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## Standardization Parameters of *Alfredia nivea* KAR.&KIR Herb

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### Abstract

The Kazakhstan flora is rich in promising poorly-studied plants, which are traditionally used in folk medicine, but their introduction into medical practice requires additional in-depth research using modern scientific methods. *Alfredia nivea* KAR.&KIR of the *Asteraceae* family, which is used in folk medicine as a neurotropic agent, is an interesting object for introduction into official medical and pharmaceutical practice.

**Aim.** To create new medicines based on *Alfredia nivea* herb, it is necessary to develop methods for quality control of this raw material, therefore, the aim of the research was to determine the parameters for standardization of the *Alfredia nivea* KAR & KIR. herb.

**Materials and methods.** The study objects were samples of the *A. nivea* herb collected in Kungei Alatau, 4.3 km southeast of the Karabulak village, Eastern Karabulak canyon, Almaty Region, Kazakhstan. The macroscopic and microscopic studies of the *A. nivea* herb were performed according to the methodology of the European pharmacopeia (EuPh) 2.8.23 "Microscopic examination of the medicinal plant raw material". The macroscopic studies were performed using a magnifying glass and a MBS-9 binocular microscope, the microscopic studies were done using MS Microscopes 10 (oculars X5, X10, X15, lenses x10, x40), Micromed XS-4130 (oculars WF15X, lenses x40/0.65, x10/0.25) with a microphotonozzle (China). Identification of the main substances was carried out by the TLC method, testing and the quantitative determination of the flavonoid content were performed according to the EuPh methods.

**Results and discussion.** Morphological and anatomical features of the *A. nivea* herb have been determined; on their basis *Identifications A and B* have been proposed; *TLC Identification C* of the main BAS of the raw material has been developed; indicators of purity tests have been determined. It has been proposed to carry out the quantitative determination by the content of flavonoids.

**Conclusions.** The parameters of the *A. nivea* herb standardization have been determined on the basis of the following indicators: macroscopic and microscopic features, TLC identification of the main BAS of the raw material (hyperoside, rutin, quercetin and chlorogenic acid), related impurities (not more than 2%), stems with a diameter of more than 20 mm (not more than 10%), the loss on drying (not more than 13%), the total ash (not more than 10%) and at least 0.5% flavonoids calculated with reference to rutin.

**Keywords:** *Alfredia nivea*; herb; standardization; quality control methods

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### Параметри стандартизації трави *Alfredia nivea* KAR.&KIR.

#### Анотація

Флора Казахстану багата на перспективні маловивчені рослини, які застосовують у народній медицині, але для їх впровадження в медичну практику потрібні додаткові глибокі дослідження, за допомогою сучасних наукових методів. Цікавим об'єктом для впровадження в офіціальну медичну та фармацевтичну практику є Альфредія снігова (*Alfredia nivea* KAR. & KIR.) родини *Asteraceae*, яку в народній медицині використовують як нейротропний засіб.

**Мета.** Для створення нових лікарських засобів на основі трави Альфредії снігової необхідно розробити методи контролю якості цієї сировини, тому метою досліджень було визначити параметри стандартизації трави *A. nivea* KAR. & KIR.

**Матеріали та методи.** Об'єктами дослідження були зразки трави Альфредії снігової (*A. nivea*), заготовлені в Кунгей Алатау, за 4,3 км південно-східніше від с. Карабулак, ущ. Східний Карабулак, Алматинської області, Казахстан. Макро- і мікроскопічні дослідження трави Альфредії снігової виконували згідно з методикою Європейської фармакопеї (ЄФ) 2.8.23 «Мікроскопічне дослідження лікарської рослинної сировини». Для макроскопічних досліджень використовували лупу та бінокулярний мікроскоп МБС-9, для мікроскопічних – мікроскопи МС 10 (окуляр х5, х10, х15, об'єктиви х10, х40), Місromed XS-4130 (окуляр WF15X, об'єктиви х40/0,65, х10/0,25) з мікрофотонасадкою (КНР). Ідентифікували основні речовини методом ТШХ, випробування та кількісне визначення за вмістом флавоноїдів здійснювали відповідно до методик ЄФ.

