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Flavonoid-profile determination for a hypoglycemic collection by high-performance liquid chromatography

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Objectives. *Herbal hypoglycemic drugs complement the conventional approach to the treatment of type-2 diabetes based on the use of synthetic prescription drugs. However, their scientifically based application and standardization are limited due to inadequate and often outdated information on their chemical composition. Accordingly, we have developed a hypoglycemic collection (HGC) consisting of common bean pods (Phaseolus vulgaris L.), bilberry shoots (Vaccinium myrtillus L.), galega herb (Gallega officinalis L.), common knotgrass herb (Polygonum aviculare L.), burdock roots (Arctium lappa L.), and cinnamon rose hips (Rosa cinnamomea L.). According to a number of researchers, the antidiabetic properties of these herbs are largely due to the presence of polyphenolic compounds, especially flavonoids. The aim of this study was to determine the profile of flavonoids in the HGC and in its total dry extract (TDE).*

Methods. *The study was performed by reverse-phase high-performance liquid chromatography with diode array and mass spectrometric detection.*

Results. *Nine individual flavonol glycosides—derivatives of myricetin, quercetin, kaempferol and kaempferide—were identified in the HGC and the TDE. The main flavonol glycosides in the studied objects were robinin and kaempferol-3-glucuronide, the contents of which in the HGC were 2.09 and 2.22 mg/g, in the TDE 4.85 and 3.84 mg/g, respectively. The other flavonol glycosides were determined in the HGC and its TDE at significantly lower concentrations.*

Conclusions. *The method developed in the study can be used to standardize HGCs and estimate their pharmacological activities.*

Keywords: *flavonoids, hypoglycemic collection, total dry extract, high-performance liquid chromatography, diode array detection, mass spectrometric detection.*

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Определение профиля флавоноидов в гипогликемическом сборе методом высокоэффективной жидкостной хроматографии

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Цели. *Гипогликемические препараты растительного происхождения успешно дополняют синтетические рецептурные лекарства, использующиеся в традиционном подходе к лечению сахарного диабета 2 типа. Однако научно обоснованное применение и стандартизация таких препаратов ограничены из-за неадекватной и часто устаревшей информации об их химическом составе. Нами был разработан гипогликемический сбор (ГГС), состоящий из створок фасоли обыкновенной (Phaseolus vulgaris L.), побегов черники обыкновенной (Vaccinium myrtillus L.), травы галеги лекарственной (Gallega officinalis L.), травы горца птичьего (спорыша) (Polygonum aviculare L.), корней лопуха большого (Arctium lappa L.), плодов шиповника коричного (Rosa cinnamomea L.). По мнению ряда исследователей, антидиабетические свойства вышеупомянутых растений во многом обусловлены присутствием в них полифенольных соединений, особенно флавоноидов. Цель данного исследования – определение профиля флавоноидов в ГГС и в суммарном сухом экстракте (ССЭ) на основе ГГС.*
Методы. *Исследование проводили методом обращено-фазовой высокоэффективной жидкостной хроматографии с диодно-матричным и масс-спектрометрическим детектированием.*
Результаты. *В ГГС и ССЭ было идентифицировано девять индивидуальных флавонолгликозидов – производных мирицетина, кверцетина, кемпферола и кемпферида. Основными флавонолгликозидами в исследуемых объектах были робинин и кемпферол-3-глюкуронид, содержание которых в ГГС составило 2.09 и 2.22 мг/г, в ССЭ – 4.85 и 3.84 мг/г, соответственно. Остальные флавонолгликозиды были обнаружены в ГГС и ССЭ в существенно более низких концентрациях.*
Выводы. *Результаты работы могут быть использованы при стандартизации ГГС и оценке его фармакологической активности.*

Ключевые слова: *флавоноиды, сбор гипогликемический, суммарный сухой экстракт, высокоэффективная жидкостная хроматография, диодно-матричное детектирование, масс-спектрометрическое детектирование.*

