Original Article

Research On The Study Of The Flavonoid Composition And End-To-End Standardization Of The Raw Material And Dosage Form Of Calendula Officinalis L. Growing In Uzbekistan

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Abstract

Herbal preparations have been used since ancient times as medicines to treat a range of ailments. Each plant synthesizes an unlimited number of very complex and unusual chemicals, the structure of which is different. There are over 120 different plant-derived chemicals currently considered important medicines.

The World Health Organization (WHO) emphasizes the importance of qualitative and quantitative methods for the characterization of herbal preparations, the quantification of biomarkers and/or chemical markers.

If the main active ingredient is known, it is most logical to quantify that compound. Where active ingredients are known to contribute to therapeutic efficacy, herbal preparations should be standardized for these compounds. If the active ingredients are not yet known, a marker substance can be selected for analytical purposes, which should be specific to the herbal preparation.

Phytochemical standardization covers all possible information obtained with respect to the chemical constituents present in a herbal medicinal product. Therefore, phytochemical evaluation for standardization purposes includes the following:

- Preliminary testing for the presence of various chemical groups.
- Quantification of chemical groups of interest (e.g. total alkaloids, total flavonoids or phenols, total triterpene acids, total tannins, etc.).
- Creation of fingerprint profiles.
- Quantification of important chemical components.

The objective of the study: to study the chemical composition and standardization of raw materials and dosage forms of Calendula officinalis L., growing in Uzbekistan.

To achieve this goal, the following **tasks** were defined:

- to study the content of the main group of biologically active compounds in the raw materials of Calendula officinalis L.;

-to determine the main component for standardization;

- to develop a method for qualitative and quantitative analysis of the content of the main substance using chromatography methods.

To study the main group of biologically active substances, dried and crushed raw materials of Calendula officinalis L. were extracted with 70% alcohol. The alcoholic extracts were thickened and the extract was successively treated with various solvents, in particular chloroform, ethyl acetate and n-butanol. In order to optimally separate flavonoids in the obtained ethyl acetate fractions of calendula, studies were carried out on the choice of a chromatographic system for TLC, the results of which showed that the solvent system consisting of ethyl acetate, formic acid and chloroform in a ratio of 2:1:2 has the most optimal separating ability. The data obtained by TLC showed that the largest number of flavonoid compounds pass into the ethyl acetate fraction of calendula - 8 adsorption zones of yellow color, flavonoid character. Also, for the separation and identification of calendula flavonoids, the HPLC method was used on HPLC by Agilent Technologies (USA) "1100 series", with the “ChemStation 3D” software, equipped with a four-gradient pump with a vacuum degasser and an autosampler, a column thermostat and a UV/VIS diode array spectrophotometric detector with a wavelength from 190 to 800 nm. We used a chromatographic column 150x3.0 mm in size filled with Zorbax Eclipse XDB C-18 sorbent with a particle size of 3.5 μm from Agilent Technologies. The analyzes were carried out in isocratic mode with a mobile phase flow rate of 0.5 ml/min, detection was performed at 250-400 nm, while simultaneously recording the spectra of each peak. Mobile phase: 50 mM phosphate buffer (pH=3) - methanol (80:20), the volume of the sample injected into the chromatograph injector was 10 μL, the duration of the analysis was 40 min. Flavonoids were identified by the retention time and the UV-correspondence factor of the spectra of standard samples solutions of the studied substances (SSS). The results of the identification of flavonoids of Calendula officinalis L. raw materials showed that it contains: hyperoside, luteolin, luteolin-7-glycoside, luteolin-3'-O-β-D-glucopyranoside, kaempferol, quercetin, isoquercetin, isorhamnetin-3-O-β-D -glycoside. With the use of this method, a method of end-to-end standardization of raw materials was developed - flowers and tincture of Calendula officinalis L., growing in Uzbekistan. Quercetin was used as a standard substance. The results of the studies show that the content of quercetin in flowers and tincture of calendula is on average 0.0146 and 0.0028%, respectively, and the relative error of the average result of the proposed method reaches up to ±4.99%.

Keywords: Calendula officinalis L. flowers, flavonoids, quercetin, thin-layer and high-performance chromatography, standardization.

Introduction

Calendula officinalis (marigold) - Calendula officinalis L. belongs to the Asteraceae family. The genus Calendula includes about 20 species of annual herbs growing wild, mainly in the Mediterranean countries.

