Chapter

Hedysarum Species from Caucasus

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Abstract

In the complex pharmacognostic studies of three species of the genus *Hedysarum* L., we have developed a method for quantitative determination by UV spectrophotometry, HPLC, and capillary electrophoresis of the sum of xanthones in terms of mangiferin. The technique takes into account the basic physicochemical properties of xanthones; is characterized by reproducibility, high accuracy, and simplicity; and allows conducting both a screening assessment of various raw materials containing mangiferin derivatives and standardization of the prepared vegetable raw materials. The developed methods are tested on the aboveground organs of *Hedysarum* species, which are collected and dried by taking into account the rules and requirements for the preparation of medicinal raw materials. As a result, it was found that the greatest quantitative content of the sum of xanthones in terms of mangiferin is distinguished by the grass *H. caucasicum* M. Bieb. ($0.62 \pm 0.021\%$). For the first time, morphological-anatomical diagnostic signs of the species *Hedysarum caucasicum M. Bieb*., *Hedysarum daghestanicum Rupr. ex Boiss*., *Hedysarum grandiflorum Pall*. are necessary for standardization of medicinal vegetal raw materials. The results show the prospect of further investigation of *Hedysarum* as an additional source of mangiferin.

Keywords: *Hedysarum*, xanthones, mangiferine, antiviral therapy

1. Introduction

To improve modern pharmacy, the study of biologically active substances exhibiting specific pharmacological activity from the natural raw materials of plant origin is of obvious interest. In our time, the problem of obtaining antiviral and antibacterial agents of plant origin is especially acute. At the moment, the problem of chronic recurrent viral diseases is very relevant. According to WHO data, about 67% of the world's population suffer from diseases of various organs and systems caused by the Herpes simplex virus.

Mangiferin (2-C-β-D-glucopyranosyl-1,3,6,7-tetraoxyxanthone) belongs to the group of xanthones proper, is the most widespread representative of C-glycosides, and has antiviral and antibacterial properties (**Figure 1**).

In species of the genus *Hedysarum*, Fabaceae, xanthones, namely mangiferin, are the main group of biologically active substances. Russian scientists have developed an antiviral drug Alpizarin, from *H. alpinum* L. and *H. flavescens* Regel & Schmalh, the active substance of which is mangiferin, which has an antiviral activity against DNA-containing viruses (*Herpes simplex, Varicella zoster, Cytomegalovirus*), immunostimulating properties, and a bacteriostatic effect on Gram-positive and Gram-negative bacteria. VILAR (Russia) produces this in two forms of production "Alpizarin ointment" and "Alpizarin tablets." In addition to domestic drugs, the

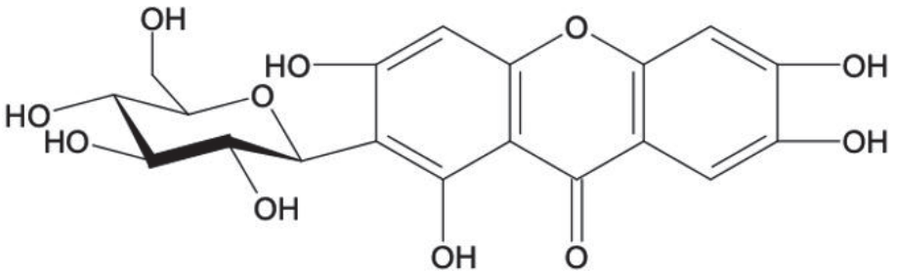

Figure 1.
Mangiferin (2-C-β-D-glucopyranosyl-1,3,6,7-tetraoxyxanthone).

foreign antiviral drug Mangogerpin (BV Pharma, Vietnam) is known, which also has two forms of release: tablets and ointment, the active substance of which is mangiferin.

There is a need to expand the raw material base for the production of broad-spectrum antiviral drugs. In this regard, a comprehensive analysis, taking into account the features of growth, biological characteristics, chemical composition, and known pharmacological properties, will make it possible to substantiate the directions of their rational use as well as the creation of additional domestic raw materials of mangiferin as a known antiviral drug based on medicinal plant raw materials. Thus, the study of species of the following genus is promising: *Hedysarum* L., *Hedysarum caucasicum* M. Bieb., *Hedysarum grandiflorum* Pall., and *Hedysarum daghestanicum* Rupr. ex Boiss., which are growing in the North Caucasus as additional raw materials for mangiferin.

The purpose of our work is to study plants of species of the genus *Hedysarum* L., including *Hedysarum caucasicum* M. Bieb., *Hedysarum daghestanicum* Rupr. ex Boiss., and *Hedysarum grandiflorum* Pall., as additional raw materials for mangiferin.

To achieve this goal, it is advisable to achieve the following objectives:

- Conduct a comparative morphological-anatomical study of these species of the genus *Hedysarum L.* (*Hedysarum caucasicum M. Bieb.*, *Hedysarum daghestanicum Rupr. ex Boiss.*, and *Hedysarum grandiflorum* Pall.) and identify the main morphological-anatomical diagnostic features.

- Develop an effective procedure for quantifying the sum of xanthones in terms of mangiferin from these species of the genus *Hedysarum* L., by methods of UV spectrophotometry, capillary electrophoresis, and high-performance liquid chromatography.

The family *Fabaceae Lindl.* (legumes) has about 650 genera and over 18,000 species, whose range of growth covers all continents of the globe. Within its range, species of the legume family are characterized by a variety of habitats. Life-form of members of the family *Fabaceae* Lindl.—perennial herbaceous plants, semiplants, and rarely trees—there are tree or herbaceous lians [1]. The leaves are complex with sills. The leaflet is next. Flowers are in finite or sinus brushes. The flowers are zygomorphic, quite large, and bright. The near-wind is double, the cup is most often actinomorphic, consists of five converged cups, and the crown consists of five petals, two of which grow into a "boat," one large—a "sail" and two small petals —"vests" remain free. The android consists of 10 stamens, nine of which usually grow together and one stamen remains free. Gynecium monocarpic tie the upper one. The fruit of legumes is monocarpium, one-, two-, or multifamily legumes, very

diverse in morphological and anatomical features. Seeds are without endosperm or with scant endosperm. Spare nutrients are deposited directly in the seed.

The genus *Hedysarum* L. combines about 285 species, which are predominantly common in Eurasia. The genus *Hedysarum* is distributed mainly in the European part, but some species are also found in Asia. *H. caucasicum* Bieb. grows in all highland areas of the North Caucasus, including the Rocky Ridge, in the alpine belt, up to 3500 m [2]. This species is endemic, growing on the highland meadows of the Caucasus: in the pre-Caucasus, Western and Eastern Transcaucasia, in Daghestan. It is described from the Caucasus type in Leningrad [1]. C. Linnaeus (1753) and B. A. Fedchenko (1902) were engaged in cystematics of the species of *Hedysarum* L. The species *Hedysarum caucasicum* Bieb we are investigating first was described in 1808 [3]. In the flora of Caucasus in 1873, the species was classified as *H. obscurum var. caucasicum* Trautv. The origin of the name of this plant is related to the shape of the fruit. Beans in appearance resemble coin meetings, and the Latin name *Hedysarum* comes from the Greek words "hedys" aroma, which literally means pleasantly smelling. Members of this genus are perennial herbs, shrubs, or semiplants of seamless or stem-shortened plants. They grow both in forests and on raw meadows, on riverbanks, and on alpine and subalpine meadows, on rocky sprouts, and in steppes.

Stems are often highly branched, branching, and sometimes completely undeveloped and the flower arrow comes out of shortened shoots developing at the neck of the rhizome; leaves are unparalleled, common: 5–9-paired, less common: 1–3-paired or even consisting of just one unparalleled leaf (**Figure 2**). The genus is characterized by simple botrioid flowers—brushes; the cup is bell-shaped, its teeth are search-and-take longer than the tube; the crown exceeds the cup; and wings are slightly or two times shorter than a boat, less often longer than it. The beans are artenic, and sometimes part of the seed does not develop and the bob consists of 1–3 artens; sprouts are flat-compressed or slightly convex, smooth, bare, or more often dried, mesh or with transverse ribs, often shrunk with short or longer bristles.

