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Research Article

Investigation of amino acids content in the herb and tubers of *Stachys sieboldii*

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Abstract

The aim of this research was the comparative study of the content of the amino acids in the herb and tubers of Stachys sieboldii. The study of the amino acid composition of the raw materials was carried out using high-performance liquid chromatography (HPLC). The results obtained have shown that the aerial parts of plants investigated have higher amino acid content than the underground organs. Free and bound L-aspartic acid, L-proline, and L-phenylalanine were present in the analyzed samples in the greatest amount. Moreover, L-cysteine was found only in Stachys sieboldii tubers in amounts (8.11 mg/g). This research established that Stachys sieboldii herb and tubers have the most suitable amino acids composition and are prospective for further pharmacological studies.

Keywords

Stachys sieboldii, herb, tubers, amino acids, HPLC

Introduction

The world pharmaceutical industry uses herbal raw materials as a basis for the creation of drugs (Feshchenko et al. 2021a; Marchyshyn et al. 2021a; Savych et al. 2021a, d). Application of herbal remedies have a number of advantages over synthetic agents, namely, they are low-toxic, and can be used for long periods without substantive side effects, are well combined with synthetic drugs (Huzio et al. 2020; Savych et al. 2020; Budniak et al. 2021a; Savych and Mazur 2021). Medicinal plants play a basic role in the development of traditional medicine, as well as actual pharmaceuticals (Marchyshyn et al. 2021c). Given the ever-growing needs of the industry in herbal raw materials for the manufacture of drugs, an important task of pharmaceutical science is to expand existing and search for new sources of the plants (Budniak et al. 2021b, c; Savych and Sinichenko 2021).

The genus *Stachys* L., a numerous member of *Lamiaceae* family, includes about 300 species, dispersing tropical and temperate regions of Asia, the Mediterranean, southern Africa, and America (Khanavi et al. 2009; Tundis et al. 2014; Salmaki et al. 2019). The plants of *Stachys* species is annual or perennial herbs or small shrubs (Goren et al. 2012; Tomou et al. 2020). Studies in the *Stachys* species showed the presence of polyphenols including tannins (Vundac et al. 2007), flavonoids (El-Ansari et al. 1995), and phenolic acids (Vundac et al. 2005). Many researches detected various activities in this genus such as antibacterial (Grujic-Jovanovic et al. 2004; Sonboli et al. 2005; Slobodianiuk et al. 2021d), anti-inflammatory (Khanavi et al. 2005; Sharifzadeh et al. 2005; Kukik et al. 2007), and antioxidant effects (Aydin et al. 2006; Matkowski and Piotrowska 2006).

Most species of this genus were previously analyzed in numerous studies concerning their pharmacological properties, therapeutic uses, and chemical composition. Nevertheless, the literature data on *Stachys sieboldii* activity are scarce, and little is known about chemical components with this plant.

Stachys sieboldii Miq. is widely distributed in Asia, North America, and Europe, and has been used for the treatment of different gastrointestinal problems, ischemic stroke, and senile dementia (Yamahara et al. 1990; Baek et al. 2004). Today, there have been no reported harmful effects associated with *S. sieboldii*. The plant contains different oligosaccharides, which could stimulate the growth of beneficial microorganisms in the human intestine and is considered a wellness-promoting food (Yin et al. 2006). *Stachys sieboldii* contains some active compounds including flavonoids (Lee et al. 2014), terpenes (Goren et al. 2011; Cho et al. 2014b), and phenolic compounds (Na et al. 2017), which are directly associated with its antimicrobial (Ryu et al. 2002; Slobodianiuk et al. 2021a), and antioxidant (Baek et al. 2003; Jeon and Park 2015; Lee et al. 2018) properties. Being a great source of oligosaccharides, proteins, and water-soluble vitamins, *S.sieboldii* tubers have been used to treat the common cold, urinary tract infections, heart disease, and tuberculosis (Feng et al. 2015; Harada et al. 2015).

