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Amino acid profiling in wild *Chamaenerion angustifolium* populations applying chemometric analysis

Kateryna Uminska[1], Zigmantas Gudžinskas[2], Liudas Ivanauskas[3], Victoriya Georgiyants[4], Alla Kozurak[5], Maria Skibytska[6], Olha Mykhailenko[4*]

[1]Zhytomyr Basic Pharmaceutical Professional College, Zhytomyr, Ukraine.
[2]Nature Research Centre, Institute of Botany, Vilnius, Lithuania.
[3]Department of Analytical and Toxicological Chemistry, Lithuanian University of Health Sciences, Kaunas, Lithuania.
[4]Department of Pharmaceutical Chemistry, National University of Pharmacy, Kharkiv, Ukraine.
[5]Carpathian Biosphere Reserve, Rakhiv, Ukraine.
[6]Botanical Garden of the Lviv National Ivan Franko University, Lviv, Ukraine.

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ABSTRACT

Evaluation of the amino acid composition of plants is a determining parameter in assessing their potential effect as food supplements. *Chamaenerion angustifolium* (Onagraceae), commonly known as "fireweed," is a traditional food and medicinal plant in Europe. Current research has focused on comparative analysis of the aerial part of 15 fireweed samples collected in Ukraine and Lithuania using Gas chromatography–mass spectrometry (GC-MS) method. The overall amino acid composition in samples of *C. angustifolium* was similar. Sulfur-containing amino acids (cysteine and methionine) were absent in all samples. The alanine content in the samples was the highest, and in the samples from Kharkiv (Ukraine), Ivano-Frankivsk (Ukraine), and Plungė district (Lithuania), it ranked first among others (its content in samples was 2.350, 6.090, and 2.44 mg/g, respectively). The high amount of free amino acids was recorded in the sample from Ivano-Frankivsk (Ukraine). The results of chemometric analysis indicated L-alanine and L-phenylalanine could be used as potential quality markers for the evaluation of the plant quality. The results indicate the potential for further pharmacological studies of fireweed raw material. Considering the content of amino acids in the aerial parts of *C. angustifolium*, its raw material could be used for development of medicines and dietary supplements.

INTRODUCTION

Amino acids are important components of metabolic processes in plants (Hildebrandt *et al*., 2015). They are part of proteins, play an important role as intermediate or end products of metabolism, and take part in the work of the tricarboxylic acid cycle (Krebs cycle) (Chahardoli *et al*., 2020; Shanaida *et al*., 2021). Those compounds play a key role in biosynthesis of secondary metabolites such as alkaloids, phenolic derivatives, terpenoids, and steroids. Furthermore, assessment of the impact of organic and amino acids on the human body as food components and pharmacological agents still continues. Amino acids, having a wide spectrum of pharmacological action, give other substances an easily digestible and harmless form, while potentiating their effect (Trumbo *et al*., 2002). It should be noted that medicinal plants are not considered as a source of an easily digestible form of amino acids in combination with other pharmacologically active compounds for the purpose of their use in the treatment of various pathologies.

Chamaenerion angustifolium (L.) Scop., widely known as *Epilobium angustifolium* L., is a widespread and variable species of the willowherb (Onagraceae) family (Constantin *et al*., 2013;

*Corresponding Author
Olha Mykhailenko, Department of Pharmaceutical Chemistry, National University of Pharmacy, Kharkiv, Ukraine.
E-mail: mykhailenko.farm @ gmail.com

Wagner *et al*., 2007). In North America and Europe, this species is commonly known as "fireweed" or "rosebay willowherb." Fireweed is a traditional food and medicinal plant in Europe.

Plants of the genus *Chamaenerion* are rich sources of ellagitannins (Ducrey *et al*., 1997; Granica *et al*., 2012; Hevesi, 2009), flavonoids (Averett *et al*., 1979; Bazylko *et al*., 2007; Ducrey *et al*., 1995; Stolarczyk *et al*., 2013), and phenolic acids and their derivatives (Granica *et al*., 2014; Shikov *et al*., 2006; Stolarczyk *et al*., 2013). This species also synthesize fatty acids and lipophilic components such as steroids (Hiermann and Mayr, 1985; Nowak and Krzaczek, 1998) and triterpenoids (Glen *et al*., 1967). Moreover, the presence of fatty acids in fireweed herb (Granica *et al*., 2012) has been reported. Some amino acids were found in the lipophilic fraction of the aerial part of *C. angustifolium* (Abudeiyh *et al*., 2010; Polezhaeva *et al*., 2007). Other organic acids of *C. angustifolium* are also poorly studied; L-ascorbic acid was identified only in its aerial parts (Shikov *et al*., 2006). The modern pharmacological studies confirmed antiproliferative (Vitalone *et al*., 2001), anti-inflammatory, antioxidant, antimicrobial (Nowak *et al*., 2022), immunomodulatory (Schepetkin *et al*., 2009), and other activities of fireweed (Granica *et al*., 2014; Vitalone and Allkanjari, 2018).

In Ukraine, fireweed is a widespread species, but it is more frequent in the northern regions of the country, in the foothills of the Carpathians (Volochai *et al*., 2021). Considering that the environment has an impact on the composition of bioactive compounds in plants (Mykhailenko *et al*., 2020a, 2020b; Silva *et al*., 2018), it is necessary to assess the composition of fireweed from Ukraine due to limited data availability. Every year, the requirements not only for the high productivity of cultivated or wild plants, but also for the compounds content in them, are increasing. Therefore, the identification of regions of Ukraine and neighboring countries with optimal ecological conditions for harvesting a promising medicinal plant of *C. angustifolium* is an urgent task.

