

Determination of Amino Acids of Cultivated Species of the Genus *Primula* L.

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Abstract: Throughout many years, plants using not only as a source of the meal but also in the fight against diseases. Among these plants are rock primrose (*Primula saxatilis* Kom.), Julia's primrose (*Primula juliae* Kusn.), and drumstick primrose (*Primula denticulata* Smith.). The aim of our research was to determine the content of amino acids in these plants with the future prospects of their application as officinal medicinal plant raw material. The amino acids of the leaves of cultivated species of the genus *Primula* L. determined by the HPLC method. 16 free and 16 bound amino acids were identified in the cultivated species of *Primula* L. High concentrations of the free amino acids that as L-glutamic acid, L-aspartic acid, and L-alanine predominate in all the plants analyzed. Among the bound amino acids in the leaves of cultivated *Primula* species prevail, L-glutamic acid, L-aspartic acid, L-lysine, and L-leucine. The results show that rock primrose, Julia's primrose, and drumstick primrose are the rich source of amino acids. The specific metabolic processes in which these amino acids are involved may be related to the medicinal properties of plants according to their use in traditional medicine, and hence may contribute to the understanding of their healing properties.

Keywords: *Primula* L.; *Primula saxatilis* Kom.; *Primula juliae* Kusn.; *Primula denticulata* Smith.; amino acids, HPLC.

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1. Introduction

Nowadays, searching for plants with a long history of usage, minor side effects, and high tolerability, regardless of the age of patients, are the objects of interest in our society [1,2]. The importance of medicinal plants did not reduce the annual increase in the number of synthetic medicines, which often model biologically active substances of plants and are their chemical equivalents [3].

The genus *Primula* L. of the family *Primulaceae* is one of the largest and unites more than 400 species of annual and perennial plants [4–8]. It is widely distributed outside of the Asian highlands and in the high altitudes of North America, Europe, and the eastern Sino-Himalayan region, and arctic areas of the Northern Hemisphere, which are considered the primary center of diversity for this genus [4,9,10]. Many *Primula* species are also grown all over the world as adornment plants [4]. These species are still poorly known in nature, but our

perception of them has been aided by the store of the material provided by plant hunters for horticultural and scientific purposes [11]. Plants of the genus *Primula* L. has long been used in folk medicine: infusion of leaves as an analgesic in case of rheumatism, as a diuretic in the kidney and bladder diseases; infusion of flowers as a diaphoretic and expectorant used for fever, bronchitis, as an anti-inflammatory – when gum inflammation, as a general tonic – for migraines, nervousness, insomnia, tachycardia [12–16]. Cowslip primrose (*Primula veris* L.), the officinal raw material of which is the plant’s underground and above-ground organs, is the most studied of the *Primula* L. genus. As the natural resources of Cowslip primrose decrease every year, it is advisable to study other species of *Primula* L. Literature sources have information about the healing properties of *Primula* L. species such as rock primrose (*Primula saxatilis* Kom.), Julia’s primrose (*Primula juliae* Kusn.) та drumstick primrose (*Primula denticulata* Smith.), which are used only in traditional medicine [17–19].

Pursuant to literature data, the plants that are rich in saponins (approximately 60 %), including primulic acid 1 (PA 1), as well as plenty of flavonoid compounds and flavonols, i.e., catechin, rutin, luteolin, and kaempferol [20–22]. A novel natural flavone, 5-hydroxy-6,2'-dimethoxyflavone, was isolated from the leaf exudate of *Primula denticulata* Smith. Its structure was elaborated by spectral researches and affirmed by synthesis [23,24]. The *Primula denticulata* Smith. to acquire a lot of triterpenoids as well as pridentigenins A–E, denticin, denticulatin, primulanin, and saxifragifolia B [25]. It is stated that *Primula* species are rich in tannins, terpenes, saponins, alkaloids, and phenolic compounds [26–29].

Considering the lack amounts of information and the lack of research about some primary metabolites, such as amino acids, is actual their – determination in some *Primula* species. Thus, the aim of our research was to determine the content of amino acids in rock primrose (*Primula saxatilis* Kom.), Julia’s primrose (*Primula juliae* Kusn.) and drumstick primrose (*Primula denticulata* Smith.) with the future prospects of their application as officinal medicinal plant raw material.

