

Hydroxycinnamic acids in the raw material of hybrid bearded iris

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Representatives of *Iris* genus (*Iridaceae*) are widespread in almost all continents. According to the literature data, *Iris* species accumulate secondary metabolites such as flavonoids, isoflavonoids, xanthones, coumarins, hydroxycinnamic acids, saponins, tannins, vitamins, organic acids.

The aim of this work was the establishment of the qualitative and quantitative composition of hydroxycinnamic acids in the raw materials of iris hybrid varieties, as well as to establish the antimicrobial activity of the dry extracts and isolation of individual compounds.

Materials and methods. The objects of study were the leaves and rhizomes of the standard dwarf bearded iris (SDB). The substances were isolated by column chromatography, nature was established by physical and physicochemical methods of analysis (UV-, IR spectroscopy, TLC). Antibacterial activity was determined by the agar well diffusion method *in vitro*.

Results. Hydroxycinnamic acids were identified in the iris raw materials by paper chromatography. The spectrophotometric method established of the hydroxycinnamic acid content in the recalculation on chlorogenic acid – was from 0.79 % to 2.83 %. The dry extracts of the leaves and rhizomes of *I. hybrida* "Little Dream" at a 1 % concentration have shown moderate inhibitory activity for Gram-positive bacteria and fungi. For the first time from the rhizomes of *Iris hybrida* "Indian Pow Waw" were isolated caffeic, chlorogenic, neochlorogenic, ferulic acids by the column chromatography and their structures were determined by spectral methods.

Conclusions. The content of hydroxycinnamic acids in the samples varies from 0.79 % to 2.83 %. The antimicrobial activity of the dry extracts of *I. hybrida* "Little Dream" leaves and rhizomes was established. For the first time from the rhizomes of *Iris hybrida* "Indian Pow Waw" were isolated 4 derivatives of hydroxycinnamic acid. The results of the study show the prospects for the use of cultivated varieties of irises as a source of hydroxycinnamic acids.

Ключові слова:

півники, гідроксикоричні кислоти, антибактеріальна активність, хроматографія.

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Гідроксикоричні кислоти в сировині гібридних бородатих півників

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Представники роду *Iris* (*Iridaceae*) поширені майже на всіх континентах. За даними фахової літератури, види півників накопичують вторинні метаболіти, як-от флавоноїди, ізофлавоноїди, ксантони, кумарини, гідроксикоричні кислоти, сапоніни, таніни, вітаміни, органічні кислоти.

Мета роботи – встановлення якісного та кількісного складу гідроксикоричних кислот у сировині гібридних сортів півників, а також антимікробної активності сухих екстрактів і виділення індивідуальних речовин.

Матеріали та методи. Об'єкти дослідження – листя, кореневища стандартних карликових бородатих півників (SDB). Речовини виділяли методом колонкової хроматографії, природу встановлювали фізичними та фізико-хімічними методами аналізу (УФ-, ІЧ-спектроскопія, ТШХ). Антибактеріальну активність визначали методом дифузії в агар *in vitro*.

Результати. Гідроксикоричні кислоти ідентифіковані в сировині півників методом хроматографії. Спектрофотометричним методом встановили вміст гідроксикоричних кислот у перерахунку на хлорогенову кислоту – від 0,79 % до 2,83 %. Сухі екстракти листя, кореневищ *I. hybrida* «Little Dream» при концентрації 1 % показали помірну інгібувальну активність на грампозитивні бактерії та гриби. Уперше колонковою хроматографією з кореневищ *Iris hybrida* «Indian Pow Waw» виділені кавова, хлорогенова, неохлорогенова, ферулова кислоти, а їхня структура встановлена інструментальними методами.

Висновки. Вміст гідроксикоричних кислот у зразках варіює від 0,79 % до 2,83 %. Встановили антимікробну активність сухих екстрактів листя та кореневищ *I. hybrida* «Little Dream». Уперше з кореневищ *Iris hybrida* «Indian Pow Waw» виділили 4 похідні гідроксикоричної кислоти. Результати дослідження свідчать про перспективність використання культивованих сортів півників як джерела гідроксикоричних кислот.