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In recent years, diabetes has become a serious global public health problem, affecting people of all ages, genders, and ethnicities. A close relationship has been established between type 2 diabetes and lack of physical activity, excessive body weight, and poor diet. To ameliorate the negative effects of diabetes, a number of synthetic antidiabetic drugs have been developed that lower blood glucose levels by various mechanisms. These include, but are not restricted to, sulfonylureas, biguanidines, and α-glucosidase inhibitors. The use of herbal pharmaceuticals and nutraceuticals can complement that of conventional synthetic prescription drugs in the treatment of this disease. Due to the lower probability of side effects and lower cost, herbal medicines are often used in the treatment of patients with type 2 diabetes [1, 2]. Such herbal collections are a valuable source of biologically active substances (BASs). However, their science-based application and standardization are limited due to inadequate and often outdated information on their chemical composition.

Informed by literature data on the antidiabetic properties of medicinal plants, we have developed a herb collection with hypoglycemic effects. The collection includes common bilberry shoots (*Vaccinium myrtillus* L.) [3, 4], common bean pods (*Phaseolus vulgaris* L.) [4], galega herb (*Gallega officinalis* L.) [5, 6], burdock roots (*Arctium lappa* L.) [7], common knotgrass herb (*Polygonum aviculare* L.) [8], and cinnamon rose hips (*Rosa cinnamomea* L.) [9, 10]. According to a number of researchers, the antidiabetic properties of these herbs are largely due to the presence of polyphenolic compounds, especially flavonoids. [11, 12].

The aim of the work was to determine the flavonoid profile of this herb collection and its total dry extract (TDE) using reverse-phase high-performance liquid chromatography (HPLC) fitted with an online diode array detector (DAD) and mass spectrometer (MS).

MATERIALS AND METHODS

Objects. The hypoglycemic collection (HGC) was obtained from plant materials produced by *Fitofarm* (Anapa, Krasnodar krai, Russia) [13, 14]. The TDE based on the collection was obtained according to the following technological scheme: extraction of the herb collection with purified water; filtering the extract; concentrating the extract; drying; and sieving the finished product.

Standards and solvents. Commercially available standard samples were used: rutin (≥94%, *Sigma-Aldrich*, USA), hyperoside (≥95%, *HWI ANALYTIK GMBH*, Germany), isoquercitrin (≥94%, *HWI ANALYTIK GMBH*, Germany), avicularin (*ChromaDex*, USA), kaempferol-3-glucoside (≥95%, *PhytoLab*, Germany), myricetin (appr. 85%, *Sigma-Aldrich*, USA), quercetin (≥98%, *Sigma-Aldrich*, USA), and kaempferol (≥99%, *Extrasynthese*, France). The following solvents and reagents were used: ultrapure water (Milli-Q® Advantage A10, *Merck*, Germany), acetonitrile UPLC/HPLC grade *AppliChem PanReac* production (Darmstadt, Germany), methanol UPLC/HPLC grade *J.T. Baker* production (*Avantor Performance Materials*, *Inc.,* Palo Alto, USA), and formic acid 98–100% *Merck* production (Darmstadt, Germany).

Sample preparation. HGC: approximately 2.0 g (exact weight) was transferred to a 100-mL round-bottom flask, and 50 mL of 60% aqueous methanol was added. The mixture was heated in a boiling water bath under reflux for 1 h. Then, the flask was allowed to cool to room temperature, placed in an ultrasonic bath for 5 min, and the extract was transferred to a 50-mL volumetric flask, made up to the mark with 60% aqueous methanol, and stirred. An aliquot was then transferred to a 1.5-mL centrifuge tube, centrifuged at 15000 rpm, and the supernatant transferred to an autosampler vial. The TDE (approximately 0.5 g accurately weighed) was transferred to a 50-mL volumetric flask, and 30 mL of 60% aqueous methanol was added. The mixture was placed in an ultrasonic bath for 10 min then made to the mark with 60% aqueous methanol and stirred. An aliquot was transferred to a 1.5-mL centrifuge tube, centrifuged at 15000 rpm, the supernatant transferred to an autosampler vial.