Calendula is used as an anti-inflammatory and antiseptic agent for sore throat, tonsillitis and other inflammatory processes of the upper respiratory tract; wound healing agent - in dentistry, gynecology; antispasmodic, choleretic, antimicrobial, sedative, hyposensitizing, antiviral, decongestant, antitoxic agent for diseases of the stomach, liver, heart, accompanied by palpitations, shortness of breath and edema [1]. In folk medicine, it is used to treat malignant tumors.

The medicinal properties of calendula are due to the presence of a BAS complex in the raw materials, namely: carotenoids, flavonoids, triterpene saponins and a number of other related substances. The main of these compounds are carotenoids and flavonoids [2].

Due to such a versatile action of calendula and its wide application, this plant, from 1961 to the present, has been studied by many, especially foreign scientists.

For example, carotenoids violaxanthin, eeaxanthin, β-carotene, lutein, neurosporin, and phytheon were isolated from the flowers of calendula officinalis, which grows in Australia [3]. On the basis of carotenoids, the preparation "Carophyllenic ointment" was obtained, which is used to treat dermatitis, eczema and trophic ulcers as an anti-inflammatory agent [1, 4].

In all organs of the calendula cultivated in Poland, sterols were found, the largest amount of which is contained in the leaves - up to 18% [5]. Along with sterols, in the leaves tocopherols were found [6, 7].

Calendula officinalis also contains triterpene compounds. They are represented by triterpene alcohols and oleanolic acid (in free form and in the form of esters, glycosides). The content of triterpene alcohols in it reaches up to 5% [8].

Coumarins such as scopoletin, umbelliferone, esculetin [9] and a monoterpene lactone, loliolid, which, according to the data [10], have antitumor activity, were isolated from the flowers of calendula officinalis. The essential oil isolated from this plant [11,12] has a high trichomonacid activity.

By chromatography on paper, 8 flavonoids were found in the inflorescences of calendula officinalis. The content of total flavonoids in raw materials varies depending on the variety and population from 0.26 to 0.91% [13].

Kurkin N. and Sharova O. for the first time isolated two flavonoids from calendula (variety "Kalta") cultivated in the Russian Federation [14].

Authors Ivasenko S.A. and others studied the quantitative content of carotenoids and flavonoids in the inflorescences of some varieties of calendula officinalis, bred at the Central Kazakhstan Research Institute of Agriculture. Then 3 varieties with a high content of total carotenoids (up to 0.65%) and 2 varieties with the same content of total flavonoids (up to 0.84%) were selected[15].

Researchers Samylina I.A. and others conducted a comparative study of flavonoids in tinctures obtained from different types of calendula [16].

The influence of drying modes on the quality of medicinal marigold raw materials was studied and it was shown that with an increase in temperature above 100^0C, the content of carotenoids and flavonoids decreases. Based on this, it is recommended to dry the raw material at a temperature of 80^0C [17].

Currently, in medical practice, the drug "Kaleflon", containing the amount of flavonoids, is used to treat gastritis, gastric and duodenal ulcers, as well as tincture as an anti-inflammatory and wound healing agent.

Meanwhile, the works devoted to the study of the local species of the above MP (medicinal plants) are few in number. Moreover, some of them have not yet found application in the practice of domestic health care.

In this regard, an in-depth study of the domestic species of the above medicinal products, provided with a sufficient raw material base, in order to develop and introduce DF on their basis into medical practice, seems to be an urgent and promising direction in pharmaceutical science.

The physiological activity of natural medicinal products obtained on the basis of MPR (medicinal plants raw materials) is determined by the synergism of the biologically active substances contained in them. Among the latter, flavonoids attract special attention due to their wide distribution in the plant world, wide pharmacological spectrum of action and traditional use, both in folk and practical medicine.

The objective of the study: to study the chemical composition and standardization of raw materials and dosage forms of Calendula officinalis L., growing in Uzbekistan.

To achieve this goal, the following **tasks** were defined:

- to study the content of the main group of biologically active compounds in the raw materials of Calendula officinalis L.;
- determine the main component for standardization;
- to develop a method for qualitative and quantitative analysis of the content of the main substance using chromatography methods.

Experimental part

Materials and research methods. The object of the study was the local raw material Calendula officinalis L., which is golden yellow or orange flowers, collected in large (4-7 cm in diameter) apical baskets. The odour is weak. The taste of the water extract is salty-bitter. Humidity - 10%.

The method of classical extraction of plant material.

Authenticity and quantitative analysis studies were carried out by classical chemical methods, as well as by thin-layer and high-performance chromatography on an HPLC instrument from Agilent Technologies (USA) “1100 series”, with “ChemStation 3D” software, equipped with a four-gradient pump with a vacuum degasser and an autosampler, a thermostat columns and UV/VIS diode array spectrophotometric detector with wavelength from 190 to 800 nm. All reagents and solvents for research were purchased from Merck (Germany).

