The investigation prospect application of alcohol-water extract of lingonberry as antimicrobial, anti-fungi and antioxidant pharmaceutical

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Infection diseases is a worldwide important problem for medicine and pharmacy. Every year 13.7 million people die from bacterial infections in the world. Thus, the search of new compounds with antimicrobial and anti-fungi activity is topical for today. The object of the study was lingonberry leaf 60% extract. The content of phenylpropanoids derivatives was evaluated by spectrophotometric method of analysis, whereas organic acids by alkalimetric method; antioxidant power was found by potentiometric method; antibacterial and antifungal activity was assessed by the method of "wells".

According to the results of the study, it was shown that the lingonberry leaf extract is inferior to the green tea leaf extract in terms of the content of phenolic compounds, catechins, hydroxycinnamic and organic acids, but flavonoids dominate in the lingonberry extract. The study found that lingonberry leaf extract has a high level of antioxidant activity. When comparing the levels of antioxidant action of lingonberry extract with green tea extract and the «gold standard» – aepigallocatechin-3-O-gallate in one concentration of 0.03 mol/L, it was found that lingonberry extract had the best result. *Escherichia coli* bacteria was the most sensitive to the extract whereas *Pseudomonas aeruginosa* was the most resistant.

This work shows that 60% ethanolic lingonberry leaf extract has potent antioxidant, antimicrobial and antifungal effects. According to the obtained results, it can be concluded that 60% *V. vitis-idaea* leaf extract is promising for the development of new antimicrobial, antifungal and antioxidant pharmaceutical.

Keywords: lingonberry; leaf; antioxidant power; antimicrobial activity; anti-fungi activity.

Лингонберри спиртінің сығындысын микробқа қарсы, саңырауқұлаққа қарсы және антиоксидантты дәрі ретінде қолдану перспективаларын зерттеу

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Жұқпалы аурулар бүкіл әлемде медицина мен фармацияның өзекті мәселесі болып табылады. Жыл сайын әлемде 13,7 миллион адам бактериялық инфекциялардан қайтыс болады. Осылайша, бүгінгі күні микробқа қарсы және саңырауқұлаққа қарсы белсенділігі бар жаңа қосылыстарды іздеу өзекті болып табылады. Зерттеу объектісі лингонжидек жапырақтарының 60% этанол сығындысы болды. Фенилпропаноид туындыларының құрамы спектрофотометриялық талдау әдісімен, органикалық қышқылдар – сілтілік әдіспен бағаланды; антиоксиданттық әсер потенциометриялық әдіспен анықталды; бактерияға қарсы және зеңге қарсы белсенділігі «құдық» әдісімен бағаланды.

Зерттеу нәтижелері бойынша лингонжидек жапырағы сығындысы фенолды қосылыстардың, катехиндердің, гидроксицинамикалық және органикалық қышқылдардың мөлшері бойынша көк шай жапырағы сығындысынан төмен екені, бірақ жидек сығындысында флавоноидтардың басым екені анықталды. Зерттеу лингонжидек жапырағы сығындысының антиоксиданттық белсенділігі жоғары екенін анықтады. Лингонжидек сығындысының антиоксиданттық әсер деңгейін жасыл шай сығындысымен және «алтын стандартты» – эпигаллокатехин-3-О-галлаттың бір концентрациясы 0,03 моль/лмен салыстырған кезде, ең жақсы нәтиже лингонжидек сығындысы екені анықталды. Ішек таяқшасының бактериялары сығындыға ең сезімтал болды, ал Pseudomonas aeruginosa ең төзімді болды.

Бұл жұмыс 60% лингонжидек жапырағы сығындысының күшті антиоксиданттық, микробқа қарсы және саңырауқұлақтарға қарсы әсері бар екенін көрсетеді. Алынған нәтижелерге сүйене отырып, 60% лингонжидек жапырағы сығындысы жаңа микробқа қарсы, саңырауқұлақтарға қарсы және антиоксиданттық препараттарды жасау үшін перспективалы деп қорытынды жасауға болады.

Түйін сөздер: лингонжидек; жапырақ; антиоксиданттық күш; микробқа қарсы белсенділік; саңырауқұлақтарға қарсы белсенділік.

