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INTERRELATION BETWEEN MITOCHONDRIAL ENZYME ACTIVITY AND ANTIOXIDANT ACTIVITY OF SECONDARY POLYPHENOL NATURE METABOLITES IN HEMIPARASITE *VISCUM ALBUM* L. LEAVES

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Antioxidants are widely used in practical medicine. Not only the search for new plant antioxidants, but also the study of the factors affecting their accumulation in plants, are relevant.

The aim is to study the interrelation between the activity of mitochondrial enzymes and the antioxidant activity of the secondary polyphenolic nature metabolites in hemiparasite *Viscum Album* L. leaves.

Materials and methods. The sampling material was *Viscum album* L. leaves, collected in winter from a host tree, *Malus domestica* Borkh. The extraction of biologically active substances was carried out with ethyl alcohol of various concentrations (90%, 70% and 50%), or purified water. The amount of total antioxidants was estimated by the amperometric method. The amount of total phenols was evaluated in the reaction with the Folin-Ciocalteu reagent. The concentration of total flavonoids was estimated by the change in the optical density of the rutin with aluminum (III) chloride complex. The antioxidant properties of the analyzed extracts were determined *in vitro* in the induced lipid peroxidation test. The activity of aconitase was assessed by a conjugated aconitase-isocitrate dehydrogenase reaction, citrate synthase – by changing the color intensity of the Ellman reagent solution, succinate dehydrogenase were determined in the reaction of succinate-dependent oxidation of 2,6-dichlorophenolindophenol.

Results. The carried out study showed that the maximum amount of total phenols (2.39±0.05%) is observed in a 50% ethanol extract from *Viscum album* L. leaves, with the content of total flavonoids equal to 1.83±0.04%, and the antioxidants equal to 0.503±0.007 mg/g (a quercetin equivalent) and 0.322±0.006 mg/g (a gallic acid equivalent). A 50% ethanol extract suppressed lipid peroxidation in the model mixture with IC_{50}=106.3±1.09 μg/ml. In *Viscum album* L. leaves, a high activity of aconitase which strongly correlated (r=0.88416) with changes in the concentration of flavonoids, has been notified.

Conclusion. The optimal extractant for obtaining extracts with a high antioxidant activity is 50% ethyl alcohol. The analysis of the mitochondrial enzymes activity showed that in *Viscum album* L. leaves collected in winter, a high activity of aconitase strongly correlated with changes in the concentration of flavonoids (r=0.88416).

Keywords: antioxidants; flavonoids; aconitase; succinate dehydrogenase; citrate synthase; *Viscum album* L.; correlation analysis; plants-hemiparasites; mitochondrial enzymes

Abbreviations: LPO – lipid peroxidation; DMSO – dimethyl sulfoxide; TBA – thiobarbituric acid; EDTA – ethylene diamine tetraacetatic acid; NADH – nicotinamide adenine dinucleotide, reduced form; NADP – nicotinamide-adenine dinucleotide phosphate; PAL – phenylalanine ammonia lyase; AU(s) – unit(s) of activity; ROS – reactive oxygen spiecies.

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ВЗАИМОСВЯЗЬ АКТИВНОСТИ МИТОХОНДРИАЛЬНЫХ ФЕРМЕНТОВ И АНТИОКСИДАНТНОЙ АКТИВНОСТИ ВТОРИЧНЫХ МЕТАБОЛИТОВ ПОЛИФЕНОЛЬНОЙ ПРИРОДЫ ЛИСТЬЕВ ГЕМИПАРАЗИТА *VISCUM ALBUM* L.

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Антиоксидантные средства находят широкое применение в практической медицине. Актуальным является не только поиск новых растительных антиоксидантов, но и изучение факторов, влияющих на их накопление в растениях.

Цель. Изучение взаимосвязи активности митохондриальных ферментов и антиоксидантной активности вторичных метаболитов полифенольной природы листьев гемипаразита *Viscum album* L.

Материалы и методы. В качестве исследуемого материала выступали листья омелы белой, собранные зимой с дерева-носителя – яблони обыкновенной. Экстракцию биологически активных веществ проводили спиртом этиловым различной концентрации (90%, 70% и 50%) или водой очищенной. Количество суммы антиоксидантов оценивали амперометрическим методом. Количество суммы фенолов оценивали в реакции с реактивом Фолина-Чокальтеу. Концентрацию суммы флавоноидов оценивали по изменению оптической плотности комплекса рутина с алюминия (III) хлоридом. Антиоксидантные свойства анализируемых извлечений определяли *in vitro* в тесте индуцированного перекисного окисления липидов. Активность аконитазы оценивали путем сопряженной аконитаза – изоцитратдегидрогеназной реакции; цитратсинтазы – по изменению интенсивности окраски раствора реактива Эллмана; сукцинатдегидрогеназы определяли в реакции сукцинат-зависимого окисления 2,6-дихлорфенолиндофенола.

