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The development of new approaches to standardization of *Cassia acutifolia* leaves.

Vladimir A. Kurkin and Anna A. Shmygareva

Abstract
There were substantiated the expediency of the using for the determination of identity of *Cassia acutifolia* Del. leaves of spectrophotometry and TLC. As the results of the chemical study of *Cassia acutifolia* leaves there were isolated the predominant components of this plant - 1,7-dihydroxy-3-carboxyanhraquinone, 8-O-β-D-glucopyranoside of torachrysone, kaempferol-3-O-gentiobioside and on this basis the methodological approaches for standardization of *Cassia acutifolia* leaves were developed. These methodological approaches consist in the determination not only of anthracene derivatives, but also other diagnostic phenolic compounds, including flavonoids and naphthalenes. There was developed the method of quantitative determination of the total anthracene derivatives by means of spectrophotometry at analytical wavelength 530 nm. The contents of the total anthracene derivatives in *Cassia acutifolia* leaves are varied with 1,21% to 1,88% (calculated on sennoside B).

Keywords: *Cassia acutifolia* Del., leaves anthracene derivatives, flavonoids, *naphthalenes, standardization.*

1. Introduction
One of the most widely used groups of drugs used in the correction of functional disorders of the digestive system, are laxatives [1]. In medical practice, widely used medicines on the basis of plant raw materials, containing anthracene derivatives [2-4], and the most popular source are two species of *Cassia* (*Senna*) - *Senna* leaves (*Cassia acutifolia* Del.)., or Alexandrian Senna (*Senna alexandrina* Mill.) and *Cassia angustifolia* Vahl.) (*Fabaceae*).
One of the unsolved full of problems from the point of view of pharmacopoeial analysis is the standardization of *Cassia* leaves. So, pharmacopoeial monograph on the leaves of *Cassia* (USSR SPh, XI Ed.) includes the section "Quality reactions", but it does not provide for the application of thin-layer chromatography (TLC) [5]. In this respect there is a foreign experience of using TLC, but in our opinion it cannot be considered successful. In fact, in this case to determine the authenticity of the raw materials used as a standardized extract of *Senna*, which does not allow the interpretation of the results from the point of view of specific values for R_f of diagnostic compounds.
The method of quantitative determination of the total anthracene derivatives in the leaves of *Cassia* [5], included in the section "Quantitative determination" of monograph on this plant material, also has several disadvantages: it is a cumbersome, multi-stage and unsafe, including such stages as acid hydrolysis, repeated extraction of raw materials, processing diethyl ether - boiling flammable solvent. In addition, the methodology used spectrophotometry providing for the measurement of absorbance at the analytical wavelength at 523 nm, and calculation of the content of total anthracene derivatives is carried out using the building cumbersome calibration graphic of a solution of cobalt chloride in terms of chrysophanol. Methods of quantitative determination of anthracene derivatives included in foreign pharmacopoeias, from the point of view of the sample preparation is comparable to the global Fund of the USSR XI edition and differ only in the value used analytical wavelength (515 nm instead of 523 nm) and approach the calculation of the content on sennoside B [6-7]. The feasibility of the introduction of new modern methods of analysis associated with the fact that existing approaches to the analysis does not fully use all the diversity of the chemical composition of raw Cassia [8-11].
In our opinion, is actually also taking into account the importance of flavonoids [9], mainly derivatives of kaempferol on both the content and the possible contribution to the pharmacological action of preparations on the basis of *Cassia* leaves.

In our opinion, is completely unjustified ignoring both the content and the possible contribution to the pharmacological effects of flavonoids, mainly kaempferol derivatives [9]. In addition, it is possible contribution to the purgative effect of drugs Cassia and derivatives of naphthalene, in particular, 8-O-β-D-glucopyranoside of torachrysone.

The aim of our study is the development of the methodical and methodological approaches to standardization of *Senna* leaves.

2. Materials and Methods

2.1. Extraction of Plant Materials

The objects of study were industrial designs of *Senna* leaves (OAO "Krasnogorskleksredstva", OOO Phytostart", OOO "ST-Medipharm", OOO "Altai kedr"). Preparative isolation of compounds there was carried out from the leaves of production of OAO "Krasnogorskleksredstva" using column chromatography.

