Original Article

Comparison of Spectrophotometric Methods of Total Flavonoid Assay Based on Complex Formation with Aluminum Chloride as Applied to Multicomponent Herbal Drug Angionorm

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Abstract

Objective: The objective was to compare the complex formation of flavones (luteolin, apigenin), flavonols (morin, quercetin, and rutin), flavanones (naringenin), flavanonols (dihydroquercetin), caffeic, and ferulic acids with aluminum ion using two spectrophotometric methods and the application of these methods to estimate the flavonoid content in the angionorm herbal product. **Materials and Methods:** Method 1 included direct complex formation with aluminum chloride, and Method 2 involved preliminary nitrozation and subsequent complex formation of nitroso-derivatives with aluminum chloride. 2,4-dinitrophenylhydrazine method was also tested. **Results:** The conjugation system increase in the chromophore structure of nitroso derivatives is reflected by the hyperchromic effect of all substances (from 1.4 to 4-fold). The largest bathochromic shift of nitroso derivatives was seen for rutin, luteolin, and dihydroquercetin (to 510–530 nm). The total flavonoids estimation in the extract of the four herbs mixture, which constitute angionorm preparation active ingredient, depends on the chosen reference substance (3.49% calculated as luteolin when measured by Method 1 or 8.71% by Method 2; 7.97% calculated as dihydroquercetin when measured by Method 1 and 4.14% by Method 2. **Conclusion:** The complex formation according to Methods 1 and 2 does not allow the identification of the selective spectral regions typical for the certain flavonoids subgroups and hydroxycinnamic acids. Neither method of complex formation is suitable for the assay of total flavonoids in unknown samples or for the flavonoid content comparison in the different herbal material.

Keywords: Flavanones, flavanonols, flavones, flavonols, nitrosation

Introduction

Flavonoids are one of the most widespread families of natural compounds, which are present in different herbal raw materials and determine the biological activity of many herbal products.[1] Quantitative characterization of the individual components in herbal raw material and herbal products, especially in multicomponent ones, is hampered by complicated composition of the natural matrix. The High-Performance Liquid Chromatography (HPLC) method allows qualitative identification of a flavonoid composition, but quantification is frequently hampered by the poor peak separation-related error. Besides, it requires a number of standard samples and is lengthy, which is not always expedient. A widespread approach to herbal material quantification is the spectrophotometric assay of total flavonoids using aluminum ion complex formation with the total content being expressed as some standard flavonoid (most often rutin, quercetin).[2] The methods of aluminum ion complexes differential spectrophotometry are simple, rapid, repeatable, and cost-effective. They are well-studied and described in multiple papers,[3,4] and included in some pharmacopeias, for example, EP 8.0 and Russian Pharmacopeia XIII.

At the same time, this approach does not allow the assessment of the different flavonoid subtypes contribution, for example., those containing conjugated or unconjugated B- and C-rings, which

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can be important due to different antioxidant properties.[5-8] Attempts were made to divide flavonoid family into flavones and flavonols or into flavans and flavanonols based on their different absorption after conjugation system modification by 2,4-dinitrophenylhydrazine derivatization[9,10] or by preliminary nitrozation and subsequent complex formation with aluminum chloride.[3,11] However, the application of these methods for flavonoid characterization in herbal raw materials and products remains uncertain. As continuation of these studies, the present work was aimed at clarification of the possibility to analyze the different flavonoid subtypes using spectrophotometric methods and to apply these methods to multicomponent phytoproducts characterization using angionorm as an example. The study objects were flavonoids with different types of hydroxy-substitution, and with single or double C2-C3 bond in C-ring, hydroxycinnamic acids (often present in herbal material together with flavonoids), and the dry extract of four herbs, which constitutes the active ingredient of angionorm drug.

Materials and Methods

Reagents

Aluminum chloride, sodium nitrite, sodium hydroxide (chemically pure grade, Khimmed, Russia), acetic acid (special purity grade), sulfuric acid (chemically pure grade, Reakhim, Russia), 2,4-dinitrophenylhydrazine (2,4-DNPH) (analytical grade, Reakhim, Russia) were used.

