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Research Article

HPLC-DAD assay of flavonoids and evaluation of antioxidant activity of some herbal mixtures

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Abstract

Medicinal plants and their combinations can influence various links of the pathogenetic mechanism of diabetes mellitus type 2 and its complications, due to the wide range of biologically active substance that they accumulate. Flavonoids deserve particular attention through their antioxidant properties. Three samples of herbal mixtures (sample 1 – *Inula helenium* rhizome with roots, *Helichrysum arenarium* flowers, *Zea mays* columns with stigmas, *Origanum vulgare* herb, *Rosa majalis* fruits, *Taraxacum officinale* roots; sample 2 – *Cichorium intybus* roots, *Elymus repens* rhizome, *Helichrysum arenarium* flowers, *Rosa smajalis* fruits, *Zea mays* columns with stigmas; sample 3 – *Urtica dioica* leaf, *Taraxacum officinale* roots, *Vaccinium myrtillus* leaf, *Rosa majalis* fruits, *Mentha* x *piperita* herb) were tested for flavonoid content and antioxidant properties.

Using HPLC-DAD analysis the content of flavonoids was evaluated and an antioxidant activity by DPPH-radicals scavenging, ferrous ion chelating capacity and ferric reducing power were established for the herbal mixtures. Rutin prevails in sample 3, its content was 2745.66±0.21 µg/g; luteolin – in samples 1 and 2, its content was 371.31±0.07 µg/g and 313.48±0.13 µg/g, respectively.

Flavonoids attribute to the antioxidant activity of the herbal mixtures, which was confirmed by DPPH radical scavenging assay, ferric reducing power assay and ferrous ion chelating assay. The highest antioxidant capacity was found for sample 3 – IC_{50} of inhibition of DPPH radicals was 301.65±2.67 µg/mL compared to control – ascorbic acid (119.24±2.35 µg/mL), the ferric reducing power was 0.382 at 100 µg/mL compared to ascorbic acid (0.412 at 100 µg/mL) and IC_{50} of chelating capacity was 206.59±2.48 µg/mL compared to EDTA-Na_2 (110.55±1.93 µg/mL).

Keywords

diabetes mellitus, herbal mixtures, flavonoids, HPLC-DAD, antioxidant activity

Introduction

Diabetes mellitus (DM) type 2 is a priority problem of World Health Organization. It requires immediate resolution as the epidemiological situation is gaining alarming proportions – the number of diabetics is increasing every year. At the same time, the number of deaths and disabilities is increasing due to the development of diabet-

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ic complications (DCs) as micro- and macro-angiopathies (Harding et al. 2019; Budniak et al. 2021a; Darzuli et al. 2021; American Diabetes Association 2022). According to the official report of International Diabetes Federation (2021) the number of diabetics will increase to 700 million by 2045. An important problem is that existing pharmacotherapy can effectively reduce hyperglycemia, but it is not always able to stabilize fluctuations in glycemic values during the day and to maintain it at an optimal glucose level (Budniak et al. 2021b; Savych and Sinichenko 2021; Savych et al. 2021a, d). As a result, it leads to a cascade of pathological processes – excessive glycation and inactivation of the body's antioxidant defense system, which triggers processes of free radical oxidation of lipids and oxidative stress that contributes to the development and progression of DCs (Skyler et al. 2017; García-Sánchez et al. 2020; Savych and Marchyshyn 2021a, b; Savych and Mazur 2021). In turn, oxidative stress causes even greater insulin resistance due to the accumulation of reactive oxygen species (ROS) and some heavy metal derivatives (iron and copper) (García-Sánchez et al. 2020; Yaribeygi et al. 2020; Savych and Polonets 2021). Therefore, the study of new therapeutic agents with antioxidant activity for the prevention and treatment of DM type 2 and DCs is a top task of pharmacy.

One of these areas can be phytotherapy, either as a monotherapy for the prevention or in the mild stages of DM type 2 or as a combination with traditional therapy in more severe forms of DM. Plant therapy is a justified method for prevention and treatment of DM type 2 (Savych 2021). It has some advantages, such as relatively low toxicity (Savych and Mala 2021), mild pharmacological effects and the possibility to be used for long periods without significant side effects (Marchyshyn et al. 2021a, 2021b, 2021c; Slobodianiuk et al. 2021a, 2021b). It is often well combined with synthetic drugs. Plant substances have a complex activity through a number of biologically active substances (BASs) (Gothai et al. 2016; Kooti et al. 2016; Governa et al. 2018; Budniak et al. 2020, 2021c, d, e; Savych and Nakonechna 2021; Savych et al. 2021g, 2022b). Particular attention deserves combinations of different medicinal plants because they principally have more BASs (Savych et al. 2021b, 2021c, 2022a) that will influence on all links of the pathogenetic mechanism of DM type 2 development.

