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Article

Chemical Characterization and In Vitro Evaluation of Antioxidant and Antimicrobial Activities of *Vaccinium myrtillus* L. Leaves from Estonia

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Abstract

Oxidative stress plays a central role in the development of chronic diseases, increasing interest in natural antioxidants and antimicrobial agents derived from plant sources. Although *Vaccinium myrtillus* L. (bilberry) fruits are well studied, the bioactive potential of its leaves remains insufficiently explored. In this study, dried leaves collected in Estonia were extracted using five solvents of different polarity (MeOH/H₂O 80:20, EtOH, H₂O, MeOH, and acetone). Antioxidant activity was evaluated in vitro using FRAP and DPPH assays, total phenolic content was determined by the Folin–Ciocalteu method, and selected extracts were qualitatively profiled by LC–ESI/MS in SIM mode. The antimicrobial activity of the aqueous extract was assessed in vitro using an agar-based colony-counting method for minimum inhibitory concentration (MIC) determination. The highest antioxidant activity and phenolic content were observed in methanol and hydroalcoholic extracts, with MeOH/H₂O (80:20) showing the lowest DPPH IC₅₀ and highest TPC values. LC–ESI/MS analysis tentatively identified phenolic acids and flavonoids, including gallic acid, caffeic acid, rutin, quercetin, and kaempferol. The aqueous extract exhibited antibacterial activity, particularly against Gram-positive bacteria (MIC 0.10–0.40 mg/mL). Statistically significant differences among solvent systems were confirmed by one-way ANOVA ($p < 0.05$). These findings support the solvent-dependent bioactivity of *V. myrtillus* leaves and their potential application in functional formulations.

Keywords: *Vaccinium myrtillus*; bilberry leaves; antioxidant activity; FRAP; Folin–Ciocalteu; DPPH; LC–ESI/MS

1. Introduction

Oxidative stress is widely recognized as a key contributing factor in the onset and progression of chronic diseases, including cardiovascular disorders, diabetes, neurodegenerative conditions, and cancer [1,2]. It arises from an imbalance between the production of reactive oxygen species (ROS) and the capacity of endogenous antioxidant defense mechanisms to neutralize them. Consequently, increasing attention has been directed toward natural antioxidants derived from plant sources, which are considered safer alternatives to synthetic antioxidants and may exert additional health-promoting effects [3]. Plants belonging to the genus *Vaccinium* are well known for their high content of bioactive compounds, particularly phenolic constituents such as phenolic acids, flavonoids, and anthocyanins [4,24]. Among them, *Vaccinium myrtillus* L. (bilberry) has attracted considerable scientific interest due to its reported antioxidant, anti-inflammatory [23], antidiabetic, and cardioprotective properties [5–7]. *Vaccinium myrtillus* L. has shown protective effects against diabetic nephropathy by improving metabolic profiles and regulating gut microbiota. In a rat model, its administration alleviated kidney

damage and inflammation, highlighting its potential as a complementary therapeutic approach for diabetic kidney disease [21]. The *Vaccinium myrtillus* aerial part extract did not directly inhibit the growth of *Escherichia coli* (*E. coli*) and *Salmonella typhimurium* (*S. typhimurium*) strains at up to 1 mg/mL concentration. However, *Vaccinium myrtillus* extract enhanced the kanamycin intake and increased its efficiency against *E. coli* strain [22]. Also, selective cytotoxic activity was shown against various cancer cell lines (human colon adenocarcinoma (HT29), human breast cancer (MCF-7) and human cervical carcinoma (HeLa) [22,26]. Extracts of *Vaccinium myrtillus* L. also inhibit melanogenesis in melanoma cells suggesting that these extracts have potential as natural agents for regulating skin pigmentation [29]. Pemmari et al. demonstrated that dried bilberry (*Vaccinium myrtillus* L.) reduced inflammation and improved adverse metabolic effects in mice fed a high-fat diet. The findings suggest bilberry may help counteract obesity-associated metabolic disturbances through its bioactive components [28]. While the fruits of bilberry have been extensively investigated and are widely consumed as functional foods, other plant parts, including the leaves, remain comparatively underexplored despite their rich phytochemical composition [8].

