INVESTIGATION OF THE ANTIBACTERIAL ACTIVITY OF BILBERRY (*VACCINIUM MYRTILLUS* L.) AND LINGONBERRY (*VACCINIUM VITIS-IDAEA* L.) LEAF DRUGS USING THE DISK DIFFUSION METHOD

Csabai Judit[1] – Hörcsik Tibor Zsolt[1] – Lengyel Anita[1] – Mária Szanyi[1] – Anzhela Kolesnyk[2] – Kolesnyk Oleksandra[2] – Kryvtsova Marina[2]

[1]University of Nyíregyháza, Institute of Engineering and Agricultural Sciences, Department of Agricultural Sciences and Environmental Management, H-4400 Nyíregyháza, Sóstói Str. 31/b
e-mail: csabai.judit@nye.hu
[2] Ungvári Nemzeti Egyetem, 3 Narodna, Ungvár, 88000

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Abstract

The increasing prevalence of antibiotic-resistant bacteria has intensified the search for natural compounds with antimicrobial activity. The aim of this study was to evaluate the antibacterial potential of leaf extracts from lingonberry (*Vaccinium vitis-idaea* L.) and bilberry (*Vaccinium myrtillus* L.) collected from different altitudes in the Gutâi Mountains (Romania). Ethanolic extracts were prepared from dried leaves and tested against Gram-positive and Gram-negative bacteria using the disk diffusion method.

The extracts showed selective antibacterial activity. The strongest inhibitory effect was observed against *Staphylococcus aureus*, with the largest inhibition zone (14 mm) recorded for one bilberry sample. Lingonberry extracts inhibited the growth of *Haemophilus influenzae*, *Staphylococcus aureus*, and coagulase-negative *Staphylococcus* spp., while no inhibitory activity was detected against *Escherichia coli*, *Enterococcus faecalis*, or *Clostridium perfringens* under the tested conditions. Differences in antibacterial activity were observed among samples collected from different locations, suggesting that environmental factors may influence the accumulation of bioactive compounds in the leaves.

The results confirm that *Vaccinium* leaf extracts possess selective antibacterial properties, particularly against Gram-positive bacteria. Although the limited number of samples does not allow conclusions regarding the effect of altitude, the findings support the potential use of lingonberry and bilberry leaves as natural sources of antimicrobial compounds. Further studies are required to identify the active constituents and determine their minimum inhibitory concentrations.

Keywords: *Vaccinium vitis-idaea*, *Vaccinium myrtillus*, leaf extracts, antibacterial activity, disk diffusion, polyphenols.

Introduction

Bilberry (*Vaccinium myrtillus* L.) and lingonberry (*Vaccinium vitis-idaea* L.) are economically and medicinally important berry species belonging to the Ericaceae family. Both species are members of the genus *Vaccinium*, which comprises more than 450 species worldwide and is recognized as an important source of bioactive compounds, particularly polyphenols and anthocyanins (Vander Kloet & Dickinson, 2009).

Lingonberry (*Vaccinium vitis-idaea* L.) is widely distributed in boreal and temperate regions, where it grows in acidic forest soils and mountainous habitats. In Hungary, it is a protected species occurring naturally in the Zemplén and Mecsek Mountains (Soó, 1980; Simon, 2000; Csapody, 1982; Huják, 1999). Its fruits are rich in phenolic compounds, flavonoids, and organic acids, which contribute to their antioxidant and antimicrobial properties (Ek et al., 2006). The official herbal drug is *Vitis-idaeae folium* (Ph. Hg. VIII.), traditionally used for the prevention and treatment of urinary tract infections (Kőszeginé Szalai et al., 2003a).

Bilberry (*Vaccinium myrtillus* L.) is a deciduous dwarf shrub reaching a height of 15–60 cm. It grows mainly on acidic, humus-rich soils in mountain forests and coniferous woodlands (Soó, 1980; Simon, 2000; Csapody, 1982; Martz et al., 2006). In Hungary, its natural populations are found primarily in the Sopron Mountains and Vas County. The fruits are particularly rich in anthocyanins, which are responsible for their high antioxidant capacity and beneficial biological effects (Kalt et al., 2020; Sekikawa et al., 2021). The official herbal drugs include *Myrtilli fructus siccus*, *Myrtilli fructus recens*, and *Myrtilli fructus recentis extractum siccum raffinatum et normatum* (Kőszeginé Szalai et al., 2003b; EMA, 2015). Bilberry fruits are widely used in the food industry as well as in traditional medicine.