2. Materials and Methods

2.1. Plant materials.

The leaves of cultivated species of the genus *Primula* L. (drumstick primrose - *Primula denticulate* Smith., Julia’s primrose - *Primula juliae* Kusn., rock primrose - *Primula saxatilis* Kom.) (Table 1). Plant raw materials reaped at the scientific and research unit of the Department of flower-ornamental plants M. M. Hryshko National Botanical Garden National Academy of Sciences of Ukraine (N 50°24'54.1" E 30°33'47.3"). The leaves were harvested during blossom, *Primula denticulate* Smith and *Primula juliae* Kusn.- May 2017, *Primula saxatilis* Kom. - June 2017.

Table 1. List of plants from cultivated species of the genus *Primula* L.

Plant	Common name	Voucher
<i>Primula denticulate</i> Smith.	Drumstick primrose	256
<i>Primula juliae</i> Kusn.	Julia’s primrose	257
<i>Primula saxatilis</i> Kom.	Rock primrose	258

2.2. Chemicals and standards.

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-glutamic acid (Glu),

L-aspartic acid (Asp), L-methionine (Met), L-hystidine (His), L-alanine (Ala), L-isoleucine (Ile), L-arginine (Arg), Glycine (Gly), L-valine (Val), L-tyrosine (Tyr), L-serine (Ser), L-cystine (Cys), L-phenylalanine (Phe), L-treonin (Thr), L-lysine (Lys), L-leucine (Leu), L-proline (Pro) (Figure 1).

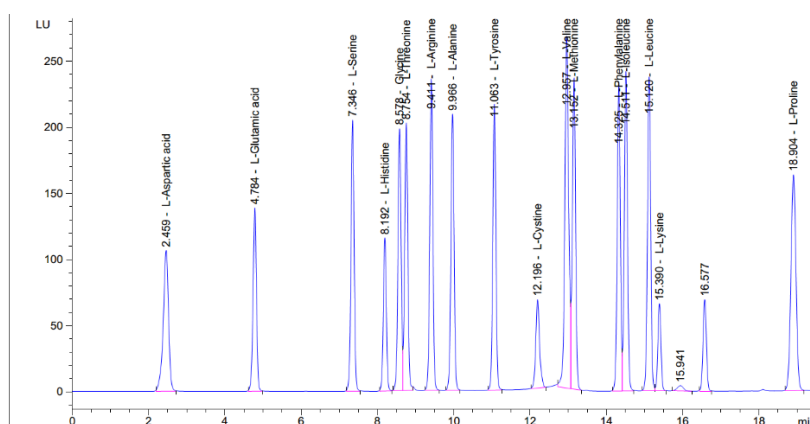


Figure 1. HPLC chromatogram of amino acid standards.

Derivatizing agents 9-fluorenylmethyl chloroformate (FMOC) and o-phthalaldehyde (OPA) were purchased from Merck. Hydrochloric acid (HCl) and Acetonitrile (ACN) were from Sigma-Aldrich (USA).

2.3. Sample preparation, HPLC determination of amino acids.

The amino acid composition of drumstick primrose, Julia's primrose, and rock primrose are determined by HPLC method with a pre-column derivatization OPA and FMOC.

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.

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 – $\text{CH}_3\text{CN}:\text{CH}_3\text{OH}:\text{H}_2\text{O}$ (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.

The pre-column derivatization was conducted with the help of automatic programmable regulations using OPA reagent and FMOC reagent. The identification of derivatized amino acids was made by a fluorescence detector [1,30,31]. For the extraction of free amino acids of powdered the raw material (to the 134 mg of drumstick primrose; to the 131 mg of Julia's primrose; to the 132 mg of rock primrose), 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 135 mg of drumstick primrose; to the 133 mg of Julia's primrose; to the 134 mg of rock primrose). 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.

The 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 peak area of the amino acids.

The number of amino acids in µg/mg was calculated according to the following equation:

$$X = \frac{C \times V}{m},$$

where: *C* – concentration, obtained from the chromatogram by calculating the reference solution and the test solution;

V – the volume of solvent for extraction;

m – is a mass of plant material [1,32].

3. Results and Discussion

The amino acid profiles of the leaves of cultivated *Primula denticulate* Smith., *Primula juliae* Kusn. and *Primula saxatilis* Kom. were evaluated using the HPLC method (Figure 2–7, Table 2).

Table 2. The content of the amino acid composition of cultivated species of the genus *Primula* L.

