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HPLC ANALYSIS OF FLAVONOIDS CONTAINED IN THE PLANT COMPONENTS OF ANTIDIABETIC MIXTURE

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Abstract

DM is a global social problem in the field of health care, due to rapid spread of this disease and the development of serious complications such as micro- and macroangiopathies, which significantly reduce the quality and life expectancy of patients.

The aim of our study was to investigate the qualitative composition and quantitative content of flavonoids in *Urticae folia, Rosae fructus, Myrtilli folia, Menthae folia and Taraxaci radices,* which are part of the antidiabetic herbal mixture with established pharmacodynamics and defined phytochemical composition in previous studies.

The study carried out by the HPLC method using an Agilent Technologies 1200 liquid chromatograph (USA). Identification and quantitative analysis were performed using CRS of rutin, isoquercetin, naringin, neohesperidin, quercetin, luteolin, naringenin, kampferol.

During the chromatographic study, 6 compounds from the class of flavonoids were identified in *Urticae folia, Menthae folia* and *Taraxaci radices;* 5 compounds in *Rosae fructus* and 4 compounds in *Myrtilli folia.* According to the results of HPLC analysis, it was found that the studied samples contain the greatest amount of rutin, the content of which is (2314.14±0.53) µg/g in *Urticae folia*, (2585.95±0.46) µg/g in *Rosae fructus,* (854.62±0.21) µg/g in *Myrtilli folia,* (952.54±0.33) µg/g in *Menthae folia* and (268.95±0.31) µg/g in *Taraxaci radices.*

A study of the qualitative composition and quantitative content of flavonoids in *Urticae folia, Rosae frucrus, Myrtilli folia, Menthae folia* and *Taraxaci radices,* substantiates the feasibility of each of these plant components in the antidiabetic mixture in order to form its antioxidant properties, which are manifested by the content of these compounds.

Keywords: *herbal mixture, flavonoids, high performance liquid chromatography, diabetes mellitus, Urticae folia, Rosae fructus, Myrtilli folia, Menthae folia, Taraxaci radices*

Introduction

Diabetes mellitus (DM) is a global social problem in the field of health care, due to rapid spread of this disease and the development of serious complications – micro- and macro-angiopathies, which significantly reduce the quality and life expectancy of patients [1, 2]. According to the official information of International Diabetes Federation (2019) the number of patients with DM type 2 is projected to increase to 642 million by 2040 [3, 4]. An important problem of pharmacovigilance is that existing pharmacotherapy can reduce hyperglycemia, but it is not always able to stabilize fluctuations of blood glucose during the day and keep it at optimal level [5, 6, 7, 8]. This leads to the formation of a cascade of pathological processes – excessive glycation and inactivation of the body's antioxidant defense system, triggering free radical oxidation of lipids and, as a consequently, the development of oxidative stress, which leads to the development and progression of diabetic complications [9, 10, 11, 12]. In turn, oxidative stress, which is the result of the accumulation of reactive oxygen species (ROS) and some derivatives of heavy metals is the main event for the development of insulin resistance [13, 14, 15, 16, 17]. This may reduce peripheral insulin sensitivity through major molecular mechanisms such as β-cell dysfunction, inflammatory responses, decreased regulation and/or localization of glucose transporter 4 (GLUT-4), mitochondrial dysfunction, and abnormal insulin signaling pathways [18, 19, 20, 21, 22]. Therefore, the optimization of pharmacotherapy, searching and study of new drugs with antioxidant activity for the prevention and treatment of DM type 2 and its dangerous complications is a top issue of pharmacy and medicine.

