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Article

Spectrophotometric and Chromatographic Assessment of Total Flavonoids Content in *Rhodendron tomentosum* Extracts

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Abstract: In the literature, the chemical composition of *Rhododendron tomentosum* is mainly represented by the study of isoprenoid compounds of essential oil. In contrast, the study of the content of flavonoids will contribute to the expansion of the pharmacological action and the use of the medicinal plant for medical purposes. The paper deals with the technology of extracts from *Rhododendron tomentosum* shoots using ethanol of various concentrations and purified water as an extractant. Extracts from *Rhododendron tomentosum* were obtained by a modified method that combined the effects of ultrasound and temperature to maximize the extraction of biologically active substances from the raw material. By the method of high-performance thin-layer chromatography in the system of solvents ethyl acetate: formic acid: water (15:1:1) the following substances have been separated and identified: rutin, hyperoside, quercetin, and chlorogenic acid in all the extracts obtained. To quantify the total flavonoids content, a method of spectrophotometric determination based on the reaction of complexation with aluminum chloride has been developed. It has been proved that the course of the complexation reaction and, accordingly, the quantification of the flavonoid structure substances by the spectrophotometric method is influenced by some factors, including the environment, pH, the structure of the complexes, etc. Regardless of the chosen method, for the further production of *Rhododendron tomentosum* extracts with a higher content of flavonoid structure substance, it is advisable to use 60%, 70%, and 80% ethyl alcohol as extractant.

Keywords: *Rhododendron tomentosum*; *Ledum palustre*; wild rosemary; rutin; hyperoside; quercetin; chlorogenic acid; aluminum chloride; UV-Vis; HPTLC

1. Introduction

Rhododendron tomentosum (formerly *Ledum palustre*) is an evergreen, squat shrub that has a significant distribution area on the territory of Ukraine throughout its northern part. The popular names of *Rhododendron tomentosum* are marsh labrador tea, northern labrador tea and wild rosemary [1,2]. Infusion and decoction of young shoots of the plant have long been used in folk medicine as an antitussive, bactericidal and diaphoretic agent in acute and chronic bronchitis, pneumonia, tuberculosis, pertussis and other diseases accompanied by cough, as well as in spastic enterocolitis, endometritis and liver diseases. The anti-inflammatory property of the raw materials is used in arthritis [1,3]. *Rhododendron tomentosum* is widespread mainly in wet and swampy coniferous and deciduous forests, sphagnum marshes, and peatlands in the northern regions of Europe, North America, including the northern parts of Canada and Alaska, Siberia, and North Asia [4]. As a raw material of *Rhododendron tomentosum*, only young shoots of the first year up to 10.0 cm long are harvested, they are crushed together with flowers and leaves. The *Rhododendron tomentosum* has a low level of regeneration, so after cutting the shoots, the biomass is restored only after 3 years [1]. *Rhododendron tomentosum* is a pharmacopeial plant raw material in France and Germany (French Pharmacopoeia 2007, German Homoeopathic Pharmacopoeia, 2000).

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Rhododendron tomentosum is considered by scientists primarily as an ethereal medicinal plant, but it also contains other groups of biologically active substances, such as diterpenoids, phenol carboxylic acids, flavonoids, polysaccharides, pectin substances [5–8]. The chemical composition of the plant in the literature is represented mainly by the study of isoprenoid compounds of essential oil. In contrast, the content of flavonoids has not been studied enough. Given the wide biological activity of flavonoids, it is important to study the technology of extracts from the shoots of *Rhododendron tomentosum*, and the qualitative and quantitative study of their flavonoid composition [9]. Thus, extraction of dried plant material with different solvents was performed and characterization of the obtained extracts was achieved by chromatographic (HPTLC) and spectrophotometric methods.

2. Results

For the research, 10 samples of extracts of Rhododendron tomentosum were prepared, which were obtained by extraction with ethanol of various concentrations from 10% to 90% in steps of 10% (EtOH10, EtOH20, etc.), and one sample - with purified water (EtOH0). The liquid extracts were obtained by the method of threefold fractional maceration in combination with the effect of ultrasound in the ratio of raw materials: extractant 1:20. The liquid extracts were evaporated to a dry extract. The higher yield of dry extracts provides the use of ethanol in concentrations of 60%, 70% and 80% and is 0.18 g, 0.17 g and 0.18 g, respectively, per 1 g of raw material (Table 2). According to organoleptic properties, dry extracts are a mass of dark brown color with a characteristic smell. For chromatographic and spectrophotometric methods of analysis, dry extracts were dissolved in methanol to equalize the influence of different concentrations of ethanol on the research results. This approach was due to the fact that ethanol is the most acceptable in pharmaceutical drug technology due to its relatively safe toxic profile, on the one hand, and other hand, the studied group of biologically active substances - flavonoids – is highly soluble in methanol, which allows them to be quantitatively transferred into methanol solution [10–12].

2.1. HPTLC-UV/Vis Method Development

The purpose of the chromatographic study was to identify flavonoids in Rhododendron tomentosum using active markers, which were subsequently used as standard samples for the quantitative determination of the total flavonoids content by the UV-Vis method. For the chromatographic separation of polyphenol compounds, several eluent systems were tested: ethyl acetate: methanol: water: formic acid (50:10:7:1), ethyl acetate: methyl ethyl ketone: formic acid: water (50-30-10-10), ethyl acetate: water: formic acid: acetic acid (68:18:7:7) (data not shown). The highest resolution of flavonoids and phenolic acids was obtained using the solvent system ethyl acetate: formic acid: water (15:1:1), the obtained HPTLC chromatograms are shown in Figures 1 and 2.

In the process of HPTLC fingerprint analysis, the identification of substances is based on the Rf value (position of the bands and color background) of the standard substances and the studied extracts [13,14]. HPTLC chromatograms visually show similarities and differences in the composition of the test samples. As can be seen from the results, on the chromatographic strips of the test samples scanned after derivatization in UV light at 366 nm, there are zones that correspond in color and Rf to the standard samples of rutin Rf (0.12), hyperoside Rf (0.32) and quercetin Rf (0.94) [15,16]. As can be seen from Figure 1, rutin, hyperoside, and quercetin are present in all extract samples regardless of the extractant, but in terms of the intensity of color and the size of the colored zones, it can be argued that samples in which the extractants were purified water and ethanol 10% are somewhat inferior. In addition to flavonoids, standards of phenolic acids, namely chlorogenic acid, rosmarinic acid, and caffeic acid, were applied to the chromatographic plate [17,18].