Qualitative content analysis, together with chemometric methods, is often used to assess the taxonomic and geographical origin of plants (Kaškonienė *et al*., 2015). Even though the distribution area of fireweed in Ukraine is more than half of the country, so far, no studies have been published comparing the composition of amino acids in fireweed from different regions, providing information on the optimal areas for harvesting raw materials. This study presents the GC-MS derivatization procedure for the free amino acids analysis in *C. angustifolium* raw materials, as well as the comparative analysis of these components. By providing chemometric amino acid profile relationships, this study could also be used to describe the geographic identity of this plant.

The objectives of this study were to determine the qualitative and quantitative amino acid composition of aerial parts of *C. angustifolium*, collected in Ukraine and Lithuania, and compare their content using chemometric approach.

MATERIALS AND METHODS

Plant material

The aerial parts of the investigated *C. angustifolium* species were collected at the flowering stage (June–July 2019) at several sites in Ukraine and Lithuania (Table 1). The samples were verified by Dr. Skibitska and Dr. Kozurak and deposited at Herbarium (LW) of the Ivan Franko National University of Lviv (LW0056625–LW0056629), Ukraine. For analysis, the raw material was dried at a temperature of 20°C–24°C and crushed to obtain particles 2–3 mm in size.

Chemicals

For analysis, L-amino acids standard mixtures were alanine, serine, valine, threonine, leucine, isoleucine, proline, aspartic acid, glutamic acid, lysine, methionine, phenylalanine, and tyrosine (Sigma-Aldrich GmbH, Steinheim, Germany). The following reagents were used for GC-MS and Thin-layer chromatography (TLC) analysis: acetonitrile (Sigma-Aldrich GmbH, Karlsruhe, Germany), N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide mit 1% tert-Butyldimethylchlorosilane (MTBSTFA) (Sigma-Aldrich, St. Louis, MO), methanol (34860, Sigma-Aldrich, Germany), butanol (W217820, Sigma-Aldrich, Germany), glacial acetic acid (A6283, Sigma-Aldrich, Germany), Ninhydrin-Reagenz 2% Solution (N7285, Sigma-Aldrich, Germany), natriumhydroxid (655104, Sigma-Aldrich, Germany), and purified water (Millipore, Bedford, MA). All reagents were of the classes "chemically pure" and "chemically pure for analysis."

Extraction

The dry plant material was weighed into a volumetric flask (0.1 g) and extracted with 50% (v/v) methanol (10 ml) on ultrasonic bath at 45°C ± 2°C for 20 minutes. The obtained plant extract was centrifuged for 10 minutes at 5,000 rpm at 25°C. An amount of 500 µl of supernatant was evaporated under nitrogen gas to dry residue. The resulting precipitate was dissolved in 100 µl of acetonitrile and 100 µl of MTBSTFA reagent. Test plant extract was heated at 100°C for 2.5 hours in a glycerol bath. Then, 1 µl of the test solution was injected into a gas chromatograph. The reference solution was prepared by taking 100 µl of the mixture of amino acid standards and drying under a stream of nitrogen to dryness. The dry pellet was mixed with 100 µl of acetonitrile and 100 µl of the MTBSTFA derivatives. The resulting solution was heated at 100°C in a glycerol bath for 2.5 hours.

Preliminary prestaining thin layer chromatography analysis

Plant samples (0.5 µl) were spotted on the TLC silica-gel F_{254} plates (Merck®, 0.25 mm thick, 10 × 10 cm). The plate with the tested samples is placed in a chamber with a mixture of solvents: *n*-butanol:acetic acid:water (3:1:1 v/v/v). Then the plates were developed at room temperature with 0.2% ninhydrin reagent. After development, the plate was dried for color development in an oven at 100°C–105°C during 10 minutes. Amino acid was dissolved in the sodium hydroxide solution (0.01 M) and used as a reference standards solution. Free amino acids appeared in the form of pink-purple spots.

GC-MS analysis

The derivatization procedure was performed according to Mykhailenko *et al*. (2020a, 2020b), described as follows: 0.1 ml of the test extract was evaporated to dryness in a stream of nitrogen gas. The dried sample was diluted with acetonitrile (0.1 ml) and MTBSTFA agent (0.1 ml). The solution was thoroughly mixed on a vortex-mixer for 1 minute and incubated at 100°C in glycerol

Table 1. Location and elevation of *Epilobium* sampling sites in Lithuania and Ukraine.

No.	Voucher specimens	Administrative location	Altitude, m	Geographical coordinators[a]
1E	LW 0056624	Ukraine, Lviv region, Shpilchina village	376	49.66174°N 24.27277°E
2E	LW 0056625	Ukraine, Transcarpathian region, Carpathian Mountains, Chornohora massif	1,203	48.15655°N 24.33370°E
3E	LW 00566210	Ukraine, Transcarpathian region, Kvass village	1,180	48.15655°N 24.33730°E
4E	LW 0056626	Ukraine, Poltava region, Pisarivka village	101	49.41504°N 34.58589°E
5E	LW 00566219	Ukraine, Poltava region, Chutove village	1,957	49.43181°N 35.10102°E
6E	LW 0056627	Ukraine, Kharkiv region, Kachalivka village	140	50.00831°N 35.21047°E
7E	LW 00566213	Ukraine, Kharkiv region, village Bohodukhiv	186	50.09425°N 35.31324°E
8E	LW 00566214	Ukraine, Kyiv, Grishka Botanical Garden	179	50.36782°N 30.49564°E
9E	LW 00566215	Ukraine, Zhytomyr region, Verkhivnia village	205	49.48161°N 29.18462°E
10E	LW 00566216	Ukraine, Volyn region, Kovel village	172	51.13091°N 24.41160°E
11E	LW 00566217	Ukraine, Chernigiv region, Pidgirne village	136	51.29282°N 31.17551°E
12E	LW 00566218	Ukraine, Ivano-Frankivsk, Volchinec	244	48.57063°N 24.44462°E
13E	LW 0056628	Lithuania, leaves, Plungė district, Pauošniai village	159	55.97995°N 21.90639°E
14E	LW 0056629	Lithuania, flowers, Plungė district, Pauošniai village	159	55.97995°N 21.90639°E
15E	LW 00566212	Lithuania, Kaunas, Valentai	110	54.00263°N 23.70746°E