Vaccinium species contain considerable amounts of anthocyanins, natural flavonoid pigments responsible for the characteristic red, blue, and purple colour of the fruits. Besides their colouring properties, anthocyanins exhibit strong antioxidant activity and have been associated with cardiovascular protection, anti-inflammatory, antimicrobial, and cytoprotective effects (Köbölkuti & László, 2016).

The total anthocyanin content of lingonberry fruits is influenced by environmental conditions. In populations from the Eastern Carpathians, values ranging from 166.49 to 324.52 mg/100 g dry weight were reported, with the highest concentrations observed in plants growing under greater light exposure. Cyanidin-3-galactoside was identified as the dominant anthocyanin, accompanied by cyanidin-3-glucoside and cyanidin-3-arabinoside (Köbölkuti & László, 2016).

Using HPLC–ESI–QTOF–MS^2 analysis, Guo et al. (2024) identified 15 anthocyanins in bog bilberry (*Vaccinium uliginosum* L.) and eight in cranberry (*Vaccinium macrocarpon* Ait.). The total anthocyanin content reached 1,286.04 mg/100 g dry weight in bog bilberry, considerably exceeding the value measured in cranberry (467.48 mg/100 g dry weight). Antioxidant assays (DPPH, ABTS, and FRAP) confirmed that higher anthocyanin and polyphenol contents were associated with greater antioxidant capacity.

These findings indicate that *Vaccinium* species represent valuable natural sources of anthocyanins. Their concentration and composition depend not only on the species but also on environmental conditions, particularly light intensity, which strongly influences antioxidant activity.

Previous studies have demonstrated that the proanthocyanins and polyphenols of *Vaccinium* species possess considerable antimicrobial activity. Proanthocyanidin fractions isolated from lingonberry (*Vaccinium vitis-idaea* L.) and cranberry (*Vaccinium macrocarpon*) were highly effective against *Staphylococcus aureus* and were also able to inhibit the adhesion of certain *Escherichia coli* strains, suggesting a role in the prevention of bacterial infections (Kylli et al., 2011).

Cioch et al. (2017) compared extracts from several medicinal plants and found that the polyphenol-rich lingonberry extract exhibited pronounced antimicrobial activity, supporting the potential use of plant extracts as natural antimicrobial agents.

Ștefănescu et al. (2020) investigated the antibacterial activity of wild bilberry and lingonberry leaf extracts and reported the strongest inhibitory effect against *Staphylococcus aureus*. The authors associated this activity with the high polyphenol content of the extracts and observed that Gram-positive bacteria were generally more susceptible than Gram-negative species.

Similarly, Elferjane et al. (2022) demonstrated that bilberry (*Vaccinium myrtillus* L.) leaves are an important source of polyphenols and that their extracts exhibit antibacterial activity against several clinically relevant microorganisms in disk diffusion assays. The authors suggested that bilberry leaves could serve as a valuable raw material for the development of natural antimicrobial products.

Kryvtsova et al. (2019, 2020) investigated the antimicrobial properties of lingonberry leaf and fruit extracts. Ethanolic and methanolic extracts showed marked activity against Gram-positive bacteria, particularly *Staphylococcus aureus*, including methicillin-resistant strains (MRSA), and

Streptococcus pyogenes. The extracts also exhibited strong antioxidant activity while having no inhibitory effect on the probiotic bacterium *Lactobacillus plantarum*. Furthermore, lingonberry leaf extracts significantly reduced *S. aureus* biofilm formation, highlighting their potential as natural antimicrobial agents (Kryvtsova et al., 2019, 2020).

Materials and Methods

Leaf samples of lingonberry (*Vaccinium vitis-idaea* L.) and bilberry (*Vaccinium myrtillus* L.) used in this study were collected from different sampling sites in the Gutâi Mountains, Maramureș County, Romania. The samples originated from different altitudes (900–1428 m above sea level), allowing the evaluation of potential habitat-related differences. The sample codes and the main characteristics of the collected material are presented in Table 1.

