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Research Article

Analysis of carbohydrates content in the plant components of antidiabetic herbal mixture by GC-MS

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Abstract

Medicinal plants and their combinations due to the wide range of biologically active substances can influence on various links of the pathogenetic mechanism of development of diabetes mellitus and its complications. One of such combinations is an antidiabetic herbal mixture (*Urticae folia*, *Rosae frucrus*, *Myrtilli folia*, *Menthae folia* and *Taraxaci radices*) with established hypoglycemic, hypolipidemic, antioxidant, hepatoprotective, pancreatoprotective activity in previous pharmacological study *in vivo*. Thus, the aim of this study was to identify and establish the content of carbohydrates in free and bound form in the plant components of antidiabetic herbal mixture. The carbohydrates were separated by gas chromatography-mass spectrometry after conversion into volatile derivatives as aldononitrile acetate. The monomeric composition of polysaccharides was studied after their hydrolysis to form monosaccharides and polyalcohols. The results of the quantitative study showed that the predominant carbohydrate in free form was saccharose in *Urticae folia*, *L*-fructose in *Myrtilli folia*, *Rosae frucrus*, *Taraxaci radices* and *Menthae folia*, *L*-glucose in *Rosae frucrus*. Concerning the determination of monomers of polysaccharides after hydrolysis, *L*-glucose prevailed in all plant components of antidiabetic herbal mixture. The chromatographic study revealed a number of polyalcohols that are important for the treatment and prevention of progression of diabetes mellitus and its complications, namely, mannitol and *myo*-inositol.

Keywords

diabetes mellitus, herbal mixture, GC-MS, carbohydrates, *Urticae folia*, *Rosae frucrus*, *Myrtilli folia*, *Menthae folia*, *Taraxaci radices*

Introduction

Diabetes mellitus is one of WHO's priorities matters, which requires immediate solutions, as the epidemiological situation is alarming – the number of patients is growing each year, leading to increased disability and mortality due to the development of diabetic angiopathies (Harding et al. 2019; American Diabetes Association 2021). According to the official information of International Diabetes Federation (2019) the number of diabetics will increase to 700 million by 2045. Therefore, the implementation of pharmacotherapy optimization, search, and study of new drugs for the prevention and treatment of this disease and its dangerous complications is a topical issue of pharmacy and medicine.

Modern pharmacotherapy increasingly considers the centuries-old experience of folk medicine with the use of herbal drugs as monotherapy and in combination with synthetic drugs. This is quite justified, because phytotherapy has a number of advantages over traditional therapy

with synthetic drugs, namely, it is low-toxic, has a mild pharmacological effect and can be used for a long period of time without significant side effects and combines well with synthetic drugs (Gothai et al. 2016; Governa et al. 2018; Slobodianiuket al. 2020). In addition, herbal medicines, as a rule, have a wide range of pharmacological properties, which is realized through different groups of phytochemicals (Oh and Jun 2014; Kooti et al. 2016; Budniak et al. 2020; Marchyshyn et al. 2020). Combinations of different medicinal plants deserve special attention as such herbal mixtures will have more biologically active substances that will affect all parts of the pathogenetic mechanism of diabetes mellitus and its complications (Savych et al. 2020a, 2021a, b, c, e, f, g).

One of such combinations is an antidiabetic herbal mixture (*Urticae folia*, *Rosae frucrus*, *Myrtilli folia*, *Menthae folia* and *Taraxaci radices*) with established hypoglycemic, hypolipidemic, antioxidant, hepatoprotective, pancreatoprotective activity in pharmacological study *in vivo* (Savych et al. 2020b, c, d, e, f, 2021d) and the defined phytochemical composition that determines such pharmacodynamics (Savych et al. 2020a, 2021a, b, c, e, f, g, h).

Biologically active substances of plant origin have a wide range of pharmacological action and various mechanisms of influence on the development of diabetes and its complications (Oh and Jun 2014; Kooti et al. 2016; Skyler et al. 2017; Marchyshyn et al. 2021b). One of the most important phytochemicals is carbohydrates that have hypoglycemic, hypolipidemic, anticholesterolemic, antioxidant, anti-inflammatory, and detoxifying effects (Chen et al. 2019; Ganesan and Xu 2019; Zhang et al. 2018). Polysaccharides stimulate the growth of beneficial bacteria in the colon, including *Bifidobacteria* and *Lactobacilli*, thereby modulating the composition of microflora. This creates an environment that protects against pathogens, toxins and free radicals resulting from lipid peroxidation (Zhang et al. 2018; Calabrese et al. 2021). Powerful antioxidant properties of polysaccharides in number of studies *in vivo* and *in vitro*, whose mechanism of action is not understood exactly, have been observed (Zhang et al. 2015; Luo et al. 2019). Plant carbohydrates have the ability to regulate the lipid metabolism by lowering of triglycerides and cholesterol, a disorder of which occurs in diabetes and leads to the development of cardiovascular diseases and microcirculatory complications – diabetic nephropathy, neuropathy and retinopathy, the formation of diabetic foot (Collins et al. 2016; Ganesan and Xu 2019; Marchyshyn et al. 2021a). The hypoglycemic activity of carbohydrates is realized by increasing of insulin secretion, inhibition of glucagon secretion, stimulation of β-cells proliferation and neogenesis (Ganesan and Xu 2019; Luo et al. 2019). In addition, they also show anti-inflammatory properties, which are manifested by reducing the parameters of inflammation such as edema, leukocyte migration and nociception (Yin et al. 2019). All these carbohydrates properties make them an important group of substances for prevention and treatment of diabetes mellitus and its extremely dangerous complications.

Aim of the research

The aim of this study was to investigate the content of carbohydrates in the free form and their monomeric composition after hydrolysis in *Urticae folia*, *Rosae frucrus*, *Myrtilli folia*, *Menthae folia* and *Taraxaci radices* as the plant components of antidiabetic herbal mixture.

Materials and methods

It was used the herbal raw materials of *Urticae folia*, *Rosae frucrus*, *Myrtilli folia*, *Menthae folia* and *Taraxaci radices* harvested from June to October 2020 in Ternopil region and Charpathians (*Myrtilli folia*) (Ukraine) during the study. The raw materials were then dried, crushed and stored according to the general GACP requirements (WHO 2003). Plants were identified by Department of Pharmacognosy with Medical Botany, Ivan Horbachevsky Ternopil National Medical University, Ternopil, Ukraine. A vouchers specimens of *Urticae folia* No. 279, *Rosae frucrus* No. 168, *Myrtilli folia* No. 254, *Menthae folia* No. 312 and *Taraxaci radices* No. 357 are kept in departmental herbarium for future record.

