Erzincan Üniversitesi
Fen Bilimleri Enstitüsü Dergisi
2025, 18(2), 391-413
ISSN: 1307-9085, e-ISSN: 2149-4584
Araştırma Makalesi

Erzincan University
Journal of Science and Technology
2025, 18(2), 391-413
DOI: 10.18185/erzifbed.1666259
Research Article

# Comprehensive Analysis of Trace Elements, Amino Acids, and Antioxidant Potential of *Verbascum orientale*

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**Received:** 27/03/2025, **Revised:** 24/07/2025, **Accepted:** 07/08/2025, **Published:** 31/08/2025

**Abstract**

This study aims to investigate the trace element composition, amino acid profile, and antioxidant activity of *Verbascum orientale*, a medicinal plant in Erzincan, Türkiye. The root and stem extracts were analyzed using ICP-MS to determine trace element levels, while LC-MS/MS was employed for amino acid profiling. Additionally, the antioxidant activities of methanol: water (70:30, v:v) extracts were evaluated using DPPH, FRAP, total phenolic content (TPC), and total flavonoid content (TFC) assays. The findings indicate that *Verbascum orientale* contains significant levels of sodium (Na) and silicon (Si), suggesting an adaptive response to arid and stressful environmental conditions. In addition, the plant exhibited high levels of amino acids such as L-proline, L-glutamine, and L-asparagine, which are essential for osmotic regulation and stress adaptation. A high iron (Fe) content suggests potential nutritional benefits, while the presence of aluminum (Al) raises concerns about possible toxicity. Despite its diverse phytochemical profile, *Verbascum orientale* exhibited moderate antioxidant activity compared to other *Verbascum* species, with the root extract demonstrating a stronger radical scavenging effect than the stem. These results underscore the importance of further research to evaluate the medicinal and nutritional applications of *Verbascum orientale*, particularly in terms of bioavailability and safety.

**Keywords:** *Verbascum orientale,* amino acids, bioactive compounds, trace elements, antioxidant activity.

# *Verbascum orientale'nin* Eser Elementleri, Amino Asitleri ve Antioksidan Potansiyelinin Kapsamlı Analizi

**Öz**

Bu çalışmanın amacı, Türkiye, Erzincan'da tıbbi bir bitki olan *Verbascum orientale*'nin eser element bileşimini, amino asit profilini ve antioksidan aktivitesini araştırmaktır. Kök ve gövde ekstraktları, eser element seviyelerini belirlemek için ICP-MS kullanılarak analiz edilmiştir; amino asit profili ise LC-MS/MS ile incelenmiştir. Ayrıca, metanol: su (70:30, v:v) ekstraktlarının antioksidan aktiviteleri DPPH, FRAP, toplam fenolik içerik (TPC) ve toplam flavonoid içerik (TFC) testleri kullanılarak değerlendirilmiştir. Bulgular, *Verbascum orientale*'nin önemli düzeyde sodyum (Na) ve silisyum (Si) içerdiğini göstermekte, bu da kurak ve stresli çevre koşullarına adaptif bir yanıt olduğunu düşündürmektedir. Ayrıca, bitki ozmotik düzenleme ve stres adaptasyonu için gerekli olan L-prolin, L-glutamin ve L-asparajin gibi amino asitleri yüksek seviyelerde sergilemiştir. Yüksek demir (Fe) içeriği potansiyel besinsel faydaları akla getirirken, alüminyum (Al) varlığı olası toksisite ile ilgili endişeleri artırmaktadır. Farklı fitokimyasal profiline rağmen, *Verbascum orientale* diğer *Verbascum* türlerine kıyasla orta düzeyde antioksidan aktivite sergilemiş, kök ekstresi gövdeye göre daha güçlü bir radikal süpürücü etki göstermiştir. Bu sonuçlar, *Verbascum orientale*'nin tıbbi ve besinsel uygulamalarını, özellikle biyoyararlanım ve güvenlik açısından değerlendirmek için daha fazla araştırma yapılmasının önemini vurgulamaktadır.

**Anahtar Kelimeler:** *Verbascum orientale*, amino asitler, biyoaktif bileşikler, eser elementler, antioksidan aktivite.

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## 1. Introductıon

Plants constitute a valuable source of traditional herbal medicine, recognized for their therapeutic heritage. Historically, the preparation of natural products for medicinal purposes has played a significant role in the development of drugs. This practice reflects a country's natural resources and continuous development. Phytoconstituents extracted from medicinal plants serve as specific agents to treat a variety of disorders [1, 2]. Drug development studies show that 50% of approved medicines since 1994 are derived from natural compounds or active elements extracted from natural sources. This highlights the significant impact of nature's compounds on modern pharmaceuticals. The World Health Organization (WHO) highlights that around 80% of the population in developing countries use traditional medicinal systems for their basic health needs [3]. Nonetheless, the demand for herbal treatments treatments remains high. WHO's projection of the herbal industry's growth, from the current global value of $62 billion to $5 trillion by 2050, highlights the increasing demand for these natural therapies [4].

Medicinal plants are not only used for disease treatment but also serve as valuable nutritional supplements because of their diverse composition of bioactive compounds, including trace elements (TE) and amino acids (AA). The concentration and levels of these constituents are influenced by various factors, such as soil composition, atmospheric deposition, and the plant's ability to absorb elements [5, 6]. TE and AA contribute significantly to both plant physiology and human health. However, medicinal plants are also at risk of absorbing harmful heavy metals due to environmental contamination. Additionally, essential elements can have toxic effects when found in high concentrations, requiring detailed detection and analysis of these components, especially with increasing environmental pollution [7, 8]. In this context, amino acid biosynthesis plays a significant role in plant growth and adaptation to abiotic stress. Certain amino acids form chelate complexes with micro- and macroelements, improving their solubility and helping them move into the plant root systems. These chelation processes improve the translocation of metal ions within plant tissues and activate metabolic pathways and enzymatic mechanisms that regulate element assimilation, further underscoring their critical role in plant nutrition and environmental interactions [9, 10]. Given their ability to accumulate essential nutrients and support ecological balance, medicinal plants are vital sources of amino acids and trace elements [11-13]. Therefore, researching these plants is important for understanding their nutritional potential and their role in environmental adaptation. The VO, a member of the Scrophulariaceae family, is of particular significance because of its high species diversity and endemic distribution. These characteristics may influence its chemical composition and adaptive mechanisms.