[a] Geographical coordinates and elevation above the sea level were identified using GPS devices (Prestigio GeoVision 5056).

bath during 2.5 hours. Each obtaining solution was analyzed by GC analysis in triplicate.

For analyses were used SHIMADZU GC-MS-QP2010 chromatography system coupled to an electron ionization ion source and a 5975C single quadrupole MS (Shimadzu Technologies). A robotic autosampler and a split/splitless injection port were used. Amino acids separation was carried out in Rxi-5 ms (Restek Corporation capillary column (30 m long, 0.25 mm outer diameter and 0.25 μm liquid–stationary phase thickness) with a liquid stationary phase 5% diphenyl and 95% polysiloxane) with helium at a purity of 99.99% as the carrier gas in a constant flow of 1.47 ml/minute. The oven temperature was programmed at 75°C for 1 minute, then it was increased to 290°C at 10°C/minute and kept for 5 minutes, and then increased to 320°C at 20°C/minute and kept for 10 minutes. The injector and the detector temperatures were maintained at 260°C and 280°C, respectively. Concentration range of standards was from 0.1 to 100 ng/ml. The MS was operated in positive mode (electron energy 70 eV). Full-scan acquisition was performed with the mass detection range set at 35–500 m/z to determine retention times of analytes, optimize oven temperature gradient, and observe characteristic mass fragments for each analyte. Collection and analysis of data were executed by G1701EA GC-MDS ChemStation (version E.02.02.1431) (Agilent Technologies). Amino acids were identified by comparing the mass spectra of the compounds with the data of the NIST14 and WRT10 libraries, and a mixture of standard samples was used to identify and quantify amino acids.

Statistical analyses

The results of descriptive statistics are presented as mean and standard deviation (mean ± SD). The normality of the data (the content of amino acids in samples) was assessed using the Shapiro–Wilk test. The content of amino acids in *C. angustifolium* samples was distributed nonnormally; therefore, nonparametric methods of statistical analysis were applied. The comparison of amino acid quantity among samples was performed by applying Kruskal–Wallis *H*-test, whereas pairwise comparisons were performed using Dunn's *z* post-hoc test. Hierarchical clustering analysis was based on qualitative composition of amino acids in samples (present or absent) applying paired group algorithm and Bray–Curtis similarity index. The effect of the geographical position of samples on the content of amino acids was assessed by applying nonmetric multidimensional scaling (NMDS). All calculations were performed using the PAST version 4.10 software (Natural History Museum, University of Oslo, Norway) (Hammer *et al*., 2001).

RESULTS AND DISCUSSION

Preliminary amino acids analysis

The main purpose of the study was to analyze the composition of amino acids which are compounds of primary metabolism necessary for the flow of important biochemical processes in a plant organism. Qualitative detection of amino acids by TLC showed the presence of 5–10 chromatographic adsorption zones in *C. angustifolium* samples by the nature of the color and the values of R_f coinciding with the standard samples of amino acids. The intensity of staining of the spots was different. Leucine, isoleucine, alanine, and phenylalanine were detected in most samples. The listed amino acids are indispensable (except for alanine) for the human body, so the studied raw materials can serve as a natural source of amino acids to a certain extent. The color of the amino acid spots, after treatment with ninhydrin, varied from pink to purple.

Qualitative amino acid composition

To study the amino acid composition of plants, combined methods of High-Performance Liquid Chromatography with Fluorescence Detection (HPLC-FD), High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) with UV, or DAD detection are frequently applied (Fremout *et al*., 2009; Azevedo *et al*., 2017; Shanaida *et al*., 2020); however, GC-MS is primarily the most common method used to characterize organic compounds in plant raw materials. Previously, we developed and presented a method for the chemical analysis of amino acids in various plant extracts. Identification is carried out by quantitative determination of free amino acids using GC-MS after derivatization (Mykhailenko *et al*., 2020a, 2020b).

The presented method for determining the amino acid composition was applied to the quantitative analysis of 15 *C. angustifolium* samples collected from different regions of Ukraine and Lithuania. A total of 10 compounds were analyzed simultaneously. Typical chemical characteristics of *C. angustifolium* samples are presented in Figure 1, and quantitative results are shown in Table 2.

The results of the study confirm the presence of 10 amino acids in *C. angustifolium* samples of which 5 are essential amino acids (valine, leucine, isoleucine, phenylalanine, and threonine) (Fig. 1). Amino acids in fireweed samples were identified by comparing the retention times of selected amino acids in specific MS chromatograms. Amino acids, such as glycine, methionine, histidine, and lysine, were not found in the analyzed samples. Among the identified amino acids, most (eight compounds) belong to the aliphatic group (Table 2); the presence of one aromatic amino acid (phenylalanine) and one heterocyclic amino acid (proline) was also recorded, which agrees with the published data on the amino acids content in plants of the genera *Chamaenerion* and *Epilobium* (Feshchenko *et al*., 2021; Näsholm Ann-Brittedfast *et al*., 1994). Aliphatic acids are represented by monoamino monocarboxylic acids (alanine, valine, isoleucine, leucine) and acids with hydroxy group (threonine, serine) compounds. Monoamino dicarboxylic acids are represented by aspartic and glutamic acids.