Results and discussion of the study.

Isolation of the flavonoids sum from MPR

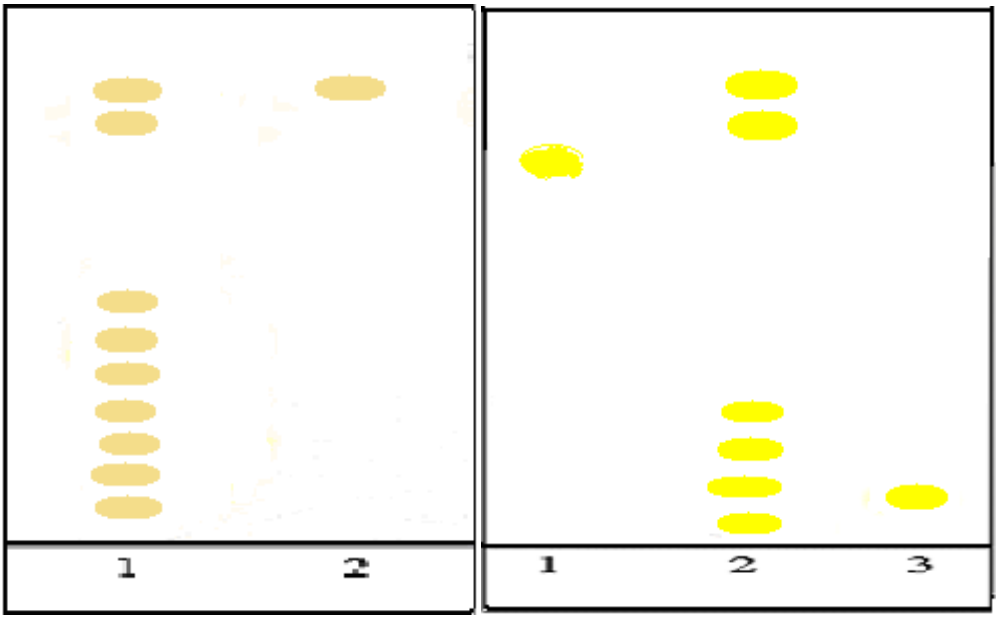
Dried and crushed raw materials (1.5 kg) of marigold flowers were extracted 3 times with 70% alcohol (10 l). The alcohol extracts were thickened on a rotary evaporator at a temperature of 40^0C. The concentrated alcoholic extract was successively treated with chloroform, ethyl acetate, and n-butanol [18,19]. The transition of flavonoids into fractions was checked by TLC.

In order to optimally separate flavonoids in the obtained ethyl acetate fractions of the studied medicinal plants, studies were carried out on the choice of a chromatographic system for TLC, the results of which are presented in Table 1. As a result

of the conducted studies (Fig. 1.), the most optimal separating ability is possessed by a solvent system consisting of ethyl acetate, formic acid and chloroform in the ratio of 2:1:2.

Table 1 **Solvent systems used for detection flavonoids in ethyl acetate fractions by TLC**

№	Solvent system	Ratio
1	Ethyl acetate: water: chloroform	1:3:4
2	Ethyl acetate: water: acetic acid	3:1:2
3	Chloroform: water: acetic acid	3:2:5
4	Ethyl acetate: water: chloroform	5:1:4
5	Ethyl acetate: formic acid: water	5:1:3
6	Ethyl acetate:butanol:chloroform	2:2:5
7	Butanol: water: formic acid	3:2:5
8	Ethyl acetate: formic acid: chloroform	2:1:2



A B

Fig. 1. Chromatogram of calendula flower extracts

A-ethyl acetate fraction
1-ethyl acetate fraction;
2-alcohol solution of quercetin SSS

B- butanol fraction
1-alcohol solution of quercetin SSS;
2-butanol fraction;
3-alcohol solution of rutin SSS.

The data obtained by TLC showed that the largest number of flavonoid compounds passes into the ethyl acetate fraction of the studied plants. Calendula chromatograms show 8 adsorption zones of yellow color, flavonoid character. Moreover, the number of adsorption zones is greater than in the butanol fraction. At the same time, the zones found in both fractions have the same R_f values. That showed the expediency of using the ethyl acetate fraction for further high-quality HPLC analysis.

The study of flavonoid composition by HPLC

Taking into account the fact that a considerable amount of solvents and time is consumed during the isolation and separation of flavonoids by column chromatography, the HPLC method [20–22] using a database of known compounds was used for the first time to separate and identify flavonoids of the above medicinal plants.