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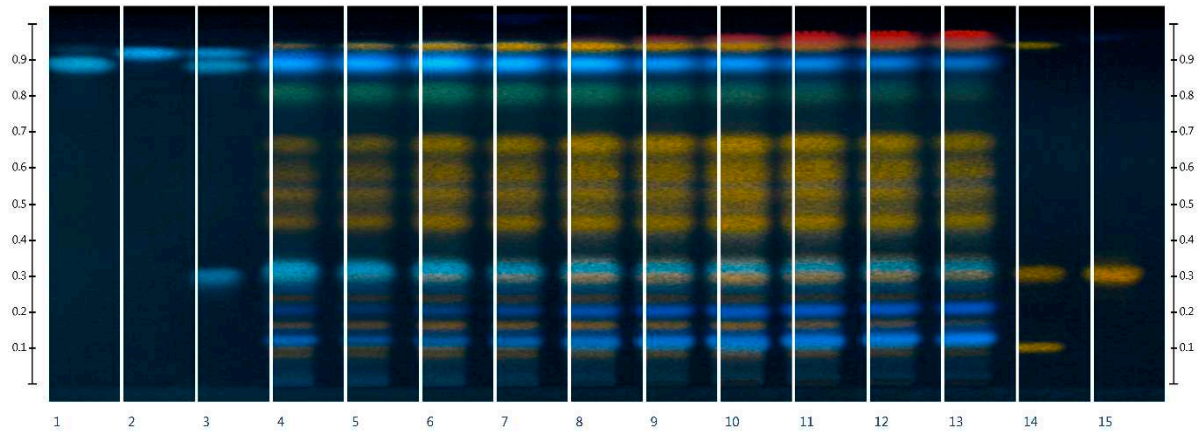

Figure 1. HPTLC fingerprints of the extracts of Rhododendron tomentosum and standards with increasing Rf at λ 366 nm after derivatization: 1 − Rosmarinic acid, 2 − Caffeic acid, 3 − Chlorogenic acid + Rosmarinic acid + Caffeic acid, 4 – EtOH0, 5 – EtOH10, 6 – EtOH20, 7 – EtOH30, 8 – EtOH40, 9 – EtOH50, 10 – EtOH60, 11– EtOH70, 12 – EtOH80, 13 – EtOH90, 14 – Rutin + Hyperoside + Quercetin, 15 – Hyperoside.

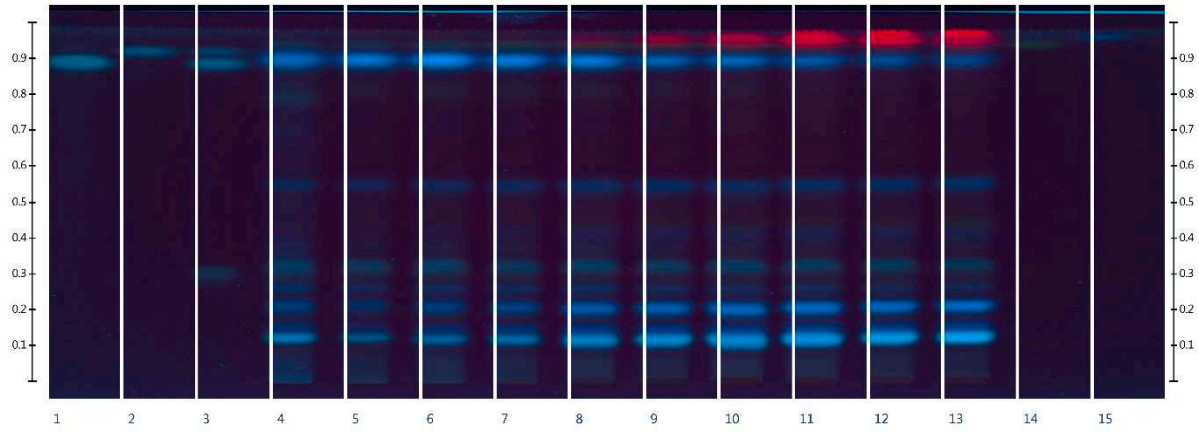

Figure 2. HPTLC fingerprints of the extracts of Rhododendron tomentosum and standards with increasing Rf at λ 254 nm before derivatization: 1 − Rosmarinic acid, 2 − Caffeic acid, 3 − Chlorogenic acid + Rosmarinic acid + Caffeic acid, 4 – EtOH0, 5 – EtOH10, 6 – EtOH20, 7 – EtOH30, 8 – EtOH40, 9 – EtOH50, 10 – EtOH60, 11– EtOH70, 12 – EtOH80, 13 – EtOH90, 14 – Rutin + Hyperoside + Quercetin, 15 – Hyperoside.

After scanning chromatographic zones before derivatization in UV light at 254 nm (Figure 2) and after detection in UV light at 366 nm (Figure 1), chlorogenic acid Rf (0.32) was identified. The substances rutin, hyperoside, quercetin and chlorogenic acid were also detected by the authors [1,5] in the raw material of Rhododendron tomentosum. The zones of rosmarinic acid and caffeic acid standard substances differ in color in UV light at 254 nm, which may indicate their absence in the raw material.