The *Verbascum* genus, known as sığırkuyruğu in Turkish, comprises 360 species worldwide, exhibiting significant diversity. However, its importance is particularly notable within Turkey, where the genus includes 245 species, categorized into 13 distinct groups. Approximately 80% of these species are endemic to the region, underscoring their distinct presence [14]. Furthermore, 30% of the species display hybrid characteristics, contributing to the complexity of the *Verbascum* genus. This genus has a rich history of use in traditional medicine for treating a wide range of ailments. Various civilizations across Africa, Central and Western Asia,

Europe, and North America have employed the flowers and leaves of *Verbascum* species in both internal and external applications, highlighting the genus' continued importance in herbal medicine. The diverse biological activities demonstrated by different *Verbascum* species, such as anticancer, wound healing, anti-inflammatory, antihelmintic, anxiolytic, preanesthetic, sedative, antifungal, and antioxidant properties [14], have been investigated in previous research. Moreover, the scientific literature includes various reports on the chemical constituents of *Verbascum* species, identifying a range of bioactive compounds, including saponins, flavonoids, iridoids, and phenylethanoids [14]. Each of these compounds is characterized by different chemical structures and contributes to the biological activity of the plant. However, to date, no studies have reported on the chemical composition or biological activity of VO.

VO is an annual, herbaceous plant, typically reaching heights between 15 and 80 cm, and is characterized by sparse indumentum. The flowers are predominantly yellow, with occasional brown spots [15]. In Turkey, this plant is commonly known as "İbrahim otu." Its fresh or dried flowers are traditionally boiled in milk for external application in the treatment of pruritic conditions affecting the urogenital region [16, 17]. VO is a plant species widely distributed in Central Asia and Anatolia and is well recognized for its therapeutic uses in traditional medicine. However, there is a significant lack of scientific literature regarding its trace element composition, amino acid profile, and antioxidant activity, all of which have been investigated for the first time.

This study aims to address these knowledge gaps by performing a comprehensive analysis of the trace element composition and amino acid profile of VO, while also evaluating its antioxidant activity to explore its potential health benefits and possible toxicological risks. The findings will improve our understanding of the plant's biological and chemical properties and provide important information about its potential applications in the pharmaceutical and nutraceutical fields. In conclusion, the biochemical analyses performed in this study could support the inclusion of VO in functional foods, dietary supplements, and new therapeutic agents, thus promoting the wider use of natural compounds in modern medicine

## 2. Material and Methods

### 2.1. Plant Material

The *Verbascum orientale* was collected from Erzincan province, in June 2021. The plant samples were authenticated by Prof. Dr. Ali Kandemir (Erzincan Binali Yıldırım University, Faculty of Art and Sciences, Department of Biology). Authentic samples (ID number VO:0000031) were deposited in the Herbarium of Erzincan Binali Yıldırım University (EBYU).

### 2.2. Extraction Procedure

The collected plant materials were separated into stem (VOS) and root (VOR) parts for analysis. Both portions were dried under room temperature conditions. The VOS and VOR parts of VO, which were dried, were then ground using a blender for further ICP-MS, antioxidant capacity, and amino acid analyses. A 10 g sample of the material was macerated overnight using a

methanol-water mixture (7:3, v:v). This extraction process was repeated in triplicate. After, the plant material was filtered. The solvent mixtures were combined, and the methanol was evaporated. Finally, the aqueous solution was then lyophilized to obtain an amorphous powder (1.5 g for the VOR and 2.1 g for the VOS).

## 2.3. Reagents

All aqueous solutions prepared for ICP-MS analysis were prepared using ultrapure water obtained from the Milli-Q Advanced A10 purification system (Millipore, USA). For plant samples, internal standards, and standard solutions, a mixture of 1% $HNO_3$ (Merck, USA) and 1% acetonitrile (Sigma Aldrich, Germany) was employed. To prevent contamination during sample preparation and analysis, all equipment, including tubes, glass bottles, and micropipette tips, were first cleaned with a 10% $HNO_3$ solution and then rinsed with deionized water. The Agilent® Trace Elements solution contained the following elements at a concentration of 100 mg $L^{-1}$: boron (B), sodium (Na), aluminum (Al), silicon (Si), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), selenium (Se), molybdenum (Mo), and cadmium (Cd). From this solution, a calibration curve for trace elements was prepared by diluting it to a concentration of 1000 µg $dL^{-1}$. The $HNO_3$ used was of ultrapure grade with a purity of 99.99%.

## 2.4. Sample Preparations for ICP-MS Analysis

To prevent contamination from atmospheric pollutants during sample preparation for ICP-MS analysis, the Milestone Connect ETHOS UP microwave system and the Direct-Q 8 UV Ultrapure Water system were employed. Microwave degradation was applied to eliminate organic compounds from plant samples and solubilize inorganic constituents. VOS and VOR samples, each weighing 200 mg, were combined with 1.8 mL of ultrapure water and homogenized using a vortex device. From these prepared mixtures, 0.5 mL aliquots were transferred to Teflon containers, to which 8 mL $HNO_3$ and 2 mL $H_2O_2$ solutions were added for microwave-assisted digestion. The ramp parameters applied in the microwave program are detailed 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.

Following the completion of the degradation process, the sample volume was diluted to 15 mL with ultra-pure water. The mixtures were then purified using 0.45 µm syringe filters. To ensure the reliability of the analytical procedure, the final samples were analyzed three times using an ICP-MS instrument, and the average values were reported. To verify the reliability of the

results, dilution factors were calculated using the formula: (final volume or weight / initial sample amount)* dilution factor.

## 2.5. ICP-MS Conditions

The elemental analysis was performed using an Agilent 7800 Quadrupole ICP-MS (Agilent Technologies, Japan), equipped with a rotary pump for operation. The sample introduction was employed by the Agilent ASX-500 Series ICP-MS Automatic Sample Injector (Agilent Technologies, Japan) in conjunction with the Integrated Sample Introduction System (ISIS 3). Instrument control and data processing were performed by the Mass Hunter 4.2 Workstation Software 7800 ICP-MS Top C.01.02. The analysis was conducted in quantitative ICP-MS mode, using a nickel sampler, a MicroMist glass concentric nebulizer, and a quartz Scott-type spray chamber. Calibration involved torch alignment, resolution calibration, standard lens adjustments, plasma optimization, spectral analysis, and performance verification. An Agilent calibration solution containing cerium, cobalt, lithium, magnesium, thallium, and yttrium at 1 $\mu g\ mL^{-1}$ was used. Trace element measurement was performed using helium collision mode with argon as the carrier gas. A 45-minute helium purification process was conducted before the measurement to ensure system accuracy. The automatic sampler tubes and probe were pretreated with 2% $HNO_3$ and 1% HCl solution. Instrument settings and parameters are presented in Table 2.