For this, the studied fractions were dissolved in methanol. The resulting solutions were filtered through a Millipore filter with a pore size of 0.45 μm and chromatographed on a 1100 series HPLC from Agilent Technologies (USA) with the "ChemStation 3D" software equipped with a four-gradient pump with a vacuum degasser and an autosampler, a column thermostat and UV/VIS diode array spectrophotometric detector with wavelength from 190 to 800 nm. We used a chromatographic column 150x3.0 mm in size filled with Zorbax Eclipse XDB C-18 sorbent with a particle size of 3.5 μm from Agilent Technologies. The analyzes were carried out in isocratic mode with a mobile phase flow rate of 0.5 ml/min, detection was performed at 250-400 nm, while simultaneously recording the spectra of each peak. Mobile phase: 50 mM phosphate buffer (pH=3) - methanol (80:20), the volume of the sample injected into the chromatograph injector was 10 μL, the duration of the analysis was 40 min. Flavonoids were identified by the retention time and the factor of conformity of UV spectra of the standard samples solutions of the substances under study (SSS). The results of identification of flavonoids of medicinal plants are presented in Table 2 and Figure 2.

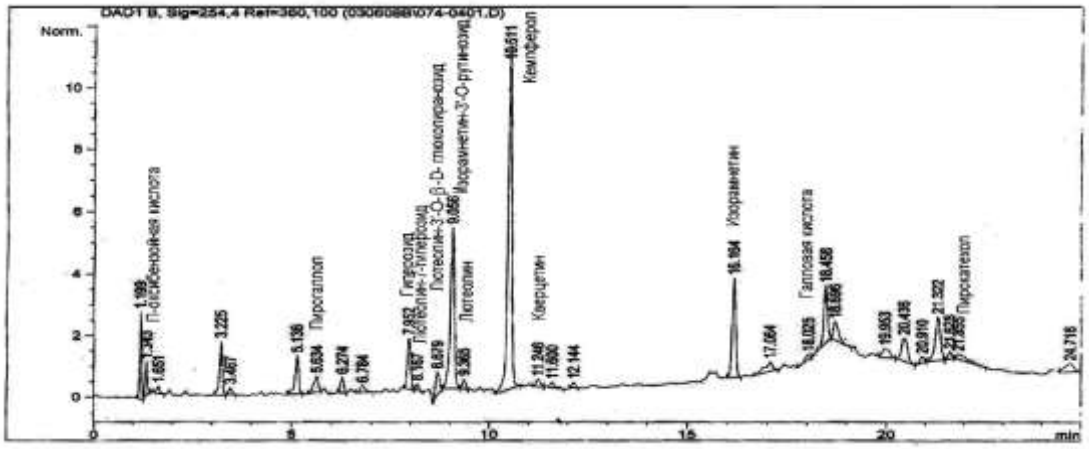


Fig.2. Chromatogram of the ethyl acetate fraction of calendula officinalis

Table 2 **Qualitative composition of phenolic compounds**

№	Name of identified substances	Calendula flowers	
		UV spectra matching factor,%	Peak area, %
1	Hyperoside (quercetin-3-β-D-galacto-pyranoside)	99.6	1.341
2	Quercetin 3-O-β-D-glucopyranoside (isoquercitrin)	99.6	6.952
3	Isorhamnetin-3-O-β-D-rutinoside (narcissin)	88.9	1.569
4	Luteolin-3-O-β-D-glucopyranoside	92.6	0.609
5	Quercetin	99	0.360
6	Luteolin-7-glucoside	92.4	0.300
7	Kaempferol	85.6	2.971
8	Luteolin	90.9	0.405

9	Isorhamnetin	89.9	1.991
10	Gallic acid	99.9	0.500
11	pyragallol	97.3	0.577
12	Pyracatechol	98.4	0.345
13	n-hydroxybenzoic acid	98.6	5.460

Table 2 data show that the raw material of calendula officinalis contains the following flavonoids: hyperoside, luteolin, luteolin-7-glycoside, luteolin-3'-O-β-D-glucopyranoside, kaempferol, quercetin, isoquercetin, isorhamnetin-3-O-β -D-glycoside.

Comparison of the flavonoid composition of the studied medicinal plants with the literature data (Table 3.) showed that luteolin-3'-O-β-D-glucopyranoside, kaempferol and hyperoside were found for the first time in the flowers of calendula officinalis, growing in the Republic of Uzbekistan.

