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TECHNOLOGY OF OBTAINING AND INVESTIGATION OF CHEMICAL COMPOSITION OF DENSE EXTRACT OF HAWTHORN FRUITS

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На фармацевтичному ринку України представлені рідкі лікарські форми глюду, які використовуються при комплексному лікуванні серцево-судинних захворювань. Українська флора нараховує понад 30 видів глюду, серед яких достатньо сировинну базу мають неофіційні дикорослі та культурні види.

Мета. Розробити технологію одержання густих екстрактів плодів неофіційних видів глюду та встановити хімічний склад одержаних екстрактів.

Методи дослідження. Для визначення складу БАР густих екстрактів плодів глюду використовували спектрофотометричний метод та метод високоэффективної рідьової хроматографії (ВЕРХ).

Результати дослідження. Розроблена технологічна схема одержання густих екстрактів плодів глюду. Встановлено вміст амінокислот, флавонійдів та гідроксикоричних кислот густих екстрактів плодів C. prunifolia Sarg., C. pseudokyrtostillla Klok. та C. leiomonogyna Klok.. Вміст флавонійдів склав від 4,27 %±0,01 до 10,94 %±0,10; гідроксикоричних кислот - від 1,45 %±0,02 до 2,56 %±0,10. Методом ВЕРХ в екстрактах ідентифіковано рутин, хлорогенову і ферулову кислоти. У густому екстракті плодів C. prunifolia Sarg. ідентифіковано апігенін-7-O-рамнозид; C. pseudokyrtostillla Klok. та C. leiomonogyna Klok. - апігенін-7-O-глікозид, апігенін, лютеолін; C. leiomonogyna Klok. - лютеолін -7-O-диацетилглікозид та кверцетин.

Висновки. Одержано густі екстракти плодів C. prunifolia, C. pseudokyrtostillla та C. leiomonogyna. Вперше методом ВЕРХ в екстрактах встановлено вміст флавонійдів та гідроксикоричних кислот. Проведено порівняльне дослідження амінокислотного складу одержаних екстрактів.

Ключові слова: глюд, плоди, густі екстракти, хімічний склад, технологія, флавонійди, гідроксикоричні кислоти, амінокислоти

1. Introduction
Genus Hawthorn (Crataegus L.) is one of the polymorphous genus of the Rosaceae L. family and its representatives are widespread in Ukraine and in the world's flora. Having analyzed the raw material base and the degree of study of Ukrainian flora hawthorns, we concluded that attention should be paid to informal wild and cultural species, as promising sources of biologically active substances (BAS).

2. Formulation of the problem in a general way, the relevance of the theme and its connection with important scientific and practical issues
As a result of the study of the chemical composition of unofficial hawthorns, we have established BAS (amino acids, phenolic compounds, organic acids), which have different types of pharmacological activity [1].
Therefore, it is relevant to obtain the biologically active substances of hawthorn fruit and to standardize them further.

3. Analysis of recent studies and publications in which a solution of the problem are described and to which the author refers

The genus Hawthorn (Crataegus L.) belongs to the subfamily of the Maloideae Focke family of Rosaceae Juss. In the world flora, there are more than 1500 species of hawthorn [2, 3].

According the results of phytochemical studies in plants of the genus Crataegus L. poly saccharides, amino acids, peptides, proteins, lipids, organic acids, simple phenols, phenolcarboxylic acids, hydroxycinnamic acids, coumarins, procyanidines, flavonoids, tannins are identified [4].

The main active compounds of the hawthorn are flavonoids [5]. More than 40 flavonoids have been identified in the leaf, fruits, flowers of hawthorn [6]. The main of which are: kaempferol, quercetin, hyperoside, bioquer cetin, 8-methoxykaempferol, apigenin and its glycosides, luteolin and its glycosides, vitexin and its derivatives [7].

