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Original Research

Development of a new spectrophotometric method for the determination of flavonoids in herb of red clover (*Trifolium pratense L*) and zigzag clover (*Trifolium medium L*)

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Abstract

Objective: Medicinal plants containing flavonoids are attracting increasing interest of researchers due to their complex therapeutic effects on the human body. Such medicinal plants include representatives of the genus Clover - red clover (*Trifolium pratense L.*) and zigzag clover (*Trifolium medium L.*). **Methods:** However, spectrophotometric techniques for the determination of the total flavonoids have numerous disadvantages, which prompted the development of a new analytical technique. As a result of our research, such a technique using gallium (III) nitrate as a staining reagent instead of the traditionally used aluminium chloride was developed, and a standard for recalculation (luteolin) was substantiated. **Results:** It has been shown that the new methodology is capable of giving satisfactory results, is characterised by excellent metrological characteristics, meets the requirements of SP RF XV ed. and the leading pharmacopoeias of the world (EP, USP, JP). **Conclusion:** The proposed methodology can be recommended for use not only for scientific purposes, but also in the field of standardisation of plant raw materials at pharmaceutical industries specialising in the production of medicinal preparations of plant origin.

Keywords: Clover herb, total flavonoid, gallium (III) nitrate, spectrophotometry

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INTRODUCTION

Representatives of the genus clover (in particular red clover) have been used in medicine for many centuries due to the confirmed favourable therapeutic effect on the human body and large natural reserves[1]. On the one hand, red clover has a high content of metals (calcium, iron and selenium, etc.) involved in maintaining the processes of normal vital activity of the organism[2], which makes it possible to consider it as a potential source of microelements. On the other hand, *Trifolium pratense* contains a huge complex of polyphenolic biologically active compounds: isoflavones, flavonoids, saponins, phenolic acids[3-8], which act on the human body as hormones, vitamins, anti-inflammatory agents, etc.[3,4,9-14]. Moreover, the composition of different groups of active substances is quite diverse. For example, the main share of isoflavones is biochanin A, formonononetin, as well as ginestein and daidzein, which have phytoestrogenic action[15-19]. This fact allows us to consider it as a raw material promising for the development of drugs for hormone replacement therapy in gynaecological disorders[20]. Moreover, additional studies have also established that phytoestrogenic compounds of red clover (biochanin A, formonononetin, genistein) are characterised by powerful neuroprotective properties in the therapy of various neurodegenerative disorders due to their systemic action on lipid metabolism[21-23]. In addition to isoflavones, the aerial part of red clover herb contains a significant amount of flavonoids, which are responsible for antioxidant, anti-inflammatory, anti-allergic, antiviral and anticarcinogenic properties: quercetin, luteolin,–rutoside, apigenin, etc.[24-30]. Therefore, this group of active substances is of particular interest for medicine.

At the same time, zigzag clover (*Trifolium medium L.*), equally widespread in nature, is not so widely used in medicine, because, according to some scattered literature data, it contains a much smaller amount of active substances[31-33]. Flavonoid compounds in medicinal plant raw materials are mainly in the form of glycosides and have therapeutic effects in a complex manner, which makes it possible not to determine each flavonoid separately, but to determine their sum. Consequently, spectrophotometry, which is a simple and accessible method, is most suitable for solving this range of problems[34,35].

The most common in pharmacognosy variant of spectrophotometric technique for the determination of the total flavonoids is the staining of test solutions with aluminium chloride solution 2%[36]. The obtained results are converted to a suitable flavonoid, the choice of which can be conditioned either by its dominance in the raw material or by the closest possible position of the absorption maxima of the standard sample solution of any flavonoid and the tested solution. However, not always the use of aluminium chloride as a staining reagent allows to obtain well reproducible results due to the peculiarities of the formation of complex compounds 'flavonoid-aluminium chloride'.

SP RF XV ed. approved pharmacopoeial monograph No. PM.2.5.0116 «Clover herb», in which a mixture of two species of clover - red clover and red clover for seeding- is used as a medicinal plant raw material[37]. In this monograph in the section 'Quantitative determination' the method of determination of the total flavonoids recalculated as rutoside is proposed, which is insufficiently justified due to the unsatisfactory choice of flavonoid for recalculation, as well as the colouring reagent-complexing agent. Analysis of the monographs of the leading pharmacopoeias of the world - European (EP), American (USP), Japanese (JP) showed the absence of satisfactory methods for the determination of the total flavonoids in the herb of red clover and zigzag clover, which makes it extremely relevant to conduct research in this area of pharmacognosy.

The aim of this work is to develop a simple, sensitive, reproducible spectrophotometric technique for the determination of the total flavonoids in herb of red clover and zigzag clover using complexing reagents new to pharmacognosy.