In this regard, the important BASs are the flavonoids (Ionus et al. 2021; Savych and Milian 2021). The pharmacological activities of these compounds depend on their structural class, degree of hydroxylation, other substitutions and conjugations, and/or their degree of polymerization. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions (Nasri et al. 2015; Panche et al. 2016; Sarian et al. 2017; Orhan et al. 2021). The antioxidant activity of flavonoids could be used the prevention and treatment of DM and DCs. These compounds can suppress ROS formation by either inhibition of enzymes or by chelating trace elements involved in free radical generation; scavenging ROS; inhibition of the enzymes involved in ROS generation – microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, nicotinamide adenine dinucleotide phosphate (NADH) oxidase, etc. (Shashank and Abhay 2013; Kawser et al. 2016).

Thus, the aim of this study was to determine the flavonoid content and to establish the antioxidant properties in three herbal mixtures (HMs) with previously studied *in vitro* and *in vivo* antidiabetic activity (Savych et al. 2021a, d, f; Savych and Sinichenko 2021).

Materials and methods

Plant materials

The raw materials were harvested from June to August 2019 in the Ternopil region (Ukraine). The plants were identified in the Department of Pharmacognosy with Medical Botany, I. Ya. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine. The materials were dried, milled and stored according to the general Good Agricultural and Collection Practice (GACP) requirements (WHO 2003). The voucher specimens of herbal raw materials (Table 1) have been deposited in the departmental herbarium for future records. For the study, three samples of HMs with established antidiabetic properties in our previous preclinical pharmacological studies (Savych and Sinichenko 2021; Savych et al. 2021a, d, f) were used. The composition of HMs is given in Table 1.

Chemicals and standards

Chemical reference substances (CRS) of luteolin, quercetin, kaempferol, naringenin, quercetin-3-rutinoside, quercetin-3-glucoside, naringenin-7-neohesperidoside, hesperetin-7-*O*-neohesperidoside were of primary reference standard grade (≥95% purity HPLC), *L*-ascorbic acid (European Pharmacopoeia reference standard, HPLC grade), ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2)

Table 1. Composition of HMs.

HMs	Component	No. of the voucher specimen	Percentage in HM, %	Relative ratio
Sample 1	*Inula helenium* rhizome with roots	275	10.0	1
	Helichrysum arenarium flowers	105	20.0	2
	Zea mays columns with stigmas	146	20.0	2
	Origanum vulgare herb	078	20.0	2
	Rosa majalis fruits	312	20.0	2
	Taraxacum officinale roots	157	10.0	1
Sample 2	*Cichorium intybus* roots	223	26.32	5
	Elymus repens rhizome	311	26.32	5
	Helichrysum arenarium flowers	105	21.05	4
	Rosa majalis fruits	312	15.79	3
	Zea mays columns with stigmas	146	10.52	2
Sample 3	*Urtica dioica* leaf	058	20.0	1
	Taraxacum officinale roots	157	20.0	1
	Vaccinium myrtillus leaf	301	20.0	1
	Rosa majalis fruits	312	20.0	1
	Mentha x *piperita* herb	126	20.0	1

(pharmaceutical secondary standard, HPLC grade) was purchased from Sigma-Aldrich Chemical Company (Germany). Methanol (≥99.9% purity, HPLC), trichloroacetic acid (TCA) (>99% purity, HPLC), acetonitrile (ACN) (HPLC grade), 0.1% (v/v) formic acid in water (LC-MS grade), phosphate-buffered saline (PBS) (pH 6.6) was purchased from Thermo Fisher Scientific (USA); ferric chloride, ferrous chloride 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p′*-disulfonic acid monosodium salt hydrate (ferrozine) were of analytical grade (≥95% purity) and purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Water used in the studies was produced by MilliQ Gradient water deionizaton system (Millipore, Bedford, MA, USA).

Extraction procedure

The samples were ground to a powder by a laboratory mill, then 500 mg (accurately weighed) were placed in a flask with 5 mL of 60% methanol (v/v). The extraction was carried out in an ultrasonic water bath at 80 °C for 4 hours with reflux condenser. The resulting extracts were centrifuged at 3000 × g and filtered through disposable membrane filters (MF-Millipore Membrane Filter, 0.22 μm pore size) (Sumere et al. 2018).

Chromatographic assay of flavonoids

Content of flavonoids in the samples was studied by high performance liquid chromatography coupled with diode array detector (HPLC-DAD) (Savych et al. 2021e) using 3D LC System from Agilent Technologies 1200 (USA) equipped with a G1313A autosampler, a G1311A quaternary pump, a G1316A thermostat and a G1315B diode array detector (Savych et al. 2021). The separation was performed on a Zorbax SB-Aq chromatographic column (4.6 mm ± 150 mm, 3.5 µm) (Agilent Technologies, USA). Chromatography conditions are shown in Table 2.

Samples were chromatographed in gradient mode (Table 3).

Table 2. Chromatographic conditions.

Flow rate	0.5 mL/min
Eluent supply pressure	10000–12000 kPa
Column temperature	30°C
Injection volume	4 µL
Detection	280 nm
Scan time	0.6 sec
Stoptime	30 min
Range of absorbance spectra	200–400 nm.
Mobile phase A	CAN
Mobile phase B	0.1% (v/v) formic acid in water

Table 3. Gradient mode.