Hawthorn flavonoids have a different pharmacological effects [8]. They have a cardiotoxic, antihypertensive, antiarrhythmic and sedative effects, increases the amplitude of heart contractions, normalize heart rhythm, accelerate blood circulation, thus improving supply the heart muscle and there is a positive inotropic effect [9]. Due to flavonoids and organic acids, the hawthorn fruit has antioxidant properties [10].

In the pharmaceutical market of Ukraine are both individual and combined drugs of hawthorn [11]. These drugs are used in the treatment of diseases of the cardiovascular system [12]. They are most often prescribed for the treatment of hypertension [13]. Due to non-toxicity of hawthorn drugs, they can be used for a long time for the prevention of cardiovascular diseases [14].

Taking into account the above, it is important to further study the representatives of the hawthorn species in order to find new sources of BAS and the creation of phytopreparations based on them.

4. The field of research considering the general problem, which is described in the article

The most studied are tincture and liquid extract of hawthorn fruit, dense extracts of fruits of unofficial hawthorns were practically not studied, although according to our research, the raw material contains a significant amount of phenolic compounds.

5. Formulation of goals (tasks) of article

To develop the technology of obtaining dense fruit extracts of unofficial hawthorn species and to determine the composition of the BAS of extracts for their further standardization.

6. Presentation of the main research material (methods and objects) with the justification of the results

The objects of the study were dense fruit extracts of C. prunifolia Sarg., C. pseudokyrtostilla Klok. and C. leiomonogyna Klok.

The dense extracts were obtained from hawthorn fruit harvested in a phase of full ripeness in August-September 2016.

One of the main conditions taken into account when developing the technology of obtaining dense extractions was to achieve the maximum output of BAS. This is done for selecting the optimal conditions of extraction. For the investigated raw material we have identified the following technological parameters: the degree of grinding, the bulk density, the specific mass, the bulk volume, the porosity, the fenestration, the free volume of the layer, the absorption coefficient of the extractant (Table 1).

Table 1

| Parameters | C. pseudokyrtostilla | C. prunifolia | C. leiomonogyna |
|------------|----------------------|--------------|-----------------|
| The bulk density, g/cm$^3$ | 0.51±0.02 | 0.52±0.03 | 0.45±0.01 |
| The specific mass, g/cm$^3$ | 1.35±0.03 | 1.50±0.02 | 1.49±0.01 |
| The bulk volume, g/cm$^3$ | 0.82±0.02 | 0.82±0.03 | 0.83±0.01 |
| The fenestration | 0.37±0.03 | 0.37±0.02 | 0.45±0.01 |
| The porosity | 0.39±0.01 | 0.45±0.02 | 0.44±0.02 |
| The free volume of the layer | 0.62±0.03 | 0.65±0.01 | 0.69±0.03 |

Extraction absorption coefficient, ml/g

| Purified water | 3.4±0.04 | 3.1±0.02 | 3.2±0.01 |
| Ethyl alcogol 50 % | 2.3±0.02 | 2.85±0.01 | 2.8±0.02 |
| Ethyl alcogol 70 % | 2.2±0.01 | 2.15±0.01 | 2.09±0.01 |
| Ethyl alcogol 96 % | 2.03±0.01 | 2.01±0.01 | 1.98±0.02 |

Experimentally, it was determined that the optimal degree of fruits grinding, in which the highest yield of extractives is achieved, was a size of particles of 1.5–3 mm. As extractant was selected 70 % ethanol, this solvent achieved a maximum output of phenolic compounds, including flavonoids.

In order to prevent the destruction of BAC and to prevent the use of large volumes of 70 % ethanol in the development of the technology for obtaining extracts, we offer a percolation method that does not provide for the heating of raw materials.

Preparation of dense extract of hawthorn fruit
The technological process of obtaining dense extracts consisted of the following stages: the raw material crushing; extraction of BAS; concentration of the extract; packaging, labeling.