Hyeon Kyung Cho et al. (2014) isolated two new triterpene saponins named sieboldii saponin B and C from the methanol extract of tubers of *Stachys sieboldii* Miq. It should be noted that the *S. sieboldii* contain a rare tetrasaccharide – stachyose, which is similar in composition and properties to inulin and has an insulin-like effect (Yin et al. 2006; Xianfeng et al. 2014; Slobodianiuk et al. 2021b).

However, few studies have focused on *Stachys sieboldii* primary metabolites and their relationship with its therapeutic properties. Amino acids are building blocks of proteins. Proteins are a fundamental part of all living animals and take part in virtually every process within the cell. Identification of amino acid sequence in a protein is of utmost importance in synthesizing new drugs for the treatment of diseases such as diabetes, cancer, and many more related to genetic disorders (Idres et al. 2014). Therefore, taking into account the possible role of amino acids as pharmacological activity, we decided to perform a comparative investigation into the content of these constituents in *Stachys sieboldii* herb and tubers.

Materials and methods

Plant materials

Herb and tubers of the *Stachys sieboldii* were collected on research grounds of Educational and Scientific Centre "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv in November 2017. The raw materials were then dried, crushed and stored according to the general GACP requirements (WHO 2003; Savych and Marchyshyn 2021a, b; Savych et al. 2021c, e). A voucher specimen was deposited in the laboratory herbarium of the Department of Pharmacognosy and Medical Botany (TNMU, Ternopil, Ukraine) (Marchyshyn et al. 2020; Marchyshyn et al. 2021b; Savych et al. 2021b; Slobodianiuk et al. 2022).

Standards and chemicals

Standards of amino acids were of analytical grade (> 99% purity). The chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and were: L-histidine, L-arginine, L-aspartic acid, L-proline, L-lysine, L-alanine, L-valine, L-isoleucine, L-tyrosine, L-glutamic acid, L-cystine, L-serine, L-methionine, L-leucine, L-threonine, L-phenylalanine, Glycine (Savych and Nakonechna 2021; Slobodianiuk et al. 2021c). Derivatizing agents' o-phthaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) were purchased in Merck. Acetonitrile (ACN), hydrochloric acid (HCl), methanol (CH_3OH), disodium hydrogen phosphate (Na_2HPO_4) and sodium hydroxide (NaOH) were of analytical grade (> 97% purity). Water used in the studies was produced by the MilliQ Gradient water deionization system (Millipore, USA).

Instrumentation and conditions of HPLC determination of amino acids

The amino acids composition in the *Stachys sieboldii* herb and tubers are determined by HPLC method with a pre-column derivatization FMOC and OPA (Budniak et al. 2021d).

HPLC analysis of amino acids was conducted using Agilent 1200 (Agilent Technologies, USA). Samples were analyzed using a column length Zorbax AAA – 150 mm, inner diameter – 4.6 mm, the diameter of sorbent grain 3 μ (Hypersil ODS (prepared by BST, Budapest, Hungary)). Mobile phase A – 40 mM Na_2HPO_4, pH 7.8; mobile phase B – CH_3CN:CH_3OH:H_2O (45:45:10, v/v/v). Gradient separation regime with a constant mobile flow rate of 1.5 mL/min. The temperature of the thermostat column is 40 °C (Table 1).

Table 1. Gradient elution scheme.

Chromatography time, min	Mobile phase A, %	Mobile phase B, %
0:00	100	0
2:00	100	0
18:00	43	57
19:00	0	100
23:00	0	100
26:00	100	0

Extraction of amino acids, pre-column derivatization

Reference solutions of free amino acids have been made with distilled water at 0.03 M concentrations of each (weighed with analytical accuracy), stored in the refrigerator and further diluted before use, in every second day.

The pre-column derivatization was conducted with a help of an automatic programmable regulations using OPA reagent and FMOC reagent. Identification of derivatized amino acids was done by a fluorescence detector (Jambor and Molnar-Perl 2009). For the extraction of free amino acids of powdered the raw material (to the 133 mg of *Stachys sieboldii* herb; to the 131 mg of *Stachys sieboldii* tubers), put in a test flask, 0.1 mol/l water solution of hydrochloric acid was added. The extraction was performed in the ultrasonic water bath at 50 °C for 3 hours. Extraction of bound and free amino acids was performed by adding 2 ml of a water solution of 6 M hydrochloric acid to the powdered of the raw material (to the 134 mg of *Stachys sieboldii* herb; to the 133 mg of *Stachys sieboldii* tubers). Hydrolysis was conducted for 24 hours in a thermostat at 110 °C.