Chemicals and standards

All applied reagents were of analytical grade (≥ 95 % purity). Chemical reference substances (CRS) of carbohydrates including *D*-mannose, *L*-rhamnose, *D*-galactose, *D*-xylose, *D*-arabinose, *D*-glucose, *D*-fructose, saccharose, *D*-mannitol, *myo*-inositol and *D*-sorbitol were purchased from Sigma-Aldrich Chemical Co. (USA), as well as methanol, trifluoroacetic acid, hydroxylamine hydrochloride, pyridine, dichloroethane, hydrochloric acid, heptanes and ethyl acetate. Water used in the studies was produced by MilliQ Gradient water deionization system (USA).

Extraction of carbohydrates and hydrolysis

The samples of the herbal raw material were grinded into a powder by laboratory mill, then about 500 mg (accurately weighed) was selected and placed into round bottom flask with 10.0 mL of methanol and internal standard – sorbitol (500 μg per sample). The extractions were carried out in the ultrasonic water bath at 80 °C for 4 hours. The resulting extracts were centrifuged at 3000 rpm and the supernatants were evaporated to dryness on a rotary evaporator. 1 mg of the extract was used for hydrolysis of carbohydrates with 5 mL of 2 (mol L^{-1}) trifluoroacetic acid at 100 °C for 6 hours. Then, 2 mL of obtained hydrolyzed solution was evaporated to dryness under 45 °C and then 1 mL of methanol was added for further evaporation and complete removal of trifluoroacetic acid. The rest of the extract was used for the determination of free carbohydrates.

Derivatization

To obtain acetylated aldonitriles 2 mL of the extract was evaporated to dryness and was added 0.3 mL of derivatization reagent [32 mg mL^{-1} hydroxylamine hydrochloride in the mixture of pyridine/methanol (4:1, *v/v*)] was added. The samples were incubated in a preheated water bath shaker at 75 °C for 25 min. Then, for acetylation of aldonitrile derivatives, 1 mL of acetic anhydride was subsequently added to the samples and incubated at 75 °C for 15 min. To the resulting reaction mixture, 2 mL of dichloroethane were added. The excess of the derivatization reagents was removed by the double extraction with 1 (mol L^{-1}) HCl and water. Dichloroethane phase was dried and dissolved into 300 μL of the mixture of heptane/ethyl acetate (1:1, *v/v)* (Marchyshyn et al. 2020; Savych et al. 2021f).

Chromatographic condition

The quantity content of carbohydrates in the samples of the herbal raw materials were studied by gas chromatography-mass spectrometry (GC-MS) using the Agilent Technologies 6890 gas chromatograph (USA) with mass spectrometry detector 5973 and capillary column (Savych et al. 2021f). Chromatography was performed under these conditions: chromatographic column – capillary HP-5MS with internal diameter 0.25 mm, length 30 m, carrier gas velocity (helium) – 1.2 mL min^{-1}, temperature of the evaporator 250 °C, interface temperature 280 °C, temperature thermostat programmable from 160 °C to 240 °C at a speed of 5 °C min^{-1}. The oven temperature was initially set at 160 °C, held for 8 min, then raised to 240°C and finally kept at this point for 6 min. Injections of 1 μL were made in the split mode 1:50. The detection was held in the SCAN mode in the range of 38–400 *m/z* (Budniak et al. 2021; Savych et al. 2021f).

To identify the components, the obtained spectra was analyzed by comparing the retention times with the data of the mass spectra libraries NIST 02. Quantitative analyses were performed by the method of internal standards. Under normal conditions of derivatization, the ketone carbohydrate (fructose) is converted into an aldo carbohydrate (glucose) (Savych et al. 2021f). According to this technique, fructose after derivatization gives 2 peaks, which are summed up during calculations.

Method validation

The method was validated for linearity, limit of detection (LOD), limit of quantitation (LOQ) and precision. Linearity of the obtained relative peak areas was determined by a calibration curve of five concentrations (25 mg mL^{-1}, 12.5 mg mL^{-1}, 5 mg mL^{-1}, 2.5 mg mL^{-1}, 1.25 mg mL^{-1}) with a threefold derivatization procedure and a single injection for each CRS of carbohydrates. The mean value and standard deviation, as well as regression analysis were calculated using Microsoft Excel software package 2016 (USA). The values for LOD and LOQ were calculated based on the data obtained during linearity testing in the low concentration range of the working in the test solution, using the following formulas: LOD = 3.3 * s / Slope; LOQ = 10 * s / Slope. Linearity testing was repeated with the same samples after a complete restart of the system with removement and re-installation of the column. Repeatability precision was determined by five-fold injection of the same sample in a row. For the resulting relative peak area of the quantifier ions the relative standard deviation (RSD) was calculated. To determine intra-day precision, five standard preparations of each CRS of carbohydrates with the same concentration were single injected and the resulting relative peak areas were used to calculate the RSD. Inter-day precision for the day of sample preparation and the two following days was specified by injecting five standard sample of each CRS of carbohydrates preparations once each on all three days. The RSD of the samples on that day together with the previous samples were calculated as above (Marchyshyn et al. 2020).

Results and discussion

The method was validated for linearity, LOD, LOQ, and precision. All CRS of carbohydrates showed acceptable linearity (R_2 > 0.99) within the concentration range 1.25–25 mg mL^{-1}. Linearity testing was based on five concentration levels and repeated with the same samples after a complete restart of the system with the removal and re-installation of the column. LODs and LOQs were estimated as 3 and 10 times the signal-to-noise ratio (*S*/*N*) for each carbohydrate. Considering all carbohydrates, in general, LODs ranged 0.11–1.15 μg mL^{-1} and LOQs 0.35–3.50 μg mL^{-1} (Table 1). Injection repeatability was

Table 1. Results of linearity data obtained for CRS of carbohydrates after GC-MS analysis.

