DETERMINATION OF CARBOXYLIC ACIDS CONTENT IN THE HERBAL MIXTURES BY HPLC

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The aim of the study was to research the qualitative composition and to investigate the quantitative content of some carboxylic acids in the herbal mixtures with established hypoglycemic, hypolipidemic and antioxidant activity in previous studies in vivo.

Materials and methods. Studies of carboxylic acid content in the herbal mixtures were performed by HPLC analysis using Agilent Technologies 1200 liquid chromatograph (USA). Identification and quantitative analysis were performed using standard solutions of carboxylic compounds (tartaric, pyruvic, isocitric, citric, succinic and fumaric acids).

Results. According to the results of chromatographic research it was found that the studied samples contain the largest amount of isocitric acid, the content of which was (45.22±0.04) mg/g in the herbal mixture No. 3, (63.65±0.06) mg/g in the herbal mixture No. 4, (7.51±0.02) mg/g in the herbal mixture No. 7, (2.54±0.01) mg/g in the herbal mixture No. 13 and (43.48±0.05) mg/g in the herbal mixture No. 19. In addition, a high content of succinic acid which is an important regulator of mitochondrial dysfunction and fumaric acid which is a powerful immunomodulatory, anti-inflammatory and antioxidant agent was found.

Conclusions. HPLC analysis of five samples of the herbal mixture with antidiabetic activity showed the presence of six carboxylic acids. The dominant acid in all samples was isocitric acid. Among the most important for the prevention and treatment of diabetes, high levels of succinic and fumaric acids have been identified and established. The obtained data indicate a correlation between the phytochemical composition of the studied herbal mixtures and their pharmacodynamics, which was previously established

Keywords: herbal mixtures, carboxylic acids, high performance liquid chromatography, diabetes mellitus, phytotherapy, isocitric acid, succinic acid, fumaric acid

1. Introduction

Diabetes mellitus is one of the priority problems of the WHO, which requires immediate solution, as the epidemiological situation is alarming – the number of patients is growing rapidly each year, leading to increased disability and mortality due to the development of macro- and microangiopathies [1, 2]. According to the official data of the International Diabetes Federation (2019), the incidence of diabetes in the world is projected to increase 1.5 times by 2030, amounting to more than 500 thousand patients [2]. Therefore, the optimization of existing antidiabetic pharmacotherapy, search and study of new drugs for the prevention and treatment of this disease and its complications is an important issue in modern pharmacy and medicine.

One of these areas is using phytomedicines in the form of monotherapy in the mild stages of the disease and for its prevention, and in combination with traditional therapy for more severe forms of the disease [3, 4]. Phytotherapy is a promising and reasonable method, as it has a number of advantages – relatively low toxicity of herbal medicines, mild pharmacological effect and the ability to use for a long time without significant side effects, the ability to combine well with synthetic drugs [5, 6]. The combinations of different medicinal plants deserve particular attention. Herbal mixtures are expected to have several biologically active substances with a wide range of pharmacological actions and a variety of mechanisms for influencing the development of diabetes and diabetic angiopathies [7, 8].

Therefore, in order to establish correlations between the phytochemical composition of the studied herbal mixtures and its antidiabetic activity, which was studied in previous research [9, 10], it is advisable to conduct phytochemical analysis, in particular, carboxylic acids as one of the influential biologically active substances in the therapy of diabetes and its complications [11, 12].

Carboxylic acids are important phytochemicals that have a positive effect on the treatment and course of diabetes. This is especially true of succinic acid, which has the ability to increase insulin secretion by closing the channels K^+-ATP, membrane depolarization and stimulating Ca^{2+} influx [13, 14]. Fumaric acid and its

esters exhibit immunomodulatory, anti-inflammatory and antioxidant activity, which are important pharmacodynamic parameters in the treatment of diabetes mellitus [15, 16].

Therefore, the aim of study was to investigate the content of carboxylic acids in some herbal mixtures with previously studied antidiabetic activity *in vivo* [9, 10].

2. Planning (methodology) of the research

In Fig. 1 a graphical representation of the research planning process is shown.

