



Optimization of Extraction of Polyphenolic Compounds from Medicinal Lungwort (*Pulmonaria officinalis* L.)

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Authors' contributions

This work was carried out in collaboration among all authors. Author LSD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AMF and VFD managed the analyses of the study. Author AYP managed the literature searches. All authors read and approved the final manuscript

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ABSTRACT

Medicinal plants are potential natural sources of biologically active substances that have geroprotective properties, slowing down the aging process. Vegetable raw materials are used as antioxidants in the food industry. *Pulmonaria officinalis* L. has a high content of biologically active substances. The purpose of this work is to determine the operating parameters for extracting the maximum amount of biologically active substances from *Pulmonaria officinalis* L. by extracting ethanol and finding sources of natural antioxidants. The antioxidant activity of several ethanol extracts of *Pulmonaria officinalis* L. – 30-70% with a step of 10 ethanol obtained from the dried root culture of the medicinal plant of biologically active substances was determined. The total content of polyphenols, flavonoids and proanthocyanidins was estimated. According to the data obtained, the relationship between the values determined for the biologically active substances presented and the values of antioxidant activity was revealed. Extracts of *Pulmonaria officinalis* L. showed a high

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yield of polyphenolic compounds-889.39±4.29 mcg of Gallic acid/ml, flavonoids-728.90±6.98 mcg of rutin/ml, proanthocyanidins-211.65±5.31 mcg of catechin/ml at an ethyl alcohol concentration of 60% and an extraction temperature of 50°C in 4 hours. The antioxidant activity using 2 methods: A spectrophotometric method DPPH (2,2-diphenyl-1-picrylhydrazyl) and the FRAP method (iron reducing/antioxidant power) was investigated. During DPPH spectrophotometric analysis, the antioxidant activity is equal to 86.96% for polyphenols, 75.47% for flavonoids, and 51.25% for proanthocyanidins. FRAP analysis showed that extracts with operating parameters had a pronounced antioxidant activity ($\tau_3= 4$ ч; $T_3= 50^\circ\text{C}$; $C_3= 60\%$)и ($\tau_3= 5$ ч; $T_3= 60^\circ\text{C}$; $C_3= 70\%$).

Keywords: *Pulmonaria officinalis* L.; extraction; polyphenols; flavonoids; proanthocyanidins; spectrophotometry; antioxidant activity.

1. INTRODUCTION

Due to adverse environmental conditions, there is a growing tendency to use plants, including wild plants containing a large amount of biologically active substances (BAS) [1,2,3]. For example, phenolic acids, flavonoids, anthocyanins, tannins, vitamins and carotenoids, which can be used as pharmacologically active products with geroprotective activity [4].

BAS of medicinal plants have a high pharmacological activity [2,3,4]. BAS include: substances of primary synthesis (vitamins, lipids, carbohydrates), substances of secondary synthesis (essential oils, glycosides, saponins, alkaloids, flavonoids, proanthocyanidins, tannins, lignans, etc.) [1,2,5].

Extraction preparations obtained from medicinal plant raw materials: tinctures, decoctions and extracts contain several groups of BAS and participate in the pharmacological effect [4,6,7]. For example, flavonoids have anti-inflammatory and antimicrobial effects, carotenoids promote rapid healing of damaged tissues, and proanthocyanidins have hepatoprotective properties [5]. For directed pharmacological action, it is necessary to fully extract individual groups of BAS from plant raw materials, carried out using a variety of techniques. When sequentially extracted from plant raw materials, you can get preparations containing different groups of BAS [8,6,7,9].

A large group of methods aimed at isolating BAS refers to traditional methods of extracting plant raw materials [10]. These are cold and hot pressing [11,12], water-steam [13], water-alcohol extraction [14]. There are modern methods of extraction: ultrasonic [15], high frequency and ultra-high-frequency [16], eletroplasmolysis and

electrodialysis [17], extraction with liquefied gases [18]. Modern extraction methods have a high yield of high-quality finished products, reduced production space, and reduced labor costs [15,16]. However, they have one significant drawback that is the high cost of equipment [17, 18]. Traditional extraction methods use simple and inexpensive equipment. The main disadvantages of traditional extraction methods are labor intensity, long duration and excessive content of ballast substances [11,12,13,14].