The different amino acid composition of *C. angustifolium* samples depends on the habitat conditions of the plants studied. L-Leucine, L-isoleucine, L-alanine, L-valine, and L-phenylalanyl were found in most samples. These amino acids

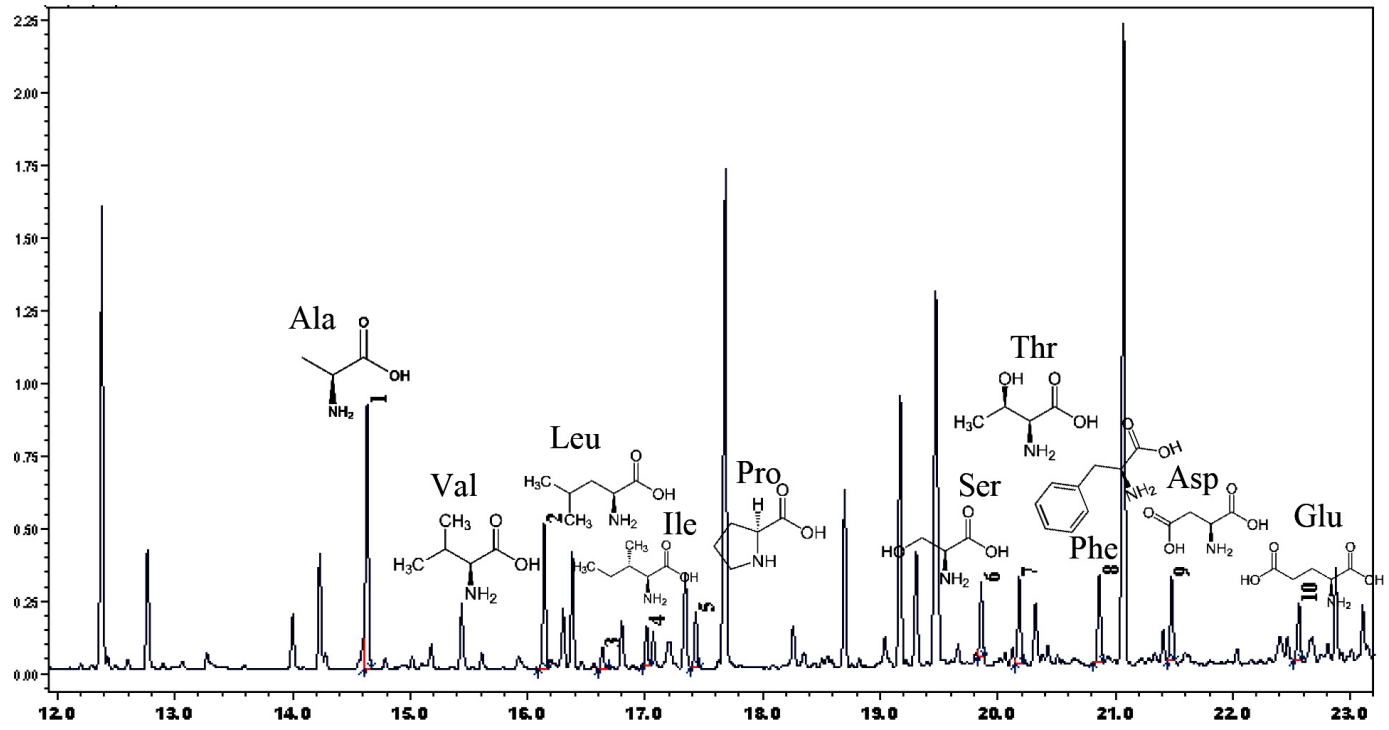

Figure 1. Topical TIC chromatogram of amino acids found in *C. angustifolium* (sample 12E). Amino acids are listed in order of release on the chromatogram: L-Alanine (**1**), L-Valine (**2**), L-Leucine (**3**), L-Isoleucine (**4**), L-Proline (5), L-Serine (**6**), L-Threonine (**7**), L-Phenylalanine (**8**), L-Aspartic acid (**9**), L-Glutamic acid (**10**).

Table 2. Amino acid content in aerial parts of *C. angustifolium*, µg/ml.