Исследование перспектив применения спиртоводного экстракта брусники в качестве противомикробного, противогрибкового и антиоксидантного лекарственного средства

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Инфекционные заболевания являются актуальной проблемой медицины и фармации во всем мире. Ежегодно в мире от бактериальных инфекций умирают 13,7 миллиона человек. Таким образом, на сегодняшний день поиск новых соединений с антимикробной и противогрибковой активностью является актуален. Объектом исследования был 60% этанольный экстракт листьев брусники. Содержание производных фенилпропаноидов оценивали спектрофотометрическим методом анализа, органических кислот – алкалиметрическим методом; антиоксидантный эффект установлено потенциометрическим методом; антибактериальную и противогрибковую активность оценивали методом «колодцев».

По результатам исследования было показано, что экстракт листьев брусники по содержанию фенольных соединений, катехинов, гидроксикоричных и органических кислот уступает экстракту листьев зеленого чая, но при этом в экстракте брусники доминируют флавоноиды. В ходе исследования было установлено, что экстракт листьев брусники обладает высоким уровнем антиоксидантной активности. При сравнений уровней антиоксидантного действия экстракта брусники с экстрактом зеленого чая и «золотого стандарта» – эпигаллокатехин-3-О-галлат в одной концентраций 0,03 моль/л было установлено, что экстракт брусники имеет наилучшей результат. Наиболее чувствительными к экстракту были бактерии *Escherichia coli*, тогда как наиболее устойчивой был *Pseudomonas aeruginosa*.

Данная работа показывает, что 60% экстракт листьев брусники обладает мощным антиоксидантным, противомикробным и противогрибковым действием. По полученным результатам можно сделать вывод, что 60% экстракт листьев брусники перспективен для разработки новых антимикробных, противогрибковых и антиоксидантных препаратов.

Ключевые слова: брусника; лист; антиоксидантная сила; антимикробная активность; противогрибковая активность.

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Article

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1. Introduction

Nowadays, the problem of bacterial infection is still relevant. According to recent statistical studies, it has been found that every year 13.7 million people per year die from bacterial infections in the world. The mortality rate for all ages was 99.6 deaths per 100.000 population. Of the pathogens studied, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* accounted for 54.9% of the 7.7 million deaths, with *S. aureus* being associated with more than 1.1 million deaths. *S. aureus* was the leading bacterial cause of death in 135 countries and was associated with the largest number of deaths among people over 15 years of age (940.000) [1]. In addition, an important threat to human populations is fungal infection. According to the latest statistics, every year 1,433,000 people suffer from systemic candidal infections, of which approximately 611 thousand people die annually [2, 3]. Thus, the search of new compounds with antimicrobial and anti-fungi activity is topical for today.

One of the richest plant sources of phenolic compounds is lingonberry. Lingonberry (*Vaccinum vitis-idaea* L.) is an evergreen shrub of the *Ericaceae* family. The distribution area is in Russia, the Baltic countries, the northern regions of Ukraine and Belarus, and Canada [4]. The chemical composition of lingonberry leaves is represented by a variety of biological active substances (BAS): hydroquinone derivatives (arbutin, methylarbutin), catechins (epicatechin, (+)-catechin), flavonoids (rutin, quercetin), hydroxycinnamic acids (ferulic and caffeic acid) and organic acids (citric and malic acid) [5, 6, 7].

Owing to rich chemical content of *V. vitis-idaea* leaf, extract has possessed variety of pharmacological activity: antimicrobial, antioxidant, anti-inflammatory, diuretic and neuroprotective actions [8, 9, 10]. In folk medicine the *V. vitis-idaea* leaf is applied in the dosage form of decoction in treatment and prevention of cystotitis, pyelonephritis, gastritis, atherosclerosis, and diarrhea [11].

Many studies have studied the antioxidant activity of aqueous and water-alcohol extracts of *V. vitis-idaea* leaf using the DPPH, ABTS and FRAP assays [12, 13], but according to literature sources, there is no studies of antioxidant activity have been carried out using the potentiometric method. In addition, in a number of recent works, the antimicrobial activity of aqueous extracts was studied [8, 13, 14], but these is no date about possibilities of inhibiting bacterial growth by alcohol-water extracts of *V. vitis-idaea* leaf.

