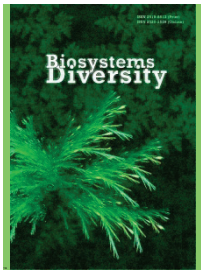

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Antibiofilm forming, antimicrobial activity and some biochemical properties of *Vaccinium vitis idaea* leaf and berry extracts on *Staphylococcus aureus*

M. V. Kryvtsova*, I. Salamon**, J. Koscova***, M. Y. Spivak*****

*Uzhhorod National University, Uzhhorod, Ukraine
**University of Prešov, Prešov, Slovakia
***University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia
****D. K. Zabolotny Institute of Microbiology and Virology of NAS of Ukraine, Kyiv, Ukraine

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Uzhhorod National University, Pidhirna st., 46, Uzhhorod, 88000, Ukraine. Tel.:+ 38-050-278-54-97. E-mail: maryna.krivcova@gmail.com

University of Prešov, Novembra st., 17, Prešov, 08116, Slovakia. Tel.: +42-191-798-40-60. E-mail: ivan.salamon@unipo.sk

University of Veterinary Medicine and Pharmacy in Košice, Komenský st., 73, Košice, 04181, Slovakia. Tel.: +42-190-548-08-97. E-mail: jana.koscova@uvlf.sk

D. K. Zabolotny Institute of Microbiology and Virology of NAS of Ukraine, Zabolotny st., 154, Kyiv, 03143, Ukraine. Tel.: + 38-044-526-23-79. E-mail: n.spivak@ukr.net

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Infections caused by *Staphylococcus* genus bacteria remain a relevant problem due to the high percentage of antibiotic-resistant biofilm-forming strains of isolates of this genus. Herbs are a promising source for many biologically active compounds with antimicrobial properties. The aim of the research was to study the antimicrobial and antibiofilm formation activity of berry and leaf extracts of *Vaccinium vitis-idaea* L. upon clinical isolates of *S. aureus*, and the main biochemical properties of these extracts. For the purpose of analysis, we used *S. aureus* isolated from the mouth cavities and pharynx of human patients suffering from inflammatory diseases. The plants for the study were gathered in Pylypets, Mizhhiria rayon, Zakarpatska oblast (Transcarpathia). From *Vaccinium vitis-idaea* L., leaf and berry extracts were produced. To determine the chemical properties of the extracts, the following constituents were investigated: total tannin, flavonoids, total phenols, anthocyanins (by spectrophotometric method), and the total amount of vitamin C in berry extract (chromatographically). The antimicrobial activity was studied by diffusion-into-agar method and determination of minimum inhibitory concentrations. The antibiofilm activity of the extracts was tested in standard 96-well microtitration plates. The main chemical composition of ethyl extracts of *Vaccinium vitis-idaea* L. berries and leaves was identified. The level of tannins in leaf extracts was established to be higher than in fruit extracts (3.50% and 0.26% per 100 g of extract, respectively). It was shown that extracts of *V. vitis-idaea* berries and leaves demonstrate high antimicrobial activity against clinical isolates of *S. aureus*. Further it was established that leaf extracts had high ability to destroy the bacterial biofilm of *S. aureus*. Leaf extracts were also able to destroy the formed biofilm. Even in the 0.01% concentration, leaf extract inhibited the formation of the biofilm by 69.9% and caused the destruction of the formed biofilm by 62.5%. Thereby, the obtained results show good prospects for the use of *V. vitis-idaea* leaf extracts as an anti-staphylococcal remedy with antibiofilm forming properties.

Keywords: antimicrobial activity; antibiofilm formation; lingonberry extract; opportunistic bacteria.