Air-dry raw material (100 g) was subjected to exhaustive extraction with 70% ethanol, combining the method of maceration (24 h) followed by extraction at a temperature of 85-90 ^{O}C. Water-alcoholic extracts was evaporated under vacuum to a thick residue (about 30 ml).

2.2. Isolation of Compounds from Plant Materials

The condensed extract was dried on silica gel L 40/100 and the obtained powder (extract + silica gel) was applied to a layer of silica gel formed in chloroform. The chromatographic column was eluated with chloroform and the mixtures of chloroform-ethanol in different ratios (99:1; 97:2; 97:3; 95:5; 93:7; 90:10; 85:15; 80:20; 70:30, 60:40, 50:50). Control over the separation of substances was performed using TLC-analysis of the plates "Sorbfil PTLC-AF-A-UV" in the systems: chloroform-ethanol (9:1), chloroform-ethanol-water (26:16:3), and n-butanol-glacial acetic acid-water (4:1:2, v/v). Fractions containing compounds **1-3**, with a view to their allocation was subjected to rechromatography on a column of silica gel L 40/100 using the mixtures of chloroform-ethanol in a gradient mode. Final purification of the compounds **3** was carried out using the rechromatography on the column of polyamide "Woelm (Germany), eluated with mixtures of water and ethanol in different ratios, and then aqueous sodium hydrogen carbonate solution, followed by precipitation of the target compounds from the eluate by acidification with 10% solution of HCl to pH about 6.0.

2.3. Methods of Structural Elucidation and Analysis of Plant Materials

^{1}H-NMR spectra were measured on spectrometer "Bruker AM 300" (300 MHz), mass spectra were determined on a mass spectrometer "Kratos MS-30", the recording the UV spectra were performed using a spectrophotometer "Specord 40" (Analytik Jena).

During the development of approaches to the standardization of the total of anthracene derivatives in the *Cassia* leaves there were studied the UV spectra of aqueous-alcoholic extracts of the studied samples of plant materials. For the development of method of qualitative analysis of *Cassia* leaves were used TLC ("Sorbfil PTLC-AF-A-UV"; solvent system: chloroform-ethanol-water, 26:16:3 (v/v). The detection of compounds on the chromatograms there were carried out in ultraviolet light at the wavelength of 254 and 366 nm, and after developing an alkaline solution of diazobenzenesulfonic acid.

3. Results and Discussion

In the course of the study of the chemical composition of *Cassia* leaves there were isolated three dominant compounds (Fig. 1), namely 8-O-β-D-glucopyranoside of torachrysone (**1**), kaempferol-3-O-gentiobioside (**2**) and 1,7-dihydroxy-3-carboxyanthraquinone (**3**).

3.1. Physical-chemical and Spectral Characteristics of Isolated Compounds

8-O-β-D-glucopyranoside of torachrysone

(1,8-dihydroxy-6-methoxy-2-acetyl-3-methylnaphthalene) (**1**). Amorphous light yellow substance, molecular formula: $C_{20}H_{24}O_{10}$. MS-EI (70 eV, 200 ^{O}C, m/z, %): M^{+} 408 (18%), M^{+} of aglycone 246 (82%), M+ aglycone-CH3 231 (100%), 217 (28%). UV spectrum (EtOH, λmax, nm): 241, 266sh, 332sh, 344. ^{1}H NMR spectrum (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 9.58 (1H, s, OH-group at C-1), 7.06 (1H, s, H-4), 6.92 (1H, d, J = 2, H-7), 6.70 (1H, d, J = 2, H-5), 5.03 (1H, d, J = 7, H-1^{1} of glucopyranose), 3.1-4.5 (6H of glucopyranose), 4.30 (3H, s, aromatic OCH_3 group at C-6), 2.53 (3H, s, SON at C-2), 2.25 (3H, s, aromatic CH_3 at C-3).