Therefore, the purpose of our study was to assess the prospects of alcohol-water extract of *V. vitis-idaea* leaf by determination the quantitative content of polyphenols and organic acids, and conducting an *in vitro* investigation of antimicrobial, antifungal and antioxidant activity.

2. Experiment

2.1 Plant material

V. vitis-idaea leaf was collected in October 2021 in the Kostivtsi village, Zhutomyr region, Ukraine (50.329417, 29.536861).

Green tea (*Camellia sinensis* L.) leaf was collected in Anhui province, China (30.634140518993203, 116.33254121482477).

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2.2 Equipment

The pH meter HANNA 2550 (Germany) with a combined platinum electrode EZDO 50 PO (Taiwan) was applied for potentiometric measurements. Quantitative analysis of biological active compounds was carried out on UV-spectrophotometer UV – 1000 (China) with matched 1 cm quarts cells. Weighing was carried out using digital analytical balance AN100 (AXIS, Poland) with d = 0.0001 g.

2.3 Extraction procedure

A 10.0 g (exact mass) of *V. vitis-idaea* leaf were grinded in the size 1-2 mm. The extraction was carried by 60% ethanol on water bath at 80ºC within 1 h on water bath with a condenser, raw material/solvent 1/20. The procedure was performed twice to provide completely extraction of biological active substances (BAS), then the filtrates were united and concentrated by vacuum evaporator to ratio of extract to raw material 1:2.

2.4 Qualitative analysis

The total content of phenolic compounds was measured by the Folin-Ciocaltau assay, the optical density was measured at 760 nm [15]. The calibration curve was plotted with interval concentrations 1.0 – 5.0 µg/ml, the calibration equation Y = 0.1055X + 0.1745 (R^2=0.9951). Expressed as gallic acid and calculated according to the following equation:

$$X(\%) = \frac{C_x \times K_{dil} \times 100}{V} \quad \text{(Eq.1)}$$

where, C_x – concentration of gallic acid according to the calibration curve, C×10^{-6}, g/ml; *V* – volume of extract, ml; K_{dil} – coefficient of dilution.

The vanillin reagent assay was applied to find out the total catechins [16], the absorbance was measured at 505 nm. The calibration curve was plotted with interval concentrations 100 – 400 × 10^{-6} g/ml, the calibration equation Y = 0.0025X – 0.0851 (R^2 = 0.9951). The total catechins content in extract, expressed as epigallocatechin-3-O-gallate, was calculated according to the following equation:

$$X(\%) = \frac{C_x \times K_{dil} \times 100}{V} \quad \text{(Eq.2)}$$

where, C_x – concentration of epigallocatechin-3-O-gallate according to calibration curve, C×10^{-6} g/ml; *V* – volume of extract, ml; K_{dil} – coefficient of dilution.

The total flavonoids were determined using assay of complex formation with $AlCl_3$, the absorbance was measured at 417 nm [17]. The total flavonoids content in extract, expressed as rutin was calculated according to the following equation:

$$X(\%) = \frac{C_x \times K_{dil} \times 100}{A_{st} \times V} \quad \text{(Eq.3)}$$

where, *A* – absorbance of analyzed solution; A_{st} – absorbance of standard solution of rutin; *V* – volume of extract, ml; K_{dil} – coefficient of dilution.

The total hydroxycinnamic acids derivatives content was measured by assay of complex formation with $NaNO_2$-Na_2MoO_4, the absorbance was measured at 505 nm [18]. The total content of hydroxycinnamic acids derivatives in extract, expressed as chlorogenic acid was calculated according to the following equation:

$$X(\%) = \frac{C_x \times K_{dil}}{188 \times V} \quad \text{(Eq.4)}$$

where, *A* – absorbance of analyzed solution; 188 – specific adsorption coefficient of chlorogenic acid; *V* – volume of extract, ml; K_{dil} – coefficient of dilution.

The total organic acids content was determined by acid-base titration with the fixation end-point by potentiometric method [19]. The total content of organic acids in extract, expressed as citric acid was calculated according to the following equation:

$$X(\%) = \frac{(V_{equiv} - V_x) \times 0.0032 \times K_{dil} \times K \times 100}{V} \quad \text{(Eq.5)}$$

where, 0.0032 – the amount of citric acid, which is equivalent to 1 ml of sodium hydroxide solution (0.05 mol/l), g; V_{equv}. is the volume of sodium hydroxide solution (0.05 mol/l), which was used for titration, ml; *Vx* – the volume of sodium hydroxide solution (0.05 mol/l), which was spent for titration in a blank experiment, ml; *V* – volume of extract, ml; K_{dil} – coefficient of dilution; *K* is correction coefficient for 0.05 mol/l sodium hydroxide solution.