Chromatography time, min	Mobile phase A, %	Mobile phase B, %
0:00	30	70
20:00	70	30
22:00	100	0
30:00	100	0

To identify the components, retention times (t_R) and DAD spectra of the standards CRS were referred to peaks in the chromatogram of the samples. Quantitative analysis was performed using peak area.

Method Validation

Validation of HPLC-DAD method was evaluated in terms of linearity, precision, repeatability, accuracy, limit of detection (LOD) and limit of quantification (LOQ) according to the International Conference on Harmonization (ICH) guidelines (2005). Linearity was performed by injecting a series of standard solutions of each CRS (5–400 µg/mL). The mean value and standard deviation were calculated and regression analysis was performed using Microsoft Excel 2016 (USA). The values for LOD and LOQ were calculated based on the data obtained during linearity testing in the low concentration range (Table 5) of the test solution, using the following formulas:

$$LOD = 3.3 \times \sigma / S; LOQ = 10 \times \sigma / S,$$

where S is the slope of the calibration curve and σ is the standard deviation of the response.

Linearity testing was repeated with the same samples after a complete restart of the system with removal and re-installation of the column. Repeatability precision was determined by five-fold injection of the same sample in a row in a day. For the resulting relative peak area the relative standard deviation (RSD) was calculated. To determine intra-day precision, three standard preparations of each reference standard 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 three standard samples of each CRS solution once each on all three days. The RSD of the samples on that day, together with the previous samples, were calculated as above (Wang et. al 2020). The accuracy of each sample was tested by recovery method. Three different levels of standard solutions (25, 50, and 100 µg/mL) were spiked into the extract. The spiked and unspiked samples were evaluated under the same condition in triplicate, then percent recoveries were calculated by comparing the measured amount of those standards with the amount added.

Antioxidant assay

DPPH radical scavenging assay

DPPH radical scavenging capacity was determined by a known method (Wu et al. 2012). One µL of the studied extracts with a concentration range (100 µg/mL, 200 µg/mL, 400 µg/mL, 800 µg/mL, 1000 µg/mL) were mixed with 2 mL of 0.04 mg/mL DPPH in ethanol. The resulting mixtures were shaken vigorously and allowed to stand at 25 °C for 30 min. Then, they were centrifuged at 1500 ×

g for 10 min, after which, the absorbance of the supernatants were measured at 517 nm. Ascorbic acid was used as a control. DPPH radical scavenging capacity was calculated using the following formula:

$$\% \text{ DPPH scavenging activity} = \frac{1-(A1-A2)}{A0} \times 100$$

where A_0 – the absorbance of the control (without extracts); A_1 – the absorbance of the sample with extracts; A_2 – the absorbance without DPPH.

Antioxidant activities of the extracts were expressed as IC_{50}, which was defined as the concentration in μg of dry material per mL (μg/mL) that inhibits the formation of DPPH radicals by 50%. Each value was determined from the regression equation.

Ferric reducing power assay

Reducing power was determined by the same known method (Wu et al. 2012). One μL of the extracts with a concentration range of 100 μg/mL, 200 μg/mL, 400 μg/mL, 800 μg/mL, 1000 μg/mL in PBS 0.2 mol/L (pH 6.6) were added to 2 mL of 1% potassium ferricyanide and incubated at 50 °C for 20 min. For the termination of reaction, 2 mL of 10% TCA was added. Then the obtained solutions were centrifuged at 3000 × g for 10 min and 2.5 mL of supernatants were mixed with 2.5 mL deionized distilled water and 0.5 mL of 0.1% ferric chloride. The mixtures were allowed to rest for 10 min and absorbance was measured at 700 nm. Ascorbic acid was used as a control.

Ferrous ion chelating assay

Ferrous ion chelating capacity was determined by the same previously reported method (Wu et al. 2012). Three μL of the extracts (100 μg/mL, 200 μg/mL, 400 μg/mL, 800 μg/mL, 1000 μg/mL) were added to 0.05 mL of 2 mmol/L ferrous chloride solution, and 0.2 mL of 5 mmol/L ferrozine solution. Then mixtures were shaken vigorously and incubated at 25 °C for 10 min. The absorbance was measured at 562 nm. EDTA-Na_2 was used as a control. Ferrous ion chelating capacity was calculated using the following formula:

$$\% \text{ chelating capacity} = \frac{AO-(A1-A2)}{A0} \times 100$$

where A_0 – the absorbance of the control (without extracts); A_1 – the absorbance of the sample with extracts; A_2 – the absorbance without EDTA-Na_2.

Chelating activity of the extracts was expressed as IC_{50}, which was defined as the concentration in μg of dry material per mL (μg/mL) that catalyzes the chelation of metal ions by 50%. Each value was determined from regression equation.

Statistical analysis

Statistical significance was calculated by one-way analysis of variance (ANOVA) using Microsoft Excel 2016 (USA). The data are expressed as a mean ± SD (n=5). Differences between values were considered significant when $p<0.05$.

