IC_{50}: 17.07 mg mL^{-1}) extracts were shown to have higher antioxidant activity compared to BHT (23.58 mg mL^{-1}). The TPC values were reported as 30.82±2.10 and 64.47±4.84 mg GAE g $extract^{-1}$, respectively [13]. When these two studies were compared, it was determined that the TPC values of both regions were similar, but the plant in Western Anatolia showed higher antioxidant activity.

In the study of Hazman et al., the most abundant TE in VL were reported as Fe (446.98 ± 94.63 ppm), Zn (20.67 ± 1.08 ppm), Mn (38.12 ± 2.39 ppm), and Na (78.38 ± 10.08 ppm). In addition, Cu and Pb elements were found at low levels, while Se and Co were not detected [13]. Compared to VL in Erzincan, the arid climate of Eastern Anatolia caused stress in the plant, resulting in higher levels of Na (6226.7 and 644.4 µg g dry $weight^{-1}$) and Si (387.0 and 593.5 µg g dry $weight^{-1}$) accumulation in the root and stem. Plants require trace amounts of elements such as Fe, Cu, Zn, and Mn in their growth and development processes [21]. However, it is known that high concentrations of these elements in medicinal plants may have carcinogenic effects [22]. In both studies, it was observed that Cu, Mn, Ni, and B elements have similar profiles at low concentrations. However, the high Fe levels of VL in Erzincan stand out as important data. On the other hand, although the carcinogenic effects of Al have not yet been definitively confirmed, it is known that it can cause plant toxicity by dissolving in acidic soils, in addition to its neurotoxic effects in humans [20,23]. In our investigation, the absence of Cd among heavy metals was regarded as a favorable safety finding. Al accumulation in roots (126.6 µg g dry $weight^{-1}$) and stems (195.9 µg g dry $weight^{-1}$) may cause a potential risk to plant development and traditional medicinal practices. This is a concern that should be carefully evaluated when using the plant for medicinal purposes.

AA biosynthesis is essential for the tolerance of plants to environmental stressors [7,8]. This study presents the first report of VL's AA profile. LC-MS/MS tests revealed that the stem part had high levels of L-proline, L-asparagine, and L-glutamine. Proline and glutamine serve as crucial defensive mechanisms against harmful environmental factors such as salt stress, dryness, and temperature variations. These AAs regulate the transport of Na ions in plants and prevent their harmful effects. Proline, in particular, promotes cell membrane integrity and restricts the entry of Al into the cell [24,25]. Glutamine and glycine help to retain and transport Fe on the root surface, making it easier to incorporate into metabolic processes [8,26]. Si generally accumulates in plants as a defense mechanism in response to stress conditions. L-asparagine helps stressed plants accumulate Na and Si while also playing an important role in nitrogen metabolism. It also protects the plant from environmental challenges by enhancing antioxidant production, such as glutathione, as well as antioxidant enzyme activity [27]. The adaptation mechanisms developed by VL in Erzincan arid climate against stress are consistent with the analysis results of the AA and the TE. The plant accumulated Na and Si to protect itself from water and salt stress. In addition, it produced large amounts of L-proline and L-asparagine to reduce Al toxicity and increase Na absorption. It also enhanced L-glutamine synthesis to support Fe absorption, which is required for metabolic functions. Although the found AA and the TE contributed to the plant's antioxidant capacity, it was determined that there was no appreciable antioxidant activity.

In conclusion, this study on *Verbascum lasianthum* from Erzincan emphasizes the importance of the AA and the TE in plant response to environmental stress situations. The accumulation of Na and Si, as well as the synthesis of L-proline, L-asparagine, and L-glutamine in the plant, were thought as a defense mechanism created against the effects of arid and stressful situations. However, it was emphasized that high levels of Al accumulation in the root and stem could offer potential risks in the medicinal use of the plant. The low antioxidant activity suggests that the pharmacological effects of this plant should be studied further for traditional medicine. These findings highlight the need to conduct a thorough investigation of chemical profiles for the safety and efficacy of medicinal plants.

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