Step 1 • Search and analysis of literature sources on the using of the herbal mixtures in the treatment of diabetes

Step 2 • Collection and procurement of the herbal mixtures components

Step 3 • Samples preparation for HPLC analysis

Step 4 • HPLC analysis of carboxylic acids

Step 5 • Identification and calculation of carboxylic acids content in the herbal mixtures

Step 6 • Justification of the effectiveness of herbal mixtures in the treatment of diabetes due to the content of carboxylic acids
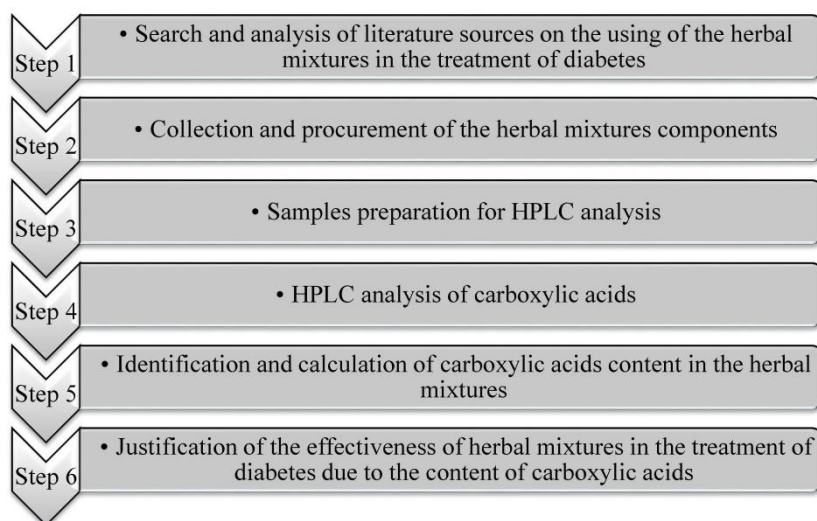
Fig. 1. Design of the experiment

3. Materials and methods

Plant materials: the herbal raw materials harvested from June to August 2019 in the Ternopil region (Ukraine) were used. After harvesting, the raw materials were dried, crushed and stored according to the general GACP requirements [17]. The plants were identified in the Department of Pharmacognosy with Medical Botany, Ternopil National Medical University named after I. Ya. Horbachevsky, Ternopil, Ukraine. The voucher specimens of herbal raw materials have been deposited in the departmental herbarium for future records. For the study, five different herbal mixtures with reliable antidiabetic activity established during pharmacological studies in vivo [9, 10] were used. The composition of the mixtures is given in Table 1.

Table 1

Composition of the herbal mixtures

Herbal mixtures	Herbal drug component	Portion in the mixture, %	Relative ratio
No. 3	*Urtica dioica* leaf	26.32	5
	Cichorium intybus roots	26.32	5
	Rosa majalis fruits	21.05	4
	Elymus repens rhizome	15.79	3
	Taraxacum officinale roots	10.52	2
No. 4	*Arctium lappa* roots	26.32	5
	Elymus repens rhizome	26.32	5
	Zea mays columns with stigmas	21.05	4
	Helichrysum arenarium flowers	15.79	3
	Rosa majalis fruits	10.52	2
No. 7	*Inula helenium* rhizome with roots	10.0	1
	Helichrysi arenarium flowers	20.0	2
	Zea mays columns with stigmas	20.0	2
	Origanum vulgari herb	20.0	2
	Rosa majalis fruits	20.0	2
	Taraxacum officinale roots	10.0	1
No. 13	*Cichorium intybus* roots	26.32	5
	Elymus repens rhizome	26.32	5
	Helichrysum arenarium flowers	21.05	4
	Rosa majalis fruits	15.79	3
	Zea mays columns with stigmas	10.52	2
No. 19	*Urtica dioica* leaf	20.0	1
	Taraxacum officinale roots	20.0	1
	Vaccinium myrtillus leaf	20.0	1
	Rosa majalis fruits	20.0	1
	Mentha piperita herb	20.0	1

Chemicals and standards: all applied reagents were of analytical grade (≥ 95 % purity). Standards of organic acids, including tartaric acid, pyruvic acid, isocitric acid, citric acid, succinic acid and fumaric acid (Fig. 2) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), as well as acetonitrile, undecanoic acid, methanol, heptane, toluene.