Kaempferol-3-O-gentiobioside

(3-O-gentiobioside of 3,5,7,4^{1}-tetrahydroxyflavone) (**2**). Yellow crystals, molecular formula: $C_{27}H_{30}O_{16}$, m.p. 174-177 ^{O}C (water-alcohol). MS-EI (70 eV, 200 ^{O}C, m/z, %): 286 (M+ of aglycone, 100%), 153 (15), 121 (22). UV spectrum (EtOH, λmax, nm): 269, 350 nm; + NaOAc 274, 368 nm; + NaOAc + H_3BO_3 272, 355 nm; + A1C1$_3$ and + A1C1$_3$ + HCl 275, 305, 395 nm. ^{1}H-NMR spectrum (300 MHz, DMSO-d6, δ, ppm, J/Hz): 12.58 (s, 1H, 5-OH), 8,03 (d, J = 9 Hz, 2H, H-2^{1},6^{1}), 6.87 (2H d, J = 9 Hz, 2H, H-3^{1},5^{1}), 6.42 (1H, d, J = 2.5 Hz, H-8), 6.20 (1H d, J = 2.5 Hz, H-6), 5.35 (1H, d, J = 7 Hz, H-1^{11} of glucose), 4.03 (1H, d, J = 7 Hz, H-1^{111} of glucose), 3.10-5.2 (m, 12H of glucose).

1,7-dihydroxy-3-carboxyanthraquinonee (**3**).

The orange crystals, molecular formula: $C_{15}H_8O_6$, MS-EI (70 eV, 200 ^{O}C, m/z, %): M+ 284 (100%), 256 (7), 254 (26), 239 (32), m.p. 305-308 ^{O}C (water). UV spectrum (EtOH, λmax, nm): 234, 264, 433 nm. ^{1}H NMR spectrum (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 11.91 (1H, s, OH-group at C-1), 8.16 (1H, br. s, H-2), 7.85 (1H, d, J = 9 Hz, H-6),7.80 (1H, br. s, H-4), 7.78 (1H, d, J = 1 Hz, H-8), 7.42 (1H, d, J = 9 Hz, H-5).

3.2. Structural Elucidation of Compounds and Analysis of Plant Materials

Using TLC there was detected (Fig. 1), that compounds **1-3** are the dominant components of *Senna* leaves. In our opinion, the detection by TLC of compounds **1-3**, diagnostically significant to *Senna* leaves, is a perspective approach for the identification of raw materials and preparations of this plant. This is especially important, due to the low content of sennoside A and B in the leaves of *Cassia* [9], the determination of these compounds by TLC is problematic. This finding is consistent with research results of foreign scientists [10], according to which dominant anthracene derivative compound of *Cassia* leaves is rhein (**4**). This draws attention to the fact that, according to our data, the dominant anthracene compound of *Cassia* leaves is not rhein (**4**), and 1,7-dihydroxy-3-carboxyanthraquinone (**3**), which is similar on chromatographic mobility, physical-chemical and spectral

characteristics to rhein. This conclusion is based on the data of the ^{1}H-NMR spectrum 1,7-dihydroxy-3-carboxyanthraquinone (**3**): presence of 11.91 ppm of one broadened singlet signal belonging to the 1-OH group, in combination with the nature of the signals of aromatic protons at C-5, C-6 and C-8. According to the literature data in the NMR spectrum of rhein are observed two characteristic singlet signal in the area of 12.0 ppm and 11.9 ppm 1-OH-group and 8-OH-group [12].

