

The Action Mechanisms, Anti-Cancer and Antibiotic-Modulation Potential of *Vaccinium myrtillus* L. Extract

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Background: Herbal medicinal products containing *Vaccinium myrtillus* L. (bilberry) fruits and fruit extracts are widely available in the market. Although bilberry leaves and stems are considered as bio-waste, they contain much higher levels of phenolic compounds than fruits. The study aimed to investigate the antimicrobial and anticancer potential of aerial part extracts from *Vaccinium myrtillus* L. (*V. myrtillus*, VM) plants harvested at high altitudes in Armenian landscape and characterize the bioactive phytochemicals.

Material and Methods: For evaluation of antioxidant properties, chemical-based tests (total phenolic and flavonoid content, and antiradical activity in 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) tests) and cellular antioxidant activity (CAA) assay were applied. Genotoxicity and anticancer properties of the extract alone and in combination with fluorouracil were explored in human cancer and normal cell lines. Antibacterial properties of *V. myrtillus* extract alone and in combination with antibiotics, as well as their effect on proton-flux rate through cell membrane were explored on bacterial strains. The characterization of active phytochemicals was done using Liquid Chromatography-Quadrupole-Orbitrap High-Resolution Mass Spectrometry (LC-Q-Orbitrap HRMS).

Results: The *V. myrtillus* aerial part extract demonstrated promising antioxidant properties in all tests. The selective cytotoxic activity was documented against various cancer cell lines (human colon adenocarcinoma (HT29), human breast cancer (MCF-7) and human cervical carcinoma (HeLa)), while it did not inhibit the growth of tested human normal primary renal mixed epithelial cells (HREC) even at 10-fold higher concentrations. The extract did not have genotoxic properties in comet assay making it a potential source for the development of anticancer preparations. The investigated 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, *V. myrtillus* extract enhanced the kanamycin intake and increased its efficiency against *E. coli* strain. The phytochemical characterization of the extract showed the presence of different groups of phenolics.

Conclusions: Based on obtained data, we suggest the aerial parts of the *V. myrtillus* plant as an alternative source of bioactive natural products for food supplements, nutraceuticals, functional foods and medicine.

Keywords: bioactive metabolites; antioxidants; antibiotic modulation; bio-waste; phenolic compounds; cytotoxicity; aerial part; metabolomics

Introduction

Nowadays, plant-origin compounds remain one of the important sources of valuable biologically active metabolites [1]. *Vaccinium myrtillus* L. (bilberry) is among the richest ones [1,2]. This plant species is common in continental Northern Europe, the British Isles, northern Asia, Japan, Greenland, Iceland, western Canada, and the Western United States (“*Vaccinium myrtillus* L”. USDA Plants

Database. Retrieved 2021-09-21). In the territory of Armenia, it grows intensively in Tavush, Syunik and Lori regions generally in subalpine and alpine zones (2800–3300 m above sea level) [3]. The fruits and aerial parts (containing stems, leaves and fruits) of this plant have been widely used in Armenian traditional medicine to treat different medical conditions [4,5].

Due to the abundance of health-promoting biologically active compounds, *Vaccinium myrtillus* L. (*V. myr-*

tillus, VM) berries are frequently referred to as “super-foods”. In particular, they are rich in anthocyanins, which in recent years have drawn study interest due to their wide range of uses. There is a vast amount of literature data mentioning the antioxidant, genoprotective, anticancer, cardioprotective, anti-inflammatory, ocular, neuroprotective and antimicrobial effects of this plant [2,6–10]. Bilberries are also good sources of different vitamins, macro- and micronutrients as well as phenolic compounds and carotenoids.