Previous studies have demonstrated that bilberry leaves contain substantial amounts of phenolic compounds, which are strongly associated with antioxidant and anti-inflammatory activities [9,10,25]. Phenolic acids and flavonoids present in bilberry leaves can scavenge free radicals, reducing metal ions, and modulating oxidative pathways, thereby contributing to their biological activity [11]. Moreover, recent research has highlighted a close link between antioxidant capacity and anti-inflammatory effects, suggesting that the inhibition of oxidative stress may underlie the anti-inflammatory potential of *Vaccinium myrtillus* extracts [12], including evidence reported for bilberry preparations [13]. The study by Petruřová and Bačkorová (2024) investigated the phytochemical quality of leaves from *Vaccinium vitis-idaea* L. and *Vaccinium myrtillus* L. collected from both polluted and non-polluted areas. The results showed that environmental pollution can significantly affect the concentration of bioactive compounds, such as polyphenols and antioxidants, in the leaves. Plants from non-polluted areas generally had higher levels of these beneficial compounds, highlighting the impact of environmental stress on plant phytochemistry and the potential implications for their use in functional foods or medicinal applications [27]. Similar antioxidant-driven bioactivity patterns have also been reported for other aromatic and medicinal plants following phytochemical characterization of phenolic-rich extracts [19].

The efficiency of phenolic compound recovery from plant matrices is strongly influenced by the extraction solvent system. Solvent polarity, the presence of water, and the use of hydroalcoholic mixtures play a crucial role in determining both the qualitative and quantitative composition of the resulting extracts [14]. Hydroalcoholic solvents have been reported to enhance the extraction of phenolic compounds by improving matrix swelling and mass transfer, leading to increased antioxidant activity compared to single-solvent systems [15,16]. In this context, recent studies employing alternative solvent systems (e.g., deep eutectic solvents) further support the concept that solvent selection critically impacts the recovery of phenolics and the resulting biological activities of plant extracts [20]. Despite growing interest in the bioactivity of *Vaccinium myrtillus*, comparative studies evaluating the impact of different extraction solvents on the antioxidant capacity and phenolic profile of bilberry leaves remain limited, especially for material originating from Northern European regions [17]. Therefore, systematic investigation of solvent-dependent extraction efficiency is necessary to better understand the antioxidant potential of bilberry leaves and to identify suitable extraction conditions for further applications. Besides, Urbonaviciene et al. (2022) showed that wild bilberries (*Vaccinium myrtillus* L.) from different geographic regions vary significantly in their levels of bioactive compounds and antioxidant activity, highlighting how location influences their nutritional and functional qualities [30]. The variability in the biologically active compounds of wild bilberries (*VACCINIUM myrtillus* L.) in different geographical locations (Norway (NOR), Finland (FIN), Latvia (LVA) and Lithuania (LTU)) has also been evaluated suggesting that genotype affects antioxidant activity [30]. Leaves and stems analysis of bilberry (*Vaccinium myrtillus* L.) cultivated in Tucumán, Argentina, identified histochemical features and strong antioxidant activity [31]. Different

Parts of Bilberry (*Vaccinium myrtillus* L.) From the Eastern Black Sea Region emphasize the significant phytochemical content and biological activities of wild bilberry extracts [32].

The aim of the present study was to investigate the antioxidant activity and phenolic composition of *Vaccinium myrtillus* L. leaves collected in Estonia using different extraction solvents (methanol/water 80:20, ethanol, water, methanol, and acetone). In addition, a preliminary evaluation of the antimicrobial activity of the extracts against selected Gram-positive and Gram-negative bacteria was performed. Antioxidant capacity was assessed using FRAP, DPPH radical scavenging, and Folin–Ciocalteu assays, while LC–ESI/MS analysis was employed for the qualitative profiling of phenolic constituents in selected extracts, following a previously reported LC–ESI–MS approach with slight modifications [18]. This integrated approach provides insight into the relationship between extraction solvent, phenolic composition, and antioxidant behavior of bilberry leaf extracts.