Stage 1. The raw material crushing. The fruits were crushed to a particle size of 1.5–3 mm, weighed, placed in cloth bags, divided into 3 equal parts and and placed in a battery of percolators.

Stage 2. Extraction of BAS. To the first percolator 70 % ethanol \( R \) was added to the "mirror" and extracted the day (Extract 1). Extract 1 was placed in a second percolator and the extractant was added to the required amount, left for extraction per day (Extract 2). Extract 2 was placed in the third percolator with the raw material, 70 % ethanol \( R \) was added to the "mirror" and left for another day (Extract 3). The liquid extract was obtained in the ratio of raw material: extractant 1: 1 (brought to a certain volume with 70 % ethanol \( R \) to achieve the required ratio of raw material: extractant).

Stage 3. Concentration of the extract. The liquid extracts were evaporated on a vacuum evaporation apparatus to a moisture content of 15 to 25 %.

Stage 4. Packaging, labeling. The resulting dense extract was packed in sterile cups of glass and covered with plastic covers.

The technological scheme of obtaining dense extracts of hawthorn fruits is shown in Fig. 1. The resulting substances were a reddish-brown mass with a characteristic smell.

Mass loss during drying for extracts was determined by the pharmacopoeial method [16]. It was established that for a dense extract of fruit \( C. \) prunifolia Sarg. the loss in mass during drying ( %) is 7,38±0,12; \( C. \) pseudokyrtostilla Klok. – 8,15±0,09; \( C. \) leiomonogyna Klok. – 6,25±0,17.

Definitions were performed three times, the results were statistically processed using the software MS Excel.

### Investigation of the chemical composition of dense extracts

The content of hydroxycinnamic acids and flavonoids in extracts was determined by spectrophotometric method and high performance liquid chromatography (HPLC) method.

**Spectrophotometric determination**

**Hydroxycinnamic acids**

### Spectrophotometric determination

**Hydroxycinnamic acids**

0.10 g of the extract was placed in a 10 ml pycnometer, was added 30 % alcohol \( P \), dissolved with shaking, adjusted to 10 ml with a solvent and stirred. 1 ml of the resulting solution was placed in a 10 ml pycnometer and added 2 ml of a 0.5 \( M \) solution of hydrochloric acid \( R \), 2 ml of a solution of 10 g of sodium nitrite \( R \) and 10 g of sodium molybdate \( R \) in 100 ml of water \( P \), 2 ml of sodium hydroxide solution diluted \( R \), water \( R \) was added to the mark.
Compensation solution. 1 ml of the stock solution was placed in a 10 ml pycnometer, mixed with 2 ml of 0.5 M solution of hydrochloric acid R and 2 ml of sodium hydroxide diluted solution R, the volume of the solution was adjusted to the mark with water R, and stirred. Optical density measured at a wavelength of 525 nm, as a comparison solution using a compensation solution [16].

The content of hydroxycinnamic acids X (%) in terms of acid chlorogenic is calculated by the formula:

\[ X = \frac{A \cdot 10 \cdot 10}{m \cdot 188} \]

where A – optical density of the tested solution at a wavelength of 525 nm; m – mass of the test sample extract, in g; 188 – specific indicator of chlorogenic acid.

Flavonoids
About 0.25 g of extract was placed in a 25 ml volumetric flask, dissolved in 20 % ethanol R while stirring, and the volume of the solution in the flask was brought to the mark with the same solvent and stirred (solution B).

2 ml of solution B added to a 25 ml volumetric flask and 2 ml of 3 % aluminum chloride solution in 96 % ethanol R was added, the volume of the solution was adjusted to 70 % ethanol R and stirred.

The optical density of the test solution was measured at a wavelength of 415 nm in a cuvette with a thickness of 10 mm.

Compared solution. A solution containing 2 ml of a solution of the PhSS rutin is brought into a volumetric flask of 25 ml capacity with 70 % ethanol R to the label and mixed. Before measuring the optical density, the tested solution and the compared solution are filtered through a paper filter, the first portions of the filtrate are discarded [17].