Among the numerous varieties of medicinal wild plants in Siberia, the plant *Pulmonaria officinalis* L. is of considerable interest [19]. The represented wildflower is a herbaceous perennial plant belonging to the *Boraginaceae* family, widely distributed in the Siberian Federal district. *Pulmonaria officinalis* L. has found traditional therapeutic use in the treatment of bronchitis, viral diseases, headache, laryngitis, kidney and respiratory diseases, as well as stomach and duodenal ulcer [20,21,22,23].

The presence of BAS in *Pulmonaria officinalis* L. contributes to the inhibition of active enzymes tyrosinase and acetylcholinextraza, which has been used in the treatment of Parkinson's and Alzheimer's diseases [23,24,25,26].

Pulmonaria officinalis L. has useful properties due to the presence in its composition of a wide range of chemical compounds, namely flavonol substances, ascorbic and silicic acid, saponins, tannins, carotene, allantoin, rutin and mucous substances [20,27,28]. The plant contains unsaturated pyrrolizidine alkaloids, so this plant is not recommended for long-term consumption [29]. As *Pulmonaria officinalis* L. has a fairly diverse chemical composition, so the proposed plant can be used to create medicines or new functional products [30,31,32,33].

Antioxidant activity is an important property of the plant extract *Pulmonaria officinalis* L., as scientists are looking for sources of natural antioxidants, which are included in many cosmetic, pharmaceutical and food formulations. In recent years the analysis of new sources of antioxidants has led to extensive research on medicinal plants [30,34,35,36].

The purpose of this work is to determine the current parameters for extracting the maximum amount of BAS from *Pulmonaria officinalis* L. by extracting ethanol and finding sources of natural antioxidants.

The scientific novelty is the selection of working parameters for the extraction of root culture *Pulmonaria officinalis* L. in order to use the extract in the creating of functional food products with geroprotective properties.

2. MATERIALS AND METHODS

Root cultures of *Pulmonaria officinalis* L. grown in vitro on liquid nutrient media in bioreactors, obtained at the early stages of the study, were used as research objects.

Matrix for matching the selected extraction parameters: Extraction temperature (T_3 , °C), extraction time (τ_3 , ч) and concentration of ethyl alcohol (C_3 , %), for *Pulmonaria officinalis* L., are presented in Table 1.

In this study, water was purified using a bidistillator of the TU 25-11.1592-81 BS brand (Russia, Labinvest).

The root culture of *Pulmonaria officinalis* L. was dried and ground at a rotary mill LZM-1M (Russia, OLIS) to the size of a fraction of 1 mm. Pulverized *Pulmonaria officinalis* L. powder taken in an amount of 3 g was extracted in 260 ml of ethyl alcohol under statistical conditions to obtain BAS. To do this, the prepared sample was placed in a round-bottomed flask of 500 ml, then poured ethyl alcohol of a certain concentration (Table 1) in an amount of 130 ml. The suspension was mixed and attached to an ascending refrigerator. The assembled structure was placed in a water bath EKROS PE-4310 (Russia, EKROSHIM) with a set temperature (Table 1).

After the set time, the extraction was filtered through a paper filter "Yellow tape" (Russia, Sartogosm) with a pore size of 8-12 microns in a measuring flask with a capacity of 250 ml so that the particles of vegetable raw materials did not fall on the filter. Next, 130 ml of ethyl alcohol was added to the vegetable raw material in the same round-bottomed flask and repeated extraction was performed under the conditions described above, and the extraction was filtered into the same measuring flask. The obtained extracts of *Pulmonaria officinalis* L. were sterilized at 85°C for 15 minutes. Samples of extracts were stored at room temperature in a dark place.