Solvents

Ethanol 95% (Merck, Germany), dimethylformamide (DMFA) (chemically pure grade, ECOS-1, Russia), and distilled water were used.

Standards

Naringenin, apigenin, luteolin, quercetin, dihydroquercetin, rutin, morin, ferulic acid, caffeic acid (all-Sigma, USA).

Dry extract of herbs mixture

Angionorm (dry extract (extracting solvent– 25% ethanol) of horse chestnut *Aesculi hippocastani* seeds (fruits), licorice (*Glycyrrhizae*) roots, hawthorn (*Crataegus*) fruits, dog rose (*Rosae*) fruits taken at 30:15:20:35 ratio (Research and Production Association PharmVILAR, batch 330416).

Equipment

UV-Vis spectrophotometer Cary 100 (Varian, Australia) was used.

Study solutions

A total of 10 mg of each substance were dissolved in 2 mL of DMFA, then 8 ml of ethanol was added (study solution). The study solution was diluted 50-fold, and the UV spectrum in the range of 250–500 nm was registered using ethanol as blank.

Nitrozation

A volume of 0.3 ml of 5% $NaNO_2$ aqueous solution was added to 0.1 ml of study solution, then 4.6 ml of ethanol was added, and the sample was mixed. UV spectrum was registered in the range of 250–500 nm 5 min after using ethanol as blank.

Complex formation with aluminum chloride (Method 1)

A volume of 5 µl of acetic acid, 0.2 ml of 10% $AlCl_3$ and 4.7 ml of ethanol was added to 0.1 ml of study solution. UV spectrum was registered in the range of 300–600 nm against the blank where the $AlCl_3$ solution was substituted with ethanol.

Nitrozation and complex formation with aluminum chloride (Method 2)

A volume of 0.3 ml of 5% $NaNO_2$ aqueous solution and 2.9 ml of ethanol were added to 0.1 ml of study solution. After 5 min, 5 µl of acetic acid and 0.2 ml of 10% $AlCl_3$ aqueous solution were added. Then, 1.5 ml of 1M NaOH was added. UV spectrum was registered in the range of 300–600 nm against the blank where the $AlCl_3$ solution was substituted with ethanol.

Samples of multicomponent herbal product angionorm were prepared as follows: 0.1 g of dry extract was dissolved in 10 ml of 25% ethanol using a magnetic stirrer for 30 min. The obtained solution was filtered through paper filter. 1 ml of the filtrate was analyzed according to above-described methods with dilution modifications. Method 1: 5 µl of acetic acid, 0.2 ml of 10% $AlCl_3$ aqueous solution, and 2.8 ml of water was added to 1 ml of the filtrate. Method 2: 0.3 ml of 5% $NaNO_2$ aqueous solution and 5 ml of water were added to 0.1 ml of study solution, after 5 min 5 µl of acetic acid and 0.2 ml of 10% $AlCl_3$ was added followed by 1.5 ml of 1M NaOH.

Total flavonoid content as dihydroquercetin or luteolin was calculated according to the following formula:

$$X = \frac{(10 \times V \times \mathrm{A})}{(m \times E_{1\mathrm{cm}}^{1\%})}$$

Where X– total flavonoid content as dihydroquercetin or luteolin, % of sample weight, V– resulting volume according to Methods 1 and 2 (4 or 8 ml respectively), A– absorbtion at 398 nm (Method 1) or 517 nm (Method 2) for luteolin, and 413 nm (Method 1) or 507 nm (Method 2) for dihydroquercetin, *m*– sample weight, g, $E_{1\mathrm{cm}}^{1\%}$ –dihydroquercetin specific absorbance = 23.1 at 413 nm (Method 1) and 134.5 at 507 nm (Method 2); or luteolin specific absorbance = 79.8 at 398 nm (Method 1) and 63.9 at 517 nm (Method 2).

Reaction with dinitrophenylhydrazine

A volume of 1 ml of 1% DNPH solution in 2% sulfuric acid was added to 1 ml of study solution diluted 10-fold with water; then, the sample was incubated for 30 min at 60°C, then 5 ml of 1% sodium hydroxide solution in ethanol was added and UV spectrum was registered in the range of 300–600 nm.

All results were obtained from three independent measurements (n = 3).