According to Flora of the USSR, in the *Hedysarum caucasicum* Bieb.—a plant 30–60 cm high, the stems are straight or ascending, not shortened, olfactory; and the leaves are 7- are 12-pairs, elliptical or egg-like long-lasting-watt, with sharpness at the top, 12–16 mm long, 7–9 mm wide. Flowers (without brush) are longer than leaves; brushes are not very thick, of 25–35 flowers; the lower tooth of the cup is equal to the tube, the rest are shorter; and the crown is dark-magenta, 16–18 mm

Figure 2.
Species of Hedysarum *from Caucasus (Russia).*

long. The fruit is a bob of 3–6 arthropods, naked or desiccated; the mushroom of the bean is oblong-elliptical, and its edge is not wide.

The microstructure of the above-ground parts of some members of the *Hedysarum* was studied by Ladygina et al. [4]. The features of the anatomical structure of leaves of five species of *Hedysarum* from the section *Gamotion* Basin were studied. These species containing mangiferin have been found to have bright golden yellow fluorescence of epidermis cells, which can be used to detect mangiferin directly in plant material [5].

We did not meet the information on the study of anatomical features of the structure of the organs in the accessible literature. The onthogenesis of species *Hedysarum* usually includes four periods and 10 age states. In the example of *H. austrosibiricum*, age spectra can be divided into four types, which probably reflect ecological-phytocenotic growth conditions [6].

During 3 years of life, alpine individuals undergo the following age conditions: in the first year—seedlings, juvenile, immature, and more than 60% of individuals are adult vegetative; in the second year, more than 90% of individuals enter a young reproductive state; and in the third year, all individuals transition to a reproductive state. Seeds of tested plants need pre-sowing treatment. Mechanical scarification and treatment with concentrated sulfuric acid are the best ways to disrupt the rest of both species [6–8].

The flowering phase of the *Hedysarum caucasicum*—the beginning and the middle of July, and the fruiting phase—the end of July—and the beginning of August [1]. For the genus *Hedysarum*, the following set of chromosomes is characteristic: 14, 16, and 48. For *Hedysarum caucasicum*, the chromosome number is 14 [9].

2. Material and methods

The object of the study was the grass of *Hedysarum caucasicum* Bieb. family legumes (Fabaceae), which were collected in the flowering phase on the southeast slope of Mount Alibek at an altitude of 2200 m (Dombay District, Russia).

Freshly harvested and dried samples of raw materials of the species of the genus *Hedysarum* L., including *H. caucasicum* M. Bieb., *H. daghestanicum* Rupr. ex Boiss., and *H. grandiflorum* Pall. were obtained (**Table 1**). The collected test raw materials were dried without direct sunlight in the air.

Research methods: comparative morphological, ecological-geographical, molecular-genetic, morphological-anatomical and histochemical, and phytochemical (UV-spectrophotometry, HPLC, chromato-mass spectrometry, capillary electrophoresis).

Species	№	Geographical coordinates	Altitude above sea level	Ecological and biological features of growth
Hedysarum caucasicum M. Bieb.	1.	N—43°43′46″, E—42°53′65”	2100 m	Open moistened areas among large boulders
	2.	N—40°30′17″, E—44°40′0”	2200 m	Southeastern slope of Alibek, gorge of Mount Alibek
	3.	N—42°19′32,21″, E—47°09′49,1”	1250 m	Deep location of river valleys between mountain spurs
Hedysarum grandiflorum Pall.	4.	N—49°45′56″, E—44°16′16”	200 m	Deep location of river valleys between mountain spurs

Species	№	Geographical coordinates	Altitude above sea level	Ecological and biological features of growth
	5.	N—42°19′32,21″, E—47°09′49,1”	1250 m	Deep location of river valleys between mountain spurs
Hedysarum daghestanicum Rupr. ex. Boiss.	6.	N—42°38′15,7″, E—46°09′45,8”	850 m	Rocky-fine-brimmed steep slope of northeastern exposition
	7.	N—42°41′38,2″, E—46°14′27,2”	1100 m	The slope of the southeast exposition, steepness 30°
	8.	N—42°59′32,9″, E—46°54′46,9”	460 m	Steepened rocky-fine-crushed steep section at the top of the slope
	9.	N—42°19′32,21″, E—47°09′49,1”	1250 m	Deep location of river valleys between mountain spurs

Table 1.
List of objects of study with indication of ecological and biological features of growth.

3. Methods for quantitative analysis of mangiferin

High-performance liquid chromatography, photocolorimetry, complexonometry, and chromato spectrophotometry are most commonly used to quantify mangiferin content in plant raw materials as well as biological fluids [10–15]. The photocolorimetric definition of mangiferin in both the raw material and crystalline powder has a number of advantages, such as shortening the analysis steps and replacing the deficient vegetable, tetrahydrofuran, with dioxane. The method is based on mangiferin's ability to produce a complex compound with chlorine iron. Mangiferin was determined from a calibration plot of the solution optical density versus mangiferin concentration. When comparing spectrophotometric and photocolorimetric methods, almost the same results were obtained [5, 10, 12, 16, 17].

As for the current method of high-performance liquid chromatography, according to literature, separation of xanthone glycosides by HPLC method could not be achieved on sorbents containing amino and cyano groups [7, 18–23]. The best results were obtained on reverse phase C18 sorbents, and methanol-water, ethanol-water, and acetonitrile-acetic acid were used as the mobile phase. In aqueous systems, the shape of the mangiferin peak deteriorated. To quantify mangiferin in biological fluids, a sensitive reverse phase HPLC technique is proposed [17, 24, 25].

The mobile phase was acetonitrile and a 3% CH_3COOH solution at a ratio of 16:84 was chromatographed at a wavelength of 254 nm using an external standard method [11, 26].

Furthermore, in the quantitative determination of mangiferin by HPLC, a system consisting of acetonitrile, water, and phosphoric acid was used as the mobile phase. The selected conditions allowed to achieve a clear separation of mangiferin and isomangiferin peaks on chromatogram. In addition to the previous systems, methanol, tetrahydrofuran, or acetonitrile, an aqueous solution of phosphoric acid in various ratios are used as the mobile phase in HPLC gradient elution. Of the latest techniques for the quantitative determination of mangiferin, liquid chromatography followed by mass spectrometric determination was used, and the method is characterized by speed and quality [27–30].

The chromate-spectrophotometric examination is based on sequential chromatography and spectrophotometry. The raw material is treated with the following

extractant system—acetone:water in ratio 1:1 with acidification with 5% hydrochloric acid followed by chromatography in the system with 15% acetic acid. The eluation of the zones with mangiferin is preparing after viewing the chromatogram in UV light. The optical density of the solutions was measured at 372 nm. The mangiferin content was calculated from the specific absorption index.

There are a number of intense absorption bands in the UV spectrum of mangiferin. The most convenient to quantify a substance is a band with a maximum at 369 nm and a specific absorption coefficient of 295 ± 0.92. In the field of working concentrations, the absorption of mangiferin solutions is subject to Lambert-Beer law [23].

Analysis of literary sources has shown that the genus *Hedysarum* L. combines about 285 species, which are predominantly common in Eurasia. *H. caucasicum* Bieb. is endemic, growing in the highland meadows of the Caucasus. In a literary search, it has been found that chemical study information relates mainly to *H. alpinum* and *H. flavescens*. Information on the chemical study of *H. caucasicum* is fragmented and insufficient. Plants of the genus *Hedysarum* are widely used in folk and waitinal medicine, as an antibacterial, antiviral, immunomodulatory, and anti-inflammatory agent [6, 8, 17, 26, 31–39]. The following methods of analysis are used to identify and quantify the main active substances of xanthones: spectrophotometry, photocolorimetry, and chromatographic methods of analysis (TLC, HPLC, and mass spectrometry) [40–42]. The study of theoretical bases of extraction of medicinal raw materials allows to find the optimal conditions of technology for creation of medicinal forms on the basis of herbal of Caucasus.