Гидроксикоричные кислоты в сырье гибридных бородатых ирисов

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Представители рода *Iris* (*Iridaceae*) распространены почти на всех континентах. По данным научной литературы, виды ирисов накапливают вторичные метаболиты, такие как флавоноиды, изофлавоноиды, ксантоны, кумарини, гидроксикоричные кислоты, сапонины, танины, витамины, органические кислоты.

Цель работы – установление качественного и количественного состава гидроксикоричных кислот в сырье гибридных сортов ирисов, а также установление антимикробной активности сухих экстрактов и выделение индивидуальных веществ.

Материалы и методы. Объекты исследования – листья и корневища стандартных карликовых бородатых ирисов (SDB). Вещества выделяли методом колоночной хроматографии, природу устанавливали физическими и физико-химическими методами анализа (УФ-, ИК-спектроскопия, ТСХ). Антибактериальную активность определяли методом диффузии в агар *in vitro*.

Результаты. Гидроксикоричные кислоты идентифицированы в сырье ирисов методом хроматографии. Спектрофотометрическим методом установлено содержание гидроксикоричных кислот в пересчете на хлорогеновую кислоту – от 0,79 % до 2,83 %. Сухие экстракты листьев и корневищ *I. hybrida* «Little Dream» при концентрации 1 % показали умеренную ингибирующую активность в отношении грамположительных бактерии и грибов. Впервые методом колоночной хроматографии из корневищ *Iris hybrida* «Indian Pow Waw» выделены кофейная, хлорогеновая, неохлорогеновая, феруловая кислоты, а их структура установлена инструментальными методами.

Выводы. Содержание гидроксикоричных кислот в образцах варьирует от 0,79 % до 2,83 %. Установлена антимикробная активность сухих экстрактов листьев и корневищ *I. hybrida* «Little Dream». Впервые из корневищ *Iris hybrida* «Indian Pow Waw» выделены 4 производные гидроксикоричной кислоты. Результаты исследования свидетельствуют о перспективности использования культивируемых сортов ирисов как источника гидроксикоричных кислот.

Ключевые слова: ирисы, гидроксикоричные кислоты, антибактериальная активность, хроматография.

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The genus *Iris* (Iridaceae) has long been known as ornamental, honey-bearing and medicinal plants, the number of which covers more than 300 species [1]. *Iris* count more than 4.000 hybrid varieties, distributed not only in Ukraine but also in Europe, Central, and Eastern Asia, North and South America [2]. According to the literature, *iris* accumulate secondary metabolites – flavonoids, isoflavonoids, xanthenes, coumarins, hydroxycinnamic acids, saponins, tannins, vitamins, organic acids [3]. In folk medicine, the rhizomes of *iris* have been used as an anti-inflammatory, antibacterial, laxative, analgesic agent [4]. Based on these data, the phytochemical study of *iris* has a practical interest in the modern pharmacy.

As a result of the previous study, the hydroxycinnamic acids were identified in the leaves and rhizomes of *Iris hungarica*, and their quantitative content was established 2.6 % in the rhizomes and 4.7 % – in the leaves [5].

The hydroxycinnamic acids belong to the class of phenolic compounds, were found in almost all plants in the free acids form, their dimers, and esters. The pharmacological activity of this group of biologically active substances is being studied, there is information about choleric, antimicrobial, tuberculostatic, antioxidant, hepatoprotective activity [6,7]. The caffeic acid and its derivatives (chlorogenic and isomers), which have an anti-inflammatory and choleric effect, are most common in nature [8]. Ferulic acid is found in plant cells. Together, chlorogenic, ferulic, caffeic, coumaric acids have a hypozotemic effect, enhance kidney function and stimulate the anti-toxic function of the liver [9].

Aim

Continuing the study of *Iris* genus plants the aim was to establish the qualitative and quantitative composition of hydroxycinnamic acids in the raw materials of *iris* hybrid varieties, as well as to establish the antimicrobial activity of dry extracts and isolation individual compounds.