Results

Different flavonoid subtypes representatives were included in the study: flavones – luteolin, apigenin; flavonols – morin, quercetin and rutin (quercetin diglycoside); flavanones – naringenin; flavanonols – dihydroquercetin. Multicomponent drugs

frequently contain hydroxycinnamic acid derivatives together with flavonoids.[12-14] Therefore, caffeic acid and ferulic acids were also used as study substances. The compounds formulas are presented in Figure 1.

Maximal absorption wavelengths of the flavonoids and the hydroxycinnamic acids in 95% ethanol are summarized in Table 1. Flavonoids with conjugated B-and C-rings have an absorption maximum of the band I (according to classification by[15] at higher wavelengths (342–393 nm) as compared to substances with unconjugated B- and C-rings (329 nm). Absorption maximum position depends on the number of hydroxyl groups and on the hydroxylation type (e.g., pyrocatechol type for quercetin, rutin and luteolin, or resorcinol type for morin). For example, apigenin, which lacks C3-hydroxyl group and has one phenolic hydroxyl group in ring B, has absorption maximum of the band I at shorter wavelength

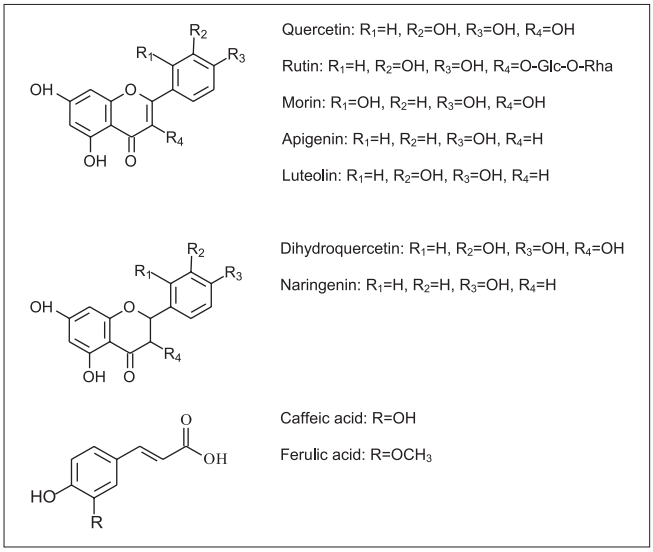

Figure 1: Structures of the studied compounds

as compared to flavonols. Caffeic and ferulic acids have chromophore structure similar to that of flavonoids (electron donating hydroxyl or alkoxyl group conjugated with an aromatic ring and double bond and electron withdrawing carboxylic group), though with a smaller conjugation system with shorter maximum absorption wavelength [Table 1].

Spectrophotometric methods of flavonoid assay in multicomponent matrix (herbal raw materials and products) are based on derivatization and complex formation with a shift of flavonoid absorption bands to longer wavelength region, where the majority of accompanying substances does not show intrinsic absorption.

Among the studied flavonoids, only naringenin (flavanone) and dihydroquercetin (flavanonol) reacted with DNPH (method according to[9,10] as demonstrated by bathochromic shift of the absorption band I wavelength from 330 nm to 490 nm.

The angionorm dry extract solution interaction with DNPH (method according to[9,10] resulted in alkaline-insoluble precipitate formation and insignificant absorption increase at 470–490 nm. This data indicate that it is not possible to control the completeness of DNPH reaction with multicomponent herbal extracts and the amount of soluble derivatives; therefore, the accuracy of the assay is impaired.

Two approaches are most commonly utilized for total flavonoids assay in herbal raw materials and preparations: direct complex formation with aluminum chloride or aluminum nitrate and preliminary nitrozation with subsequent complex formation with aluminum ion. UV spectra of flavonoid nitroso derivatives were obtained to compare both approaches applied to multicomponent herbal extract. Flavonoid nitrozation is performed rapidly in the conditions described by[16] due to the orientation effect of the side groups in rings A and C. At the same time; it is individual whether mono- or di-nitrozation occurs and as well as the position of nitrosyl group(s).