Table 1. Sampling sites and characteristics of the bilberry (*Vaccinium myrtillus* L.) and lingonberry (*Vaccinium vitis-idaea* L.) samples used in the study

Sample	**Species**	**Sampling site**	**Coordinates**	**Estimated altitude (m a.s.l.)**
V/1	*Vaccinium vitis-idaea*	Kakastaréj Peak (Creasta Cocoșului), Gutâi Mountains	47°42′14.59″ N, 23°50′31.13″ E	1428
V/4	*Vaccinium vitis-idaea*	Masa de Piatra Waterfall (Cascada Masa de Piatra)	47°41′58.33″ N, 23°49′58.55″ E	1180
F/1	*Vaccinium myrtillus*	Masa de Piatra Waterfall (Cascada Masa de Piatra)	47°41′58.33″ N, 23°49′58.55″ E	1180
F/5	*Vaccinium myrtillus*	Poiana Boului, Gutâi Mountains	47°41′23.64″ N, 23°47′19.65″ E	900

Ethanolic extracts were prepared from dried and homogenized leaves of lingonberry (*Vaccinium vitis-idaea* L.) and bilberry (*Vaccinium myrtillus* L.). For each sample, 1.0 g of plant material was extracted with 10 mL of 70% ethanol. The extraction was carried out for 24 h at room temperature in the dark with occasional shaking. The extracts were then filtered, and the ethanol was completely removed under reduced pressure using a rotary evaporator with a water bath maintained at 35–40 °C. The resulting dry extracts were reconstituted in distilled water and used for the antibacterial assays.

Gram-positive (*Staphylococcus aureus, Enterococcus faecalis,* and coagulase-negative *Staphylococcus* spp.), Gram-negative (*Escherichia coli* and *Haemophilus influenzae*), and anaerobic (*Clostridium perfringens*) bacterial strains were used in the study (Table 2).

Table 2. Bacterial strains used in the antibacterial assay

Group	**Bacterial strain**
Gram-negative	*Escherichia coli*
Gram-negative	*Haemophilus influenzae*
Gram-positive	*Enterococcus faecalis*
Gram-positive	*Staphylococcus aureus*
Gram-positive	Coagulase-negative *Staphylococcus* spp.

Group	Bacterial strain
Anaerobic	*Clostridium perfringens*

The plant extracts were diluted with distilled water under sterile conditions at a 1:2 ratio (500 µL extract and 1000 µL distilled water), resulting in a final volume of 1500 µL. The mixtures were homogenized using a vortex mixer and subsequently used for the antibacterial assays (Figure 1).

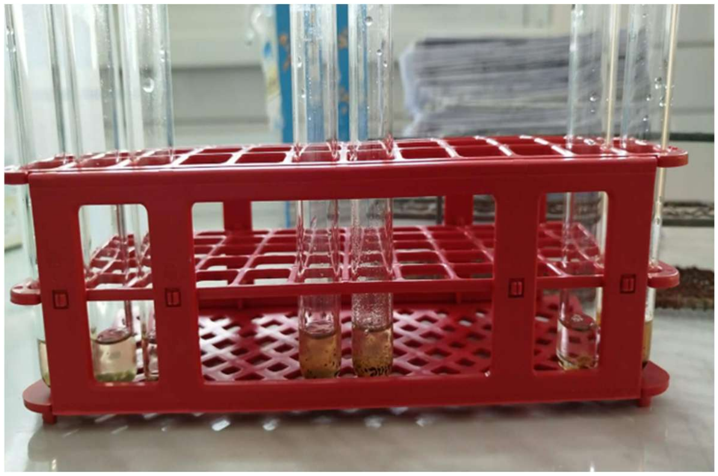

Figure 1. Plant extract and distilled water prior to vortex mixing during the dilution procedure.

Antibacterial activity was evaluated using the disk diffusion method. Sterile paper discs (6 mm in diameter) were impregnated with 30 µL of plant extract and dried at 37 °C (Figure 2). The dried discs were placed onto agar plates previously inoculated with bacterial lawns and incubated at 37 °C for 18–24 h. Antibacterial activity was assessed by measuring the diameter of the inhibition zones surrounding the discs. Paper discs impregnated with distilled water served as the negative control.

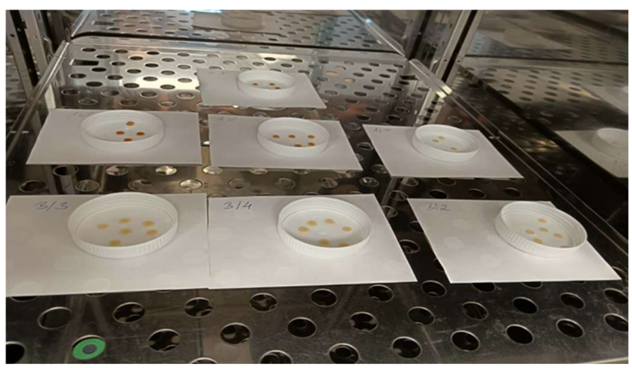

Figure 2. Drying of paper discs impregnated with plant extracts prior to the disk diffusion assay.