**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 $min^{-1}$ |
| continued | |
| 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 the analysis of the plant samples, the instrument was calibrated and validated to confirm the accuracy of the method. This process used Agilent-certified reference materials, following the manufacturer's guidelines and daily quality control checks. After, data analysis and the calculation of measurements were conducted using the Mass Hunter 4.2 Workstation Software 7800 ICP-MS Top C.01.02.

## 2.6. Chromatographic and Mass Spectroscopic Conditions for Amino Acids Analysis

The separation of amino acids was conducted using the LC-MS/MS technique, following the protocol detailed in previous studies [18]. The experiments were performed on an Agilent 6470 triple quadrupole LC-MS/MS system (Agilent Technologies, Santa Clara, CA, USA), which comprised a 1290 high-speed pump (G7120A), a 1290 autosampler (G7129A), and a 1260 multi-column thermostat (G7116A). Electrospray ionization (ESI) was employed as the ionization method. Quantification of amino acid concentrations in the VOS and VOR extracts was performed using the Jasem Quantitative Amino Acids LC-MS/MS Analysis Kit (Altium International Lab. Cih. A.Ş., Istanbul, Türkiye). This CE-IVD-certified and fully validated kit includes calibration curves from seven different standards, a mixture of 27 isotope-labeled amino acids serving as internal standards (IS), two sets of quality control (QC) samples, and a dilution reagent (Reagent-1).

The standards, quality control samples, and extract samples were prepared following the sample preparation protocol provided by the assay kit. For the calibration standards, 50 µL of the standard solution was added to a vial, followed by 50 µL of the internal standard (IS) and 700 µL of Reagent-1. The vial was vortexed for 5 seconds and later transferred to the autosampler for LC-MS/MS analysis. For the extract samples, 10 mg of the extract was dissolved in Jasem's mobile phase (A: 3% formic acid, 5% methanol, 30 mM ammonium formate; B: acetonitrile; 1:1, v/v). Following filtration, 50 µL of the extract solution was combined with 50 µL of IS and 700 µL of Reagent-1. The mixture was vortexed for 5 seconds and then centrifuged at 3600 g for 5 minutes at room temperature. The resulting supernatant was transferred to a vial for LC-MS/MS analysis.

The chromatographic separation of amino acids was performed using the analytical column, gradient elution program, and mobile phases in the kit protocol. A constant environment of 30°C was maintained for the analytical column, and 3 µL of standard, control, and sample solutions were injected into the HPLC system. Jasem's mobile phases A and B were employed at a flow rate of 0.7 mL $min^{-1}$, in accordance with the gradient elution program detailed in Table 3. The total analysis time is 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

Quantification of amino acids was performed using mass spectrometry in positive ion multiple reaction monitoring (MRM) mode. The mass spectrometry parameters used in the analytical method are as follows: capillary voltage 2000 V, drying gas temperature 150 °C, drying gas

flow rate 10 L $min^{-1}$, nebulizer pressure 40 psi, sheath gas temperature 400 °C, and sheath gas flow rate 10 L $min^{-1}$. MS/MS analyses were conducted using collision-induced dissociation (CID) where precursor ions were fragmented into product ions. For each ion transition, MRM conditions for amino acids were optimized by modifying the fragmentor voltage (FV) and collision energy (CE) values. The determination of internal standards (IS) and monitoring of transitions were performed during this optimization process. The summary of mass spectrometry parameters and precursor/product ion transitions for each analyte is presented in Table 4.

**Table 4.** MRM conditions of amino acids.

| 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 |
| continued | | | | | | |
| 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 |
| continued | | | | | | |
| *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.

Calibration curves were employed to quantify the analytes. During the LC-MS/MS analysis, data acquisition, characterization, and quantification were performed using Agilent MassHunter software (versions 10.1 and 10.0).

## 2.7. Antioxidant activity assays

### 2.7.1 DPPH free radical scavenging activity

The free radical scavenging activity of plant extracts was evaluated using the method determined by Akman et al. [19]. First, a 0.26 mM DPPH solution and stock solutions (1 mg $mL^{-1}$) were prepared. These stock solutions were diluted in methanol to different concentrations (20-1000 µg $mL^{-1}$). Each diluted extract solution was mixed with 1 mL of DPPH solution, vortexed, and incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm, and the % activity was calculated. Finally, $IC_{50}$ values (µg $mL^{-1}$) were

determined, and the antioxidant activities of the extracts were evaluated by comparison with Trolox, ascorbic acid, BHT, and BHA.

### 2.7.2 Ferric-reducing power activity (FRAP)

The reducing power activity of the samples was evaluated according to the method of Albayrak et al. [20]. Solutions and standard stock solutions (1 mg $mL^{-1}$) were prepared. For the assay, 100 µL of the sample was diluted with 1.25 mL of 0.2 M phosphate buffer (pH 6.6). Then, 1.25 mL of 1% $K_3Fe(CN)_6$ was added and the mixture was incubated at 50 °C for 20 minutes. After incubation, 1.25 mL of 10% TCA and 0.25 mL of 0.1% $FeCl_3$ were added. Absorbance was measured at 700 nm, and the results were expressed as mg TE $g^{-1}$ extract according to the Trolox calibration curve. The antioxidant activity of extracts was compared with ascorbic acid, BHT, and BHA.

### 2.7.3 Total phenolic content (TPC)

The total phenolic content of the extracts was determined using the Folin-Ciocalteu method, determined by Gözcü et al. [21]. A 100 µL sample solution was diluted with 4.5 mL of distilled water, then 100 µL of Folin-Ciocalteu reagent and 300 µL of a 2% $Na_2CO_3$ solution were added. The mixture was incubated for 2 hours, and absorbance was measured at 760 nm. The results were expressed as mg GAE $g^{-1}$ extract.