MATERIALS AND METHODS

Object of the study

Red clover herb was collected on the territory of Taldomsky District, Moscow Region (GPS coordinates: N 56.682488º, E 37.732892º) in July 2021. Herb of zigzag clover was collected in the territory of Sverdlovsky District of Moscow Region (GPS coordinates: N 55.898819º, E 38.177013º) in July 2021. The herb was dried by air-shade drying method. The dried herb was stored in kraft bags and shredded before analysis.

Reagents

The standard sample solutions used in the study were: luteolin (CAS No. 491-70-3, ≥ 98 %, Sigma-Aldrich, Germany), rutoside (CAS No. 153-18-4, ≥ 97+ %, Acros Organics, Belgium), quercetin (CAS No. 117-39-5, ≥ 98+ %, Sigma-Aldrich, Germany). The staining reagents were: aluminium chloride, anhydrous (CAS No. 7446-70-0, ≥ 98.5 %, Acros Organics, Belgium), zirconyl chloride hydrate (CAS No. 15461-27-5, 99.99 %, Sigma-Aldrich, Germany), gallium (III) nitrate hydrate (CAS No. 69365-72-6, 99.999 %, Sigma-Aldrich, Germany). Staining solutions of 0.5; 1; 2; 4 % were prepared by dissolving the suspensions of the respective staining reagents in ethanol 96% in a 100.0 ml measuring flask. Ethanol (CAS № 64-17-5, 96%, neoFroxx, Germany), glacial acetic acid (CAS № 64-19-7, CH, Himmed, Russia) were also used.

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Apparatus

To determine the optical density we used spectrophotometer PE-5400UV (Ekroschem LLC, Russia) with software - 'SC5400' for Windows. Analytical scales ViBRA AJ-320CE (SHINKO DENSHI Co., Ltd., Japan) were used in the study.

A new general methodology for the determination of flavonoids in red clover and zigzag clover herbs in terms of luteolin

Luteolin standard sample solution

About 0.05 g (exact weight) of luteolin previously dried to constant weight at 100-105°C was placed in a 100.0 ml measuring flask and dissolved in 50 ml of 70% ethanol while heating on a water bath. After cooling to room temperature, the contents of the flask were brought to the mark with the same solvent and stirred (solution A). 1.0 ml of solution A was placed in a 25.0 ml volumetric flask, 3 ml of gallium nitrate (III) 4% solution in ethanol 96% and 1 drop of acetic acid diluted 30% were added, the volume of the solution was brought to the mark with ethanol 96%, stirred and incubated for 20 min (solution B).

Preparation of the test solution

About 1.0 g (exact weight) of crushed raw material (particle size - 0.5 mm) was placed in a 250 ml flask with a slurry, 80 ml of ethanol 70% was added. The flask with the contents was connected to a reflux condenser and heated on a water bath for 15 min. After cooling to room temperature, the extract was filtered through a "blue-ribbon" paper filter into a 100.0 ml volumetric flask, brought to the mark with the same solvent and stirred (solution C). 1.0 ml of solution C was placed in a 25.0 ml volumetric flask, added 3 ml of gallium nitrate (III) 4% solution in ethanol 96% and 1 drop of acetic acid diluted 30%, brought the volume of the solution with ethanol 96% to the mark, stirred, incubated for 20 min (solution D).

Measurements

The optical density of solutions B and D was measured sequentially on a spectrophotometer at a wavelength of 403 nm in a cuvette with a layer thickness of 1 cm relative to the reference solution. A solution consisting of 1 ml of solution C, 1 drop of acetic acid diluted 30%, brought to the mark with ethanol 96% in a measuring flask with a capacity of 25.0 ml was used as a reference solution.

The content of the total flavonoids recalculated as luteolin in absolutely dry raw material in per cent (X, %) was calculated according to the formula:

where: A - optical density of the test solution, mAU; A_0 - optical density of luteolin standard sample solution, , mAU; a - raw material suspension, g; a_0 - luteolin standard sample suspension, g; P - luteolin content of a standardised sample of luteolin, %; W - raw material humidity, %.

RESULTS AND DISCUSSION

It is known from literature data that the herb of red clover and zigzag clover predominantly contains the following flavonoids: rutoside, luteolin, quercetin[7,24-30]. Therefore, in order to select a flavonoid that could be used for recalculation of the analysis results, a preliminary study consisting in staining with aluminium chloride solution of 2% the standard sample solutions with the same concentration of the above listed flavonoids and the tested solutions according to the method described in PM.2.5.0116 "Clover herb" was carried out[37]. Absorption spectra are presented in Figure 1.