Table 1: Results of spectrophotometry of study substances' solutions before and after sodium nitrite addition

Substance	*c* (mmol/L)	λ_{max} (nm) (95% ethanol)	λ_{max}, nitroso-derivative (nm)	Shift λ_{max} (nm)	Increase of optical density at λ_{max} (fold)
Flavonoids with double C_2-C_3 bond					
Quercetin	0.0715	376	363	−13	1.63
Rutin	0.0334	368	357	−11	3.25
Morin	0.0768	393	364 405 (shoulder)	−29	1.40
Apigenin	0.0755	342	348	6	4.02
Luteolin	0.0727	353	354	1	2.94
Flavonoids with single C_2-C_3 bond					
Dihydroquercetin	0.0657	329	341	12	2.21
Naringenin	0.0735	328	341	13	2.50
Phenolic acids					
Caffeic acid	0.1177	317	317	0	1.39
Ferulic acid	0.1092	314	314	0	1.34
Extract of herbs mixture					
Angionorm extract	1.34 mg/mL	278	280 353	2 75	1.29 1.06

The results of study substances' solutions spectrophotometry before and after sodium nitrite addition are presented in Table 1.

Bathochromic shift of maximal absorption wavelengths after nitrozation was noted for flavonoids with unconjugated B-and C-rings (dihydroquercetin and naringenin). Flavonoids with double C2-C3 bond are characterized by hypsochromic shift of maximal absorption wavelengths (except for apigenin). Absorption band I in flavone and flavonol spectra is probably caused by intramolecular charge transition from donor phenolic hydroxyl group through conjugation system to electron withdrawing carbonyl group. Withdrawing nitrosyl group introduction into the molecule creates competition to this transfer.

The conjugation system increase in the nitroso derivatives' chromophore structure is reflected by the hyperchromic effect of all substances. Among flavonoids with double C2-C3 bond, apigenin (hydroxyl group in position 4′ of B-ring, no hydroxyl group in C3 position) and rutin (pyrocatechol type of hydroxyl groups in B-ring, glycosylated hydroxyl group in C3 position) exhibited the largest absorption increase after nitrozation: 4-fold and 3.25-fold, respectively. Morin (resorcin type of hydroxyl groups in 2′,4′ position of B-ring) nitrozation demonstrated the least increase of optical density (1.4-fold). Luteolin and quercetin nitrozation resulted in the 2.94-fold and 1.63-fold maximal absorption increase, respectively.

Nitrozation of dihydroquercetin and naringenin (unconjugated B-and C-rings) resulted in a small bathochromic shift and the 2.21-fold and 2.50-fold absorption increase, respectively. Nitrozation of caffeic and ferulic acids practically did not result in a maximal absorption wavelengths shift though the optical density increased 1.39-fold and 1.34-fold, respectively.

Complex formation of the studied substances with aluminum ion was performed according to,[3] and complex formation with preliminary nitrozation – according to.[3,17] The results are presented in Table 2, and spectra are shown in Figures 2 and 3.

In general, complex formation of flavones and flavonols with aluminum ion results in longer maximal absorption wavelengths (390–430 nm); it is noteworthy that the maximal absorption wavelength for dihydroquercetin also falls into this range (413 nm). Naringenin and caffeic acid have shorter maximal absorption wavelengths. It seems difficult to choose a wavelength for an analysis of a mixture of flavonoids with different structure, as the contribution of an individual flavonoid will be underestimated.

Among the flavonoids with double C2-C3 bond, rutin and luteolin (which do not have free hydroxyl group in position 3) showed the largest maximal absorption wavelengths bathochromic shifts on aluminum ion complex formation after preliminary nitrozation: To 531 and 517 nm, respectively. In contrast, dihydroquercetin (flavanonol with unconjugated B-and C-rings) had maximal absorption wavelength close to that or rutin and luteolin (507 nm).

Complex formation of caffeic acid with aluminum ion according to Method 2 depends on the pH of the medium. Absorption is not found in wavelength region above 450 nm in conditions similar to that used for flavonoids (95% ethanol with addition of acetic acid, pH = 4.5). In the absence of acetic acid (pH = 6.3) in reaction medium, nitrozation with subsequent complex formation results in absorption band development with absorption maximum wavelength above 500 nm [Figure 3].