Introduction

The rapid development of resistance of microorganisms to antibiotics has become a difficult issue for biology and medicine (Nikolaidis et al., 2012; Nikolaidis et al., 2014; Belbase et al., 2017). Over the past years, special attention has been paid to the mechanisms and causes of development of resistance to antimicrobial preparations and to the ways of overcoming it. At the same time, existence of bacteria in the form of biofilm as an elaborate community that occurs both in the environment and in the human organism is one of the causes of formation of chronic persisting inflammatory processes (Romanova & Gintsburg, 2011). Biofilm formation complicates the course of the infection process because bacteria in the biofilm structure acquire resistance to such environmental factors as temperature, pH indicators, on the one hand, and to antibiotics used to treat infections, on the other hand. The discovery of bacteria in the biofilm culture marked a major breakthrough in the understanding of peculiarities of the course, therapy and prevention of infectious diseases (Donlan, 2002; Sidashenko et al., 2015). Among the opportunistic bacteria, *Staphylococcus* genus microorganisms remain among the most widespread infectious agents which are complicated with the formation of biofilm (Plata et al., 2009; Pottinger, 2013). Staphylococci are known to be the pathogens of a considerable part of community-acquired and nosocomial infections (Power Coombs et al., 2013; Deyno et al., 2017). The main infections are caused by *S. aureus*, which may colonize and affect human organs and tissues, demonstrating a broad spectrum of adaptive possibilities. At the same time, the role of *S. haemolyticus* and *S. epidermidis* in the formation of inflammatory processes has also been growing over the past years (Patel et al., 2012; Fey & Olson, 2008).

This problem is especially vital for inflammatory diseases of the oral cavity, where most of the microorganisms are in the form of biofilm, and the course of the disease is often of a chronic and persistent type (Pérez-Chaparro et al., 2014). Medicinal plants that contain a wide spectrum of biologically active substances which have an additive antimicrobial activity constitute a mighty source for antimicrobial substances (Nazzaro et al., 2013; Sánchez et al., 2016). Plants as the source of antimicrobial substances have a number of advantages due to the low probability of side effects and high antioxidant properties that encourage the increase of the organism's resistance, and contain a wide choice of biologically active substances, vitamins, minor and major nutrients.

Although the antimicrobial activity of medicinal plants has been actively studied and used in folk and traditional medicine, it is crucial to investigate the influence of the substances of plant origin upon the biofilm-forming microorganism strains and their potential ability to destroy biofilms – because overcoming the resistance of biofilm to antimicrobial preparations and inhibiting its formation is considered as a promising way to develop new medicines with antimicrobial activity.

The purpose of this work was to study the antimicrobial and antibiofilm formation activity of fruit and leaf extracts of *Vaccinium vitis-idaea* L. upon clinical isolates of *S. aureus* and the main biochemical properties of the given extracts.

Materials and methods

Extract manufacturing techniques. The plant materials were collected in the territory of Pylypets, Mizhhiria rayon, Zakarpatska oblast (Trancarpathia), dried at the temperature of 30–35 °C in the shade, then ground and placed into tightly closed containers.

The leaves and fruit of *V. vitis-idaea* were used as the work material to obtain ethyl extracts. A 10 g batch of dry plant material was ground to powdery mass. In an Erlenmeyer flask, 10 g of plant material were blended with 200 mL of or 96º ethyl (Sigma, Germany). The flask neck was closed with a food wrap to avoid evaporation. Following a 30-minute-long incubation in the ultrasonic bath (Kraintek) at 35 °C, the blend was filtered through Whatman No. 1 filter paper. The clear solution was placed in an evaporative device (16–17/32" × 34–59/64" G5B, Coated Dry Ice Condenser Rotary Evaporator) to obtain pure alcoholic extract at 50 °C, 82 rpm. Then, the extracts were subjected to evaporation under the reduced pressure at 40 °C to remove the ether ethyl. The resulting extracts contained less than 1% of ethanol.

Antimicrobial assay. The antimicrobial activity of the extracts was determined using the agar diffusion test (Balouiri, 2016). The bacterium inocula 100 μL in the physiological solution were adjusted to the equivalent of 0.5 McFarland standard, and evenly spread on the surface of Muller-Hinton agar (Himedia, India) (incubated at 37 ± 2 °C for 24 hours). Optical density was determined on a Biosan densitometer. The extracts (10 μL) were introduced into wells 6 mm in diameter. The diameters of the inhibition zones were measured in millimetres including the diameter of the well. Each antimicrobial assay was performed at least three times.

As test cultures, the following reference bacterial strains were used: *S. aureus* ATCC 25923, *S. aureus* CCM 4223, a biofilm forming strain. We also used clinical strains of *S. aureus* (n = 20) and Methicillin-resistant *Staphylococcus aureus* (MRSA) (n = 20) isolated from the oral cavities of patients suffering from inflammatory periodontium and pharynx. As positive control, gentamicin (10 mg/disk) for Gram-negative bacteria, ampicillin (10 mg/disk) for Gram-positive bacteria, nystatin (100 UI) for *Candida* were used. As negative control, dimethyl sulfocide (DMSO) was used.