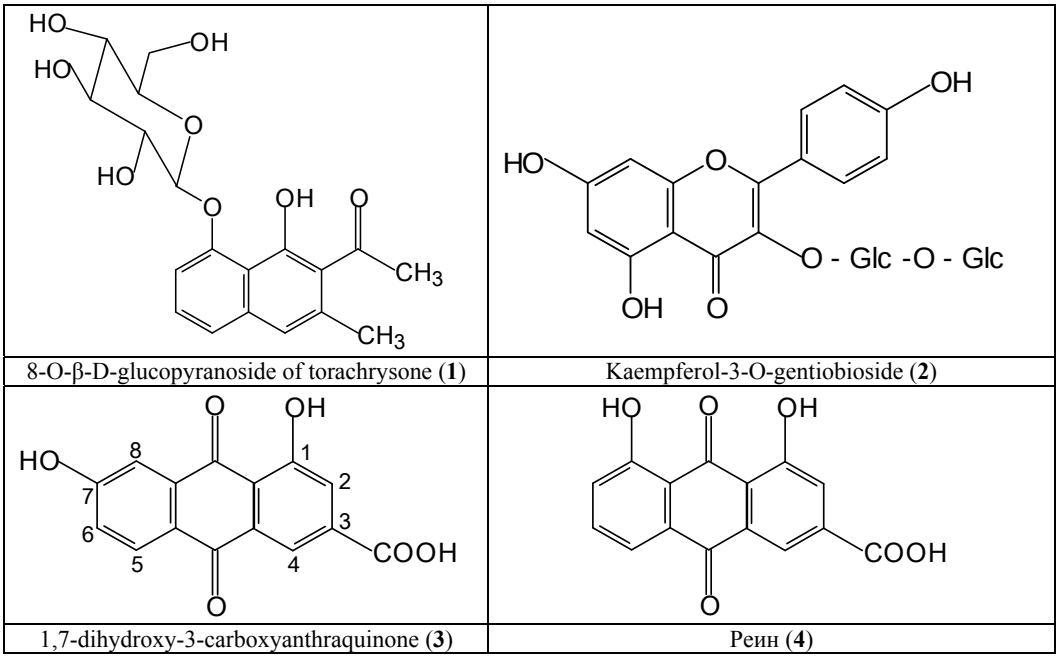


Fig 1: The compounds of water-alcoholic extraction from *Cassia acutifolia* leaves.

The study of UV spectra of water-alcohol extraction from *Cassia acutifolia* leaves was showed (Fig. 1) that the characteristic parameters are the presence of maximum absorption at 270±2 nm and 360±2 nm. These characteristics according to the flavonoids, namely kaempferol-3-O-gentiobioside (**2**), which is predominant compound of water-alcohol extraction from *Cassia acutifolia* leaves (Fig. 2).

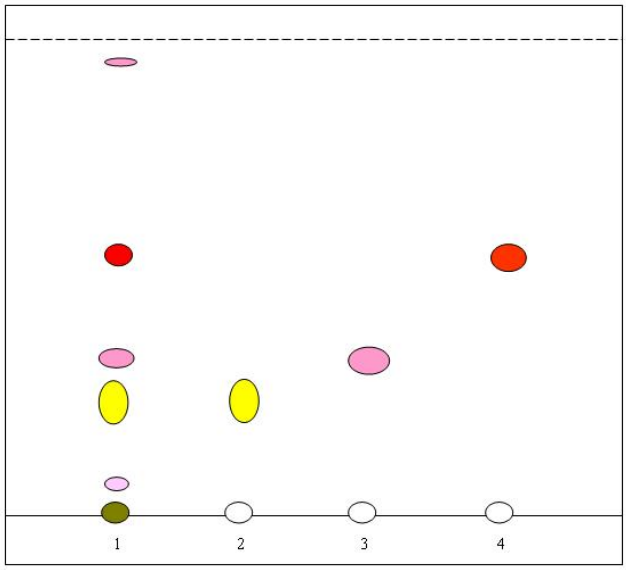


Fig 2: Chromatographic profile of a water-alcohol extract of *Cassia acutifolia* leaves;1 - water-alcoholic extract of *Cassia acutifolia* leaves; 2 - kaempferol-3-O-gentiobiose; 3 - 1,7-dihydroxy-3-carboxyanthraquinone; 4 - torachrysone-8-O-glucoside.

The predominant phenolic components are also 8-O-β-D-glucopyranoside of torachrysone (**1**) and kaempferol-3-O-gentiobiose (**2**) (Fig. 2), which, in our opinion, have diagnostic value for the identification of *Cassia* leaves. It is interesting that, according to foreign scientists, and in conditions of HPLC kaempferol-3-O-gentiobiose (**2**) is the dominant compound [9].

Great interest from the point of view of standardization is the fact that the curve of the electronic absorption spectrum of aqueous-alcoholic extract of the leaves *Cassia acutifolia* (λmax 270 nm and 350 nm) is mainly determined by the flavonoids, in particular, 3-O-gentiobiose kaempferol (Fig. 3).