0.5 ml of centrifuged extract was vaporized on a rotary evaporator and then rinse three times with purified water to eliminate hydrochloric acid. The product received was resuspended in 0.5 ml water and filtered through membrane filters from restored cellulose with pores of 0.2 μm. Before recording the samples into the chromatographic column in the automatic software mode, fluorescence derivative amino acids were obtained.

Identification and calculation amino acids by HPLC

Identification of amino acids was performed according to their hold-up time (using standards as a reference) at 265 nm. The quantitative content of amino acids is calculated from the value of the of the peak area of the amino acids. The content of bound amino acids was determined by subtracting the content of free amino acids from their total content (Feshchenko et al. 2021b).

Validation of the method

The analytical procedure has been validated to confirm its reliability. The validation method and the analysis procedure of the amino acid content were performed according to validation guides for EURACHEM analytical methods.

To evaluate the sensitivity and linearity of the signal in relation to the concentration, 8 linear calibrations were generated for each amino acid. The calibration curves of each amino acid were plotted in the 0.015–0.625 μmol/ml range, and the linearity range for which the correlation coefficient that characterizes the regression line R^2 was obtained, was examined visually.

The method was validated for linearity, limit of detection (LOD), limit of quantitation (LOQ) and precision. The performance parameters of the reference amino acid method, concentrations, limit of detection (LOD), limit of quantification (LOQ) and calibration curves were statistically calculated using Statistica v 10.0 (StatSoft I nc.) program and are shown in Table 2. All statistical tests were performed at a confidence level of 95% and k = 2 (Feshchenko et al. 2021b). Linearity testing was repeated with the same samples after a complete restart of the system with removement and re-installation of the column. Repeatability precision was determined by eight-fold injection of the same sample in a row. For the resulting relative peak area of the quantifier ions the relative standard deviation (RSD) was calculated. To determine intra-day precision, eight injections of amino acids reaction mixtures with the same concentration were single injected and the resulting relative peak areas were used to calculate the RSD. Inter-day precision for the day of sample preparation and the two following days was specified by injecting eight standard sample of amino acids reaction mixtures once each on all three days. The RSD of the samples on that day together with the previous samples were calculated as above.

Table 2. Performance parameters of the amino acid determination method.

Amino acid	Correlation coefficient R^2	Limit of detection LOD, μmol/ml	Limit of quantification LOQ, μmol/ml	Retention time (SD±0.01)
L-aspartic acid	0.9999	0.005437	0.01779	2.46
L-glutamic acid	0.9997	0.001589	0.005342	4.78
L-serine	0.9999	0.004365	0.014549	7.35
L-histidine	0.9989	0.001235	0.005238	8.19
Glycine	0.9994	0.002345	0.004895	8.58
L-threonine	0.9996	0.01817	0.060565	8.75
L-arginine	0.9998	0.010724	0.035745	9.41
L-alanine	0.9987	0.003456	0.013567	9.97
L-tyrosine	0.9996	0.004678	0.014356	11.06
L-cystine	0.9995	0.001592	0.005308	12.19
L-valine	0.9999	0.002622	0.00874	12.96
L-methionine	0.9996	0.01785	0.06543	13.15
L-phenylalanine	0.9995	0.004532	0.01356	14.33
L-isoleucine	0.9999	0.01235	0.05426	14.51
L-leucine	0.9989	0.002897	0.018652	15.12
L-lysine	0.9999	0.096521	0.321737	15.39
L-proline	0.9998	0.003978	0.013261	18.91

Results and discussion

Appropriate to the polar nature of amino acids, derivatization is required prior to HPLC analysis (Jarukas et al. 2018). Higher sensitivity can be reached during the analysis of amino acids for the HPLC method by using derivatization agents, such as FMOC (9-fluorenylmethyl chloroformate) and OPA (o-phthalaldehyde).

