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Development of methods of standardization of medicinal plants – Pyrola rotundifolia leaf

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Abstract
The study object was medicinal herb - Pyrola rotundifolia leaf. To identify biologically active substances in medicinal plants, they used the thin-layer chromatography (TLC). TLC studies used a chromatographic plate Silica Gel 60 F254 (Mersk, Germany), a chromatographic chamber CAMAG, a samples applicator Linomat 5 (CAMAG, Switzerland), a UV lamp to view chromatograms CAMAG, and standard examples of flavonoids and phenolcarbonic acids (Sigma-Aldrich). Accurately weighed quantities of standard identification samples were dissolved in appropriate volumes of methanol and rutin - to quantify in ethanol (70% (v / v)). For quantitative determination of flavonoids, they used the differential spectrophotometry method with a spectrophotometer Cary-50. Content of flavonoids was converted in rutin.
Applying modern approaches to standardization of medicinal plants, they processed methods of identification and quantification of flavonoids in Pyrola rotundifolia leaf. An identification criterion to be incorporated into the quality control technique applicable to medicinal plants is TLC determined flavonoids. They substantiated and experimentally proved that a quantitative indicator of Pyrola rotundifolia leaf is flavonoid content, a quantitative criterion of which still to be determined through analysis of more samples.
Based on modern approaches to standardization of herbal medicines, they have processed methods for identification and quantification of flavonoids in medicinal plants – Pyrola rotundifolia leaf.
They offered incorporating the TLC-based flavonoids identification method into the quality control procedures applicable to Pyrola rotundifolia leaf; identification markers shall be availability of four flavonoids, including hyperoside and luteolin-7-O-glucoside, and three phenol-glycosides, which may be arbutin derivatives.
Methods to control quality of medical plant shall include quantitative determination of flavonoids by the spectrofluorimetric method. However, to establish quantitative criterion, further research is required using more samples of Pyrola rotundifolia leaf from different vegetation regions.

Keywords: Pyrola rotundifolia leaf, medical plant, standardization, flavonoids

1. Introduction
Diseases of the genitourinary system rank fourth (5.5%) in the overall morbidity rate in Ukraine, second only to cardiovascular diseases (31%), respiratory (19.8%) and digestive system (9.9%). The structure of chronic kidney disease is dominated by chronic pyelonephritis and glomerulonephritis. Each year, the number of secondary nephropathies increases - kidney lesions in other diseases, especially diabetes and hypertension. According to statistics, about 10% of the world population suffers chronic kidney disease, which in many cases leads to disability and death. In connection with development of chronic kidney failure, the number of patients in need of the substitutive renal therapy grows from year to year (that is, hemodialysis, peritoneal dialysis, kidney transplantation). Such methods are extremely expensive and their use is the global economic burden. Therefore, it is very important to prevent, detect and adequately treat development of kidney diseases [1].
Search for effective remedies to prevent and treat the genitourinary system diseases is a pressing issue. It is especially important to develop drugs having combined pharmacological effects, namely combining antimicrobial, anti-inflammatory, and renoprotective properties. The development of drugs based on herbal materials was steadily growing in the past decade as they have mild pharmacological effect, low toxicity, long-term applicability and the lack of severe side effects. In this regard, an interesting object for research is Pyrola rotundifolia - a medicinal herb, which can be used for treatment of urogenital diseases [2, 3]. No drug composing Pyrola rotundifolia has been registered in Ukraine; also, it does not offered as herbal teas [4].

A number of foreign researchers studied properties of the medicinal plant - Pyrola rotundifolia leaf; they established a wide spectrum of pharmacological activity of the herbal raw material. The traditional medicine uses Pyrola rotundifolia leaf and grass as a diuretic, antiseptic, anti-inflammatory, hemostatic, stomachic, astringent, analgesic, and healing treatment [5]. From the literature it is known that leaf of Pyrola rotundifolia contain phenols and their derivatives (arbutin, hydroquinone, homo-arbutin, iisohomoarbutin, metylarbutin, dimeric-phenolic-glycoside-pyrolaside A, and trimeric phenolic-glycoside-pyrolaside B), flavonoids (kaempferol, quercetin, hyperoside), phenolic and hydroxycinnamic acids (protocatechic, trioxybenzoic, methoxycinnamic), tanning agents (in fall laves up to 18.6%) [5-7].