2. Materials and Methods

2.1. Plant Material and Extraction

Dried leaves of *Vaccinium myrtillus* L. were collected in Valga County, Estonia, in June 2023 by M. Kulm (Tartu Health Care College, Estonia). The botanical identity of the plant material was verified, and a voucher specimen was recorded under the code MK-01. The collected leaves were air-dried under shade at 35 °C in the dark for 48 h until constant weight. The dried plant material was stored in paper bags in a dry environment at room temperature during transport and subsequently kept in airtight containers at 4 °C protected from light until extraction. The dried leaves were ground into a fine powder using a laboratory grinder. An aliquot of 100 mg of plant powder was extracted with 1.0 mL solvent, corresponding to a solid-to-solvent ratio of 1:10 (w/v). Five solvent systems of different polarity were used: methanol/water (80:20, v/v), ethanol (EtOH), water (H₂O), methanol (MeOH), and acetone. Extraction was performed using an ultrasonic bath (Tierratech LT-100 PRO, 100 W) for 20 min at room temperature. The extracts were then centrifuged at 3200 rpm for 10 min, and the supernatants were collected as crude extracts for further analysis. Each extraction was performed using three independent biological replicates, and all subsequent spectrophotometric analyses were conducted in triplicate measurements [20].

The extraction yield was calculated using the following equation: Extraction yield (%) = (mass of dried extract / mass of plant material) × 100. The average extraction yield obtained under the applied extraction conditions was 17.48 ± 1.5%.

2.2. FRAP Assay

Antioxidant capacity was measured by the ferric reducing antioxidant power (FRAP) assay at 593 nm using a UV–Vis spectrophotometer (Ultrospec 2100 pro). The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ solution, and FeCl₃ solution in a 10:1:1 (v/v/v) ratio. Ascorbic acid standards (10–0.156 mM) were prepared from a 0.01 M stock solution (1.76 g in 1,000 mL ddH₂O). For standards, 40 µL were mixed with 1,200 µL FRAP reagent and incubated for 4 min at 42 °C (Binder WTC), followed by absorbance measurement at 593 nm. Incubation at 42 °C was used to ensure rapid and complete development of the Fe²⁺–TPTZ chromophore prior to absorbance measurement. Such minor temperature adjustments are occasionally applied in modified FRAP protocols to accelerate reaction kinetics without affecting the principle of the assay. For extracts (100 mg/mL), 20 µL extract and 20 µL ddH₂O were mixed with 1,200 µL FRAP reagent, incubated for 4 min at 42 °C, and measured at 593 nm against the corresponding solvent blank. All measurements were performed in triplicate.

The calibration curve was constructed using ascorbic acid standards, and the coefficient of determination (R²) was calculated from the linear regression analysis. The linearity of the assay was verified within the concentration range of 0.156–10 mM, which produced a proportional increase in absorbance at 593 nm. To minimize potential interference from co-extracted reducing substances, solvent blanks corresponding to each extract were used during absorbance measurements.

2.3. Total Phenolic Content (Folin–Ciocalteu)

Total phenolic content (TPC) was determined by the Folin–Ciocalteu assay at 765 nm (Ultrospec 2100 pro) using gallic acid as standard. A gallic acid stock (10 mg/mL) was prepared (10 mg in 1.0 mL MeOH) and serial dilutions were made by 1:1 mixing (50 μ L standard + 50 μ L ddH₂O). Na₂CO₃ (2%, w/v) was prepared (2 g in 100 mL ddH₂O). For standards, 1.0 mL Na₂CO₃ was added, incubated 5 min at room temperature, then 50 μ L Folin–Ciocalteu reagent were added and mixtures were incubated for 30 min at 37 °C prior to reading at 765 nm. For extracts, 50 μ L sample were treated identically (1.0 mL Na₂CO₃, 5 min; + 50 μ L Folin–Ciocalteu, 30 min at 37 °C) and measured against the corresponding solvent blank. All measurements were performed in triplicate.

2.4. DPPH Radical Scavenging Activity

DPPH scavenging activity was measured at 517 nm. Extracts were serially diluted and reacted with 0.1 mM DPPH solution prepared in methanol, incubated for 1 h in the dark, and absorbance was recorded at 517 nm. Radical scavenging activity was calculated as: $\text{RSA (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$.