One of these areas is phytotherapy, as it has several advantages over traditional therapy with using oral synthetic agents, namely, it is low-toxic, has a mild pharmacological effect and possibility to be used for long time without significant side effects [23, 24, 25, 26, 27], is well combined with other drugs, has a complex activity through a numerous of biologically active compounds [28, 29, 30, 31, 32, 33, 34]. The combination of different medicinal plants deserves special attention because such herbal mixtures are expected to have more biologically active substances that influence on all links of the pathogenetic mechanism of DM type 2 and its complications development [35, 36, 37, 38]. One of such combinations is an antidiabetic herbal mixture (*Urticae folia*, *Rosae fructus*, *Myrtilli folia*, *Menthae folia* and *Taraxaci radices*) with established hypoglycemic, hypolipidemic, antioxidant, hepatoprotective, pancreatoprotective activity in pharmacological study *in vivo* and *in vitro* [39, 40, 41, 42] and the defined phytochemical composition that determines such pharmacodynamics [43, 44, 45].

Plant biocompounds have a wide range of pharmacological action and a variety influencing mechanisms on DM type 2 development (pathogenesis consists of development of insulin resistance, relative insufficiency of insulin, decreased secretory activity of β-cells of the pancreas) [46, 47, 48] and its angiopathies (pathogenesis consists of inactivation of antioxidant protection system, activation of lipid peroxidation and development of oxidative stress) [49, 50, 51, 52, 53, 54].

In this regard, the important biologically active substances are hydroxylated polyphenolic compounds, namely flavonoids [55, 56, 57, 58].

Therefore, the aim of our study was to investigate the flavonoids content in *Urticae folia*, *Rosae fructus*, *Myrtilli folia*, *Menthae folia* and *Taraxaci radices* as the plant components of antidiabetic herbal mixture.

Methods

Plant materials: The herbal raw materials harvested in June to August 2020 in Ternopil region and Charpathians (*Vaccinium myrtillus* leaf) (Ukraine) were used. After harvesting, the raw materials were dried, crushed and stored according to the general GACP requirements [59]. The plants were identified by Prof. S. M. Marchyshyn. The voucher specimens of herbal raw materials have been deposited in Herbarium of Pharmacognosy with Medical Botany Department, I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine.

Chemicals and standards: All applied reagents were of analytical grade (≥ 95 % purity) and purchased from the Ltd. Sfera Sim (Lviv, Ukraine). Chemical reference substance (CRS) of flavonoids, including rutin, isoquercetin, naringin, neohesperidin, quercetin, luteolin, naringenin, kaempferol were of analytical grade (≥ 95 % purity HPLC) and purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The water used in the studies was produced by MilliQ Gradient water deionizaton system (Millipore, Bedford, MA, USA).

Extraction procedure: The samples of herbal raw materials were ground into a powder by laboratory mill, then about 500 mg (accurately weighed) was selected and placed into flask with 10 mL of 70 % methanol. The extractions were carried out in an ultrasonic water bath at 80°C for 5 hours. The resulting extracts were centrifuged at 3000 rpm and filtered through disposable membrane filters with pores of 0.22 µm [48].

Conditions of HPLC analysis: Flavonoids content in the plant samples was studied by high performance liquid chromatography (HPLC) using 3D LC System from Agilent Technologies 1200 (USA) [60]. The separation was performed on a Zorbax SB-C18 chromatographic column (4.6 mm ± 150 mm, 3.5 µm) (Agilent Technologies, USA) with thermostat temperature 30°C, injection volume of samples 4 µL and flow rate 0.25 mL/min at the gradient elution with the mobile phases – acetonitrile (A) and 0.1 % solution of formic acid in water (B). Elution was performed in a gradient mode: 0 min – A (30 %): B (70 %); 20 min – A (70 %): B (30 %); 22 min – A (100 %): B (0 %); 30 min – A (100 %): B (0 %). The registration of signal was done at 280 nm and 365 nm and fixation of absorption spectra in the range of 210-700 nm.

To identify the components, the obtained spectra were analysed by comparing the retention times (t_R) of CRS of flavonoids. Quantitative analyses were performed using the peaks areas.