Results and discussion

The results of qualitative and quantitative analyses of flavonoids in HMs are presented in Table 4. Three flavonoids (flavonols – quercetin and luteolin and a flavanone – naringenin) were identified in sample 1. Four flavonoids (flavonols – isoquercetin and quercetin, flavone – luteolin and flavanone – naringenin) were quantified in sample 2. In sample 3 four flavonoids (flavonol glycosides – rutin and isoquercetin, flavonols – quercetin and luteolin were determined (Figs 1–3).

The quantitative determination of flavonoids showed that predominant in sample 1 and sample 2 was luteolin, its content was 371.31±0.07 μg/g and 313.48±0.13 μg/g, respectively (Table 4). Luteolin, a flavone, exhibits a numerous of pharmacological effects: antioxidant, antihyperglycemic, antidiabetic, anti-inflammatory, hepatoprotective, antiallergic, antiosteoporotic, anticancer, antiplatelet and vasodilatory properties (Bumke-Vogt et al. 2014; Li et al. 2015).

Rutin was determined as the major component in sample 3 – 2745.66±0.21 μg/g (Table 4). Rutin (quercetin-3-rutinoside) has numerous peripheral activities: antioxidant, anti-inflammatory, cardiovascular, neuroprotective, antidiabetic, and anticancer (Sarian et al. 2017; Enogieru et al. 2018).

The second dominant flavonoid in all investigated HMs was quercetin, its content was 200.70±0.18 μg/g in sample 1, 164.68±0.15 μg/g in sample 2 and 273.25±0.16 μg/g in sample 3 (Table 4). The content of isoquercetin was 47.11±0.03 μg/g in sample 2 and was 72.81±0.04 μg/g in sample 3 (Table 4). Flavonols, such as quercetin and its glycosides (rutin, isoquercetin), that were detected during HPLC-DAD analysis have powerful antioxidant activities, which are manifested due to their chemical structure, which provides the cleavage of hydrogen atoms. In addition, flavonols increase the production of glutathione (GSH) and antioxidant enzymes – superoxide dismutase (SOD) and catalase (CAT), as well as inhibit xanthine oxidase, which is involved in the generation of ROS (Dabeek and Marra 2019; Xu et al. 2019; Chouikh 2020).

It was established the quantitative content of naringenin in sample 1 and sample 2, it was 57.98±0.08 μg/g and 53.10±0.05 μg/g, respectively (Table 4).

Table 4. HPLC-DAD assay of flavonoids in HMs.

No. of peak	t_R, min (SD±0.02)	Identified compound	Content in HMs, μg/g		
			Sample 1	Sample 2	Sample 3
1.	3.51	quercetin-3-rutinoside	–	–	2745.66±0.21
2.	4.65	quercetin-3-glucoside	–	47.11±0.03	72.81±0.04
3.	5.97	naringenin-7-neohesperidoside	–	–	–
4.	7.90	hesperetin-7-*O*-neohesperidoside	–	–	–
5.	12.78	quercetin	200.70±0.18	164.68±0.15	273.25±0.16
6.	13.23	luteolin	371.31±0.07	313.48±0.13	264.10±0.08
7.	15.47	naringenin	57.98±0.08	53.10±0.05	–
8.	17.05	kaempferol	–	–	–

