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Carboxylic Acids and Amino Acids of *Galium pseudomollugo* Herb

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ABSTRACT

In Galium pseudomollugo Klokov herb, by means of gas chromatography-mass spectrometry 30 carboxylic acids have been identified and quantified, including 23 monobasic acids, 6 dibasic acids and 1 tribasic acid. By the radical linked to the carboxyl group, the acids were classified as 9 aromatic acids and 21 aliphatic acids, including 12 fatty acids, 9 of which were saturated and 3 unsaturated; 1 keto acid was identified. The dominant was levulinic acid. By HPLC in G. pseudomollugo herb 21 amino acids have been identified; the free amino acid content equaled 0.98%, 0.25% of which fell on essential amino acids, whereas the content of bound amino acids amounted to 5.75%, 2.51% of which fell on essential amino acids. Identified as prevailing in the total amino acid content were aspartic acid, glutamic acid and proline. Dominant among free amino acids were aspartic acid, asparagine and proline.

Keywords: *Galium pseudomollugo Klokov*, Organic dibasic acids, Aromatic acids, Fatty acids, Amino acids

INTRODUCTION

Among *Galium* L. genus species (Madder family, Rubiaceae Juss.) *Galium pseudomollugo Klokov* is described only for Ukrainian flora (section *Eugalium* DC, serie *Erecta* Pobed.) and replaces *G. mollugo* species [1]. According to the current data *G. pseudomollugo Klokov* species is one of the numerous synonyms of *G. mollugo*. The comparison of the herbarium specimens of the species harvested in the Kharkiv region with the herbarium specimens of *G. mollugo* at Pharmacognosy Department revealed distinctive features of the species under study, namely narrow-pyramidal inflorescence crowded on the main stem (whereas *G. mollugo* bears spreading paniculate inflorescence), differences in middle stem leaves structure as well as in the number of leaves in the middle stem whorls and in bract whorls, which enabled identifying the species under study as *G. pseudomollugo Klokov*, restricted to woodland and forest-steppe areas of southern Ukraine along the Vinnitsa–Bila Tserkva–Poltava–Kharkiv axis [1,2].

Currently, *Galium pseudomollugo* is used in homeopathy (in comprehensive treatment of chronic bronchitis, chronic sinusitis, bronchial asthma, neurodermatitis or eczema) and in folk medicine as a diuretic, choleretic, sedative, antispasmodic, or in epilepsy treatment [3].

It was previously established that the underground parts of the plant accumulate anthraquinone derivatives of the alizarin type, whereas the aerial parts accumulate phenolcarboxylic acids, flavonoids, and tannins [3]. We studied a lipophilic complex of *G. pseudomollugo* herb, which proves to produce a pronounced antibacterial and antifungal activity and determined its chemical profile [4,5]. As is known, hydroxy acids and dibasic carboxylic acids show an antimicrobial activity, whereas fatty acids display an antifungal activity against a number of pathogenic fungi [6]. Amino acids are an independent class of biologically active substances (BAS) and can affect the bioavailability and activity of other BAS classes.

The aim of the present research was to study the composition of the carboxylic acids and amino acids of *Galium pseudomollugo Klokov* herb.

MATERIALS AND METHODS

The plant material was harvested in the Kharkiv oblast (Novo-Vodolazhskiy region) in June 2015 (herbarium specimen No 17/15 stored at the Herbarium of Pharmacognosy Department of the National University of Pharmacy).

For the analysis of the content of carboxylic acids in the air-dried plant material (50 mg) in a 2 ml vial, an internal standard (50 mg of tridecane in hexane) was added as well as 1.0 ml of a methylating agent (BCl_3 in methanol, 14% solution, Supelco 3-3033) [7].

For the extraction and hydrolysis of fats and other esters as well as for a simultaneous methylation of fatty acids and other organic acids, the mixture was heated in a sealed vial for 8 h at 65ºC. Then, the reaction mixture was decanted from the plant material and the precipitate was diluted in 1 ml distilled water, from which methyl esters of the carboxylic acids were extracted with 0.2 ml methylene chloride. The mixture was gently shaken several times within an hour and then the obtained extract of the methyl esters was chromatographed. The sample injection (2 μl) was performed in a splitless mode at an injection rate of 1.2 ml/min per 0.2 min.

The analysis of the amino acids was performed with the use of a high-performance liquid chromatograph, Agilent Technologies 6890, equipped with a mass-spectrometric detector 5973. The chromatographic conditions were as follows, the INNOWAX capillary chromatographic column of i.d. 0.25 mm, length 30 m; the carrier gas (helium) flow rate of 1.2 ml/min; and the inlet heater temperature of 250ºC with the oven temperature program from 50ºC to 250ºC at the rate of 4°C/min. The components identification was performed with the use of mass spectra libraries NIST 05 and Willey 2007 with a total of more than 470,000 spectra in combination with AMDIS and NIST identification software. The content of compounds was calculated with the internal standard taken into account.