4. Results

Morphological study: Life-form—a perennial herbaceous plant 40–50 cm high, and underground organs reach 30 cm length. The escape is elongated, branched, straight-standing, or raised. The number of leaflets located on rachis varies from 11 to 15. The leaves have an egg shape, a rounded base, a whole edge, and a spiky top. Decaying of leaflets is insignificant. The flower is simple botriode, brush. The flower is zygomorphic. The cup consists of five cups, a moth-type crown, and the color of the petals is pink-purple. The android consists of nine converged tangles and one free. Ginecey is monocarpal. Tie the top one. Fruit: according to morphogenetic classification of fruits, it refers to monocarp; according to morphological—bob, flat, oblong in shape, and consists of rounded arthropods. Number of squads range from 3 to 5.

The leaf is amphistomatic. The upper epidermal has weak and almost straight anticline cell walls. The mouth is abnormal, surrounded usually by 3–5 near-oral cells. Trichomes were not detected on the abaxial side of the leaf. The lower epidermal has strong anticline cell walls. An anomocytic type oyster is surrounded by usually 3–5 parotid cells. The embossing is formed by simple single-cell hairs located either in the region of the veins or on the edge of the leaf plate. When considering the sheet microreparation from the surface, rhombic calcium oxalate crystals located in large veins are found. They form a characteristic crystalline lining of the veins. On the cross section, the leaves have a characteristic dorsoventrale structure (**Figure 3**).

The palisade mesophyll localizes only under the upper epidermis in one layer, its cells are stretched, tightly pressed to each other. The mechanical fabric is a collenchym which is located both under the upper and lower epidermis in the region of the core. In the central part of the main core, there is a large collateral conducting bundle, and from the dorsal side to the conducting bundle there is a

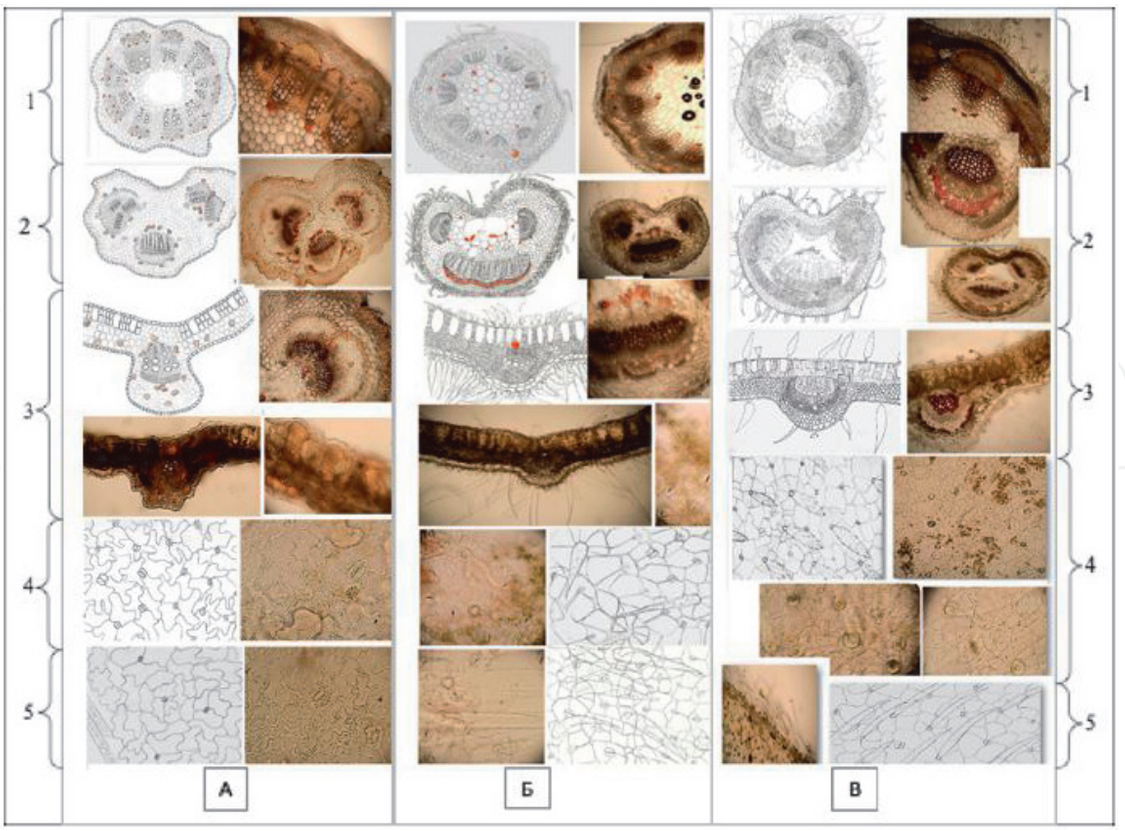

Figure 3.
Microscopic signs of the above-ground organs of Hedysarum caucasicum *M. Bieb. (A)* Hedysarum daghestanicum *Rupr. ex Boiss. (B)* Hedysarum grandiflorum *Pall. (C) 1. Transversal section of the stem in the lower part; 2. transversal section of the leaf petiole in the lower part; 3. transverse slice of leaf plate; 4. the adaxial epidermis of the leaf blade; and 5. the abaxial epidermis of the leaf blade.*

sclerenchym of pericyclic origin. The leaflets are arranged on small cherries, which have cylindrical shape on the cross section. The covering tissue of the cherry is represented by an epidermal, under it there is a collenchym in 1–2 layers, and the main volume occupies chlorenchyma. The collateral conducting bundle surrounded by sclerenchym is located in central part of cut. The rachis at the bottom has a grooved shape with a deep horseshoe spoon on the abaxial side. Collateral conducting bundles of 6–8 are arranged in an arc-like manner. It is interesting that idioblasts with yellow content are found in the phloem, in the pericyclic zone, and in the parenchyma of the core adjacent to the xylem portion of the conductive beam.

The stem has a polyhedral shape on the cross section. A cavity may form in the central part of the stem. Under the epidermal continuous ring in 1–2 layers of cells, there is a plate-type collenchym. In the projection regions, the number of layers of collenchym cells increases to 5–7. Chlorenchym is located following collenchym in discontinuous regions in 2–3 layers of cells. The pericyclic zone is represented by sclerenchymal fibers arranged in discontinuous areas above the conducting beams. Conducting system is of bundle type. Conducting beams are of open collateral type in an amount of 14–16 (**Figure 3**).

The cambium forms secondary conductive beams and thick-walled parenchyma, which is subsequently strongly ligated. Idioblasts are found, as in the cherry, in the phloem part of the conducting beam, in the pericyclic zone, as well as in the parenchyma of the core. The abovementioned micromorphological characteristics of the above-ground organs of the *Hedysarum caucasicum* can be used in drawing up regulatory documentation for the Hedysarum caucasicum grass as an additional raw material source of xanthone glycoside mangiferin.

A comparative morphological-anatomical study of three species of the genus *Hedysarum* L., growing in the North Caucasus, was carried out. The most significant diagnostic morphological features have been established, which provide to establish the species belonging of the medicinal raw materials specified in **Table 2**.

A comparative analysis of the anatomical structure of the stem revealed common features, which include the multifaceted shape of the stem on the cross section, and the pubescence is represented by simple bicellular hairs, which have a narrowed base and an expanded structure, a characteristic feature of the hairs is the wariness of the cuticle (**Figure 3**).

In a comparative study of the anatomical structure of the caulifoliar system, the main attention was drawn to the anatomical structure of the petiole and rachis of a complex leaf. The following differences were revealed—the shape on the cross section of the petiole of a complex sheet changes from a clearly saddle-shaped to a rounded-triangular shape, the number of ribs on the abaxial side changes.