The authors [8] revealed that Pyrola rotundifolia leaf contain hyperoside, rutin, quercetin, luteolin, dihydroquercetin, luteolin-7-glucoside. Among phenolic acids and hydroxycinnamic ascids, Pyrola rotundifolia contains gallic, chlorogenic, chicory, ferulic, neochlorogenic, cinnamylic acids. The experiment found the presence of analgesic and anti-inflammatory activity in ursul acid and himafilin present in Pyrola rotundifolia leaf [9] and antibacterial properties of pyrolaside B [6].

Any herbal plant having a pharmacological effect and prospects for new drug development should be standardized, which would allow monitoring the quality and obtaining the expected efficacy and safety. Selection and quality profiling of raw materials is one of the most important stages of standardization. This is a top propriety issue for non-pharmacopoeial species of medicinal plants not covered by a corresponding monograph in SPU or foreign Pharmacopoeias. Pyrola rotundifolia of Ericaceae bloodline is of such kind of herbal plants.

Research of phenolic compounds conducted at different times by many scientists allows distinguishing three main classes of compounds - phenolic-glycosides (arbutin and derivatives), flavonoids, proanthocyanidins. Recent papers devoted to the comparative pharmacognostical study of three types of Pyrola (Pyrola minor, Pyrola media and Pyrola rotundifolia) revealed that these species could be distinguished by composition of flavonoids, which differ for the three types more than phenolic-glycosides [5].

No systematic studies have been conducted on the basis of pharmacopoeia approaches to research and standardization of Pyrola rotundifolia leaf. Therefore, the study of standardization of the Pyrola rotundifolia raw leaf is of high relevance, especially by identifying and determining the content of flavonoids.

The aim is develop methods for identification and quantification of flavonoids in Pyrola rotundifolia leaf.

2. Materials and methods

To study flavonoids of Pyrola rotundifolia leaf, they have selected 5 samples of raw materials from Ukraine and Russia (see Table 1). Content of flavonoids and their identification was examined by the thin-layer chromatography method (TLC), which is generally accepted pharmacopoeial approach. Among the mobile phases for TLC-based research of flavonoids, as described in the Pharmacopoeia, the optimal one is a solvent mixture of anhydrous formic acid R - water R - ethyl acetate R (10:10:80).

Methods of identification of the medicinal herbal substance - Pyrola rotundifolia leaf.

Test solution: Place 1.0 g of powdered material in a 50 ml conical flask, add 25 ml of methanol R, boil in water bath under reflux for 1 h and filter.

Reference solution: 2.5 mg of rutin, hyperoside, luteolin-7-O-glucoside and quercitrin each dissolved in 10.0 ml methanol.

Apply 20 ml test solution and 5 ml reference solution on a start line of a chromatographic plate in 10 mm strips. Place a plate in a chamber with a mixture of solvents - formic acid anhydrous R - water R – ethyl acetate R (10:10:80). When a solvent front passes 12 cm from the starting line, a plate is removed from the camera.

Drying: dry in air and then maintained at a temperature 100 ° C to 105 ° C for 2 min.

Extraction: spray a warm plate with a 10 g/l aminoethyl diphenylborinate solution R in methanol R, air dry and view in ultraviolet light with a 365 nm wavelength.

Results: the chromatogram of reference solution shows four fluorescent orange zones, corresponding to rutin, hyperoside, luteolin-7-O-glucoside and quercitrin (bottom-up).

The test solution chromatogram should show two intense orange fluorescent zones corresponding to the hyperosideand luteolin-7-O-glucoside zones in the reference solution chromatogram; a very intense blue fluorescent zone slightly above the rutin zone in the reference solution chromatogram; two weaker fluorescent orange zones above the quercitrin zone in the reference solution chromatogram, two intense blue zones - one zone located below the quercitrin zone in the reference solution chromatogram and another one above the two upper orange zones of flavonoids in the test solution chromatogram. The following is the zones sequence in the test solution and reference solution chromatograms (Fig. 1).