Results and Discussion

The results of qualitative detection of flavonoids and their quantitative content in the plant component of the antidiabetic mixture are shown in Figs. 1-5 and Table 1. During HPLC analysis it was identified 6 flavonoids, including 2 anthoxanthins – quercetin and luteolin; 2 flavonols in the form of a glycoside – isoquercetin and rutin; 1 flavanone in the form of a glycoside – neohesperidin and 1 flavanone – naringenin in the three herbal raw materials, such as *Urticae folia, Menthae folia* and *Taraxaci radices* (Figs. 1, 4, 5). According to the results of chromatographic detection it was identified 5 flavonoids, including 2 flavonol glycosides – quercetin and luteolin; 1 flavanone – naringenin and 2 flavonol glycosides – isoquercetin and rutin in *Rosae frucrus* (Fig. 2). Through the research it was found 4 flavonoids, including 1 flavonol glycoside – rutin; 2 anthoxanthins – quercetin and luteolin and 1 flavanone – naringenin in *Myrtilli folia* (Fig. 3).

The quantitative determination of flavonoids showed that the predominant polyphenol in all herbal raw materials was rutin, *Urticae folia* it contained 2314.14±0.53 µg/g, *Rosae fructus* – 2585.95±0.46 µg/g, *Myrtilli folia* – 854.62±0.21 µg/g, *Menthae folia* – 952.54±0.33 µg/g *and Taraxaci radices* – 268.95±0.31 µg/g. Rutin, as a quercetin detivative, has numerous peripheral activities, such as antioxidant, anti-inflammatory, cardiovascular, neuroprotective, antidiabetic, and anticancer [19, 61]. Flavonols, such as quercetin and its glycosides (rutin, isoquercetin), that were detected during HPLC analysis have powerful antioxidant activities, which are manifested due to their chemical structure and provides the cleavage of hydrogen atoms [62]. In addition, flavonols increase the production of glutathione (GSH) and antioxidant enzymes – superoxide dismutase (SOD) and catalase (CAT), as well as inhibits xanthine oxidase, which is involved in the generation of ROS [63, 64].

The second dominant flavonoids was luteolin, *Urticae folia* contained it 356.36±0.17 µg/g, *Rosae fructus* – 301.41±0.13 µg/g, *Myrtilli folia* – 524.16±0.08 µg/g, *Menthae folia* – 713.42±0.22 µg/g *and Taraxaci radices* – 353.16±0.15 µg/g (Table 1). Luteolin, a flavone, which exhibits a series of pharmacological effects, such as antioxidant, antihyperglycemic, antidiabetic, anti-inflammatory, hepatoprotective, antiallergic, antiosteoporotic, anticancer, antiplatelet and vasodilatory properties [19, 61].

The results show that all plant components, such as *Urticae folia, Rosae fructus Myrtilli folia*, *Menthae folia* and *Taraxaci radices* of the antidiabetic herbal mixture have a high content flavonoid, which are very important active substances for the prevention and treatment of DM, as they have powerfull antioxidant activity that will regulate the antioxidant defense system and reduce oxidative stress, which is key in the development and progression of diabetic complications [2, 5, 65].

The chemical structure of flavonoids is based on fifteen-carbon skeleton consisting of two benzene rings linked via a heterocyclic pyrane ring [56]. The pharmacological activities of this biocompounds depend on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization. In particular, the functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions [63, 64].

Antioxidant activity of flavonoids in the treatment and prevention of diabetes and its complications is important as they can include suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation; scavenging ROS; inhibition the enzymes involved in ROS generation – microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, nicotinamide adenine dinucleotide phosphate (NADH) oxidase, and so forth [9, 63, 64].

Conclusions

We identified and established the quantity content of flavonoids in *Urticae folia, Rosae fructus*, *Myrtilli folia*, *Menthae folia* and *Taraxaci radices*, which are plant components of antidiabetic herbal mixture with hypoglycemic, hypolipidemic, antioxidant, hepatoprotective, pancreatoprotective activity and defined phytochemical composition. The results of the quantitative study showed that the predominant flavonoid was rutin in all herbal raw materials. Its content was 2314.14±0.53 μg/g in *Urticae folia*, 2585.95±0.46 μg/g in *Rosae fructus*, 854.62±0.21 μg/g in *Myrtilli folia*, 952.54±0.33 μg/g in *Menthae folia* and 268.95±0.31 μg/g in *Taraxaci radices.*

This phytochemical study is a confirmation of flavonoids content in the antidiabetic herbal mixture in general, which was studied in previous researches and shows which plant components it is formed. This indicates the possibility of including each component in the antidiabetic mixture to form the antioxidant activity required for the complex therapy of DM type 2.