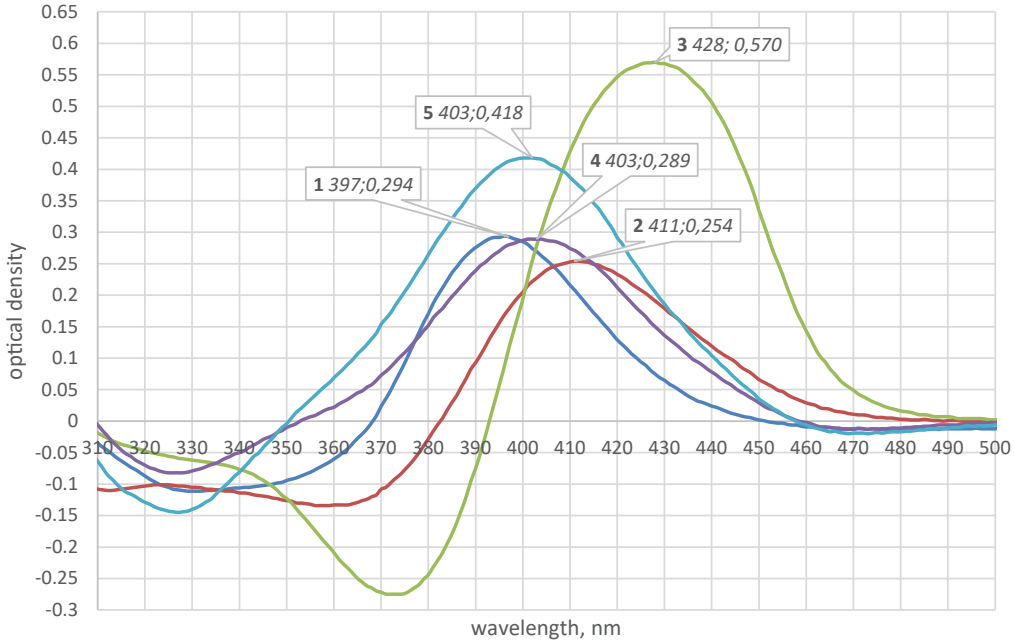

Figure 1. Absorption spectra of standard sample solutions of flavonoids ($C_{flavonoid}$ = 500 µg/ml) and test solutions stained with aluminium chloride 2%: 1 - luteolin, 2 - rutoside, 3 - quercetin, 4 - red clover, 5 - zigzag clover.

Extraction conditions optimization

The obtained spectra show that the absorption maxima of the formed complexes 'flavonoid-$AlCl_3$' in the tested solutions of red clover and zigzag clover are at a wavelength of 403 nm. At the same time absorption maxima of complexes in solutions of rutoside, quercetin and luteolin with $AlCl_3$ in the spectra of the standard sample flavonoids solutions are present at wavelengths 411, 428, 397 nm, respectively. Thus, the obtained data indicate that the application of aluminium chloride solution of 2% traditionally used in pharmacognosy does not allow obtaining satisfactory results, because in this case none of the studied flavonoids provides absorption maximum close to the absorption maximum observed in the tested solution. In particular, recalculation for rutoside, recommended in SP RF XV ed.[37], is not correct due to the presence of a large error of analysis, which is due to the non-coincidence of absorption maxima. At the same time, in our opinion, it is more rational to use luteolin as a standard substance, because of all the flavonoids studied, it provides the minimum difference between the absorption maxima of the complexes in the standard and test solutions (about 6 nm).

It is mentioned in[38] that the use of gallium nitrate (III) and zirconyl chloride solutions as staining reagents is more promising. The author notes that compared to aluminium chloride, these reagents allow to achieve higher sensitivity of determination. However, no research results are given. This initiated the continuation of search studies in this area. Thus, Figure 2 and Figure 3 show absorption spectra of solutions of luteolin complexes and tested solutions with gallium nitrate (III) 2%, zirconyl chloride 2%, aluminium chloride 2%.

Spectrophotometric measurements

The spectra presented in Figure 2 and Figure 3 show that gallium nitrate (III) allows to provide an acceptable coincidence of absorption maxima of complexes in luteolin solutions and tested solutions (the difference is about 1 nm) and, thus, to balance spectral errors of determination. The influence of $Ga(NO_3)_3$ concentration, volume of the complexing agent solution, and reaction time was studied to select the most optimal conditions for the formation of the analytical signal. The investigations were carried out as follows: to 1 ml of luteolin standard sample solution placed in a 25.0 ml volumetric flask, $Ga(NO_3)_3$ staining solutions in ethanol 96% with characteristics indicated in Table 1 were added. Then 1 drop of acetic acid diluted 30% was added and the volume of the solution was brought to the mark with ethanol 96% and stirred. The data are presented in Table 1. The data presented in Table 1 allow us to conclude that for the formation of a complex, providing a stable analytical signal and sensitivity of determination, it is necessary to use 3 ml of $Ga(NO_3)_3$ 4% solution in 96% ethanol; the time of colour development is 20 minutes, wavelength - 403 nm.