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

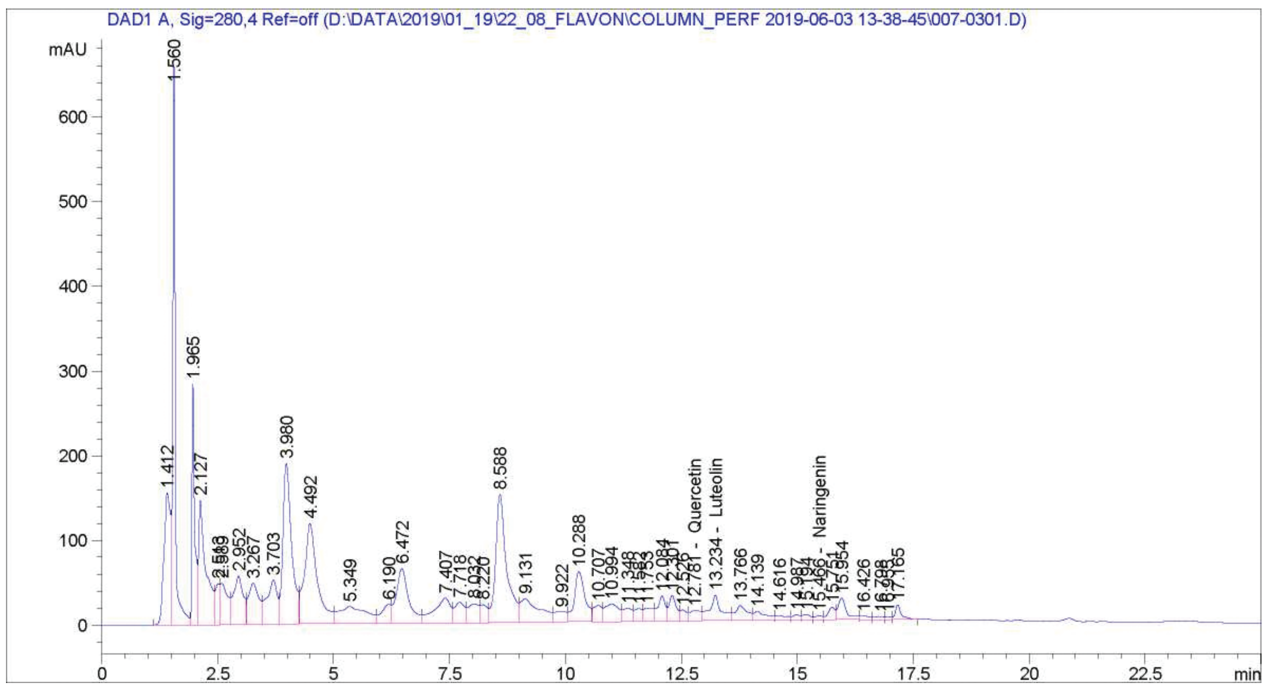

Figure 1. HPLC-DAD chromatogram of flavonoids identified in sample 1, λ=280 nm.

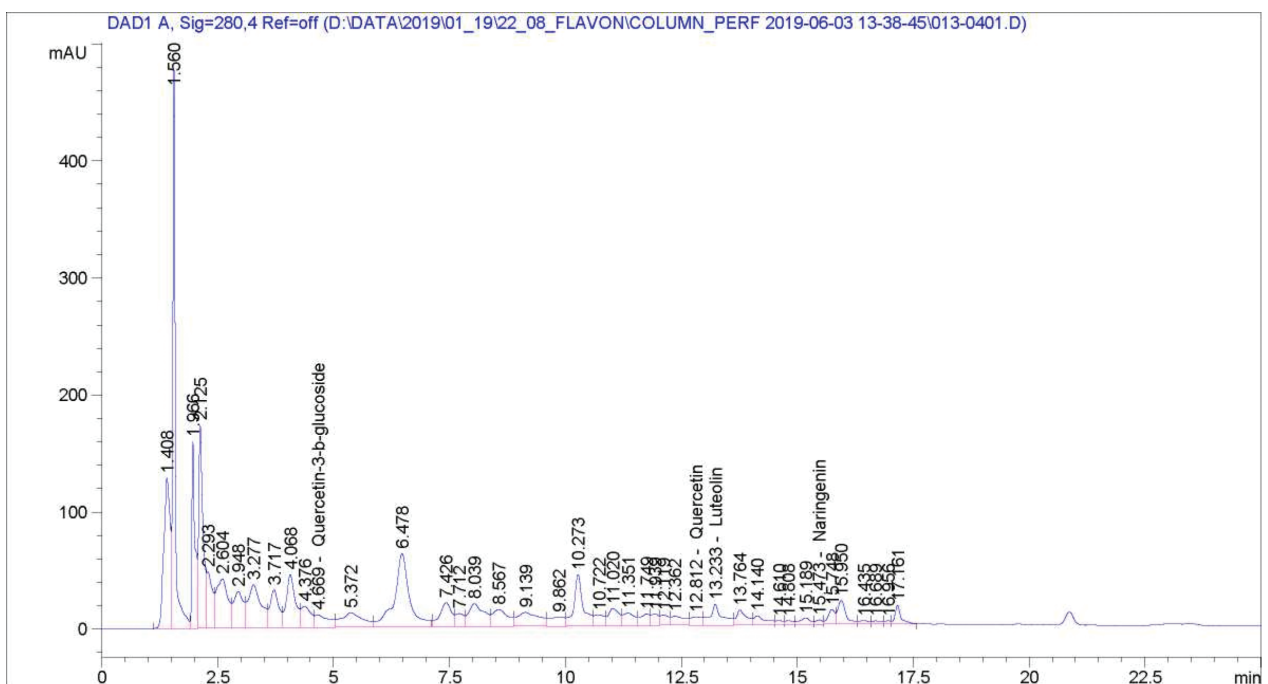

Figure 2. HPLC-DAD chromatogram of flavonoids identified in sample 2, λ=280 nm.

Naringenin, a flavanone, has antioxidant, anti-inflammatory, immunomodulatory, hepatoprotective, nephroprotective, neuroprotective, anticancer, antiatherosclerotic, and antidiabetic actions (Hernández-Aquino and Muriel 2018; Den Hartogh and Tsiani 2019; Zheng et al. 2019).

The chromatographic method was validated by evaluating linearity range, precision, repeatability, accuracy, LOD and LOQ. The linearity of the method was appreciated by studying its ability to obtain an analyte response linearly proportional to its concentration in a given range. To determine linearity parameter, calibration curves were generated by injection of standard solutions of each CRS at six concentration levels in triplicate and their correlation coefficients were calculated. As shown in Table 5, the linearity of the HPLC-DAD method was good, R^2 was in the range of 0.996 – 0.999.