Результаты. Проведенное исследование показало, что максимальное количество суммы фенолов (2,39±0,05%), наблюдается в 50% этанольном извлечении из листьев омелы белой, при содержании суммы флавоноидов 1,83±0,04% и антиоксидантов 0,503±0,007 мг/г (эквивалент кверцетина) и 0,322±0,006 мг/г (эквивалент галловой кислоты). 50% этанольное извлечение подавляло перекисное окисление липидов в модельной смеси с IC_{50}=106,3±1,09 мкг/мл. В листьях омелы белой отмечена высокая активность аконитазы, сильно коррелировавшая (r=0,88416) с изменением концентрации флавоноидов.

Заключение. Оптимальным экстрагентом для получения извлечений с высокой антиоксидантной активностью является спирт этиловый 50%. Анализ активности митохондриальных ферментов показал, что в листьях омелы белой, собранных зимой, отмечена высокая активность аконитазы, сильно коррелировавшая с изменением концентрации флавоноидов (r=0,88416).

Ключевые слова: антиоксиданты; флавоноиды; аконитаза; сукцинатдегидрогеназа; цитратсинтаза; *Viscum album* L.; корреляционный анализ; растения гемипаразиты; митохондриальные ферменты

Список сокращений: ПОЛ – перекисное окисление липидов; ДМСО – диметилсульфоксид; ТБК – тиобарбитуровая кислота; ЭДТА – этилендиаминтетраацетат натрия; НАДФН – никотинамидадениндинуклеотид фосфат восстановленный; НАДФ – никотинамидадениндинуклеотид фосфат; ФАЛ – фенилаланин-аммиак лиаза; ЕД – единицы действия; АФК – активные формы кислорода.

INTRODUCTION

About 100 species of evergreen semi-parasitic plants belong to the Mistletoe genus, and only *Viscum album* L. and *Viscum coloratum* (Kom.) Nacai are common in Russia. *Viscum album* L. is an evergreen hemispherical forking shrub that grows on the trunks and branches of many deciduous trees as a parasitic form. The roots of *Viscum album* L. form branches that penetrate under the bark and into the wood of the host tree, forming numerous special attachment organs – haustoria in it[1,2].

According to the taxonomic position and according

[1] Plant resources of the USSR. Flowering plants, their chemical composition, use: Families *Rutaceae* – *Elaeagnaceae* / Academy of Sciences of the USSR, Botan. in-t im. V.L. Komarova; [Compiled by I.B. Sandina and others]; Rep. ed. P.D. Sokolov. – L.: Nauka: Leningrad. department, 1988: 356 p. Russian

[2] Liakh Y., Yurel D. [Mistletoe (*Viscum album*) and its ecological significance in the republic of Belarus]. Sakharov readings 2018: environmental problems of the XXI century: materials of the 18th int. scientific conf. Minsk: Information Center of the Ministry of Finance. 2018; 2: 152–54.

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to the classification proposed by Takhtadzhyan A.E. and Gilyarova M.S., *Viscum album* L. is assigned to the Mistletoe genus (*Viscaceae* Miers.), the santal order *(Santalales).* According to Elenevskaya A.G. and others' classification – to the *Loranthaceae* genus (*Loranthaceae* Juss.); according to Tsitsin N.V.'s one – to the *Loranthaceae* Juss. genus[3,4].

Between *Viscum album* L. and other parasitic plants is that *Viscum album* L. does not destroy host cells to obtain nutrients, but rather binds to the vascular system of the host tree. For its vegetation, a *Viscum album* L. tree uses a significant part of the host tree's nutrients, which leads to the loss of its resources, thereby increasing its susceptibility to various diseases. The resulting morphological disturbances reduce protective functions of the host tree, which leads to hypertrophic processes, resulting in dying off a part of the tree[5].

In the structure of *Viscum album* L., there is a variety of biologically active compounds – lectins, viscotoxins, viscerin, flavonoids, nitrogen-containing substances, oleanolic and ursolic acids, alcohols, amines and other active compounds that determine its unique therapeutic properties [1, 2].

Three lectins from *Viscum album* L. have been investigated, the most studied of which is lectin ML-1, the immunomodulating protein which increases the activity of the immune system, and herewith the therapeutic effects are manifested in extremely small doses [3]. Selectively interacting with the immune cells (monocytes, lymphocytes), lectin stimulates the secretion of interferon proteins, interleukins and a tumor necrosis factor by cells. [4–6]. In addition to lectins, viscotoxins have been isolated from *Viscum album* L. extracts; their action in large doses is cytotoxic, which is a prerequisite for an anticancer activity [1, 5].

Flavonoids and phenolcarboxylic acids found out in *Viscum album* L., are marked by an antioxidant activity, they are characterized by a structural diversity, a high biological activity, and a low toxicity [6].

Some of the most effective antioxidants are: fat-soluble polyphenols (tocopherol and its derivatives, ubiquinones, some steroid hormones), phenolic compounds (flavonoids, phenolic acids). In their chemical structure and mechanism of action, these substances are close to the most active antioxidants of a synthetic origin: hydroquinone, agidol, naphthols [6–9].