The antibacterial activity of the studied extracts was also assessed by the minimum inhibitory concentration (MIC) coefficient. To study the MICs of plant extracts, their following solutions in beef-extract broth were produced: 100, 50, 25, 22.5, 20.0, 17.5, 15.0, 12.5, 10.0, 7.5, 5.0, 3.5, 2.5, 2.25 mg/mL. The bacterial suspension was introduced into each test-tube in the amount of 100 μL, which corresponded to 0.5 McFarland standard (1.5×10^8 CFU/mL) from a 24-hour culture of microorganisms in sterile physiological solution. The test-tube was incubated for 24 hours at 37 °C, whereupon part of the contents of each test-tube was inoculated into the beef-extract broth. The last test-tube, whose inoculations did not show any growth of microbial culture, was taken as MIC. The negative controls were the following: bacterial suspension + dimethyl sulfocide; bacterial suspension + alcohol.

Determination of antibiofilm activity. The antibiofilm activity of the plant extracts was tested in standard 96-well microtitration plates (Greiner-BioOne, Austria) using modified staining method according to O'Toole (2011). With the purpose of studying the antibiofilm formation activity, an 18-hour culture of the reference *S. aureus* CCM 4223 grown at 37 °C was used. Into the wells, 180 μL of bacterial suspension, McFarland in broth (Tryptic soy broth (TSB), Himedia, India) was introduced. The *V. vitis-idaea* leaf and berry extracts were adjusted to the concentrations of 1%, 5% and 10% in DMSO (Sigma-Aldrich, USA) and introduced into the wells in the amount of 20 μL per well. Upon the addition of the bacterial suspension, the concentrations of plant extracts in the broth were equal to 0.10%, 0.05% and 0.01%, respectively. The wells with only 180 μL of broth and 20 μL of 10% DMSO served as control. Following a 24-hour-long incubation in the thermostat at 37 °C, the supernatant was withdrawn and washed 3 to 5 times with distilled water. Following a 30-minute-long incubation, it was dyed with 200 μL of 0.1% solution of crystal violet; then the dye was withdrawn, and the supernatant washed 3 to 5 times with distilled water. Into every well, 200 μL of 30% acetic acid was added and incubated for 10 minutes. Optical density was measured on the Synergy HT (Biotek, USA) spectrophotometer at 550 nm. The mean absorbance ($OD_{550\ nm}$) of the samples was determined and percentage inhibition obtained using Eq. 1. (Sandasi et al., 2011).

Negative controls: 180 μL of bacterial suspension + 20 μL of alcohol (ethyl or merthyl, respectively); 180 μL of suspension + 20 μL of dimethyl sulfocide.

When a 50% reduction in absorbance was observed, it was considered as significant inhibition (Raut et al., 2014).

Destruction of the biofilm on the formed microbial biofilms was determined by introducing extracts on a 48-hour biofilm and incubating it for 24 hours.

Tannins were determined spectrophotometrically (Galavo et al., 2018) with the use of a Folin-Ciocalteu reagent. The optical density was measured at 750 nm (A), using a Beckman Coulter DU 530v spectrophotometer (USA); water was used as the solution for comparison. The percentage of tannins was expressed compared with the activity of pyrogallol (Medini et al., 2014; Ganjiwale et al., 2007).

Determination of the total number of flavonoids. The contents of flavonoids was determined by absorption spectrophotometry. For quantitative determination, the spectrophotometric methods based on the measurement of absorption of the complex: aluminium chloride with flavonoids, was used. The quantitative content was recounted into rutin, and simultaneously the absorption of the standard rutin solution (the comparison solution) was measured. The total number of flavonoids was determined by aluminium chloride spectrophotometric method (Koolen et al., 2013; Vronska, 2018). The optical density was determined on the Beckman Coulter DU 530 spectrophotometer.