**Результати та їх обговорення.** Визначено морфологічні й анатомічні ознаки трави Альфредії снігової, на основі яких запропоновано *Ідентифікації А та В*, розроблено ТШХ *Ідентифікацію С* основних БАР сировини, визначено показники випробувань на чистоту, а також запропоновано проводити кількісне визначення за вмістом флавоноїдів.

**Висновки.** Визначено параметри стандартизації трави Альфредії снігової за такими показниками: макро- та мікроскопічні ознаки, ТШХ ідентифікація основних БАР сировини (гіперозид, рутин, кверцетин та хлорогенова кислота), сторонні домішки (не більше 2%), наявність стебел діаметром понад 20 мм (не більше 10%), втрата в масі під час висушування (не більше 13%), загальна зола (не більше 10%) і не менше 0,5% флавоноїдів у перерахунку на рутин.

**Ключові слова:** Альфредія снігова; *Alfredia nivea*; трава; стандартизація; методи контролю якості

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## ■ Introduction

The Kazakhstan flora is rich in promising poorly-studied plants, which are traditionally used in folk medicine, but their introduction into medical practice requires additional in-depth research using modern scientific methods [1, 2]. *Alfredia nivea* KAR&KIR of the *Asteraceae* family, which is used in folk medicine as a neurotropic agent, is an interesting object for introduction into official medical and pharmaceutical practice in Kazakhstan [3].

*A. nivea* is a perennial plant of 25–70 cm height with leathery, oblong-lanceolate, pinnately lobed leaves. The venation is pinnate, the lateral veins at the ends turn into sharp yellowish spines. The leaf blade is grayish-green on the upper side, whitish-pubescent on the lower side. The inflorescence is a spherical head of pink tubular flowers with a multi-row involucre. The plant blooms in July, fruits ripen in August – September. It grows in the subalpine and alpine belts of the mountains, along the stepped rocky slopes, in spruce-fir forests. It is found in Tarbagatai, Dzungarian, Trans-Ili and Küngöy Ala-Too Range Alatau [4–6]. The plant's raw material reserves are sufficient for the needs of Kazakhstan.

Plants of the *Alfredia* genus are insufficiently studied. Most of the studies are devoted to *A. cernua* L. Thus, extracts of this species have been shown to affect the central nervous system and improve memory [7, 8]. It has also been found

that *A. cernua* extracts have nootropic properties, contribute to the improvement of indicators of orientation-exploratory behavior, the preservation of the passive escape reflex during hypoxic shock, and the increase of physical performance in mice [9, 10]. The most pronounced effect was observed with the use of the *A. cernua* extract obtained with 95% ethanol [10, 11]. It has also been shown that the extract of the *A. nivea* above-ground part in 95% ethanol in the dose of 100 mg kg<sup>-1</sup> restores exploratory behavior and reflex safety after hypoxic trauma [12]. Thus, the *A. nivea* herb is a promising raw material for creating new neurotropic medicines.

Previously, the standardization of the *A. cernua* herb [13], the macroscopic and microscopic study of the *A. nivea* herb were carried out, and its diagnostic signs were determined [14]. These data were taken into account when developing a project of quality control methods for the *A. nivea* herb.

Therefore, the aim of the research was to determine the parameters of the standardization of the *A. nivea* KAR. & KIR. herb.

## ■ Materials and methods

The study objects were samples of the *A. nivea* herb collected in Kungei Alatau, 4.3 km south-east of the Karabulak village, Eastern Karabulak canyon, Almaty Region, Kazakhstan (1762 m above the city, N 43°02'30.2", E 078°34'16.0") July

06, 2021. The identity of the plant was determined by professor Tetiana M. Gontova, D. Sc., National University of Pharmacy (Kharkiv, Ukraine) [4, 15]. Voucher specimens were deposited at the School of Pharmacy, Asfendiyarova Kazakh National Medical University (Almaty, Kazakhstan, No. 432–435). The raw material was dried at room temperature [16, 17] in a well-ventilated area for ten days and stored in paper bags [18, 19].