Water used in the studies was produced by MilliQ Gradient water deionizaton system (USA).
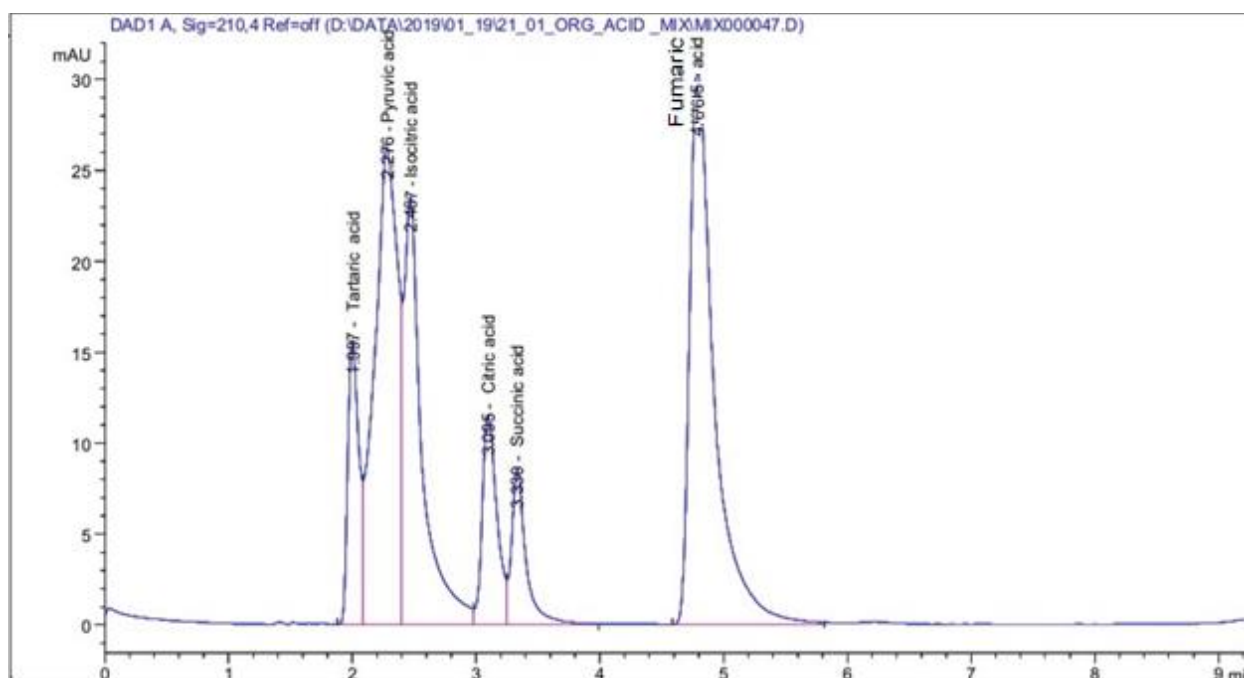

Fig. 2. HPLC chromatogram of a standard mixture of carboxylic acids

Instrumentation and conditions: HPLC analysis of carboxylic acids was performed using Agilent 1200 (Agilent Technologies, USA) [17, 18]. Mobile phase A – acetonitrile (ACN); mobile phase B – 0.1% solution of phosphoric acid in water (1:99, v/v). Elution was performed in isocratic mode. Separation was performed on a Zorbax SB-Aq chromatographic column (4.6±150 mm, 3.5 μm) (Agilent Technologies, USA), column flow rate 0.5 mL/min, the temperature of the thermostat column is 30 °C, volume injection 3 μl. Detection was performed using a diode-matrix detector with signal registration at 210 nm and fixation of absorption spectra in the range of 210–700 nm.

Standard solutions (1000 ppm) of tartaric, pyruvic, isocitric, citric, succinic and fumaric acids were prepared in the mobile phase consisting of 0.1% phosphoric acid solution. Stock solutions of every carboxylic acid were made in the mobile phase by corresponding dilutions.