### 2.7.4 Total flavonoid content (TFC)

The total flavonoid content of the BC was determined using the method by Gözcü et al. [22]. Sample and standard stock solutions (100 µL) were diluted to 4.8 mL with methanol. Then, 100 µL of 1 M ammonium acetate ($NH_4CH_3COO$) and 100 µL of 10% aluminum chloride ($AlCl_3$) were added. The mixture was vortexed and incubated for 45 minutes at room temperature. Absorbance was measured at 415 nm, and results were expressed as mg QE $g^{-1}$ extract.

## 3. Results and Discussion

### 3.1. ICP-MS Analysis

This study determined the trace element concentrations in the VOS and VOR using a validated ICP-MS method [23]. Analysis showed varying levels of trace elements between the VOS and VOR (Table 5). Silicon (Si) was found to be the highest concentration trace element in the VOR at 1492.7 µg $g^{-1}$ dry weight (dw), while sodium (Na) was the most abundant in the VOS at 1854.3 µg $g^{-1}$ dw. There was also a significantly high concentration of aluminum (Al) in the VOR at 1471.1 µg $g^{-1}$ dw. Iron (Fe) was detected in significant concentrations, with 1445.5 µg $g^{-1}$ dw in the VOR and 174.3 µg $g^{-1}$ dw in the VOS. Additionally, boron (B), manganese (Mn), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), and selenium (Se) were present in both parts, but at lower concentrations. Molybdenum (Mo) was detected in low levels in both parts, while cadmium (Cd) was not detected in both parts.

**Table 5** Trace element concentrations in VOS and VOR (µg $g^{-1}$ dw)

| Parts | B | Na | Al | Si | Mn | Fe | Co | Ni | Cu | Zn | Se | Mo | Cd |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***VOS*** | 19.7 | 1854.3 | 232.1 | 330.6 | 10.1 | 174.3 | 0.034 | 7.4 | 7.0 | 213.6 | 0.2 | 0.3 | <0.000 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***VOR*** | 15.3 | 687.3 | 1471.1 | 1492.7 | 33.5 | 1445.5 | 3.3 | 16.5 | 11.3 | 66.1 | 0.1 | 0.9 | <0.000 |

The ICP-MS results indicated that VO contains high levels of sodium (Na), silicon (Si), and iron (Fe) in its VOS and VOR. The high concentration of Si (1492.7 µg $g^{-1}$ in the VOR) suggests that the plant may use silica-based structural support as an adaptive strategy against environmental stress. Similarly, the high levels of Fe in both the VOR (1445.5 µg $g^{-1}$) and VOS (174.3 µg $g^{-1}$) indicate that VO could serve as a natural dietary source of iron, which is essential for hemoglobin synthesis and overall human health. These findings correspond with previous studies on *Verbascum* species, which have reported high Fe levels as a common trait among members of this genus, particularly in species adapted to iron-rich soils [24, 25]. However, the presence of aluminum (Al) in the VOR (1471.1 µg $g^{-1}$) highlights potential neurotoxic effects, indicating the need for further toxicological evaluation before considering medicinal or dietary use [26, 27]. High Al concentrations have been previously reported in some *Verbascum* species, but the levels observed in this study are among the highest documented, highlighting the need for detailed safety evaluations [28, 29].

The presence and concentration of specific trace elements may significantly influence the antioxidant capacity of VO, either by directly participating in redox reactions or by serving as essential cofactors in antioxidant enzymatic systems. Elements such as Fe, Mn, Zn, Cu, and Se are known to participate in redox reactions and act as cofactors for antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [30, 31]. The elevated levels of iron (Fe) in both the VOR (1445.5 µg $g^{-1}$ dw) and VOS (174.3 µg $g^{-1}$ dw) may be associated with the antioxidant activity of the extracts, as supported by the stronger activity observed in the VOR. Although Fe can catalyze the formation of reactive oxygen species (ROS) via the Fenton reaction, it is also essential in cellular oxidative balance, depending on its bioavailability and chelation state [32, 33]. Similarly, manganese (Mn) and copper (Cu), albeit present at lower concentrations, are important constituents of antioxidative defense systems and may synergistically support free radical scavenging. Zinc (Zn), known for stabilizing cell membranes and preventing oxidative damage, was present at a higher concentration in VOS (213.6 µg $g^{-1}$) compared to VOR (66.1 µg $g^{-1}$), which may partially explain the higher flavonoid content in the stem extract, as Zn has been implicated in secondary metabolite synthesis [33, 34]. Although present at low concentrations, selenium (Se) plays a key role in redox homeostasis as an essential component of selenoproteins, and may support antioxidant defense mechanisms even in minimal quantities [35, 36]. The presence of aluminum (Al) at a high concentration in the VOR (1471.1 µg $g^{-1}$) may pose potential toxicological risks, highlighting the need for further safety evaluation. While Al is not essential for human metabolism and may interfere with cellular redox balance by inducing oxidative stress, its impact depends on the form, solubility, and bioaccumulation potential [37, 38]. Therefore, while V. orientale demonstrates a favorable mineral profile with potential antioxidant significance, additional research is required to determine the bioavailability and toxicological margins of these elements in the context of therapeutic or nutritional use.

### 3.2. LC-MS/MS Analysis for Amino Acids

A highly sensitive, precise, and valid LC-MS/MS analytical method was employed to evaluate the amino acid profiles in the VOS and VOR. A comprehensive quantitative analysis of 43 amino acids was performed. Figure 2 presents the MRM chromatograms resulting from the LC-MS/MS analysis of the VOS and VOR extracts.

