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Hydroxycinnamic and organic acids of snowdrops (*Galanthus* L.)

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ABSTRACT

The composition and content of hydroxycinnamic (HCA) and organic (OA) acids were investigated by HPLC with UV detection in Voronov's snowdrop (*Galanthus woronowii* Losinsk.) and common snowdrop (*Galanthus nivalis* L.) crude herbal drugs. *G. woronowii* HCA composition is presented by neo chlorogenic, chlorogenic and cryptochlorogenic acids. Neo chlorogenic acid is absent in *G. nivalis*, but *G. nivalis* also contains chlorogenic and cryptochlorogenic acids. The total HCA content in *G. woronowii* was 84.1 ± 6.7 mg / 100 g, and in *G. nivalis* – 62.0 ± 5.0 mg / 100 g (0.06-0.08%). OA profile of two snowdrop species is presented by succinic, oxalic and malic acids. The total OA content in *G. woronowii* was 237.7 ± 2.4 mg / 100 g, and in *G. nivalis* – 405.1 ± 4.7 mg / 100 g (0.2-0.4%). The obtained data concerning minor specific biologically active substances composition can be used in the standardization and activity evaluation of drugs produced from snowdrops.

INTRODUCTION

Snowdrop (*Galanthus* L.) genus comprises herbaceous ephemeroid bulbous plants of the *Amaryllidaceae* J.St.-Hilfamily, it includes about 19 species and 2 hybrids of natural origin (World Checklist of Selected Plant Families). Voronov's snowdrop (*Galanthus woronowii* Losinsk.) and common snowdrop (*Galanthus nivalis* L.) are most abundant in Russia (Bokov *et al.*, 2015). Despite the fact that the snowdrops are the plants listed in the Red data Book, they are easy to cultivate on a commercial scale (Maisuradze *et al.*, 2000). Snowdrops became widely known thanks to the containing a number of Amaryllidaceae alkaloids, other groups of biologically active substances (BAS) remain poorly investigated. Snowdrops BAS complex, containing organic and hydroxycinnamic acids, exhibits a wide range of biological activities. *G. woronowii* and *G. nivalis* are used as sources of homeopathic crude herbal drugs in Russia and other countries (Bokov *et al.*, 2015). Hydroxycinnamic acids (HCA) – are the most common polyphenolic acids in higher plants. HCA are generally presented in forms of glycosylated derivatives or esters of ferulic, coumaric, caffeic, and sinapic acids with tartaric, quinic or shikimic acids and complexes with proteins, lignin and cellulose (Bartosz, 2013). In vitro tests of HCA showed strong antiradical and antioxidant properties; they also possess anti-inflammatory, antiviral and immunostimulatory activity (Medvedev *et al.*, 2010). HCA was determined both in herbal drugs and in foods (Dimitrios, 2006; Maruta *et al.*, 1995). Organic acids (OA) of plant origin is another class of compounds exhibiting a wide range of biological activities. Citric, malic, fumaric, succinic, tartaric, shikimic, acetic, oxalic and other acids are among them. They are characterized by anti-inflammatory, immunomodulatory, antioxidant activity, involved in metabolism. OA have a positive effect on the food digestion thanks to the creation of favorable conditions for the living of beneficial gut bacteria (Nollet *et al.*, 2015).

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Also, worth noting is that succinic acid plays a special role, it is a universal intermediate metabolite in the Krebs cycle. Succinic acid exhibits antioxidant properties, has anti acidosic and anti-hypoxic action, being a natural factor of multifactor resistence of the body. Succinate ion is found in all cells capable for aerobic respiration while generating capacity of ATP synthesis at the oxidation of succinic acid is much higher than in a oxidation of any other substrate (Wiesner *et al.*, 1986).

The aim of the study was to determine the minor BAS (HCA and OA) in the snowdrops crude herbal drugs.