The amino acid profiles of the herb and tubers of *Stachys sieboldii* were evaluated using the HPLC method (Figs 1–4, Table 3). The HPLC method established the presence of 17 amino acids in *Stachys sieboldii* herb and tubers. According to a comparative analysis of the composition of amino acids in examined *Stachys sieboldii* plants parts, it can be seen that the aboveground organs have a higher content of amino acids than their underground organs (Table 3).

The results show that the herb of *Stachys sieboldii* has the highest concentration of certain free amino acids, which possibly explains the presence of a pronounced

antioxidant effect in the herb (Jeon and Park 2015). The predominant amino acids in *Stachys sieboldii* herb were L-phenylalanine (0.73 mg/g), L-proline (0.64 mg/g), and L-glutamic acid (0.63 mg/g) (Fig. 1). Phenylalanine, an amino acid, is a "building block" of protein. In current treatment, phenylalanine is prescribed as an anti-depressant agent (Akram et al. 2020). L-Proline is a proteinogenic amino acid with the α-amino group present as an auxiliary amine which is essential for primary metabolism (Meena et al. 2019). Proline contributes to stabilizing sub-cellular structures, scavenging free radicals, and buffering cellular redox potential under stress conditions (Serraj and Sinclair 2002; Hayat et al. 2012). Glutamic acid is used as fuel in the metabolic reaction in the body and the synthesis of all proteins for muscle and other cell components, and it is essential for proper immune function (Mamuad and Lee 2021).

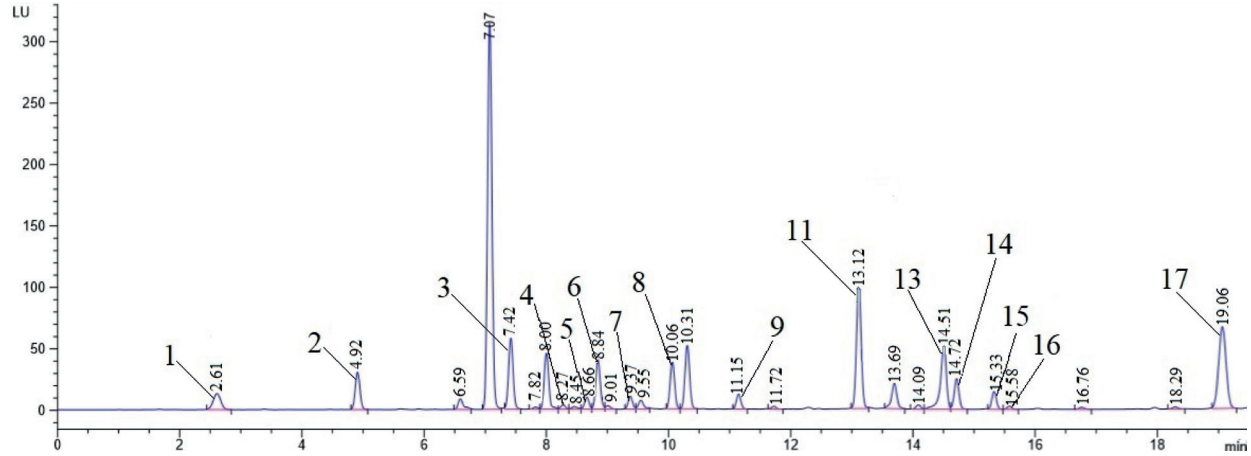

Figure 1. HPLC chromatogram of free amino acids of *Stahys sieboldii* herb.