The macroscopic and microscopic studies of the raw material were performed according to the methodology described in the European Pharmacopoeia (EuPh) 2.8.23 “Microscopic examination of the medicinal plant raw material” [18, 20]. The macroscopic studies were conducted using a magnifying glass and a MBS-9 binocular microscope. The study of the anatomical structure of the *A. nivea* herb was carried out on samples of the whole and cut raw material according to the requirements of the EuPh. The herb was fixed in a mixture of 96% ethanol *R* – glycerin *R* – purified water *R* (1:1:1). The structure of stems and leaves was studied on transverse sections. The epidermal of the organs was considered from the surface according to generally accepted methods [18, 21]. The raw material was crushed according to the requirements of the EuPh monograph 2.9.12 “Sit analysis” and clarified with the help of chloral hydrate *R* [18, 20]. The studies of transverse and longitudinal sections, epidermis and preparations from the surface were carried out using MS Microscopes 10 (oculars X5, X10, X15, lenses x10, x40), Micromed XS-4130 (oculars WF15X, lenses x40/0.65, x10/0.25) with a microphotonozzle (China). The results of the study were recorded using the Canon IXUS 220 HS camera.

To develop the identification method for the main biologically active substances (BAS) in the *A. nivea* herb, the TLC method was used [18, 22]. Merck Silica Gel F254 plates were used for chromatography. Purity of solvents used for the preparation of chromatographic systems were of chemically pure and analytical grades. The ratio of solvents indicated by numbers were taken in volumetric units.

The content of related impurities (2.8.2), the loss on drying (2.2.32), the total ash content (2.4.16) were determined according to the requirements of the EuPh [18, 23, 24].

*Assay of flavonoids.* The assay of flavonoids was determined in accordance with the methodology of the EuPh by the spectrophotometric method calculated with reference to rutin [18, 25].

About 5.0 g of *Alfredia* herb (accurate weight) crushed to the size of particles passing through a sieve with a diameter of 2 mm was placed in a 200 mL ground joint flask, 50 mL of 70% ethanol solution was added. The flask was connected to a condenser and heated on a water bath for 30 min, periodically shaken to wash off particles of the raw material from the walls. The mixture was cooled and filtered through cotton wool, so that the particles of the raw material did not get on the filter. The cotton wool was transferred to the ground joint flask for extraction, and the new portion of the extractant was added. The extraction was repeated twice under the conditions described above, and the extract was filtered in the same flask. The combined extract was evaporated up to  $\frac{1}{4}$  of the initial volume. The evaporated extract was quantitatively transferred to a 50.0 mL measuring flask, cooled and was diluted with 70% ethanol solution to the volume (Solution A).

2.0 mL of Solution A was placed into a measuring 25 mL flask, 2.0 mL of 3% aluminum chloride solution in 96% ethanol *R* was added, the volume was diluted with 70% ethanol solution and mixed.

In 30 min, the solution was filtered through the paper filter, throwing away the first portions of the filtrate, and the optical density was measured on a Specol 1500 spectrophotometer (Switzerland) at a wavelength of 410 nm in a 10 mm cuvette. *Reference solution* contained 2.0 mL of Solution A, diluted with 70% ethanol to the volume in a 25 mL flask.

In parallel, experiment with a *Standard solution* of rutin (pharmacopoeial standard) was carried out in the same conditions. 1.0 mL of 3% aluminum chloride solution was added to 1.0 mL of the *Standard solution* of rutin, and the volume was diluted to 25.0 mL with 70% ethanol. As a reference, 1.0 mL of the *Standard solution* of rutin diluted with 70% ethanol to the volume of 25 mL was used.