CRS of carbohydrates	Regression equations	R^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
D-rhamnose	$y = 0.0159x + 0.1246$	0.9924	0.19	0.59
D-arabinose	$y = 0.0133x + 0.0874$	0.9928	0.83	2.46
D-fucose	$y = 0.0163x + 0.1314$	0.9921	0.22	0.69
D-xylose	$y = 0.0249x + 0.1878$	0.9920	0.54	1.65
D-mannose	$y = 0.0285x + 0.0888$	0.9975	1.15	3.50
D-glucose	$y = 0.0229x + 0.0062$	0.9923	0.37	1.15
D-galactose	$y = 0.0232x + 0.0572$	0.9951	0.29	0.90
D-fructose	$y = 0.0103x + 0.0412$	0.9921	0.11	0.35
myo-inositol	$y = 0.018x + 0.1778$	0.9921	0.23	0.70
D-mannitol	$y = 0.0176x + 0.1269$	0.9920	0.18	1.55
saccharose	$y = 0.0188x + 0.1396$	0.9933	0.78	2.42

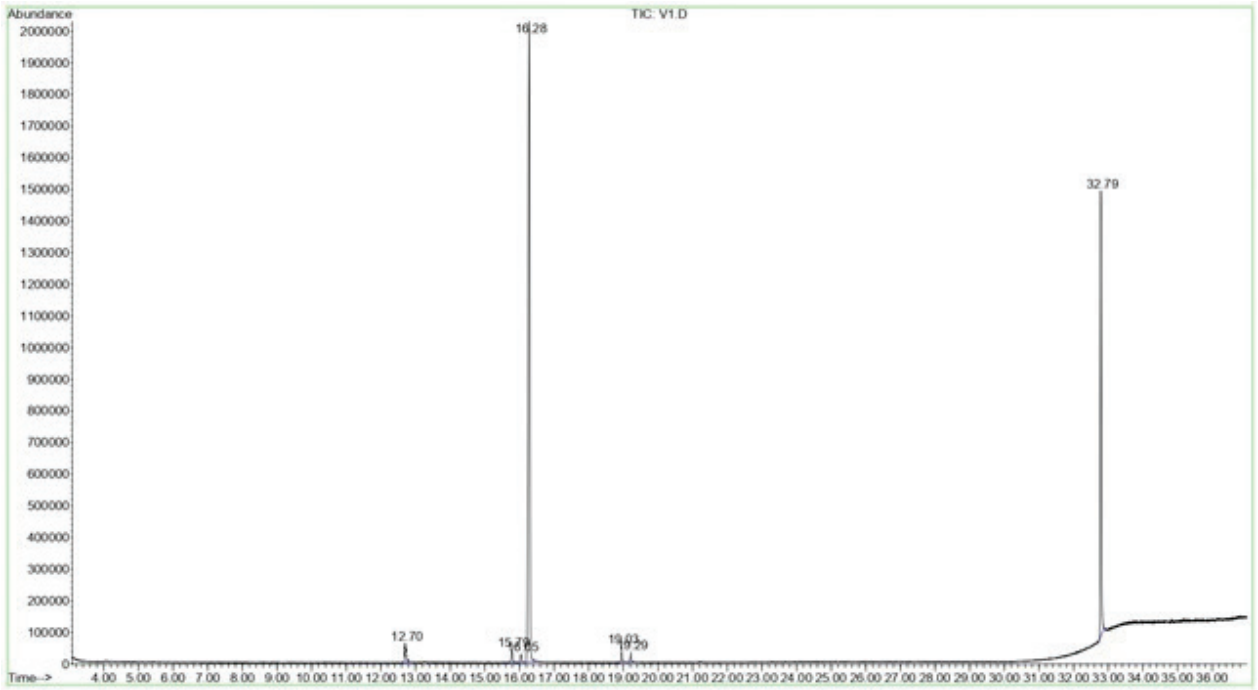

Figure 1. GC-MS chromatogram of derivatives of free carbohydrates in *Urticae folia.*

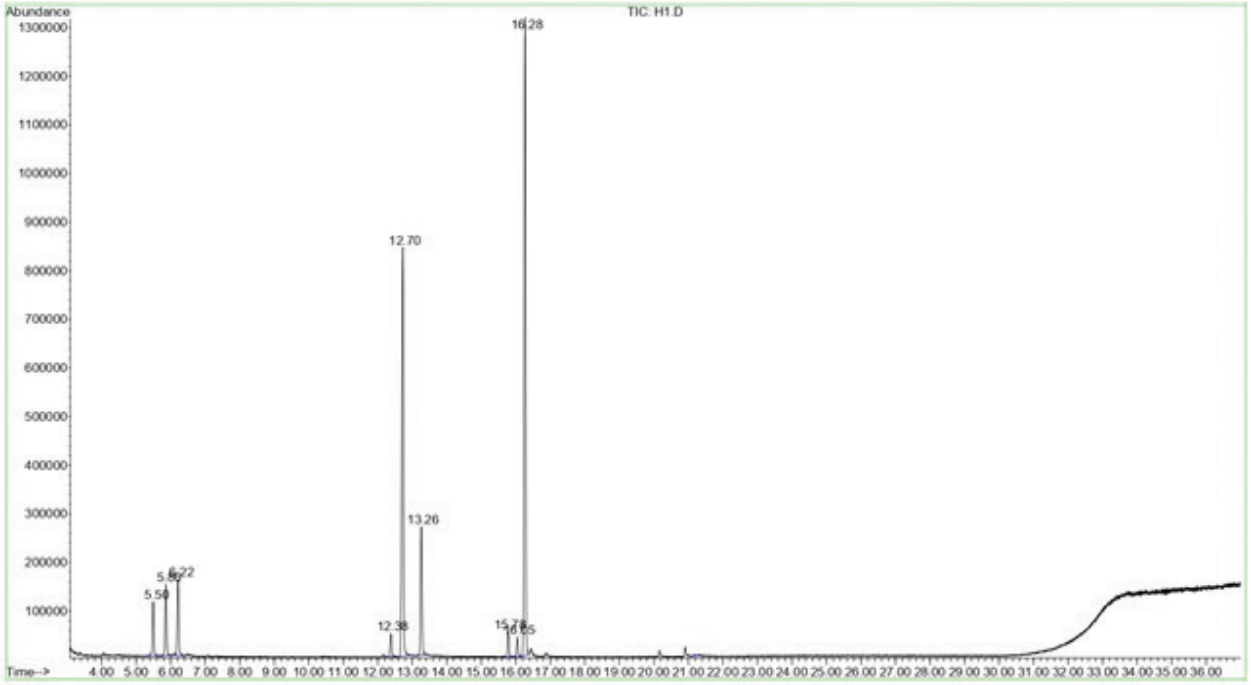

Figure 2. GC-MS chromatogram of derivatives of carbohydrates after hydrolysis in *Urticae folia*.

determined by a five-fold injection of the same sample. To determine intra-day precision, five samples of the same concentration of each CRS of carbohydrates were single injected to calculate RSD. Inter-day precision for the day of sample preparation and the two following days was specified by injecting five samples of each reference standard, once each, on all three days. Intra- and inter-day RSD ranged from 4.8 to 6.6%.