Table 3 **Comparative data on the flavonoid composition of calendula officinalis**

№	substances	Domestic raw materials of calendula officinalis	Literary data of flowers of calendula officinalis
1	Quercetin	+	+
2	Kaempferol *	+*	-
3	Luteolin	+*	
4	Luteolin 3'-0-β-D-glucopyranoside *	+*	-
5	Luteolin-7-glucoside	+*	-
6	Hyperoside	+*	-
7	Narcissin (3-O-rutinoside isorhamnetin)*	+	+
8	Isoquercetin (3-O-β-D-glucopyr-noside)*	+	+
9	Isorhamnetin (3,5,7,4'-tetrahydroxy-3'-methoxyflavonol)	-	+
0	Isorhamnetin 3-glucoside	-	+
1	Isorhamnetin 3-β-glucopyranoside (3,5,7,4'-tetrahydroxy-3'-methoxyflavonol)*	-	+
2	Isorhamnetin 3-β-D-glucopyranoside *	+	+
3	Quercetin-3-β-D-glucopyranoside (3,5,7,3′,4′-pentahydroxyflavone)*	-	+
1	Myricetin - 3-rutinoside	-	-

*- first discovered substances.

More than 4,500 species of plants grow on the territory of the Republic of Uzbekistan, 1,000 of which have medicinal properties, and at present, more than 83 species of medicinal plants are allowed to be used for medical purposes.

Today, the medicinal plant calendula officinalis is used in folk and scientific medicine in the form of infusions and decoctions, as well as tinctures.

At the first stage of standardization of dosage forms of plant origin, studies are carried out to ascertain biologically active substances that determine the therapeutic effect.

Literature data and the results of preliminary studies have shown that among the biologically active substances that determine the therapeutic effect of calendula officinalis tincture, one of the leading groups are flavonoids. Therefore, it was expedient to determine the authenticity and quantitative analysis of DF by flavonoids.

Based on the data presented, classical qualitative reactions were used to determine the presence of flavonoids. At the same time, the reactions of interaction of flavonoids with solutions of alkali, ammonia, aluminum chloride, iron (III) chloride, etc. were tested. However, the use of qualitative reactions does not allow us to conclude that all components are present.

Therefore, when developing the "Authenticity" section, the possibility of using spectrophotometry and chromatography was studied.

Based on chromatographic studies of tincture of calendula officinalis, a method for determining their authenticity in a thin layer of silica gel (TLC) is proposed. Chromatography is carried out on plates "Silufol UV-254" (Czech Republic) in solvent systems: n-butanol - acetic acid - water - 4:1:5. Under these conditions, the main adsorption zones appear on the chromatograms, by which it is possible to identify not only the authenticity of drugs, but also their qualitative composition.

To determine the authenticity of each DF during the quality control of its production, the most appropriate methods of analysis were recommended.

In order to determine the authenticity of the object under study, we have developed and included in the TPA the following methods of qualitative analysis.

Methods for determining the authenticity of calendula tincture. To 1 ml of the preparation 1 ml of concentrated hydrochloric acid and 0.1 g of magnesium cuttings are added. The mixture is heated; a red color appears.

10 ml of the drug is evaporated in a porcelain cup in a water bath. 2 drops of concentrated sulfuric acid are added to the residue, a red-violet color appears.

2. 0.015 ml of calendula tincture is applied to a “Silufol UV 254” plate (15x15 cm). The plate with the applied sample is dried in air, placed in a chamber with a mixture of solvents n-butanol - acetic acid - water - 4:1:5 and chromatographed in an ascending manner. When developing with ammonia vapor or spraying with a solution of aluminum chloride, yellow spots should appear.

To standardize the tincture due to informativeness, expressiveness and reliability to stepwise quality control, methods of qualitative and quantitative analysis using the HPLC method have been developed.

It is known that the separation of flavonoids is carried out, as a rule, on reversed phases by elution with various mixtures, in particular acetonitrile-water or methanol-water with a small content of phosphoric acid. These mobile phases are convenient for separating complex mixtures of both flavonoids and their glycosides under isocratic and gradient elution conditions and allow the use of UV detectors. Since the acidity constants of flavonoids pK_a correspond to the range of 8.6 ± 0.5, therefore, at $pH < pK_a$, all compounds of the class under study exist in solutions only in the OH form. The fulfillment of this condition ensures the necessary standardization of the UV spectra and, as a consequence, obtaining sufficiently reproducible results.