IC₅₀ values were calculated by regression analysis of RSA (%) versus extract concentration. All measurements were performed in triplicate.

2.5. LC–ESI/MS Analysis

Qualitative profiling of the ethanol and methanol extracts was performed by LC–ESI/MS (LC-20AD coupled to LCMS-2020) using a Synchronis™ C18 column (150 $\times$ 4.6 mm, 5 μ m) at 45 °C and 0.8 mL/min. Mobile phases were solvent A (95% ddH₂O, 5% MeOH, 0.2% acetic acid) and solvent B (50% acetonitrile/50% ddH₂O). MS conditions were: gas temperature 350 °C; nebulizer gas flow 1.5 L/min; drying gas flow 15 L/min; nebulizer pressure 45 psig; capillary voltage +4000 V; injection volume 50 μ L; dwell time 0.5 s. SIM was acquired in negative mode at m/z 285, 125, 109, 224.05, 609, 191, 366.8, 1060.8, 163, 149.06, 169.17, and 179.15, and in positive mode at m/z 159, 104, and 300.5. Identification was supported by external calibration using mixed and individual standards (0.05–20 μ g/L). Standards and samples were analyzed in triplicate [18].

2.6. In Vitro Evaluation of Antimicrobial Activity

For antimicrobial evaluation, a preparative-scale extraction was performed to obtain sufficient material for microbiological testing. Dried leaves of *Vaccinium myrtillus* L. were ground into a fine powder using a mechanical blender. Powdered plant material (10 g) was macerated in 100 mL of a hydroalcoholic solvent consisting of 90% distilled water and 10% ethanol (solid-to-solvent ratio 1:10, w/v). The extraction was carried out for two weeks at room temperature (22–25 °C) in the dark, with daily manual agitation to ensure adequate solvent–matrix interaction, following previously described extraction procedures with minor modifications [32].

After filtration, the extract was centrifuged at 7000 $\times$ g for 15 min, and the supernatant was collected. Solvent removal was performed using a rotary evaporator at 50 °C for 2 h. The resulting residue was re-dissolved in sterile distilled water and stored at 2 °C in airtight containers protected from light until antimicrobial testing.

The in vitro antimicrobial activity of the aqueous leaf extract of *Vaccinium myrtillus* L. was evaluated against four reference bacterial strains: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 13883. Fresh bacterial cultures were prepared and adjusted to an inoculum density equivalent to a 0.5 McFarland standard (approximately 1.0×10^8 CFU/mL). The inoculum density was verified by plate counting to confirm the viable cell concentration.

The extract was dissolved in sterile distilled water to obtain final concentrations ranging from 0.001 to 0.8 mg/mL. Equal volumes of standardized bacterial suspension and extract solution were mixed thoroughly. Aliquots (25 μ L) of each mixture were inoculated onto selective chromogenic agar

plates (Bioprepare, Athens, Greece) and incubated aerobically at 37 °C for 24 h. Antimicrobial activity was evaluated by colony counting. The minimum inhibitory concentration (MIC) was defined as the lowest extract concentration at which no visible microbial growth was observed, based on the absence of colony formation.

A growth control (bacterial suspension without extract) and a solvent control (sterile distilled water) were included in all experiments to verify that bacterial growth inhibition was attributable to the plant extract and not to the solvent. All experiments were performed in triplicate independent assays, and results were expressed as mean ± standard deviation (SD). The preparative extract used for antimicrobial evaluation corresponded to the hydroalcoholic (90:10) extract and was not subjected to LC–ESI/MS profiling; therefore, its phytochemical composition is inferred from the solvent-dependent antioxidant extracts and is acknowledged as a methodological limitation.

2.7. Statistical Analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (SD). IC₅₀ values for the DPPH assay were calculated from concentration–response curves using regression analysis. Normality of the data was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene’s test. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. Statistical significance was set at $p < 0.05$. Data analysis was performed using IBM SPSS Statistics (version 26).