According to the results of the GC-MS analysis, it was identified six carbohydrates in free form in *Urticae folia* (Fig. 1), five carbohydrates in *Myrtilli folia* (Fig. 3), *Taraxaci radices* (Fig. 7) and *Menthae folia* (Fig. 9), four carbohydrates in *Rosae frucrus* (Fig. 5). GC-MS analysis of carbohydrates after hydrolysis showed that the largest number of carbohydrate monomers was contained in *Urticae folia* (Fig. 2) and it was eight, and *Myrtilli folia* (Fig. 4)

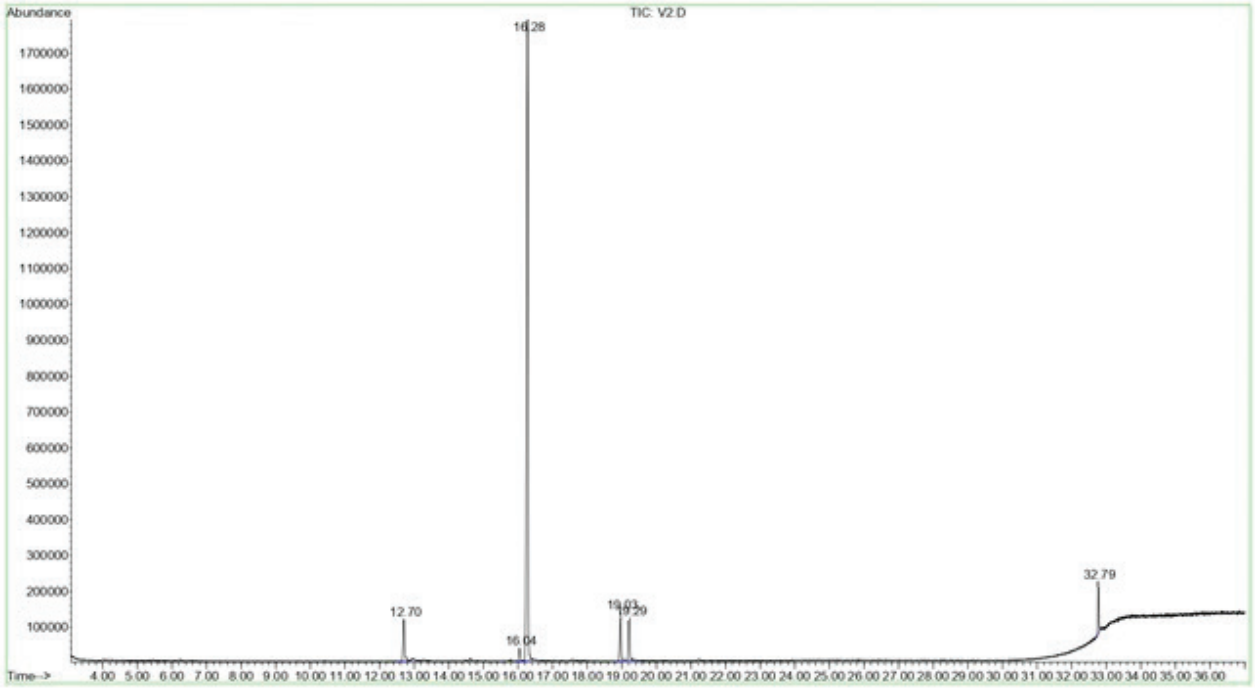

Figure 3. GC-MS chromatogram of derivatives of free carbohydrates in *Myrtilli folia.*

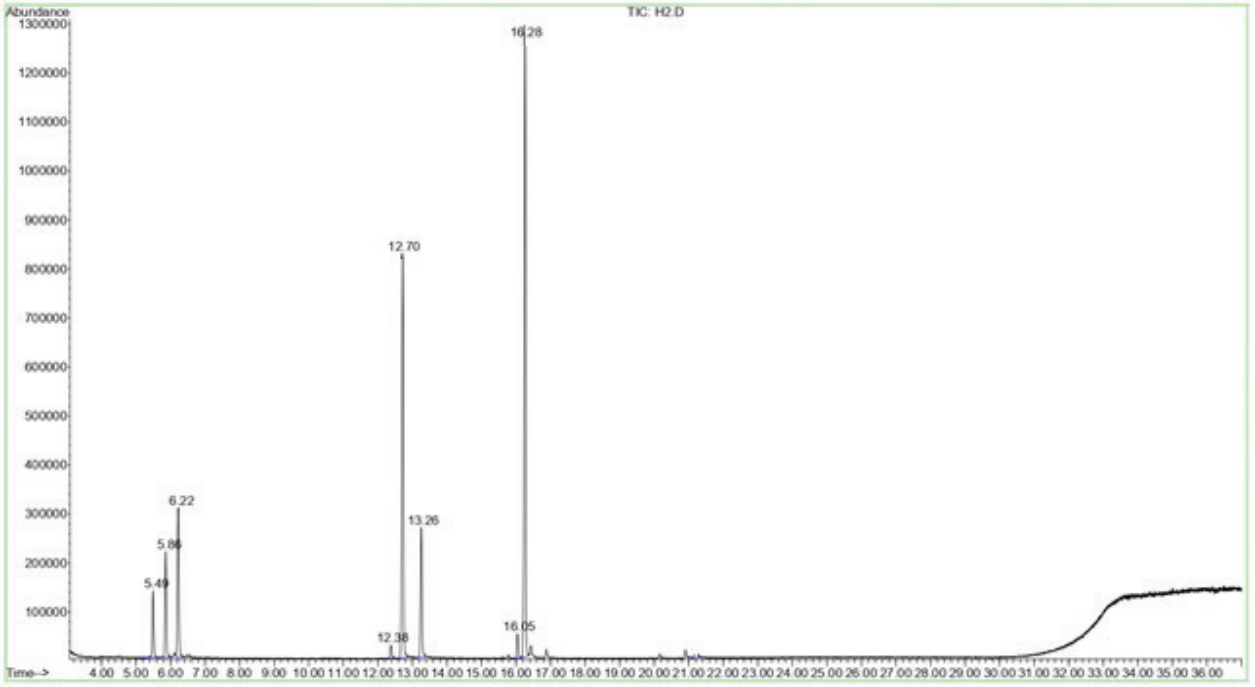

Figure 4. GC-MS chromatogram of derivatives of carbohydrates after hydrolysis in *Myrtilli folia* **.**

contained seven carbohydrates, *Rosae frucrus* (Fig. 6) and *Menthae folia* (Fig. 10) – six carbohydrates and *Taraxaci radices* (Fig. 8) – five carbohydrates.