2.1 Determination of Polyphenol Content

The total content of polyphenols in the extract of *Pulmonaria officinalis* L. was determined by the Folin-Ciocalteu method [37]. The essence of the method is a reducing agent of phosphoric-tungstic acids contained in the Folin-Ciocalteu reagent (Spain, Panreac Applichem). These acids interact with oxidizing OH groups of phenol.

0.3 ml of distilled water was added to 0.2 ml of the extract and mixed with 0.5 ml of the Folin-Chocalteu reagent. Then 2.5 ml of 20% sodium carbonate solution (Ukraine, SILUR) was added to the mixture. In 30 minutes, light absorption was measured at room temperature using a UV-1800 spectrophotometer (Germany, Shimadzu) at a wavelength of 725 nm. Subsequently, a calibration curve was constructed based on data from standard solutions of Gallic acid (France, "AixLab") in the range from 0.05 to 0.40 mg/ml.

The total polyphenol content of the extract was calculated and stated in milligrams of gallic acid equivalents per gram of dry weight based on the standard gallic acid curve ($y = 6,35; A = -0,052; R^2 = 0,9994$, where y – concentration in mg/ml, A – absorption).

2.2 Determination of Flavonoid Content

The concentration of flavonoids in the extract of *Pulmonaria officinalis* L. was determined by colorimetric method on a UV-1800 spectrophotometer (Germany, Shimadzu), at an optical density of 510 nm, using an aluminum chloride solution (Russia, REACHIM) [38].

Table 1. Matrix of compliance of extraction current parameters

| Extraction current parameters | 1 | 2 | 3 | 4 | 5 |
|--|----------|----------|----------|----------|----------|
| Content of ethyl alcohol, C ₃ , (%) | 30 | 40 | 50 | 60 | 70 |
| Time, τ ₃ , (h) | 2 | 3 | 4 | 5 | 6 |
| Temperature, T ₃ , (°C) | 30 | 40 | 50 | 60 | 70 |

To determine flavonoids, a reaction mixture was prepared containing an untreated extract in the amount of 0.2 ml, deionized water 3.35 ml and a solution of 0.05 g/ml of sodium nitrate 0.15 ml (Russia, B2bito). In 5 minutes, 0.3 ml 0.1 g/ml of aluminum chloride was added to the mixture, and in 6 minutes, 1 ml of sodium hydroxide 1 mol/l (Ukraine, SILUR) was added, and then the reaction mixture was mixed.

The content of flavonoids was expressed in micrograms of rutin equivalent (France, AixLab) per 1 ml of extract.

2.4 Determination of Proanthocyanidins

Determination of proanthocyanidins was performed using vanillin analysis in icy acetic acid HC (Russia, Hermeon) [39] with minor modifications. The vanillin reagent contained 4% of concentrated HCl (Russia, Logosib) and 0.5% vanillin in methanol (France, AixLab). The absorption was read on a UV-1800 spectrophotometer (Germany, Shimadzu) at 500 nm using a 1 cm cell. The results were expressed in catechin equivalents (Spain, Panreac Applichem) per 1 ml of the extract.

2.5 Determination of Antioxidant Activity of Extracts

a) DPPH (2,2-diphenyl-1-picrylhydrazyl) Spectrometric method

Flavonoids have a fairly strong radical activity based on their ability to act as hydrogen or electron donors [22,29,34]. The antioxidant properties of phenolic compounds depend directly on their structure. Phenolic compounds contain at least one aromatic ring that carries one or more hydroxyl groups, and these compounds are able to destroy free radicals, while forming phenoxyl radicals [23,29,35].

The DPPH radical is a stable free radical that is widely used as a tool for evaluating the antioxidant activity of DPPH. Antioxidants have the ability to restore the stable radical DPPH to yellow diphenylpicrihydrazine. The effect of an

antioxidant on DPPH is related to the ability to break off hydrogen.