Sample	Country	ALA[a]	VAL	LEU	ILE	PRO	SER	THR	PHE	ASP	GLU	Total per sample
1E	UKR	1.251 ± 0.52	0.481 ± 0.20	0.107 ± 0.02[b]	0.225 ± 0.01	nd	0.230 ± 0.02	nd	0.670 ± 0.01	nd	1.868 ± 0.40	4.832 ± 0.15
2E	UKR	0.591 ± 0.40	0.449 ± 0.40	0.124 ± 0.01	0.222 ± 0.02	nd	nd	nd	0.633 ± 0.20	nd	nd	2.019 ± 0.22
3E	UKR	1.098 ± 0.64	0.793 ± 0.52	0.136 ± 0.19	0.292 ± 0.15	nd	0.291 ± 0.15	nd	1.051 ± 0.45	0.408 ± 0.10	nd	4.069 ± 0.80
4E	UKR	2.726 ± 1.12	1.421 ± 0.83	0.481 ± 0.41	0.756 ± 0.40	nd	nd	nd	3.843 ± 1.15	nd	4.927 ± 1.55	14.154 ± 0.05
5E	UKR	0.713 ± 0.55	0.168 ± 0.42	0.046 ± 0.04	0.074 ± 0.01	nd	nd	nd	nd	nd	nd	1.001 ± 0.01
6E	UKR	2.350 ± 0.15	2.125 ± 0.55	0.236 ± 0.02	0.648 ± 0.04	0.786 ± 0.04	1.079 ± 0.22	nd	2.853 ± 0.22	nd	3.030 ± 0.10	13.10 ± 0.05
7E	UKR	0.659 ± 0.02	0.570 ± 0.04	0.109 ± 0.10	0.293 ± 0.02	nd	0.369 ± 0.01	0.540 ± 0.02	0.670 ± 0.60	nd	nd	3.21 ± 0.10
8E	UKR	1.565 ± 0.54	0.570 ± 0.03	0.241 ± 0.22	0.315 ± 0.10	nd	0.192 ± 0.03	0.269 ± 0.01	2.117 ± 0.82	nd	nd	5.269 ± 0.80
9E	UKR	0.498 ± 0.06	0.118 ± 0.02	0.074 ± 0.02	0.089 ± 0.05	nd	nd	nd	0.221 ± 0.04	nd	nd	1.002 ± 0.06
10E	UKR	0.799 ± 0.08	0.254 ± 0.01	0.059 ± 0.04	0.134 ± 0.04	1.300 ± 0.10	0.215 ± 0.04	0.142 ± 0.01	nd	nd	1.070 ± 0.15	3.973 ± 0.04
11E	UKR	0.504 ± 0.04	0.103 ± 0.01	0.073 ± 0.03	0.065 ± 0.12	nd	0.099 ± 0.01	nd	nd	nd	nd	0.844 ± 0.02
12E	UKR	6.090 ± 1.02	5.418 ± 1.05	1.139 ± 0.25	2.302 ± 0.32	0.721 ± 0.04	3.674 ± 0.14	4.430 ± 0.20	5.857 ± 0.02	4.431 ± 0.04	2.024 ± 0.20	36.086 ± 1.35
13E	LT	2.449 ± 0.05	1.660 ± 0.82	0.110 ± 0.04	0.373 ± 0.10	0.894 ± 0.02	0.806 ± 0.04	nd	0.968 ± 0.05	1.026 ± 0.05	nd	8.286 ± 0.82
14E	LT	nd	0.598 ± 0.05	0.179 ± 0.32	0.254 ± 0.04	1.201 ± 0.064	0.646 ± 0.04	nd	0.830 ± 0.01	nd	nd	3.708 ± 0.16
15E	LT	nd	0.258 ± 0.02	0.045 ± 0.12	0.107 ± 0.02	nd	0.083 ± 0.02	0.130 ± 0.02	0.253 ± 0.02	nd	nd	0.876 ± 0.02
Mean content of amino acid		1.638 ± 0.82	0.999 ± 0.64	0.222 ± 0.32	0.432 ± 0.50	0.980 ± 0.54	0.699 ± 0.43	1.102 ± 0.65	1.664 ± 0.84	1.955 ± 0.75	2.584 ± 0.88	

[a] ALA, alanine; VAL, valine; LEU, leucine; ILE, isoleucine; PRO, proline; SER, serine; THR, threonine; PHE, phenylalanine; ASP, aspartic acid; GLU, glutamic acid.
[b] All values are expressed as mean ± standard deviation; nd – compound was not detected.

exhibit high metabolic activity in the brain and stimulate redox processes and protein metabolism, changing the functional state of the endocrine and nervous systems. Thus, glutamic acid is an important component in the treatment of human neuropsychiatric diseases (Zhou and Danbolt, 2014), and the search for sources of raw materials with these amino acids is very relevant. In addition to glutamic acid, L-phenylalanine and L-valine, which are also found in significant amounts in the studied samples, are necessary for the normal functioning of the central nervous system and the brain.

Hierarchical clustering analysis

The results of hierarchical clustering analysis performed according to the contents of the 10 amino acids in *C. angustifolium* samples revealed no clear grouping by their geographical origin (Fig. 2). Regarding the qualitative composition of the amino acids, all samples tested had a similarity of 75% or more. The samples from Western Ukraine (1E–3E, 10E, and 12E), Eastern Ukraine (4E–7E), Central Ukraine (8E–9E and 11E), and Lithuania (13E–15E) formed mixed groups according to the qualitative parameters of amino acids. The samples from Lithuania also did not form a well-defined group and were grouped together with the samples from all parts of Ukraine (Fig. 2). The samples from (7E) Kharkiv and (8E) Kyiv, as well as the samples from (2E) Transcarpathia and (9E) Zhytomyr, had the same amino acid composition (Fig. 2). The results of the analysis indicated that *C. angustifolium* samples could be distinguished from each other based on the chemical profile. In fact, the samples differ only in the presence or absence of several amino acids: threonine (found only in samples 7, 8, 10, 12, and 15), aspartic acid (found only in 3, 12, and 13), and glutamic acid (found in 1, 4, 6, 10, and 12).