Extraction conditions optimization

The influence of reduced in size degree, ethanol content in the extractant, the ratio of raw materials:extractant, extraction time, extraction rate on the completeness of flavonoids

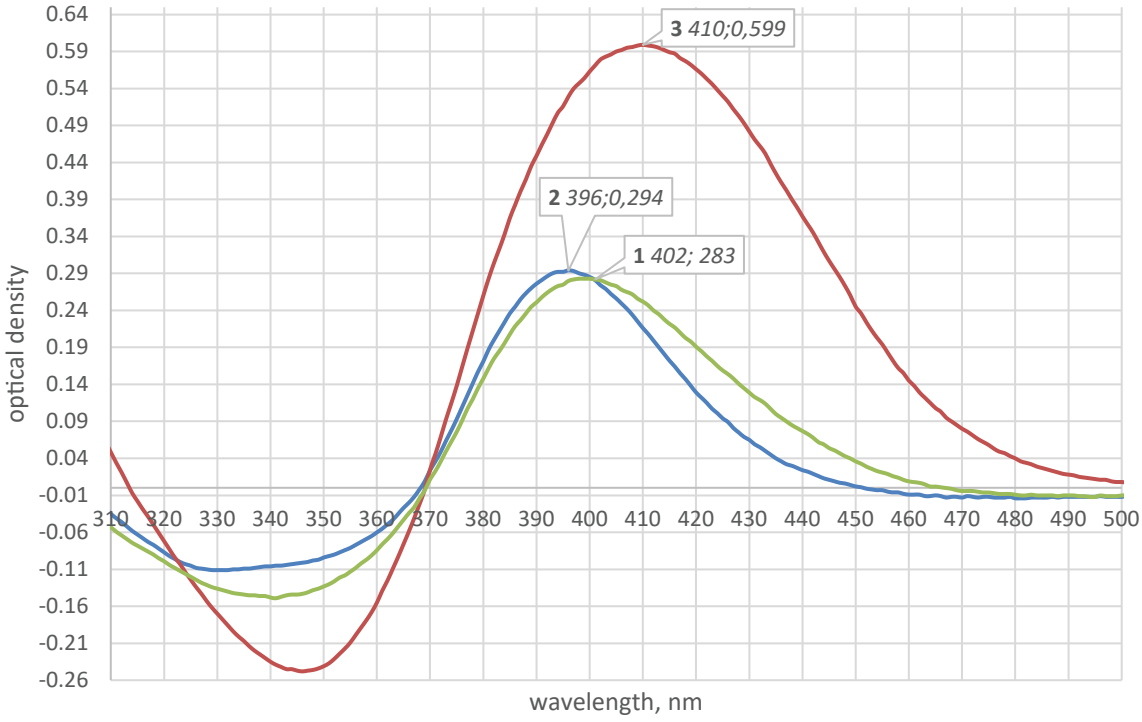

Figure 2. Absorption spectra of luteolin complexes: 1 - with $Ga(NO_3)_3$ (2% solution), 2 - with $AlCl_3$ (2% solution), 3 - with $ZrOCl_2$ (2% solution), $C_{Luteolin}$ = 500 µg/ml