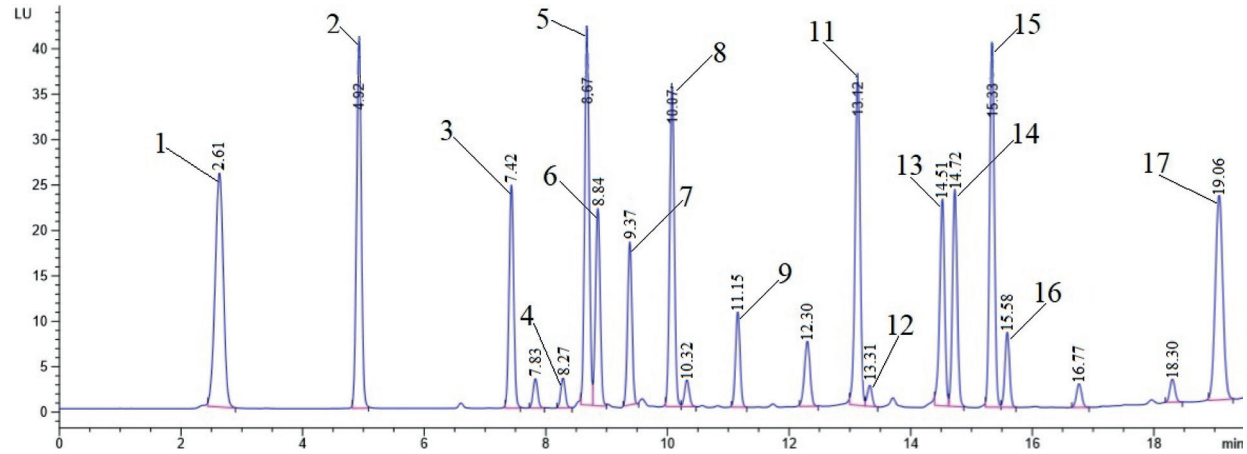

Figure 2. HPLC chromatogram of amino acids after hydrolysis of *Stahys sieboldii* herb.

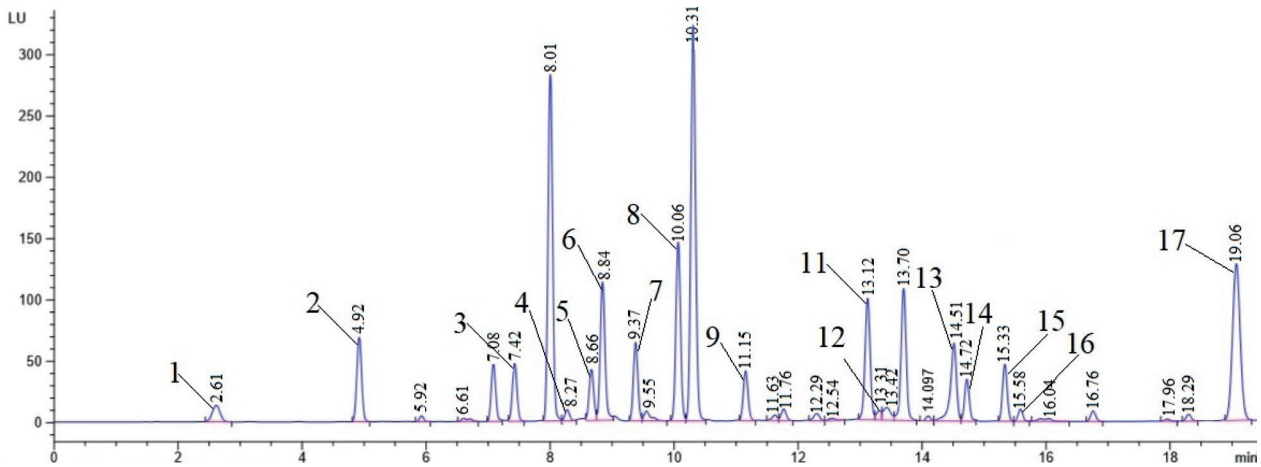

Figure 3. HPLC chromatogram of free amino acids of *Stahys sieboldii* tubers.