The precision of the method was evaluated by injecting the same sample spiked with three concentration levels (covering the specific range for each compound) five

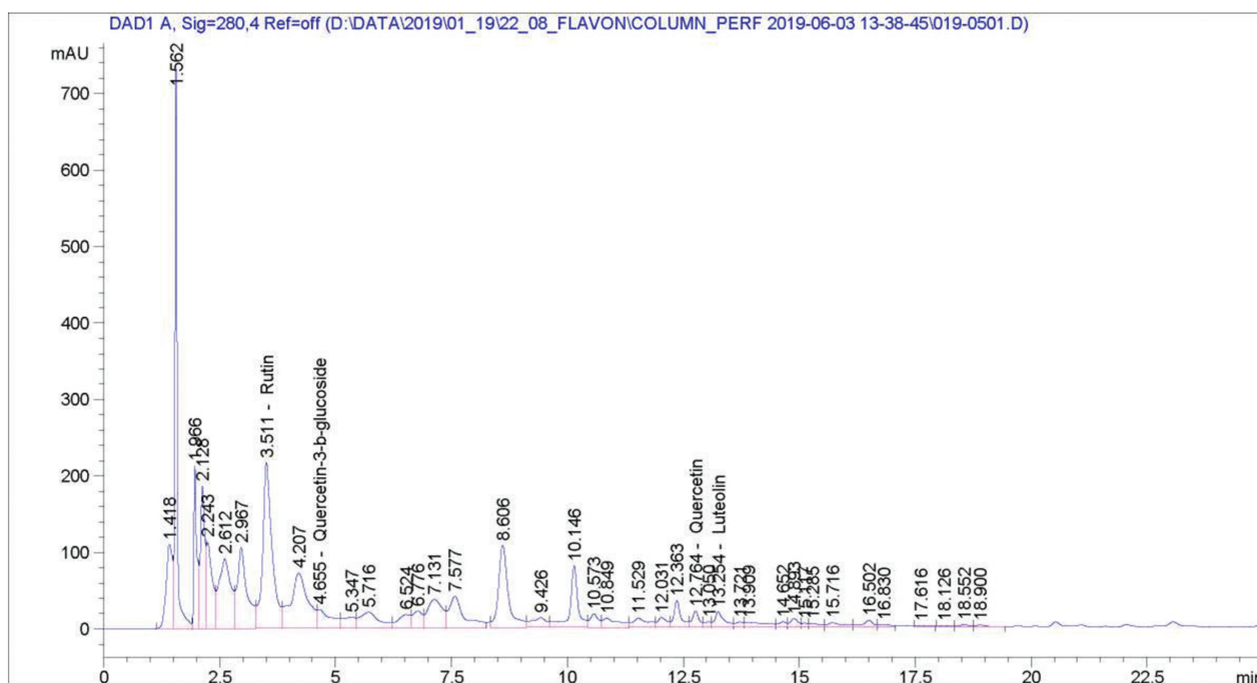

Figure 3. HPLC-DAD chromatogram of flavonoids identified in sample 3, λ=280 nm.

Table 5. Analytical data of linearity, sensitivity, precision for HPLC-DAD method.

Compound	Linear range, μg/mL	R^2	Regression equation	LOD, μg/mL	LOQ, μg/mL	Precision, % RSD	Repeatability, % RSD
quercetin-3-rutinoside	5–300	0.998	y = 162.41× – 46.78	0.2	0.7	1.91	1.33
quercetin-3-glucoside	5–400	0.999	y = 190.98× – 14.96	0.1	0.3	1.41	0.89
naringenin-7-neohesperidoside	5–400	0.997	y = 72.67× – 29.44	0.3	1.0	2.38	1.27
hesperetin-7-O-neohesperidoside	5–400	0.996	y = 75.81× – 33.14	0.2	0.5	1.42	0.91
quercetin	5–300	0.999	y = 245.77× – 13.57	0.1	0.2	0.64	0.42
luteolin	5–400	0.999	y = 368.06× – 53.26	0.2	0.5	1.73	1.23
naringenin	5–300	0.999	y = 244.51× – 19.13	0.1	0.2	1.54	1.28
kaempferol	5–400	0.999	y = 195.98× – 9.65	0.2	0.5	0.39	0.21

times, during three consequent days. Repeatability was calculated by analysing five times the same solution of each CRS. Both parameters were evaluated by RSDs that were in the range of 0.39% – 2.38% for inter-day precision and were from 0.21% to 1.28% for intra-day precision (Table 5).

HPLC-DAD method allowed the detection of flavonoids in the range of 0.1 – 0.3 μg/mL and the quantification in the range of 0.2 – 1.0 μg/mL, as it is shown in Table 5.

The accuracy of HPLC-DAD method was evaluated by the recovery test. In this way, previously analyzed samples of CRS, were spiked at three concentration levels (25, 50, and 100 μg/mL) with the target compounds and were injected in triplicate. The recoveries of all compounds ranged between 99.22% and 101.25% (Table 6).

The antioxidant activities *in vitro* of HMs with a concentration range 100–1000 μg/mL were evaluated by DPPH radical scavenging activity, ferrous ion chelating capacity and ferric reducing power.