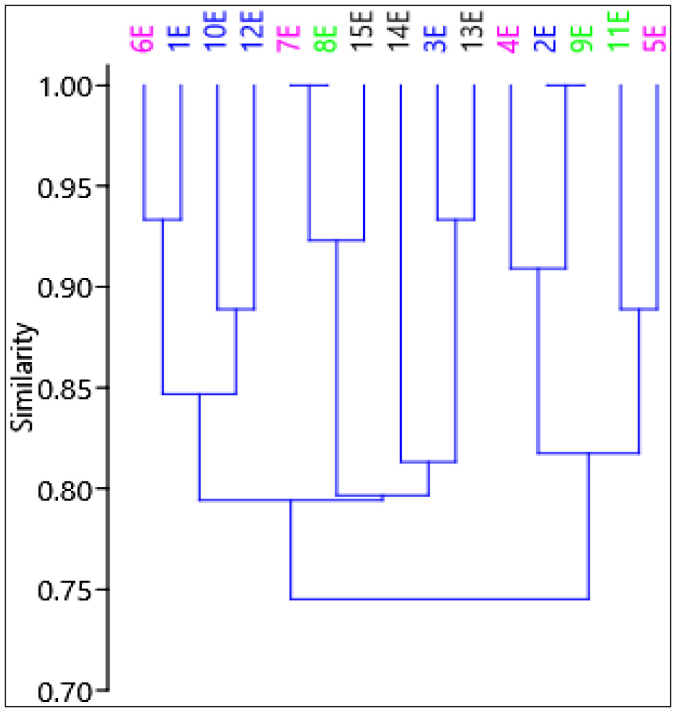

Figure 2. Dendrogram of hierarchical clustering analysis based on qualitative composition of amino acids in *C. angustifolium* samples applying paired group algorithm and Bray–Curtis similarity index. The sample codes are the same as in Table 1. The blue color indicates samples from West Ukraine, magenta from East Ukraine, green from Central Ukraine, and black from Lithuania.

The qualitative analysis suggests that there are no clear regional differences in the amino acid composition of *C. angustifolium* aerial parts and that the differences that do exist may be due to local ecological conditions or to genetic differences in the population.

Quantitative amino acid composition

Although the amino acid profile in the samples was similar, the content of compounds varied greatly depending on the location of the sampled plants. One of the criteria for obtaining high-quality herbal raw materials is the selection of appropriate conditions for plant growing or collecting raw materials in the wild, which are based on the study of the influence of environmental conditions on the accumulation of the bioactive compounds by plant species. Therefore, it is important to determine factors that under natural conditions have a positive effect on the biosynthesis of the primary and secondary plant metabolites. It is the content of the bioactive compounds that determines the value of herbal raw materials. The chemical composition of the plant is subject to significant fluctuations and depends on many factors. The same plant may contain different chemical compounds in different climatic and geographical zones. The content of bioactive compounds in plants is subject to changes depending on species, population and stage of plant vegetation, the soil type, its physical properties and chemical composition, geographical location of the growth area, climatic and meteorological conditions, and other ecological factors as well as raw material processing technologies. Thus, the analysis of the influence of environmental factors on the content of amino acids in the raw material of *C. angustifolium* is crucial for the use of the plant as a medicine or food supplement. The quantitative content of identified amino acids is presented in Table 2.

The lowest content of amino acids in terms of absolutely dry raw materials was found in sample 11E from Ukraine, Chernigiv (0.844 µg/ml), and the highest content was in the sample 12E from Ukraine, Vinnytsia (36.086 µg/ml). Previously, Feshchenko *et al.* (2021) analyzed the amino acid composition of *C. angustifolium* from Ternopil region (Ukraine) by HPLC. However, the current work is the first comparative analysis of the aerial part of *C. angustifolium* from nine different regions of Ukraine and Lithuania, where this plant grows widely in the wild. Samples from Ukraine showed higher content of amino acids. The aboveground part from the mountainous area stood out by the quantity of amino acids, although this difference is possibly associated with the lagging of the phenological phases in comparison with flat regions of Ukraine and Lithuania (Table 1).

Aliphatic glutamic acid was found only in four samples of *C. angustifolium* from Ukraine, but in a large amount, ranging from 1.868 to 4.927 µg/ml. This nonessential amino acid belongs to the group of neurotransmitter amino acids (Albrecht *et al.*, 2010). The high content of the studied raw material was also noted for such amino acids as alanine (0.498~2.726 µg/ml), leucine (0.016~1.139 µg/ml), and isoleucine (0.032~2.302 µg/ml), which are most often included in the composition of nootropic drugs.

L-phenylalanine is also predominant compound in most samples. The amino acid is precursor in phenylpropanoid pathway (Fraser and Chapple, 2011) that explains its high content rich in flavonoid fireweed aerial part. L-phenylalanine is involved in the production of important hormones and molecules in the body such as norepinephrine, epinephrine, tyrosine, and dopamine. All these substances take part in the work of the central nervous system of humans, improve memory, and regulate mood. The amino acids profile plays an important role in chemical properties, so it is useful information for further research.

Further, the chemometric approaches were applied based on the characteristics of the content of 10 amino acid compounds in the analyzed *C. angustifolium* samples for their comparison. The data obtained will provide more information about the chemical difference of the samples depending on the growth place.

The results of the quantitative amino acid analysis showed significant differences between all samples tested (Kruskal–Wallis $H = 53.49$, $p < 0.001$). The most remarkable of all was a sample (12E) from Ivano-Frankivsk, which was significantly different in amino acid quantity from all other samples except sample (6E) from Kharkiv (Dunn's post-hoc $z = 1.82$, $p = 0.069$). Similar amino acid levels were found in samples from (1E) Lviv, (9E) Zhytomyr, (11E) Chernigiv, and (15E) Kaunas. No significant differences were also found between the two samples (13E and 14E) from Plungė, where flowers and leaves were analyzed separately ($z = 1.25$, $p = 0.209$). It should be noted that the sample (14E) from Plungė, which consisted of flowers only, was not significantly different in amino acid content from all the other samples analyzed, except for sample (12E) from Ivano-Frankivsk.