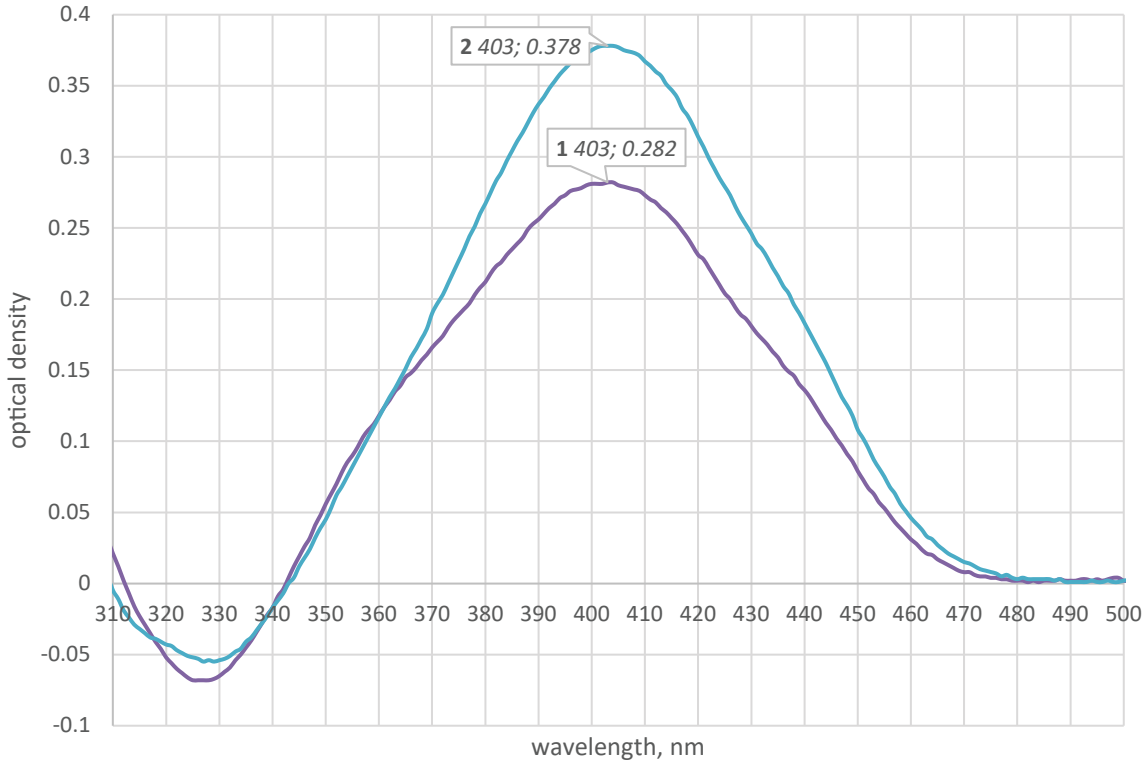

Figure 3. Absorption spectra of 'flavonoid-$Ga(NO_3)_3$' complexes in tested solutions: 1 - red clover, 2 - zigzag clover

extraction from samples of red clover and zigzag clover are presented in Table 2 and Table 3. Based on the data presented in Table 2 and Table 3, it was found that the highest extraction of flavonoids from herb of red clover and zigzag clover is observed under the following conditions: reduced in size degree of raw materials - 0.5 mm; extractant - ethanol 70%; ratio of raw materials:extractant - 1:80; extraction time - 15 min; extraction rate - 1. Thus, the conducted studies allowed us to develop a simple, sensitive method for quantitative determination of the total flavonoids in *Trifolium pratense* herb and *Trifolium medium* herb. At the same time, Ga^{3+} was chosen as a complexing ion instead of Al^{3+} traditionally used in pharmacognosy, and luteolin was chosen as a standard.

Validation parameters (Linearity, Accuracy, Repeatability)

To evaluate the specificity of the proposed new methodology, spectra of luteolin standard sample solutions and test solutions prepared for quantitative determination were obtained (see above section 'New general methodology for the determination of the total flavonoids in herb of red clover and zigzag clover recalculated as luteolin'). The range of spectra recorded was from 250 to 500 nm. Typical absorption spectra of luteolin standard sample solutions and tested solutions are shown in Figure 4. The obtained data testify to high specificity of the new method, since absorption spectra of the tested solutions in the range from 250 to 500 nm correspond to the absorption spectrum of luteolin standard sample solution in the same area. Moreover, the local absorption maximum is also located at the same wavelengths - 403±2 nm.

Table 1. Effect of $Ga(NO_3)_3$ concentration, volume of the complexing agent solution and time on the completeness of the reaction of 'luteolin - $Ga(NO_3)_3$' complex formation

Concentration $Ga(NO_3)_3$, %	**wavelength, nm**	**optical density**
0,5	396	0,158
1	396	0,229
2	399	0,317
4	401	0,399
Volume $Ga(NO_3)_3$, ml	**wavelength, nm**	**optical density**
0,25	399	0,214
0,5	399	0,287
1	399	0,360
2	402	0,386
3	402	0,435
5	402	0,436
Reaction time, min	**wavelength, nm**	**optical density**
10	403	0,422
20	403	0,417
30	403	0,415
40	403	0,414
50	403	0,411
60	403	0,411