3. Results

3.1. FRAP Reducing Power

An ascorbic acid calibration curve (0.156–2.5 mM) was constructed using absorbance at 593 nm (Table 1). FRAP values for the extracts were expressed as mM ascorbic acid equivalents (AAE), calculated from the calibration curve and corrected for the 1:2 dilution applied prior to measurement. Among the tested solvents, the methanol extract showed the highest FRAP value (7.444 ± 0.041 mM AAE), followed by methanol/water (80:20) (7.103 ± 0.040 mM AAE) and water (7.061 ± 0.060 mM AAE). Ethanol yielded a slightly lower reducing power (6.098 ± 0.210 mM AAE), whereas acetone exhibited markedly reduced FRAP activity (2.426 ± 0.185 mM AAE).

Table 1. Ascorbic acid standards used for FRAP calibration (593 nm).

Ascorbic acid (mM)	Absorbance (593 nm)
2.5	1.855
1.25	1.023
0.625	0.550
0.312	0.292
0.156	0.178

3.2. Total Phenolic Content (Folin–Ciocalteu)

Total phenolic content (TPC) differed across solvent systems (Table 2). The highest TPC was obtained for methanol/water (80:20) (835.95 ± 0.054 µg/mL GAE), followed by methanol (825.91 ± 3.64 µg/mL GAE) and water (823.185 ± 0.009 µg/mL GAE). Ethanol resulted in intermediate TPC values (614.1 ± 0.011 µg/mL GAE), while acetone produced the lowest TPC (508.64 ± 34.87 µg/mL GAE).

3.3. DPPH Radical Scavenging Activity

Radical scavenging activity, expressed as IC₅₀ (mg/mL), varied substantially among extracts (Table 2). The methanol/water (80:20) extract displayed the strongest scavenging activity (IC₅₀ = 0.859 mg/mL), followed by water (IC₅₀ = 1.193 mg/mL) and ethanol (IC₅₀ = 1.698 mg/mL). The methanol

extract exhibited a higher IC₅₀ (6.03 mg/mL), indicating lower scavenging efficiency under the applied conditions, whereas the acetone extract showed the weakest activity (IC₅₀ = 37.6 mg/mL).

3.4. LC–ESI/MS Qualitative Profiling

Qualitative profiling by LC–ESI/MS (SIM mode) supported the presence of phenolic constituents in the analyzed extracts. In the ethanol extract, the detected profile was dominated by phenolic-acid-type compounds and small polar metabolites (Table 3), including citric acid, gallic acid, caffeic acid, coumaric acid, cinnamic acid, catechol, and quercetin. In the methanol extract, a broader profile was observed (Table 4), comprising phenolic acids (e.g., gallic, caffeic, coumaric, cinnamic acids) as well as flavonoid-related constituents such as quercetin, kaempferol, and rutin. Overall, the MeOH/H₂O (80:20) extract showed the highest TPC and the lowest DPPH IC₅₀, whereas the acetone extract exhibited the lowest FRAP and TPC values and the highest DPPH IC₅₀ among the tested solvent systems. Pearson correlation analysis was performed to examine the relationships between total phenolic content (TPC), FRAP antioxidant capacity, and DPPH IC₅₀ values. A very strong positive correlation was observed between TPC and FRAP values ($r = 0.99$), indicating that phenolic compounds contribute significantly to the reducing power of the extracts. Strong negative correlations were observed between TPC and DPPH IC₅₀ values ($r = -0.96$) and between FRAP and DPPH IC₅₀ values ($r = -0.95$), suggesting that extracts with higher phenolic content and reducing capacity exhibited stronger radical scavenging activity.

Table 2. Summary of antioxidant indices of *Vaccinium myrtillus* leaf extracts (mean ± SD, n= 3).