2.2. UV/Vis Method Development

The most commonly used method for quantifying the total flavonoids content is the method of differential spectrophotometry after the complexation reaction with $AlCl_3$ [19,20]. Numerous scientific publications are devoted to the study of the spectrophotometric behavior of flavonoids in reactions with $AlCl_3$, which demonstrate that the differential spectra of flavonoids (wavelength and intensity of maximum absorption) depend on the chemical nature of the flavonoids, the

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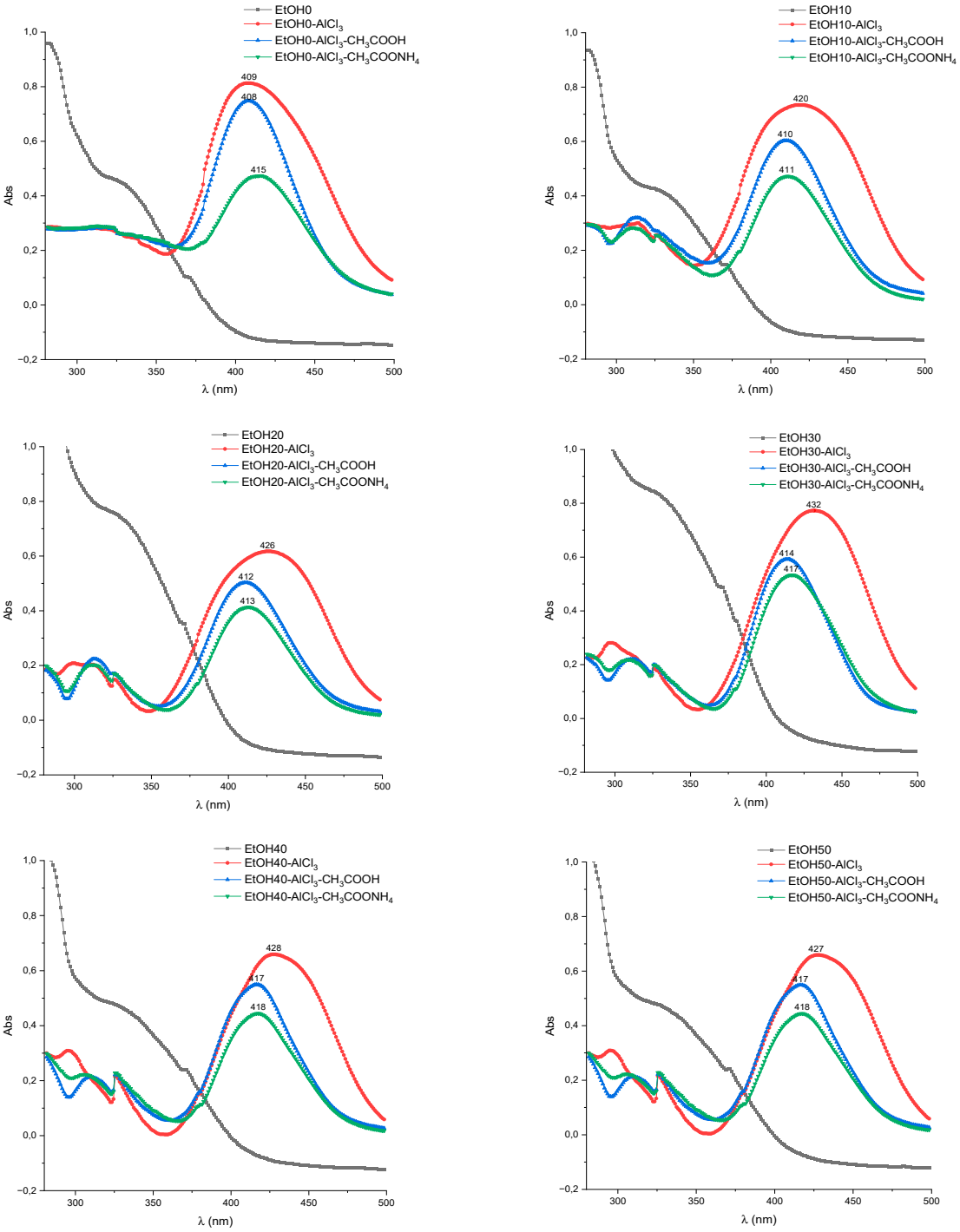
stoichiometric ratio of $AlCl_3$ molecules and flavonoids, the pH of the medium, the time and conditions (in darkness or daylight) of the reaction, etc. [21–26]. Due to the multifactorial dependence of the results of spectrophotometric quantification of flavonoids, the reproducibility of the technique receives special attention. Therefore, at the stage of the methodology development, the electronic absorption spectra of extracts and the spectra obtained by three methods that differed in conditions were taken (Figure 3). The values of the wavelengths of the spectra at which the maximum absorption occurs are indicated in Table 1.

All samples were tested under the same sample preparation conditions: reaction time of 30 minutes at room temperature without direct sunlight [27].



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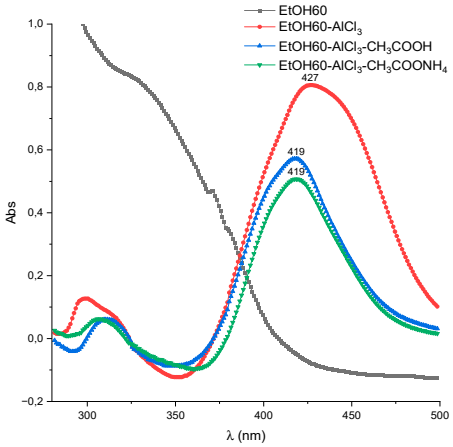