Metal ions play a special role in the initiation of peroxide processes, herewith, among metals with a variable valence, iron and copper most often take part in oxidation-reduction reactions. Ionized iron is a component of the active sites of many enzymatic systems, such as aconitase, proline hydroxylase, or the antioxidant defense enzyme – catalase. A biological role of iron is associated with its ability to accept and donate electrons at a high reaction rate, transforming, respectively, into divalent (Fe^{2+}) or trivalent (Fe^{3+}) forms [6, 11].

Being secondary plant metabolites, flavonoids perform many vital biological functions, offering a valuable metabolic and genetic model for studying the transcriptional control of the gene expression. Flavonoid biosynthesis includes many well-characterized enzymatic and regulatory proteins that control flavonoid biosynthesis by activating the early stages of biosynthesis [12, 13].

THE AIM. To study the interrelation between the activity of mitochondrial enzymes and the antioxidant activity of the secondary polyphenolic nature metabolites in hemiparasite *Viscum Album* L. leaves.

MATERIALS AND METHODS

Study objects

The object under study was *Viscum album* L. leaves growing on *Malus domestica* Borkh., collected in the vicinity of Stavropol, Russia. The raw materials were collected during the winter period (01/21/2022). The choice of time for harvesting raw materials was based on the preliminary studies results of a comparative analysis of the active components accumulation in *Viscum album* L. leaves depending on the season for harvesting raw materials; the maximum content of biologically active compounds is observed in winter.

Determination of antioxidants total content

The total content of antioxidants was determined by a amperometric method on a Tsvet Yauza 01-AA liquid chromatograph (NPO Khimavtomatika, Russia) using a calibration plot of the dependence of the output signal on the concentration of quercetin and gallic acid [14, 15].

Obtaining extracts under study

A precisely weighed amount of the raw material dried by the air-shadow method and crushed with office scissors (1 g), was placed in a flask with a capacity of 100 ml; 30 ml of ethyl alcohol of the appropriate concentration (50%, 70% and 90%) or purified water was added, boiled in the water bath for 60 minutes. The contents of the flask were filtered through a paper filter into a 100 ml volumetric flask. The extraction by the above method was repeated twice, the filter was washed with the appropriate extractant and the volume of the filtrate was brought to the mark. If necessary, the sample was diluted [14–17].

The total content of antioxidants (mg/g) was determined by formula 1:

$$X = \frac{X_G \cdot V_n \cdot N}{m_n \cdot 1000}, \quad (1)$$

where: X_G is a mass concentration of antioxidants found according to the calibration chart, mg/l; V_n is an extraction volume, ml; m_n is a sample of raw materials, g; N is a dilution factor.

[3] Ibid.
[4] Plant resources of the USSR. Flowering plants, their chemical composition, use: Families *Rutaceae – Elaeagnaceae*, 1988.
[5] Turova AD. [Medicinal plants and their use]. M.: Medicine, 2013: 203 p. Russian

Evaluation of antioxidant activity of extracts under study on iron (II)-induced lipid peroxidation model

The antioxidant effect of the extracts under study on the model of Fe^{2+}-induced lipid peroxidation (LPO) was investigated *in vitro*. Lecithin liposomes acted as a model medium for LPO. The intensity of liposomes lipid peroxidation was evidenced by a change in the concentration of the products that react with 2-thiobarbituric acid (TBA-active products). The content of lipid liposomes in the medium under study was 40 mg/ml [18–20].

The antioxidant activity was evaluated after a preliminary evaporation of the extract in the water bath and drying in the oven at the temperature not exceeding 60°C [18–22].

The reaction of Fe^{2+}-induced LPO was carried out in the water bath at 37°C with continuous bubbling. The studied dried extracts were preliminarily dissolved in dimethyl sulfoxide (DMSO), added to the liposome suspension and a ferrous iron solution and incubated for 15 min. Then 0.5 ml of a 20% trichloroacetic acid solution was added and centrifuged for 15 minutes at 3 thousand rpm. Next, 1 ml of a 0.5% thiobarbituric acid solution was added to the supernatant and heated in the water bath for 15 minutes at 100°C. The content of TBA-active products was measured on an SF-102 spectrophotometer (Aquilon, Russia) at 532 nm [23]. The percentage of the LPO inhibition was calculated with respect to the control sample (solvent) using formula 2 [24]:

$$AOA = \frac{\Delta A_k - \Delta A_{op}}{\Delta A_k} \cdot 100\%, \quad (2)$$

$$\Delta A_k = A_{k15} - Ak;\ \Delta A_{op} = A_{op15} - A_{op}$$

where: *AOA* is an antioxidant activity, %; A_k and A_{op} are optical densities before the incubation; A_{k15} and A_{op15} are optical densities after 15 min of the incubation.

In this work, the semi-inhibition coefficient (IC_{50}) of lipid peroxidation, which had been calculated by the regression analysis method [18–20], served as a criterion indicator that made it possible to determine the antioxidant activity level of the extracts under study.