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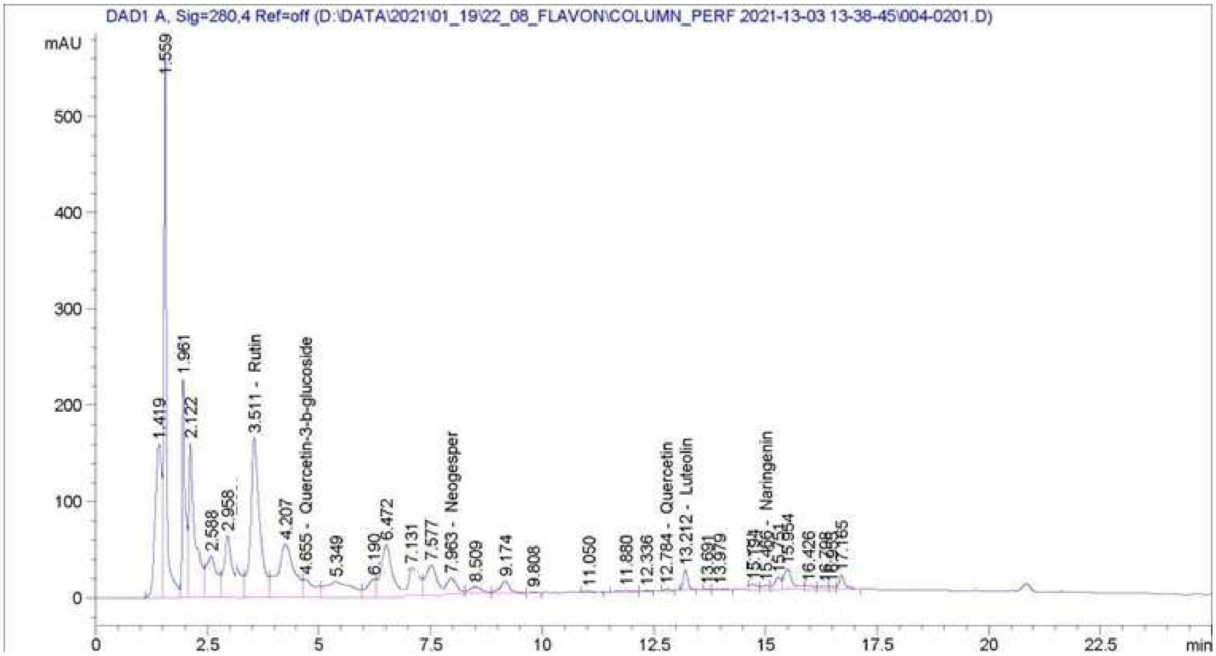

Figure 1. *HPLC chromatogram of flavonoids in Urticae folia. Values are expressed as mean ± SD (n=5).*