Complex formation according to Methods 1 and 2 was applied to characterize angionorm dry extract. Preliminary HPLC analysis of the extract showed the presence of different types of flavonoid glycosides including quercetin and naringenin glycosides and phenolic acids. As the results of the Method 2 interactions depend on the reaction medium, analysis was

Table 2: Spectrophotometric measurements results according to methods 1 and 2

Substances	*c* (mmol/L)	λ_{max} (method 1)	A (method 1)	λ_{max} (method 2)	A (method 2)	λ_{max} shift (method 2-method 1)
Flavonoids with double C_2-C_3 bond						
Quercetin	0.0715	430	1.681	477	0.105	47
Rutin	0.0334	409	0.227	531	0.029	122
Morin	0.0768	423	1.709	Precipitate	-	-
Apigenin	0.0755	389	0.281	389	0.062	0
Luteolin	0.0727	398	0.166	517	0.133	119
Flavonoids without double C_2-C_3 bond						
Dihydroquercetin	0.0657	413	0.046	507	0.269	94
Naringenin	0.0735	379	0.055	-	-	-
Phenolic acids						
Caffeic acid	0.1177	360	0.021	-	-	-
Caffeic acid*	0.1177	338	1.023	512	0.656	174
Ferulic acid	0.1092	Precipitate	-	Precipitate	-	-
Extract of herbs mixture						
Angionorm extract	1.34 mg/mL	411	0.246	493	0.748	82

*Modified conditions without acetic acid

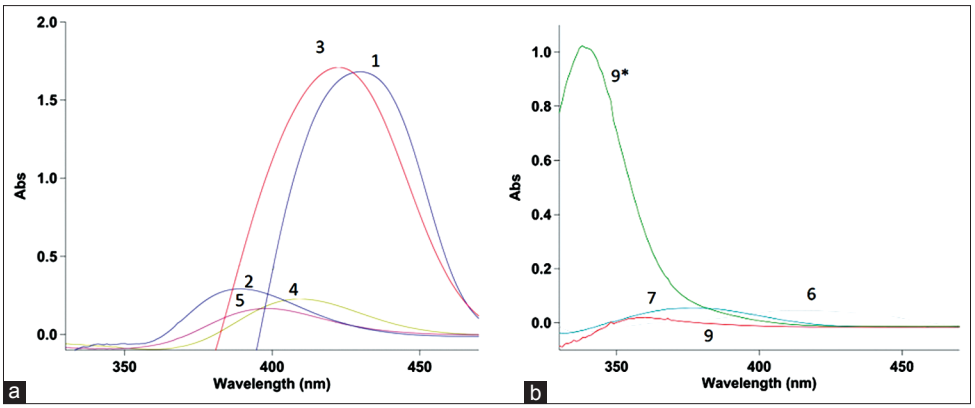

Figure 2: (a and b) Spectra of the studied substances, method 1: 1 – quercetin; 2 – apigenin; 3 – morin; 4 – rutin; 5 – luteolin; 6 – dihydroquercetin; 7 – naringenin; 9 – caffeic acid; 9* - caffeic acid, modified conditions without acetic acid

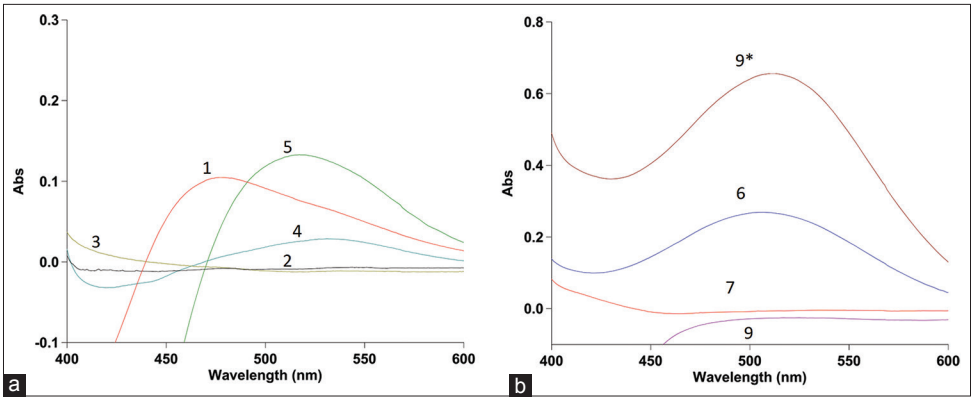

Figure 3: (a and b) Spectra of the studied substances, method 2: 1 – quercetin; 2 – apigenin; 3 – morin; 4 – rutin; 5 – luteolin; 6 – dihydroquercetin; 7 – naringenin; 9 – caffeic acid; 9* - caffeic acid, modified conditions without acetic acid