Extraction solvent	FRAP (mM AAE)	TPC (µg/mL GAE)	DPPH IC ₅₀ (mg/mL)
MeOH/H ₂ O (80:20)	7.103 ± 0.040	835.95 ± 0.054	0.859
EtOH	6.098 ± 0.210	614.1 ± 0.011	1.698
H ₂ O	7.061 ± 0.060	823.185 ± 0.009	1.193
MeOH	7.444 ± 0.041	825.91 ± 3.64	6.03
Acetone	2.426 ± 0.185	508.64 ± 34.87	37.6

Values are expressed as mean ± SD (n = 3). Different superscript letters indicate statistically significant differences between extracts according to one-way ANOVA followed by Tukey’s post hoc test (p < 0.05).

Table 3. LC–ESI/MS (SIM) tentative identification in the ethanol extract of *Vaccinium myrtillus* leaves.

Compound	m/z	tR of standard (min)	tR in EtOH extract (min)
Pyrogalllic acid	125	5.40	5.39
Sodium salicylate	159	17.81	17.76
Catechol	109	32.053	32.14
Quercetin	300.5	22.926	23.07
Citric acid	191	2.70	2.04
Coumaric acid	163	24.80	24.91
Cinnamic acid	149.06	2.90	3.18
Gallic acid	169.15	4.40	4.08
Caffeic acid	179.15	16.083	16.01
Tartaric acid	104	31.60	31.05

Table 4. LC–ESI/MS (SIM) tentative identification in the methanol extract of *Vaccinium myrtillus* leaves.

Compound	m/z	tR of standard (min)	tR in MeOH extract (min)
Pyrogalllic acid	125	5.40	5.56
Sodium salicylate	159	17.81	17.43
Catechol	109	32.053	32.89
Tartaric acid	104	31.60	31.65
Rutin	609	32.165	32.23
Quercetin	300.5	22.926	22.96

Citric acid	191	2.70	2.66
Curcumin	366.8	62.50	62.52
Tannic acid	1060.8	26.65	26.98
Kaempferol	285	50.00	50.21
Coumaric acid	163	24.80	25.25
Cinnamic acid	149.06	2.90	3.18
Gallic acid	169.15	4.40	4.22
Caffeic acid	179.15	16.083	16.11

3.5. In Vitro Evaluation of Antimicrobial Activity

The aqueous extract of *Vaccinium myrtillus* leaves exhibited measurable antimicrobial activity against all tested reference strains, with MIC values ranging from 0.10 to 0.40 mg/mL. Gram-positive bacteria were generally more susceptible, with *Staphylococcus aureus* showing the lowest MIC value (0.10 ± 0.03 mg/mL), followed by *Enterococcus faecalis* (0.14 ± 0.04 mg/mL). Among Gram-negative strains, *Klebsiella pneumoniae* displayed comparable sensitivity (0.14 ± 0.04 mg/mL), whereas *Escherichia coli* was less susceptible (0.40 ± 0.10 mg/mL).

Table 5. Minimum inhibitory concentration (MIC, mg/mL) of the aqueous *Vaccinium myrtillus* leaf extract against reference bacterial strains (mean $\pm$ SD, n = 3).

Microorganism	Gram	MIC (mg/mL)
<i>Staphylococcus aureus</i> ATCC 25923	+	0.10 ± 0.03
<i>Enterococcus faecalis</i> ATCC 29212	+	0.14 ± 0.04
<i>Escherichia coli</i> ATCC 25922	–	0.40 ± 0.10
<i>Klebsiella pneumoniae</i> ATCC 13883	–	0.14 ± 0.04

4. Discussion

4.1. FRAP Reducing Power

FRAP results demonstrated substantial reducing capacity for the majority of *Vaccinium myrtillus* leaf extracts, with the highest value observed for methanol (7.444 ± 0.041 mM AAE), followed closely by methanol/water (80:20) (7.103 ± 0.040 mM AAE) and water (7.061 ± 0.060 mM AAE). Ethanol exhibited slightly lower reducing power (6.098 ± 0.210 mM AAE), while acetone yielded markedly reduced activity (2.426 ± 0.185 mM AAE). These findings confirm that solvent polarity strongly affects the recovery of compounds contributing to electron-transfer reducing capacity.