2.5 Antioxidant activity assay

Antioxidant activity of extract was evaluated by potentiometric method [20, 21]. Antioxidant activity was calculated according to the following equation and expressed as mmol-equiv./$m_{dry\ res.}$:

$$AOA = \frac{C_{OX} - \alpha \times C_{red}}{1 + \alpha} \times K_{dil} \times 10^3 \times \frac{m_1}{m_2} \quad \text{(Eq.6)}$$

where, $\alpha = C_{ox}/C_{red} \times 10^{(\Delta E - E\text{ethanol})nF/2.3RT}$; C_{ox} – concentration of $K_3[Fe(CN)_6]$, mol/l; C_{red} – concentration of $K_4[Fe(CN)_6]$, mol/l; $E_{ethanol}$ – $0.0546 \cdot C_{\%} - 0.0091$; $C_{\%}$ – concentration of ethanol; *ΔE* – change of potential; *F* = 96485.33 C/mol – Faraday constant; n = 1 – number of electrons in electrode reaction; *R* = 8.314 J/molK – universal gas constant; *T* – 298 K; K_{dil}– coefficient of dilution; m_1 – mass of dry residue; m_2 – mass of dry residue in 1.0 ml of extract.

Epigallocatechin-3-*O*-gallate, 60% extract of *C. sinensis* leaf were used as the standard.

2.6 Test organisms

Museum strains of *Staphylococcus aureus ATCC 25923*, *Staphylococcus aureus ATCC 6538*, *Escherichia coli ATCC 25922*, *Proteus vulgaris NTCS 4636*, *Pseudomonas aeruginosa ATCC* 27853 and *Candida albicans ATCC 885/653* were used in accordance with the recommendations for the assessment of antimicrobial activity of drugs**.**

2.7 Antimicrobial activity assay

In our study, we used solution of extract, the solvent of which were 60% ethanol. The method of diffusion of the drug into agar carried out using the method of “wells” [22]. Studies of

antibacterial activity performed using the method of wells. Preparation of microorganisms suspensions with determined concentrations of microorganisms (optical density) was carried out by the standard of turbidity (0.5 units according to scale of McFarland) with using of equipment of Densi-La-Meter (Czech, wavelength 540 nm). Suspensions were prepared according to equipment and information list. Colony forming unit was 107 microorganisms at 1 ml of growth medium and determined by standard of McFarland). On solidified agar, using a pipette under sterile conditions in Petri dishes made 1 ml of a suspension of microorganisms. After uniform distribution of microorganisms over the entire surface of the agar, the plates were incubated at room temperature for 15-20 min. Next, wells with a diameter of 6 mm were made in the cups, into which solutions of the test substances were introduced. The samples incubated at 37°C for 16-24 hs. After incubation, the plates were placed upside down on a dark matte surface so that light fell on them at an angle of 45° (accounting in reflected light). The diameter of the growth retardation zones measured using a caliper [23].

Gentamycin, and fluconazole were used as reference drugs for assessing antimicrobial and anti-fungal activity.

2.8 Statistical analysis

For all the experiments, two samples were analyzed and all the assays were carried out in 5 times. The results were expressed as mean values with confident interval. The MS EXCEL 7.0 and STATISTIKA 6.0 were used to provide statistical analysis.

3. Results and Discussion

The quantitative content of polyphenols, catechins, flavonoids and hydroxycinnamic acids in the analyzed *V. vitis-idaea* leaf extract was carried out using the spectrophotometric method, whereas organic acids were by titrimetric method. The *C. sinensis* leaf extract was chosen as "gold standard", due to its high content of polyphenols.