It is known that flavonoid solutions have intense absorption in the UV region of the spectrum with a maximum at 320-380 nm (first absorption band) and 240-280 nm (second absorption band). Wavelengths of 254 and 220 nm are fairly common standards for UV detection, and the value of 360 is consistent with the absorption maxima characteristic of flavonoids in the long wavelength region (362±14 nm). We found that the UV absorption spectrum taken for the study of objects has a maximum at 370 ± 5 nm. A similar absorption spectrum has a solution of quercetin, the most accessible and known as SSS. Therefore, in the future, alcohol-water solutions of quercetin with known optical absorption spectra were used as a standard for research. Optical absorption spectra of quercetin (C_{15} $H_{10}O_7$ $2•H_2O$) with absorption maxima in the wavelength range of 220–270 and 350–390 nm were recorded on an Agilent Technologies (USA) brand 8453 spectrophotometer.

Chromatography was performed on an Agilent Technologies (USA) liquid chromatograph (Agilent 1100 series) with “Chemstation 09.03.a” software, an isocratic pump, and a spectrophotometric detector. Separation was carried out on Zorbax columns. Detection was carried out at a wavelength of 370 nm, which is the characteristic λ_{max} of quercetin. The flow rate of the eluent was 1 ml/min, the volume of the injected sample was 20 µl. Chromatography temperature - 20^0C.

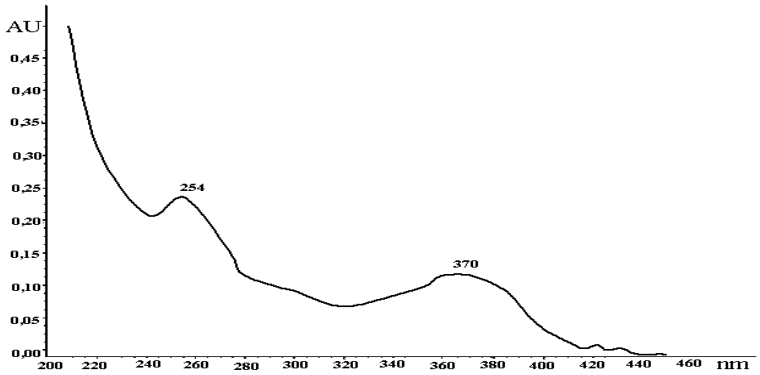


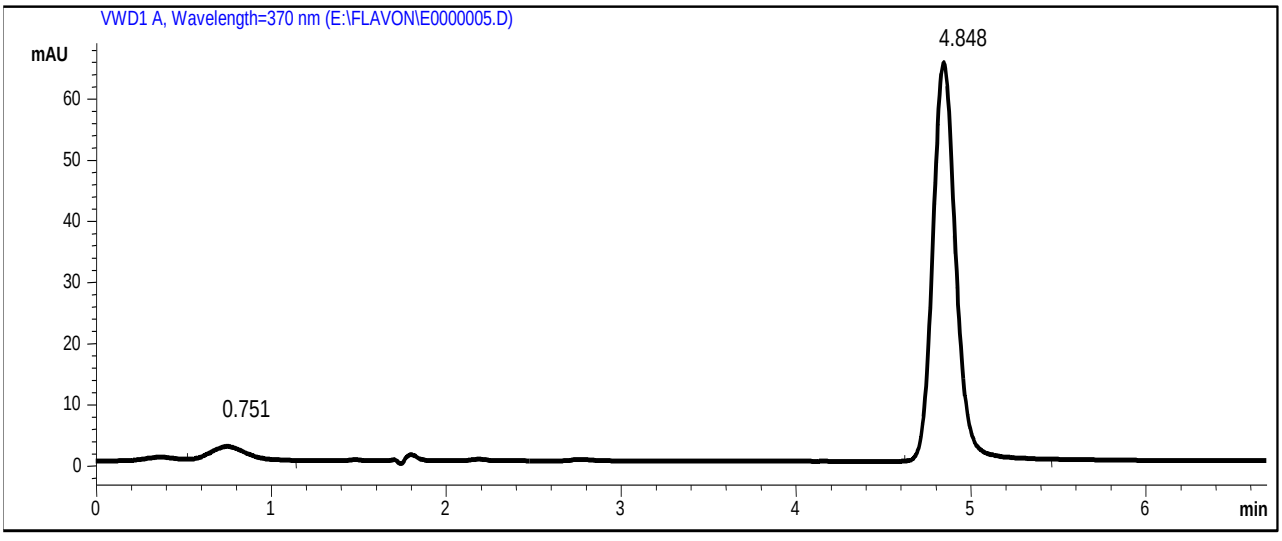
Fig. 3. UV absorption spectrum of the quercetin Standard sample solution

The suitability of the chromatographic system, where reproducible results are obtained, was checked by the number of theoretical plates, the degree of separation of the peaks and the relative standard deviation.

The developed HPLC method was tested on raw materials of local origin and industrial samples of calendula tincture.