performed in neutral and weakly acidic media. The obtained spectra are shown in Figure 4, and the spectrophotometric data are summarized in Table 2.

The maximal absorption wavelength of the aluminum ion complex obtained after preliminary nitrozation shows the bathochromic shift of 82 nm. The band absorption under the Method 2 conditions depends on the medium pH. The maximum absorption without acetic acid in the medium is 1.44 times higher than that in the presence of acetic acid. Taking into account the above data [Figure 3 and Table 2], the intensity difference is suggested to be caused by caffeic acid and its esters in the reaction medium. Therefore, caffeic acid derivatives can possibly contribute to the total absorption.

Total flavonoids assay in dry ethanolic (25% ethanol) extract of the 4 herbs mixture (Angionorm) was performed according to Methods 1 and 2 and significantly depended on the reference substance chosen for the calculation. Among the studied flavonoids rutin, dihydroquercetin and luteolin show the closest maximal absorption wavelengths to that of angionorm extract when processed by Method 1. Dihydroquercetin, quercetin and luteolin show the closest maximal absorption wavelengths to that of angionorm extract when processed by Method 2. Total flavonoid content calculated as dihydroquercetin is equal to

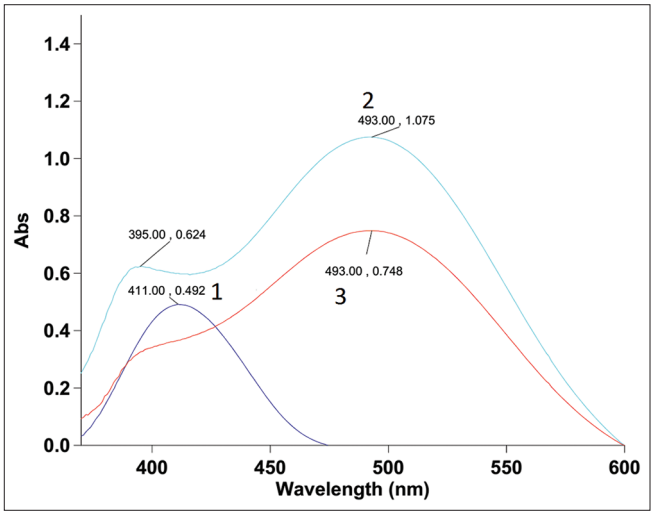

Figure 4: Application of the methods 1 and 2 (with and without acetic acid) to the Angionorm dry extract analysis: 1 – method 1; 2 – method 2 without acetic acid; 3 – method 2 with acetic acid

7.97% when analyzed by Method 1 and 4.14% when analyzed by Method 2. Total flavonoid content calculated as luteolin is 3.49% and 8.71% when measured by Methods 1 and 2, respectively.

Discussion

Flavonoids are an important group of antioxidant substances, and they are abundant in many medicinal herbs. Flavonoids can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and by acting on oxidative enzymes.[1] Flavones, flavanoles and their glycosides (A, B, and C rings are conjugated) differ significantly from flavanes and flavanoles (A and B rings are not conjugated) in antioxidant activity.[5-8] Some other biological activity is also different. Therefore, flavonoid subgroups assay in medicinal herbs is important.

Flavonoids as carbonyl group-containing compounds are potentially capable of reaction with DNPH, which must result in conjugation chain elongation and the consequent bathochromic shift of absorption bands. A number of authors[9,18] demonstrated that this interaction under certain conditions (heating with 1% DNPH at 50°C for 50 min with subsequent addition of 1% potassium hydroxide) can occur selectively with flavanones and flavanonols, but does not occur with flavones and flavonols.