Equipment. The analysis was performed using an Ultimate 3000 liquid chromatography system equipped with a degasser, a three-channel pump, a column thermostat, a thermostatically controlled autosampler, a DAD, and an TSQ Endura triple-quadrupole mass spectrometer (MS) (*Thermo Scientific*, USA).

HPLC conditions: stationary phase: Waters NovaPak® C18 ID 4 μм 4.6 × 150 mm column; mobile phase A: 0.1% aqueous solution of formic acid, mobile phase B: acetonitrile; flow rate: 0.5 mL/min; gradient elution: 0–30 min 10–25% B, 30–40 min 25–50% B; column regeneration: 40–45 min 50–10% B, 45–50 min 10% B; column temperature: 25°C; sample volume: 10 μL; autosampler temperature: 20°C; detection was performed at four different analytical wavelengths: 70, 350, 338, and 290 nm. Spectra were recorded in the wavelength range 190–400 nm.

MS conditions: heated electrospray ionization in positive ion detection mode (HESI-MS+) was performed over the m/z range 100–1000 Da. Scanning speed: 100 Da/s; Q1 quadrupole resolution: 0.7. Operating parameters of the ionization source: capillary voltage: 3500 V, fragmentation voltage: 5 V, desiccant gas (N_2) flow: 50 arbitrary units; auxiliary gas flow: 15 arbitrary units; purge gas flow: 2 arbitrary units; ion transfer tube temperature: 325°C, evaporator temperature: 350°C.

Data processing was carried out using the Thermo Xcalibur 3.0.63 Qual browser program.

RESULTS AND DISCUSSION

The peaks in the chromatograms were identified by comparing their retention times and ultraviolet and mass spectra with those of standards as well as on the basis of published data on the flavonoid profiles of the constituent plants.

Chromatograms of the HGC extract and the TDE are presented in Fig. 1 and Fig. 2.

Nine individual flavonol glycosides—derivatives of myricetin, quercetin, kaempferol, and kaempferid—were identified in the HGC and TDE (Table 1). According to published data, robinin, 3-glucuronides

of quercetin, and kaempferol are characteristic of common beans [15]. We have previously identified 3-glucuronides of myricetin, quercetin, kaempferol and kaempferide as well as 3-arabinoside kaempferol in common knotgrass (*Polygonum aviculare*). In addition, according to the results of our previous studies, quercetin and kaempferol 3-glucuronides are present in blueberry shoots. With a high degree of probability, quercetin-3-robinoside-7-rhamnoside and quercetin-3-pentosyl glucuronide are flavonol glycosides of common beans, the contents of which have not been previously reported in the literature.

The quantitative contents of the flavonoids identified were determined by an external standard method: quercetin tri- and diglycosides are expressed in terms of rutin, myricetin- and quercetin-3-glucuronides are expressed in terms of isoquercitrin, and kaempferol glycosides are expressed in terms of keampferol-3-glucoside (Table 2).

As can be seen from Table 2, the main flavonoids in the studied objects are robinin and kaempferol-3-glucuronide, the contents of which in the HGC are 2.09 and 2.22 mg/g, and in the TDE are 4.85 and 3.84 mg/g, respectively. Quercetin-3-robinoside-7-rhamnoside, campferol-3-robinoside, quercetin-3-glucuronide, campferid-3-glucuronide, and campferol-3-arabinoside were detected in HGC and TDE at significantly lower concentrations than those of the main flavonoids