The content of flavonoids in the raw material was calculated with reference to rutin (%) by the following equation [28, 29]:

$$X, \% = \frac{A_1 \times a_0 \times 50 \times 1 \times 25 \times 100 \times 100}{A_0 \times a_1 \times 25 \times 2 \times 50 \times (100 - w)} ,$$

where  $A_1$  – is the optical density of the *Test solution*;

$A_0$  – is the optical density of the *Standard solution* of rutin;

$a_1$  – is the accurate weight of raw material, g;

$a_0$  – is the accurate weight of rutin, g;

$w$  – is the loss on drying, %.

*Preparation of the standard solution of rutin.* About 0.01 g (accurate weight) of rutin (pharmacopoeial standard FS 42-2508-87) dried at 135 °C to the constant mass, was placed into a 25.0 mL measuring flask, dissolved in 96% ethanol, diluted with the same solvent to the volume and mixed.

*Preparation of a 3% aluminum chloride solution in 96% ethanol.* According to the State standard 3759-85, 3.0 g of aluminum chloride (chemically pure) was dissolved in 96% ethanol in a 100.0 mL measuring flask, diluted with the same solvent to the volume and mixed.

## ■ Results and discussion

**Source.** The raw material is a dried herb, which includes whole or cut leaves, flowers and stems of *A. nivea* KAR. & KIR. (*Asteraceae*). The herb was collected in summer, the below ground parts were removed, and then the soil was dried to obtain the intact form. Alternatively, the herb was sliced while fresh, and dried immediately to obtain the sliced form of the *A. nivea* herb.

Based on the results of the macroscopic analysis of the *A. nivea* herb [14], such macroscopic signs, which served as the basis for the development of *Identification A*, were determined.

The stem is usually unbranched in the lower part, slightly branched in the upper part, rough, slightly ribbed, 20–60 cm long, 5 mm in diameter. It is externally grayish-green, tomentose-pubescent. The texture is hard. Rosette leaves are 15–35 cm long and 2.5–6.0 cm wide; stem leaves are short-petiolate or sessile, 5.0–7.0 cm long and 1.5–2.5 cm wide, leathery, linear-lanceolate and lanceolate, pinnatipartite, with a pinnate venation with prominent lignified veins and long sharp ends; non-tomentose and green on the adaxial surface and grayish green, abundant tomentose-pubescent on the abaxial surface. The petiole of the rosette leaf is elongated-triangular in outline with elongated “wings” directed upwards at an angle of 45 degrees, narrow at the ends.

The inflorescence is a spherical drooping flower head up to 6.0 cm in diameter with a 3–5-row, tiled involucre made of hard lanceolate, pointed leaflets with filmy fringed-outgrowths on the sides. The tubular pink flowers are 5-tooth, thin, up to 3.0 cm in length; numerous sepals are modified to thin long hairs; stamens and the pistil protrude from the corolla tube; the cypselas are up to 6.0 mm, pale yellowish with variegated brown spots and stripes on the surface.

Based on the results of the microscopic analysis of the *A. nivea* herb [14], such microscopic signs, which served as the basis for the development of *Identification B*, were determined.

The stem and leaves are tomentose-pubescent with covering and rarely glandular trichomes; covering trichomes are of three types – long filamentous hairs with a 2-cell base, large multicellular wide lumen hairs with collapsed cells; and multicellular narrow lumen hairs with collapsed cells; glandular hairs are of 2 types – 1-headed with a 1-celled stalk, and headed with a multicellular stalk and a multicellular head with collapsed cells. The stem is rounded with slightly protruding ribs. The cells of the epidermis are covered with a layer of a folded cuticle; prosenchymatous cells are straight-walled with slightly thickened walls and simple straight pores. The central cylinder is of transitional type. The collenchyma is angular and lacunar; parenchyma cells are collenchymatous; the endoderm is distinct; open collateral bundles are with a well-developed sclerenchyma, xylem vessels are porous and spiral.

Leaf is dorsiventral; the cells of the upper and lower epidermis above the veins are prosenchymatous, straight-walled, with slightly thickened walls and straight pores; between the veins the cells of the upper epidermis are 5–6-angle, isodiametric and on the lower – parenchymal, sinuous-walled, with slightly thickened walls; the stomata are frequent only on the lower epidermis. The stomatal apparatus is anomocytic.