Antioxidant activity was determined spectrophotometrically by the method of DPPH (2,2-diphenyl-1-picrylhydrazyl) by measuring the decrease in the maximum absorption of the DPPH radical (Russia, Stimulus) at 517 nm after 3 minutes [39]. The percentage of DPPH activity in samples of *Pulmonaria officinalis* L. extract was calculated as follows:

$$\text{Antioxidant activity, (\%)} = \frac{A_B - A_A}{A_B} * 100,$$

where A_B – control optical density;
 A_A – optical density of the sample.

b) FRAP Method (iron reducing/antioxidant power)

The antioxidant activity was determined by FRAP [40]. Samples of *Pulmonaria officinalis* L. extracts in an amount of 0.1 ml were mixed with 2.5 ml of 200 mm / l sodium-phosphate buffer (pH 6.6) (Italy, Bio-optica) and 2.5 ml of 1% potassium ferricyanide (France, AixLab). The mixture was shaken intensively, then incubated at 50°C for 20 minutes. After the set time, 2.5 ml of 10% trichloroacetic acid (Russia, REACHIM), 2.5 ml of deionized water and 0.5 ml of 0.1% iron chloride (Russia, Himsnab) were added to the prepared mixture. Absorption was measured by spectrophotometric method at 700 nm.

2.6 Statistical Analysis

The analysis of the studies was carried out in three-fold repetition. For statistical analysis, we used Microsoft Office Excel 2016 software. Statistical analysis of the data was performed using a one-way pair Studens test. The differences were considered statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

Determination of the total content of polyphenols, flavonoids and proanthocyanidins.

The total content of BAS-polyphenols, flavonoids and proanthocyanidins under various operating conditions for extracting extracts of *Pulmonaria officinalis* L. is shown in Table 2.

According to the results of the analysis, it is clear that the maximum allocation of BAS – polyphenols, flavonoids and proanthocyanidins from extracts of *Pulmonaria officinalis* L. was carried out at a temperature of 50-60°C with a concentration of ethyl alcohol of 60-70% for 4 hours. The analyzed extracts of *Pulmonaria officinalis* L. showed a high yield of polyphenolic compounds-889.39±4.29 mcg of Gallic acid/ml, flavonoids – 728.90±6.98 mcg rutin/ml, proanthocyanidins – 211.65±5.31 micrograms of catechin/ml at an ethyl alcohol concentration of 60% and an extraction temperature of 50°C for 4 hours.

At the maximum temperature range (60-70°C) of *Pulmonaria officinalis* L. extraction and the maximum extraction time (5-6 h), BAS destruction occurred. At the minimum temperature limits of extraction (30-40°C) and the minimum time (2-3 h), a small amount of BAS was formed.

Pulmonaria officinalis L. extract has a beneficial effect on human health as antimicrobial, anti-inflammatory, anti-diabetic, anti-cholesterol, antioxidant and anti-cancer agents [27,26]. The resulting extracts contain a significant amount of polyphenols, compared to other BAS (Table 2), which formed an important group of secondary metabolites.

Literature related to pre - screening of antioxidant activity for isolated *Pulmonaria officinalis* L. extracts used in the food industry has shown a high value of antioxidant activity, which may be associated with a high content of flavonoids [22, 23,29,35].

3.1 Determination of Antioxidant Activity

a) Antioxidant activity of DPPH

A significant relationship ($p < 0.05$) was found between the antioxidant activity (determined by DPPH and FRAP) and the total content of polyphenols and flavonoids, which indicates a significant contribution of these compounds to the overall antioxidant activity observed for these plant extracts of *Pulmonaria officinalis* L.[29].