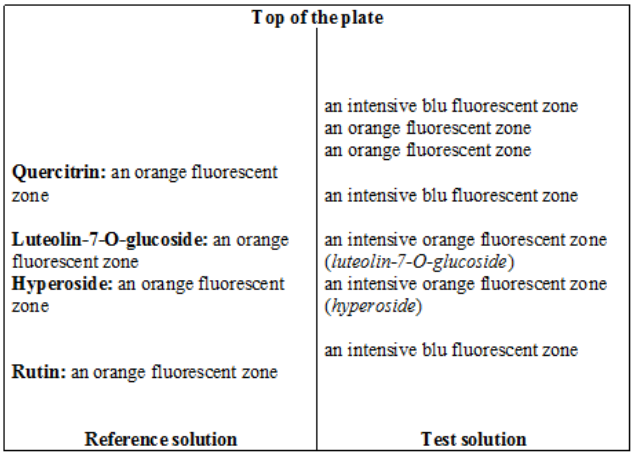

Fig 1: Scheme of chromatograms for identification of flavonoids in Pyrola rotundifolia leaf after treatment with aminoethyl diphenylborinate solution and macrogol 400 solution when viewed in the ultraviolet light with 365 nm wavelength.

Methods of quantitative determination of flavonoids in Pyrola rotundifolia leaf

Primary solution. They place 1.0 g (exact weight) of powdered materials in 100 ml flat-bottomed flask, add 60.0 ml ethanol (70% (v / v)) and heat in water bath under reflux for 40 minutes. Then cool down a flask, filter the resulting extract through a paper filter in a 100 ml volumetric flask, and decant liquid. Add 20.0 ml ethanol (70% (v / v)) to a solvent

cake and continue boiling in water bath under reflux for 20 minutes. Then cool down a flask, filter an extract in the same volumetric flask, combining the filtrate. Add 15.0 ml of ethanol (70% (v / v)) to a solvent cake in the flask and continue boiling in water bath under reflux for 15 minutes. Then cool down a flask, filter the extract in the same volumetric flask, combining the filtrate. Wash a cake and the filter with ethanol (70% (v / v)), bring the filtrate volume in a volumetric flask to the mark, mix.

Test solution. They put an aliquot (V_a = 2.5-5.0 ml) of the *primer solution* in a 25 ml volumetric flask, add 3.0 ml 3% alcohol (70% (v / v)) solution of aluminum chloride and dilute the obtained solution with ethanol (70% (v / v)) to the mark, mix.

Compensation solution. They put an aliquot (V_a = 2.5-5.0 ml) of the *primer solution* in a 25 ml volumetric flask and dilute the obtained solution with ethanol (70% (v / v)) to the mark, mix.

Standard sample solution of rutin. They put 0.05 g (exact weight) of the standard rutin sample (SRU) in a 100 ml volumetric flask, add 70 ml of ethanol (70% (v / v)), dissolve and dilute with the same solvent to the mark and mix.

Reference solution. They put 1.0 ml *standard rutin sample solution* in a 25 ml volumetric flask, add 3.0 ml of 3% alcohol (70% (v / v)) solution of aluminum chloride and dilute with *ethanol* solution (70% (v / v)) to the mark, mix.

Compensation solution. They put 1.0 of *standard rutin sample solution* in a 25 ml volumetric flask, and dilute with *ethanol* solution (70% (v / v)) to the mark, mix.

Optical density of the test solution and reference solution is measured in 45 minutes after preparing at a wavelength 410 nm ± 2 in relation to compensation solutions for each respective solution.

The percentage content of flavonoids amount (X) in terms of rutin and dry raw material is calculated using the formula:

$$X = \frac{A_X \cdot m_0 \cdot 100 \cdot 100}{V_a \cdot A_0 \cdot m \cdot (100 - W)}$$, where

A_X - optical density of the test solution;

A_0 - the optical density of the reference solution;

m_0 - mass of the standard rutin sample, in grams;

m - mass of Pyrola rotundifolia leaf sample, in grams;

V_a - aliquot volume of the primary solution taken for analysis, in milliliters;

W - loss in weight on drying Pyrola rotundifolia leaf, in percentage.

3. Results and discussion

Chromatograms obtained for methanol extractions from the study material, shown a clear division of flavonoid zones, which allowed selecting this solvent system to identify unknown BAS as identification markers for Pyrola rotundifolia leaf. Chromatograms of test solutions for five investigated samples of materials and standard flavonoid solutions are presented in Figure 2.