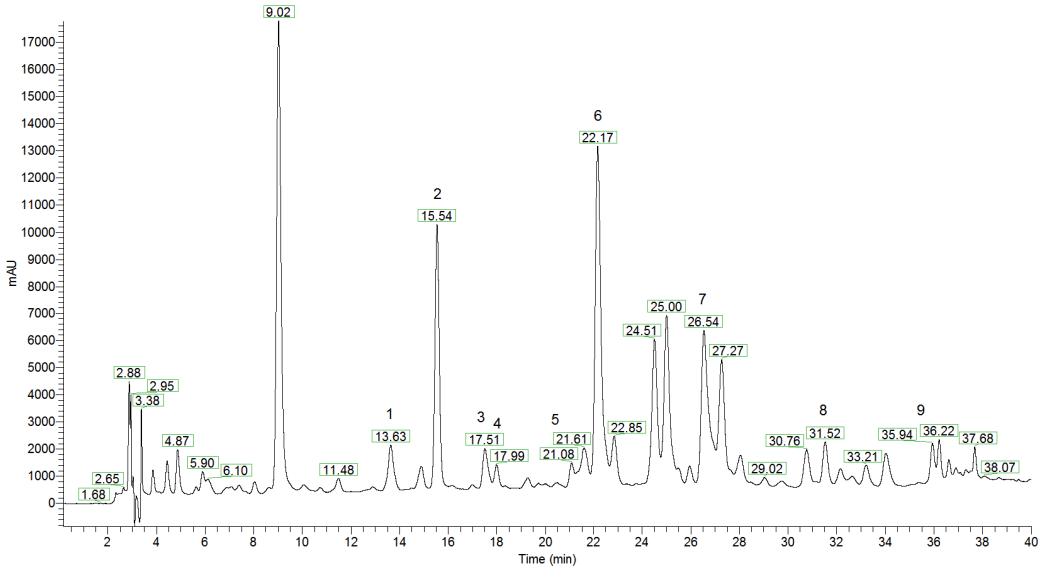

Fig. 1. Chromatogram of the HGC extract at λ = 350 nm.
The peak numbers correspond to the flavonoid numbers in Tables 1 and 2.

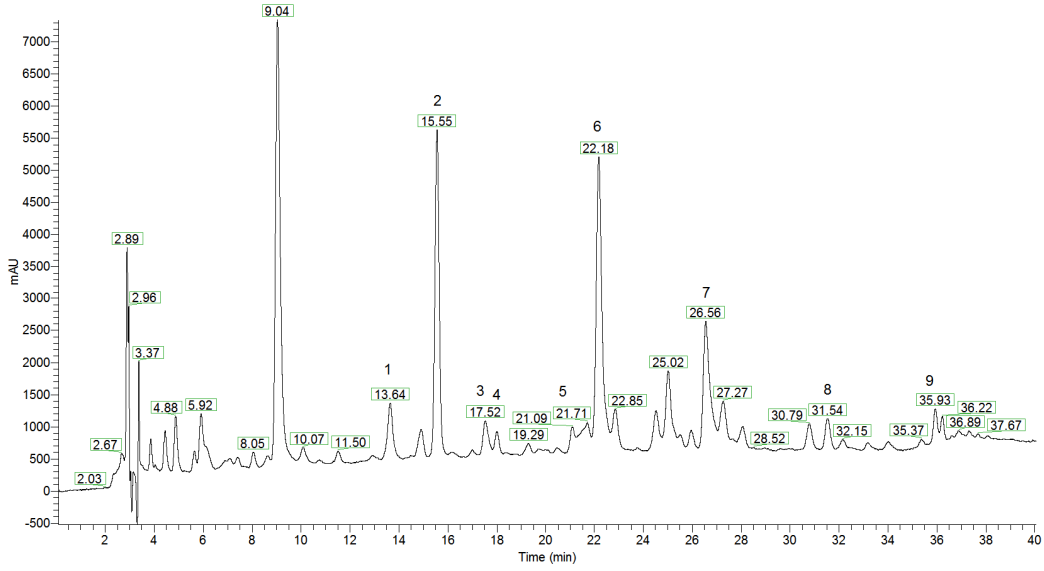

Fig. 2. Chromatogram of the TDE at λ = 350 nm.
The peak numbers correspond to the flavonoid numbers in Tables 1 and 2.