The name of the amino acid	The content of the amino acid, µg/mg					
	<i>Primula denticulata</i> Smith. leaves		<i>Primula juliae</i> Kusn. leaves		<i>Primula saxatilis</i> Kom. leaves	
	Free	Bound	Free	Bound	Free	Bound
Asp	1.35	9.96	0.95	10.47	3.78	6.37
Glu	3.38	16.23	1.11	16.55	4.74	10.65
Ser	0.52	4.46	0.52	4.23	1.21	3.01
His*	0.14	3.32	0.36	2.98	0.43	2.34
Gly	0.32	6.67	0.22	6.48	2.08	3.93
Thr*	0.38	5.08	0.58	4.61	0.99	3.38
Arg*	0.76	6.10	4.84	2.89	1.42	3.94
Ala	1.70	5.76	2.18	4.81	2.42	3.66
Tyr	0.25	2.96	0.44	2.58	0.65	1.88
Val*	0.43	5.51	0.75	4.89	1.08	3.86
Met*	0.06	0.58	0.07	0.74	0.16	0.62
Phe*	0.50	6.16	0.75	5.52	1.10	4.24
Ile*	0.27	5.76	0.63	5.17	0.89	4.09
Leu*	0.39	9.86	0.61	8.91	1.38	6.93
Lys*	0.36	8.65	0.60	7.90	1.32	6.57
Pro	0.26	5.64	0.29	5.01	1.18	3.73

* essential amino acid

The HPLC method identified 16 free amino acids of the leaves of cultivated *Primula* species (drumstick primrose, Julia’s primrose, and rock primrose) (Figure 2, 4, 6). Free glutamic acid and aspartic acid were present of the leaves in *Primula* species in the greatest amount (3.38 µg/mg and 1.35 µg/mg in the *Primula denticulata* Smith.; 1.11µg/mg and 0.95 µg/mg in the *Primula juliae* Kusn.; 4.74 µg/mg and 3.78 µg/mg in the *Primula saxatilis* Kom.). L-aspartic acid is an endogenous four-carbon amino acid present in nervous tissues, endocrine glands, and used not only as a stand-alone food additive but also as a raw material for the pharmaceutical and food industries [33,34]. Glutamic acid participates in more metabolic reactions than any other amino acid. It is a source of glucose, which is the main source of fuel. It holds the normal blood glucose level and the acidity as well [35]. It acts as a source of fuel for the intestinal epithelium.

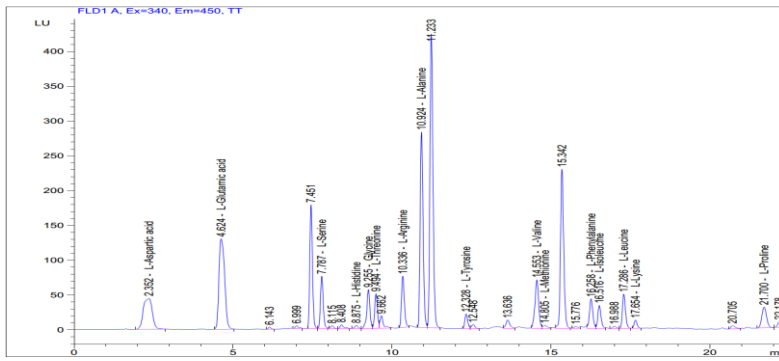


Figure 2. HPLC chromatogram of free amino acids of *Primula denticulata* Smith.

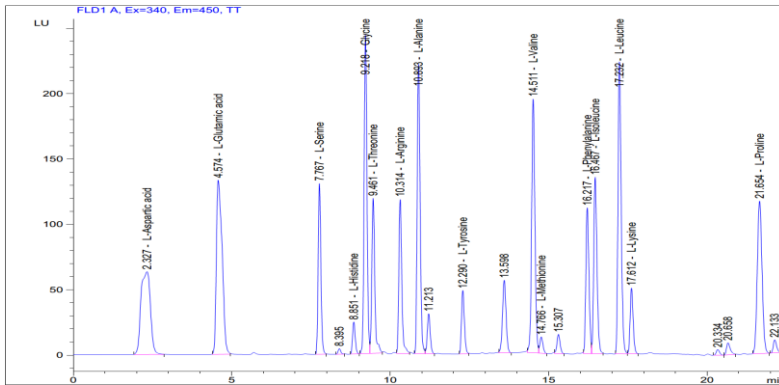


Figure 3. HPLC chromatogram of bound amino acids of *Primula denticulata* Smith.