It should be noted that we included two samples from Lithuania, Plungė district, in the experiment: 13E was only *C. angustifolium* leaves, and sample 14E was only *C. angustifolium* flowers. The analysis revealed that the content of amino acids in the flowers is lower, so there is no need to separate the flowers from the leaves, but more studies on a larger number of samples are needed to confirm this presumption.

NMDS results indicate that the geographical location of the collection of *C. angustifolium* samples influences the amino acid content of the raw material. It was found that the amino acid content of the raw material increases with increasing latitude (scores were 0.06 and 0.52 for Coordinate 1 and Coordinate 2, respectively). Longitude (scores were −0.07 and −0.43 for Coordinate 1 and Coordinate 2, respectively) has a negative effect, whereas the effect of altitude (scores were 0.32 and −0.18 for Coordinate 1 and Coordinate 2, respectively) was less expressed (Fig. 3). Samples from Lithuania nested separately, whereas samples from West, North, and East Ukraine were interlinked (Fig. 3). This suggests that the amino acid composition of *C. angustifolium* is dependent on environmental conditions, but further research is needed to determine which conditions have the greatest influence on amino acid composition. It cannot be excluded that the geographical races of the species may also influence the amino acid composition. Larger scale studies involving other regions are needed to test this assumption.

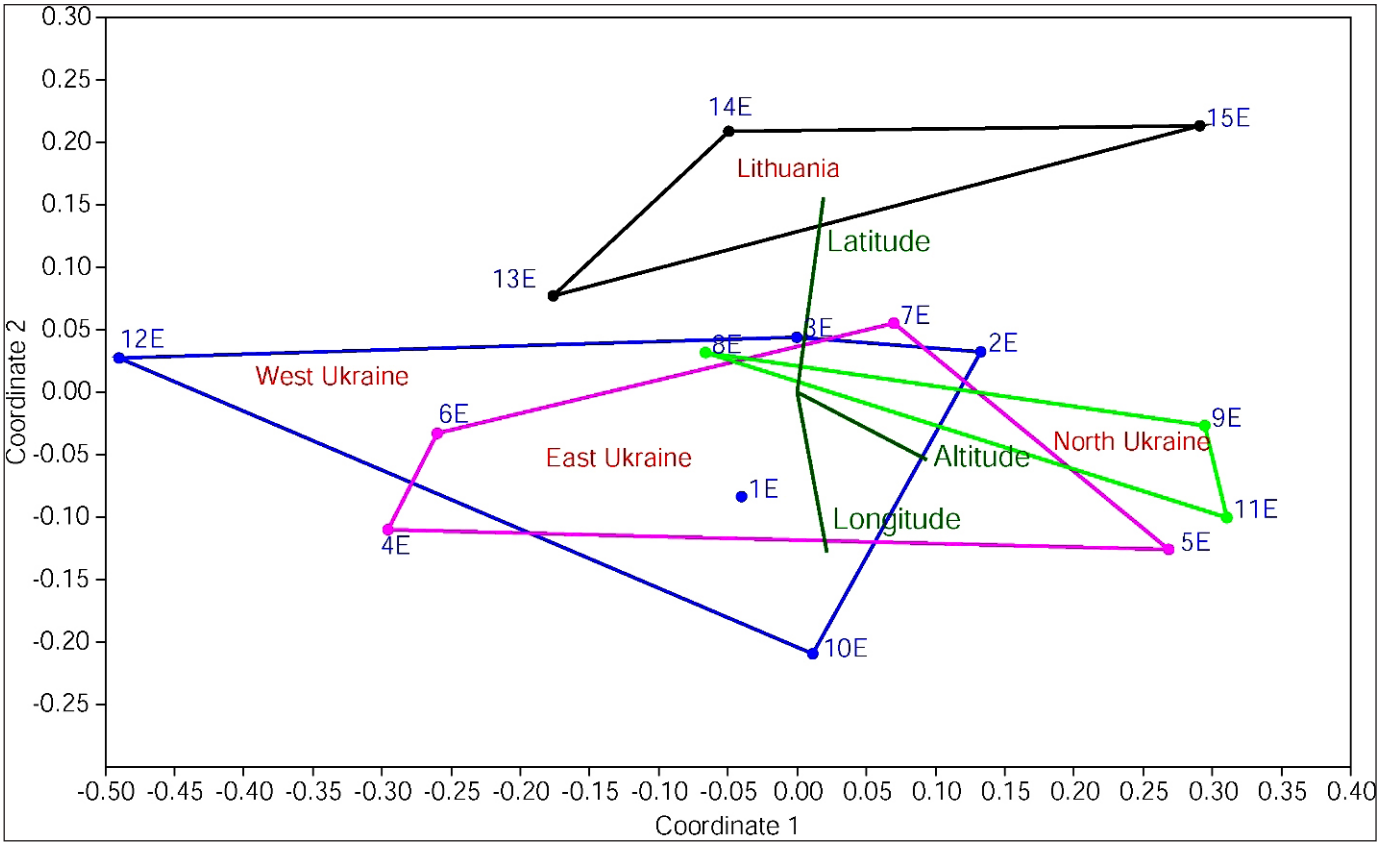

Figure 3. NMDS of *C. angustifolium* samples according to the content of amino acids and geographical position (longitude, latitude, and elevation) of the locality.