Table 2. Content of polyphenols, flavonoids and proanthocyanidins in analyzed extracts of *Pulmonaria officinalis* L

| Time, τ_3 , (h) | Temperature, T_3 , (°C) | Content of ethyl alcohol, C_3 , (%) | Polyphenols (mcg of Gallic acid/ml) | Flavonoids (mcg of routine/ml) | Proanthocyanidins (mcg of catechin/ml) |
|----------------------|---------------------------|---------------------------------------|-------------------------------------|--------------------------------|--|
| 2 | 30 | 30 | 156,25 ± 5,26 | 85,05 ± 3,98 | 20,98 ± 1,83 |
| | | 40 | 105,63 ± 3,27 | 111,60 ± 8,35 | 11,92 ± 4,24 |
| | | 50 | 98,50 ± 4,21 | 133,89 ± 9,34 | 18,92 ± 8,29 |
| | | 60 | 215,36 ± 3,45 | 145,62 ± 5,67 | 39,27 ± 5,37 |
| | | 70 | 301,26 ± 4,78 | 158,37 ± 4,29 | 51,98 ± 4,01 |
| 3 | 40 | 30 | 187,28 ± 6,87 | 214,38 ± 2,37 | 60,67 ± 3,81 |
| | | 40 | 202,89 ± 5,15 | 208,21 ± 3,56 | 62,80 ± 5,29 |
| | | 50 | 190,62 ± 6,74 | 259,11 ± 6,52 | 78,81 ± 3,27 |
| | | 60 | 385,27 ± 2,32 | 299,26 ± 7,89 | 99,63 ± 8,30 |
| | | 70 | 402,10 ± 4,12 | 301,38 ± 1,89 | 100,47 ± 6,38 |
| 4 | 50 | 30 | 523,85 ± 7,28 | 603,16 ± 3,64 | 187,96 ± 3,24 |
| | | 40 | 656,78 ± 6,47 | 569,97 ± 5,32 | 157,93 ± 6,95 |
| | | 50 | 541,63 ± 8,11 | 623,74 ± 3,27 | 189,47 ± 4,15 |
| | | 60 | 889,39 ± 4,29 | 728,90 ± 6,98 | 211,65 ± 5,31 |
| | | 70 | 745,74 ± 5,27 | 662,31 ± 6,34 | 208,98 ± 6,24 |
| 5 | 60 | 30 | 395,31 ± 8,13 | 356,20 ± 7,29 | 80,45 ± 7,48 |
| | | 40 | 500,37 ± 2,37 | 128,45 ± 9,32 | 75,96 ± 3,12 |
| | | 50 | 420,36 ± 6,31 | 195,35 ± 7,94 | 52,20 ± 3,94 |
| | | 60 | 402,10 ± 4,94 | 211,39 ± 6,67 | 70,21 ± 4,24 |
| | | 70 | 576,62 ± 6,32 | 272,81 ± 9,21 | 61,50 ± 6,23 |
| 6 | 70 | 30 | 251,85 ± 1,87 | 120,63 ± 9,37 | 37,93 ± 2,31 |
| | | 40 | 165,89 ± 3,14 | 202,98 ± 3,75 | 40,87 ± 5,28 |
| | | 50 | 194,63 ± 8,19 | 105,47 ± 7,48 | 35,64 ± 1,54 |
| | | 60 | 209,91 ± 2,01 | 230,74 ± 7,34 | 55,32 ± 0,85 |
| | | 70 | 264,10 ± 8,09 | 217,32 ± 4,25 | 69,37 ± 8,37 |

For analysis, we selected an extract of *Pulmonaria officinalis* L., with operating parameters— $\tau_3=4$ ч; $T_3=50^\circ\text{C}$; $C_3=60\%$ with the highest content of polyphenols (889.39 ± 4.29 mcg of Gallic acid/ml), flavonoids (728.90 ± 6.98 mcg of rutin/ml) and proanthocyanidins (211.65 ± 5.31 mcg of catechin/ml) showed the highest antioxidant activity in both methods used.

Using the DPPH analysis found that the value of polyphenols for antioxidant activity DPPH was 86,96% (at 3 mg/ml), which corresponds to the high content of polyphenols in the extract *Pulmonaria officinalis* L.

The antioxidant activity of proanthocyanidins is 51.25% (at 3 mg / ml), which corresponds to the maximum content of proanthocyanidins in the extract of *Pulmonaria officinalis* L. that is 211.65 ± 5.31 micrograms of catechin/ml. Consequently, ethanol plant extracts have a high inhibitory activity against DPPH free radicals.