Some authors[9,10] applied this approach to determine flavone content calculated as naringenin in samples of propolis (contribution of this flavonoid type exceeds that of other flavonoids when measured by this method). However,[19] demonstrated that the reaction with DNPH at 80°C for 15 min in strongly acidic medium (15% HCl) is nonspecific, and flavones and flavonols also showed the development of absorption bands at 470 nm. The DNPH interaction specificity is possibly determined by the difference of intramolecular hydrogen bond strength in flavones and flavonols on the one hand and flavanones and flavanonols on the other hand. The free carbonyl group of any flavonoid can possibly react with DNPH in more harsh conditions.[20] carried out the reaction according to method of[9] and showed that both quercetin and naringenin reacted with DNPH and quercetin absorption at 430 nm was 48% of that of naringenin, i.e., the reaction was not specific for flavanones and flavanonols. As demonstrated above, DNPH derivatization of a complex four herbs mixture (Angionorm dry extract) does not allow separate flavonoid subgroups assay. Studied flavonoids and hydroxycinnamic acids nitrozation result in absorption increase, but the shifts of maximum absorption wavelengths are different. Spectral data for the studied nitroso derivatives allows concluding that there is no selective spectral region that can be used for selective flavonoid subgroups and related hydroxycinnamic acids analysis. At the same time, the absorption increase after flavonoid nitrozation can have positive impact on the analysis sensitivity.

Complex formation of individual flavonoids with aluminum ion was already studied.[15,21,22] The stoichiometry of complex formation was determined, and the aluminum ion binding sites were identified as pyrocatechol structure of B-ring and carbonyl group primarily associated with hydroxyl group in C3 position of ring C, or, if absent, in C5 position of ring A. The complexes composition depends on the medium and differs in neutral (methanol), acidic (chloric acid addition) and alkaline (potassium acetate addition) media.[15,21]

The spectral data analysis indicates that aluminum ion complex formation with any method does not result in specific interaction which would be characteristic for certain flavonoid types and hydroxycinnamic acids, and it is not possible to identify specific spectral regions which can be used for their subgroup quantification.

Nitroso derivatives complexation with aluminum ion does not allow identifying specific flavonoid subgroups spectral regions. Maximal absorption wavelengths of quercetin and rutin (flavonoids with the same chromophores) differ significantly, which is also noted.[3] In the herbal mixture extract spectrum upon nitrozation and aluminum ion complexation [Angionorm, Figure 2], there is an absorption band in the region above 520 nm. This allows supposing that the extract contains quercetin, rutin, luteolin, and dihydroquercetin, but this assumption should be confirmed with chromatographic methods.

A wide-spread flavonoid spectrophotometric quantification method is using some standard flavonoid. In some studies, the flavonoid content estimated with such method correlates with biological activity, particularly with antioxidant activity, and usually with low correlation coefficient.[17] The poor correlation source may be the difficulty to select the flavonoid standard and the analytical wavelength when different structure flavonoids are present in a sample. In any case, some flavonoids will be over- or under-estimated. As demonstrated above, our flavonoid content estimations in the same sample vary depending on the standard flavonoid and complexation method.

Assay results calculated as any flavonoid are reproducible (relative measurement error falls within the range 1.3%–2.5%), but the specific figures depend on the chosen compound and assay conditions. Therefore, neither method of aluminum ion complex formation unequivocally reflects flavonoid content in herbal material and drug products, and the comparison of the values in different herbal material is indirect and can be performed only in strictly identical conditions.

Conclusion

The flavonoid assay spectrophotometric methods in herbal material based on DNPH derivatization and on aluminum ion complex formation with or without preliminary nitrozation do not allow unequivocal separation of flavonoids into subgroups with different structure (hydroxylation degree, B- and C-rings conjugation).

Total flavonoids estimation in herbal material depends on the selected method and the reference compound used for calculation. Spectrophotometric methods based on complex formation with aluminum ion are simple, reproducible and can be used for the specific herbal material standardization in strictly identical conditions, but they are not suitable for comparison of total flavonoid content in the different herbal material.

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Nil

Conflicts of interest
There are no conflicts of interest.

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