Determination of the level of anthocyanins in lingonberry fruit was performed spectrophometrically. To a 0.25 g sample, 20 mL of 0.1% methanol solution were added, then the composition was stirred for 30 min., filtered, and made up to 25 mL with methanol. Then 0.5 mL of the solution was mixed with 0.1% solution of hydrochloric acid in methanol. The optical density was measured with the wavelength of 528 nm. As control, 0.1% solution of hydrochloric acid in methanol was used.

Sum of lingonberry fruit and leaf polyphenols. The quantitative content of polyphenols was determined following the Procedures of the State Pharmacopoeia of Ukraine 1.2, "Determination of tannins in medicines of plant origin" (State Pharmacopoeia of Ukraine, 2015). The measurements were performed by spectrophotometric method with the wavelength of 760 ± 2 nm, in terms of pyrogallol. The optical density of the solution was measured at the wavelength of 760 nm, using water as a compensation solution.

Ascorbic acid was determined chromatographically (HPLC/DAD) with the use of the Dionex UltiMate 3000.

Organic acids were assessed by titrimetric method in terms of malic acid, according to the standard procedures (Logvinova et al., 2015).

The reagents used for the biochemical research were made by Sigma (Germany).

Data obtained were expressed as mean ± standard deviation (SD) of three measurements. The Tukey test was applied for comparisons of means; differences were considered significant at $P < 0.05$.

Results

The research has shown that lingonberry fruit and leaf extract has high antimicrobial activity against clinical isolates of *S. aureus* (Table 1). It is worth noting that the extracts demonstrated activity against MRSA *S. aureus*. The MIC of leaf and berry extracts against typical isolates equalled 2.5 mg/mL, whereas it grew by 1.5 times reaching 3.75 mg/mL when taken against MRSA.

The results of the study of the antibiofilm formation activity of the extracts are shown in Figures 1 and 2. The study of the antibiofilm formation activity of ethyl extract of *V. vitis-idaea* berry showed that it hardly affects the processes of biofilm formation. The introduction of a 0.1% solution of the extract reduced the biofilm forming ability by 28%, which fact statistically significantly did not differ from the activity of a 0.05% solution, and

the introduction of a 0.01% extract reduced the formation of biofilm by 18%. The investigation of the activity of *V. vitis-idaea* leaf extracts showed their high antibiofilm forming effect. The leaf extract reduced the formation of biofilm in the concentrations of 0.10%, 0.05% and 0.01%, this fact proved its significant antibiofilm forming effect. It is worth noting that the antibiofilm formation activity of the extract did not reduce significantly with the reduction of its concentration. Thus, we recorded an expressive antibiofilm formation activity of *V. vitis-idaea* leaf extract, whereas reduction of its concentrations would not affect significantly its antibiofilm formation activity.

Table 1

Antimicrobial activities of *Vaccinium vitis-idaea* L. berry and leaf extract against typical and clinical isolates of *S. aureus*, zone inhibition (mm, x ± SD)

Test culture	Berry extract	Leaf extract
S. aureus ATCC 25923	24.2 ± 0.15^{a}	24.4 ± 0.30^{a}
S. aureus CCM 4223 (biofilm formation)	23.6 ± 0.33^{a}	23.4 ± 0.50^{b}
S. aureus (clinical), oral cavity isolate, n = 10	24.5 ± 0.25^{a}	24.3 ± 0.54^{a}
MRSA (clinical), oral cavity isolate, n = 10	19.3 ± 0.52^{c}	20.7 ± 0.52^{c}
S. aureus (clinical), pharynx isolate, n = 10	24.3 ± 0.58^{a}	24.7 ± 0.58^{a}
MRSA (clinical), pharynx isolate, n = 10	22.0 ± 0.56^{b}	20.0 ± 0.51^{c}
Ethanol	–	–

Notes: the data were statistically significant as compared with the control ($P < 0.05$); ethanol control (extraction solvent) – no inhibition.