Differences were identified regarding the structure and location of collenchyma, sclerenchyma, and the conducting system. The conductive system of the petiole and

Species	Life-form	Leaves	Flowers	Fruits
Hedysarum caucasicum M. Bieb.	High plant, 30–50 cm tall. The lower internodes are not shortened	The leaves are scattered throughout the stem, from 7 to 12 pairs of elliptical or ovate oblong leaflets with a pointed tip at the apex. Pubescence is not developed	The brushes on long legs, 11/2–2 times longer than the leaves, are not very thick. The lower tooth of the calyx is equal to the tube, the rest are shorter. Dark-purple petals	The segments are not large, smooth, or slightly toothed
Hedysarum grandiflorum Pall.	Perennial 20–40 cm tall. Stemless or almost stemless. Stipules are large, membranous, fused, brown, and scattered-hairy	The leaves are petiole, short pressed-hairy and, long spaced pubescent. Leaflets 1–4-paired, ovate, or broadly elliptical, and large, slightly hairy above, less often glabrous, densely silvery-silky below	The brushes are multi-flowered, with deflected flowers. Bracts are lanceolate, light brown, hairy, bell-shaped calyx, its teeth are linear-awl-shaped, densely thawed-hairy, Corolla yellow	Beans 2–5-segmented, segments rounded, densely white-haired, mesh-ribbed, along the edges with spines bent inside
Hedysarum daghestanicum Rupr. ex Boiss.	Stem-free rod-root perennial. Peduncles with brushes 10–25 cm long. All parts of the plant have a grayish plaque	Ten leaves with 3–4 pairs of leaflets, leaflets oblong, up to 18 mm long and up to 8 mm wide. The apical leaf is larger. Accrete stipules	The brushes are small-flowered, thick. The flowers are large, creamy white or purple. The calyx is four times shorter than the corolla. The boat is shorter than the flag and two times longer than the wings. The flowers are large, cream-white	Beans from 2 to 4 segments. Lentil-shaped, warty segments

Table 2.
Comparative morphological characterization of Hedysarum *L. species growing in the North Caucasus.*

rachis of the complex sheet is represented by three large and 2–7 small conducting bundles (**Figure 3**).

All conducting bundles have a pronounced collateral structure, while one large, dorsal conducting bundle is located on the adaxial side, and it has a characteristic rounded or semilunar shape and is reinforced with sclerenchymal fibers on the phloem side. Two ventral conducting bundles are smaller and are usually located in the rib zone, also reinforced by sclerenchyma on the phloem side.

Thus, the rachis zone is usually characterized by 2–4 additional conductive beams, or they are completely absent. The number and arrangement of conducting beams seem to us most interesting and more informative from the point of view of diagnostics of this species, growing under various environmental conditions (**Figure 1**). When conducting a comprehensive morphological-anatomical study of three species of the genus *Hedysarum* L., the morphological-anatomical diagnostic features were revealed, which are presented in this chapter.

5. Determination of humidity

By the moisture content of the raw material, it is meant a loss in mass due to hygroscopic moisture and volatile substances, which is determined by drying to a constant mass. Medicinal plant raw materials should not contain moisture above permissible standards. Moisture content of the analyzed raw material was determined by the method of drying by Pharmacopea XIV [14]. The weight loss on drying was 8.60% (average of two parallel determinations).

6. Determination of total ash

The ash of plant raw materials refers to the residue of inorganic substances obtained after burning the raw materials and then calcining the residue to a constant weight. Plant ash (total ash) consists of a mixture of various inorganic substances in the plant itself and mineral impurities (earth, sand, dust, and stones) that can enter the raw materials when collected and dried. The common ash most commonly contains the following elements: Na, K, Ca, Mg, Fe, Si, F, P, and C, which are in the form of oxides or salts of carbonic, phosphoric, sulfuric, and other acids. Ash determination of total (x) was carried out by Pharmacopea of Russia. The total ash content was 4.04% (average of two parallel determinations).

7. Microbiological purity test

For research, the grass of the *Hedysarum caucasicum* Bieb. family legumes (*Fabaceae*) were collected in the flowering phase on the southeast slope of Mount Alibek (Dombay Gorge District, CHR) and used. According to amendment No. 3 to the article of Pharmacopea of the publication "Methods of microbiological control of medicines," introduced on June 19, 2003, the studied plant raw materials belong to the category 4Б—medicinal plant preparations and medicinal plant raw materials "angro," prepared without the use of boiling water. The requirements for this category are as follows:

- Total number of aerobic bacteria—no more than 105 in 1 g or 1 ml.
- Total number of fungi—not more than 104 in 1 g or in 1 ml.

- Enterobacteria and other gram-negative bacteria—not more 103 1 g or 1 ml.
- Absence of *Escherichia coli*—in 1 g or 1 ml.
- Absence of *Salmonella* in 10 g or 10 ml.

Before preparing the dosage form, the studied vegetable raw material of the *Hedysarum caucasicum* Bieb. was tested for microbiological purity. The presented results make it possible to conclude that according to the indicator "microbiological purity," the sample of vegetable raw materials of the *Hedysarum caucasicum Bieb.*, presented for analysis, meets the requirements for medicinal vegetable raw materials "angro," used without thermal treatment.

8. High-quality phytochemical analysis

Determination of tanning substances: About 1.0 g of raw material was poured with 100.0 g of water, heated for 20–30 min in a water bath and filtered. The following reactions were carried out with the resulting solution [15]: Several drops of iron ammonium alum were added to 2 ml of the solution, and black and green staining appeared, indicating the presence of condensed tanning agents; a few drops of a 1% solution of quinine hydrochloric acid were added to 2 ml of the solution, and opalescence appeared.

Determination of polysaccharides: For qualitative detection of polysaccharides, water extraction was prepared from 2.0 g of alpine penny roots in a water bath for 30 min. Then it was filtered off and the filter was washed with hot water. The recovery was evaporated to 1/5 volume and three times the volume of 96% ethanol was added thereto. As a result, a loose curd precipitate of the polysaccharide complex was formed. The precipitate was separated, dissolved in water, reprecipitated, washed with alcohol, and dried. The obtained polysaccharide complex is an amorphous mass soluble in water. In the composition of water-soluble polysaccharides of the penny, mucous substances predominate [43].

Definition of the restoring sugars: About 1.0 of the milled raw material was placed in a 25 ml flask, poured with 10 ml of water, and refluxed for 0.5 h. The solution was filtered through gauze and washed with water. 5 ml of the resulting solution was transferred to a tube and 15 ml of 95% ethyl alcohol was added. Precipitation of the bulk precipitate was observed. The solution was filtered, the precipitate was transferred to a test tube, 5 ml of diluted hydrochloric acid was added, boiled for several minutes, 5 ml of Feling reagent was added and boiled again, and orange-red staining was observed [14].

Determination of free organic acids: A 1:10 decoction was prepared from Caucasus penny grass while heating in a water bath for 1 h. Broth was filtered. Five drops of digestion were placed in the tube and adjusted to 1 ml with purified water. One drop of the methyl red indicator was added, and red staining was observed, indicating the presence of organic acids [15].

Definition of amino acids: For qualitative detection of amino acids, reaction with 0.1% solution of ninhydrin in n-butanol on filter paper was used, and characteristic blue-violet staining appeared in formation of Rueman complex [15].

Determination of flavonoids and xanthones: In order to determine flavonoids, it was necessary to obtain an alcohol extract from the raw material. Extraction was carried out with 80% ethyl alcohol. About 1 g of the feed was placed in a 25 ml flask, 10 ml of 80% ethyl alcohol was added and heated in a water bath for 10–15 min

under reflux. The resulting solution was filtered through a paper filter after cooling. Reactions were carried out with the resulting solution.

Cyanidine sample: 0.1 g of magnesium dust, 2 ml of concentrated hydrochloric acid were added to 1 ml of extraction, heated in a water bath for 2–3 min, and after some time red-orange staining was observed; 2 drops of 2% basic lead acetate solution were added to 1 ml of the recovery, and yellow-lemon staining appeared; 1 ml of a 10% ammonia solution was added to 1 ml of the recovery, and yellow staining turned orange on heating appeared. 1 ml of a 2% solution of aluminum chloride in 96% ethyl alcohol was added to 1 ml of the recovery, and lemon-yellow staining was observed [31].