Extraction of carboxylic acids: 700 mg of each powdered raw material was placed in a vial and extracted with 10 mL of 0.1% solution of phosphoric acid. Extraction was performed in the ultrasonic bath at 80 °C for 4 hours. Then 8.3 mL of the obtained extracts were centrifuged at 3000 rpm/min for 30 minutes and filtered through Supelco Discovery DSC-18 filter and then concentrated to the residual volume of the extract of 20 μl.

Identification and calculation by HPLC: was performed using standard solutions of carboxylic compounds (tartaric, pyruvic, isocitric, citric, succinic and fumaric acids). The content of organic acids in μg/g was calculated by the following equation:

$$X = \frac{C \times V_{solv} \times V_{inj}}{m \times V_{actual\ inj} \times 1000},$$

where C – concentration, obtained from the chromatogram by calculating the reference solution and the test solution;

V_{solv} – volume of solvent for extraction;
V_{inj} – volume of injection standard of organic acid;
$V_{actual\ inj}$ – volume of injection of extract;
m – mass of the raw material.

4. Result

The results of qualitative and quantitative analyses of short chain carboxylic acids in the herbal mixtures are represented in Figs. 3–7 and in Table 2.
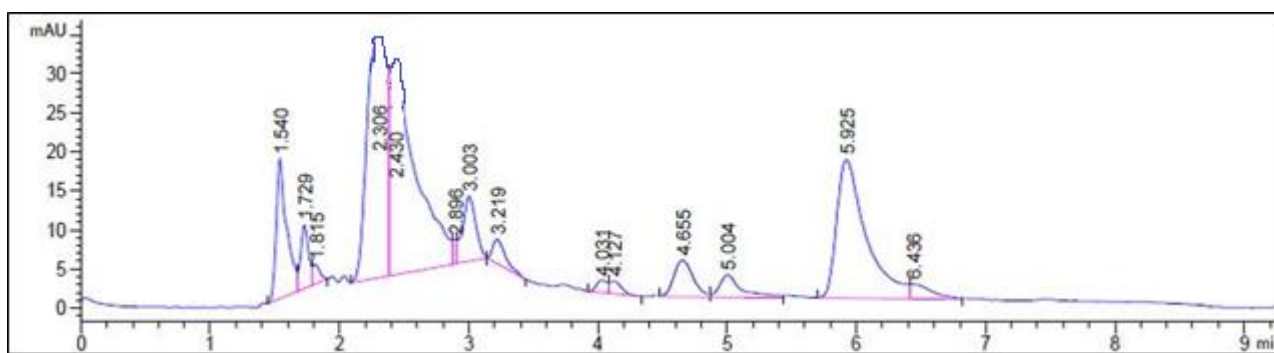
Fig. 3. HPLC chromatogram of carboxylic acids in the herbal mixture No. 3
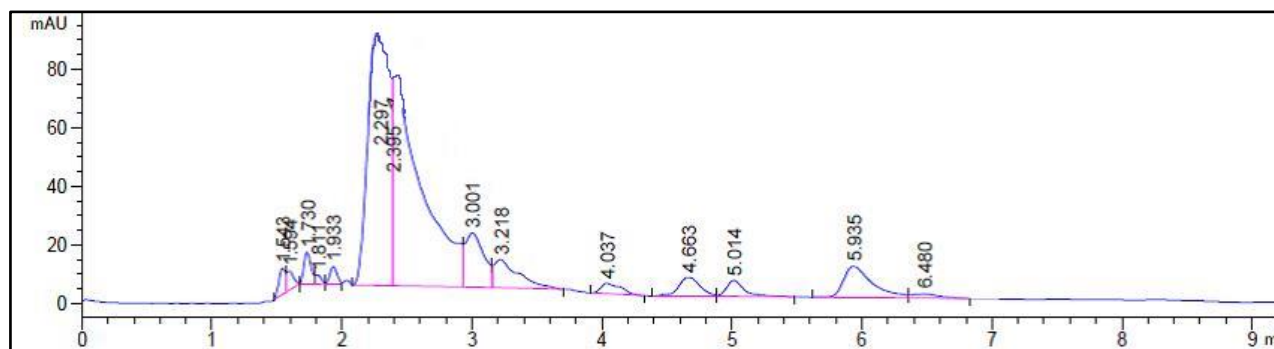