An association between increased DPPH radical scavenging activities and the concentration of methanol extracts of HMs has been established. It was established that IC_{50} of sample 1 was 377.49±2.98 μg/mL; IC_{50} of sample 2 – 349.26±3.21 μg/mL and IC_{50} of sample 3 – 301.65±2.67 μg/mL. The IC_{50} value of the control – ascorbic acid was 119.24±2.35 μg/mL (Fig. 4a).

According to the result of *in vitro* assay, the value of ferric reducing power of sample 1 was 0.324 at 100 μg/mL and 0.692 at 1000 μg/mL; the value of sample 2 – 0.364 at 100 μg/mL and 0.713 at 1000 μg/mL; the value of sample 3 – 0.382 at 100 μg/mL and 0.744 at 1000 μg/mL. Ascorbic acid exhibited only slightly higher activity, with a value of ferric reducing power of 0.412 and 0.791 at 100 μg/mL and 1000 μg/mL, respectively (Fig. 4b).

For the chelating capacity, it was established that IC_{50} of sample 1 was 314.50±2.74 μg/mL; IC_{50} of sample 2 – 284.08±2.69 μg/mL; IC_{50} of sample 3 – 206.59±2.48 μg/mL. IC_{50} value of positive control – EDTA-Na_2 was 110.55±1.93 μg/mL (Fig. 4c).

The *in vitro* study showed that HMs can reduce the oxidative stress by capturing free radicals and binding heavy metal ions with free radical activity. Among three studied HMs, the best antioxidant properties had sample 3

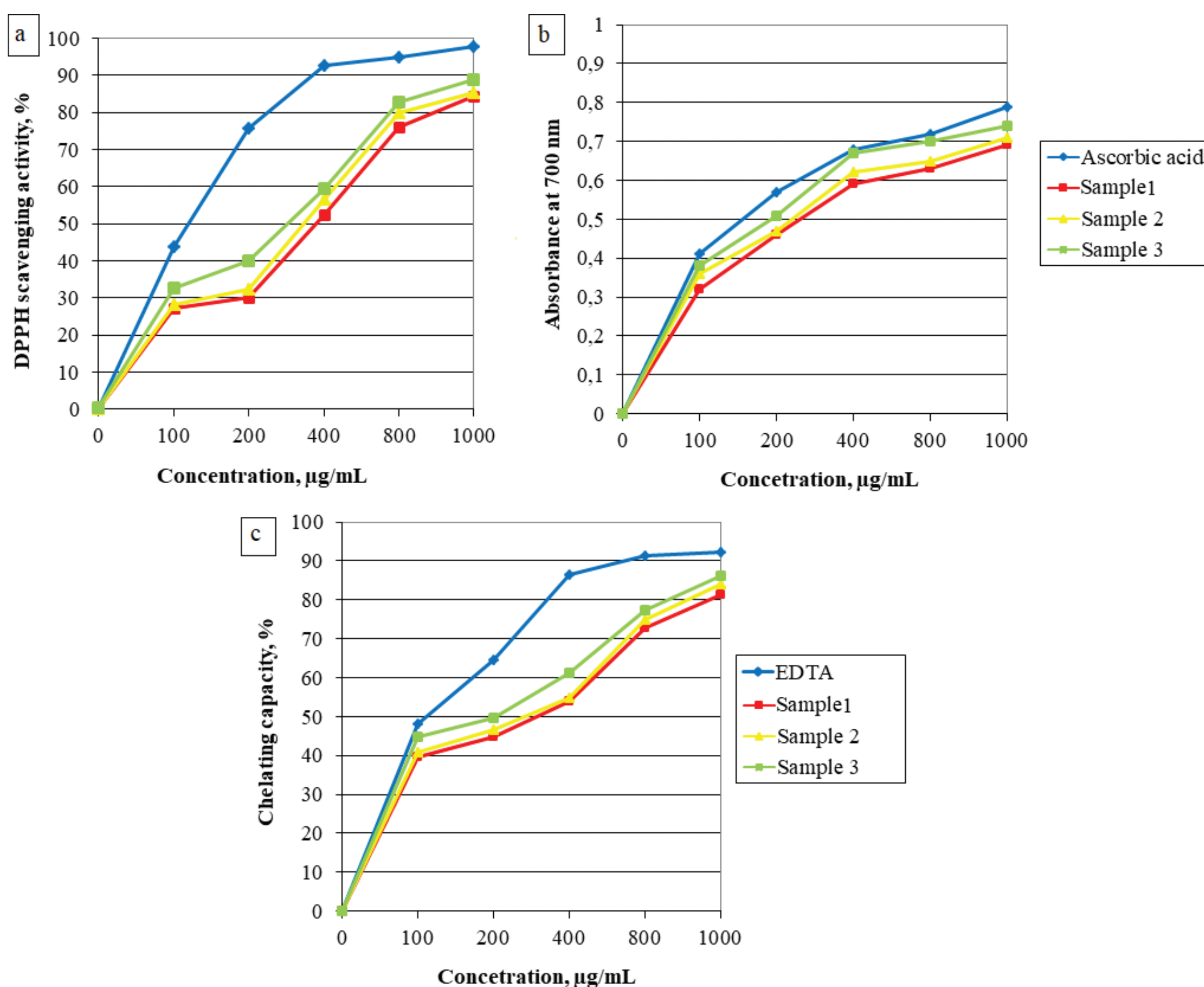

Figure 4. DPPH radical scavenging activities of the methanol extracts of HMs and ascorbic acid (**a**); Ferric reducing power of the methanol extracts of HMs and ascorbic acid (**b**); Ferrous ion chelating activities of the methanol extracts of HMs and EDTA. Values are expressed as a mean ± SD (n=5).