As seen from the chromatograms, all studied samples coming from different places of growth have identical chromatographic profiles, allowing us to suggest such profile for identification of Pyrola rotundifolia raw leaf. Our studies confirm the presence of hyperoside and luteolin-7-O-glucoside in all samples of raw materials, so these flavonoids have been offered as markers for identification of raw materials.

When developing methods for TLC identification of flavonoids, they selected methanol as a solvent that dissolves studied BAS and provides rapid coating. A comparison solution was hyperoside and luteolin-7-O-glucoside (cynarozid) as marker substances being identified in all samples, and quercitrin (quercitrozide) and rutin. The two latter compounds were applied to clarify the chromatographic position of some unidentified flavonoids and phenolic compounds, which may be phenolic-glycosides - arbutin derivatives. This approach allows uniquely characterizing flavonoid and phenolic- glycosidic composition of identification materials.

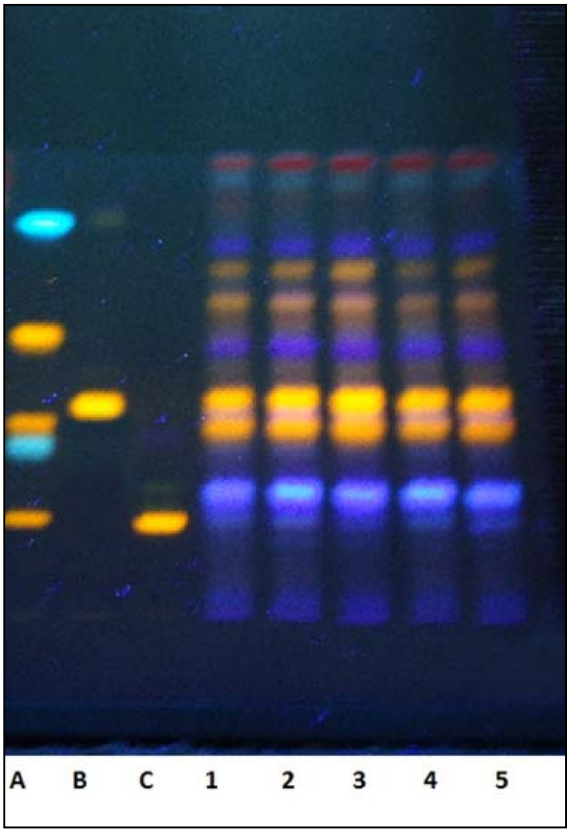

Fig 2: Chromatogram obtained for different samples of herbal substances - Pyrola rotundifolia leaf during identification of flavonoids: A – standard sample solution of rutin, chlorogenic acid, hyperoside, quercetin, caffeic acid; B - standard sample solution of luteolin-7-O-glucoside; C - caffeic acid of rutin; 1-5 - test solutions for samples 1-5; chromatogram processing with aminoethyl diphenylborinate solution; viewed at a wavelength of 365 nm.

Chromatographic results and literature data [5] point to the high content of flavonoids in raw materials that justifies their use as a quantitative measure for Pyrola rotundifolia leaf quality.

Electronic absorption spectra of alcohol extractions from the raw demonstrate the maximum absorption range 410-412 nm in the presence of aluminum chloride (Fig. 3), coinciding with maximum rutin absorption in similar conditions. The latter allows recalculating of the flavonoids amount into rutin, which is an available standard.

Previously, they researched into influence of ethanol concentration in an extractant to flavonoid extraction, and selected 70% ethanol for sample preparation. Also, they selected a weighted amount yielding the optimal optical density of the test solution.