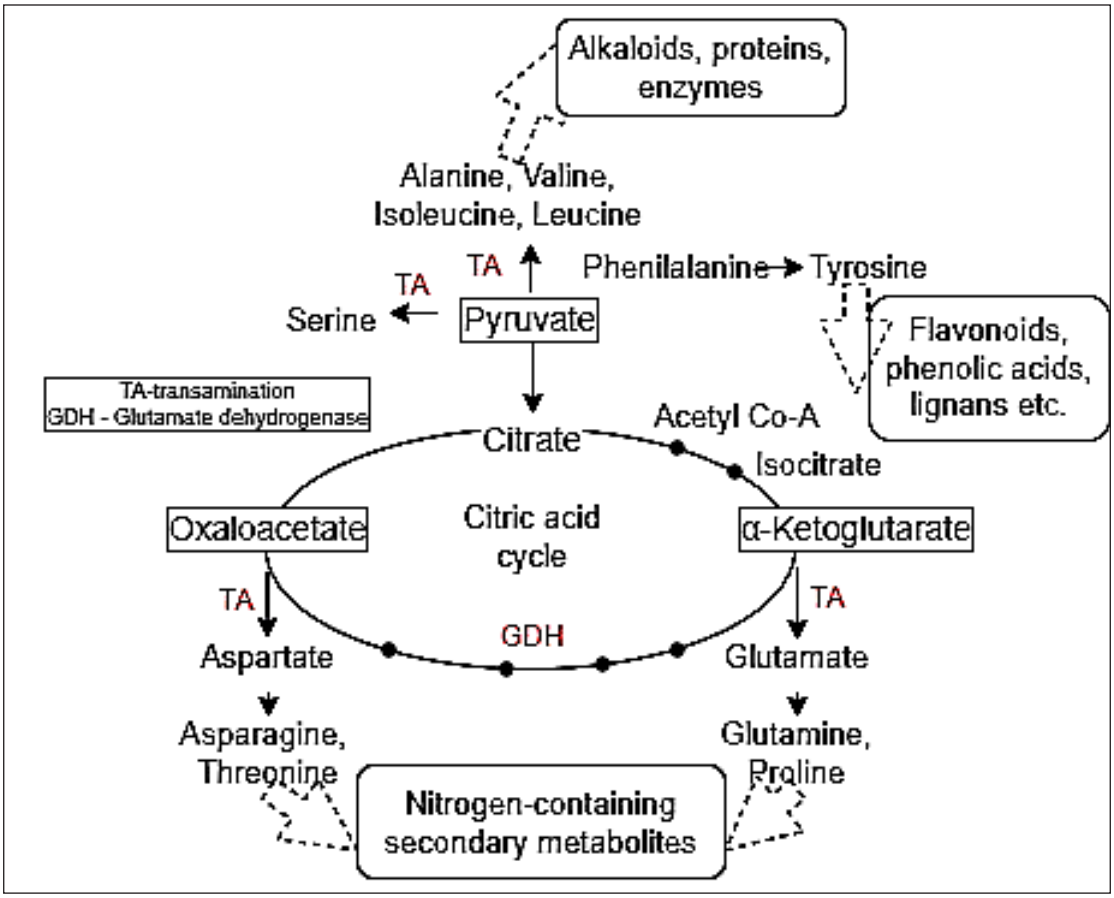

Figure 4. Biosynthetic pathways for some of the nonessential amino acids identified in *C. angustifolium* and possible further bioactive compound classes.

Glutamic acid (average content in the samples was 2.584 µg/ml), aspartic acid (1.955 µg/ml), alanine (1.638 µg/ml), and phenylalanine (1.664 µg/ml) were predominant in the plant raw materials. In a plant cell, these amino acids play a key role in the metabolism of compounds and the activity of key enzymes (nitrate reductase, glutamine synthetase, glutamate dehydrogenase, glutamine synthase, asparagine synthetase, and others). Thanks to these enzymes, nitrogen is metabolized from soil nitrates and their gradual transformation into secondary metabolites (hydroxycinnamic acids and flavonoids). Alanine is the first amino acid formed in the plants roots, then dicarboxylic amino acids (aspartic and glutamic acids) are formed after a relatively short time (Parthasarathy *et al*., 2019). The synthesis of diamino acids and aromatic amino acids occurs much later in leaves, apparently due to the amine groups of alanine and dicarboxylic amino acids as a result of transamination reactions (Santosh *et al*., 2021). Environmental factors (such as light and location) affect the conversion and recycling of key amino acids by regulating key enzyme gene expression and activity.

Comparison of the results of qualitative and quantitative analysis suggested that most of the amino acids in the studied *C. angustifolium* samples are in a bound form in the composition of proteins, peptides, and other chemical compounds. The very study of the amino acid composition of *Epilobium* and *Chamaenerion* is of practical interest, since it increases the number of sources for obtaining amino acid complexes. As is known, the amino acids themselves and their complex preparations have a wide range of pharmacotherapeutic activity (hypotensive, immunomodulatory, cytokinetic, hepatoprotective, anti-inflammatory, detoxifying effects, etc.). At the same time, amino acids are the main substances in the biosynthesis of other biologically active compounds.

CONCLUSION

The chemical and chemometric analyses of *C. angustifolium* occurring in Ukraine and Lithuania applying NMDS and hierarchical cluster analysis indicated that the geographic origin of samples of this species can be differentiated by the two amino acids, L-alanine and L-phenylalanine. These amino acids can be also used as chemical markers for the evaluation of the quality of *C. angustifolium* raw material. Additionally, analysis showed existence of different chemotypes of *C. angustifolium* and their relation to the geographic origin. In combination with other groups of biologically active compounds (phenolic compounds, polysaccharides, macro- and microelements, and others), this emphasizes the therapeutic significance of *C. angustifolium* samples and makes it possible to develop new affordable drugs of combined action based on a promising type of medicinal plant material. The comparative amino acid composition of the studied plant was analyzed for the first time.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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The authors have no conflicts of interest to declare.

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This study does not involve experiments on animals or human subjects.

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