Bacterial suspensions were prepared in 0.9% physiological saline (one colony per 3 mL) and pre-incubated at 37 °C for 15 min. Subsequently, 400 µL of each suspension was spread evenly onto the surface of agar plates using a sterile spreader to obtain a uniform bacterial lawn. Before and after placing the extract-impregnated paper discs on the agar surface, the plates were left at room temperature for 15 min to facilitate the diffusion of the extracts. The plates were then incubated at 37 °C for 24 h. Anaerobic conditions for *Clostridium perfringens* were established using a gas-generating sachet in a sealed anaerobic jar.

The disk diffusion assay and the interpretation of the results were performed in accordance with the international guidelines for antimicrobial susceptibility testing (EUCAST, 2025; CLSI, 2024).

Results

After 24 h of incubation, the diameters of the inhibition zones were measured in millimetres (Table 3). Leaf extracts of lingonberry (*Vaccinium vitis-idaea* L.) and bilberry (*Vaccinium myrtillus* L.) exhibited selective antibacterial activity against the tested bacterial strains.

The largest inhibition zone (14 mm) was observed for the F/5 bilberry sample against *Staphylococcus aureus*. Lingonberry extracts inhibited the growth of all susceptible bacterial strains (*Haemophilus influenzae, Staphylococcus aureus*, and coagulase-negative *Staphylococcus* spp.), whereas bilberry extracts showed antibacterial activity only against Gram-positive *Staphylococcus* strains.

No inhibition zones were detected for *Escherichia coli, Enterococcus faecalis,* or *Clostridium perfringens*, indicating that these bacteria were not susceptible to the tested extracts at the applied concentration.

Our findings are consistent with previous studies reporting that polyphenol-rich leaf extracts of *Vaccinium* species exhibit stronger antibacterial activity against Gram-positive bacteria than against Gram-negative species (Kylli et al., 2011; Ștefănescu et al., 2020; Elferjane et al., 2022). Differences observed among the samples suggest that ecological conditions at the sampling sites may influence the quantity and composition of bioactive secondary metabolites. However, the limited number of samples in the present study does not allow firm conclusions regarding the independent effect of altitude on antibacterial activity.

Table 3. Inhibition zones produced by the Vaccinium leaf extracts against the tested bacterial strains

Bacterial strain	**Extract code**	**Inhibition zone (mm)**
Haemophilus influenzae	V/1	10
Haemophilus influenzae	V/4	11
Staphylococcus aureus	V/1	13
Staphylococcus aureus	V/4	12
Staphylococcus aureus	F/5	14
Coagulase-negative *Staphylococcus* spp.	V/1	9
Coagulase-negative *Staphylococcus* spp.	V/4	14
Coagulase-negative *Staphylococcus* spp.	F/5	9

Conclusions

The results of this study demonstrate that leaf extracts of lingonberry (*Vaccinium vitis-idaea* L.) and bilberry (*Vaccinium myrtillus* L.) possess selective antibacterial activity. The strongest inhibitory effect was observed against *Staphylococcus aureus*, whereas lingonberry extracts exhibited a broader antibacterial spectrum by also inhibiting the growth of *Haemophilus influenzae*. No antibacterial activity was detected against *Escherichia coli, Enterococcus faecalis,* or *Clostridium perfringens* at the tested concentration.

Our findings are consistent with previous studies showing that polyphenol-rich extracts of *Vaccinium* leaves are more effective against Gram-positive bacteria. The observed differences among the samples suggest that environmental conditions at the sampling sites may influence the composition of bioactive compounds and, consequently, their antibacterial activity. However, further studies involving a larger number of samples are required to clarify the effect of altitude and other ecological factors. Future research should focus on the identification of the active compounds, determination of the minimum inhibitory concentration (MIC), and further evaluation of the antibacterial potential of *Vaccinium* leaf extracts.

Table 4. Comparison of the antibacterial activity of *Vaccinium* leaf extracts collected from different altitudes

Sample	**Estimated altitude (m a.s.l.)**	***H. influenzae***	***S. aureus***	**Coagulase-negative *Staphylococcus* spp.**
V/1	1428	10 mm	13 mm	9 mm
V/4	1180	11 mm	12 mm	14 mm
F/5	1180	–	14 mm	9 mm
F/1	900	–	–	–

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