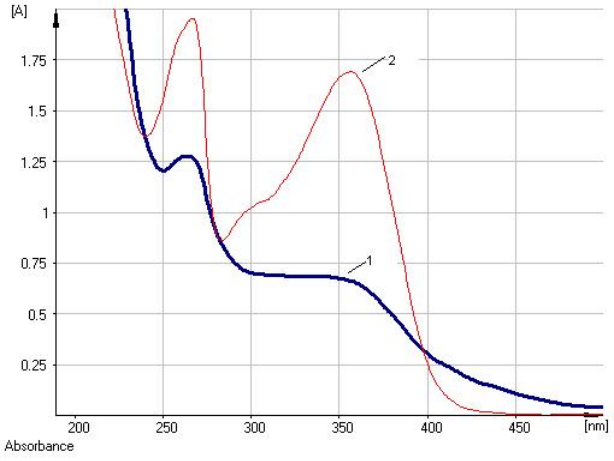


Fig 3: UV spectra of aqueous-alcoholic extract from the leaves of *Cassia acutifolia*: 1 - aqueous-alcoholic extract from the leaves of *Cassia acutifolia*; 2 - 3-O-gentiobiose of kaempferol.

Diagnostic value has also the curve of the absorption of alkali-ammoniac solution of aqueous-alcoholic extract from the leaves of *Cassia*: absorption maximum at 430 nm ± 3 nm (anthracene derivatives) (Fig. 4).

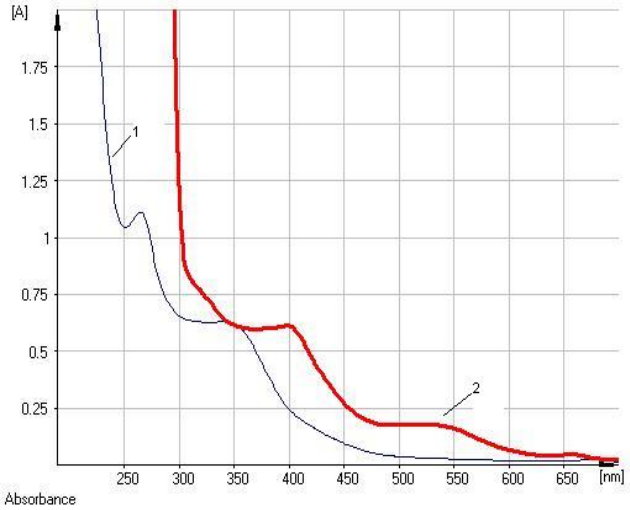
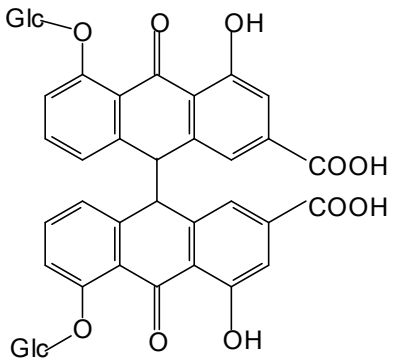


Fig 4: UV spectra of initial solution (1) and an alkali-ammoniac solution of aqueous-alcoholic extract from the leaves of *Cassia acutifolia* (2).

3.3. Method of quantitative determination of the total anthracene derivatives in the leaves of *Cassia acutifolia*

With the aim of developing methods of quantitative determination of the total anthracene derivatives the optimal conditions for extraction of anthracene derivatives from the leaves of *Cassia* there was studied: extractant - 70% ethanol; the ratio of the raw material-extractant - 1:30; extraction time in a boiling water bath for 60 min (table 1). As the analytical wavelength recommended value 530 nm, although at the present time for the purpose of standardization is used, and other values of the wavelength [5-8].

In accordance with the European Pharmacopoeia for the calculation of the content of total anthracene derivatives it is advisable to use the theoretical value of theoretical value of the specific absorption index (240) for standard sample of sennoside B [6].



Sennoside B

Table 1: The dependence of the degree of extraction of anthracene derivatives from the leaves of *Cassia acutifolia* on various factors

Extractant	The ratio of raw material: extractant	Extraction time, min	Contents of total anthracene derivatives calculated on Sennoside B and absolutely dry raw material (in %)
40% ethanol	1:30	60	1,49± 0,02
50% ethanol	1:30	60	1,50± 0,02
60% ethanol	1:30	60	1,78± 0,03
60% ethanol	1:50	60	1,86± 0,04
70% ethanol	1:30	30	1,52± 0,02
70% ethanol	1:30	45	1,62± 0,03
70% ethanol	1:30	60	1,87± 0,02
70% ethanol	1:30	90	1,81± 0,02
70% ethanol	1:30	120	1,48± 0,02
70% ethanol	1:50	60	1,88± 0,03
70% ethanol	1:100	60	1,87± 0,04
80% ethanol	1:30	60	1,70 ± 0,03
95% ethanol	1:30	60	1,44± 0,02