MATERIALS AND METHODS

Chemicals

Standards (≥98.0%, HPLC) of HCA (neo chlorogenic, chlorogenic acid and cryptochlorogenic) and OA (succinic, oxalic and malic acids) were purchased from Sigma-Aldrich Company Ltd (St. Louis, USA). The organic solvents – acetonitrile (Panreac Quımica, S.A., Barcelona, Spain)and methanol (J.T. Baker, Netherlands) – were HPLC grade. High purity water was prepared by using Millipore Milli-Q plus water purification system. All other chemicals and reagents such as tetrabutylammonium hydroxide, potassium dihydrogen phosphate, formic acid, and phosphoric acid were obtained from Merck Laboratories (Darmstadt, Germany). All other chemicals were of analytical grade purity.

Plant material collection

The objects of the study were whole fresh plants of *Galanthus woronowii* and *G. nivalis* (aboveground and underground parts) collected at flowering stage in the Botanical Garden of I.M. Sechenov First Moscow State Medical University (Moscow, Russia) in March-April 2016.

Determination of HCA

HCA determination was performed by RP-HPLC method with UV detection at 330 nm wavelength in a gradient elution mode (Tutelyan *et al.*, 2010). HCA concentration was determined with the aid of external calibration (HCA standard of Sigma-Aldrich), based on peak areas. To prepare the HCA standard solutions with a concentration of 0.005 mg/ml, 5 mg of substance was placed in 100 ml flask, dissolved in 20 ml of methanol and diluted to the mark with methanol, and then 5 ml aliquot was transferred to a 50 ml flask and diluted to the mark with methanol. Snowdrops plant material (2.0 g) was grounded in a mortar to a mushy state. Twofold extraction was performed by 50% ethyl alcohol (60 and 40 ml) in a boiling water bath under reflux for 40 and 20 minutes. Extracts analysis was carried out using Agilent 1100 Series liquid chromatography system (Agilent Technologies, USA). It was consisted of liquid chromatograph Agilent 1100 Series (Agilent Technologies, USA) equipped with degasser, pump for simultaneous supply of 2 solvents, autosampler with thermostat (samples temperature – 15 ° C), thermostat of chromatographic columns, a diode-array detector (Agilent 1100 Series Diode Array (DAD)), ChemStation processing chromatographic software data. Stationary phase – ProteCol™-P C18 HPH125 (250 × 4.6 mm, 5 µm) column. Mobile phase "A" – 0.1% formic acid solution, The mobile phase "B" – acetonitrile. The gradient elution mode is represented in Table 1. The mobile phase flow rate – 1 mL/min. Column temperature – 25 °C. Injected sample volume – 10 µl.

Determination of OA

OA determination was performed by ion-pair HPLC with UV- detection at 210 nm wavelength in the isocratic elution mode. OA concentration was determined with the aid of external calibration (OA standard of Sigma-Aldrich), based on peak areas (Daood *et al.*, 1994). Three standard solutions were prepared (concentration of 0.1, 0.05 and 0.025 mg/ml) for each organic acid (succinic, oxalic and malic). The solution samples of malic and oxalic acid were prepared by dissolving 50.0 ± 0.1 mg of the standard at 25 °C, and succinic – at 60 ° C in the mobile phase, and then were diluted to the desired concentration. Snowdrops plant material (4.0 g) was grounded to a mushy state in a mortar. Twofold extraction by 1M NaOH solution (60 and 40 ml, pH = 8.5) was performed in a boiling water bath under reflux for 40 and 20 minutes, then it was neutralized to pH = 5.5 by 1 M HCl solution. 2 ml of this extract was evaporated by vacuum centrifuge Concentrator plus (Eppendorf) at 30 ° C and recovered with 2 ml of mobile phase. Extracts analysis was carried out using Agilent 1260 Infinity Quaternary liquid chromatography system (Agilent Technologies, USA). It was consisted of liquid chromatograph Agilent 1260 Series(Agilent Technologies, USA) equipped with micro vacuum degasser, quaternary pump for generating gradients from four individual solvent channels, autosampler with thermostat, chromatographic column thermostat, UV detector (Agilent 1260 Infinity Diode Array Detector (DAD)) and the ChemStation chromatographic data processing software. Stationary phase – Waters Atlantis™ dC18 (250×4.6 mm, 5 µm) column.