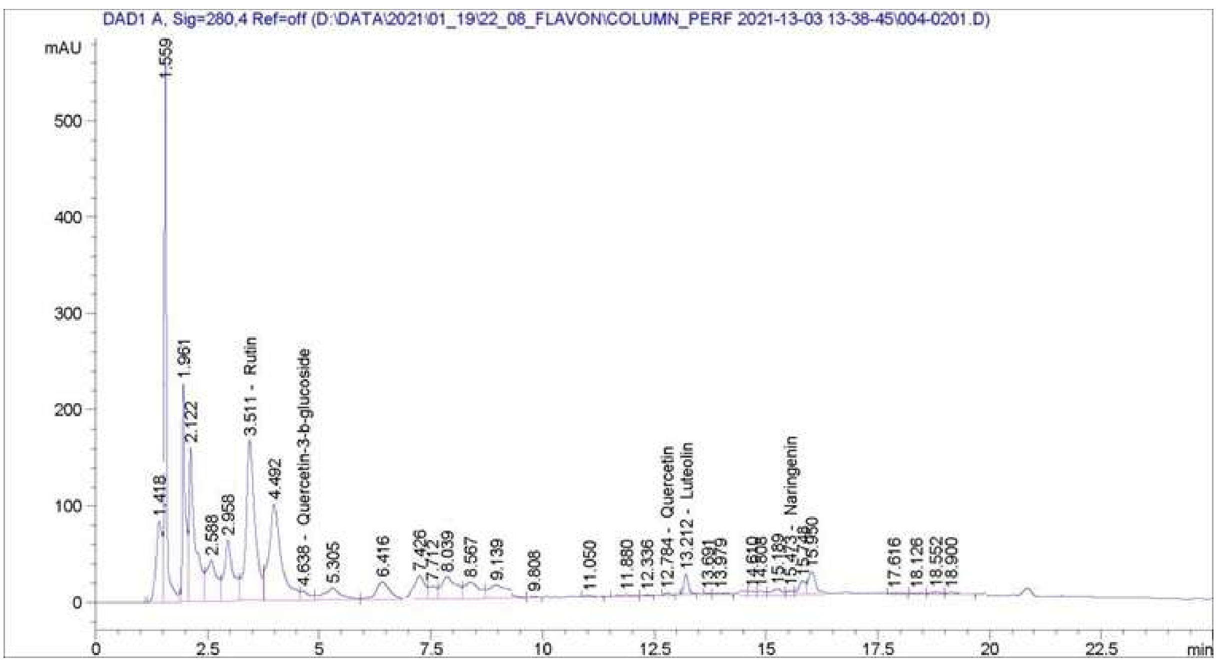

Figure 2. *HPLC chromatogram of flavonoids in Rosae fructus. Values are expressed as mean ± SD (n=5).*