Content of the amount of flavonoids (%) was calculated by the formula:

\[ X = \frac{A_1 \cdot a_0 \cdot 25 \cdot 25 \cdot 100}{a_1 \cdot 25 \cdot 25 \cdot (100 - W)} \]

where A – optical density of the tested solution; A_0 – optical density of the PhSS rutin solution; a_1 – mass of the test extract, in g; a_0 – mass of the PhSS rutin, in g; W – loss in mass during drying, in percentages.

The results of the study of hydroxycinnamic acids and flavonoids are shown in Table 2

| Substances                        | The sum of hydroxycinnamic acids, % | The sum of flavonoids, % |
|----------------------------------|-------------------------------------|-------------------------|
| Extract of C. prunifolia Sarg. fruits | 2.56±0.10                           | 10.94±0.10              |
| Extract of C. pseudokyrtostilla Klok. fruits | 2.15±0.03                           | 3.92±0.007              |
| Extract of C. leiomonogyna Klok. fruits | 1.45±0.02                           | 4.27±0.01               |

As seen from the results of research, the content (%) of hydroxycinnamic acids in the extracts ranged from 1.45±0.02 to 2.56±0.10; flavonoids – from 4.27±0.01 to 10.94±0.10.

**Study of phenolic compounds by HPLC method**

The studies were carried out on an Agilent 1200 3 DLC System Technologies (USA) chromatograph, complete with Supelco DiscoveryC18 chromatography column (250 × 4.6 mm), sorbent-silica gel. As standard compounds used chlorogenic acid, caffeic acid, ferulic acid, rutin, apigenin, quercetin-3-glycoside, luteolin, quercetin. The standards were introduced in a volume of 5 µl, test extracts - 10 µl [18].

Chromatography was carried out under the following conditions: mobile phase solution A – 0.1 % solution of H_3PO_4 in water R, B – MeOH in ratios of 90:10, 70:30 or 20:80; feed rate of the mobile phase – 0.25 ml/min [19]. Working pressure of the eluent was 240–300 kPa; the temperature of thermostat column – 32 °C; sample volume – 5 µl [20]. The gradient mode was use (Table 3). Detection parameters: scale of measurement – 1.0; scan time – 0.5 sec.; parameters for removing the spectrum – each peak is 190–600 nm [21].

| Time, min. | A %, (0.2 % TFA) | B % 70 % MeOH, (0.2 % TFA) | C % 100 % MeOH |
|------------|------------------|-----------------------------|----------------|
| 0          | 92               | 8                           | 0              |
| 8          | 62               | 38                          | 0              |
| 24         | 0                | 100                         | 0              |
| 24.1       | 0                | 0                           | 100            |
| 29         | 0                | 0                           | 100            |

Phenolic compounds were identified by retention time and spectral characteristics in comparison with standards.

Chromatograms of phenolic compounds of dense extract of hawthorn fruits are shown in Fig. 2–4, the identified compounds are shown in Table 4.
Fig. 2. HPLC chromatogram the fruit dense extract of *C. prunifolia*

Fig. 3. HPLC chromatogram the fruit dense extract of *C. pseudokyrtostilla*
Fig. 4 HPLC chromatogram the fruit dense extract of C. leiomonogyna

| Substance                        | Content, mg/100 g |
|----------------------------------|-------------------|
|                                  | Chlorogenic acid  | Apigenin-7-O-rhamnoside | Ferulic acid | Rutin | Apigenin-7-O-glycoside | Apigenin | Luteolin-7-O-diglycoside | Quercetin |
| Extract of **C. prunifolia** fruits | 535.6           | 825.1                  | 1523.6       | 1721.0 | –                  | –         | –                         | –         |
| Extract of **C. pseudokyrtostilla** fruits | 1205.1          | –                      | 315.2        | 1805.2 | 202.1              | 476.3     | 252.2                     | –         |
| Extract of **C. leiomonogyna** fruits | 1630.4          | –                      | 415.7        | 1825.2 | –                  | 175.3     | 152.1                     | 223.5     | 165.7 |

The total content of identified phenolic compounds in extract of fruits **C. prunifolia** Sarg. was 4.6 %; fruits **C. pseudokyrtostilla** Klok. – 4.3 %; fruits **C. leiomonogyna** Klok. – 4.6 %.