The mobile phase was prepared as follows: 1.0 ml of a 20% tetrabutylammonium hydroxide and 30 ml of methanol were added to 970 ml of 0.01M potassium dihydrogen phosphate solution and adjusted the pH to 2.75 ± 0.1 with 85% phosphoric acid (H_3PO_4 85% by weight). Flow rate (isocratic mode) – 1.5 mL/min. Column temperature – 25 °C. Injected sample volume – 10 µl.

RESULTS AND DISCUSSION

Hydroxycinnamic acids

The total HCA content in *G. woronowii* was 84.1 ± 6.7 mg / 100 g, and in *G. nivalis* – 62.0 ± 5.0 mg / 100 g (0.06-0.08%). The HPLC-chromatogram of *G. woronowii* extract (neo chlorogenic, chlorogenic acid and cryptochlorogenic) is shown at Fig. 1. Chlorogenic and cryptochlorogenic acids were found in *G. nivalis* (Fig. 2). The results of HCA determining are shown in Table 2.

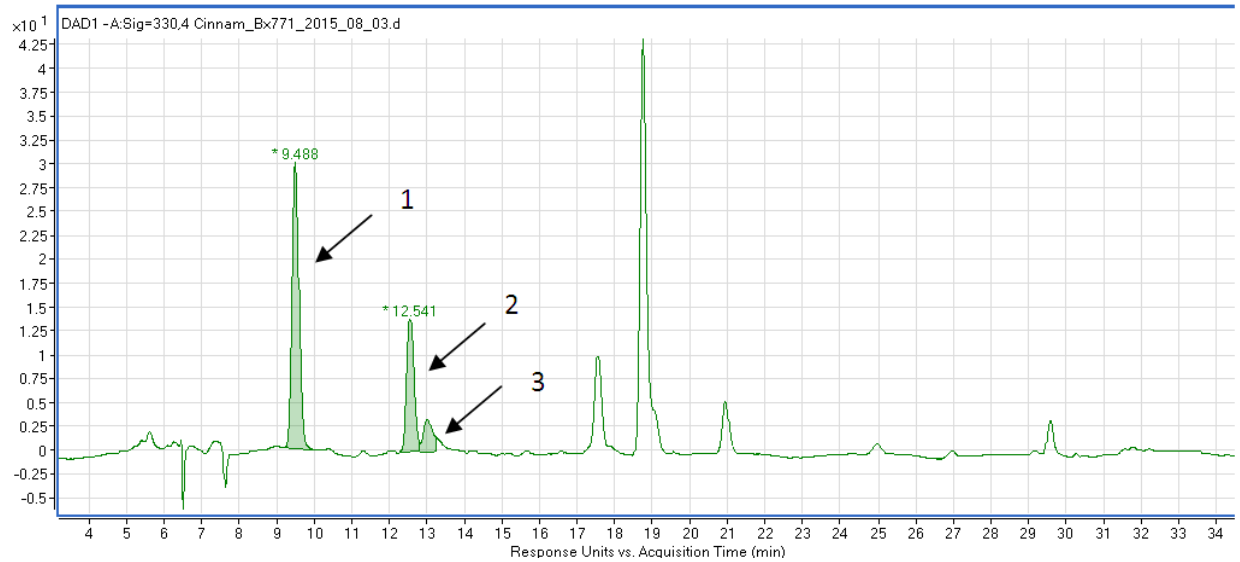

Fig. 1: HPLC-UV chromatogram of *G. woronowii* HCA: 1 – neo chlorogenic acid, 2 – chlorogenic acid, 3 – cryptochlorogenic acid.