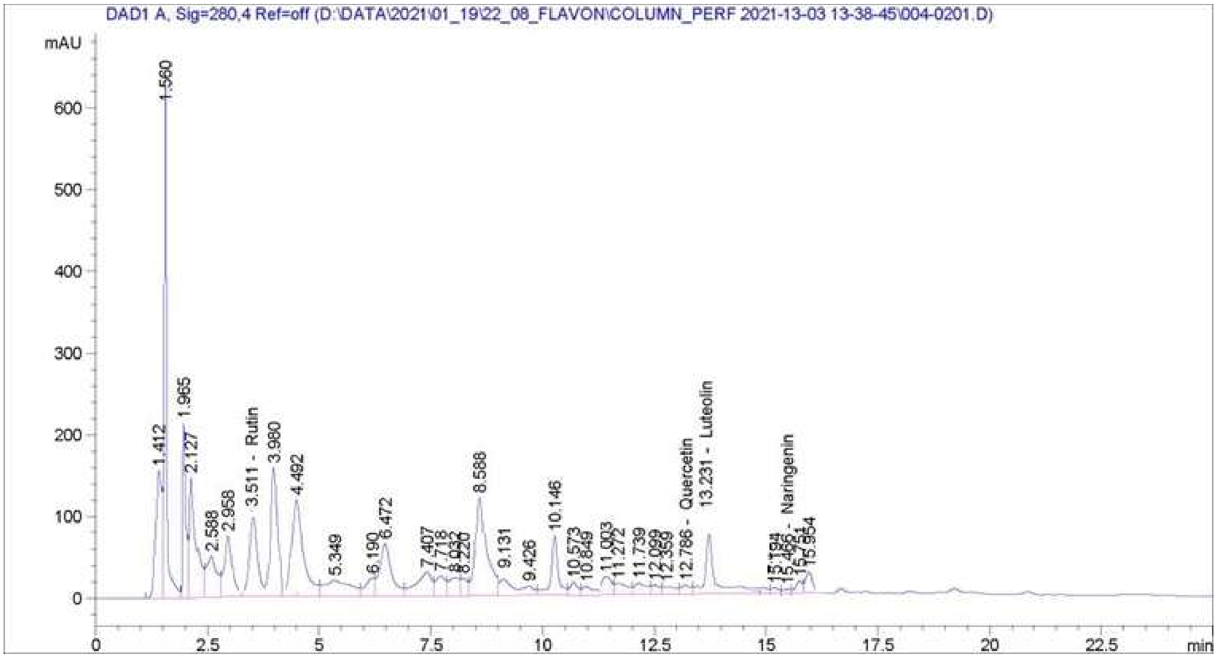

Figure 3. *HPLC chromatogram of flavonoids in Myrtilli folia. Values are expressed as mean ± SD (n=5).*

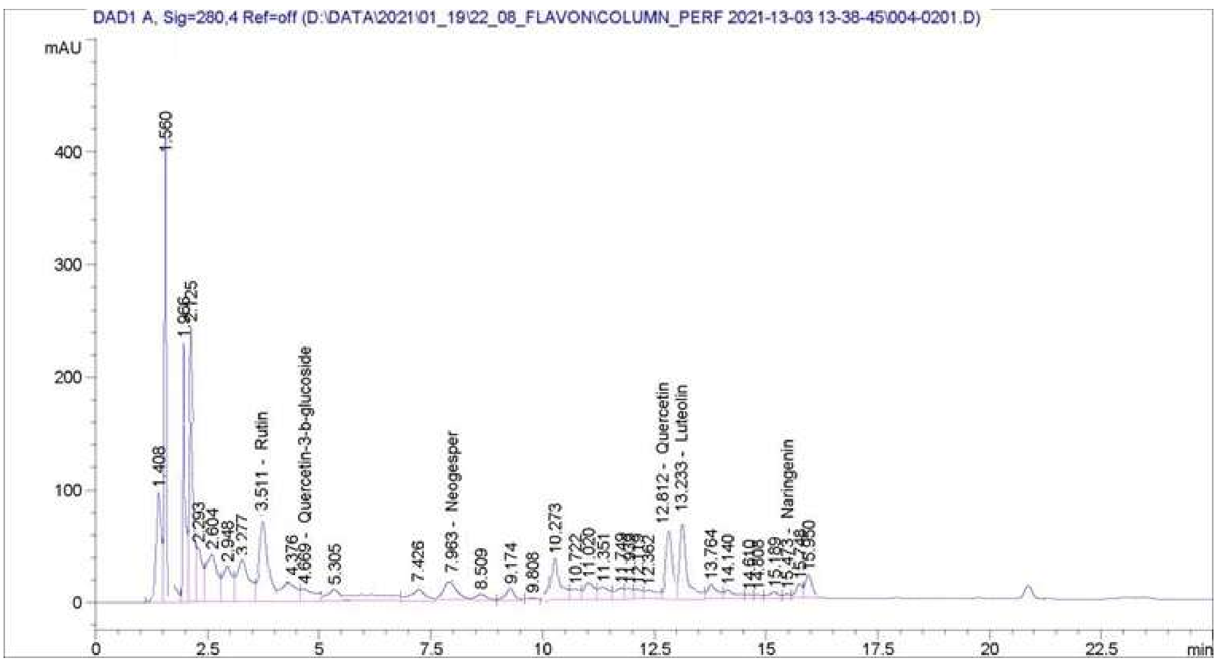

Figure 4. *HPLC chromatogram of flavonoids in Menthae folia. Values are expressed as mean ± SD (n=5).*