4.2. Total Phenolic Content

TPC values were highest for methanol/water (80:20) (835.95 ± 0.054 μ g/mL GAE) and remained comparably high for methanol (825.91 ± 3.64 μ g/mL GAE) and water (823.185 ± 0.009 μ g/mL GAE), whereas ethanol and acetone extracts showed lower phenolic content. The improved performance of hydroalcoholic systems is consistent with the general observation that adding water to organic solvents can increase matrix swelling and mass transfer, thereby enhancing the extraction of phenolic constituents relative to neat solvents.

4.3. DPPH Radical Scavenging Activity

DPPH scavenging activity (IC₅₀) varied markedly among solvent systems. The methanol/water (80:20) extract showed the lowest IC₅₀ (0.859 mg/mL), indicating the strongest radical scavenging activity, followed by water (1.193 mg/mL) and ethanol (1.698 mg/mL). In contrast, methanol displayed substantially weaker scavenging efficiency (IC₅₀ = 6.03 mg/mL), despite showing high FRAP and TPC values, while acetone presented the weakest activity (IC₅₀ = 37.6 mg/mL). This divergence highlights that different assays probe different antioxidant mechanisms and may respond

differently to the qualitative composition of the extracts (e.g., phenolic subclasses and other co-extracted constituents).

Although the methanol extract exhibited the highest FRAP reducing capacity, its DPPH radical scavenging activity was comparatively lower than that of some other extracts. This difference may be attributed to the distinct mechanisms measured by the two assays, as FRAP evaluates reducing power while DPPH reflects radical scavenging ability. Variations in the composition of phenolic and non-phenolic reducing compounds may therefore influence the results obtained by the two methods.

Pearson correlation analysis was performed to further examine the relationships between total phenolic content (TPC), FRAP antioxidant capacity, and DPPH IC₅₀ values. A very strong positive correlation was observed between TPC and FRAP values ($r = 0.99$), indicating that phenolic compounds contribute significantly to the reducing power of the extracts. Strong negative correlations were observed between TPC and DPPH IC₅₀ values ($r = -0.96$) and between FRAP and DPPH IC₅₀ values ($r = -0.95$), suggesting that extracts with higher phenolic content and reducing capacity exhibited stronger radical scavenging activity.

4.4. LC-ESI/MS Qualitative Profiling

LC-ESI/MS profiling of the ethanol and methanol extracts supported the presence of phenolic constituents in *Vaccinium myrtillus* leaves, with signals corresponding mainly to phenolic-acid-type compounds (e.g., gallic, caffeic, coumaric, cinnamic acids), along with flavonoid-related constituents in the methanol extract (e.g., rutin, quercetin, kaempferol). Overall, the LC-ESI/MS results are consistent with the chemical assays, supporting that the antioxidant potential of the studied extracts is associated with a phenolic-rich composition. The presence of curcumin was tentatively suggested based on retention time comparison with available standards. However, curcumin is primarily associated with *Curcuma longa* and has not been widely reported in *Vaccinium myrtillus* leaves. Therefore, this identification should be interpreted with caution and requires further analytical confirmation.

4.5. In Vitro Evaluation of Antimicrobial Activity

The aqueous extract of *Vaccinium myrtillus* leaves exhibited notable antimicrobial activity against all tested reference strains, with Gram-positive bacteria being more susceptible than Gram-negative species. This behavior is consistent with the structural differences in bacterial cell walls, as Gram-positive bacteria lack an outer lipopolysaccharide membrane, facilitating the penetration of bioactive compounds [35,36].

The pronounced activity against *Staphylococcus aureus* and *Enterococcus faecalis* may be attributed to the high content of phenolic acids, flavonoids, and tannins identified by LC-ESI/MS analysis. Compounds such as gallic acid, caffeic acid, quercetin, and tannic acid are known to exert antimicrobial effects through membrane disruption, enzyme inhibition, and interference with nucleic acid synthesis [37–40]. In contrast, the lower susceptibility of *Escherichia coli* can be explained by the presence of an outer membrane barrier, which limits the diffusion of hydrophilic and polyphenolic compounds [38]. Interestingly, *Klebsiella pneumoniae* exhibited sensitivity comparable to Gram-positive strains, suggesting strain-dependent permeability and susceptibility patterns, as previously reported for plant-derived phenolic extracts [41].