Determination of phenolic compounds total content by Folin-Ciocalteu method

In the solutions under study, the concentration of phenolic compounds in terms of gallic acid was determined according to the calibration curve, and the percentage (X) in terms of absolutely dry raw materials was calculated using formula 3:

$$X = \frac{C \cdot W_1 \cdot W_2 \cdot 100}{a \cdot V_a \cdot (100 - W)}, \quad (3)$$

where: *C* is a concentration of phenolic compounds in the extract under study, calculated according to the calibration curve, g/100 ml; *a* – sample of raw materials, g; V_a – aliquot volume, ml; W_1, W_2 – volumes of volumetric flasks, ml; W is a weight loss during drying of raw materials, % [25–27].

The quantitative determination of flavonoids was carried out by the spectrophotometric method on an SPh-102 spectrophotometer. When calculating the total flavonoids content, the value of the specific absorption index of the rutin with aluminum chloride complex was used, because the extracts from *Viscum album* L. leaves in the presence of a 2% aluminum (III) chloride alcohol solution have a maximum absorption at the wavelength of 415±2 nm[6].

The total content of flavonoids in percent (X) in terms of rutin and absolutely dry raw materials is calculated by formula 4:

$$X = \frac{A \cdot 25 \cdot 100 \cdot 100}{A_{1cm}^{1\%} \cdot a \cdot 2 \cdot (100 - W)}, \quad (4)$$

where: *A* is the optical density of the test solution; $A_{1cm}^{1\%}$ is a specific absorption index of the rutin with aluminum chloride complex at the wavelength of 415±2 nm equal to 248; *a* is a weighed amount of raw materials, g; *W* is the raw material moisture content, % [35].

Preparation of plant materials for determining activity of mitochondrial enzymes

The activity of mitochondrial enzymes was evaluated in the cytosolic fraction enriched with mitochondria, for which 1.0 g of precisely weighed crushed raw materials was placed in a 50 ml conical flask and filled with 10 ml of a buffer system (composition: 50 mM/l HEPES + 2 mM/l magnesium (II) chloride + 40 mM/l potassium chloride + 1 mM/l EDTA + 0.1% bovine serum albumin + 1% polyvinylpyrrolidone 40 000 + 10% glycerol + 50 μg/ml trypsin and potassium hydroxide up to pH 7.8). The resulting mixture was kept at the temperature of 37°C for 30 minutes. Then it was filtered through a glass filter and the filtrate was centrifuged at 1 800 g for 10 minutes. The resulting supernatant was taken into sterile Eppendorf tubes, layered with a 10% Percoll solution and centrifuged again at 18 000 g for 10 minutes. The secondary supernatant was removed for the analysis [28].

[6] Russian State Pharmacopeia XIV edition. Publisher: Ministry of Health of the Russian Federation, 2018; 2, 4. Available from: http: // http: // femb.ru/femb/pharmacopea.php

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Assessment of aconitase activity

An aconitase activity was assessed spectrophotometrically using a PROMECOLAB PE-5300V spectrophotometer (Shanghai Mapada Instruments Co., Ltd., China) in the course of a conjugated aconitase–isocitrate dehydrogenase reaction by detecting NADH at 340 nm. The course of the analysis was as follows: 0.1 mg/ml (10 µl) of a sodium citrate solution was added to the medium containing 0.03 U/l isocitrate dehydrogenase, 0.32 mg/ml NADP, 55 µl phosphate-buffered saline and 50 µl of the test biomaterial. The change in the optical density of the obtained solutions was recorded against the mixture of reagents without the biomaterial under study within 2 minutes. An aconitase activity was calculated according to the change in the absorbance using an extinction coefficient of 0.0313µM-1. The enzyme activity was expressed as U/mg protein. In the analyzed samples, the protein content was determined by the Bradford method [29].

Assessment of citrate synthase activity

The citrate synthase activity was evaluated in the medium consisting of a 5,5'-di-thiobis-2-nitrobenzoic acid solution (Ellman's reagent) 100 mM Tris-HCl buffer with pH 7.8 and higher; acetyl-CoA 100 mm; 0.1% Triton-x 100 µl and 4 µl of the test biomaterial. The reaction was started by adding 100 µl of oxaloacetate. After 3 minutes, the change in the optical density of the medium at 412 nm was evaluated. The citrate synthase activity was also expressed as U/mg of protein. In the samples under study, the protein content was determined by the Bradford method [30].

Assessment of succinate dehydrogenase activity

The activity of succinate dehydrogenase was evaluated spectrophotometrically (spectrophotometer PROMEKOLAB PE-5300V) in the reaction of succinate-dependent oxidation of 2,6-dichlorophenolindophenol at 600 nm. During the analysis, standard kits from Abcam were used.

Statistical analysis

The statistical analysis was performed using the STATISTICA 6.0 Software package. The results were expressed as M ± SEM (mean ± standard error of the mean). A further analysis was performed by a one-way analysis of variance (ANOVA) with Newman-Keuls post-processing in the presence of a Gaussian distribution and the Kruskal-Wallis test in the absence of the Gaussian distribution. The distribution normality was tested using the Shapiro-Wilk test. The differences between the study groups were considered statistically significant at $p < 0.05$. The correlation analysis was performed using the Spearman's test as interpreted by the Chaddock's scale.