The adaxial surface of the leaf is rarely pubescent with thin long hairs, and abaxial – densely pubescent with filamentous hairs with a 2-cell base and less often multicellular hairs with collapsed cells. The cells of the outer and inner epidermis of the involucre leaflets are elongated, slightly thickened and straight-walled with light brown contents. The inner epidermis is covered with filamentous trichomes. The cells of the petal epidermis are prosenchymatous, thin-walled or heavily thick-walled, straight-walled with colorless, rarely brown contents; the epidermis is covered with a weakly expressed folding cuticle; vessels are thin spiral. The style of the pistil is densely covered with short conical hairs. Pollen grains are rounded, yellow-brown, the structure of ectine is slightly spiny.

**The powdered raw material.** The powder is yellowish-green in color, examined under a microscope using *Chloral hydrate solution R*. The powder shows the following diagnostic signs: fragments of epidermis (Figure 1, A) composed of cells

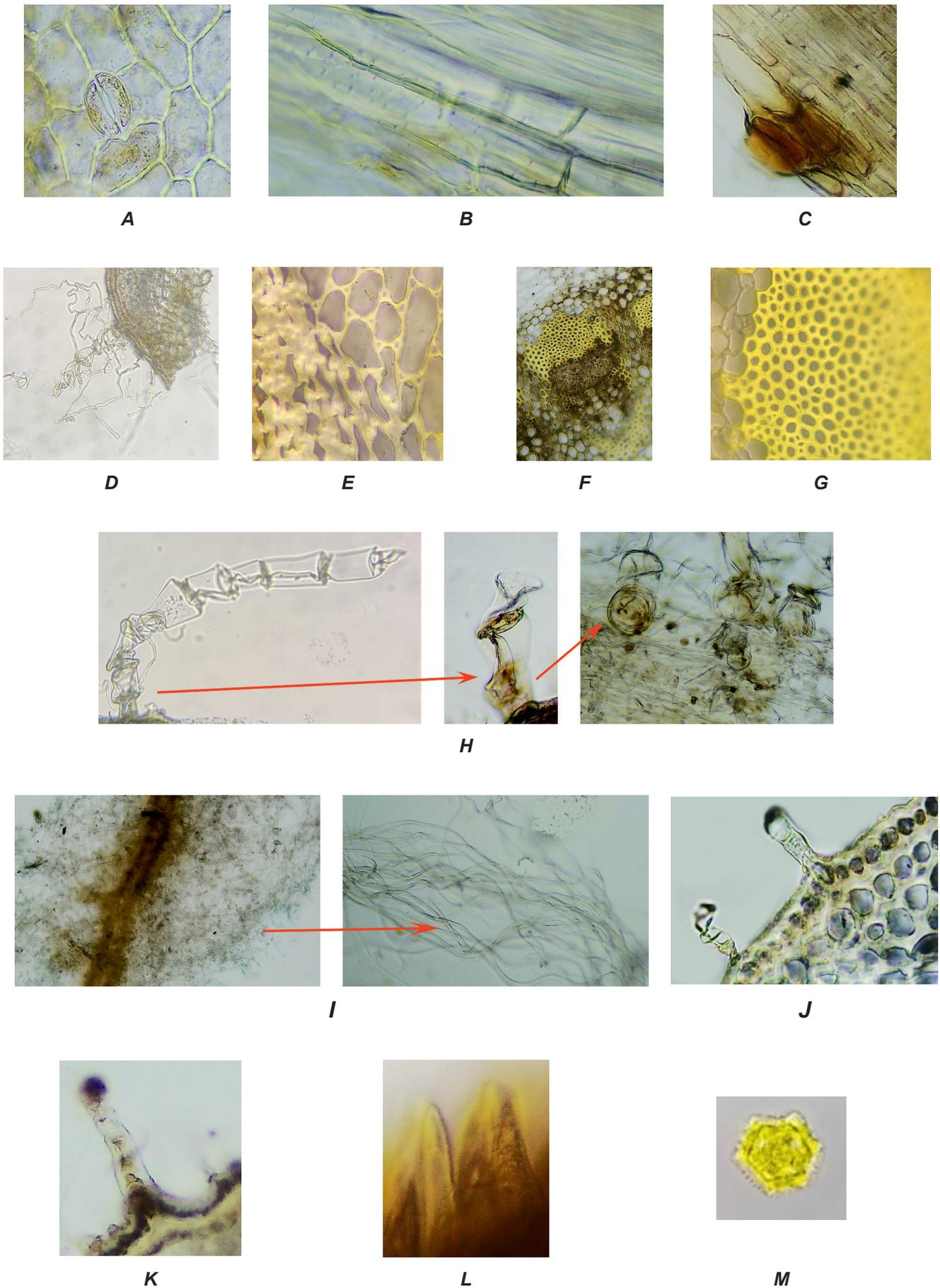


Figure 1. Microscopic features of the powder

with prosenchymatous straight-walled and slightly thick-walled cells with simple straight pores (Figure 1, B); cells with light brown contents (Figure 1, C); stomata of the anomocytic type (Figure 1, A); fragments of the angular and lacunar collenchyma (Figure 1, D); collenchymatous cells of the parenchyma (Figure 1, E); open collateral bundles (Figure 1, F); sclerenchyma (Figure 1, G), porous and spiral vessels; long filamentous hairs with a 2-cell base, large multicellular wide lumen hairs with collapsed cells (Figure 1, H), and multicellular narrow lumen hairs with collapsed