The results of the quantitative study showed that the predominant carbohydrate in free form was saccharose in *Urticae folia* (20.30±0.09 mg g^{-1}), *L*-fructose in *Myrtilli folia* (78.60±0.12 mg g^{-1}), *L*-fructose in *Rosae frucrus* (586.18±0.29 mg g^{-1}), *L*-fructose in *Taraxaci radices* (162.69±0.23 mg g^{-1}) and *L*-glucose in *Rosae frucrus* (586.18±0.29 mg g^{-1}), *L*-fructose in *Menthae folia* (40.65±0.11 mg g^{-1}) (Table 2). As for carbohydrates after hydrolysis, the predominant carbohydrate monomer was *L*-glucose in all plant components of antidiabetic herbal mixture, it content was 464.76±0.31 mg g^{-1} in *Urticae folia*, 653.37±0.38 mg g^{-1} in *Myrtilli folia*, 869.28±0.63 mg g^{-1} *Rosae frucrus*, 809.95±0.38 mg g^{-1}

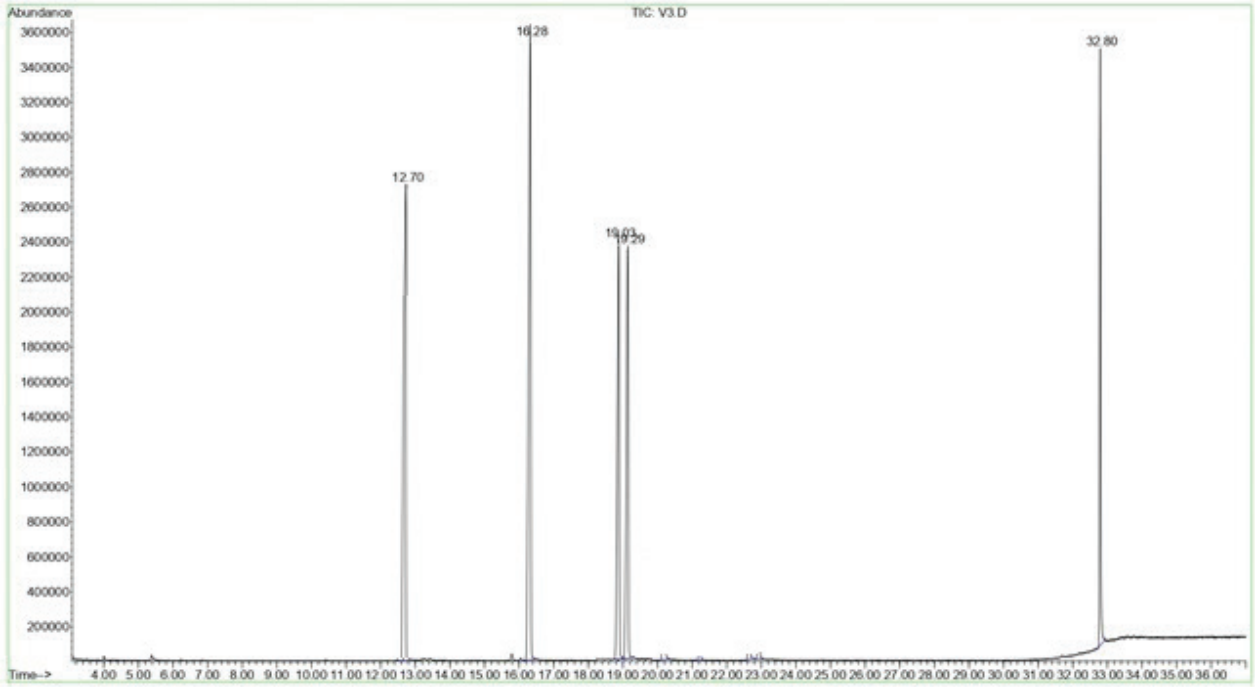

Figure 5. GC-MS chromatogram of derivatives of free carbohydrates in *Rosae frucrus*.

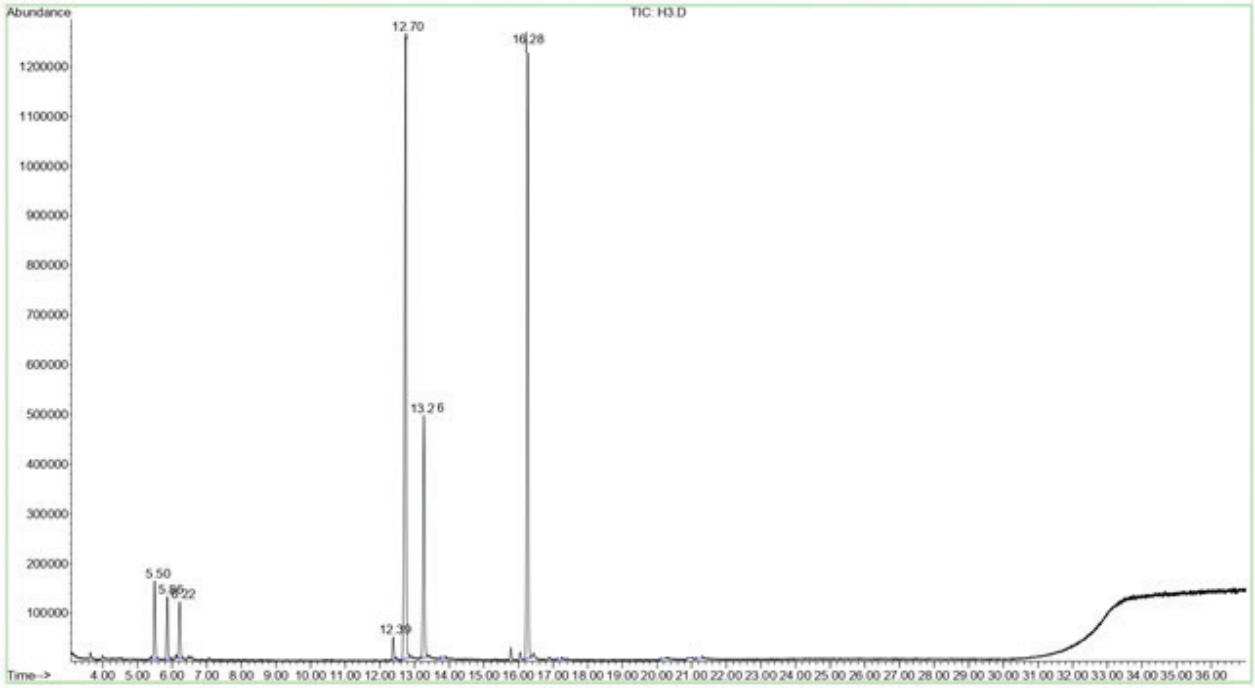

Figure 6. GC-MS chromatogram of derivatives of carbohydrates after hydrolysis in *Rosae frucrus*.