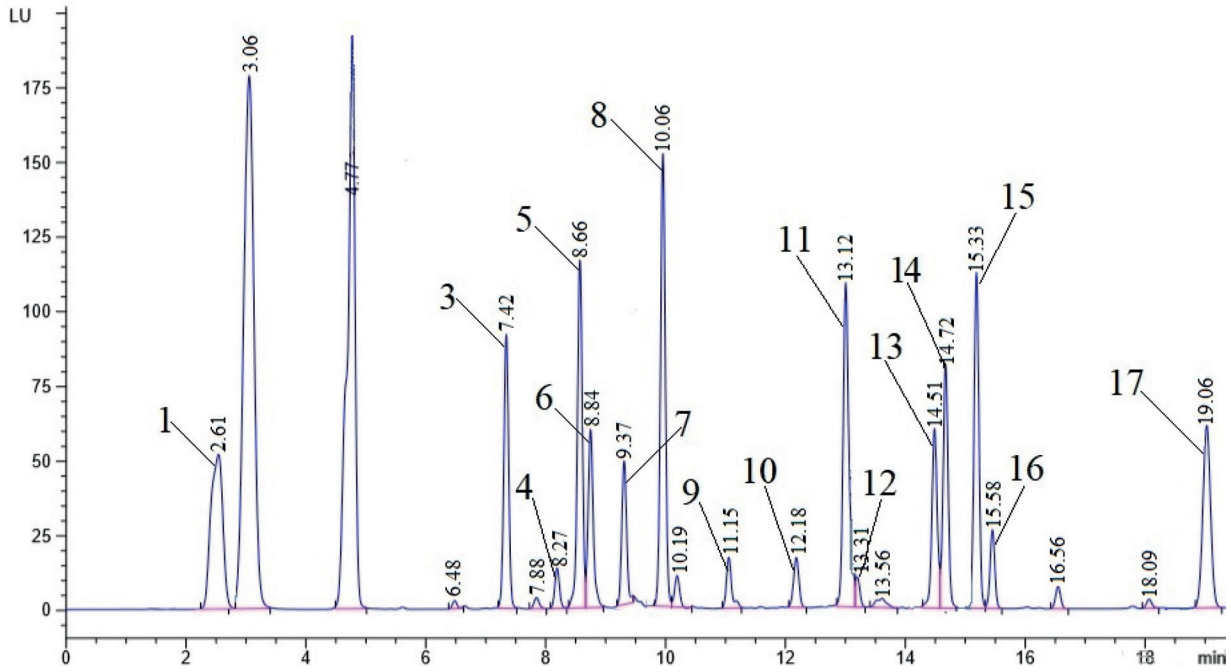

Figure 4. HPLC chromatogram of amino acids after hydrolysis of *Stahys sieboldii* tubers.

Experimental data showed that the herb of *S. sieboldii* is characterized by the highest content among bound amino acids, L-aspartic acid (16.22 mg/g), L-serine (14.39 mg/g), and L-lysine (15.31 mg/g) (Fig. 2). L-Aspartic acid is a four-carbon amino acid that is used not only as a stand-alone food additive but also as a raw material for the pharmaceutical and food industries (Choi et al. 2015). Aspartic acid is used to bolster immune function and as a natural combatant to depression. Its capacity to aid in energy production, DNA and RNA synthesis, fatigue resistance, and liver detoxification give it broad clinical use (Kumar et al. 2017). Moreover, it is used as an intermediary substrate in the manufacture of pharmaceuticals, serving as the building block molecule for active pharmaceutical ingredients (Appleton and Rosentrater 2021). L-serine has a diversity of functions and roles from primary protein structure to cell signaling, the latter primarily via post-translational modification by phosphorylation. Deficiencies in L-serine may have profound health effects from embryo to geriatrics (Metcalf et al. 2018). Dietary supplementation with L-serine is now being clinically researched as a possible therapy for progressive neurodegenerative diseases including Alzheimer's disease and ALS (Levine et al. 2017). L-Lysine is an integrant building block for all proteins in the human body. Its plays an important role in calcium absorption, building muscle protein, recovering from sports injuries or surgery, and the body's production of enzymes, antibodies, and hormones (Singh et al. 2011).