cells (Figure 1, I); glandular hairs of 2 types – 1-headed with a 1-celled stalk (Figure 1, J), and headed with a multicellular stalk and a multicellular head with collapsed cells (Figure 1, K); styles with short conical hairs (Figure 1, L); rounded, yellow-brown pollen grains with slightly spiny ectine (Figure 1, M).

**Identification C** of BAS of the *A. nivea* herb was developed using the TLC method with standards of hyperoside, quercetin, rutin and chlorogenic acid in the system: ethylacetate *R* – aqua *R* – formic acid *R* – acetic acid anhydrous *R* (72:14:7:7).

For this analysis, 300 mg of the *Alfredia* herb was extracted with 3 mL of methanol for 30 min at 40 °C on a water bath with ultrasound irradiation. 15 µL of the extract was applied to the chromatogram. The standards were prepared by

dissolving 1 mg of the standards in 10 mL of pure methanol. After spraying the plate with diphenylborinic acid 2-aminoethyl ester *R*, macrogol 400 *R* the zones at the level of quercetin, hyperoside, rutin and chlorogenic acid were detected in UV light [28].

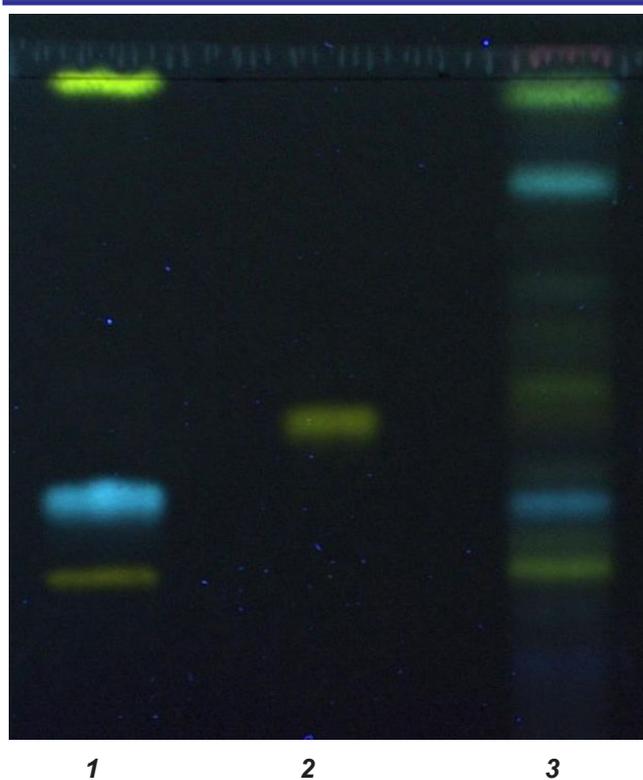
A photo of the sequence of zones on the chromatogram of the *A. nivea* the test extract and the reference solutions are presented in Figure 2. The main substances found in the TLC chromatogram are quercetin, chlorogenic acid, rutin and hyperoside. Other additional fluorescent zones can be found on the chromatogram of the test extract, but they do not play an important role when identifying the raw material. Also, using the chromatogram obtained in the same conditions, after spraying the plate with the Drangendorff reagent [29], it was determined that there were no alkaloids in the raw material.