3.4 Method of quantitative determination of the total anthracene derivatives in the leaves of *Cassia acutifolia*

An analytical sample of the raw material is crushed to the size of the particles with a diameter of 1 mm. About 1 g of the crushed material (the exact sample) is placed in a flask with a volume of 100 ml, add 30 ml of 70% ethanol. Stoppered flask and weighed on a calibrated scale accurate to ±0.01 g. The flask is attached to the back of the fridge and heated in a boiling water bath (moderate boiling) for 60 min and then cooled for 30 min, cover the same tube, weighed again and fill in the missing extractant to the initial mass, and then removing the filter.

The test solution is prepared as follows: 1 ml of extract obtained is placed in a flask with a volume of 25 ml and the volume was adjusted solution to the mark alkali-ammoniac solution, prepared according to the pharmacopoeial method [5]. The test solution is placed in a flask with a volume of 25 ml and heated for 15 min in a boiling water bath under reflux. After cooling measure the optical density of the test solution on the spectrophotometer at a wavelength of 530 nm. As the reference solution there was using purified water.

The content of total anthracene derivatives in the leaves of *Senna* calculated on sennoside B in and absolutely dry raw materials in percentage (X) are carried out according to the formula:

$$X = \frac{A * 30 * 25 * 100}{m * 240 * (100 - W)}$$

A - absorbance of the test solution;
m - the mass of raw material, g;
W - loss of mass on drying in percentage;
240 - specific absorption of Sennoside B.

Metrological characteristics of the methodology of quantitative measurement of the total anthracene derivatives in the leaves of *Senna* presented in table 2. The relative degree of the determination of the total anthracene derivatives in developed method with confidence probability 0,95 is no more than ±3,27% (table. 2).

Table 2: Metrological characteristics of the methods of quantitative determination of the total anthracene derivatives in *Cassia acutifolia* leaves

f	$\overline{X}$	*S*	*P, %*	*t (P,f)*	*ΔX*	*E, %*
10	1,88	0,0276	95	2,23	±0,061	±3,27

Using the developed methods we analyzed a number of sample practical (table 3) and determined that the contents of the total anthracene derivatives varies from 1.21% to 1.88%.

Table 3: The content of total anthracene derivatives in various samples of *Cassia acutifolia* leaves

No.	**Characteristics of the sample of herbal materials**	**Contents of total anthracene derivatives calculated on Sennoside B and absolutely dry raw material (in %)**
1.	*Cassia* leaves (filter package) (OAO "Krasnogorskleksredstva")	1,56± 0,02
2.	*Cassia* leaves OAO "Krasnogorskleksredstva"	1,88± 0,02
3.	*Cassia* leaves (OOO "ST-Medipharm")	1,86± 0,03
4.	*Cassia* leaves (OOO "Fitostart")	1,21± 0,02
5.	*Cassia* leaves (OOO "Altai kedr")	1,40± 0,03

Thus, as results of the phytochemical investigations of *Cassia acutifolia* leaves there were developed new approaches to standardization, consisting in the determination of anthracene derivatives (1,7-dihydroxy-3-carboxyanthraquinone), flavonoids (kaempferol-3-O-gentiobioside) and derivatives of naphthalene (8-O-β-D-glucopyranoside of torachysone) using TLC and spectrophotometry.

4. Conclusions

From the leaves of *Cassia acutifolia* there were isolated the dominant components of this plant - 1,7-dihydroxy-3-carboxyanthraquinone (anthracene derivatives), 8-O-β-D-glucopyranoside of torachysone (naphthalene derivatives) and kaempferol-3-O-gentiobiose (flavonoid), which has diagnostic value. Methodological approaches to standardization of leaves of *Cassia acutifolia*, consisting in the determination of anthracene derivatives, flavonoids and derivatives of naphthalene using the method of thin-layer chromatography and spectrophotometry. The method of quantitative determination of the total anthracene derivatives using the analytical spectrophotometry at a wavelength of 530 nm. The content of total anthracene derivatives in samples of raw materials varies from 1.21% to 1.88% (calculated on sennoside B).

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