RESULTS

Estimation of antioxidants total amount in objects under study

The total content of antioxidants in hydroalcoholic and aqueous extracts from *Viscum album* L. leaves was expressed as the equivalent of quercetin and gallic acid. The results are presented in Table 1.

By the amperometric method, it was found out that the maximum amount of antioxidants in the extracts from *Viscum album* L. leaves was notified with the use of 50% ethanol as an extractant, which amounted to 0.503±0.007 mg/g in terms of quercetin and 0.322±0.006 mg/g in terms of gallic acid.

Determination of antioxidant effect on Fe^{2+}-induced lipid peroxidation model

The extracts from *Viscum album* L. leaves were added to the medium under study in the form of water-alcohol and aqueous solutions in the final concentrations of two-fold dilutions: 200 µg/ml – 12.5 µg/ml. The extract from *Viscum album* L. leaves, obtained by the extraction with 50% ethanol, exhibits the highest antioxidant activity.

The maximum decrease in lipid peroxidation is observed in the extracts from *Viscum album* L. leaves (the extractant is 50% ethyl alcohol) collected in winter, at the concentration of 200 µg/ml, and it is 73.64±1.43%. A closer result for quercetin was observed at the concentration of 25 µg/ml.

The calculated IC_{50} values, numerically equal to the concentration of the substances in the analyzed medium, causing a 50% decrease in the intensity of lipid peroxidation, were 19.27±0.97 µg/ml for quercetin and 106.3±1.09 µg/ml for the 50% alcohol extraction from *Viscum album* L. leaves under study.

The data obtained when determining the total content of antioxidants on a liquid chromatograph Tsvet Yauza 01-AA by the amperometric method, are consistent with the data obtained when determining the antioxidant effect of the extracts from *Viscum album* L. leaves on Fe^{2+}-induced LPO in the liposomal system.

Determination of phenolic compounds amount using Folin-Ciocalteu reagent

For the quantitative determination of phenolic compounds, the equation of the calibration curve was used: y = 1746x+0.002. The results of determining the total content of phenolic compounds are presented in Fig. 1 and Table 3.

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Table 1 – Total amount of antioxidants in *Viscum album* L. extracts

Raw materials used	Extractants	Dilution ratio	Content of antioxidants, mg/g in terms of	
			Quercetin (n=6)	Gallic acid (n=6)
Viscum album L. leaves harvested in winter	ethanol 90%	–	0.127±0.002	0.078±0.001
	ethanol 70%	–	0.374±0.003	0.244±0.003
	ethanol 50%	**2**	**0.503±0.007**	**0.322±0.006**
	purified water	–	0.261±0.003	0.167±0.002

Table 2 – Effect of extracts from *Viscum album* L. leaves on Fe^{2+}-induced LPO in liposome system

Final concentration, μg/ml	% decrease in LPO, n=3			
	Extractants			
	Ethanol 90%	Ethanol 70%	**Ethanol 50%**	Purified water
12.5	8.22±1.28	9.05±1.05	**12.08±1.36**	11.28±1.27
25	10.16±1.89	17.45±1.24	**22.48±1.23**	22.17±2.89
50	29.75±1.76	39.53±1.49	**41.77±2.19**	37.04±1.54
100	41.17±2.35	51.61±2.32	**55.71±2.04**	44.71±2.77
200	56.01±1.63	68.71±1.77	**73.64±1.43**	62.26±2.69
IC_{50}	160.52±2.26	120.07±1.94	**106.30±1.09**	137.02±2.16

Note: A 95% alcohol solution of quercetin was used as a reference, which was added in final concentrations of two-fold dilutions: 3.125 μg/ml, 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml. The decrease in the accumulation of LPO products was as follows: 13.89±1.56%, 31.04±2.41%, 47.04±2.21%, 69.91±2.07%, 88.97±1.37%.

Table 3 – Amount of phenolic compounds from *Viscum album* L. leaves

Raw material name	*Viscum album* L. leaves	
Extractants used	Ratio of extract sample and Folin-Ciocalteu reagent	Content of phenolic compounds, % (n=6)
Ethanol 90%	1:1	1.74±0.03
Ethanol 70%	1:2	2.02±0.05
Ethanol 50%	**1:1**	**2.39±0.05**
Purified water	1:1	1.75±0.04

Note: When determining the total content of phenolic compounds, V_a = 0.5 ml was used for *Viscum album* L. leaves extracts.