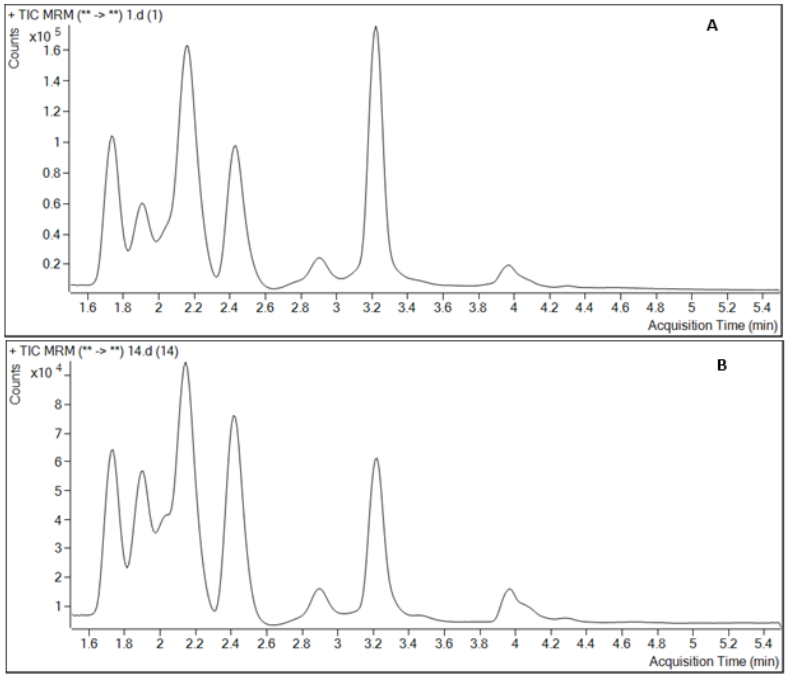


**Figure 2.** The MRM chromatograms of the A) VOS and B) VOR extracts

The amino acid concentrations in these extracts were measured in nmol $mL^{-1}$. Table 6 presents the linear regression equations, correlation coefficients ($R^2$), and limit of quantification (LOQ) values for the amino acids.

**Table 6.** Calibration curve equations, correlation coefficients, and LOQ values for amino acids in the LC-MS/MS method

| Amino Acids | Calibration curve equations | $R^2$ | LOQ (nmol $L^{-1}$) |
|---|---|---|---|
| Taurine | y= 0.001442x – 0.001064 | 0.9979 | 5.0 |
| L-tryptophan | y= 0.028557x - 0.053366 | 0.9990 | 1.0 |
| L-phenylalanine | y= 0.020012x + 0.067728 | 0.9996 | 1.0 |
| L-tyrosine | y= 0.013461x - 0.019332 | 0.9995 | 1.0 |
| L-leucine | y= 0.001315x - 7.336834E-004 | 0.9980 | 5.0 |
| L-isoleucine | y= 0.002582x - 0.007951 | 0.9976 | 1.0 |
| L-methionine | y= 0.021032x + 0.025020 | 0.9966 | 1.0 |
| 3-aminoisobutyric acid | y= 0.005320x + 0.006929 | 0.9979 | 1.0 |
| Gamma-aminobutyric acid | y = 0.039074x + 0.058776 | 0.9962 | 1.0 |
| 2-aminoadipic acid | y= 0.012476x - 0.005961 | 0.9924 | 0.5 |
| L-valine | y= 0.002968x + 0.014195 | 0.9989 | 5.0 |
| L-norvaline | y= 0.002458$x^2$ + 00.042305x + 0.014883 | 0.9975 | 0.1 |

| | | | |
|---|---|---|---|
| L-glutamic acid | y= 0.017219x - 0.038986 | 0.9994 | 5.0 |
| Ethanolamine | y= 0.386430x + 1.384151 | 0.9961 | 2.0 |
| 2-aminobutyric acid | y= 0.164021x - 0.304197 | 0.9969 | 2.5 |
| Beta-alanine | y= 0.006016x + 0.002325 | 0.9970 | 0.2 |
| L-aspartic acid | y = 0.020336x – 0.028037 | 0.9995 | 1.0 |
| L-threonine | y= 0.010757x + 0.078723 | 0.9953 | 2.5 |
| L-serine | y= 0.013133x + 0.214409 | 0.9962 | 5.0 |
| L-alanine | y= 0.002234x - 0.003392 | 0.9991 | 1.0 |
| Glycine | y= 0.001120x - 0.008773 | 0.9955 | 5.0 |
| Sarcosine | y= 0.050509x + 0.228475 | 0.9991 | 5.0 |
| L-asparagine | y= 0.012480x - 0.008593 | 0.9962 | 2.0 |
| Trans-4-hydroxy L-proline | y= 0.005091x - 0.003910 | 0.9993 | 1.0 |

continued

| | | | |
|---|---|---|---|
| L-proline | y= 0.005376x + 0.024883 | 0.9979 | 5.0 |
| L-glutamine | y= -1.686812E-006$x^2$ + 0.006569x + 0.054237 | 0.9994 | 5.0 |
| L-citrulline | y= 0.018775x + 0.039661 | 0.9986 | 0.5 |
| Homocitrulline | y= 0.004492x - 0.007928 | 0.9986 | 2.0 |
| *o*-phosphoserine | y= 5.074562E-004x - 7.213929E-006 | 0.9999 | 0.25 |
| *o*-phosphoryl ethanolamine | y= 9.059945E-004x - 1.944540E-004 | 0.9997 | 0.25 |
| Homocystine | y= 0.039396x + 0.012211 | 0.9998 | 0.25 |
| L-cystathionine | y= 0.101093x + 0.001578 | 0.9998 | 0.1 |
| Argininosuccinic acid | y= 0.013154x - 7.593827E-004 | 0.9992 | 1.0 |
| L-arginine | y= 0.012304x + 7.375877E-004 | 0.9994 | 2.0 |
| L-cystine | y= 0.010862x - 0.007446 | 0.9987 | 2.0 |
| 5-hydroxylysine | y= 0.003114x + 0.003675 | 0.9971 | 1.0 |
| L-histidine | y= 0.010063x + 0.072616 | 0.9984 | 5.0 |
| L-lysine | y= 0.025117x + 0.130604 | 0.9984 | 1.0 |
| L-ornithine | y= 0.013625x + 0.526848 | 0.9993 | 2.0 |
| L-carnosine | y= 0.025459x - 0.002913 | 0.9989 | 1.0 |
| 1-methylhistidine | y= 0.134550x + 0.030534 | 0.9963 | 1.0 |
| L-anserine | y= 0.020276x + 0.003041 | 0.9994 | 0.4 |

| | | | |
|---|---|---|---|
| 3-methylhistidine | y= 0.007255x + 0.009899 | 0.9970 | 1.0 |

*$R^2$: Correlation coefficient

In this study, a comprehensive analysis was performed to investigate the amino acid profiles of the VOS and VOR. The findings demonstrated significant variations in the amino acid concentrations between these two parts. Specifically, the data presented in Table 7 demonstrate that the VOS showed a higher concentration of amino acids in comparison to the VOR. This differential distribution of amino acids is indicative of the distinct physiological roles performed by the VOS and VOR.