Qualitative reactions with these reagents showed the presence of flavonoid substances in the grass of the Caucasus penny, which allowed us to use the chromatography method for further analysis, which is widely used for their detection and identification. Chromatographic separation of the sum of flavonoids and xanthones was carried out in the preparation of the extracts, and ethyl alcohol of 96, 80, 60, and 40% concentration was used as the extractant. 0.05 ml of the *Hedysarum caucasicum* Bieb. extracts were applied to a 40 × 40 cm Whatman chromatographic paper and subjected to ascending chromatography in a solvent system: butanol-glacial acetic acid-water in a ratio of 4:1:5 compared to witness substances. When viewing the chromatogram in UV light, three main spots were found in extracts of the following concentrations of ethanol 96:80:60%. First spot corresponds to mangiferin, second to hyperoside, and third to campferol. Further, chromatograms were sprayed with alcohol solution $AlCl_3$, and a change in stain color was observed.

9. Qualitative detection and quantification of xanthones

Thin layer chromatography was used for qualitative detection of mangiferin. Chromatography was carried out in systems: n-butanol-acetic acid-water (4:1:5); chloroform-methanol-water (13:7:2); and 15% acetic acid, on "Sorbfil PTCC-AF-A" plates 10 × 10 cm and 10 × 15 cm.

The development of the plates was carried out by spraying with the following reagents: a solution of iron(III) chloride of 2%, an alcoholic solution of aluminum chloride of 1%, and ammonia vapors and UV radiation (fluorescent lamp UV-A). As a result of the TLC study of the extraction of raw materials there are seventy percent ethyl alcohol compared to standard samples indicated that xanthone glycoside-mangiferin was present in these samples (**Table 3**).

10. Quantification of the sum of xanthones mangiferin by UV spectrophotometry

The content of the sum of xanthones in the test subjects in terms of mangiferin was calculated in two ways, using the optical density of the solution of the standard sample of mangiferin and the value of the specific absorption index of mangiferin under similar conditions. *Determination of specific value of mangiferin uptake:* A precise suspension of a standard mangiferin sample (about 0.01 g) was placed in a measuring flask with a capacity of 25 ml, 20 ml of 70% ethyl alcohol was added, stirred until the standard sample was completely dissolved, and the volume of solution was adjusted to a mark in the flask. Aliquots from the resulting solution were placed in measuring flasks with a capacity of 25 ml and labeled with the same solvent. The optical density was measured on a spectrophotometer at a wavelength

Value R_f	Color of chromatographic area									
	***H. caucasicum* M. Bieb.**		***H. grandiflorum* Pall.**		***H. daghestanicum* Rupr. ex Boiss.**		**Standard mangiferin (Sigma-Aldrich)**		**Alpizarin**	
	Before detecting	**After detecting**	**Before detecting**	**After detecting**	**Before detecting**	**After detecting**	**Before detecting**	**After detecting**	**Before detecting**	**After detecting**
System 1: n-butanol-acetic acid-water (4:1:5)										
0.51	Pale yellow	Bright yellow	Pale yellow	Bright yellow	Pale yellow	Bright yellow	Pale yellow	Bright yellow	Pale yellow	Bright yellow
System 2: chloroform-methanol-water (13:7:2)										
0.56	Pale yellow	Bright green	Pale yellow	Bright green	Pale yellow	Bright green	Pale yellow	Bright green	Pale yellow	Bright green
System 3: acetic acid 15%										
0.38	Pale yellow	Bright yellow	Pale yellow	Bright yellow	Pale yellow	Bright yellow	Pale yellow	Bright yellow	Pale yellow	Orange

Table 3.
Results of chromatography of Hedysarum *L. species together with the standard mangiferin (Sigma-Aldrich).*

of 365 nm in the resulting solutions. Ethyl alcohol 70% was used as a comparison solution. **Tables 4** and **5** shows the results of the experiment.

The obtained results were statistically processed; the relative error of determination was 1.4%, which makes it possible to conclude the reliability of the obtained results. The optical density of solution B was measured on a spectrophotometer at a wavelength of 365 ± 2 nm in a cuvette with a layer thickness of 10 mm. As the comparison solution, 70% ethyl alcohol was used. The quantitative content of the sum of xanthones in terms of mangiferin was calculated using the optical density value of the standard sample of mangiferin(I) and the value of the specific absorption index of mangiferin(II) established by us experimentally. **Table 5** shows the results of quantification of the sum of xanthones in terms of mangiferin in the raw material (**Table 5**).

As a result of complex chromatographic studies of three species of the genus *Hedysarum*, it was revealed that the maximum content of the sum of xanthones in terms of mangiferin is 0.62 ± 0.021%, and this is observed in the grass of *Hedysarum* (**Table 6**). The obtained results indicate the prospect of further study of the above-ground part, which allows us to consider this species as an additional raw material source of mangiferin.

Aliquot, ml	Concentration of mangiferin, % $\times 10^{-4}$	Optical density, A	Calculated value of specific key figure absorption, $A_{1cм}^{1\%}$	Metrological characteristics
0.25	4	0.1278	319.5	$S_{\bar{x}}$=18,433 $\Delta X = 4.52$ $t_{0,95} = 2.45$ 325.1 ± 4.52 $\varepsilon = 1.4\%$
0.50	8	0.2603	325.4	
0.75	12	0.3945	328.8	
1.00	16	0.5178	325.7	
1.25	20	0.6427	321.4	
1.50	24	0.7718	321.5	
1.75	28	0.9338	333.5	
			$\bar{A}_{1cм}^{1\%}$=325.1	

Table 4.
Results of determination of specific value of mangiferin absorption (exact weight 0.0101 g).

Mass, g	Value of optical density, A_x (λ = 364 nm)	Maintenance of the sum of xanthones, % (A_0)	Maintenance of the sum of xanthones, % ($A_{1cm}^{1\%}$)	Metrological characteristics	
		I	II	I	II
10.015	0.4621	0.640	0.637	$\acute{X}$ =0.629	$\acute{X}$ = 0.624
10.018	0.4672	0.647	0.644	$S_{\acute{x}}$ =0.0830	$S_{\acute{x}}$ =0.0830
10.100	0.4671	0.642	0.639	Δx =0.021	Δx =0.021
0.9989	0.4329	0.601	0.599	$\acute{x} \pm \Delta x =$	$\acute{x} \pm \Delta x =$
0.9996	0.4358	0.605	0.602	0.629 ± 0.021	0.624 ± 0.021
0.9898	0.4504	0.632	0.629	ε =3.39%	ε =3.42%

Table 5.
Content of the sum of xanthones in terms of mangiferin (a_o = 0.0101 г; A_o = 0.5178; $A_{1см}^{1\%}$ =325.1; w = 7.23%).

Species	The content of the sum of xanthones in terms of mangiferin, %
Hedysarum caucasicum M. Bieb.	0.62 ± 0.02
Hedysarum grandiflorum Pall.	0.60 ± 0.02
Hedysarum daghestanicum Rupr. ex Boiss.	0.56 ± 0.01

Table 6.
The content of the sum of xanthones in terms of mangiferin in the above-ground part of species of the genus Hedysarum *L. by the value of the specific absorption index of mangiferin.*

11. Quantification of mangiferin in grass of *Hedysarum* species by capillary electrophoresis

For the study of these species of the genus *Hedysarum* capillary electrophoresis "Kapel - 105m" (Lumex Marketing OJSC, Russia), quartz capillary (L_{eff}/L_{tol} = 50/60 cm, ID = 75 μm) was used. The quartz capillary was previously washed successively with purified water, 1 M aqueous solutions of sodium hydroxide and hydrochloric acid.