According to the obtained results of the study presented in Table 1, it was shown that the content of phenolic compounds was 2.13±0.04%, catechins – 1.39±0.02%, flavonoids – 0.65±0.01%, hydroxycinnamic acids – 0.21±0.04% and organic acids – 0.48±0.01%. Comparing with *C. sinensis* leaf extract it was found that sum of phenolic compounds, catechins, hydroxycinnamic acids and organic acids were 79, 87, 63 and 56% less than in *C. sinensis* leaf extract, respectively. Whereas, the content of flavonoids was higher in 50% than in *C. sinensis* leaf extract.

The content of BAS groups in the 60% extract of *V. vitis-idaea* leaf was in the following order: in the first place were polyphenols, in the second place were catechins, in the third place were flavonoids, in the fourth place were organic acids and in the last place were hydroxycinnamic acids.

A recent study of Bujor O.-C. *et al.* [24], they have investigated phenolic profile of lingonberry leaf from July to September. They found that sum of phenolic compounds content was 1.03, 1.55 and 2.50% in 50% ethanolic extract for July, August and September period, respectively. Compared to our results, in our study the sum of phenolic compounds was 2.13%, in our case we collected at September. Our obtained result is less than in mentioned research, but the difference in the content of phenolic compounds is, in our opinion, is associated with different brewing times, leaves/extractant ratio used, species, climate, and geographical position.

The level of antioxidant activity was assessed using the potentiometric method. The reference standard was *C. sinensis* leaf extract. To assess the "strength" of the antioxidant effect of the studied samples, it was used recently developed conditional classification of the level of antioxidant activity [25], which based on the "gold standard" epigallocatechin-3-O-gallate. Table 2 shows that the level of antioxidant activity of *V. vitis-idaea* leaf extract was 148.39±2.00 mmol-eqv./$m_{dry\ res.}$, and the "strength" of antioxidant effect according to conditional classification was high. The level of antioxidant activity of *V. vitis-idaea* leaf extract was 73% lower than *C. sinensis* leaf extract.

Table 2 – The level of antioxidant activity of *V. vitis-idaea* leaf liquid extract

Sample	Antioxidant activity, mmol-eqv./$m_{dry\ res.}$ ±SD	Conditional term of antioxidant level
60% extract of *V. vitis-idaea* leaf	148.39±2.00	High level
60% extract of *C. canensis* leaf	548.79±2.00	Very high level

Note: SD – standard deviation**,** n=3.

Table 1 – The sum of phenolic compounds, catechins, flavonoids, hydroxycinnamic acids and organic acids in *V. vitis-idaea* leaf liquid extract

Sample	Amount of polyphenols, %±SD	Amount of catechins, %±SD	Amount of flavonoids, %±SD	Amount of hydroxycinnamic acids, %±SD	Amount of organic acids, %±SD
60% extract of *V. vitis-idaea* leaf	2.13±0.04	1.39±0.02	0.65±0.01	0.21±0.04	0.48±0.01
60% extract of *C. sinensis* leaf	10.10±0.05	10.47±0.05	0.31±0.01	0.56±0.02	1.08±0.01

Note: SD – standard deviation**,** n=3.

Further, it was prepared solutions (in terms of the amount of polyphenols expressed as gallic acid) of extracts with 0.03 M concentration of *V. vitis-idaea* leaf extract, *C. sinensis* leaf extractand epigallocatechin-3-O-gallate. As a result of the study, it was found that the level of antioxidant activity of *V. vitis-idaea* extract was 10 and 15% higher of epigallocatechin-3-O-gallate and green tea leaf extract, respectively (Table 3).

Table 3 – The level of antioxidant activity of *V. vitis-idaea* leaf liquid extract at concentration 0.03 mol/L

Sample	Molar concentration, mol/L	Antioxidant activity, mmol-eqv./$m_{dry\ res.}$ ±SD
60% extract of *V. vitis-idaea* leaf		34.27±1.00
60% extract of *C. canensis* leaf	0.03[a]	27.49±1.00
Epigallocatechin-3-O-gallate		30.78±1.00

Note: SD – standard deviation, n=3, a – molar concentration of *V. vitis-idaea* and green tea leaf extracts was calculated as total phenolic compounds expressed as gallic acid.