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Table 2. Influence of various factors on the completeness of extraction of the total flavonoids from red clover herb

size, mm	Ethanol content, %	Raw material to extractant ratio	extraction time, min	Extraction rate	X, %
1	96	1:40	60	1	2,86
1	70	1:40	60	1	3,12
1	40	1:40	60	1	3,04
1	water	1:40	60	1	0,97
1	70	1:20	60	1	2,62
1	70	1:40	60	1	3,09
1	70	1:80	60	1	3,59
1	70	1:120	60	1	3,31
0,5	70	1:80	60	1	3,44
1	70	1:80	60	1	2,89
2	70	1:80	60	1	2,04
5	70	1:80	60	1	1,19
0,5	70	1:80	15	1	4,06
0,5	70	1:80	30	1	3,68
0,5	70	1:80	60	1	3,22
0,5	70	1:80	90	1	2,61
0,5	70	1:80	15	1	4,60
0,5	70	1:80	15	2	4,60

Table 3. Influence of various factors on the completeness of extraction of the total flavonoids from the herb of zigzag clover

size, mm	Ethanol content , %	Raw material to extractant ratio	extracyion time, min	Extraction rate	X, %
1	96	1:40	60	1	2,44
1	70	1:40	60	1	2,55
1	40	1:40	60	1	1,88
1	Вода	1:40	60	1	1,42
1	70	1:20	60	1	1,98
1	70	1:40	60	1	2,77
1	70	1:80	60	1	3,27
1	70	1:120	60	1	2,91
0,5	70	1:80	60	1	2,80
1	70	1:80	60	1	2,38
2	70	1:80	60	1	1,46
5	70	1:80	60	1	0,41
0,5	70	1:80	15	1	3,02
0,5	70	1:80	30	1	2,86
0,5	70	1:80	60	1	2,70
0,5	70	1:80	90	1	2,10
0,5	70	1:80	15	1	2,96
0,5	70	1:80	15	2	2,96

To determine the linearity of the method, a series of luteolin standard sample solutions were prepared in the concentration range from 125 to 1000 μg/ml and their optical densities were measured. The obtained results are reflected in Table 4. According to the obtained results (Table 4), the graph of dependence of optical density of standard sample solutions on luteolin concentration was plotted and processed by the least squares method. To confirm linearity, the correlation coefficient (r) was calculated. The graph shown in Figure 5 confirms the linear relationship between optical density and concentration of luteolin standard sample solutions in the content range from 125 to 1000 μg/ml, as the value of correlation coefficient (r) is 0.9987.

The accuracy of the method was studied by the spiking method, by adding the exact amount of luteolin standard sample to the extracts, which were pre-diluted 2 times so that the obtained concentrations of analytes after the addition did not exceed the linearity range of the method. Based on the results of these studies, the recovery was calculated as the ratio between the experimentally found value of the determined content (after addition) and its theoretical value.

The results of recovery determination are given in Table 5 and Table 6. To determine the repeatability of the method, the coefficient of variation was calculated from the results of the total flavonoids determination in the analysed samples (in 6 repetitions) using the developed method. The results are given in Tables 7-8. The coefficient of variation of the total flavonoids determination results in red clover herb (P=0.95; n=6) does not exceed 1%.

The coefficient of variation of the total flavonoids determination results in zigzag clover herb (P=0.95; n=6) does not exceed 1%. Thus, according to the results of metrological studies, the new method demonstrated high specificity, accuracy (coefficient of variation is in the range of 1.0-2.0%), repeatability (coefficient

Table 4. Linearity assessment of the methodology

№	A_1	A_2	A_3	Aa.v.	$C_{luteolin}$, μg/ml
1	0,120	0,118	0,119	0,122	125
2	0,209	0,209	0,207	0,205	250
3	0,413	0,414	0,414	0,413	500
4	0,569	0,571	0,571	0,573	700
5	0,859	0,860	0,860	0,860	1000

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Table 5. Accuracy of determination of the content of the total flavonoids (recalculated as luteolin) in red clover ($A_{taste\ solutin}$ = 0.102*; $m_{taste\ solutin}$ =127.5 µg)

$m_{add.\ standard\ solutionn.}$, µg	$A_{add.\ standard\ solutionn}$	$A_{theoretical}$	$m_{theoretical}$ µg	$A_{practical}$	$m_{practical}$, µg	Recovery, %
125,0	0,100	0,202	252,5	0,198	247,6	98,02
125,0	0,100	0,202	252,5	0,198	247,6	98,02
125,0	0,100	0,202	252,5	0,195	243,9	96,53
250,0	0,200	0,302	377,5	0,293	365,7	96,85
250,0	0,200	0,302	377,5	0,288	360,6	95,49
250,0	0,200	0,302	377,5	0,290	362,3	95,94
350,0	0,280	0,382	477,5	0,374	467,6	97,90
350,0	0,280	0,382	477,5	0,364	455,6	95,38
350,0	0,280	0,382	477,5	0,369	461,6	96,64