It has been established that flavonoid glycosides predominate in qualitative composition, quercetin derivatives, apigenin and hydroxycinnamic acids predominate in quantification.

In all investigated dense extracts predominates rutin, whose content from the amount of phenolic compounds (%) was: for the dense extract **C. prunifolia** Sarg. 37.3; **C. pseudokyrtostilla** Klok. – 42; **C. leiomonogyna** Klok. – 39.7 %. The highest content of chlorogenic acid detected in extract of **C. leiomonogyna** Klok.

In the dense extract **C. prunifolia** Sarg. contains 2 % hydroxycholic acids and 2.6 % flavonoids; **C. pseudokyrtostilla** Klok. – 1.5 % hydroxycholic acids and 2.8 % flavonoids; **C. leiomonogyna** Klok. – 2 %, hydroxyccinnamic acids and 2.6 % flavonoids.

**Amino acids**

On the basis of the method is extraction of free amino acids from the raw material and acid hydrolysis with HPLC analysis with precolumn derivatization of 9-fluorenylmethoxycarbonyl chloride and o-phthalic aldehyde with fluorescence detector detection.

The chromatographic separation of the components was carried out on a Agilent 1200 (Agilent Technologies, USA) liquid chromatograph under the following conditions: a Zorbax AAA column of 150 mm in length and an internal diameter of 4.6 mm, a sorbent grain diameter of 3 μm; moving phase A – 40 mM Na2HPO4 pH 7.8; B-ACN: MeOH: water (45:45:10, v/v/v); gradient separation mode: 0 min. – 12 % "B", 30 min. 25 % "B", 33 min. 25 % "B", 38 min. 30 % "B", 40 min. 40 % "B", 41 min. 80 % "B", 49–60 min. 12 %; constant flow rate of 1.5 ml/min; temperature thermostat column 40 °C. The derivatization was performed automatically using FMOC reagent (Agilent 5061-3337) and OPA reagent (Agilent 5061-3335). Detection of amino acids was carried out using a fluorescence detector [22].

**Sample preparation of raw materials.** To determine the free amino acids, the weight of the extract was placed in the vial and added 2 ml of aqueous solution of 1 M hydrochloric acid R , the mixture was kept in ultrasonic bath at 50 °C for 3 hours.

The determination of the total amino acids was carried out as follows: the weight of extract was added to the vial and added 2 ml of aqueous solution of 6 M hydrochloric acid acid R , the mixture was kept for 24 hours in the thermostat at 110 ° C (hydrolysis). 0.5 ml of a hydrolyzate (pre-centrifuged) was evaporated on a
rotary evaporator and washed three times with purified water R to remove the hydrochloric acid. Resuspended in 0.5 ml of purified water R and filtered through membrane cellulose filters with pores in diameter of 0.2 μm. The fluorescence derivatives were obtained in an automatic programmable mode before entering the sample into a chromatographic column.

Amino acids were identified by comparing the retention time compounds with a mixture of standard amino acids (Agilent 5061-3334). The quantitative content of amino acids was determined by the area of their chromatographic peaks. The content of linked amino acids was determined by subtracting the content of free amino acids from the total amount. A typical HPLC chromatogram of free amino acids (on the example of C. prunifolia fruit extract) is shown in Fig. 5.

Free amino acids identified in the dense hawthorn fruit extracts are presented in Table 5.