The optical density of the prepared solution was measured on a spectrophotometer in the wavelength range of 200–500 nm. For this purpose, an aliquot of 1 ml was placed in two measuring flasks with a capacity of 25 ml. In one of the measuring flasks, the solutions were labeled with alcohol 70% ethyl, and in the other with borate buffer solution 0.01 M. As comparison solutions, ethyl alcohol 70% was used in the first case, and in the second case, borate buffer solution 0.01 M was used. A shift of the maximum light absorption of mangiferin from 365 nm to 383 nm in the borate buffer solution was observed, which may be due to the formation of a complex of mangiferin with sodium tetraborate.

Solution B with the following concentrations (mg/ml): 0.35; 0.25; 0.15; and 0.05. For this, aliquots of solution B (1; 0.7; 0.5; 0.3; and 0.1 ml) were placed in 1 ml Eppendorf tubes. 0, 0.3, 0.5, 0.7, and 0.9 ml of 70% ethyl alcohol were added. Centrifugation of the solutions was carried out for 5 min at 8000 rpm.

Capillary electrophoresis analysis was carried out at +20 kV, with capillary temperature of + 20°C, and detection was carried out spectrophotometrically at a wavelength of 383 nm, and the analysis time was 10 min. As an electrolyte, a borate buffer solution 0.01 M with a pH of 9.2 ± 0.02 was used, prepared in accordance with GOST 4919.2-2016 "Reagents and especially pure substances. Methods for the preparation of buffer solutions." Previously the capillary was washed consistently with solutions of acid hydrochloric 1 M and sodium hydroxide 1 M.

Washing between acid and alkali solutions, as well as final washing before analysis, was carried out with purified water. Washing solutions and electrolyte solutions were filtered through a Vladipor paper filter of type with a membrane diameter of 25 mm. The buffer solutions, like the test solutions, were centrifuged at 8000 rpm for 5 min. The results were processed and a calibration plot was plotted (**Figure 4**).

The preparation of purified alcohol extracts from the raw material was carried out in accordance with the procedure developed by us to determine the sum of xanthones in terms of mangiferin by UV spectrophotometry for the samples under study (the procedure described above in the UV spectrophotometry section) (**Figures 5–7**). Centrifugation of solution A was carried out for 5 min at 8000 rpm.

Based on the experimental data obtained, it can be concluded that the Caucasian penny is the largest content of mangiferin among the studied species of the genus,

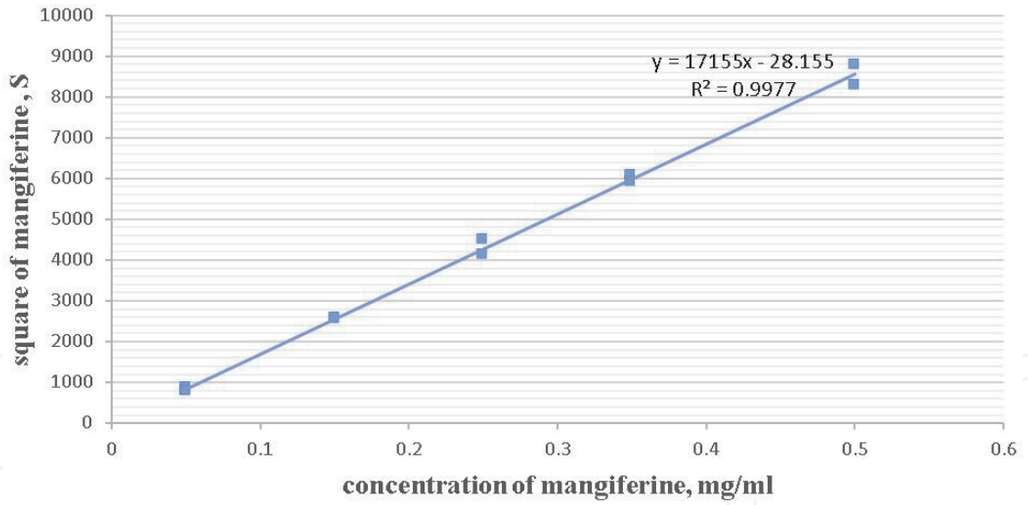

Figure 4.
Calibration graph of peak area versus mangiferin concentration in solution.

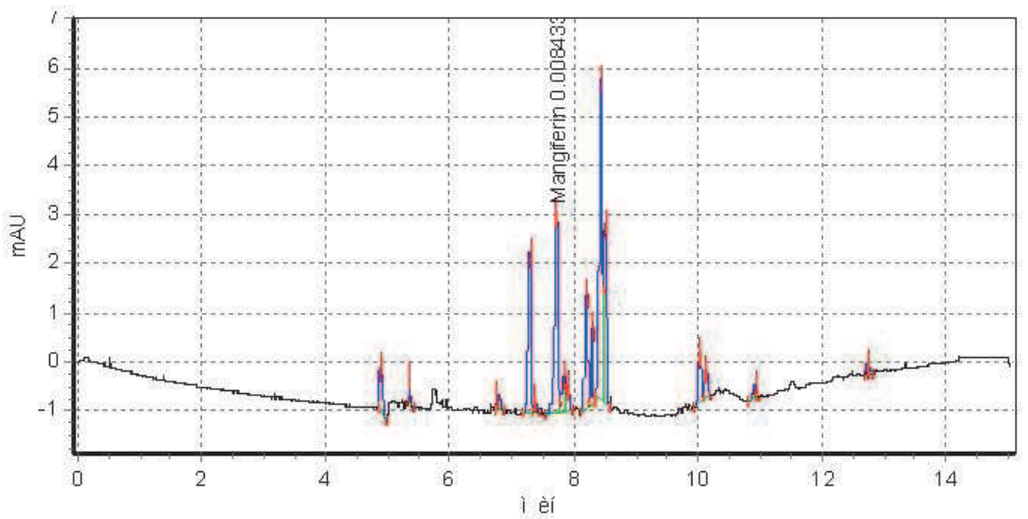

Figure 5.
Electrophoregram of extraction of Hedysarum caucasicum.