The most sensitive to *V. vitis-idaea* extract was the bacterial strain *E. coli* (22.00±0.20 mm), and the least sensitive was *P. aeruginosa* (18.00±0.20 mm) (Table 4). *V. vitis-idaea* leaf extract inhibited bacterial growth by 9%, 13%, 25%, and 34% less than the reference standard gentamicin for *S. aureus*, *E. coli*, *P. vulgaris*, and *P. aeruginosa*, respectively. In the case of a study of antifungal activity, it was determined that *V. vitis-idaea* leaf extract inhibits the growth of the fungus *C. albicans* at the level of the fluconazole standard. In research of Kryvtsova et *al.* [26] was investigated the antimicrobial activity of *V. vitis-idaea* leaf extract obtained with 96% ethanol against *S. aureus* by the «well» method. As result, it was established that retention zone of extract was 24.4 mm. Comparing with our data, our *V. vitis-idaea* leaf extract interferer on 18%. It may have associated with a different amount of phenolic compounds, the content of sum phenolic compounds in Kryvtsova *et al.* in two times higher than in our obtained extract.

Ștefănescu. *et al.* [27] investigated antimicrobial activity of *V. vitis-idaea* leaf extract as a solvent was used 40% ethanol as well as raw material was collected on the territory of Romania. It was shown that *V. vitis-idaea* leaf extract was more active against Gramm-positive than Gramm-negative bacteria. Comparing results, there was not such relation found between Gramm-positive and Gramm-negative strains, in our view, it may have associated with different geographical location, extraction and cultivars.

At first glance it can be considered that the antimicrobial and antifungal activity of *V. vitis-idaea* leaf extract is significantly inferior to the action of gentamicin and fluconazole, because their concentration of solutions was significantly lower than the content of phenolic compounds in the extract.

However, we would like to note that gentamicin has serious toxicity to the auditory nerve, kidneys and liver, which can lead to serious complications of the disease [28, 29, 30]. Comparing the antifungal effects of fluconazole and *V. vitis-idaea* leaf extract, it was found that they inhibited the growth of the fungal strain at the same level, while the concentration of fluconazole was also lower, like gentamicin.

We can declare that fluconazole is a leader as anti-fungi medicine, but at the same time it weakly inhibits the growth of gram-negative and gram-positive bacteria, but to *V. vitis-idaea* leaf extract both strains of bacteria and fungus are sensitive. Thus, *V. vitis-idaea* leaf extract is a combined pharmaceutical that affects different mechanisms of vital activity of bacteria and fungi, thereby having a wide spectrum of action against different strains of bacteria and fungi, and at the same time not possessing serious toxicity.

4. Conclusion

A research was shown that *V. vitis-idaea* leaf alcohol-water extract contain high content of polyphenols and organic acids. This work shows that 60% *V. vitis-idaea* leaf extract has potent antioxidant, antimicrobial and antifungal effects. *E. coli* and *C. albicans* were the most sensitive to *V. vitis-idaea* leaf extract. The antioxidant activity of 60% *V. vitis-idaea* leaf extract is higher than 60% *C. sinensis* leaf extract. According to the obtained results, it can be concluded that 60% *V. vitis-idaea* leaf extract is promising for the creation of new antimicrobial, antifungal and antioxidant pharmaceutical.

Table 4 – Results of antimicrobial and antifungal activity of *V. vitis-idaea* leaf liquid extract

Sample	Concentration, mmol/L	Diameter of the growth retardation zone, mm				
		S. aureus ATCC *25923*	*E. coli* ATCC *25922*	*P. vulgaris ATCC 4636*	*P. aeruginosa* ATCC *27853*	*C. albicans ATCC 653/885*
60% extract of *V. vitis-idaea* leaf	0.04	20.00±0.20	22.00±0.20	20.00 ±0.20	18.00±0.20	20.00±0.10
Gentamycin	0.003	22.00±0.20	25.33±0.33	25.00±0.20	25.67±0.67	12.00±0.20
Fluconazole	0.003	18.00±0.20	14.33±0.33	12.33±0.33	10.00±0.20	20.00± 0.50

Authors' Contributions

Maslov O. Y.: Writing – Original draft, Methodology, Investigation; Komisarenko M.A.: Data curation, Investigation, Visualization; Ponomarenko S.V.: Data curation, Investigation, Visualization; Osolodchenko T.P.: Conceptualization, Supervision; Kolisnyk S.V.: Conceptualization, Supervision; Golik M.Yu.: Writing, Review & Editing.

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