**Table 7.** Amino acid levels in the VOS and VOR extracts (nmol $mL^{-1}$)

| Amino Acids | VOS | VOR | Amino Acids | VOS | VOR |
|---|---|---|---|---|---|
| Taurine | 0.8411 | 0.7903 | L-asparagine | 273.6796 | 43.4524 |
| L-tryptophan | 35.1069 | 5.1213 | Trans-4-hydroxy L-proline | 1.2753 | 1.0179 |
| continued | | | | | |
| L-phenylalanine | 10.9487 | 0.3949 | L-proline | 486.6640 | 20.4339 |
| L-tyrosine | 22.0438 | 6.5473 | L-glutamine | 450.9776 | 166.8947 |
| L-leucine | 42.1122 | 17.2956 | L-citrulline | 0.6039 | 0.0000 |
| L-isoleucine | 41.9483 | 0.0000 | Homocitrulline | 1.7904 | 1.8314 |
| L-methionine | 0.2457 | 24.2901 | *o*-phosphoserine | 0.0355 | 0.0577 |
| 3-aminoisobutyric acid | 185.9262 | 86.1140 | *o*-phosphoryl ethanolamine | 0.2907 | 0.3217 |
| Gamma-aminobutyric acid | 154.3363 | 70.5706 | Homocystine | 0.0000 | 0.0000 |
| 2-aminoadipic acid | 7.8850 | 7.7302 | L-cystathionine | 0.2574 | 0.0040 |
| L-valine | 117.0627 | 48.3234 | Argininosuccinic acid | 1.5614 | 0.2526 |
| L-norvaline | 5.6832 | 2.5113 | L-arginine | 20.3570 | 6.2985 |
| L-glutamic acid | 83.2595 | 108.9549 | L-cystine | 0.9450 | 1.3747 |
| Ethanolamine | 80.8343 | 47.5464 | 5-hydroxylysine | 0.0000 | 0.0000 |
| 2-aminobutyric acid | 2.6014 | 4.4729 | L-histidine | 18.0353 | 0.0000 |
| Beta-alanine | 2.6786 | 1.1306 | L-lysine | 6.8172 | 4.4661 |
| L-aspartic acid | 16.9282 | 53.9895 | L-ornithine | 0.0000 | 0.0000 |
| L-threonine | 72.0305 | 16.9403 | L-carnosine | 0.1737 | 0.1949 |
| L-serine | 126.3642 | 45.4642 | 1-methylhistidine | 0.0000 | 0.0000 |
| L-alanine | 253.7771 | 101.6304 | L-anserine | 0.0000 | 0.0993 |
| Glycine | 26.9997 | 17.7640 | 3-methylhistidine | 0.0000 | 0.0000 |

| | | |
|---|---|---|
| Sarcosine | 8.9103 | 0.0000 |

As a result of the analysis, a total of 36 amino acids were identified in the VOS part of VO, while 34 amino acids were detected in the VOR part. In the VOS part, the amino acids with the highest concentrations were L-proline (486.66 nmol $mL^{-1}$), L-glutamine (450.98 nmol $mL^{-1}$), and L-asparagine (273.68 nmol $mL^{-1}$), respectively. The typical and mass chromatograms of the VOS and VOR are presented in Figures 3 and 4. In the VOR part, the highest concentrations were observed for L-glutamine (166.89 nmol $mL^{-1}$), L-glutamic acid (108.95 nmol $mL^{-1}$), and L-alanine (101.63 nmol $mL^{-1}$), respectively. Despite the detection of L-anserine in trace concentrations (0.0993 nmol $mL^{-1}$) in the VOR, it was not detected in the VOS. In contrast, L-isoleucine, sarcosine, L-citrulline, and L-histidine were found in the VOS but not detected in the VOR (0.6039-41.9483nmol $mL^{-1}$). Amino acids such as L-tryptophan, L-phenylalanine, L-tyrosine, L-leucine, L-threonine, L-serine, L-valine, ethanolamine, gamma-aminobutyric acid, 3-aminoisobutyric acid, and L-arginine were detected in lower concentrations in the VOR but were found at significantly higher concentrations in the VOS (5.1213–185.9262 nmol $mL^{-1}$). However, L-ornithine, which has been previously reported as a highly concentrated amino acid in the roots of some plants, was not detected in both parts. Additionally, amino acids such as Taurine, 2-aminoadipic acid, 2-aminobutyric acid, L-norvaline, Beta-alanine, Trans-4-hydroxy L-proline, Homocitrulline, o-phosphoserine, o-phosphoryl ethanolamine, L-cystathionine, Argininosuccinic acid, L-cystine, L-lysine, and L-carnosine were detected in low concentrations (0.0040–7.8850 nmol $mL^{-1}$) in both the VOR and the VOS. Furthermore, Homocystine, 5-hydroxylysine, L-ornithine, 1-methylhistidine, and 3-methylhistidine were not detected in both the VOS and VOR of the plant.