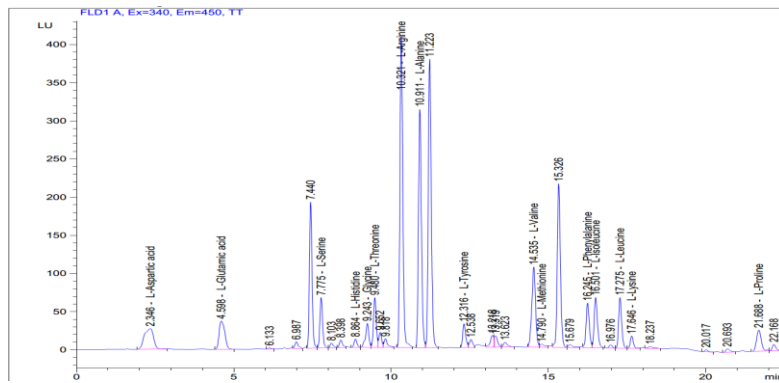


Figure 4. HPLC chromatogram of free amino acids of *Primula juliae* Kuhn.

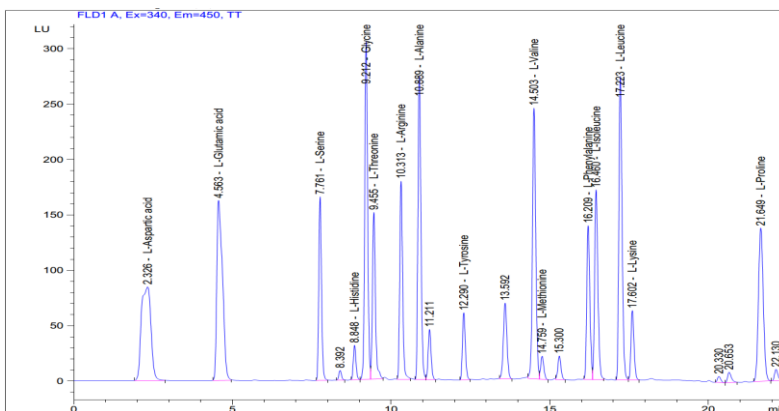


Figure 5. HPLC chromatogram of bound amino acids of *Primula juliae* Kuhn.

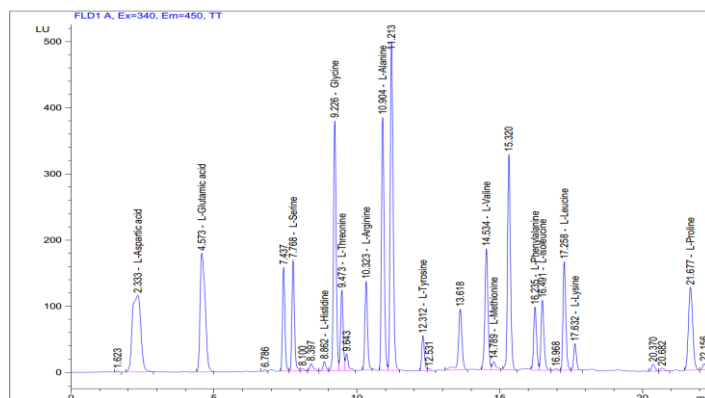


Figure 6. HPLC chromatogram of free amino acids of *Primula saxatilis* Kom.

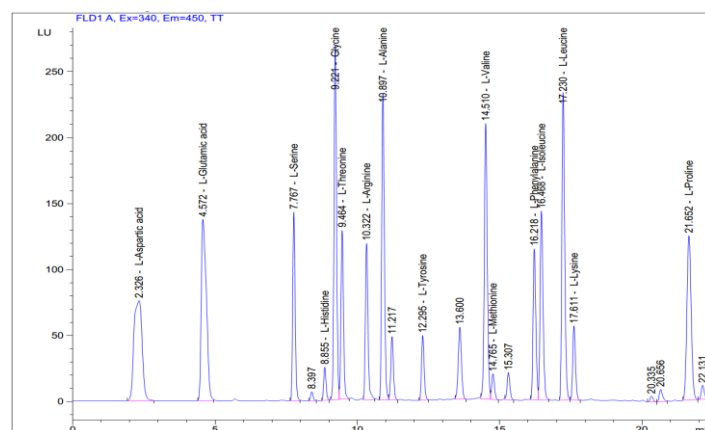


Figure 7. HPLC chromatogram of bound amino acids of *Primula saxatilis* Kom.

Glutamic acid is used as a conjugate because it improves the effectiveness of anticancer remedy and decreases its toxicity in relation to normal cells. The synthetic amides of L-glutamic acid display activity with respect to Ehrlich ascites carcinoma [36,37].

Also, among the free amino acids, Ala was present in drumstick primrose (1.70 $\mu\text{g}/\text{mg}$), Julia's primrose (2.18 $\mu\text{g}/\text{mg}$), and rock primrose (2.42 $\mu\text{g}/\text{mg}$) in the greatest amount. L-alanine serves as an intermediary between protein catabolism and carbohydrate synthesis. It can be easily synthesized from the alpha-keto acid pyruvate and has close ties to several metabolic fate, including glycolysis, gluconeogenesis, and the citric acid cycle. Together with lactate, it has the capability of obtaining glucose from muscle protein through gluconeogenesis in the liver. Thus, alanine plays a leading role in the metabolism of muscle protein and is the main criterion in nitrogen metabolism [38–40]. The number of other amino acids was fewer.