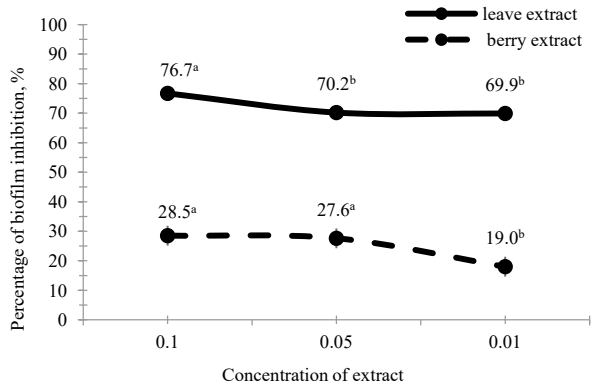

Fig. 1. Influence of leaf and berry extracts of *Vaccinium vitis-idaea* L. upon the formation of *S. aureus* biofilm: control – *S. aureus* suspension in broth + dimethylsulfoxide was taken as 100% and used as control; OD = 3.82 ± 0.20, n = 3

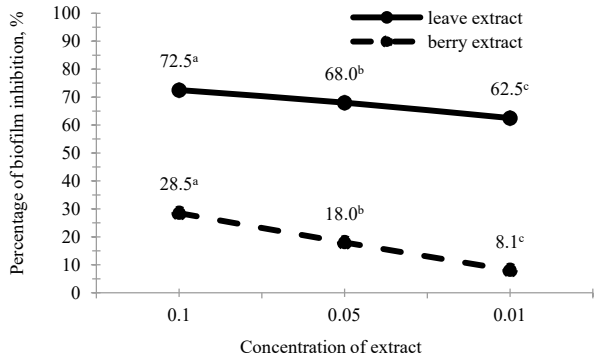

Fig. 2. Influence of leaf and berry extracts of *Vaccinium vitis-idaea* L. upon the formed *S. aureus* biofilm: control – *S. aureus* suspension in broth + dimethylsulfoxide were taken as 100% and used as control; OD = 3.82 ± 0.20, n = 3

The study of the influence of the extracts upon the formed biofilm showed that the berry extract was not capable of destroying the *S. aureus* biofilm. At the same time, the leaf extract demonstrated an expressive antibiofilm formation activity. Thus, a 0.10% leaf extract caused biofilm reduction by 72.5%, a 0.05% extract – by 68.0%, and a 0.01% extract – by 62.5%. Figure 3 shows the effect of the 0.10% leaf extract upon the formed biofilm. Tables 2–4 show characteristics of the chemical composition of lingonberry fruit and leaf extracts. A comparative analysis of the berry and leaf extracts allowed us to establish the following regularities. The leaf extract was characterized by a higher content of tannins, whose level in the leaves was by 13.5 times higher than in the berry. The same tendency was characteristic of the polyphenolic substances, whose content in the leaves was by 1.5 times higher than in the berry. The flavonoid contents in the leaves and in the berry did not differ statistically. Anthocyanins, vitamin C and organic acids, including malic acid, were found in the berry extracts, but they were absent in the leaf extracts.

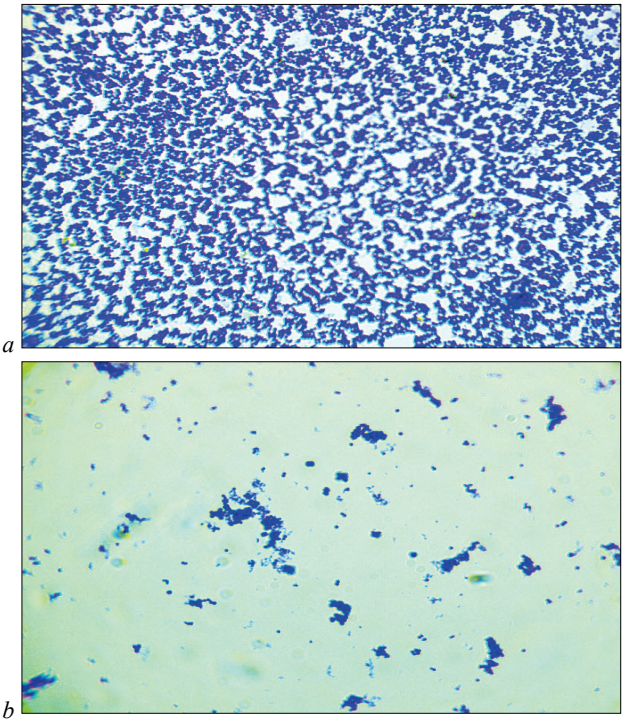

Fig. 3. A microphotography of biofilm: a – prior to, and b – after the introduction of *Vaccinium vitis-idaea* L. (leaf) extract upon the formed *S. aureus* biofilm