The analysis of the amino acid composition of the samples under study was carried out with the use of a HPLC chromatograph, Agilent Technologies (Model 1100), equipped with a G1379A flow-vacuum degasser, a G13111A four-channel low-pressure gradient pump, a G1313A automated injector, a G13116A column thermostat and a G1316A diode-matrix detector. With the view to determining the free amino acids, a 10 ml vial was placed on analytical scales and 0.30 g of finely powdered plant material was weighed. Then, 3 ml of 0.1 N aqueous solution of hydrochloric acid with 0.2% of β-mercaptoethanol was added. The vial was sealed and placed in an ultrasonic bath for 2 h at 50ºC. With the view to quantifying a total of free and bound amino acids, a 10 ml vial was placed on the analytical scales and 0.20 g of finely powdered raw material was weighed. Then, 3 ml of 6 N aqueous solution of hydrochloric acid with 0.4% of β-mercaptoethanol was added. The vial was sealed and placed in the ultrasonic bath for 24 h at 110ºC [8]. The contents of the vials were centrifuged and filtered. The prepared filtrates were added into 2 ml vials, i.e., 100 μl of filtrate for the free amino acids and 20 μl of filtrate for the assay of the total amino acid content, which were placed in a vacuum desiccator at 40-45ºC and under the pressure of less than 1.5 mm of mercury until the complete removal of hydrochloric acid. *Via* an automatic dispenser, 200 μl of 0.8 M borate buffer pH 9.0, 200 μl of 20 mM solution of 9-fluorene methoxycarbonyl chloride in acetonitrile was added into the reaction vials, followed by 20 μl of 150 mM solution of amantadine hydrochloride in a 50% aqueous solution of acetonitrile after 10 min. For the assay of the amino acids, the ZORBAX-SB C-18 chromatographic column (4.6 × 50 mm) filled with octadecylsilyl sorbent (in 1.8 μm particles) and a guard column as well as standard solutions of amino acids (TC 6-09-3147-83) were used. The chromatographic conditions were described in a previously published article [9].

The identification of the amino acids was performed by comparison of their retention time with those of the standards. The calculation of the content of the bound amino acids was made by subtracting the free amino acids from the total amino acid content. However, it should be taken into account that in the course of the acid hydrolysis, asparagine and glutamine almost quantitatively convert to glutamic acid and aspartic acid, respectively. Thus, the calculation of the bound amino acid content, i.e., asparagine and aspartic acid, and glutamine and glutamic acid was made by their total content, respectively.

RESULTS AND DISCUSSION

In *Galium pseudomollugo* herb 30 carboxylic acids have been identified (Table 1).

Identified carboxylic acids included 23 monobasic acids, 6 dibasic acids and 1 tribasic acid. By the radical linked to the carboxyl group the acids were classified as 9 aromatic acids and 21 aliphatic acids, including 12 fatty acids (9 saturated fatty acids and 3 unsaturated fatty acids) and 1 keto acid.

The content of dibasic acids totaled 6413.3 mg/kg, the aromatic acid content totaled 1839.0 mg/kg, whereas the content of fatty acids was 6272.4 mg/kg. The content of the saturated fatty acids equaled 56.39% of the total fatty acid content, and the unsaturated fatty acid content was 43.61%. The dominating acids (of the total carboxylic acid content) were levulinic acid (27.01%)–monobasic γ-ketoacid, tribasic citric acid (16.93%) and, among the dicarboxylic acids, dominating was oxalic acid (12.14%). Among aromatic acids, prevailing were hydroxycinnamic acids, namely ferulic acid (1.98%) and *p*-coumaric acid (1.17%) and, among fatty acids, palmitic acid (5.98%) and linoleic acid (3.45%) dominated.

The total carboxylic acid content in *Galium pseudomollugo* herb was 3.52%. Note worthily, levulinic, fumaric and succinic acids display antimicrobial and fungicidal properties. In *Galium pseudomollugo* herb, 21 amino acids have been identified, including 20 proteinogenic acids, 8 of which were essential and 12 were non-essential (including 2 conditionally essential amino acids) and 1 non-proteinogenic amino acid (Table 2).