Materials and methods

Experimental. For determination of the received substances structures physical and physicochemical methods of analysis (UV-, IR spectroscopy, chromatography in a thin layer of sorbent (Silufol UV-254 plates; silica gel 60 F₂₅₄ TLC plates), column chromatography on silica gel (230–400 mesh, Merck, Germany) were used. Melting point (MP) was determined on the Kofler block (Franz Kustner ngch

K:G:Dresden; N.K.70/3314k). The substances were dried on rotary evaporator “Heidolph 2 WB eco, Laborata400 efficient” (Germany). UV-absorption spectra and optical density were recorded with a spectrophotometer module of Thermo Scientific Evolution 60S UV (USA) in cells with a layer thickness of 10 mm. IR-spectra were recorded on the instrument Tensor 27, UR20 (GDR) in the tablets of potassium bromide.

Plant material. The objects of the study were leaves and rhizomes of varietal bearded irises, harvested in the vegetation phase in 2017 in the M. M. Gryshko National Botanical Gardens of the National Academy of Sciences of Ukraine (Kyiv, Ukraine): *Iris hybrida hort.* “Bright white”, *Iris hybrida hort.* “Indian Pow Waw”, *Iris hybrida hort.* “Galleon Gold”, *Iris hybrida hort.* “Mini Dynamo”, *Iris hybrida hort.* “Little Dream”. These varieties belong to the group of standard dwarf bearded irises, which are widely cultivated and valuable for their high decorative properties. The objects of research were identified by one of the authors (Yu. V. Buidin)

Plant material was dried to air-dry condition, stored in a cool well-ventilated place. The raw materials were crushed to a particle size of 2–3 mm for qualitative, quantitative analysis and for the isolation of substances.

Qualitative analysis. Analytical samples of leaves and rhizomes of varieties *iris* were crushed to 2–3 mm particles, an extracting agent (70 % ethanol) was added in a ratio of 1 : 10, a reflux condenser was added and heated in a water bath at 90 °C for 1 hour to obtain extracts. After cooling, the extracts were filtered through a paper folded filter. Qualitative analysis was performed by paper and thin-layer chromatography in the solvent systems of 15 % acetic acid, 2 % acetic acid.

On the chromatograms, the substances were detected by the characteristic fluorescence in UV light at a wavelength of 365 nm and 254 nm. Ammonia vapors, 2 % alcohol solution of aluminum chloride, 10 % sodium/potassium hydroxide solution, 5 % alcohol solution of diazotized sulfanilic acid (diazo reactive) were used as developers, which allows obtaining zones with brighter and more characteristic fluorescence in UV light. The R_f of spots was also determined for the identification of substances and compared with standard samples of phenol carboxylic acids – chlorogenic, neochlorogenic, coffee, ferulic, cinnamic, hydroxycinnamic, p – coumaric.

Quantitative analysis. To establish the quantitative content of the amount of hydroxycinnamic acids in the leaves and rhizomes of varieties irises the spectrophotometry

method was used ("Herb Egeronum Canadian" (TPM 42-U-6/37-323-96) [10]: about 1.0 gram (an exact weight) of the crushed raw material was placed in a 200 ml flask, 50 ml of 40 % ethyl alcohol was added, a reflux condenser was added and heated in a water bath for 1 hour, maintaining a gentle boil. After cooling, the extract was filtered through a paper filter. 1 ml of the obtained extract was transferred to a 50 ml volumetric flask and the volume was adjusted to the mark with 20 % ethyl alcohol. 20 % ethyl alcohol was used as a compensation solution. Spectra were measured at a wavelength of 327 nm. The content of hydroxycinnamic acids in terms of chlorogenic acid and absolutely dry raw materials was calculated by the formula, using the specific absorption rate of chlorogenic acid:

$$X, \% = \frac{A * 50 * 50 * 100}{531 * m_n * 1 * (100 - W)}$$

where **A**: the optical density of the test solution; **m_n**: sample weight, g; **W**: weight loss on drying, %; **531**: specific absorption rate of chlorogenic acid.