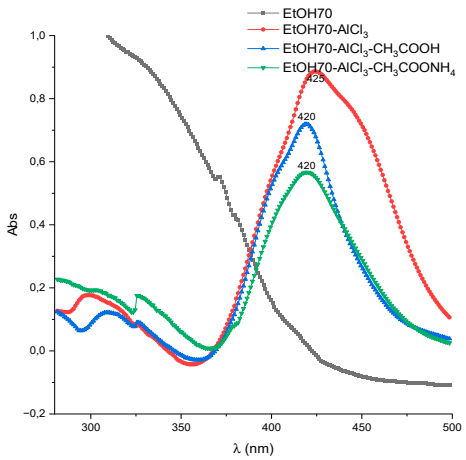

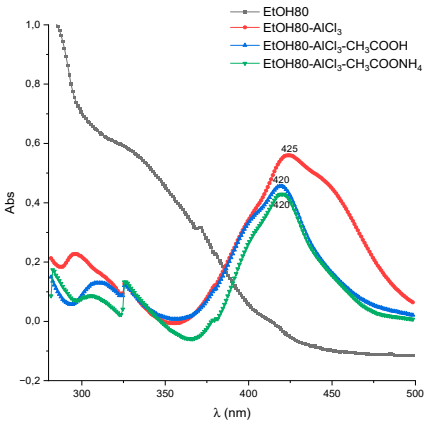

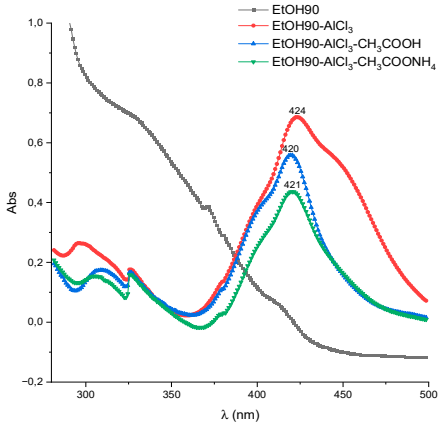

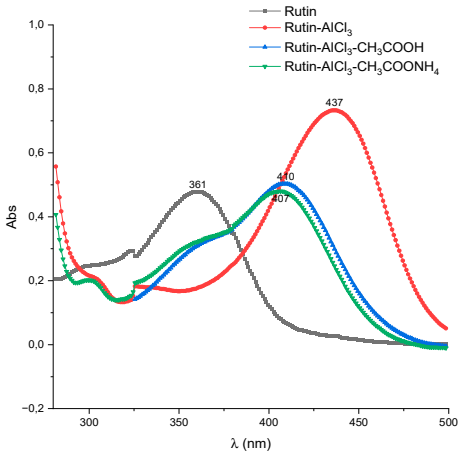

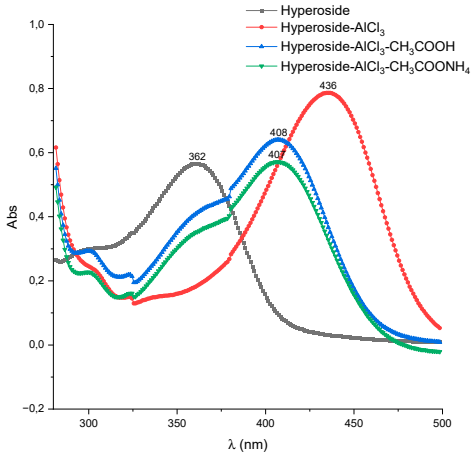

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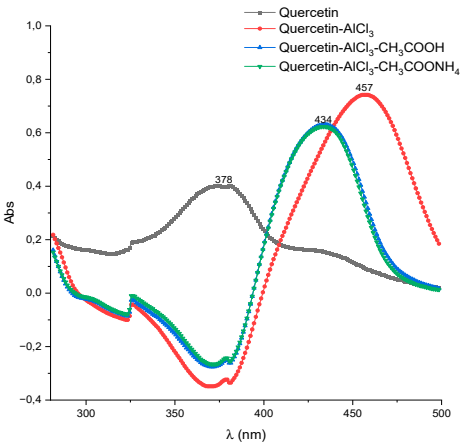

Figure 3. Absorption spectra of the test samples and aluminum chloride complexes.

Table 1. Wavelengths at which maximum absorption is observed.

Test specimen	Band I, λmax nm	Shift of band I, λmax nm		
		Method I ($AlCl_3$)	Method II ($AlCl_3$-CH_3COOH)	Method III ($AlCl_3$-CH_3COONH_4)
EtOH0	336-338	399-403	404-408	409-413
EtOH10	336-340	417-423	407-411	408-413
EtOH20	340-344	424-428	410-413	411-414
EtOH30	346-349	427-431	413-416	415-419
EtOH40	345-347	429-432	412-415	415-418
EtOH50	338-341	423-427	414-417	417-421
EtOH60	345-350	426-428	417-419	417-420
EtOH70	347-351	423-425	418-420	418-420
EtOH80	337-342	422-426	418-420	419-421
EtOH90	339-344	422-424	419-421	419-422
Rutin	358-361	435-439	407-410	404-408
Hyperoside	360-362	434-437	406-408	405-408
Quercetin	380	454-458	432-435	431-434

The study of the total flavonoids content in the analyzed samples, depending on the extractant used, was carried out in comparison with such standard samples as rutin, hyperoside, and quercetin. The total content flavonoids of Rhododendron tomentosum determined in dry extracts under the conditions of three methods in terms of rutin, hyperoside and quercetin are shown in Figure 4 and Table 2.

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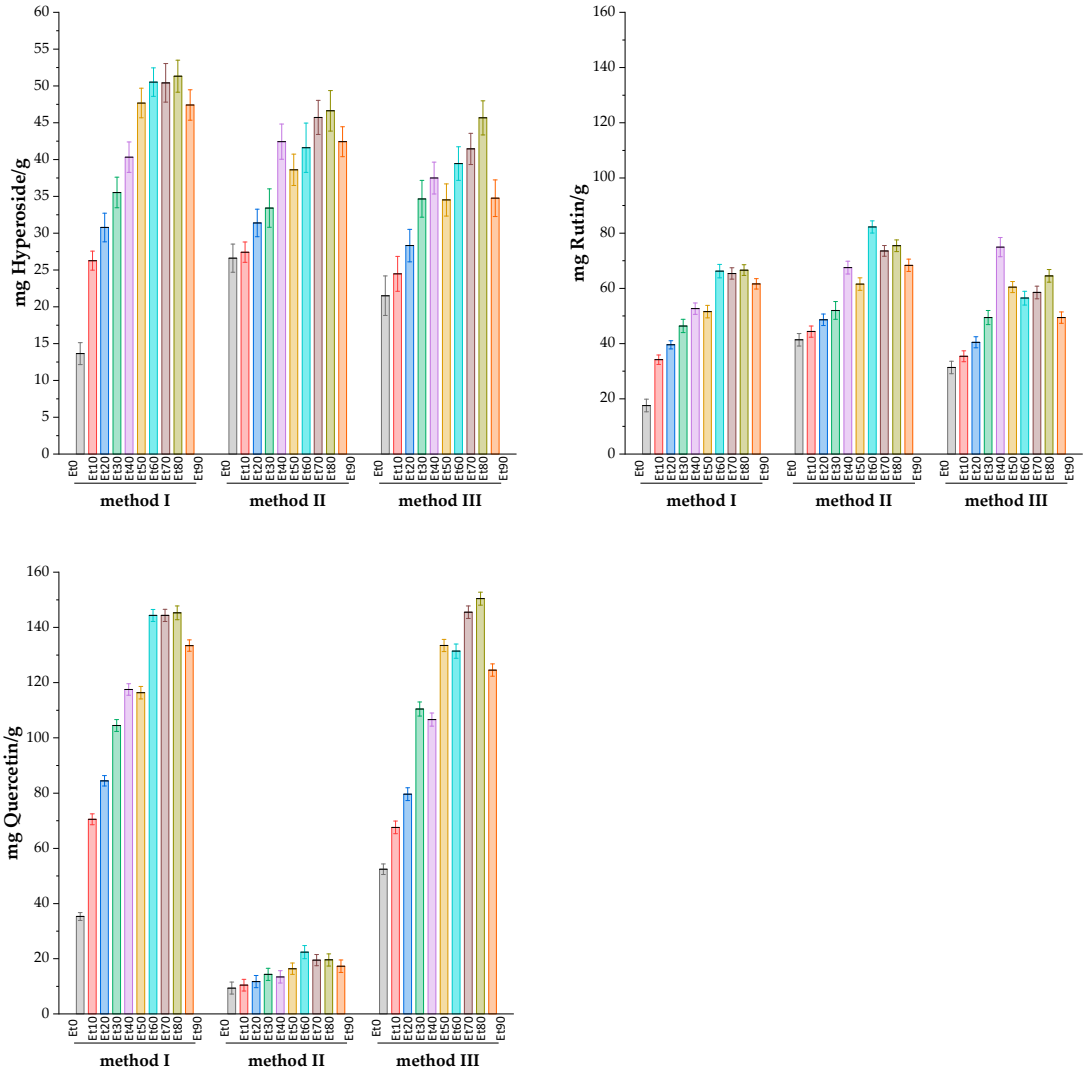