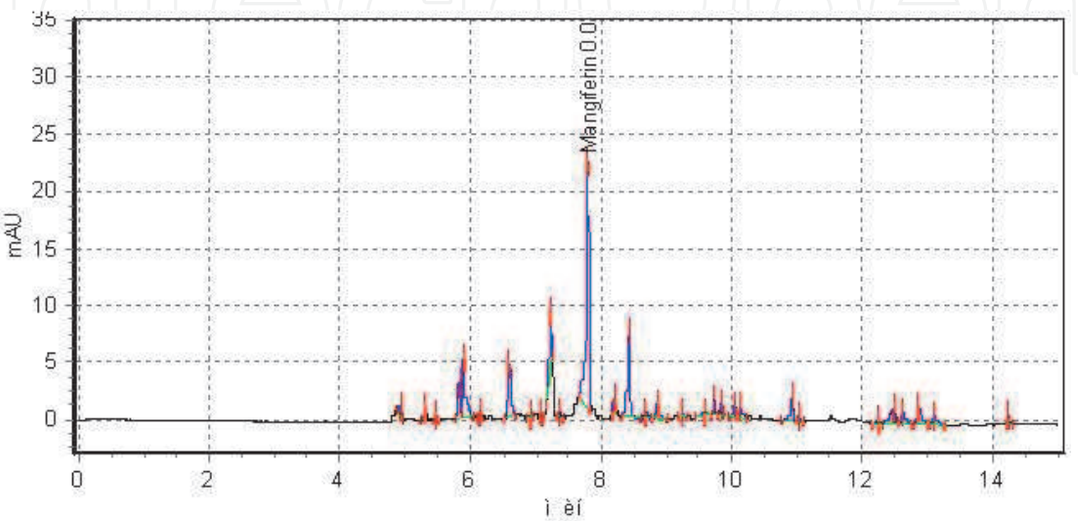

Figure 6.
Electrophoregram of extraction of Hedysarum grandiflorum *pall.*

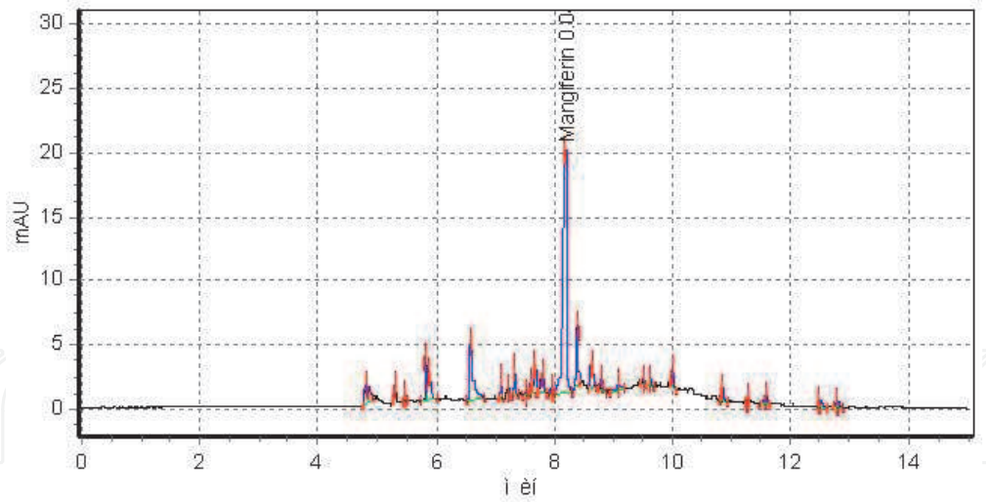

Figure 7.
Electrophoregram of extraction of Hedysarum daghestanicum *Rupr. ex Boiss.*

which confirms the assumption based on molecular genetic studies, since it is this species that belongs to the Obscura section, as well as the alpine penny used to obtain mangiferin.

12. Quantification of mangiferin in the grass of *Hedysarum caucasicum* M. Bieb. by HPLC

High-performance liquid chromatography was used to quantify mangiferin in the subjects under study. Registration of electronic spectra was carried out on a liquid chromatograph Shimadzu Prominence LC-20 AD with a degasser DGU-20A3R. A sample of mangiferin (Sigma-Aldrich, cate. M3547, USA, 2017) was used as a standard sample.

Standard mangiferin solution: A slurry of 0.0050 g (based on 100% substance) of mangiferin was placed in a measuring flask with a capacity of 25 ml, dissolved in 70% ethyl alcohol, and then the volume was adjusted with the same solvent to a mark and mixed. An aliquot of 1.0 ml of the resulting solution was placed in a measuring flask with a capacity of 10 ml, dissolved in the mobile phase, and then the volume was adjusted with the same solvent to a mark and stirred. The solution was filtered through a 0.45 μm membrane filter (**Figure 8**).

Buffer solution: In a 1000 mL measuring flask, 15.6 g of sodium phosphate of monosubstituted dihydrate was placed, which was dissolved with 200 mL of purified water and the volume adjusted to a mark, stirred. The potentiometric method determined the value of pH, which should be 4.40 ± 0.05.

Mobile phase: A mixture of buffer solution, acetonitrile, and methanol in a ratio of 81:16:3.

The developed method of quantitative determination of mangiferin in the above-ground organs of the studied type takes into account the main physicochemical properties of xanthones, and it is characterized by reproducibility, high accuracy, and allows conducting both screening assessment of various raw materials containing mangiferin derivatives and standardization of prepared medicinal plant raw materials of the *Hedysarum* L.

As a result, it was found that the largest in the above-ground organs of *Hedysarum caucasicum* found was 0.148 ± 0.003% mangiferin (**Figures 9** and **10**). The method of quantification of mangiferin by HPLC has been developed, which is

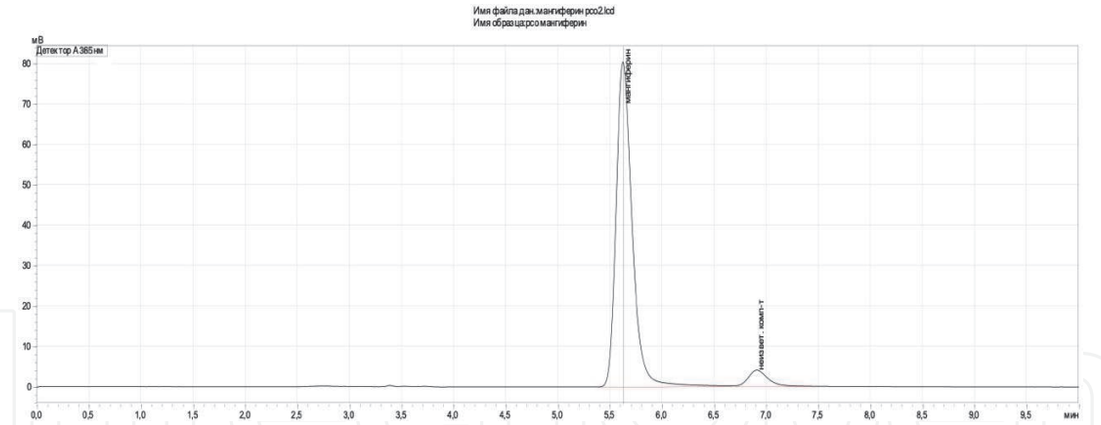

Figure 8.
Chromatogram of standard solution of mangiferin.

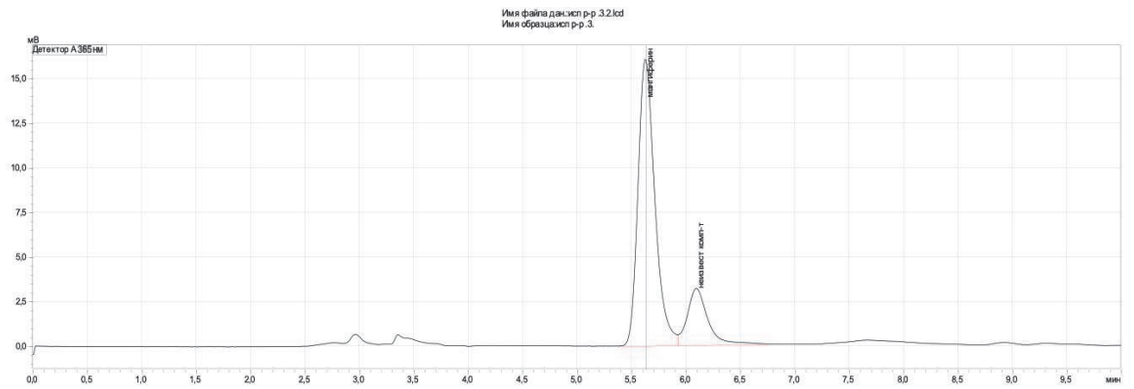

Figure 9.
Chromatogram of solution 1.

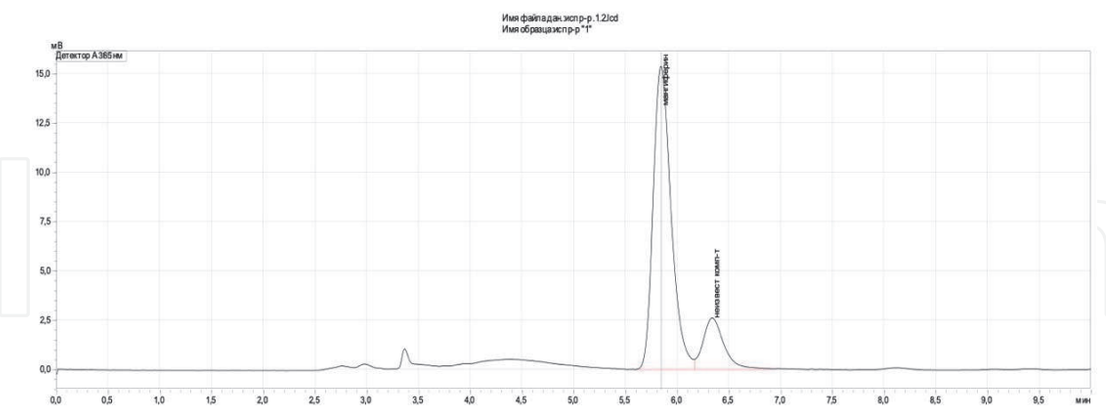

Figure 10.
Chromatogram of solution 2.

characterized by good reproducibility and tested at raw materials. The obtained results can be used in the preparation of regulatory documentation for plant raw materials. As a result, in species *Hedysarum caucasicum* M. Bieb., *Hedysarum grandiflorum* Pall., and *Hedysarum daghestanicum* Rupr. ex Boiss., biologically active substances such as polysaccharides, triterpene compounds, flavonoids, saponins, polyphenol compounds, leucoanthocyanins, and tannins were found. The quantitative content of tannins in *Hedysarum caucasicum M. Bieb*. was 5.43 ± 0.15%.