To obtain reliable results of the analysis, the preparation of a sample from the raw material for HPLC was carried out as follows: about 5 g (accurately weighed) of calendula flowers were placed in a round-bottom flask with a 100-mL section, and a 70% ethanol solution was added in a ratio of 1:10. The flask is attached to a reflux condenser and heated in a boiling water bath for 40 minutes, after which the mixture is cooled to room temperature. The alcohol extract is filtered through a paper filter into a 100 ml volumetric flask. Extraction is carried out again. The second extract obtained is also filtered into the same flask as the first. Then the raw material is washed with 10 ml of 70% ethanol, filtered into the same volumetric flask. The volume of liquid in the flask was adjusted to the mark with 70% ethanol and mixed thoroughly. The resulting extract of 20 µl is injected into the liquid chromatograph injector. Separation of flavonoids was carried out on a Zorbax column 3×150 mm in size filled with Eclipse XDBC-8 sorbent with a particle size of 5 µm. Detection was carried out at λ=370 nm characteristic of quercetin. The eluent flow rate was 1 ml/min, the chromatography temperature was -20^{0}C.

Under similar conditions, calendula tincture and quercetin SS solution were subjected to chromatography in parallel. Quercetin was identified by comparing the retention times of the peaks in the chromatograms of the studied samples and its SSS (Fig. 4 and Table 4).



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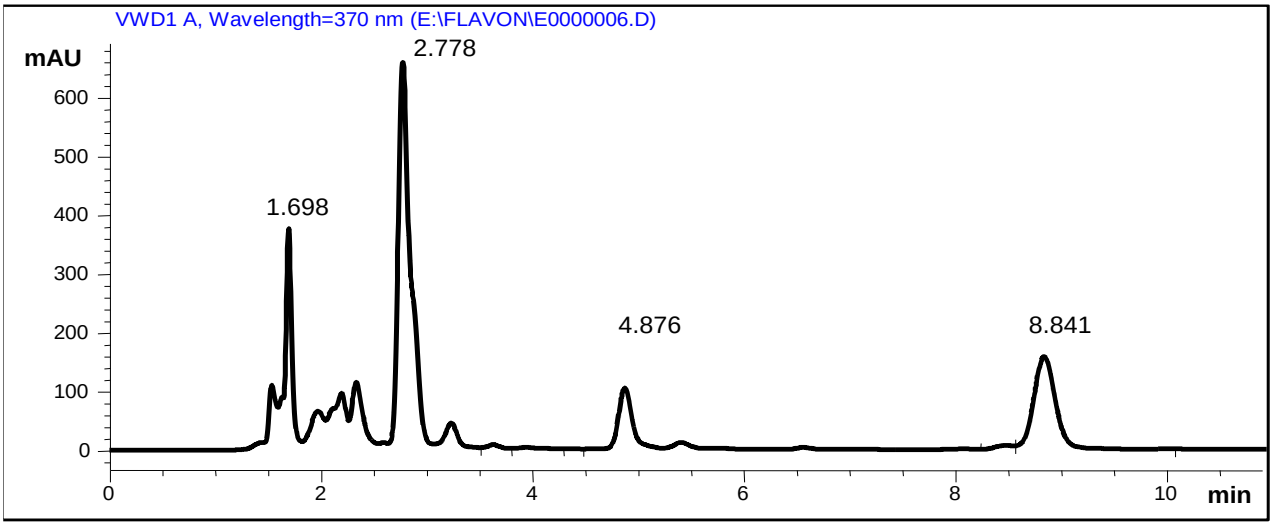

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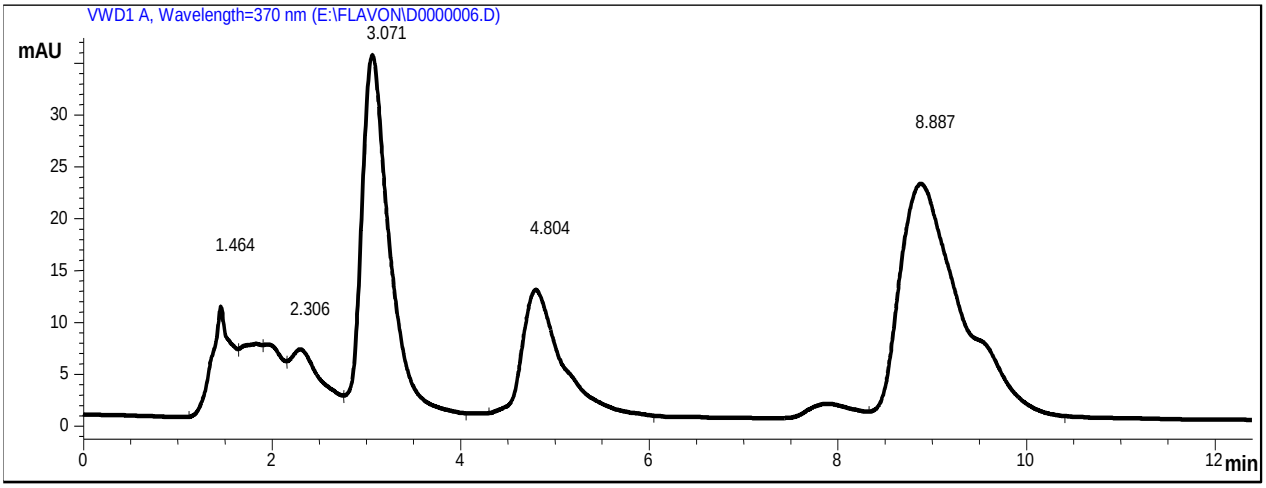