16 bound amino acids were identified in the leaves of cultivated species of the genus *Primula* L (Figure 3, 5, 7). The contents of bound Glu, Asp, Leu, and Lys were the greatest in the *Primula denticulata* Smith., *Primula juliae* Kusn., *Primula saxatilis* Kom. Leu contributes to energy metabolism (glucose uptake, mitochondrial biogenesis, and fatty acid oxidation) to render energy for protein synthesis while inhibiting protein degradation [41]. Leucine plays a rare signaling role in skeletal muscle and adipose tissue [42,43], as well as other cell kinds, as well as placental cells [44–46] and mammary epithelial cells [47]. L-lysine is a necessary amino acid in human food, and thus the body cannot produce it. Therefore, it must be received through diet or supplementation. Lysine is prominent for proper growth, and it plays a significant role in the production of carnitine, a nutrient responsible for converting fatty acids into energy and helping to decrease cholesterol [48–50].

4. Conclusions

Using the HPLC method determined the amino acids in the leaves of cultivated *Primula* species. The results suggested that the drumstick primrose, Julia's primrose, and rock primrose content significant amounts of free and bound amino acids. High concentrations of the free amino acids that as L-glutamic acid, L-aspartic acid, and L-alanine predominate in all the plants analyzed. Among the bound amino acids in the leaves of cultivated *Primula* species prevail, L-glutamic acid, L-aspartic acid, L-lysine, and L-leucine. This allowed these amino acids to be considered distinguishing markers of the *Primula denticulata* Smith., *Primula juliae* Kusn., *Primula saxatilis* Kom. Character metabolic processes in which these amino acids take part may be associated with the therapeutic properties of plants pursuant to their use in traditional medicine and, therefore, may contribute to the understanding of their therapeutic properties.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Slobodianiuk, L.; Budniak, L.; Marchyshyn, S.; Basaraba, R. Determination of amino acids and sugars content in *Antennaria dioica* Gaertn. *IJAP* **2019**, *11*, 39-43, <https://doi.org/10.22159/ijap.2019v11i5.33909>.
2. Darzuli, N.; Budniak, L.; Hroshovyi, T. Selected excipients in oral solid dosage form with dry extract of *Pyrola rotundifolia* L. *IJAP* **2019**, *11*, 210-6, <https://doi.org/10.22159/ijap.2019v11i6.35282>.
3. Stoiko, L.; Kurylo, K. Development of optimal technology of alcohol extract *Centaurium erythraea* Rafn. herb. *Archives Of The Balkan Medical Union* **2018**, *53*, 523-8, <https://doi.org/10.31688/ABMU.2018.53.4.06>.
4. Singh, S.; Ali, S.; Singh, M. Biological screening of plants extract showing hypoglycaemic and wound healing properties: *Capparis zeylanica* and *Primula denticulata*. *Am J Phytomed Clin Ther.* **2014**, *12*, 1338-45.
5. Wu, Z.-K.; Zhao, F.-W.; Chen, J.-H.; Huang, Y. *Primula dongchuanensis* (Primulaceae), a new species from northern Yunnan, China. *PhytoKeys* **2019**, *130*, 171-81, <https://doi.org/10.3897/phytokeys.130.35047>.
6. Colombo, P.S.; Flamini, G.; Christodoulou, M.S.; Rodondi, G.; Vitalini, S.; Passarella, D.; Fico, G. Farinose alpine *Primula* species: Phytochemical and morphological investigations. *Phytochemistry* **2014**, *98*, 151-9, <https://doi.org/10.1016/j.phytochem.2013.11.018>.
7. Budniak, L.I.; Vasenda, M.M. The effect of dispersity on the removal of biologically active substances of primrose *denticulata* leaves. *Medical and Clinical Chemistry* **2019**, *21*, 156-61, <https://doi.org/10.11603/mcch.2410-681X.2019.v.i4.10853>.
8. Liu, T.J.; Zhang, C.Y.; Yan, H.F.; Zhang, L.; Ge, X.J.; Hao, G. Complete plastid genome sequence of *Primula sinensis* (Primulaceae): structure comparison, sequence variation and evidence for accD transfer to nucleus. *Peer J.* **2016**, *28*, <https://doi.org/10.7717/peerj.2101>.
9. Kovtonyuk N.; Durand-Andro, V. Typification of *Primula* L. taxa names (Primulaceae), described by A. Franchet in 1886 from China and Eastern Tibet. *Adansonia* **2019**, *41*, 75-89, <https://doi.org/10.5252/adansonia2019v41a9>.
10. Ren, T.; Yang, Y.; Zhou, T.; Liu, Z. Comparative Plastid Genomes of *Primula* Species: Sequence Divergence and Phylogenetic Relationships. *International Journal of Molecular Sciences* **2018**, *19*, <https://doi.org/10.3390/ijms19041050>.
11. Mast, A.R.; Kelso, S.; Richards, A.J.; Lang, D.J.; Feller, D.M.S.; Conti, E. Phylogenetic relationships in *Primula* L. and related genera (Primulaceae) based on noncoding chloroplast dna. *Int J Plant Sci.* **2001**, *162*, 1381-400, <https://doi.org/10.1086/323444>.