In the lower part of the chromatogram of the reference solutions, a yellow fluorescent zone corresponding to rutin, as well as the blue fluorescent zone corresponding to chlorogenic acid, and the yellow fluorescent zone corresponding to hyperoside, were found. The yellow fluorescent zone corresponding to quercetin was detected in the upper part of the chromatogram. On the chromatogram of the test extract, the intense fluorescent yellow zone at the level of the reference solution of rutin was detected. At the level of the reference solution of chlorogenic acid the intense blue fluorescent zone was also determined.

Since the zones of rutin and quercetin are most clearly identified and have a significant area during the TLC analysis, it has been noticed that they dominate in the raw material, therefore, the quantitative determination of BAS in the *A. nivea* herb should be carried out by the assay of flavonoids calculated with reference to rutin adapting the methodology of the EuPh [18].

The results of determining related impurities, indicators “loss on drying during drying”, “total ash” and the quantitative content of flavonoids are given in Table.

The tests were proposed to be carried out according to the following indicators: related impurities (2.8.2), namely, stems with a diameter of more than 20 mm – not more than 10%; other impurities – not more than 2%; the loss on drying (2.2.32) – not more than 13.0% (1.000 g of the raw material crushed into a powder at a temperature of 105°C for 2 h); the total ash (2.4.16) – not more than 10%.



**Figure 2.** The TLC chromatogram of *A. nivea* phenolic compounds: 1. the standards of quercetin, chlorogenic acid, rutin; 2. hyperoside; 3. the *A. nivea* herb extract

**Table.** The results of the analysis of the *A. nivea* herb

Indicators	Standardization	Samples of the raw material					
		1	2	3	4	5	6
Description	According to the parameters developed	+	+	+	+	+	+
Identification A. External signs – macroscopy	According to the parameters developed	+	+	+	+	+	+
Identification B. Anatomical signs – microscopy	According to the parameters developed	+	+	+	+	+	+
Identification C. TLC method	According to the parameters developed	+	+	+	+	+	+
Impurities	Stems with a diameter of more than 20 mm not more than 10%	8.7	6.2	9.6	7.1	8.5	8.0
	Related impurities – not more than 2%	1.5	1.8	1.0	1.5	1.0	0.5
Loss on drying	Not more than 13.0%	8.7	9.3	7.2	9.7	8.5	11.0
Total ash	Not more than 10%	7.2	6.6	8.6	5.3	8.2	8.9
The content of flavonoids calculated with reference to rutin	Not less than 0.5%	0.74	0.69	0.63	0.76	0.60	0.58

Note: «+» – meets the requirements

The assay was proposed to be carried out by the content of flavonoids – at least 0.5% calculated with reference to rutin (C<sub>27</sub>N<sub>30</sub>O<sub>16</sub>; M.M. 610.5) in the dry raw material.

## Conclusions

The parameters of the *A. nivea* herb standardization have been determined on the basis

of the following indicators: macroscopic and microscopic features, TLC identification of the main BAS of the raw material (hyperoside, rutin, quercetin and chlorogenic acid), related impurities (not more than 2%), stems with a diameter of more than 20 mm (not more than 10%), the loss on drying (not more than 13%), the total ash (not more than 10%) and at least 0.5% flavonoids calculated with reference to rutin.

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