Figure 4. The total content flavonoids of *Rhododendron tomentosum* determined in dry extracts under the conditions of three methods in terms of rutin, hyperoside and quercetin.

Table 2. The total content of flavonoids in *Rhododendron tomentosum* extracts.

Sample	Weight of the dry extract sample, g/g	Total Flavonoids in dry extract of *Rhododendron tomentosum* in terms of standard, mg/g								
		Method I			Method II			Method III		
		mg R/g	mg H/g	mg Q/g	mg R/g	mg H/g	mg Q/g	mg R/g	mg H/g	mg Q/g
EtOH0	0.13	17.99±1.18	13.89±0.76	31.67±0.72	41.03±1.16	26.36±0.98	9.38±1.11	31.29±1.15	21.52±1.37	52.42±0.99
EtOH10	0.09	34.34±0.87	26.37±0.66	70.97±1.02	44.05±1.04	28.20±0.70	10.43±1.08	35.52±1.00	24.56±1.21	67.25±1.16
EtOH20	0.15	39.76±0.76	30.46±0.99	84.34±0.97	48.72±1.06	31.11±0.95	11.90±1.13	40.68±1.02	28.23±1.13	79.67±1.20
EtOH30	0.16	46.77±1.23	35.80±1.06	104.22±1.09	52.39±1.64	33.11±1.33	14.29±1.13	49.49±1.30	34.54±1.28	110.18±1.31
EtOH40	0.16	52.64±1.05	40.25±1.05	117.82±1.07	67.83±1.18	42.65±1.22	13.53±1.14	74.84±1.77	37.37±1.11	106.79±1.21

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EtOH50	0.18	51.56±1.16	47.96±1.02	116.76±1.14	61.77±1.13	38.85±1.09	16.24±1.06	60.43±1.02	34.68±1.11	133.36±1.14
EtOH60	0.17	66.05±1.23	50.60±0.98	144.51±1.11	82.10±1.12	41.08±1.70	22.31±1.22	56.43±1.27	39.57±1.17	131.02±1.32
EtOH70	0.18	65.35±1.05	50.18±1.33	144.38±1.11	73.62±0.99	45.78±1.18	19.63±1.03	58.94±1.16	41.34±1.08	145.39±1.17
EtOH80	0.11	66.79±0.99	51.31±1.11	145.35±1.27	75.45±1.09	46.86±1.40	19.79±1.12	64.98±1.17	45.75±1.18	150.77±1.19
EtOH90	0.13	61.96±0.96	47.71±1.05	133.80±1.04	68.21±1.13	42.20±1.04	17.35±1.15	49.48±1.05	34.88±1.26	124.61±1.13

3. Discussion

Flavonoids are a numerous class of secondary metabolites of plant raw materials, which today has about 8,000 identified individual compounds [28]. Flavonoids are classified on the basis of chemical structure into groups of flavones, flavonols, isoflavons, flavanones (dihydroflavones), flavanonols (dihydroflavonols), aurones, chalkones (Figure 5) [19].

Accordingly, each group of flavonoids differs in their chemical and spectral properties, which makes it impossible to use standard methods of quantification without the procedure for adapting the methodology for specific raw materials [29].