(*Urtica dioica* leaf, *Taraxacum officinale* roots, *Vaccinium myrtillus* leaf, *Rosa majalis* fruits, *Mentha* x *piperita* herb) in terms of inhibition of DPPH radicals, ferric reducing power and ferrous ion chelating activities. This result is explained by the fact that this HM contains the highest total content of flavonoids according to HPLC-DAD analysis (Table 4). In addition, the high antioxidant activity of all HMs was confirmed in the studies *in vivo*, due to the regulation between the antioxidant defense system and the lipid peroxidation system (Savych and Sinichenko 2021; Savych et al. 2021a, d).

Thus, studies on the detection of flavonoids by HPLC-DAD in HMs and analysis of their antioxidant *activity in vitro* by DPPH radical scavenging assay, ferric reducing power assay and ferrous ion chelating assay prove their antioxidant capacity. This gives us reason to believe that these HMs may be promising additions in the complex treatment of DM type 2 and DCs.

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Table 6. Analytica data of accuracy for HPLC-DAD method.

Compound	Added amount, µg/mL	Found amount*, µg/mL	Recovery*, %	RSD, %
quercetin-3-rutinoside	25	25.03±0.06	100.12±0.24	0.34
	50	50.55±0.02	101.10±0.04	0.66
	100	99.45±0.04	99.45±0.04	0.46
quercetin-3-glucoside	25	25.11±0.03	100.45±0.11	0.42
	50	50.63±0.04	101.25±0.22	0.89
	100	100.89±0.06	100.89±0.06	0.51
naringenin-7-neohesperidoside	25	25.12±0.05	100.49±0.49	0.44
	50	49.61±0.07	99.22±0.12	0.54
	100	99.39±0.04	99.39±0.04	0.48
hesperetin-7-O-neohesperidoside	25	25.30±0.02	101.21±0.07	0.75
	50	49.81±0.05	99.61±0.11	0.45
	100	100.94±0.05	100.94±0.05	0.59
quercetin	25	44.80±0.04	99.41±0.08	0.49
	50	70.25±0.04	100.26±0.05	0.37
	100	120.53±0.06	100.38±0.05	0.41
luteolin	25	62.56±0.04	100.69±0.07	0.52
	50	87.44±0.05	100.36±0.05	0.40
	100	138.52±0.08	101.02±0.05	0.62
naringenin	25	30.91±0.06	100.35±0.20	0.39
	50	55.61±0.07	99.66±0.12	0.35
	100	105.28±0.08	99.51±0.07	0.38
kaempferol	25	25.27±0.03	101.09±0.11	0.65
	50	50.19±0.03	100.39±0.05	0.41
	100	100.47±0.04	100.47±0.04	0.52

*Values are the mean ± SD (n=3)

Conclusions

The identification and determination of quantity content of flavonoids in three samples of HMs was carried out by HPLC-DAD assay. The calibration curves of eight CRS were linear (R^2>0.996) over the range of 5–400 µg/mL. LODs and LOQs were in the range of 0.1–0.3 µg/mL and 0.2–1.0 µg/mL, respectively, RSDs were from 0.39% to 2.38% for inter-day precision and from 0.21% to 1.28 for intra-day precision. The recoveries of all compounds ranged between 99.22% and 101.25%. During HPLC-DAD analysis were identified three flavonoids: luteolin, quercetin and naringenin in sample 1 (*Inula helenium* rhizome with roots, *Helichrysum arenarium* flowers, *Zea mays* columns with stigmas, *Origanum vulgare* herb, *Rosa majalis* fruits, *Taraxacum officinale* roots); four flavonoids: luteolin, quercetin, isoquercetin and naringenin in sample 2 (*Cichorium intybus* roots, *Elymus repens* rhizome, *Helichrysum arenarium* flowers, *Rosa majalis* fruits, *Zea mays* columns with stigmas); four flavonoids: rutin, isoquercetin, luteolin, quercetin in sample 3 (*Urtica dioica* leaf, *Taraxacum officinale* roots, *Vaccinium myrtillus* leaf, *Rosa majalis* fruits, *Mentha* x *piperita* herb). The flavonoids that have been identified explain the antioxidant activity of three samples of HMs, which has been confirmed by *in vitro* studies – DPPH radical scavenging assay, ferric reducing power assay and ferrous ion chelating assay.

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