13. Amino acid analysis

Amino acid analysis in the grass of species of the genus *Hedysarum* was carried out on the basis of the FSBOU VO of the Stavropol GAU by column liquid chromatography on an amino acid analyzer according to the procedure indicated in the guest 32,195–2013. Qualitative and quantitative analysis of amino acids was carried out on an amino acid analyzer, AminoAcid Analyzer AAA 339 M (Mikrotechna). The comparative amino acid composition of the three studied samples of species of the genus growing in the North Caucasus showed that amino acids such as aspartic (1.84–2.68%) and glutamic acid (1.29–1.33%) as well as proline (0.83–1.39%), leucine (0.86–0.90%), and phenylalanine (0.58–0.63%) were found in significant quantities in the above-ground organs of the studied species. **Table 7** shows the results of a comparative analysis of the amino acid composition of the species under study.

The study of the amino acid composition during complex pharmacognostic analysis is one of the mandatory areas of research, since amino acids are involved in the biogenesis of many biologically active compounds, including xanthones. It is known that the main components of xanthone biosynthesis are acetyl-CoA,

Amino acid type		**Species**		
Types	**Structure**	**№1**	**№2**	**№3**
Monoamino dicarboxylic acids				
Asparaginic	α-Aminosuccinic acid	1.86	2.68	1.84
Glutaminic	α-Aminoglyutaroic acid	1.33	1.31	1.29
Monoamine monocarboxylic acids				
Leucine*	α-Aminoisocapronic acid	0.90	0.89	0.86
Valine*	α-Aminoisovaleric acid	0.68	0.74	0.71
Alanine	α-Aminopropionic acid	0.67	0.65	0.63
Serine	α-Amino-βoxypropionic acid	0.62	0.66	0.60
Phenylalanine*	α-Amino-β-phenylpropionic acid	0.62	0.63	0.58
Glycine	α-Aminoacetic acid	0.55	0.58	0.57
Threonine*	α-Amino-β-hydroxy-butyric acid	0.54	0.51	0.50
Thyrosine	α-Amino-β-oxy phenylpropionic acid	0.52	0.63	0.54
Isoleucine*	α-Amino-β-ethyl-β-methylphenylpropionic acid	0.48	0.58	0.54
Methionine*	α-Amino-γ-methylthion-n-butyric acid	0.04	0.11	0.10
Monoamine monocarboxylic acids				
Lysine*	α, ε-Aminocapronic acid	0.74	0.73	0.72
Arginine	α-Amino-σ-guanidine-n-valeric acid	0.55	0.56	0.56
Heterocyclic compounds				
Proline	Pyrrolidine-α-carboxylic acid	0.83	1.39	1.19
Gystidine	α-Amino-β-imidazolyl-propionic acid	0.33	0.42	0.35

**№1—Hedysarum caucasicum M. Bieb.; №2—Hedysarum grandiflorum Pall.; and №3—Hedysarum daghestanicum Rupr. ex Boiss.*

Table 7.
Comparative analysis of amino acid composition in three samples of Hedysarum L. species growing in the North Caucasus, %.

Element title	Raw material content, %	Element title	Raw material content, %
Macroelements			
K	1.47	Na	0.10
Ca	0.29	P	0.49
Mg	0.10		
Microelements			
Al	0.049	Mn	0.0025
Ba	0.0029	Cu	0.00098
B	0.00098	Ti	0.0073
Fe	0.029	Cr	0.0009
Si	0.29	Zn	0.0049

Table 8.
Elemental composition of herb Hedysarum caucasicum *M. Bieb.*

mevalonic, and shikimic acids, from which phenylalanine is further synthesized. The obtained research results can be further used in preparing a complex metabolomic evaluation of medicinal plant raw materials of *Hedysarum* species.

Spectral analysis of the above-ground organs of *Hedysarum caucasicum* M. Bieb. was carried out at the Central Testing Laboratory of Caucasian Geologic Survey JSC in Essentuki. Sample preparation of grass raw material *Hedysarum caucasicum* M. Bieb. was carried out according to the methodology of the Pharmacopea of Russian Federation. Ash was studied according to the method of the MP plant 4C atomic emission spectrometry on diffraction spectrograph.

The ash sample was evaporated into the graphite electrode cell by means of an electric arc. In equilibrium processes of excitation and reverse transition to the basic state of electrons of element atoms, emission (emission) spectra were recorded. The results are shown in **Table 8**.

Spectral analysis of herb *Hedysarum caucasicum* M. Bieb. represented by macroelements (potassium, calcium, magnesium, sodium, phosphorus) and trace elements (manganese, iron, zinc, copper, and silicon). The obtained results of elemental composition are necessary in the complex analysis of the sum of active substances of the plant.

14. Conclusion

For the first time, morphological-anatomical diagnostic signs of the species are necessary for standardization of medicinal vegetal raw materials. Indicators of caulifolar micromorphology were introduced to diagnose plant objects using the example of the genus *Hedysarum* L. Molecular genetic studies were carried out and morphometric indicators were determined, which make it possible to establish correlations between morphological, molecular genetic, and phytochemical indicators of species assigned to certain sections of the genus, as well as to predict the accumulation of xanthones in previously unearthed species.

Introduction studies of *Hedysarum causicum M. Bieb.*, *Hedysarum grandiflorum Pall.*, and *Hedysarum daghestanicum Rupr. ex Boiss.* on the territory of the Botanical Garden of the PMFI and in the Dagestan Scientific Center of the Mining Botanical Garden of the Russian Academy of Sciences, including the main phases of development, phenological spectra, were compiled.

Within the framework of phytochemical screening, BAVs such as polysaccharides, triterpene compounds, flavonoids, saponins, polyphenolic compounds, leucoanthocyanins, and tannins were found in the objects we studied. For the first time in the grass of three species of the genus *Hedysarum*, the presence of amino acids, the main part of which belongs to the group of essential amino acids, and the presence of proline and phenylalanine prove the presence of xanthones. The obtained research results can be further used in preparing a complex metabolomic evaluation of medicinal plant raw materials of *Hedysarum* species.

A comprehensive study of the qualitative analysis and quantitative content of the sum of xanthones and, in fact, mangiferin was carried out using thin-layer and paper chromatography, UV spectrophotometry, capillary zone electrophoresis, as well as high-performance liquid chromatography.

In the framework of complex pharmacognostic studies of three species of the genus *Hedysarum*, we have developed a method for quantitative determination by UV spectrophotometry of the sum of xanthones in terms of mangiferin. The technique takes into account the basic physicochemical properties of xanthones; is characterized by the reproducibility, high accuracy, and simplicity of execution; and allows conducting both a screening assessment of various raw materials containing mangiferin derivatives and standardization of the prepared vegetable raw materials.

The developed methods are tested on the above-ground organs of *Hedysarum* species, collected and dried taking into account the rules and requirements for the preparation of medicinal raw materials. As a result, it was found that the greatest quantitative content of the sum of xanthones in terms of mangiferin is distinguished by the grass *Hedysarum caucasicum* M. Bieb. ($0.62 \pm 0.021\%$). The results show the prospect of further investigation of *Hedysarum caucasicum* M. Bieb. herb as an additional source of mangiferin.

Conflict of interest

The authors declare no conflicts of interest.

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