in *Taraxaci radices* and 453.50±0.41 mg g^{-1} in *Menthae folia* (Table 2).

The chromatographic study revealed a number of sugar alcohols, which are often used as sweeteners for diabetics because they have fewer calories and are poorly digested carbohydrates that are only partially absorbed from the small intestine and are not metabolized. Most often, mannitol is used as a sugar substitute owing to its low-calorie content (1.6 calories per gram) (Gupta 2018) and our research showed that *Urticae folia* and *Myrtilli folia* contain this polyol in free and bound form (Table 1). *Myo*-inositol, which is present in *Urticae folia* and *Menthae folia* in the free and bound form (Table 1), together with *D*-chiro-inositol are two inositol stereoisomers, which are acting like insulin mediators. These polyalcohols are involved in increasing insulin sensitivity of body tissues, which reduces insulin

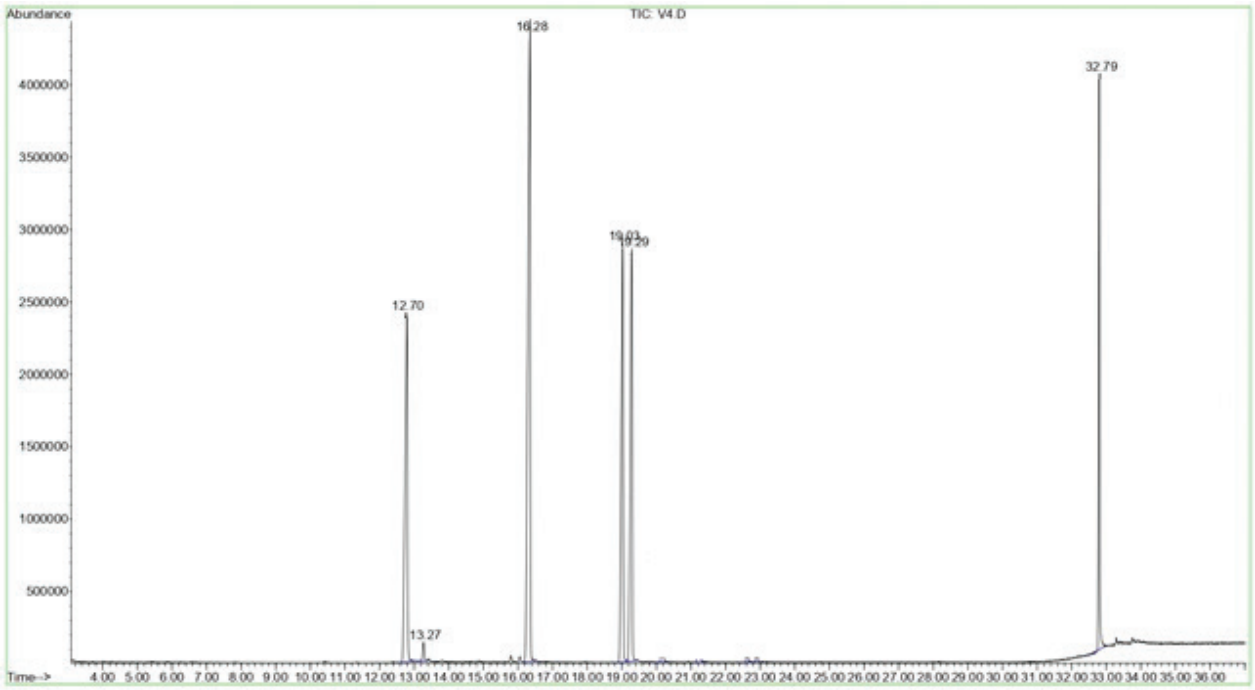

Figure 7. GC-MS chromatogram of derivatives of free carbohydrates in *Taraxaci radices*.