Table 2

Description of leaf and berry extracts of *Vaccinium vitis-idaea* L. (in % per 100 g of extract, x ± SD, n = 3)

Index	Leaves	Berry
pH (1% water solution)	5.2 ± 0.10	3.0 ± 0.10
Refraction index	1.4 ± 0.01	1.4 ± 0.01
Dry matter	35.0 ± 1.20	52.0 ± 1.12
Loss on drying	65.0 ± 1.15	48.0 ± 1.20
Ashes	1.0 ± 0.20	0.6 ± 0.20
Ethanol	<1	<1

Table 3

Certain chemical peculiarities of berry extract of *Vaccinium vitis-idaea* L. (in % per 100 g of extract, x ± SD, n = 3)

Index	In liquid extract	In dry residue
Tannins + polyphenols	0.94 ± 0.05	1.80 ± 0.05
Tannins	0.26 ± 0.05	0.50 ± 0.01
Polyphenols	0.68 ± 0.04	1.30 ± 0.03
Anthocyanins, cyanidine-3-glucoside chloride	0.14 ± 0.03	0.27 ± 0.02
Organic acids, including malic acid	13.00 ± 1.12	25.00 ± 1.20
Ascorbic acid	0.33 ± 0.05	0.63 ± 0.11
Flavonoids	0.03 ± 0.01	0.05 ± 0.01

Table 4

Certain chemical peculiarities of leaf extract of *Vaccinium vitis-idaea* L. (in % per 100 g of extract, x ± SD, n = 3)

Index	In liquid extract	In dry residue
Tannins + polyphenols	4.50 ± 0.02	12.90 ± 0.31
Tannins	3.50 ± 0.03	19.00 ± 0.52
Polyphenols	1.00 ± 0.05	2.90 ± 0.34
Flavonoids	0.03 ± 0.01	0.09 ± 0.01

Discussion

Scientific publications have described the antimicrobial properties of lingonberry fruit and showed its impact upon the biofilm of the oral cavity formed by *S. mutans* and *F. nucleatum* (Riihinen et al., 2014). It also established the fact of inhibition of the growth of *E. coli*, isolated from the urogenital tract when affected by berry extract of *V. vitis-idaea* (Wojnicz, 2012). The antibacterial activity of lingonberry leaf extract against *E. coli* was ascertained (Lahiri et al., 2019). The anti-adhesive properties were shown for the use of proto-anthocyanidines of *Vaccinium macrocarpon* Aiton berry (Huber et al., 2003).

The antimicrobial activity of berry extracts of *Vaccinium* genus plants upon *P. fluorescens*, *P. aeruginosa*, *B. cereus*, *S. aureus*, *S. marcescens*, *B. subtilis*, and *L. monocytogenes* was shown (Laslo & Kobolkuti, 2017). At the same time, data are available about the antimicrobial activity of lingonberry tincture upon Gram-positive cultures of *M. luteum* and *S. aureus*. It was also established that bilberry flavonoids exhibit antibiofilm formation activity against oral streptococci (Riihinen et al., 2014). The review of literature (Cowan, 1999; Ginovyan et al., 2017) analyzed the known biologically active substances contained in plants, and the mechanism of their antimicrobial activity. In particular, the authors noted the antimicrobial effect of tannins and flavonoids. The work by Hayashi et al. (2008) showed that tannins from *V. vitis-idaea* showed an antibacterial effect against *Porphyromonas ginigivalis* and *Prevotella intermedia*, and an inhibiting effect against *Listonella anguillarum* (serotypes O1 and O2), *Yersinia ruckeri*, *Photobacterium damselae* subsp. *piscicida*, and *Lactococcus garvieae* (Ho et al., 2001). The authors assumed that tannins isolated from *V. vitis-idaea* with antimicrobial activity could potentially be used for the treatment of periodontal disease.

Lingonberry leaves are an officinal plant preparation with described disinfectant and diuretic activity, which makes for their use for the treatment of urolithiasis, podagra, pyelonephritis, and cystitis. Its antibacterial activity is related to the presence of arbutin and methylarbutin (Vernigorova & Buzuk, 2019). It was also established that anthocyanins from plants of *Vaccinium* genus are both valuable as a food product and as a therapeutic means, including for diabetes (Karcheva-Bahchevanska et al., 2017).