Isolation of individual compounds. *Iris hybrida* "Indian Pow Wow" raw material was used for the experiment, collected in the M. M. Gryshko National Botanical Garden in 2017. Air – dry raw materials (rhizomes) in the total mass of 1 kg were extracted with 70 % ethyl alcohol (10 L). The extract (8.5 L) concentrated to a volume of 800–900 ml and left for 24 hours to precipitate chlorophylls and gums (5–10 °C). The tar precipitate was filtered and washed by hot water (0.5 L). The resulting extract was evaporated and treated sequentially with chloroform, ethyl acetate, butanol. The fractions were evaporated until the solvents were removed.

The qualitative composition of the fractions was identified by paper and thin-layer chromatography. Chloroform and ethyl acetate extraction had a similar component composition, so they were combined. For this, the chloroform extraction was evaporated to a minimum amount, washed with 96% ethyl alcohol, and then treated with ethyl acetate.

The combined fraction was evaporated, mixed with 5 g of silica gel and applied to the column (d = 4.5; h = 80). The substances were eluted with chloroform and a mixture of chloroform: ethanol with an increasing concentration of alcohol. 120 fractions were obtained.

The composition of all the obtained fractions was controlled by paper and thin-layer chromatography: solvent system BAW (n-butanol – acetic acid – water) 4 : 1 : 2, 15 % acetic acid, chloroform – methanol 8:2. Similar fractions were combined. In fractions 41–46 and 74–78 substances previously classified as phenol carboxylic acid derivatives were detected.

Spectral data

Caffeic acid. C₉H₈O₄, yellow substance, mp 194–195 °C. UV λ max (C₂H₅OH) nm: 325, 203. Rf 0.32 (solvent system – 2 % acetic acid). IR (KBr), ν, cm⁻¹: 3400, 3235, 2975 (OH), 1647, 1630 (C = O), 1607, 1540 (Ar), 880, 860 (substituted benzene).

Chlorogenic acid. C₁₆H₁₈O₉, mp 203–205 °C. UV λmax (C₂H₅OH) nm: 325, 273. Rf 0.66 (solvent system – 2 % acetic acid); IR (KBr), ν, cm⁻¹: 2970, 2900, 2780 (OH), 1740–1710 (ester group), 1660, 1625 (C = O), 1605–1550 (Ar), 840 (substituted benzene).

Neochlorogenic acid. C₁₆H₁₈O₉, amorphous solid; UV λmax (C₂H₅OH) nm: 325, 300. Rf 0.70 (solvent system – 2 % acetic acid).

Ferulic acid. C₁₀H₁₀O₄, mp 168–170 °C. UV λ max (C₂H₅OH) nm: 320, 274 should. Rf 0.43 (solvent system – 2 % acetic acid); IR (KBr), ν: 3450 cm⁻¹ (carbonic acid OH), 1690 cm⁻¹ (C = O), 1275 cm⁻¹ and 1510 cm⁻¹ (carboxylic acid C-O stretching), 1605 cm⁻¹ (aromatic C = C) confirms the skeleton of ferulic acid.

Extraction procedure of plant for bioassay. To determine the antibacterial activity previously was obtained dry extracts from the leaves and rhizomes of irises. 100.0 g of crushed raw materials were placed in a round-bottom flask, poured 1000 ml of solvent (distilled water), heated in a boiling water bath for 2 hours. The first portion of the extract was taken, after which 500 ml of distilled water was added to the raw material and heated in a water bath for another 2 hours. The combined extract was filtered through a Buchner funnel and evaporated to dryness.

Antibacterial activity. According to the WHO recommendations, antibacterial activity was determined by agar well diffusion method *in vitro*. The inoculum suspension was prepared using a Densi-La-Meter apparatus (PLIVA-Lachema, 540 nm). The cultures were synchronized using low temperature (4 °C). The microbial load was 10⁷ cells per 1 ml of the medium and was determined according to the McFarland standard [11]. The 18–24 – hour culture of microorganisms was used for the test. Mueller-Hinton agar was used ("Himedia Laboratories Pvt. Ltd India", India) for bacteria. The strains of *Candida albicans* were cultivated using Sabouraud agar ("Himedia Laboratories Pvt. Ltd India", India). The standard medium was prepared according to the requirements of the manufacturer. When determining microorganisms' sensitivity to the samples of iris take into account zones of growth of bacteria strains. An alcoholic solution of chlorophyllipt at a concentration of 10 mg/ml was used as a reference drug (GNZLS "OZ").