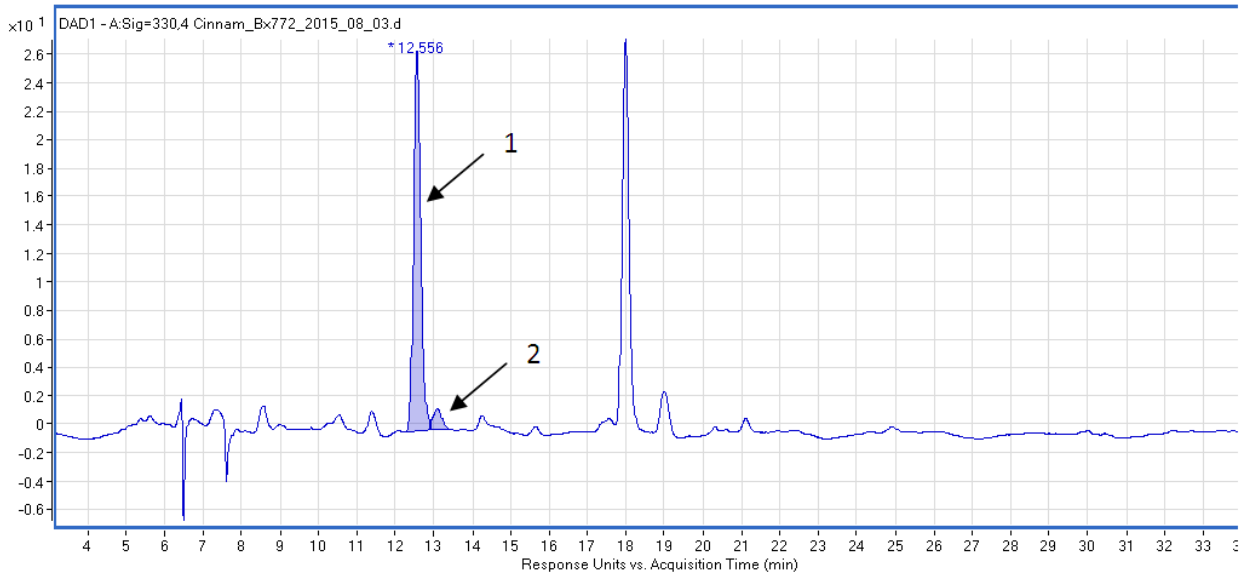

Fig. 2: HPLC-UV chromatogram of *G. nivalis* HCA: 1 - chlorogenic acid, 2 - cryptochlorogenic acid.

Table 1: The gradient elution mode of hydroxycinnamic acids.

Time, min	0	18	30	35
A,%	95	77	70	50
B,%	5	23	30	50

Table 2: Hydroxycinnamic acids of snowdrops (M ± m, n = 5).

HCA	**Retention time, t_R**	**Content, mg / 100 g (calculated to dry material)**	
		G. woronowii	***G. nivalis***
neo chlorogenic	9,5	51,7±4,1	–
chlorogenic	12,6	24,6±2,0	58,2±4,7
cryptochlorogenic	13,0	7,8±0,6	3,8±0,3
Total amount		**84,1±6,7**	**62,0±5,0**

Organic acids

The total OA content in *G. woronowii* was 237.7 ± 2.4 mg / 100 g, and in *G. nivalis* – 405.1 ± 4.7 mg / 100 g (0.2-0.4%). Succinic, malic and oxalic acids are the main OA that were detected (Figs 3, 4). The results of OA determining are shown in Table 3. Metrological characteristics of HCA and OA determination in *G. woronowii* and *G. nivalis*are presented in Table 4.

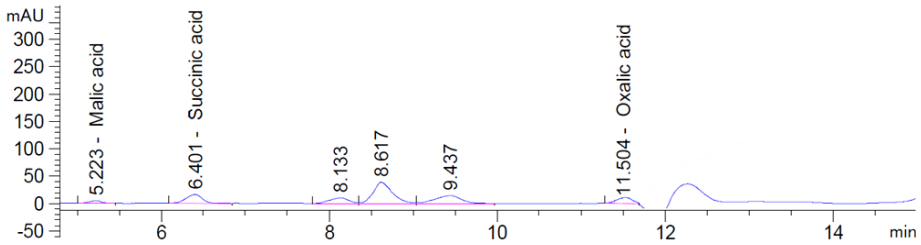

Fig. 3: HPLC-UV chromatogram of *G. woronowii* OA: malic, succinic, oxalic acid.