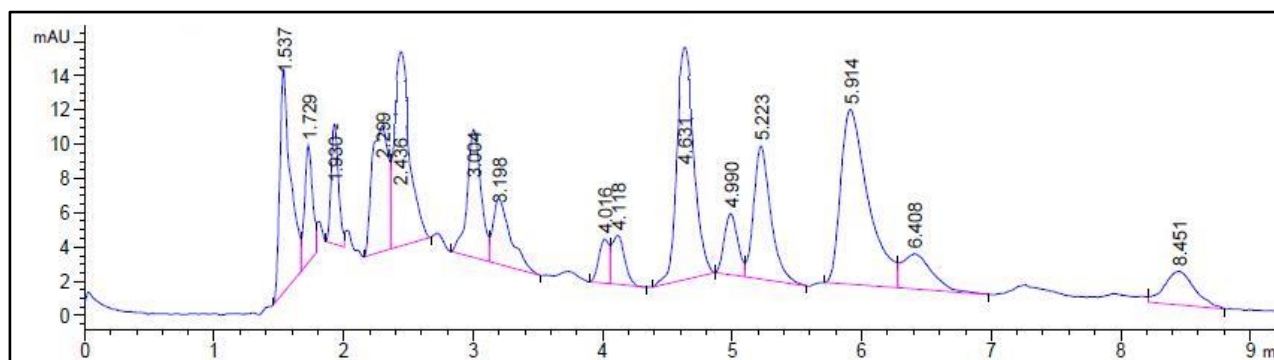Fig. 4. HPLC chromatogram of carboxylic acids in the herbal mixture No. 4

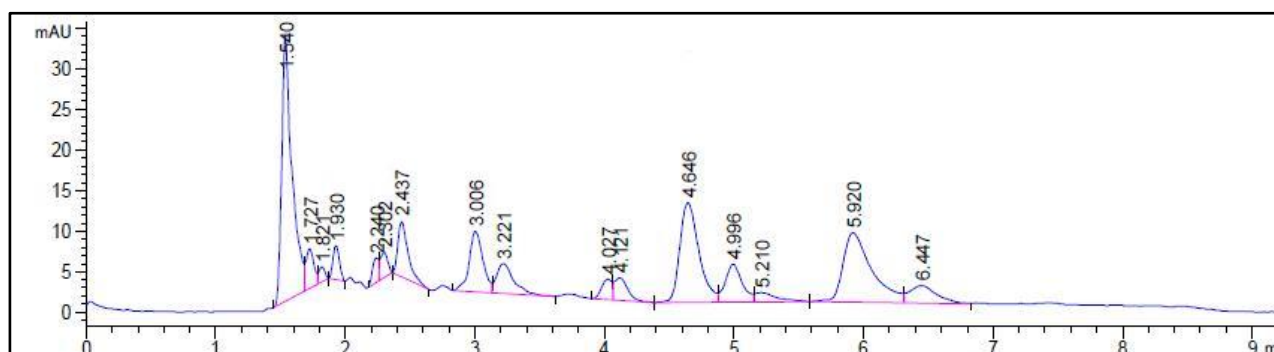Fig. 5. HPLC chromatogram of carboxylic acids in the herbal mixture No. 7