The antioxidant activity of DPPH extract of *Pulmonaria officinalis* L. is shown in Fig. 1.

The percentage of antioxidant activity of DPPH decreases in proportion to the concentration of the analyzed sample.

b) FRAP Method (iron reducing/antioxidant power)

This analysis evaluated the transformation of Fe^{3+} - Fe^{2+} in the presence of *Pulmonaria officinalis* L. extracts. The regenerative capacity of *Pulmonaria officinalis* L. extracts, which contained the largest amount of BAS and ascorbic acid, was evaluated. Ascorbic acid was used as a control connection.

For FRAP analysis, extracts of *Pulmonaria officinalis* L. with the maximum content of BAS were taken. These extracts had the following extraction parameters:

- Extract № 1 – $\tau_3=4$ ч; $T_3=50^\circ\text{C}$; $C_3=60\%$ with the content of polyphenols- 889.39 ± 4.29 mcg of Gallic acid/ml, flavonoids- 728.90 ± 6.98 mcg of rutin/ml and proanthocyanidins- 211.65 ± 5.31 mcg of catechin/ml;
- Extract № 2 – $\tau_3=4$ ч; $T_3=50^\circ\text{C}$; $C_3=70\%$ with the content of polyphenols- 745.74 ± 5.27 mcg of Gallic acid/ml, flavonoids- 662.31 ± 6.34 mcg of rutin/ml and proanthocyanidins- 208.98 ± 6.24 mcg of catechin/ml;
- Extract № 3 – $\tau_3=5$ ч; $T_3=60^\circ\text{C}$; $C_3=60\%$ with the content of polyphenols- 402.10 ± 4.94 mcg of Gallic acid/ml, flavonoids- 211.39 ± 6.67 mcg of rutin/ml and proanthocyanidins- 70.21 ± 4.24 mcg of catechin/ml;
- Extract № 4 – $\tau_3=5$ ч; $T_3=60^\circ\text{C}$; $C_3=70\%$ with the content of polyphenols- 576.62 ± 6.32 mcg of Gallic acid/ml, flavonoids- 272.81 ± 9.21 mcg of rutin/ml and proanthocyanidins- 61.50 ± 6.23 mcg of catechin/ml.

The results of the FRAP method for determining the antioxidant activity of *Pulmonaria officinalis* L. extracts are shown in Fig. 2.

In comparison with ascorbic acid, ethanol extracts of *Pulmonaria officinalis* L. showed high activity. In the analysis, extracts of *Pulmonaria officinalis* L. had a relationship between the FRAP method and the content of polyphenols, flavonoids and proanthocyanidins in them.

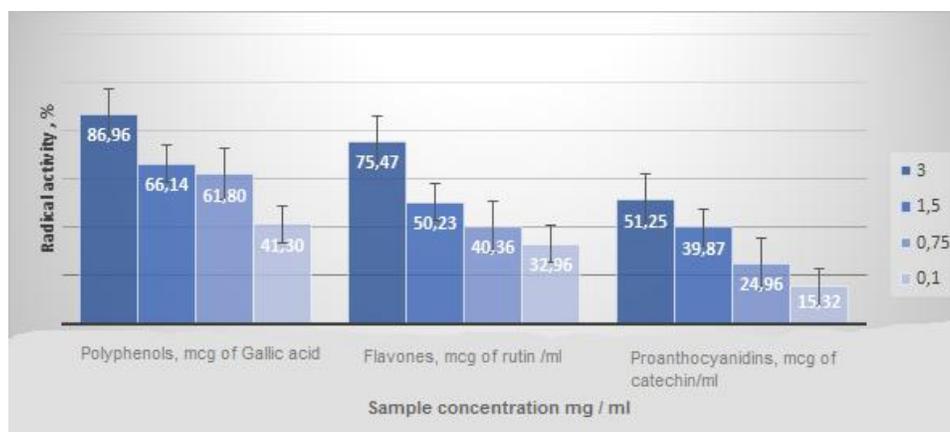


Fig. 1. Antioxidant activity of DPPH (p <0.05) extract of *Pulmonaria officinalis* L.