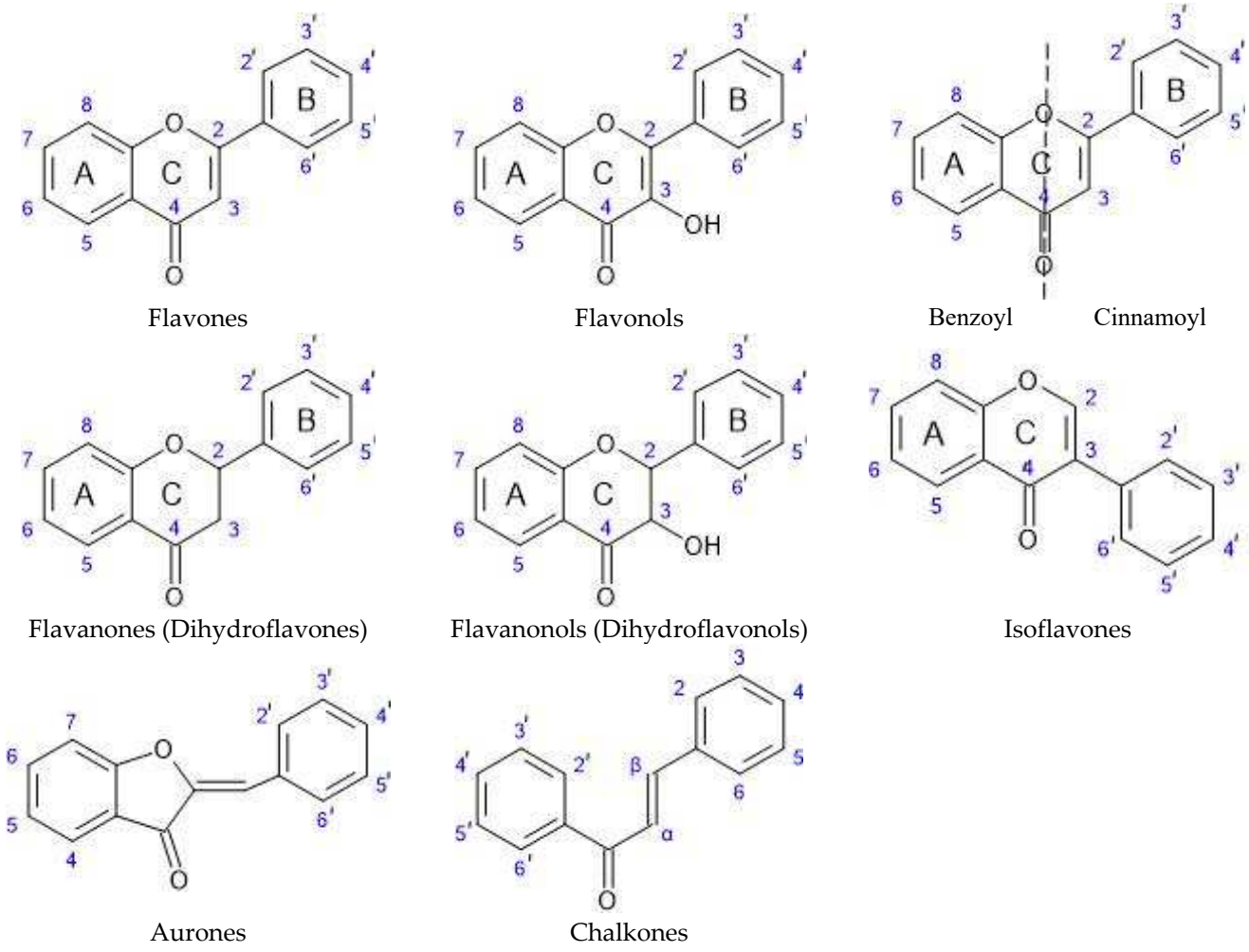

Figure 5. Basic chemical structure of flavonoids.

According to the literature, the spectra of flavonoids show two main absorption maxima in the 240-400 nm region [19]. These two maxima are commonly referred to as Band I (300-380 nm) and

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Band II (240-280 nm) and are present in the extracts under study (Figure 3). It is believed that Band I is associated with the absorption due to cinnamoyl system of the B-ring, and Band II is associated with the absorption due to the benzene system of the A-ring (Figure 5). The characteristic wavelength range for Band I for flavones is 304-350 nm, for flavonols (3-hydroxyl substituted) - 328-357, for flavonols (free 3-hydroxyl) - 352-385 nm [19]. Of the obtained by scanning the differential spectra of the extracts, the maximum absorption of a range of Band I is in the above range, which indicates the presence of flavones and flavonols in the extracts (Table 2). The spectra of the selected active flavonoid markers also have a maximum absorption in the specified range without the addition of $AlCl_3$.

According to the scientific literature, the reaction of complexation with $AlCl_3$ can potentially have several centers for the course, the localization of which is determined by the presence of a nearby hydroxyl and carbonyl group in the C-4 position of the C-ring [19,20,24,28]. 3-OH and C=O group ("3-4 site") or 5-OH and C=O group ("5-4 site") or a pair of 3'-OH and 4'-OH group of ring B ("3'-4' site") can participate in the binding of metal ions. Figure 6 shows possible ways of complexation using the example of the quercetin molecule [19].

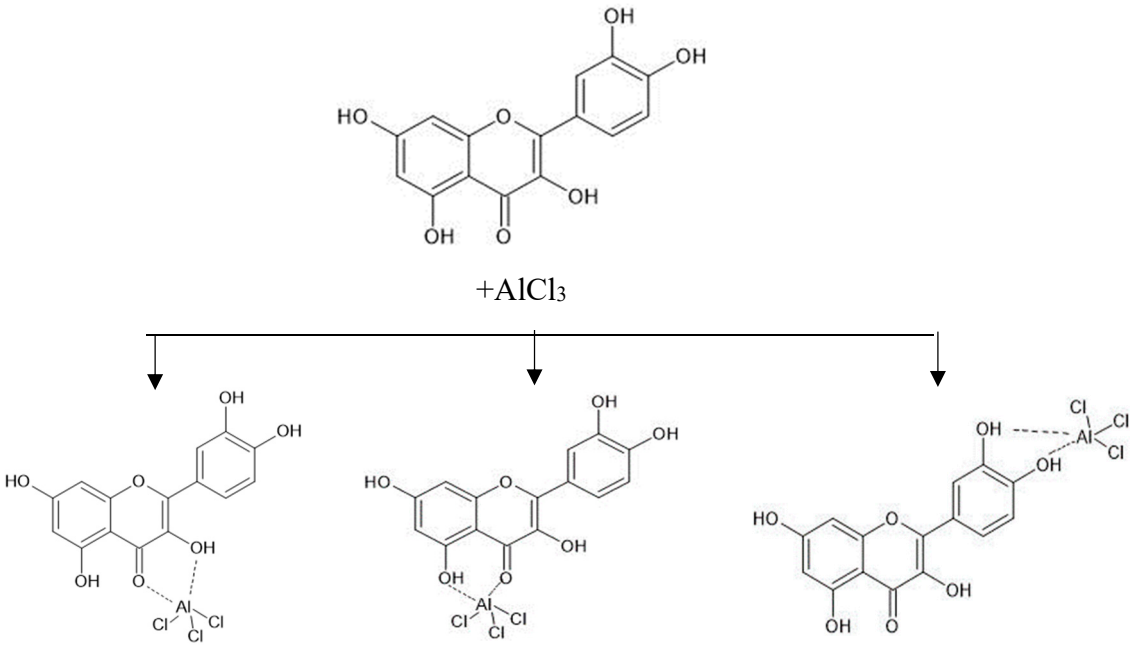

Figure 6. Diagram of possible focal points of binding between the quercetin molecule and $AlCl_3$.