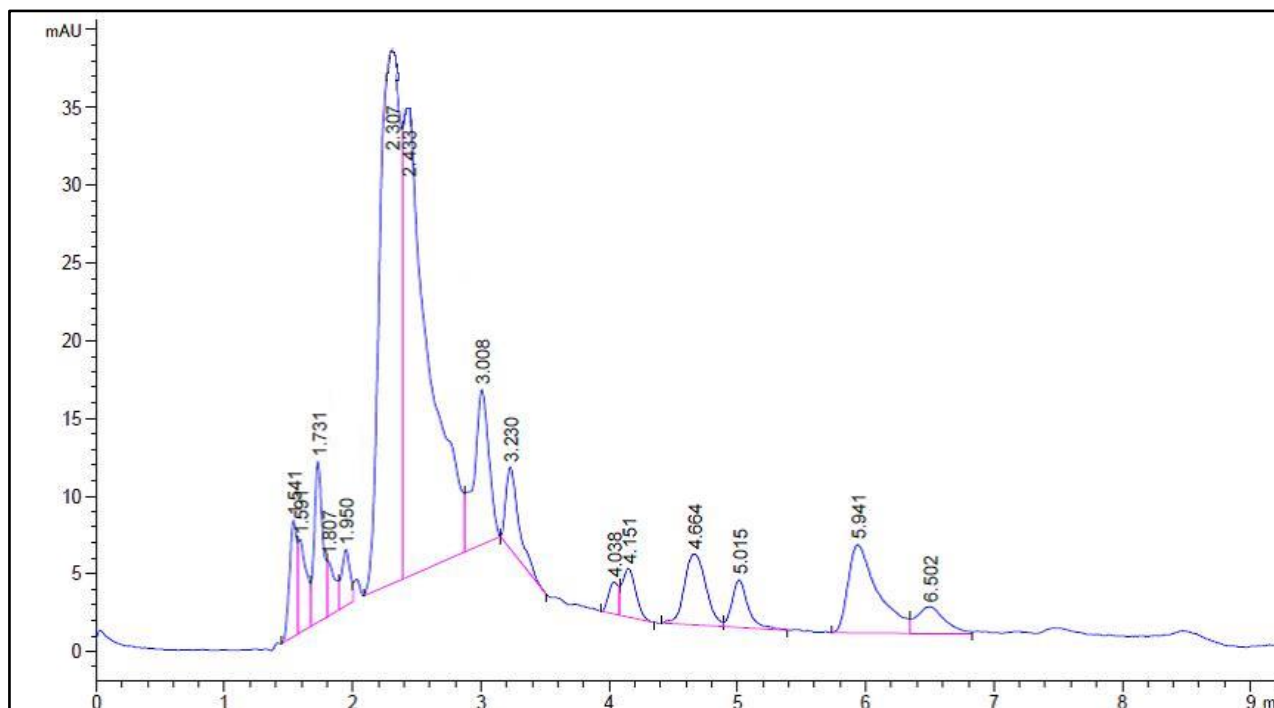Fig. 6. HPLC chromatogram of carboxylic acids in the herbal mixture No. 13

Fig. 7. HPLC chromatogram of carboxylic acids in the herbal mixture No. 19

Table 2

The results of the comparative analysis of carboxylic acids in the herbal mixtures

No.	tR	Common name of carbox-ylic acid (IUPAC)	Molecular formula	Quantitative content the herbal mixtures, mg/g				
				No. 3	No. 4	No. 7	No. 13	No. 19
1.	1.930	Tartaric (2,3-dihydroxybutanedioic) acid	C4H6O6	n/d	0.45±0.02	0.49±0.02	0.29±0.01	0.33±0.01
2.	2.299	Pyruvic (2-oxopropanoic) acid	C3H4O3	1.76±0.01	4.62±0.03	0.30±0.01	0.08±0.01	1.76±0.02
3.	2.436	Isocitric (1-hydroxypropane-1,2,3-tricarboxylic) acid	C6H8O7	45.22±0.04	63.65±0.06	7.51±0.02	2.54±0.01	43.48±0.05
4.	3.004	Citric (2-hydroxypropane-1,2,3-tricarboxylic) acid	C6H8O7	1.40±0.01	3.52±0.03	1.08±0.01	1.17±0.01	1.71±0.02
5.	3.198	Succinic (butanedioic) acid	C4H6O4	1.04±0.01	4.60±0.03	1.37±0.02	1.38±0.01	1.53±0.02
6.	4.665	Fumaric ((2E)-but-2-enedioic) acid	C4H4O4	0.06±0.01	0.07±0.01	0.10±0.01	0.12±0.01	0.06±0.01

Note: 1. n/d – not detected; 2. Values are expressed as mean ± SD (n=5)

During HPLC analysis it was found that the herb-al mixture No. 3 contains 5 components of carboxylic acids (pyruvic, isocitric, citric, succinic and fumaric acid), and the herbal mixtures No. 4, No. 7, No. 13 and No. 19 – 6 components of carboxylic acids (tartaric, pyruvic, isocitric, citric, succinic and fumaric acid) (Figs. 3–7).