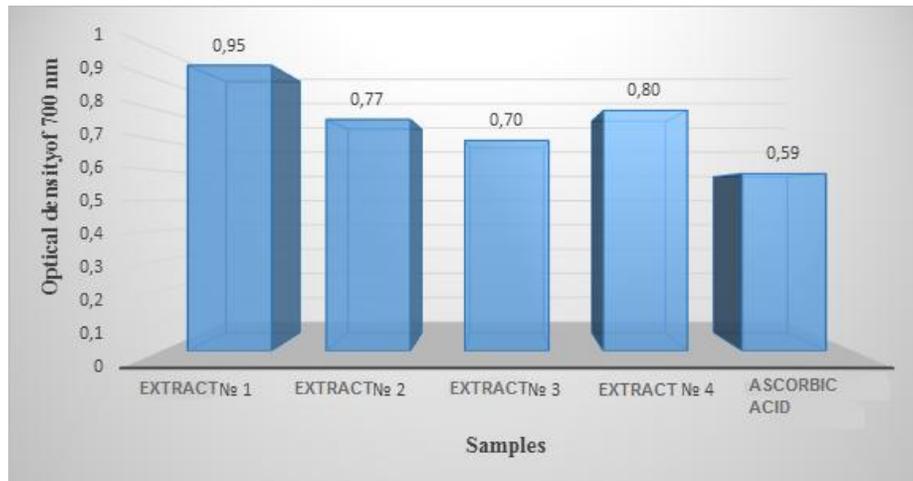


Fig. 2. Antioxidant activity by FRAP method of *Pulmonaria officinalis* L.

The results obtained for the determination of antioxidant activity by the FRAP method interact with the results obtained by the DPPH radical antioxidant activity method. Thus, extract № 1 had the most pronounced antioxidant activity ($\tau_3 = 4$ ч; $T_3 = 50^\circ\text{C}$; $C_3 = 60\%$) followed by extract № 4 ($\tau_3 = 5$ ч; $T_3 = 60^\circ\text{C}$; $C_3 = 70\%$) (Fig. 2).

4. CONCLUSIONS

In this study, ethanol extracts of *Pulmonaria officinalis* L. were obtained with selected appropriate extraction parameters (alcohol concentration, temperature, and time). BAS – polyphenols, flavonoids, and proanthocyanidins were determined by spectrophotometric method in the obtained extracts.

As a result of research, it was found that the working parameters for the extraction of *Pulmonaria officinalis* L. were: the extraction temperature ($50\text{--}60^\circ\text{C}$), the alcohol concentration ($60\text{--}70\%$) and the extraction duration of 4 hours, since it is at these extraction parameters that the maximum formation of BAS occurs. For example, at an ethyl alcohol concentration of 60% and an extraction temperature of 50°C , the extraction duration of 4 hours, the yield of polyphenolic compounds is 889.39 ± 4.29 micrograms of Gallic acid/ml, flavonoids – 728.90 ± 6.98 micrograms of rutin/ml, proanthocyanidins – 211.65 ± 5.31 micrograms of catechin/ml. For extracts with a high content of BAS, the antioxidant properties were determined by two different methods. When analyzing the antioxidant activity of the DPPH radical, it was found that for polyphenols the activity value is 86.96%, for flavonoids the

activity is 75.47%, and for proanthocyanidins it is 51.25%. When analyzing FRAP, it was found that extracts № 1 ($\tau_3 = 4$ ч; $T_3 = 50^\circ\text{C}$; $C_3 = 60\%$) и № 4 ($\tau_3 = 5$ ч; $T_3 = 60^\circ\text{C}$; $C_3 = 70\%$) had a pronounced antioxidant activity.

Based on the presented data, *Pulmonaria officinalis* L. is a useful medicinal source of BAS, which have geroprotective properties.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

FINANCING

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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