### Table 5

| Amino acid       | Content, mg/100 g |
|------------------|-------------------|
|                  | Dense extract of C. prunifolia fruit | Dense extract of C. pseudokyrtostilla fruit | Dense extract of C. leiomonogyna fruit |
| L-Asparagine     | 125.3             | 175.8             | 215.2             |
| L-Threonine      | 11.0              | 11.4              | 8.3               |
| L-Serine         | 0.0               | 12.3              | 7.9               |
| L-Glutamic acid  | 275.4             | 175.6             | 198.4             |
| L-Proline        | 5.2               | 0.0               | 7.8               |
| L-Glutamyne      | 8.3               | 35.6              | 16.8              |
| L-Methionine     | 10.4              | 0.0               | 0.0               |
| L-Leucine        | 10.2              | 7.8               | 0.0               |
| L-Tirozine       | 5.2               | 24.0              | 10.5              |
| L-Phenylalanine  | 11.3              | 9.8               | 5.2               |
| L-Histidine      | 0.0               | 10.3              | 14.7              |
| L-Lysine         | 12.5              | 0.0               | 7.25              |
| L-Arginine       | 320.5             | 386.5             | 175.8             |
| The sum          | 784.3             | 851.8             | 667.85            |

In the dense extract of fruits of C. prunifolia Sarg. and C. pseudokyrtostilla Klok. identified 10 free amino acids, C. leiomonogyna Klok. – 11 amino acids. The highest amino acid content is determined in the dense extract of C. pseudokyrtostilla Klok. It has been established that the amino acid composition of dense extracts is differ, depending on the type of hawthorn. So, in the dense extract of C. prunifolia Sarg., there are no serine and histidine, while in others these amino acids are identified. Methionine and leucine are not identified in the dense extract of C. leiomonogyna Klok. The leucine and methionine are not contained in the extracts of C. pseudokyrtostilla Klok.

### 7. Conclusions from the conducted research and prospects for further development of this field

The technological parameters of raw materials are established and the scheme of obtaining of dense extracts of fruit C. prunifolia Sarg., C. pseudokyrtostilla Klok. and C. leiomonogyna Klok. was developed. For obtained extracts was detected mass loss in drying, which for a dense fruit extract C. prunifolia Sarg. was 7.38 % ± 0.12; C. pseudokyrtosylla Klok. – 8.15 % ± 0.09; C. leiomonogyna Klok. – 6.25 % ± 0.17. According to the results of the spectrophotometric determination, defined that according the content of flavonoids and hydroxycinnamic acids the dense fruit extract C.
prunifolia Sarg. the most promising. For the first time, the HPLC method determined chlorogenic, ferulic acids and rutin in all investigated extracts.

Apigenin-7-O-rhamnoside is founded in the C. prunifolia Sarg. fruits extract; C. pseudokyrtostilla Klok. - apigenin-7-O-glycoside; C. leiomonogyna Klok. – luteolin-7-O-diglycoside and quercetine. Also, apigenine and luteolin have been identified in C. pseudokyrtostilla Klok. and C. leiomonogyna Klok. extracts of fruits. In a comparative study of the content of free amino acids of extracts was found that in all investigated substances are dominated asparagine, glutamic acid and arginine.

The obtained results are the basis for further study of the obtained substances in order to create medicinal drugs.

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Diabetes mellitus (DM) is one of the most common diseases with a steady tendency to increase, "a non-infectious epidemic of the 21st century" [1]. It is projected that by 2030 the number of patients will reach 552 million (9.9 % or 1 diabetes patient in 10 healthy adults), and by 2035 – to 592 million (10.1 %) [2]. DM is one of the main causes of cerebrovascular complications. So, the risk of stroke, in patients with diabetes, is 3–4 times higher. In 10 % of cases, vascular complications in patients with DM, lead to lethal effects [3]. The authors of numerous scientific papers in experimental and clinical studies have identified the leading role of oxygen and nitrogen radicals in the pathogenesis of diabetes and its complications, in particular, diabetic encephalopathy [4, 5].