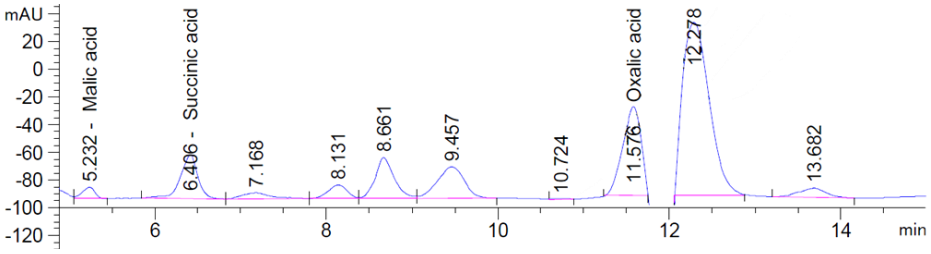

Fig. 4: HPLC-UV chromatogram of *G. nivalis* OA: malic, succinic acid, oxalic acid.

Table 3: The organic acid of snowdrops (M ± m, n = 5).

OA	Retention time, t_R	Content, mg / 100 g (calculated to dry material)	
		G. woronowii	*G. nivalis*
Succinic	6,4	195,2±1,2	334,7±2,3
malic	5,2	29,3±0,8	42,4±1,8
oxalic	11,5	13,2±0,4	28,0±0,6
Total amount		**237,7 ±2,4**	**405,1±4,7**

Table 4: Metrological characteristics of HCA and OA determination in snowdrop species (n = 5, f = 4, P = 95%, T (f, P) = 2.7764).

Object	Biologically active substance	$\bar{X}$	S^2	S	$S_{\bar{x}}$	ΔX	E,%
HCA							
G. woronowii	neo chlorogenic	51,7±4,1	11,061	3,326	1,487	4,13	7,98
	chlorogenic	24,6±2,0	2,699	1,643	0,735	2,04	8,31
	cryptochlorogenic	7,8±0,6	0,243	0,493	0,220	0,61	7,84
G. nivalis	chlorogenic	58,2±4,7	14,497	3,808	1,703	4,72	8,12
	cryptochlorogenic	3,8±0,3	0,076	0,276	0,124	0,34	9,02
OA							
G. woronowii	succinic	195,2±1,2	1,001	1,001	0,448	1,24	0,63
	malic	29,3±0,8	0,434	0,659	0,295	0,81	2,79
	oxalic	13,2±0,4	0,124	0,353	0,158	0,44	3,31
G. nivalis	succinic	334,7±2,3	3,508	1,873	0,838	2,33	0,69
	malic	42,4±1,8	2,169	1,473	0,659	1,83	4,31
	oxalic	28,0±0,6	0,234	0,483	0,216	0,60	2,14

Note. *n* – number of repeat tests, *f* – number of degrees of freedom, *P* % – confidence figure, *T*(*f, P*) – Student's coefficient, $\bar{X}$ – mean value, S^2 – dispersion, S – standard deviation, $S_{\bar{x}}$ – the standard deviation of the mean value, ΔX –confidence interval, E,% – relative error.

CONCLUSION

In the present study, using HPLC-UV analytical method, organic and hydroxycinnamic acids, the main indicators characterizing the content of biologically active compounds in *G. woronowii* and *G. nivalis* crude herbal drugs of Russian origin, are identified for the first time. The data obtained can be used to assess biological activity, as well as establishing a potential pharmacological activity, determining chemotaxonomic indicator components and standardization of drugs, prepared from snowdrops.

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Conflict of Interests: There are no conflicts of interest.

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