Furthermore, the observed antimicrobial activity may be enhanced by synergistic interactions among different phenolic constituents present in the extract. Such synergistic effects have been widely documented for polyphenol-rich plant matrices and are considered critical for their biological efficacy [41,42]. Similar susceptibility patterns have been reported for phenolic-rich extracts derived from *Vaccinium* species and other medicinal plants, supporting the relevance of the present findings [42]. Overall, these results suggest that bilberry leaf extracts may represent a promising source of natural antimicrobial compounds and warrant further investigation.

4.6. Limitations and Future Work

This study has several limitations that should be considered when interpreting the findings. First, antioxidant activity was assessed exclusively through in vitro chemical assays (FRAP, Folin–Ciocalteu, and DPPH), which capture different reaction mechanisms and may not directly translate to biological efficacy. Second, the Folin–Ciocalteu method provides an estimate of total reducing capacity and is not fully specific to phenolic compounds, while assay outcomes may also be influenced by matrix effects and co-extracted non-phenolic constituents. Third, LC–ESI/MS analysis was performed in SIM mode and supports tentative compound identification based on *m/z* and retention behavior; further structural confirmation (e.g., MS/MS fragmentation and/or high-resolution MS) would strengthen compound assignment.

In addition, expressing results per dry weight of plant material (e.g., mg GAE/g DW and AAE equivalents per g DW) would facilitate comparison with literature data and improve reproducibility across laboratories. Another limitation is that the extract used for antimicrobial testing was prepared under different extraction conditions from those used for antioxidant and phytochemical analyses; therefore, direct correlation between antimicrobial activity and the identified phenolic constituents cannot be established. Future work should therefore include quantitative LC–MS/MS profiling of key phenolic markers, evaluation of additional antioxidant assays to broaden mechanistic coverage, and standardized extraction and reporting protocols to enable robust comparisons between geographic origins and plant matrices. Future antioxidant studies should also include a reference compound such as Trolox as a positive control. In addition, future antimicrobial investigations should incorporate standard antibiotics as positive controls and determine minimum bactericidal concentrations (MBC) to further characterize the antimicrobial potential of the extracts.

5. Conclusions

The present study evaluated the antioxidant properties and preliminary antimicrobial activity of *Vaccinium myrtillus* L. leaf extracts obtained using solvents of different polarity. The results demonstrate that solvent selection significantly influences the recovery of phenolic compounds and the measured antioxidant capacity. Hydroalcoholic and polar solvents, particularly MeOH/H₂O (80:20) and water, yielded extracts with higher total phenolic content and stronger antioxidant activity as determined by FRAP and DPPH assays.

Qualitative LC–ESI/MS analysis tentatively identified several phenolic acids and flavonoids, including gallic acid, caffeic acid, rutin, quercetin, and kaempferol, which are known contributors to antioxidant activity. Similar phenolic profiles and antioxidant properties have been reported for bilberry plant materials in previous studies [30,33].

The aqueous extract also exhibited inhibitory effects against the tested bacterial strains, with Gram-positive bacteria showing greater susceptibility than Gram-negative species. However, the antimicrobial evaluation performed in this study represents a preliminary assessment, and further microbiological investigations are required to confirm these observations.

Overall, the findings highlight the importance of solvent selection in the extraction of phenolic compounds from *Vaccinium myrtillus* leaves and support their relevance as a phenolic-rich plant matrix with notable antioxidant potential. However, further studies including quantitative LC–MS analysis and standardized antimicrobial testing are required to confirm these findings.

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Abbreviations

The following abbreviations are used in this manuscript:

AAE Ascorbic acid equivalents
 ATCC American Type Culture Collection
 CFU Colony-forming units
 DPPH 2,2-Diphenyl-1-picrylhydrazyl
 FRAP Ferric reducing antioxidant power
 GAE Gallic acid equivalents
 LC–ESI/MS Liquid chromatography–electrospray ionization/mass spectrometry
 MIC Minimum inhibitory concentration
 ROS Reactive oxygen species
 SD Standard deviation
 SIM Selected ion monitoring
 TPC Total phenolic content

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