*- optical density of the test solution diluted by a factor of 2

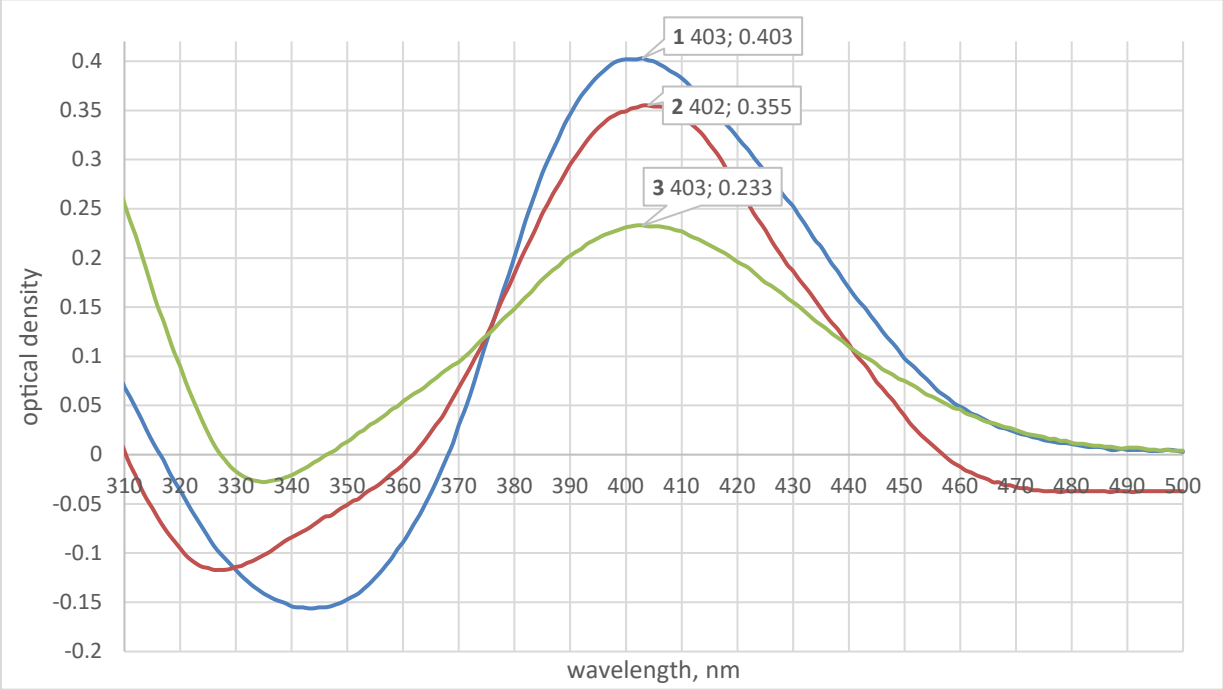

Figure 4. Typical absorption spectra of luteolin standard sample solution ($C_{luteolin}$ = 500 µg/ml) and test solutions stained with $Ga(NO_3)_3$ 4% solution: 1 - luteolin, 2 - red clover, 3 - zigzag clover.

Table 6. Accuracy of determination of the content of the total flavonoids (recalculated as luteolin) in zigzag clover ($A_{taste\ solutin}$ = 0.080*; $m_{taste\ solutin}$ =100.0 µg)

$m_{add.\ standard\ solutionn.}$, µg	$A_{add.\ standard\ solutionn}$	$A_{theoretical}$	$m_{theoretical}$ µg	$A_{practical}$	$m_{practical}$, µg	Recovery, %
125	0,100	0,180	225,0	0,179	223,4	99,22
125	0,100	0,180	225,0	0,179	223,4	99,22
125	0,100	0,180	225,0	0,182	226,9	100,8
250	0,200	0,280	350,0	0,273	340,7	97,31
250	0,200	0,280	350,0	0,280	350,0	100,0
250	0,200	0,280	350,0	0,269	336,0	95,97
350	0,280	0,360	450,0	0,362	452,9	100,6
350	0,280	0, 360	450,0	0,371	463,8	103,0
350	0,280	0, 360	450,0	0,367	458,3	101,8