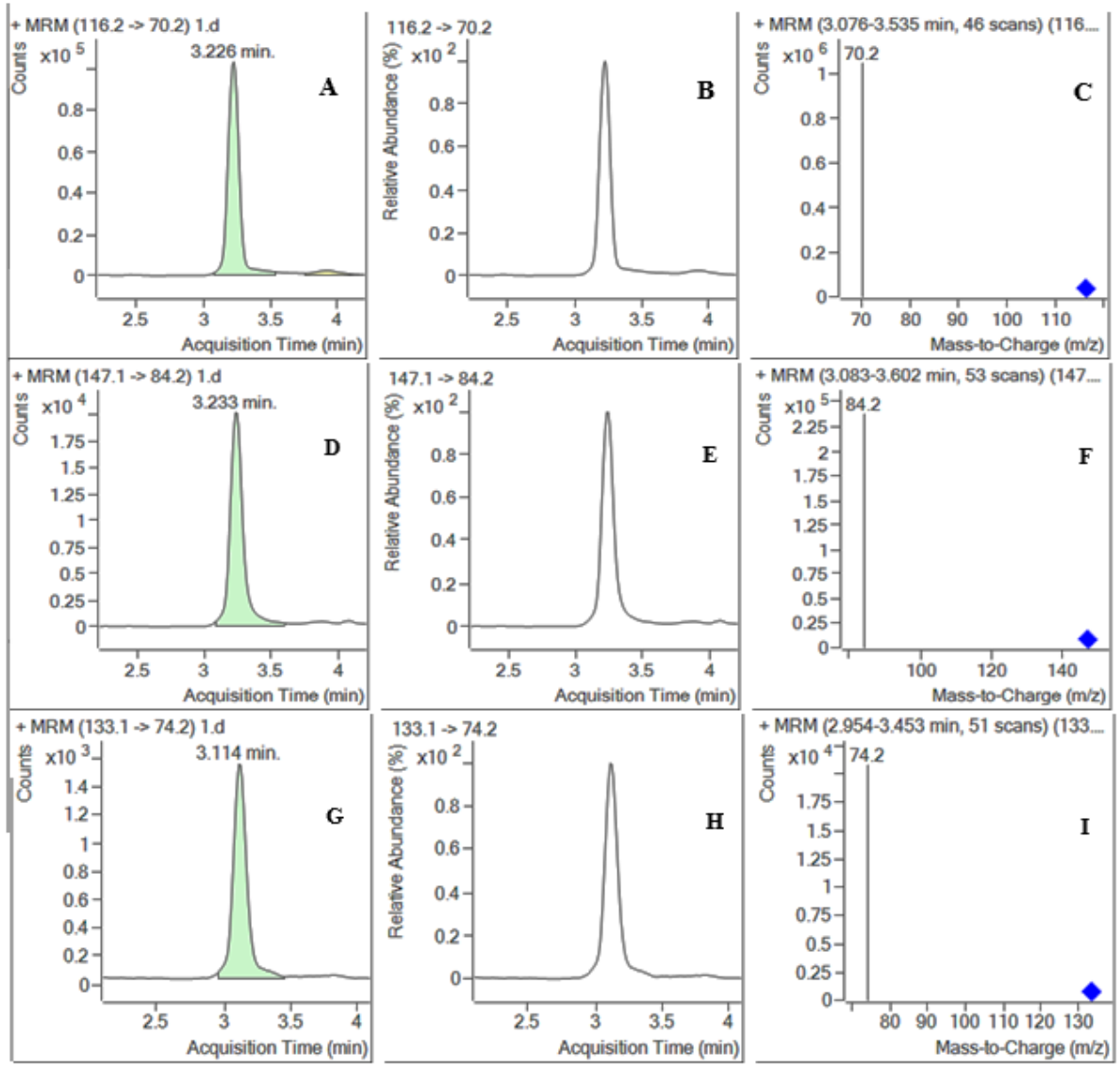


**Figure 3.** The typical chromatograms of the VOS showed the peak area and concentration, respectively, of A, B) L-proline; D, E) L-glutamine; and G, H) L-asparagine. The mass spectrums of C) L-proline; F) L-glutamine; and I) L-asparagine.

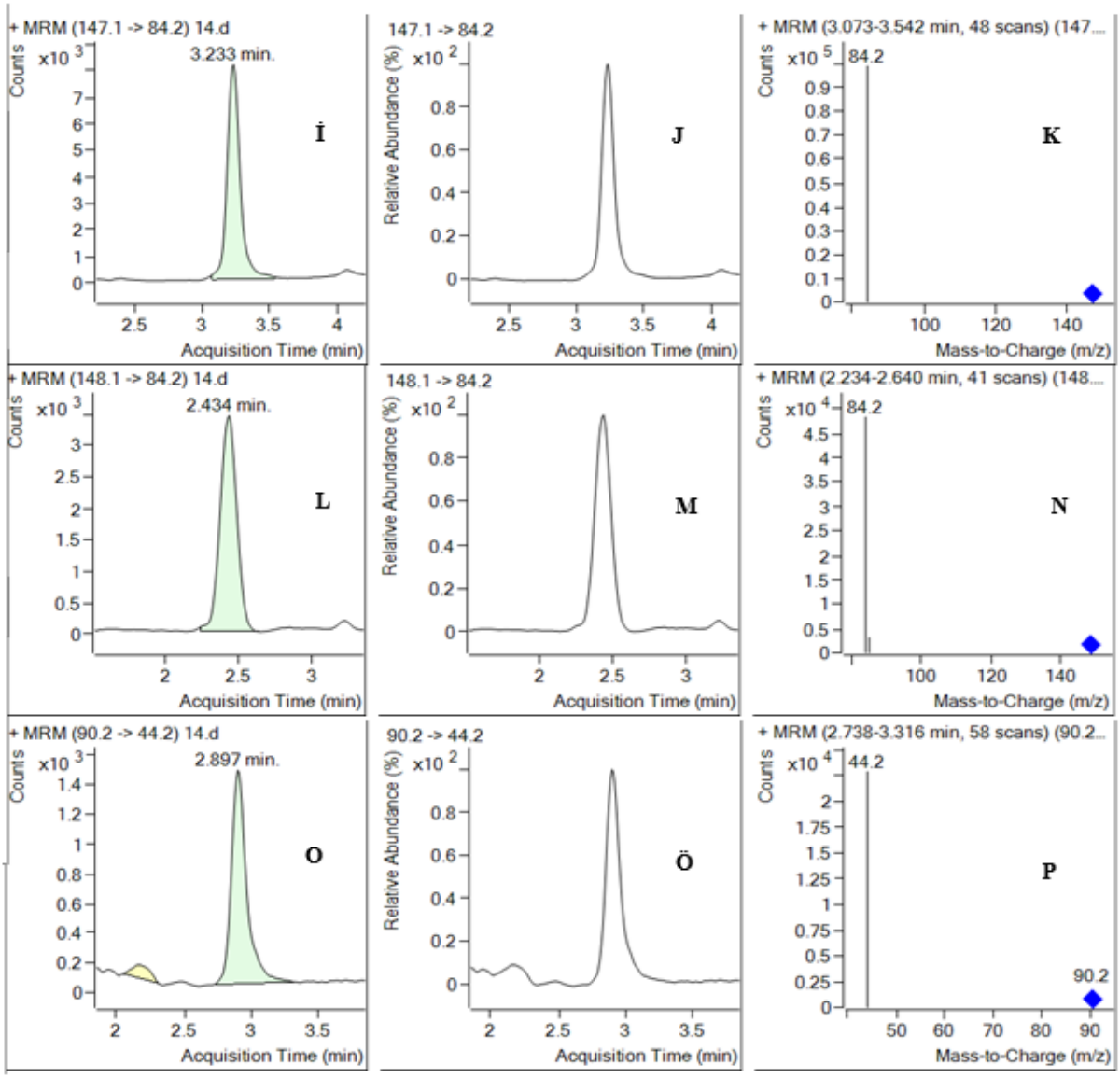


**Figure 4.** The typical chromatograms of the VOR showed the peak area and concentration, respectively of İ, J) L- glutamine, L, M) L-glutamic acid, and O, Ö) L-alanine. The mass spectrums of K) L- glutamine, N) L-glutamic acid, and P) L-alanine.