12. Marchyshyn, S.M.; Sinichenko, A.V. Investigation of phenolic compounds about ground organs of cultivated species genus *Primula* L. *The Pharm Innov J.* **2016**, *5*, 38-42.
13. Belaeva, T.N.; Butenkova, A.N. Leaf anatomy of valuable species of genus *Primula*. *Ukrainian Journal of Ecology* **2019**, *9*, 150-55, https://doi.org/10.15421/2019_72.
14. Demir, S.; Turan, I.; Aliyazicioglu, R.; Yaman, S.O.; Aliyazicioglu, Y. *Primula vulgaris* extract induces cell cycle arrest and apoptosis in human cervix cancer cells. *J Pharm Anal.* **2018**, *8*, 307-311, <https://doi.org/10.1016/j.jpha.2018.05.003>.
15. Shostak, L.G.; Marchyshyn, S.M.; Kozachok, S.S.; Karbovska, R.V. Investigation of phenolic compounds of *Primula veris* L. *J Educ Health Sport.* **2016**, *6*, 424-32, <http://dx.doi.org/10.5281/zenodo.56701>.
16. Turan, I.; Demir, S.; Aliyazicioglu, R.; Aliyazicioglu, Y. Evaluation of antioxidant and cytotoxic properties of *Primula vulgaris* leaf extract. *KSU J Nat Sci.* **2017**, *20*, 361-7, <http://doi.org/10.18016/ksudobil.288175>.
17. Sinichenko, A.V.; Marchyshyn, S.M.; Stoiko, L.I.; Slobodianiuk, L.V. content of saponins in leaves and rhizomes with roots of cultivated plants of *Primula* L. *Med and Clin Chem.* **2018**, *20*, 125-9, <https://doi.org/10.11603/mcch.2410-681X.2018.v0.i4.9824>.
18. Budnyak, L.I.; Vasenda, M.N.; Sokhatsky, V.I. The technological properties of *Primula denticulata* Smith. *Science and Innovation* **2019**, *4*, 107-10.
19. Sinichenko, A.V.; Marchyshyn, S.M.; Sira, L.M.; Lykanyuk, M.I. Investigation of morphological and anatomical underground parts structure of genus *Primula* L. cultivated species. *Ukrainian biopharmaceutical journal* **2018**, *54*, 55-63, <https://doi.org/10.24959/ubphj.18.157>.
20. Colombo, P.S.; Rodondi, Gr.; Flamini, G.; Giuliani, C. Phytochemistry of European *Primula* species. *Phytochem.* **2017**, *143*, 132-44, <https://doi.org/10.1016/j.phytochem.2017.07.005>.
21. Sinichenko, A.V.; Marchyshyn, S.M.; Slobodianiuk, L.V.; Budniak, L.I. Investigation of phenolic compounds in the rhizomes with roots of *Primula denticulate* Smith., *Primula juliae* Kuhn., *Primula saxatilis* Kom. *Phytotherapy.Chasopys* **2019**, *3*, 26-31.
22. Tarapatsky, M.; Kapusta, I.; Gumienna, A.; Puchalski, C. Assessment of the Bioactive Compounds in White and Red Wines Enriched with a *Primula veris* L. *Molecules* **2019**, *24*, <https://doi.org/10.3390/molecules24224074>.
23. Wollenweber, E.; Tanaka, T.; Iinuma, M.; Mizuno, M. 5-Hydroxy-6,2'-dimethoxyflavone from *Primula denticulate*. *Phytochem.* **1990**, *29*, 633-7, [https://doi.org/10.1016/0031-9422\(90\)85132-Y](https://doi.org/10.1016/0031-9422(90)85132-Y).
24. Tokalov, S.V.; Kind, B.; Wollenweber, E.; Gutzeit, H.O. Biological effects of epicuticular flavonoids from *Primula denticulata* on human leukemia cells. *J Agric Food Chem.* **2004**, *52*, 239-45, <https://doi.org/10.1021/jf0347160>.