*- optical density of the test solution diluted by a factor of 2

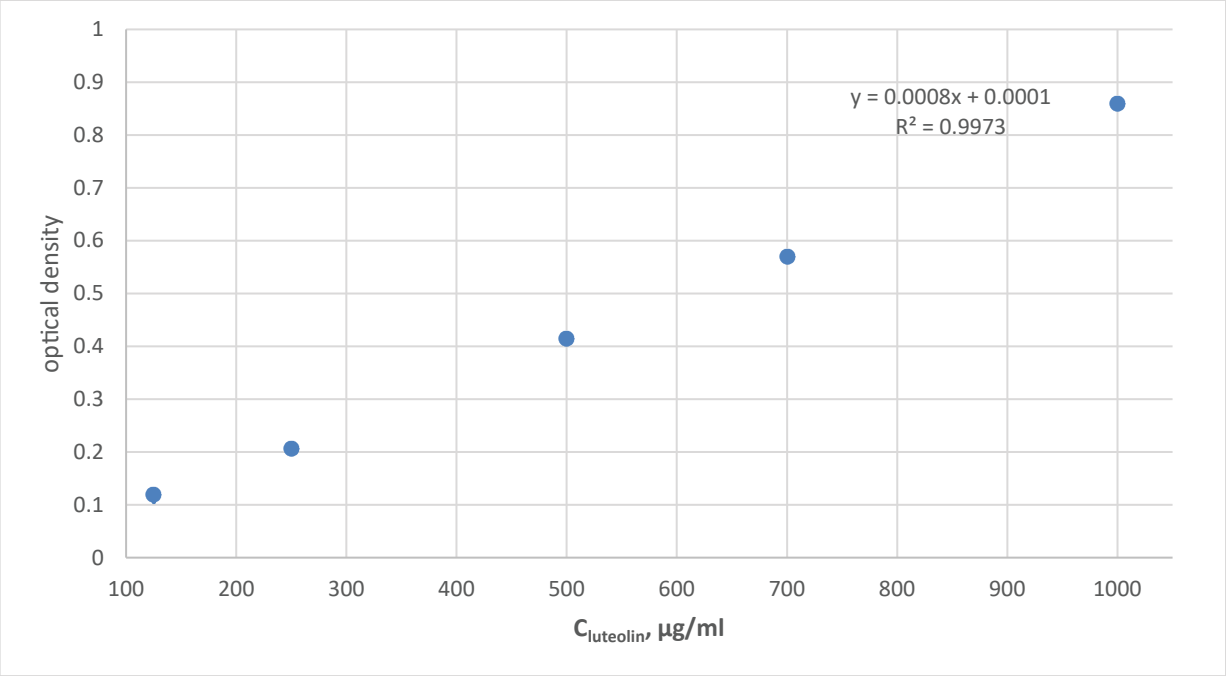

Figure 5. Dependence of optical density of standard solutions on luteolin concentration

Table 7. Repeatability of the method for the total flavonoids determination (recalculated as luteolin) in red clover herb						
Parameters	**1**	**2**	**3**	**4**	**5**	**6**
A_1	0,355	0,350	0,358	0,354	0,353	0,352
A_2	0,350	0,353	0,355	0,351	0,355	0,357
A_3	0,356	0,357	0,356	0,352	0,360	0,355
Average A	0,354	0,353	0,356	0,352	0,356	0,355
X, %	4,50	4,49	4,53	4,48	4,53	4,51

Table 8. Repeatability of the method for the total flavonoids determination (recalculated as luteolin) in zigzag clover herb						
Parameters	**1**	**2**	**3**	**4**	**5**	**6**
A_1	0,227	0,226	0,227	0,228	0,226	0,229
A_2	0,227	0,227	0,227	0,225	0,226	0,228
A_3	0,227	0,226	0,229	0,227	0,226	0,228
Average A	0,227	0,226	0,228	0,227	0,226	0,228
X, %	2,90	2,88	2,91	2,90	2,88	2,91

of variation does not exceed 1.0%). The relative error of the determinations did not exceed 0.7%. The limit of detection estimated as (2-3)σ, the limit of quantification estimated as (5-10)σ were 2.0 and 10.0, respectively.

CONCLUSION

Based on the results obtained, a new specific repeatable spectrophotometric method for the determination of the total flavonoids in the herb of red clover and zigzag clover was developed. Luteolin is proposed as a standard for recalculation, as a staining reagent - ethanol solution of gallium nitrate (III) 4%, which allows almost completely balance errors caused by mismatch of absorption maxima of the standard flavonoid solution and the tested solution. Consequently, the presented method allows to obtain more accurate results in comparison with existing methods, where aluminium is used as a metal-complexing agent and rutoside as a standard. With the help of the developed methodology the content of flavonoids in plant raw material - herb red clover (the content of the total

flavonoids in terms of luteolin is (4,51±0,03)%) and herb zigzag clover (the content of the total flavonoids in terms of luteolin is (2,90±0,02)%) was determined. The methodology can be recommended for application not only in scientific research, but also for quality control purposes of herbs of red clover and zigzag clover at pharmaceutical production facilities of pharmacognostic profile.

AUTHORS CONTRIBUTIONS

All authors contributed equally in this manuscript. All authors have read and agreed to the final version of this manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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