The literature also mentions the composition of phenolic compounds of alcohol extract from lingonberry leaves; in particular, it identifies arbutin; two phenolcarbonic acids – gallic and ellagic; three hydroxycinnamic acids – chlorogenic, coumaric and ferulic; four coumarins; three flavonoid aglycons – luteolin, kaempferol and quercetin; gallotannins and ellagotannins (Vernigorova & Buzuk, 2019). At the same time, according to Quave et al. (2012), ellagic acid isolated from *Rubus ulmifolius* Schott. inhibits the formation of *S. aureus* biofilm and increases their antibiotic sensitivity. It was established that ellagic acid acts as an inhibitor of bioiflm formation (Fontaine et al., 2017), which fact may explain the mechanism of antibiofilm formation activity of lingonberry leaf extract.

The level of phenolic compounds and antioxidant activity of the plants growing in mountainous localities, including high-altitude swards in the Carpathians, was shown to be higher than that of the plants growing in lowlands. This fact is explained by the high adaptive potential of the former plants to temperature fluctuations and other environmental factors. These results draw us to the conclusion that the difference in climatic conditions, in addition to the different geographical location, does affect the adaptation of lingonberry plants with different biochemical peculiarities. High antioxidant activity of lingonberry leaves may appoint them as probable candidates for a role in the chemical prophylactics and treatment of cancer (Wang et al., 2005; Poorva et al., 2015).

Research (Bhullar & Rupasinghe, 2015) has also shown the high antioxidant and cytoprotective activities of polyphenols of lingonberry fruit. The authors also pointed to the gene-protective and anxiolytic activities of berry extracts from *Vaccinium* genus plants upon the rats' cognitive function following a 30-day feeding regime. What is more, the said extract reduces the oxidative damage of the brain DNA tissue (Karcheva-Bahchevanska et al., 2017).

The high level of ascorbic acid revealed in the berry may explain the high antioxidant activity of the berry extracts. The studies proved the synergetic action of the ascorbic acid and flavonoids. Ascorbic acid is known to play a considerable part in the metabolic processes of the human organism. In plants, ascorbic acid accumulates in the protein complex, the so-called ascorbigen. A peculiar feature of ascorbic acid is its participation in the oxidation-reduction reactions in human organisms. It was established that together with certain water-soluble antioxidants – flavonoids – ascorbic acid may much more efficiently prevent the oxidation processes; and taken in much lower concentrations, it may inhibit the formation of free radicals. It is indicative of a synergetic activity which significantly exceeds its cumulative effect. Flavonoids prevent oxidation of ascorbic acid; the ascorbic acid – bioflavonoid complex affects positively the functional state of the capillaries and improves the muscular performance. Ascorbic acid prevents oxidative inactivation of flavonoids, which in their turn inhibit the activity of ascorbate oxidase enzyme (Karpova et al., 2019).

It is known from literary sources that ascorbic acid intensifies the antioxidant activity of α-tocopherol and improves its regeneration in the organism. Intensification of this effect was also noted in the presence of biflavonoids.

Conclusion

Ethyl extract of *Vaccinium vitis-idaea* L. leaves demonstrated the highest antimicrobial activity, combined with the antibiofilm formation properties. Berry extract also had a high antimicrobial activity, but it was not able to destroy the *S. aureus* biofilm. It is worth noting that lingonberry leaf extract exerted antimicrobial activity upon both typical and clinical isolates of *S. aureus*, including the methicillin-resistant strains. Of all extracts analyzed, ethyl leaf extract demonstrated the highest level of tannins. The same tendency was characteristic of polyphenolic substances. The level of flavonoids in the ethyl leaf extract was equal to that in the berry extract. The berry extract also contained ascorbic acid and anthocyanins. The antimicrobial activity of the extracts to a greater extent correlated with the contents of tannins and polyphenols, whose level was the highest in the lingonberry leaf extract. At the same time, only the lingonberry leaf extract had an antibiofilm formation activity.

Therefore, *V. vitis-idaea* leaf extract was characterized by high antimicrobial activity against *S. aureus*, combined with the ability to cause biofilm destruction.

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