25. Singh, S.; Farswan, M.; Ali, S.; Afzal, M.; Al-Abbasi, F.A.; Kazmi, I.; Anwar, F. Antidiabetic potential of triterpenoid saponin isolated from *Primula denticulate*. *Pharm Biol.* **2014**, *52*, 750-5, <https://doi.org/10.3109/13880209.2013.869759>.
26. Demir, S.; Turan, I.; Aliyazicioglu, Y. Antioxidant properties of *Primula vulgaris* flower extract and its cytotoxic effect on human cancer cell lines. *KSU J Agric Nat.* **2019**, *22*, 19-25, <https://doi.org/10.18016/ksutarimdogavi.460242>.
27. Mostafa, F.A.; Gamal, M.A.; Sabrin, I.R.M.; Ehab, E.S. Antioxidant and anti-inflammatory activities of phenolic constituents from *Primula elatior* L. aerial part. *IJPPR* **2014**, *6*, 74-8.
28. Włodarczyk, M.; Pasikowski, P.; Osiewała, K.; Frankiewicz, A.; Dryś, A.; Gleńsk, M. In Search of High-Yielding and Single-Compound-Yielding Plants: New Sources of Pharmaceutically Important Saponins from the Primulaceae Family. *Biomolecules* **2020**, *10*, <https://doi.org/10.3390/biom10030376>.
29. Tugce Ozkan, M.; Aliyazicioglu, R.; Demir, S.; Misir, S.; Turan, I.; Yildirmis, S.; Aliyazicioglu, Y. Phenolic characterisation and antioxidant activity of *Primula vulgaris* and its antigenotoxic effect on fibroblast cells. *Jundishapur J Nat Pharm Prod.* **2017**, *12*, <https://doi.org/10.5812/jjnpp.40073>.
30. Jambor, A.; Molnar-Perl, I. Quantitation of amino acids in plasma by high performance liquid chromatography: Simultaneous deproteinization and derivatization with 9-fluorenylmethylloxycarbonyl chloride. *J Chromatogr A.* **2009**, *1216*, 6218-23, <https://doi.org/10.1016/j.chroma.2009.06.083>.
31. Hanczkó, R.; Jám bor, A.; Perl, A.; Molnár-Perl, I. Advances in the o-phthalaldehyde derivatizations: Comeback to the o-phthalaldehydeethanethiol reagent. *J Chromatogr A.* **2007**, *1163*, 25-42, <https://doi.org/10.1016/j.chroma.2007.06.013>.
32. Jám bor, A.; Molnár-Perl, I. Amino acid analysis by high-performance liquid chromatography after derivatization with 9-fluorenylmethylloxycarbonyl chloride literature overview and further study. *J Chromatogr A.* **2009**, *1216*, 3064-77, <https://doi.org/10.1016/j.chroma.2009.01.068>.
33. Choi, S.; Song, C.W.; Shin, J.H.; Lee, S.Y. Biorefineries for the production of top building block chemicals and their derivatives. *Metab Engin.* **2015**, *28*, 223-39, <https://doi.org/10.1016/j.ymben.2014.12.007>.
34. D'aniello, G.; Tolino, A.; D'aniello, A.; Errico, F.; Fisher, G.H.; Di Fiore, M.M. The role of D-aspartic acid and n-methyl-D-aspartic acid in the regulation of prolactin release. *Endocrinology* **2000**, *141*, 3862-70, <https://doi.org/10.1210/endo.141.10.7706>.
35. Okasha, R.M.; Al-Shaikh, N.E.; Aljohani, F.S.; Naqvi, A.; Ismail, E.H. Design of Novel Oligomeric Mixed Ligand Complexes: Preparation, Biological Applications and the First Example of Their Nanosized Scale. *International journal of molecular sciences* **2019**, *20*, <https://doi.org/10.3390/ijms20030743>.