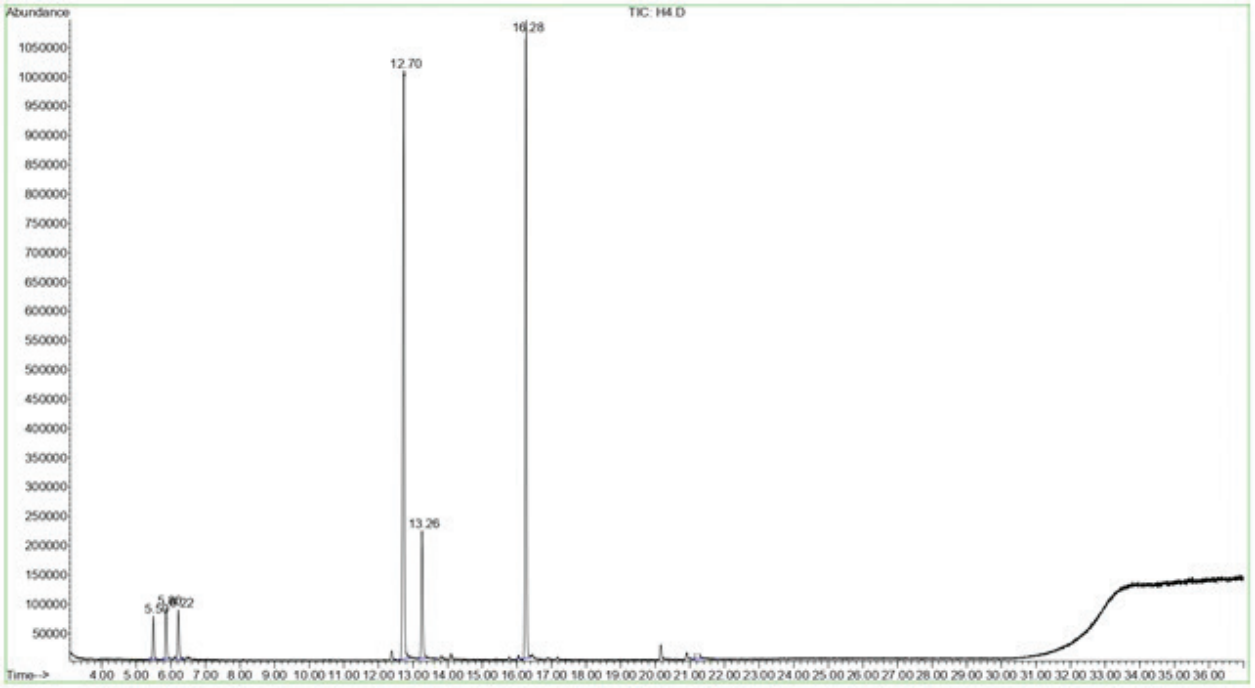

Figure 8. GC-MS chromatogram of derivatives of carbohydrates after hydrolysis in *Taraxaci radices*.

resistance as one of the main pathogenetic mechanisms of development of diabetes mellitus type 2 (Chhetri 2019).

The results show that all plant components, such as *Urticae folia*, *Rosae frucrus Myrtilli folia*, *Menthae folia* and *Taraxaci radices*, of the antidiabetic herbal mixture have a high content of carbohydrates, which provide a number of pharmacological properties of this phytomixture. The carbohydrates obtained from plants are very important active substances for the prevention and treatment of diabetes mellitus and diabetic angiopathies, because they have hypoglycemic, hypolipidemic, anticholesterolemic, antioxidant, anti-inflammatory and detoxifying activities.

This phytochemical study is a confirmation of carbohydrates content in the antidiabetic herbal mixture (*Urticae folia*, *Rosae frucrus*, *Myrtilli folia*, *Menthae folia* and *Taraxaci radices*) as a whole, which was studied in previous

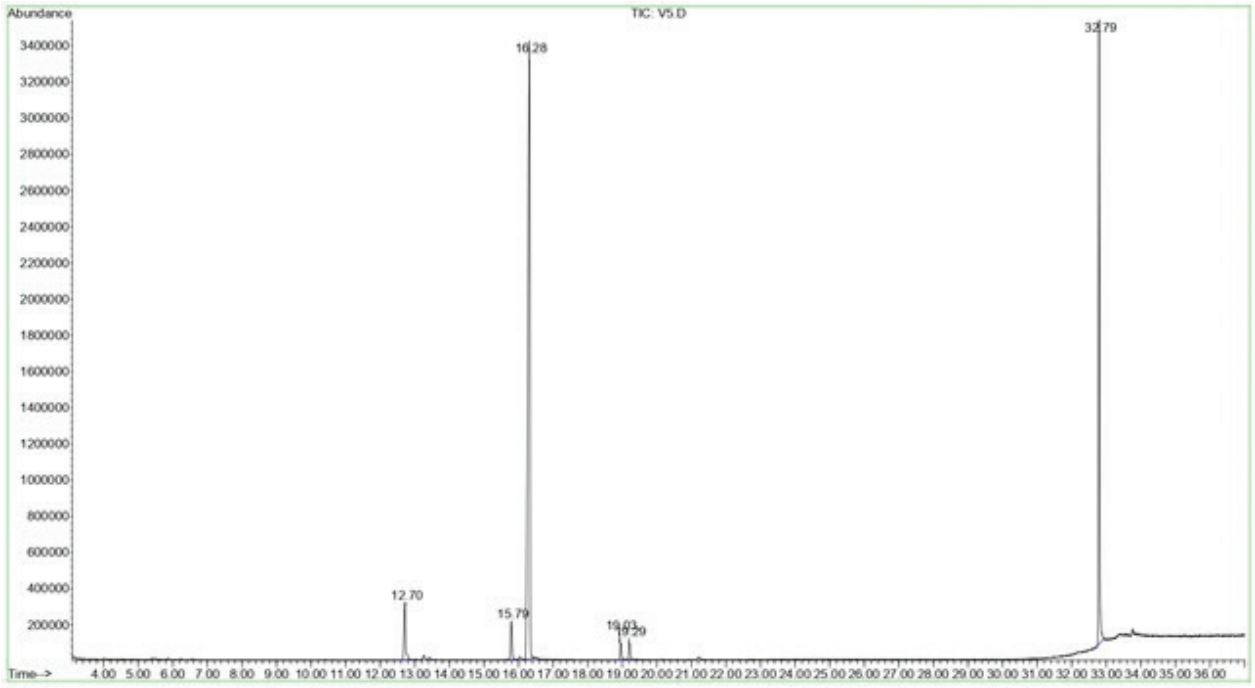

Figure 9. GC-MS chromatogram of derivatives of free carbohydrates in *Menthae folia*.