Table 1. HPLC-DAD-MS results for the HGC and its extract

No.	Flavonoid	R_t, min	λ_{max}, nm	m/z	Ion detected
1	Quercetin-3-robinoside-7-rhamnoside	13.6	255, 265, 355	757 611 449 303	$[M + H]^+$ [M – rhamnose* + H]$^+$ [M – robinose + H]$^+$ [M – rhamnose – robinose + H]$^+$
2	Kaempferol-3-robinoside-7-rhamnoside (Robinin)	15.5	265, 350	741 595 433 287	$[M + H]^+$ [M – rhamnose + H]$^+$ [M – robinose + H]$^+$ [M – rhamnose – robinose + H]$^+$
3	Myricetin-3-glucuronide	17.5	255, 270, 355	495 319	$[M + H]^+$ [M – glucuronic acid + H]$^+$
4	Quercetin-3-pentosylglucuronide	18.0	255, 270, 355	611 435 303	$[M + H]^+$ [M – glucuronic acid + H]$^+$ [M – pentose + H]$^+$
5	Kaempferol-3-robinoside	21.0	270, 345	595 433 287	$[M + H]^+$ [M – rhamnose + H]$^+$ [M – robinose + H]$^+$
6	Quercetin-3-glucuronide	22.1	255, 265, 355	479 303	$[M + H]^+$ [M – glucuronic acid + H]+
7	Kaempferol-3-glucuronide	26.5	265, 345	463 287	$[M + H]^+$ [M – glucuronic acid + H]$^+$
8	Kaempferol-3-arabinoside	31.5	265, 245	419 287	$[M + H]^+$ [M – arabinose + H]$^+$
9	Kaempferide-3-glucuronide	35.9	270, 345	477 301	[M + H]+ [M – glucuronic acid + H]$^+$

**Hereinafter:* for dehydrated carbohydrate or glucuronic acid ($–H_2O$), water is lost upon forming a glycosidic bond.

Table 2. Flavonoids content in HGC and TDE

No.	Flavonoid	Content, mg/g	
		HGC	TDE
1	Quercetin-3-robinoside-7-rhamnoside	0.10	0.31
2	Robinin	2.09	4.85
3	Myricetin-3-glucuronide	0.07	0.16
4	Quercetin-3-pentosylglucuronide	0.05	0.15
5	Kaempferol-3-robinoside	0.33	0.93
6	Quercetin-3-glucuronide	0.43	0.72
7	Kaempferol-3-glucuronide	2.22	3.84
8	Kaempferol-3-arabinoside	0.64	1.63
9	Kaempferide-3-glucuronide	0.53	1.37
	Total flavonoids	**6.46**	**13.96**

(0.10–0.64 and 0.31–1.63 mg/g, respectively). The contents of minor flavonol glycosides, which include quercetin-3-pentosyl glucuronide and myricetin-3-glucuronide, are less than 0.10 mg/g in the collection and less than 0.20 mg/g in the extract. The total content of flavonoids in the collection is 6.46 mg/g (or 0.646%) and that in the extract is 13.96 mg/g (or 1.396%).

CONCLUSIONS

The flavonoid profiles of HGC and its TDE were determined by HPLC-DAD-MS. Nine individual flavonol glycosides were identified, and these were found to be derivatives of myricetin, quercetin, kaempferol and kaempferide, which are characteristic for common bean pods (*Phaseolus vulgaris*), common knotgrass (*Polygonum aviculare*), and bilberry shoots. Robinin and kaempferol-3-glucuronide predominated in HGC and TDE. Quercetin-3-robinoside-7-rhamnoside and quercetin-3-pentosyl glucuronide were identified for the first time and assigned as bean flavonoids. The total flavonoid content in the HGC was 6.46 mg/g (or 0.646%) and that in the TDE was 13.96 mg/g (or 1.396%).

This method and its results can be used to standardize hypoglycemic collections and evaluate their pharmacological activities.

The authors declare no conflicts of interest.

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