The LC-MS/MS analysis indicated that VO is abundant in various amino acids, notably L-proline, L-glutamine, and L-asparagine, with significantly higher concentrations found in the VOS compared to the VOR. Specifically, L-proline (486.66 nmol $mL^{-1}$) and L-glutamine (450.98 nmol $mL^{-1}$) are recognized for their roles in osmotic regulation and stress response [39-41], suggesting that VO uses these amino acids as a protective mechanism against abiotic stressors. Similar findings have been observed in other stress-resistant medicinal plants, wherein elevated proline levels were associated with drought tolerance and enhanced metabolic adaptation [42-44]. The presence of L-glutamic acid, an amino acid essential for neurotransmitter function and nitrogen metabolism, further underscores the plant's adaptive significance [45, 46]. Compared to other species of *Verbascum*, VO demonstrates a distinct amino acid profile, with relatively higher concentrations of stress-related amino acids, possibly attributed to its specific growth conditions in Erzincan's semi-arid environment [47, 48].

### 3.3. Antioxidant Activity Assay

The antioxidant potential of VOS and VOR extracts was determined by their DPPH• radical scavenging activity, TPC, TFC, and FRAP, with the results presented in Table 8. The DPPH• radical scavenging activity of the extracts was quantitatively determined and expressed as $IC_{50}$ values (μg $mL^{-1}$). The VOR extract exhibited the highest radical scavenging activity with an $IC_{50}$ value of 24.42±0.38 μg $mL^{-1}$, followed by the VOS extract at 78.51±1.54 μg $mL^{-1}$. In comparison to synthetic antioxidants BHA (8.04±0.69 μg $mL^{-1}$), BHT (10.70±0.73 μg $mL^{-1}$), and ascorbic acid (9.91±0.87 μg $mL^{-1}$), the extracts demonstrated moderate antioxidant potential, with the VOR extract showing particularly significant activity. In addition, the TPC was found to be the highest in the VOR extract (86.91±1.40 mg GAE $g^{-1}$ extract), while the VOS extract showed a lower value (42.07±0.80 mg GAE $g^{-1}$ extract). A similar trend was observed in the TFC, with the VOS extract showing the highest flavonoid concentration (15.71±0.61 mg QE $g^{-1}$ extract), while the VOR extract had a lower flavonoid content (10.72±0.95 mg QE $g^{-1}$ extract). The reducing power of the extracts was evaluated by their Trolox equivalent (TE) values. The highest reducing power was observed in the VOR extract, with a value of 102.53±1.94 mg TE $g^{-1}$ extract, followed by the VOS extract at 29.85±2.97 mg TE $g^{-1}$ extract. Synthetic antioxidants ascorbic acid (394.17±0.98 mg TE $g^{-1}$ extract), BHA (338.57±0.31 mg TE $g^{-1}$ extract), and BHT (257.80±1.24 mg TE $g^{-1}$ extract) showed significantly higher reducing capacities.

**Table 8.** Antioxidant activity, total phenolic and flavonoid contents in the VOS and VOR extracts

| 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 $Extract^{-1}$) |
|---|---|---|---|---|
| *VOS* | 78.51±1.54 | 42.07±0.80 | 15.71±0.61 | 29.85±2.97 |
| *VOR* | 24.42±0.38 | 86.91±1.40 | 10.72±0.95 | 102.53±1.94 |
| 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 |

Despite the content of bioactive amino acids and trace elements, the antioxidant activity of VO was found to be comparatively low compared to other *Verbascum* species. The DPPH radical scavenging assay indicated an $IC_{50}$ value of 24.42 μg $mL^{-1}$ for the VOR extract, while the VOS extract exhibited a significantly lower antioxidant capacity with an $IC_{50}$ value of 78.51 μg $mL^{-1}$. These findings are consistent with previous studies that have reported variable antioxidant activity among different *Verbascum* species, with some demonstrating strong radical scavenging potential due to higher polyphenolic content [49, 50]. The total phenolic and flavonoid content data further support this observation, showing that the VOR extract contains a higher phenolic content (86.91 mg GAE $g^{-1}$ extract) compared to the VOS (42.07 mg GAE $g^{-1}$ extract), while the VOS exhibited a relatively higher flavonoid content (15.71 mg QE $g^{-1}$ extract) than the VOR (10.72 mg QE $g^{-1}$ extract). In comparison to the *Verbascum* genus, which has been reported to contain higher flavonoid and phenolic concentrations and show stronger antioxidant activity [4, 47, 51, 52], VO has a moderate antioxidant profile. This suggests that its therapeutic potential may be more attributable to its trace element and amino acid composition rather than its antioxidant capacity.

## 5. Conclusion

This study offers a comprehensive analysis of the antioxidant activity, trace element composition, and amino acid profile of VO, a medicinal plant traditionally used. The findings indicate that VO shows different trace element distribution patterns, with high concentrations of silicon and sodium, which suggests its adaptation to dry environments. However, the increased levels of aluminum present potential neurotoxic effects, highlighting the necessity for controlled use. Amino acid analysis indicates that VO synthesizes significant amounts of L-proline, L-glutamine, and L-asparagine, compounds known for their roles in stress adaptation and metabolic processes. These findings demonstrate the plant's biochemical strategies for survival and its potential nutritional contributions. Despite the presence of bioactive elements and amino acids, the antioxidant activity of VO was found to be moderate compared to other *Verbascum* species. While the VOR extract demonstrated comparatively higher antioxidant capacity, both extracts did not reach the potency of synthetic antioxidants. In conclusion, these findings improve our comprehension of VO's phytochemical profile, highlighting its ethnobotanical significance and at the same time its potential limitations. Future research should focus on a comprehensive analysis of its secondary metabolite profile and in vivo biological activities to fully understand its pharmacological potential and confirm its safety.

## Ethics in Publishing

There are no ethical issues regarding the publication of this study.

## Acknowledgements

The authors extend their special thanks to Prof. Dr. Ali KANDEMİR for his invaluable assistance in the taxonomic identification and collection of plant specimens.

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