Statistical data processing was carried out according to the requirements of the State Pharmacopoeia of Ukraine using software (Microsoft Office Excel 7.0) [12].

Results

Qualitative analysis. According to the results of qualitative reactions with alcoholic solutions of sodium hydroxide, aluminum chloride and diazoreactant on chromatograms with extracts *Iris hybrida hort.* "Bright white", *Iris hybrida hort.* "Indian Pow Wow", *Iris hybrida hort.* "Galleon Gold", *Iris hybrida hort.* "Mini Dynamo", *Iris hybrida hort.* "Little Dream" was found more than 8–21 substances of phenolic nature. At the same time, absorption zones with blue, violet, and blue-violet fluorescence characteristic of phenol carboxylic acids were identified.

Chromatograms differed in the number of spots, their chromatographic behavior, the color of the spots before and after their treatment in the UV light, the values of Rf.

According to the chromatographic behavior and comparison with the standards in the all iris varieties were identified caffeic, cinnamic, hydroxycinnamic, *p*-coumaric, fumaric, chlorogenic, and neochlorogenic acids (Table 1).

Quantitative analysis. The quantitative content of hydroxycinnamic acids in the leaves and rhizomes of varieties

Table 1. The identification parameters of hydroxycinnamic acids in the raw materials of iris varieties

Hydroxycinnamic acids	UV fluorescence			Rf	The type of the raw material, in which the acid was identified		
	before treatment	after treatment NH ₃	after treatment with diazoreactive		solvent system: 2 % acetic acid	leaves	rhizomes
chlorogenic	blue	blue – green	red – brown	0.66		in the all objects	in the all objects
neochlorogenic	blue	blue – green	dark – red	0.70		in the all objects	in the all objects
ferulic	blue	blue-violet	red – brown	0.43		<i>I. hybrida</i> "Galleon Gold" <i>I. hybrida</i> "Little Dream" <i>I. hybrida</i> "Indian Pow Waw"	<i>I. hybrida</i> "Galleon Gold" <i>I. hybrida</i> "Little Dream" <i>I. hybrida</i> "Indian Pow Waw"
cinnamic	pale – blue	blue-violet	brown	0.54		in the all objects	in the all objects
hydroxycinnamic	brown	brown	red – brown	0.58		in the all objects	in the all objects
caffeic	pale – blue	bright – blue	red – brown	0.32		<i>I. hybrida</i> "Bright white" <i>I. hybrida</i> "Indian Pow Waw"	<i>I. hybrida</i> "Mini Dynamo" <i>I. hybrida</i> "Little Dream"
p-coumaric	blue	blue-violet	dark – red	0.48		<i>I. hybrida</i> "Bright white" <i>I. hybrida</i> "Little Dream"	<i>I. hybrida</i> "Bright white" <i>I. hybrida</i> "Little Dream"

irises was established by the spectrophotometric method, the data are presented in Table 2.

Antibacterial activity. Hydroxycinnamic acids exhibit many pharmacological activities, including antibacterial. Based on the data obtained, in the leaves and rhizomes of *I. hybrida* "Little Dream" contains the maximum amount of hydroxycinnamic acids, antimicrobial activity was established by the method of diffusion into agar (Table 3).

Discussion

Fumaric acid was found in the leaves and rhizomes of *I. hybrida* "Galleon Gold", *I. hybrida* "Little Dream", *I. hybrida* "Indian Pow Waw"; *n*-coumaric – in the leaves and rhizomes of *I. hybrida* "Bright white", *I. hybrida* "Little Dream"; caffeic – in the leaves *I. hybrida* "Bright white", *I. hybrida* "Indian Pow Waw", and in the rhizomes of *I. hybrida* "Mini Dynamo", *I. hybrida* "Little Dream". Chlorogenic, neochlorogenic, cinnamic and hydroxycinnamic acids were found in all objects.