Table 4 – Stability of 50% ethanol extraction complexes with Folin-Ciocalteu reagent in dynamics

Raw material	Exposure time, min											
	5	10	15	20	25	30	35	40	45	50	55	**60**
Optical density	0.613	0.664	0.686	0.704	0.710	0.713	0.716	0.720	0.733	0.762	0.767	**0.770**
Phenols content	1.902	2.061	2.129	2.185	2.204	2.219	2.223	2.235	2.275	2.366	2.381	**2.391**

Table 5 – Total content of phenolic compounds from *Viscum album* L. leaves

Raw material name	*Viscum album* L. leaves	
Extractants used	Stabilization time (min) of optical density	Content of flavonoids, % (n=6)
Ethanol 90%	30	0.327±0.004
Ethanol 70%	35	1.46±0.03
Ethanol 50%	30	1.83±0.05
Purified water	35	0.282±0.003

Note: when determining the total content of phenolic compounds, V_a = 5.0 ml was used for extracts from *Viscum album* L. leaves growing on *Malus domestica* Borkh.

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Table 6 – Study of complexes color stability of 50% ethanol extract with 2% aluminum (III) chloride alcohol solution in dynamics

Raw material name	Exposure time, min								
	5	10	15	20	25	30	35	40	45
Optical density	0.796	0.811	0.820	0.823	0.832	**0.833**	0.832	0.818	0.816
Phenols content	1.744	1.777	1.80	1.804	1.823	**1.825**	1.823	1.793	1.788

Table 7 – Changes in mitochondrial enzymes activity in *Viscum album* L. leaves

Index	*Viscum album* L. leaves collected in winter
Aconitase, U/g protein	3.36±0.14
Citrate synthase, U/g protein	0.54±0.07*
Succinate dehydrogenase, U/g protein	1.12±0.06*

Note: * – statistically significant in relation to the activity of aconitase (Kruskell-Wallis test, p <0.05).

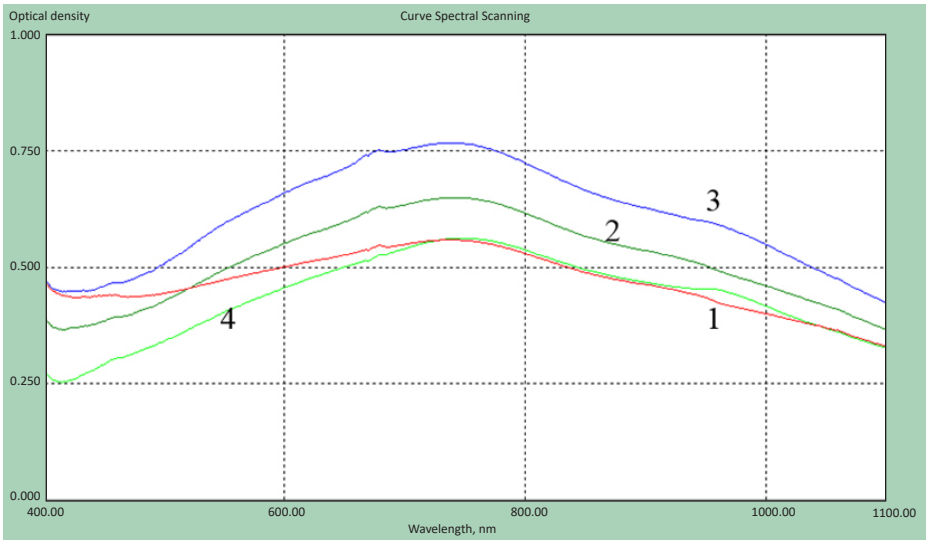

Figure 1 – UV absorption spectra of extracts complexes from *Viscum album* L. leaves with Folin-Ciocalteu reagent

Note: 1 – extract obtained by extraction with 90% ethanol; 2 – extract obtained by extraction with 70% ethanol; 3 – extract obtained by extraction with 50% ethanol; 4 – extract obtained by extraction with purified water.

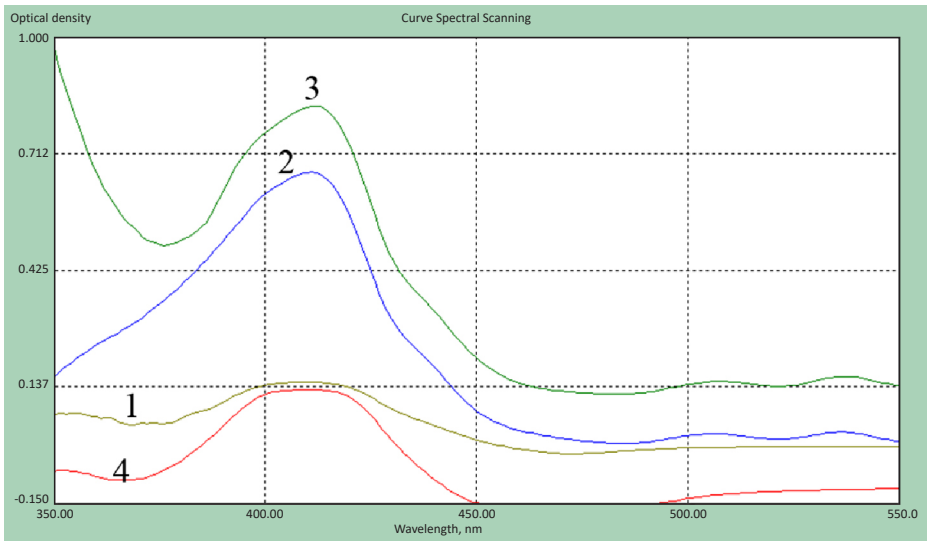

Figure 2 – UV absorption spectra of extracts complexes from *Viscum album* L. leaves with 2% alcohol solution of aluminum (III) chloride

Note: 1 – extract obtained by extraction with 90% ethanol; 2 – extract obtained by extraction with 70% ethanol; 3 – extract obtained by extraction with 50% ethanol; 4 – extract obtained by extraction with purified water.