According to the results of chromatographic re-search it was found that in the studied herbal mixtures contain the largest amount of isocitric acid that was (45.22±0.04) mg/g in the herbal mixture No. 3, (63.65±0.06) mg/g in the herbal mixture No. 4, (7.51±0.02) mg/g in the herbal mixture No. 7, (2.54±0.01) mg/g in the herbal mixture No. 13 and (43.48±0.05) mg/g in the herbal mixture No. 19. In addi-tion, a high content of succinic acid was found, which is an important regulator of mitochondrial dysfunction, (1.04±0.01) mg/g in the herbal mixture No. 3,

(4.60±0.03) mg/g in the herbal mixture No. 4, (1.37±0.02) mg/g in the herbal mixture No. 7, (1.38±0.01) mg/g in the herbal mixture No. 13 and (1.53±0.02) mg/g in the herbal mixture No. 19. Regard-ing fumaric acid, which exhibits immunomodulatory, anti-inflammatory and antioxidant activity, its content in the studied plant samples was – (0.06±0.01) mg/g in the herbal mixture No. 3, (0.07±0.01) mg/g in the herbal mixture No. 4, (0.10±0.01) mg/g in the herbal mixture No. 7, (0.12±0.01) mg/g in the herbal mixture No. 13 and (0.06±0.01) mg/g in the herbal mixture No. 19 (Figs. 3–7, Table 2).

5. Discussion
Carboxylic acids play an important role in the progression of diabetes and in the formation of diabetic angiopathies, in particular, succinic acid, which has the ability to reduce mitochondrial dysfunction that plays a

leading role in the development of neurodegenerative diseases [21, 22]. In addition, succinic acid has an effect on the development of inflammation by reducing the neurotransmitters of inflammation [23, 24]. The high content of succinic acid found in the studied herbal mixtures may indicate the ability of these mixtures to prevent the development of mitochondrial dysfunction, and at the same time the development of oxidative stress, which is the main pathogenic mechanism of diabetic angiopathies. Fumaric acid has pronounced antioxidant properties, which is expressed by the cytoprotective ability against the harmful effects of lipid peroxidation and the development of oxidative stress, which underlie the development and progression of diabetic complications [24, 25]. Fumaric acid, which has been identified and quantified in all studied herbal mixtures, also has the ability to increase the function of the immune system and reduce inflammation [26, 27]. The obtained data testify to the expediency of using the studied herbal mixtures in order to optimize antidiabetic pharmacotherapy.

Study limitations. The research needs additional study of other carboxylic acids, as some acid compounds were not detected during the study.

Prospects for further research. According to the obtained research results, further screening of pharmacodynamic properties and development of parameters for standardization of plant mixtures is planned.

6. Conclusions

The results of chromatographic examination indicate a sufficient content of carboxilyc acids, in particular fumaric acid in five herbal mixtures, that has immunomodulatory, anti-inflammatory, antioxidant activity and succinic acid that has the ability to regulate mitochondrial dysfunction, which confirms the antidiabetic efficacy of five investigated samples in the pharmacological studies in vivo. The predominant carboxilyc acid was isocitric acid in all samples of the studied mixtures, its content was (45.22 ± 0.04) mg/g in the herbal mixture No. 3, (63.65 ± 0.06) mg/g in the herbal mixture No. 4, (7.51 ± 0.02) mg/g in the herbal mixture No. 7, (2.54 ± 0.01) mg/g in the herbal mixture No. 13 and (43.48 ± 0.05) mg/g in the herbal mixture No. 19. The obtained phytochemical studies may indicate a correlation between the component composition and content of carboxylic acid in the samples of studied mixtures and their effectiveness in the treatment and prevention of diabetes mellitus and its complications.

Conflict of interests

The authors declare that they have no conflicts of interest.

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