The content of hydroxycinnamic acids in the samples varies from 0.79 % to 2.83 %. The smallest content in the rhizomes is from 0.79 % to 2.49 %, which is due to the peculiarities of the metabolism of herbaceous plants. However, it should be noted that in the rhizomes of *I. hybrida* "Little Dream" the content of hydroxycinnamic acids is relatively high – 2.49 %. The largest amount of hydroxycinnamic acids is observed in the leaves of *I. hybrida* "Little Dream" (2.83 %), *I. hybrida* "Galleon Gold" (2.82 %), *I. hybrida* "Mini Dynamo" (2.55 %); the smallest is in the leaves of *I. hybrida* "Indian Pow Waw".

The data in Table 3 indicate that dry extracts of *I. hybrida* "Little Dream" leaves and rhizomes exhibit antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Candida albicans* and a weak effect on *Proteus vulgaris* and *Pseudomonas aeruginosa*.

Conclusions

1. In the raw materials of 5 dwarf bearded irises varieties, hydroxycinnamic acids were identified.

2. The quantitative content of hydroxycinnamic acids in terms of chlorogenic acid was established. It is noted that hydroxycinnamic acids accumulate in larger amounts in the leaves of irises.

3. In *I. hybrida* "Little Dream" raw materials the content of hydroxycinnamic acids is the highest: 2.49 % – in rhizomes and 2.83 % – in leaves.

Table 2. The content of hydroxycinnamic acids in the raw material of varieties iris in terms of chlorogenic acid

The hydroxycinnamic acids content, %		Varieties
leaves	rhizomes	
2.82 ± 0.03	0.79 ± 0.09	<i>I. hybrida</i> "Galleon Gold"
2.83 ± 0.07	2.49 ± 0.11	<i>I. hybrida</i> "Little Dream"
2.44 ± 0.10	1.25 ± 0.07	<i>I. hybrida</i> "Bright white"
2.55 ± 0.13	1.77 ± 0.08	<i>I. hybrida</i> "Mini Dynamo"
1.14 ± 0.08	0.97 ± 0.12	<i>I. hybrida</i> "Indian Pow Waw"

Table 3. The activity of dry extracts of *I. hybrida* "Little Dream" leaves and rhizomes to microorganisms

Microorganism strains	The diameters of the growth inhibition zones at 1% concentration, mm			
	leaves	rhizomes	reference drug	control
<i>Staphylococcus aureus</i> ATCC 25923	17.66 ± 0.10	16.66 ± 0.08	17.63 ± 0.11	growth
<i>Escherichia coli</i> ATCC 25922	16.00 ± 0.08	15.66 ± 0.13	16.20 ± 0.09	growth
<i>Proteus vulgaris</i> ATCC 4636	14.66 ± 0.12	13.66 ± 0.08	14.34 ± 0.12	growth
<i>Pseudomonas aeruginosa</i> ATCC 27853	14.66 ± 0.15	13.00 ± 0.14	14.85 ± 0.10	growth
<i>Bacillus subtilis</i> ATCC 6633	18.66 ± 0.07	17.00 ± 0.09	18.40 ± 0.13	growth
<i>Candida albicans</i> ATCC 653/885	17.00 ± 0.10	16.00 ± 0.13	17.63 ± 0.09	growth

4. The antimicrobial activity of the dry extracts of *I. hybrida* "Little Dream" leaves and rhizomes was established: the preparations are highly sensitive to *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*; weak activity on *Proteus vulgaris* and *Pseudomonas aeruginosa*.

5. The method of column chromatography was used to isolate 4 substances, derivatives of phenol carboxylic acids: caffeic, chlorogenic, neochlorogenic, ferulic acids, the structure of which was proved by spectral methods of analysis.

6. The results of the study show the prospects for the use of cultivated varieties of irises as a source of hydroxycinnamic acids.

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