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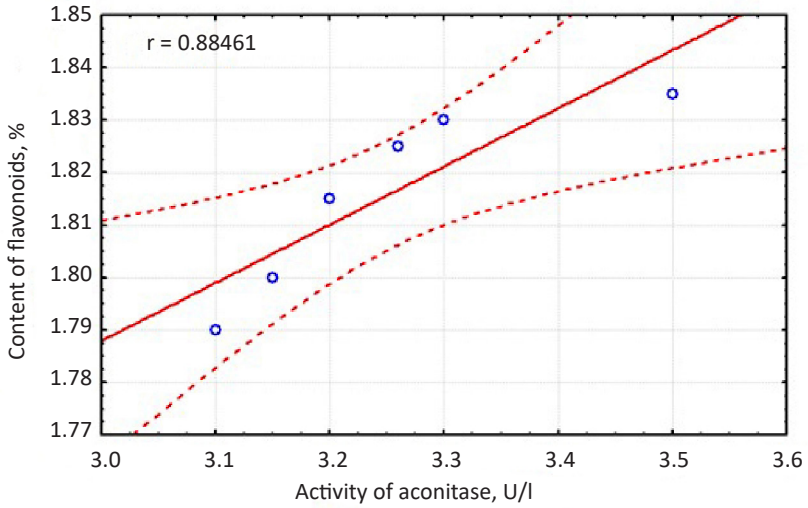

Figure 3 – Dependence of the change in the concentration of flavonoids on the activity of aconitase in *Viscum album* L. leaves

Note: the figure shows the determined indicators are highlighted in blue; the correlation trend line is solid; the data scatter boundaries are highlighted in dotted lines.

The maximum amount of total phenols was notified in the extract from the *Viscum album* L. leaves obtained by extraction with 50% ethyl alcohol, and it was 2.39 ± 0.05%. The optical density stabilization time was 60 min.

Due to the fact that the reaction develops in dynamics, it has been found out that the optical density value stabilizes within 60 min, which is optimal for the analysis.

Qualitative and quantitative analyses of flavonoids

A quantitative determination of flavonoids was carried out in terms of rutin in 6 repetitions. The results of determining the total content of flavonoids are shown in Fig. 2 and Table 5.

The data presented in Table 5 show that the maximum amount of total flavonoids in the extracts from *Viscum album* L. leaves is observed during the extraction of raw materials with 50% ethyl alcohol, and it is 1.83 ± 0.04%. The stabilization time of the optical density in the analyzed solutions is from 30 to 35 min.

Due to the fact that the reaction evolves in time, it has been found out that the optical density value stabilizes within 30 min, which is optimal for the analysis [26].

Evaluation of changes in the activity of mitochondrial enzymes in *Viscum album* L. leaves

The study of changes in the activity of enzymes of the mitochondrial origin in *Viscum album* L. leaves showed that the activity of aconitase was higher than the activity of citrate synthase and succinate dehydrogenase by 6.2 times ($p < 0.05$) and 3.0 times ($p < 0.05$), respectively.

As a result of the correlation analysis, it was found that the content of flavonoids, as the main polyphenolic antioxidants, in *Viscum album* L. leaves strongly correlates with the changes in the aconitase activity with a correlation coefficient of r=0.88416 (Fig. 2). In case of assessing the dependence of changes in the concentration of flavonoids on the activity of citrate synthase and succinate dehydrogenase, a weak correlation was notified (r=0.23681 and r=0.33984).

DISCUSSION

Flavonoids are a class of structurally diverse secondary metabolites that perform many functions in plants. For example, the leading role of flavonoids in the growth regulation, pigment biosynthesis, transmission of intracellular signals, a plant protection from ultraviolet radiation and the action of reactive oxygen species have been established. Today, flavonoids are one of the most studied groups of natural compounds, while most of the ongoing investigations are aimed at evaluating their pharmacological activity. At the same time, the features of biosynthesis and accumulation of flavonoids are also of certain scientific interest [31].

Biosynthesis of all secondary metabolites in plants is a controlled and deterministic process based on a strictly defined sequence of enzymatic reactions. While the pathways for the synthesis of carbohydrates, fats, proteins, and nucleic acids, or simply primary metabolites, are essentially the same, there is a

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wide variety of synthesis mechanisms for secondary metabolites in all living organisms. Understanding these metabolic processes is critical to regulating the biosynthesis of the desired phytochemicals and their potential use in the food or medical industry. Enzymatic control of flavonoid biosynthesis reactions implies the intensification of synthesis under certain conditions, which, as applied to a plant, depend on growing conditions and the season [32].