A possible simultaneous substitution along the rings A and B, leads to a bathochromic shift of both bands and an increase in the intensity of absorption [19]. Rutin and hyperoside flavonoid molecules differ from quercetin in the presence of a glycosidic substituent attached to the oxygen atom at position 3, so rutin and hyperoside have a "5-4 site" and a "3'-4' site" to form complexes with aluminum ions.

The bathochromic shift of absorption maxima in the electronic spectra of both the test samples and the standard substances of rutin, hyperoside and quercetin compared to the published data in scientific articles [20,22,23] can be explained by the use of ammonium acetate instead of potassium or sodium acetates (method III) and different, twice greater concentration of $AlCl_3$ in all three methods.

Under the conditions of an increase in the concentration of $AlCl_3$ compared to those described in the literature [23], a significant bathochromic shift is observed, as a possible result of interaction at several binding coordination centers in the flavonoid molecule. According to the authors [25], the interaction of flavonoid molecules with aluminum ions occurs sequentially in the order of binding "3'-4' site" - "3-4 site" - "5-4 site" [25,30]. This means that the binding of aluminum ions to the site "3-4 site" will take place when all "3 '-4' sites" are occupied.

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Analysis of the obtained data (Figure 3 and Table 1) indicates that the introduction of donor substituents into the reaction mixture leads to a bathochromic shift of the range of Band I in all test samples. The value of bathochromic shift for extracts obtained by method I is in the range of 65-85 nm and maxima are observed at 420-431 nm. The values of bathochromic shift for test samples obtained by methods II and III practically coincide and are slightly less than 65-80 nm, and the absorption maxima are in the wavelength range of 405-420 nm.

The difference in the spectral behavior of flavonoids can be explained by the fact that flavones and flavonols from $AlCl_3$, which contain hydroxyl groups at C-3 and/or C-5 and C-4 ketol groups, form acid-resistant complexes, and acid-labile complexes with ortho-dihydroxyl systems (3′-OH and 4′-OH groups of ring B) [19,25]. The complexes formed between $AlCl_3$ and the ortho-dihydroxyl groups of ring B decompose in the presence of acid. Therefore, the presence of ortho-dihydroxyl groups in the B-ring of flavones and flavonols can be detected by comparing the spectrum of the flavonoid in the presence of $AlCl_3$ with the spectrum obtained from $AlCl_3$ in the presence of acetic acid, while a hypsochromic shift of Band I [19] is observed. The presence of the ortho-dihydroxyl system (3′-OH and 4′-OH groups of ring B) in the flavonoid structure is of great importance in the mechanism of implementation of the antioxidant action of flavonoids [29,31,32]. The presence of a 3'-4' -dihydroxyl group in isoflavones, flavanones, and dihydroflavonols cannot be detected using $AlCl_3$ in the UV spectrum, as the B-ring has little or no conjugation with the main chromophore – ring C [19].

It is somewhat more difficult to find an explanation in the literature regarding the role of acetic acid salts (sodium acetate and potassium acetate) in the reaction medium. Comparing the data of the scientific literature, it can be assumed that the addition of acetic acid salts to the reaction medium leads to the ionization of mainly 7- 4'- 3-OH groups of flavonoids, which also leads to a bathochromic shift of the range of Band II. In the case of substitution of 7-OH group, bathochromia is not observed even in the presence of free 4'- 3-OH groups [19,33,34].

Thus, by performing the complexation reaction of flavonoids with $AlCl_3$ in various reaction media under these experimental conditions, it can be assumed that there are such groups of flavonoids as flavones and flavonols with a potential antioxidant effect [35].

The total content flavonoids of Rhododendron tomentosum determined in dry extracts under the conditions of three methods in terms of rutin, hyperoside and quercetin are shown in Figure 4 and Table 2. As can be seen from the data, the use of quercetin as a standard substance to recalculate the total amount of flavonoids in Rhododendron tomentosum extracts under these experimental conditions can give both false-positive and false-negative results. The question of the dependence of the results of determining the total flavonoids content on the choice of the standard of flavonoids, experimental conditions and selected wavelengths for calculations is of critical importance and is discussed by the authors in publications [22–24]. Thus, the question of the dependence of the results of determining the amount of flavonoids on the experiment conditions and the selected wavelengths for calculations is of critical importance and affects the final result of the determination.

For the raw material of Rhododendron tomentosum shoots, according to the results of chromatographic and spectrophotometric studies, it is advisable to use rutin or hyperoside as a standard substance. Regardless of the selected markers and method, the largest amount of flavonoids was found in the samples obtained using 60%, 70% and 80% ethyl alcohol as an extractant.

4. Materials and Methods

4.1. Chemicals

All the chemicals and reagents used were of analytical grade. Ethanol, methanol, ethyl acetate, formic acid, ammonium acetate, glacial acetic acid and aluminum chloride hexahydrate were purchased from Poch S.A. (Gliwice, Poland). The standards of rutin, hyperoside, quercetin, rosmarinic acid, caffeic acid and chlorogenic acid were purchased from Sigma-Aldrich (Poland). HPTLC analyses were performed on 20cm×10cm HPTLC silica gel 60 F254 plates (Merck, Darmstadt, Germany).

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4.2. Plant Material

The shoots of *Rhododendron tomentosum* (*Ledum palustre*) were collected at the fruiting stage in the forest belts of the Rivne region (Ukraine, 51.302381 °N,26.509154 °E). The identity of the raw material was established by Nadiia Kovalska, assoc. prof. of the Department of Pharmacognosy and Botany of Bogomolets National Medical University. The voucher specimens of the plant have been deposited in the herbarium of the Department [36–38]. The shoots were shade dried at 25-35°C and stored in tightly closed containers.