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Fig. 4. Chromatogram of a quercetin SSS (1), an extract obtained from vegetable raw materials (2) and calendula tincture (3)

As can be seen from the presented chromatograms, peaks with a retention time of 4.804 correspond to quercetin.

The content of quercetin (X) in raw materials in terms of air-dry raw materials as a percentage was calculated by the formula:

$$X = \frac{S \cdot V_1 \cdot a_{ñò.} \cdot 100 \cdot 100}{S_{ñò.} \cdot a \cdot V_2 \cdot (100 - W)} \quad ,$$

where, S, S_{st} are the areas of quercetin peaks on the chromatograms of the test sample and the quercetin SS solution, mAu*sec; V_1 is the volume of quercetin SSS, ml; V_2 is the volume of dilution of the test sample, ml; a, a_{st} – weighed portions of the studied sample and SSS of quercetin, g; W- loss in mass during drying of raw materials, %.

The content of quercetin in the tincture (X, %) was calculated from the area of the peaks by the following formula:

$$X = \frac{S \cdot V_1 \cdot a_{ñò.} \cdot 100}{S_{ñò.} \cdot a \cdot V_2} \quad ,$$

where, S, S_{st} are the areas of the quercetin peaks of the tested sample and quercetin SSS, mAu*sec; V_1 is the volume of quercetin SSS, ml; V_2 is the volume of dilution of the tested sample, ml; a, a_{st} – weighed portions of the studied sample and quercetin SSS, g;

Table 4

The results of the quantitative determination of quercetin in raw materials and calendula tincture by HPLC (at f=4; t=2.78; P=95%)

№	Sample name	$\overline{X}$,%	Metrological characteristics of the technique			
			S^2	S	ΔX	$\pm\overline{\varepsilon}$,%
1	Calendula flowers	0.0146	0.00000016	0.000400	0.000509	3.48
2	Tincture of calendula	0.0028	0.000000013	0.000114	0.000142	4.99

Where $\overline{X}$ is the average content of quercetin.

The results of the studies in Table 5 show that the content of quercetin in flowers and tincture of calendula is on average 0.0146 and 0.0028%, respectively, and the relative error of the average result of the proposed method reaches up to ±4.99%.

A unified method for the qualitative and quantitative analysis of raw materials and calendula tincture using HPLC has been developed. This technique allows you to simultaneously judge the authenticity, purity and, ultimately, the quantitative content of active substances. The HPLC technique can be used at the stage of research and study in the development of flavonoid-containing drugs, as well as in quality control at the stage of their production.

Conclusion

Thus, as a result of the research:

1. On the basis of a comprehensive study of the flavonoid composition of domestic plant raw materials of the flora of Uzbekistan, it was ascertained that calendula does not differ significantly from its analogues growing abroad in terms of the number and content of flavonoids. A comparative study of the qualitative composition of this plant made it possible to identify a variety of substances of a flavonoid nature. The results of the study of the flavonoid composition served as the basis for the development of methods for quality control and standardization (according to the content of the total flavonoids) of the DF obtained from them and the corresponding regulatory documentation.

2. As a result of experimental data, methodological approaches have been developed for the “end-to-end” standardization of raw materials and dosage forms derived from it - tincture of calendula officinalis flowers in terms of the content of the flavonoids amount. The developed HPLC technique will allow an objective assessment of their quality. Methods for qualitative and quantitative analysis of calendula tincture have been introduced into the practice of control and analytical laboratories of phytochemical industries.

3. The possibility of using the technique for step-by-step quality control of phytopreparations containing flavonoids has been shown.

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