The majority of secondary metabolites, including flavonoids, primarily perform a protective function in plants, and their synthesis is activated under the influence of unfavorable external factors. In connection with this, in this study, the content of secondary metabolites of the polyphenolic structure in *Viscum album* L. leaves collected in winter, has been estimated, i.e. under the conditions most predisposing to their synthesis in large quantities. As a result, it was shown that the maximum extraction of flavonoids was notified when using 50% ethyl alcohol while the content of the desired substances was 1.83 ± 0.05%. A high content of these substances in *Viscum album* L leaves collected in winter, can be explained by the fact that low temperatures stimulate the expression of genes for enzymes responsible for the synthesis of flavonoids: PAL (Phenylalanine-ammonia lyase), chalcone synthase, flavanone-3-hydroxylase, dihydroflavonol- 4-reductase, anthocyanidin synthase, flavonoid-3-O-glucosyltransferase, leukoanthocyanidin reductase [12, 13], which lead to an increase in the synthesis and accumulation of flavonoids and other polyphenols. A strong correlation has also been notified between the change in the content of flavonoids and the activity of aconitase (r=0.88416) in *Viscum album* L. leaves, while the dependence in relation to citrate synthase (r=0.23681) and succinate dehydrogenase (r=0.33984) was weak.

Aconitase, also known as aconitate hydratase, is an iron-sulfur cluster protein enzyme that catalyzes the reversible isomerization of citrate to isocitrate via cis-aconitate in the tricarboxylic acid cycle. In mammals, the enzyme is found out in two-cell compartments – mitochondria and cytoplasm. The mitochondrial isoform is directly involved in the Krebs cycle, while the role of the cytoplasmic isoenzyme is reduced to the regulation of the stability of certain transcripts upon cleavage of the iron-sulfur cluster (in this case, aconitase acts as an RNA-binding protein) [33].

In plants, cytoplasmic and mitochondrial enzymes have similar characteristics and cannot be differentiated with a high degree of selectivity. Plant aconitase is involved in the reactions of the glyoxylate cycle and sucrose metabolism, as well as in several important physiological processes, including the inactivation of peroxides and nitroxides. Recent studies have shown an important role of aconitase in the biosynthesis of secondary metabolites, including flavonoids. Thus, some hybrids demonstrating a reduced concentration of flavonoids compared to the maternal line, have a deficient aconitase phenotype [34].

Citrate synthase also plays a significant role in the synthesis of secondary plant metabolites. Zhao H. et al., showed that mitochondrial citrate synthase is a key regulator of anthocyanin synthesis in *Petunia hybrida hort. ex E. Vilm* [35]. In its turn, succinate dehydrogenase is responsible for the synthesis of organic acids in plant cells [36]. Apparently, different roles of mitochondrial enzymes in the biosynthesis of secondary plant metabolites can explain their interrelation established in this study.

Thus, it can be assumed that, under conditions of a temperature stress, protective enzymatic and non-enzymatic mechanisms (aconitase and increased synthesis of flavonoids, respectively) are activated in *Viscum album* L. leaves, the action of which is aimed at maintaining cell viability. It has been established that the impact of low temperatures as a strong stressor on a plant cell leads to the accumulation of reactive oxygen species (ROS): hydroxyl radicals, superoxide anion, singlet oxygen and hydrogen peroxide. High levels of ROS have a detrimental effect on cell structures, leading to the DNA damage, lipid peroxidation, protein denaturation, reduced photosynthesis, an impaired enzyme activity, and a cell death. Therefore, maintaining a moderate level of ROIS is necessary for the protection against various abiotic and biotic stresses. It is the activation of oxidative stress that can be associated with an increased expression of aconitase from *Viscum album* L. leaves and the synthesis intensification of secondary metabolites [37].

CONCLUSION

The content of antioxidants, phenols, flavonoids in the leaves of *Viscum album* L. growing on *Malus domestica* Borkh., has been established. The optimal extractant is 50% ethyl alcohol. The analysis of the mitochondrial enzymes activity showed that in *Viscum album* L. leaves collected in winter, a high activity of aconitase which strongly correlated with the changes in the concentration of flavonoids (r = 0.88416), was notified, while there was no interrelation between the activity of succinate dehydrogenase, citrate synthase and the content of flavonoids in *Viscum album* L. leaves.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

SLA – development of the study concept, obtaining analyzed extracts, conducting an *in vitro* study, preparing the final version of the manuscript; DIP – development of the study concept, assessment of the mitochondrial enzymes activity, statistical processing of the study results, preparing the final version of the manuscript; NMCh – development of the study concept, obtaining analyzed extracts, conducting an *in vitro* study, preparing the final version of the manuscript; EOK – determination of the antioxidant effect, analysis of the literature; ETO – development of the study concept, analysis of the literature, preparing the final version of the manuscript.

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