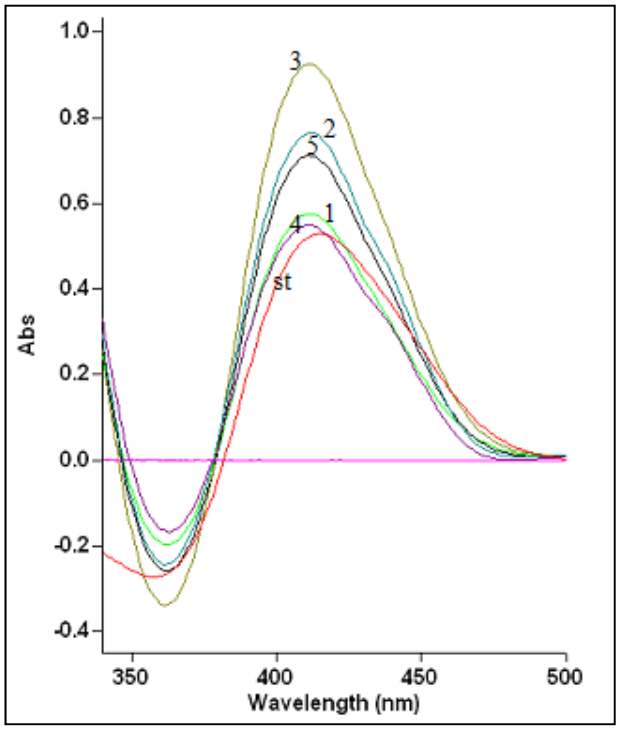

Fig 3: Electronic absorption spectra of test solutions (1.5) and a standard rutin sample (st), obtained during quantitative determination of flavonoids in Pyrola rotundifolia leaf

Table 1: Results of the quantitative determination of flavonoids in Pyrola rotundifolia leaf

Sample	Content of flavonoids amount in terms of rutin, %	Loss in weight on drying Pyrola rotundifolia leaf, %
1. Narodofarma, Kropyvnytskyi town (Ukraine)	1.52 ± 0.02	5.42 ± 0.02
2. Planeta Trav, "Kropyvnytskyi town (Ukraine)	2.11 ± 0.01	8.51 ± 0.01
3. Herbs, Lutsk city (Ukraine)	2.45 ± 0.02	9.77 ± 0.01
4. Horst, Barnaul city (Russia)	1.47 ± 0.02	7.01 ± 0.02
5. Ivano-Frankivsk region, Ukraine	1.93 ± 0.01	7.90 ± 0.01

When comparing results of study of flavonoids in Pyrola rotundifolia leaf with the literature, one may claim the confirmed presence of luteolin-7-O-glucoside (cynaroside) and hyperoside in the raw material. Authors [10] have revealed that Pyrola rotundifolia contains luteolin-6-O-glucoside (stereolensin). Two flavonoids were found in butanol fractions in the study [11], including hyperin (quercetin-3-O-β-D-galactoside) and quercetin-3-O- β-D-galactoside-2"-gallate.

As regards the quantitative content, results well coincide with the spectrophotometric data analysis on raw material samples from different regions of growth. Studied five samples of Pyrola rotundifolia leaf contain 1.5-2.5% flavonoid in terms of rutin, while according to the authors [5], content was 1.96% in terms of rutin for samples growing in Brest and Grodno regions of Belarus; from 0.605% to 2.290% in terms of rutin for samples collected in Belgorod and Yaroslavl regions of Russia. In total, they have analyzed 19 samples of raw materials [8]; from 0.93% to 2.79% in terms of quercitrin for samples originating from different areas of the Altai Territory; 6 samples of raw materials were analyzed 6 [3]. Somewhat higher value obtained by [3], is explained by different spectral characteristics of aglycone - aluminum chloride complex. To establish a quantitative criterion of quality, one should continue research on more samples from different regions of growth.

Based on the results of research of Pyrola rotundifolia leaf, it was resolved to standardize it by flavonoids. To this end, they have developed a TLC-based identification method and the spectrophotometric method of quantitative determination.

4. Conclusions

Based on modern approaches to standardization of herbal medicines, they have processed methods for identification and quantification of flavonoids in medicinal plants – Pyrola rotundifolia leaf.

They offered incorporating the TLC-based flavonoids identification method into the quality control procedures applicable to Pyrola rotundifolia leaf; identification markers shall be availability of four flavonoids, including hyperoside and luteolin-7-O-glucoside, and three phenol-glycosides, which may be arbutin derivatives.

Methods to control quality of medical plant shall include quantitative determination of flavonoids by the spectrofluorimetric method. However, to establish quantitative criterion, further research is required using more samples of Pyrola rotundifolia leaf from different vegetation regions.

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