The presented results prove that L-proline (1.16 mg/g), L-threonine (0.96 mg/g), L-alanine (0.87), and L-phenylalanine (0.84) were predominates among free amino acids in *Stachys sieboldii* tubers (Fig. 3). L-threonine is an essential branched-chain amino acid and has been widely used in the food and pharmaceutical industries (Wang et al. 2019). Alanine is incorporated into pantothenate (Vitamin B5), and therefore, is a predecessor of Coenzyme A (CoA) and acyl-carrier protein, which shuttle carbon within the cell (Voet et al. 2006). It's a component of carnosine, a dipeptide concentrated in brain and muscle tissue, which repose the wide use of alanine in humans as a strength-enhancing supplement (Parthasarathy et al. 2019).

Among the bound amino acids, the content of L-aspartic acid (9.15 mg/g) and L-arginine (8.44 mg/g) were the highest (Fig. 4). L-Arginine is a semi-essential amino acid that is the substrate for the enzyme nitric oxide synthase (NOS), which is responsible for the production of nitric oxide. The use of L-arginine provides significant positive benefits for reducing systolic and diastolic blood pressure in hypertensive adults, reducing diastolic blood pressure in pregnant women with gestational hypertension (McRae 2016). The number

Table 3. The amino acid composition content of *Stachys sieboldii*.

Number of peak on chromatogram	Amino acid Name	Amino acids content of *Stachys sieboldii*, mg/g			
		Herb		Tubers	
		Free	Bound	Free	Bound
1	L-aspartic acid	0.24±0.02	16.22±0.06	0.23±0.02	9.15±0.04
2	L-serine	0.33±0.02	14.39±0.05	0.48±0.02	n/d
3	L-glutamic acid	0.63±0.01	8.11±0.04	0.71±0.01	2.91±0.02
4	L-histidine	0.13±0.01	3.53±0.03	0.29±0.01	0.85±0.01
5	Glycine	0.05±0.01	7.10±0.06	0.20±0.01	2.48±0.02
6	L-threonine	0.36±0.02	5.88±0.05	0.96±0.02	2.48±0.01
7	L-arginine	0.12±0.01	6.48±0.05	0.67±0.02	8.44±0.05
8	L-alanine	0.24±0.03	7.15±0.06	0.87±0.01	2.87±0.03
9	L-tyrosine	0.16±0.02	4.33±0.03	0.51±0.02	1.66±0.01
10	L-cysteine	n/d	n/d	n/d	8.11±0.07
11	L-valine	0.54±0.02	6.10±0.04	0.52±0.02	4.09±0.03
12	L-methionine	n/d	0.73±0.02	0.07±0.01	0.33±0.02
13	L-phenylalanine	0. 73±0.02	8.13±0.04	0.84±0.02	2.04±0.03
14	L-isoleucine	0.21±0.03	6.27±0.03	0.27±0.02	2.55±0.02
15	L-lysine	0.17±0.01	15.31±0.07	0.50±0.02	3.46±0.04
16	L-leucine	0.06±0.01	6.21±0.04	0.21±0.03	4.42±0.03
17	L-proline	0.64±0.02	6.49±0.04	1.16±0.03	2.49±0.03

Note: n/d – not detected.

of other amino acids was fewer. Nevertheless, L-cysteine has been found only in *Stachys sieboldii* tubers in amounts (8.11 mg/g). L-cysteine is used as a supplement for various purposes, for example, to boost the immune system, promote skin and hair health, and combat inflammatory-related problems. It induces the synthesis of GHS, which is a powerful natural antioxidant (Lubna et al. 2018).

Conclusions

As a result of this work, the comparative analyses of amino acids in the herb and tubers of *Stachys sieboldii* using a sensitive HPLC method were carried out for the first time. It should be noted that the amino acid content in the aboveground organs is higher compared to their underground organs. High concentrations of the free amino acids such as L-aspartic acid, L-proline, and L-phenylalanine predominate in the analyzed samples. Due to the fact that amino acids take part in different metabolic processes in the body, it is important to study the pharmacological activities of *Stachys sieboldii* herb and tubers. Moreover, the predominant hydrophobic amino acid group (proline, alanine, isoleucine, leucine, and phenylalanine) in the herb and tubers of *Stachys sieboldii* is an important factor, that affects the antioxidant properties of the plant. Attention should be paid to future pharmacological studies on the nootropic activity of the analyzed raw materials.

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