4.3. Extracts Preparation

Extracts from Rhododendron tomentosum shoots were obtained using a modified method that combined the effects of ultrasound and temperature to maximize the extraction of biologically active substances from the raw material [11,39–41]. Extracts were prepared using different concentrations of hydroalcoholic mixture and purified water to determine the effect of ethanol concentration on the extraction of flavonoids in the form of aglycones and glycosides since glycosidic and aglyconic forms of flavonoids have different solubility [10,42,43]. The use of ethanol as an extractant is the most acceptable in the pharmaceutical industry for the manufacture of herbal medicines and does not require a test for residual amounts of extractants in plant extracts. About 5.0 g of raw material (exact weight), crushed to the size of particles passing through a 60 mesh sieve, placed in a flask with a thin section with a capacity of 100 mL, added 50 mL of the corresponding extractant. As an extractant ethanol of various concentrations was used: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or purified water. The flask was sealed and left to infuse for 24 hours at room temperature (21±1) °C. After infusion, ultrasonic extraction was performed by placing the flasks in an ultrasonic bath (Elmasonic S30H, Elma Schrnidbauer GmbH) for 30 min. Under the influence of ultrasound, there is an unauthorized increase in temperature, which at the end of the ultrasonic extraction of raw materials was (45±0.5) °C. The resulting extract was filtered through cotton wool so that the particles of raw materials did not fall on the filter. Extraction was carried out twice more under the conditions described above with 25 mL of fresh extractant, filtering the extracts into the same flask. For analytical purposes, the combined extracts were evaporated to a dry extract on a rotary evaporator (Heating Bath B-100, BUCHI) at a temperature of (45±0.2) °C and a vacuum of 0.01 MPa [44]. The resulting dry extracts were dissolved in 25 mL of methanol, filtered through a paper filter, and used for research as initial test samples.

For chromatographic studies, extracts (2 mL) were additionally filtered through a Millipore filter with a pore size of 0.45 μm. For the UV-Vis analysis, the original test samples were diluted with methanol, so that the maximum absorption at the wavelengths of interest (λmax) was within the range of 0.6-0.8.

4.4. Chromatographic Analyses

The test samples and standard substances were applied to the plates using an automatic HPTLC application device (Linomat 5, CAMAG, Muttenz, Switzerland). The sample application volume was 5 μL. Methanol solutions of standard substances were prepared at a concentration of: Rutin 1 mg/mL, Hyperoside 1 mg/mL, Quercetin 0.2 mg/mL, Rosmarinic Acid 1 mg/mL, Caffeic Acid 1 mg/mL and Chlorogenic acid 0.2 mg/mL.

Chromatographic separation was performed on HPTLC plates in a vertical glass chamber (CAMAG). Mobile phase ethyl acetate: formic acid: water (15:1:1) [14,45,46]. 70 mL of the mobile phase was used, the chamber saturation time with the mobile phase was 40 minutes. After the eluent covered the distance from the start line to the finish line, the plates were removed from the chamber and dried in an oven (105±2) °C. Detection was based on natural fluorescence before and after derivatization by sequentially spraying 2-aminoethyl diphenylborate (10 g/L) and macrogol 400 (50 g/L) in UV light at 254 and 366 nm. The obtained chromatographic images were analyzed using HPTLC software (visionCATS, CAMAG).

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4.5. Method for determining the total flavonoids content

The content of flavonoids was determined using differential spectrophotometry based on the formation of complexes of aluminum ions with flavonoids in a different reaction medium according to the method described in article [24]. Determination was carried out in methanol according to three methods that differed in the reaction medium - the first with the addition of only $AlCl_3$, the second - $AlCl_3$ and CH_3COOH and the third - $AlCl_3$ and CH_3COONH_4 [23,47,48].

Method I: 2 mL of methanol, 0.5 mL of the test extract sample (or standard) and 0.20 mL of a 10% solution of $AlCl_3$ in methanol were successively added to a 5.0 mL flask, held for 3 minutes and the volume of methanol was adjusted to 5.0 mL.

Method II: 2 mL of methanol, 0.5 mL of the test extract sample (or standard) and 0.20 mL of a 10% solution of $AlCl_3$ in methanol were successively added to a 5.0 mL flask, and held for 3 min. Next, 0.2 mL of a 1M solution of glacial acetic acid in methanol was added and the volume was adjusted to 5.0 mL with methanol.

Method III: 2 mL of methanol, 0.5 mL of the test extract sample (or standard) and 0.20 mL of a 10% solution of $AlCl_3$ in methanol were successively added to a 5.0 mL flask, and held for 3 min. Next, 0.2 mL of a 1M solution of ammonium acetate in methanol was added and the volume was adjusted to 5.0 mL with methanol.

Compensation solutions were prepared similarly but without the addition of $AlCl_3$.

The reaction mixtures were thoroughly stirred, held for 30 minutes at room temperature and subjected to spectrophotometric analysis in the range of 280 to 500 nm (U-3900/3900H Hitachi).

The total content of flavonoids in the studied extracts was calculated by the standard method in terms of rutin or hyperoside, or quercetin according to the formula:

$$X, mg/g = \frac{A \cdot m_{St} \cdot K}{A_{St} \cdot m} \cdot 1000 \quad (1)$$

where X is the total content of flavonoids in terms of rutin or hyperoside, or quercetin, mg/g;

K – dilution factor;

A – absorption at 437 nm (method I), 409 nm (method II), 406 nm (method II) for conversion to rutin; at 436 nm (method I), 407 nm (method II), 407 nm (method II) for conversion to hyperoside; at 456 nm (method I), 434 nm (method II), 433 nm (method II) for conversion to quercetin;

Ast – absorption of solutions of standard samples of flavonoids at the same wavelengths.

4.6. Statistical analysis

The mean and standard deviation (SD) were calculated according to the monograph "Statistical Analysis of the Results of a Chemical Experiment" of the State Pharmacopoeia of Ukraine [49]. The average value was established based on 3 measurements. Values of the confidence interval were calculated using Student's criterion limit. The data are presented as the mean ± SD [46].

5. Conclusions

Using an inexpensive, fast, and reproducible technique HPTLC and using test markers, the presence of substances of flavonoid structure: rutin, hyperoside and quercetin, and hydroxycinnamic acids chlorogenic acid were established in all the studied samples. The obtained results show that the HPTLC method of choice for the analysis of plant herbal extracts.

Spectrophotometric techniques are proposed that are based on the reaction of complexation of flavonoids with aluminum chloride, using appropriate standard samples of flavonols rutin, hyperoside, and quercetin. It has been proved that the course of the complexation reaction and, accordingly, the quantification of the flavonoid structure substances by the spectrophotometric method is influenced by a number of factors, including the environment, pH, the structure of the complexes, etc.

Regardless of the chosen method, for the further production of *Rhododendron tomentosum* extracts with a higher content of flavonoid structure substance, it is advisable to use 60%, 70%, and 80% ethyl alcohol as extractant.

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