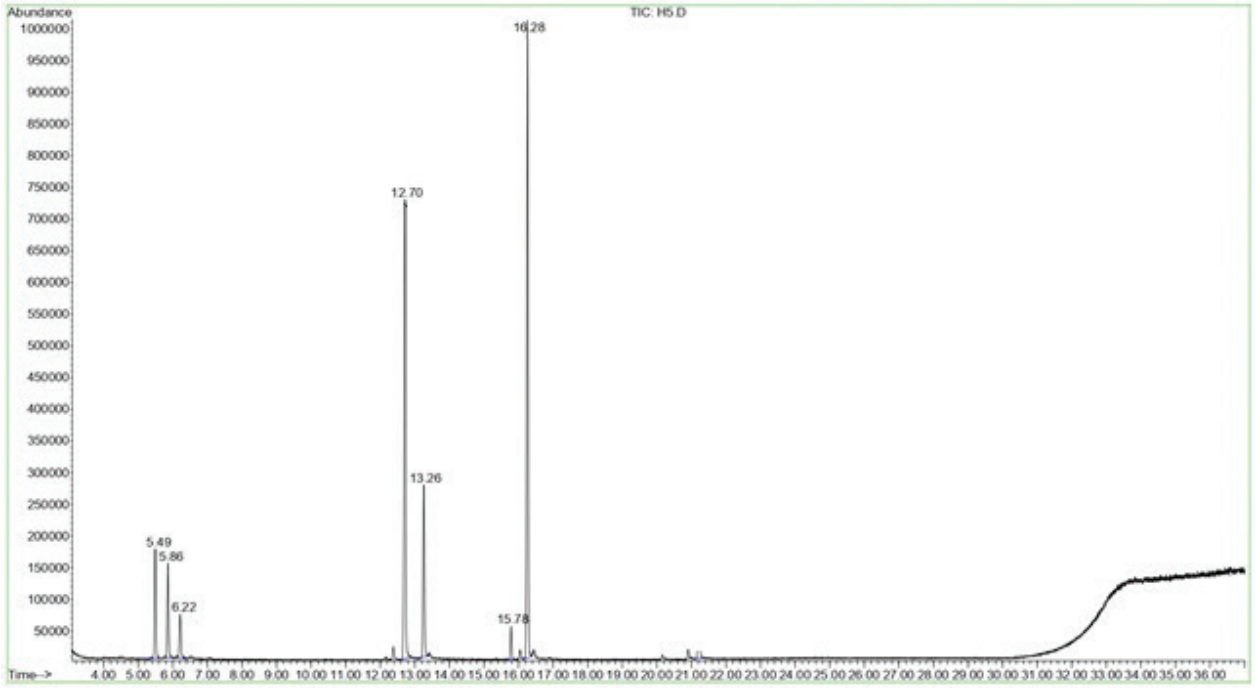

Figure 10. GC-MS chromatogram of derivatives of carbohydrates after hydrolysis in *Menthae folia*.

research (Savych et al. 2020) and shows due to which plant components it is formed. This indicates the feasibility of including each component in the antidiabetic mixture in order to form hypoglycemic, hypolipidemic, anticholesterolemic, antioxidant, anti-inflammatory, and detoxifying activities, which is necessary for the complex therapy of diabetes (Chen et al. 2019; Ganesan and Xu 2019; Zhang et al. 2018).

Conclusion

We identified and established the quantity content of carbohydrates in free and bound form in *Urticae folia*, *Rosae frucrus*, *Myrtilli folia*, *Menthae folia* and *Taraxaci radices*, which are plant components of antidiabetic herbal mixture with hypoglycemic, hypolipidemic, antioxidant, hepatoprotective, pancreatoprotective activity and defined

Table 2. The results of the GC-MS analysis of carbohydrates in the plant components of antidiabetic herbal mixture.

t_R, min (SD±0.01)	Identified substance	Derivatization products	Content in the herbal raw materials (mg g^{-1})				
			Urticae folia	*Myrtilli folia*	*Rosae frucrus*	*Taraxaci radices*	*Menthae folia*
Carbohydrates – free form							
12.70	Glucose	2,3,4,5,6-Penta-*O*-acetyl-*D*-gluconitrile	1.51±0.12	49.32±0.12	376.08±0.38	70.45±0.12	40.65±0.11
13.26	Galactose	2,3,4,5,6-Penta-*O*-acetyl-*D*-galactonitrile	–	–	–	8.48±0.23	–
15.78	*Myo*-inositol	*Myo*-inositol hexa-acetate	0.89±0.11	–		–	38.93±0.12
16.05	Manitol	*D*-mannitol hexa-acetate	0.62±0.11	25.61±0.12	–	–	–
16.28	Sorbitol	*D*-sorbitol hexa-acetate			**Internal standard**		
19.03	Fructose	Naphthalene-1-carboxylic acid-4-butylamino-6,7-dimethoxy-2-methyl- ethyl ester	0.48±0.12	35.97±0.14	318.06±0.31	89.54±0.22	17.09±0.12
19.29	Fructose	1-Nitro-4-phenoxyanthraquinone	0.65±0.08	42.63±0.13	268.12±0.27	76.15±0.24	16.48±0.08
32.79	Saccharose	Saccharose octa-acetate	20.30±0.09	34.72±0.14	452.64±0.34	24.64±0.23	34.54±0.12
Carbohydrates after hydrolysis							
5.50	Ramnose	2,3,4,5-Tetra-*O*-acetyl- *D*-ramnononitrile	55.37±0.19	73.64±0.17	85.95±0.31	54.95±0.25	105.53±0.18
5.86	Arabinose	2,3,4,5-Tetra-*O*-acetyl- *D*-arabinononitrile	88.48±0.32	95.82±0.19	73.97±0.22	56.36±0.21	81.36±0.12
6.22	Xylose	2,3,4,5-Tetra-*O*-acetyl- *D*-xylonitrile	68.95±0.11	128.86±0.24	70.85±0.21	55.73±0.17	42.15±0.11
12.36	Manose	Methyl 2,3,4,6-tetra-*O*-acetyl-α-*D*-manno-hexopyranoside	18.08±0.11	17.84±0.18	65.36±0.11	–	–
12.70	Glucose	2,3,4,5,6-Penta-*O*-acetyl-*D*-gluconitrile	464.76±0.31	653.37±0.38	869.28±0.63	809.95±0.41	453.50±0.41
13.26	Galactose	2,3,4,5,6-Penta-*O*-acetyl-*D*-galactonitrile	105.24±0.23	253.17±0.26	346.90±0.23	220.89±0.32	130.30±0.22
15.78	*Myo*-inositol	*Myo*-inositol hexa-acetate	19.63±0.12	–	–	–	34.75±0.11
16.05	Manitol	*D*-mannitol hexa-acetate	14.72±0.12	21.84±0.13	–	–	–
16.28	Sorbitol	*D*-sorbitol hexa-acetate			**Internal standard**		

Values are expressed as mean ± SD (n=5).

phytochemical composition by GC-MS method. The results of the quantitative study showed that the predominant carbohydrate in free form was saccharose in *Urticae folia*, *L*-fructose in *Myrtilli folia*, *Rosae frucrus*, *Taraxaci radices* and *Menthae folia*, *L*-glucose in *Rosae frucrus*. As for carbohydrates after hydrolysis, the predominant carbohydrate monomer was *L*-glucose in all plant components of antidiabetic herbal mixture. It content was 464.76±0.31 mg g^{-1} in *Urticae folia*, 653.37±0.38 mg g^{-1} in *Myrtilli folia*, 869.28±0.63 mg g^{-1} *Rosae frucrus*, 809.95±0.38 mg g^{-1} in *Taraxaci radices* and 453.50±0.41 mg g^{-1} in *Menthae folia*. This indicates the feasibility of including each component in the antidiabetic mixture in order to form hypoglycemic, hypolipidemic, anticholesterolemic, antioxidant, anti-inflammatory, and detoxifying activities, which is necessary for the complex therapy of diabetes.

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