In *Galium pseudomollugo* herb, the free amino acid content totaled 0.98%, 0.25% of which fell on essential amino acids; whereas the content of bound amino acids amounted to 5.75%, 2.51% of which fell on essential amino acids. In the plant material under study, the total of free and bound amino acids equaled 6.72%, 3.03% of which fell on essential amino acids.

Among essential proteinogenic amino acids, prevailing was aspartic acid (with 16.21% of the total of free and bound amino acids), whereas among non-essential proteinogenic amino acids, dominating were glutamine (11.90%) and proline (9.60%).

Among free non-essential amino acids, prevailing was aspartic acid (with 7.46% of the total of free amino acids), and among free amino acids, prevailing were asparagine (28.18%) and proline (20.06%).

Table 1: Carboxylic acids of *Galium pseudomollugo* herb

No.	Ret. time, min	Acid	Content, mg/1000 g
1	5.815	Caproic (N-Hexanoic)	35.56
2	10.856	Oxalic (Ethanedioic)	4278.13
3	13.182	Malonic (Propanedioic)	1055.25
4	14.002	Fumaric (Butenedioic)	153.99
5	14.682	Levulinic (4-Oxopentanoic)	9523.06
6	15.128	Succinic (Butanedioic)	585.12
7	15.731	Benzoic	47.44
8	18.887	Phenylacetic	19.45
9	19.2	Salicylic (2-Hydroxybenzoic)	88.45
10	22.272	2-Hydroxy-3-methylglutaric (2-Hydroxy-3-methylpentanedioic)	133.71
11	23.895	Malyc (2-Hydroxybutanedioic)	5237.38
12	23.99	Myristic (Tetradecanoic)	233.70
13	26.371	Azelaic (Nonanedioic)	207.11
14	27.883	Palmitic (Hexadecanoic)	2109.09
15	31.268	Citric (2-Hydroxypropane-1,2,3-tricarboxylic)	5967.26
16	31.43	Stearic (Octadecanoic)	201.42
17	31.764	Oleic (9Z)-Octadec-9-enoic acid)	459.04
18	32.584	Linoleic (9Z,12Z)-9,12-Octadecadienoic)	1216.23
19	33.66	Linolenic	1060.13
20	34.291	Vanillic (4-Hydroxy-3-methoxybenzoic acid)	327.69
21	34.737	Arahidic (Eicosanoic)	217.46
22	34.887	2-Hydroxypalmitic (2-Hydroxyhexadecanoic)	92.87
23	37.826	Behenic(Docosanoic)	227.34
24	38.423	*p*-Coumaric (E)-3-(4-Hydroxyphenyl)-2-propenoic)	412.21
25	39.425	*p*-Hydroxybenzoic	29.59
26	39.739	Syrringic (4-Hydroxy-3,5-dimethoxybenzoic)	103.24
27	40.303	Gentisic (2,5-Dihydroxybenzoic)	113.16
28	40.738	Lignoceric (Tetracosanic)	310.82
29	42.55	Ferulic (E)-3-(4-Hydroxy-3-methoxy-phenyl)-prop-2-enoic)	697.70
30	44.429	Pentacosanic	108.77

Table 2: Amino acids of *Galium pseudomollugo* herb

Amino acid	Content, mg/100 g of the plant material		
	Total of bound and free amino acids	Free amino acids	Bound amino acids
Essential proteinogenic amino acids			
Val	281.1	34.3	246.8
Ile	210.0	18.3	191.7
Leu	440.3	16.7	423.6
Lys	285.9	10.6	275.3
Met	76.8	35.3	41.5
Asp	1089.8	72.7	742.3
Thr	341.4	40.1	301.3
Phe	302.5	17.7	284.8
Total	3027.8	245.7	2507.3
Non-essential proteinogenic amino acids			
Gly	391.8	7.4	384.4
Ala	454.7	26.2	428.5
Ser	461.3	85.9	375.4
Arg*	379.0	15.4	363.6
Glu	800.1	26.5	765.4
Asn	0	274.8	0
Pro	645.7	195.6	450.1
4-Hyp	133.6	5.6	128.0
Cys	8.3	0	8.3
Tyr	201.9	39.7	162.2
His*	166.0	17.7	148.3
Gln	0	8.2	0
Total	3642.4	703.0	3214.2
Non-proteinogenic amino acids			
γ-Abu (GABA)	53.5	26.4	27.1
Total	6723.7	975.1	5748.6

Note: *Conditionally essential amino acids.

CONCLUSION

Presence of levulinic, ferulic and succinic acids may account for antimicrobial and fungicidal effect in the substances of *Galium pseudomollugo* herb. Whereas presence of aspartic acid may account for sedative effect. Carboxylic acids and amino acids in *Galium pseudomollugo* herb were first studied.

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