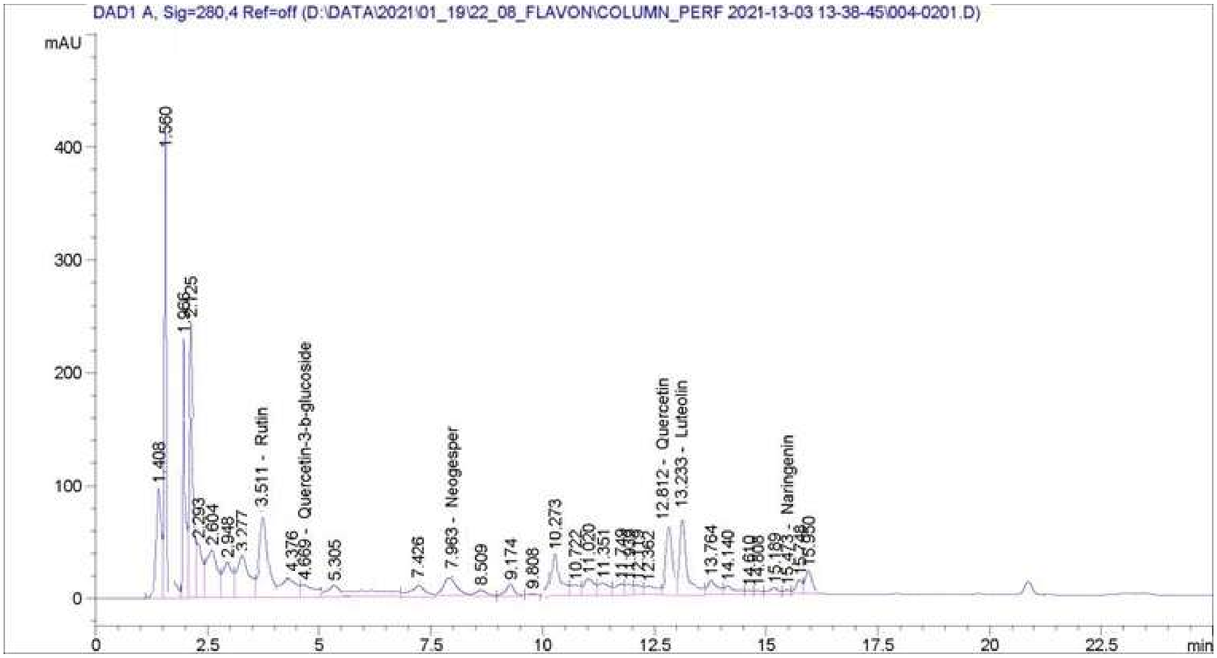

Figure 5. HPLC chromatogram of flavonoids in Taraxaci radices. Values are expressed as mean ± SD (n=5).

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Table 1. The results of HPLC analysis of flavonoids in the plant component of the antidiabetic mixture

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

t_R, min (SD± 0.02)	**Identified substance**	**Content in the plant component of the antidiabetic mixture, µg/g**					**Group of flvonoids**
		Urticae folia	*Rosae fructus*	*Myrtilli folia*	*Menthae folia*	*Taraxaci radices*	
3.51	rutin	2134.14 ±0.53	2585.95 ±0.46	854.62 ±0.21	952.54 ±0.33	268.95 ±0.31	flavonol glycoside
4.65	isoquercetin	209.78 ±0.24	287.19 ±0.23	n/d	69.94 ±0.21	172.79 ±0.22	flavonol glycoside
5.97	naringin	n/d	n/d	n/d	n/d	n/d	flavanone glycoside
7.90	neohesperidin	215.97 ±0.21	n/d	n/d	168.44 ±0.16	321.36 ±0.19	flavanone glycoside
12.78	quercetin	187.70 ±0.18	269.16 ±0.15	68.29 ±0.16	643.18 ±0.27	86.93 ±0.17	flavonol
13.23	luteolin	356.36 ±0.17	301.41 ±0.13	524.1 6±0.08	713.42 ±0.22	353.16 ±0.15	flavone
15.47	naringenin	112.65 ±0.18	97.10 ±0.15	57.92 ±0.13	119.34 ±0.28	68.12 ±0.11	flavanone
17.05	kaempferol	n/d	n/d	n/d	n/d	n/d	flavonol