36. Dutta, S.; Ray, S.; Nagarajan, K. Glutamic acid as anticancer agent. *Saudi Pharm J.* **2013**, *21*, 337-43, <https://doi.org/10.1016/j.jsps.2012.12.007>.
37. Vila, J.; Thomasset, N.; Navarro, C.; Dore, J.F. In vitro and in vivo anti-tumor activity of L-glutamic acid c-mono-hydroxamate against L1210 leukemia and B16 melanoma. *Int J Cancer.* **1990**, *45*, 737-43, <https://doi.org/10.1002/ijc.2910450428>.
38. Holvik, K.; Froyland, L.; Haugem, M.; Henjuman, S.; Lovik, M.; Stea, T.H.; Strand, T.A. Risk assessment of “other substances” – L-alanine. Opinion of the Panel on Nutrition, dietetic products, Novel Food an Allergy of the Norwegian Scientific Committee for Food Safety. (*VKM*) **2017**, *6*.
39. Hatazawa, Y.; Qian, K.; Gong, D.W.; Kamei, Y. PGC-1 α regulates alanine metabolism in muscle cells. *PLOS ONE* **2018**, *13*, <https://doi.org/10.1371/journal.pone.0190904>.
40. Tomé, D. Protein: what’s on in research on clinical nutrition. *Eur J Clin Nutr* **2018**, *72*, 1215–20, <https://doi.org/10.1038/s41430-018-0240-9>.
41. Duan, Y.; Li, F.; Li, Y.; Tang, Y.; Kong, X.; Feng, Z.; Anthony, G.T.; Watford, M.; Hou, Y.; Wu, G.; Yin, Y. The role of leucine and its metabolites in protein and energy metabolism. *Amino Acids* **2016**, *48*, 41-51, <https://doi.org/10.1007/s00726-015-2067-1>.
42. Li, F.; Yin, Y.; Tan, B.; Kong, X.; Wu, G. Leucine nutrition in animals and humans: mTOR signaling and beyond. *Amino Acids* **2011**, *41*, 1185-93, <https://doi.org/10.1007/s00726-011-0983-2>.
43. Carpentier, A.C. Branched-chain Amino Acid Catabolism by Brown Adipose Tissue. *Endocrinology* **2020**, *161*, <https://doi.org/10.1210/endocr/bqaa060>.
44. Kim, S.W.; Mateo, R.D.; Yin, Y.L.; Wu, G.Y. Functional amino acids and fatty acids for enhancing production performance of sows and piglets. *Asian Austral J Anim.* **2007**, *20*, 295-306, <https://doi.org/10.5713/ajas.2007.295>.
45. Kim, J.Y.; Burghardt, R.C.; Wu, G.; Johnson, G.A.; Spencer, T.E.; Bazer, F.W. Select nutrients in the ovine uterine lumen: VII. Effects of arginine, leucine, glutamine, and glucose on trophectoderm cell signaling, proliferation, and migration. *Biol Reprod.* **2011**, *84*, 62-9, <https://doi.org/10.1095/biolreprod.110.085738>.
46. Li, P.; Yin, Y.L.; Li, D.F.; Kim, S.W.; Wu, G.Y. Amino acids and immune function. *Br J Nutr.* **2007**, *98*, 237-52, <https://doi.org/10.1017/S000711450769936X>.
47. Lei, J.; Feng, D.Y.; Zhang, Y.L.; Zhao, F.Q.; Wu, Z.L.; San Gabriel, A.; Fujishima, Y.; Uneyama, H.; Wu, G. Nutritional and regulatory role of branched-chain amino acids in lactation. *Front Biosci.* **2012**, *17*, 2725-39, <https://doi.org/10.2741/4082>.
48. Singh, M.; Rao, D.M.; Pande, S.; Battu, S.; Mahalakshmi, K.; Dutt, K.R.; Ramesh, M. Medicinal Uses of L-Lysine: Past and Future. *Int J Res Pharm Sci.* **2011**, *2*, 637-42.
49. Ghoreyshi, S.M.; Omri, B.; Chalghoumi, R.; Bouyeh, M.; Seidavi, A.; Dadashbeiki, M.; Lucarini, M.; Durazzo, A.; van den Hoven, R.; Santini, A. Effects of Dietary Supplementation of L-Carnitine and Excess Lysine-Methionine on Growth Performance, Carcass Characteristics, and Immunity Markers of Broiler Chicken. *Animals (Basel)* **2019**, *9*, <https://doi.org/10.3390/ani9060362>.
50. Tufarelli, V.; Mehrzad-Gilmalek, H.; Bouyeh, M.; Qotbi, A.; Amouei, H.; Seidavi, A.; Paz, E.; Laudadio, V. Effect of Different Levels of L-carnitine and Excess Lysine-Methionine on Broiler Performance, Carcass Characteristics, Blood Constituents, Immunity and Triiodothyronine Hormone. *Agriculture* **2020**, *10*, <https://doi.org/10.3390/agriculture10040138>.