Various bilberry chemo-types differ from each other in the quantity and quality of their phytonutrient composition. According to a number of scientific data, this phenomenon can be explained by the influence of different climatic factors, soil composition and other agents that can change the course and intensity of plant metabolic pathways [11–14]. It was reported that the total phenolic content of bilberry harvested from areas exposed to the sunlight was higher compared with plants growing under the shadow [15]. Many other scientists also discuss the geographic variability of biologically active compounds of *V. myrtillus* [16–20]. For example, it was reported that total phenolic content was higher in altitudes above 1500 m [15]. In another study, it was shown that the chemical composition of *Vaccinium* spp. varied between the plants harvested from different altitudes above sea level: the flavanols levels were higher in leaf extracts of plants growing at higher elevations [21]. The amount of arbutin in leaves varied between 1000 mg/100 g dry weight (DW) to 2400 mg/100 g DW; the content of flavonoids amounted from 400 to 600 mg/100 g DW and the anthocyanins’ content varied from 490 to 1640 mg/100 g DW and organic acids from 3300 to 8800 mg/100 g DW depends on the growing area of plants [22].

The bilberry fruits and fruit extracts are currently widely distributed in the market as herbal medicinal products. Moreover, European Medicines Agency (EMA) has adopted herbal monographs regarding these products [23, 24]. *Vaccinium* berries are now dominant plant products in commercial use, while their leaves and stems are generally regarded as a byproduct or waste of the berry industry [15,25]. On the other hand, comparative studies on bilberry indicate that the quantity of phenolic compounds in leaves and stems is much higher than in fruits. These findings have drawn increased attention to these raw materials for their potential health benefits associated with their high levels of phenolic content and biological activity [15,25]. As the main components flavanols (catechin and its derivatives, procyanidin dimer I, procyanidin dimer II and procyanidin trimer), hydroxycinnamic acids (chlorogenic acid, feruloylquinic acid, caffeoylarbutin and dicaffeoylquinic acid), flavonols (quercetin derivatives), and anthocyanins (cyanidins, petunidins, malvidins, delphinidins, and peonidins) were identified in the leaves of bilberry applying High-Performance Liquid Chromatography

with Diode-Array Detection-Electrospray ionization-Mass Spectrometry (HPLC-DAD-ESI-MS) and other techniques [2,21]. On the other hand, there is a growing interest in utilizing bio-residues from the agri-food industry, as it is becoming increasingly important to the global economy [26]. To enhance their use and promote the concept of a circular economy, researchers are exploring new ways to transform these residues into high-value raw materials with industrial importance.

Considering the high differences in phytochemical composition and biological properties of VM variations depending on altitude and other environmental factors, we hypothesized that VM plants wildly growing in the mountains of Armenia (≥ 2800 m above sea level) may have distinct characteristics that require exploration since no scientific data exist on this variation. The objective of the study was also to characterize and compare the chemical composition of the Armenian plant variant with that of growing in other regions. On the other hand, despite the huge amount of information concerning the biological properties of the extracts of blueberries, there is a lack of data about their antibiotic-modulating properties. Considering the richness of blueberry extract with biologically active phytochemicals it was of practical and scientific interest to explore antibiotic-modulatory properties of aerial part extract with both anticancer and antimicrobial preparations.

Thus, the objectives of this study were to explore different biological properties (including their antioxidant, anticancer, genotoxic, antimicrobial, and antibiotic modulatory properties) of aerial parts of wild *V. myrtillus* plants growing in high attitudes of Armenia, as well as to characterize their active phytochemicals using advanced chromatographic techniques.

Materials and Methods

Study Design

The study aimed to evaluate the bioactive properties of the *V. myrtillus* aerial part extract which are generally considered as bio-waste, however, it can have good potential as a source of bioactive components. Therefore, we explored its antioxidant properties in chemical-based tests (total phenolic and flavonoid content, and antiradical activity in 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) tests) and cellular antioxidant activity (CAA) assay in human colon adenocarcinoma (HT29) cell line. Genotoxicity (Comet assay) and anticancer properties of the extract (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay) alone and in combination with fluorouracil were explored in human cancer and normal cell lines. Antibacterial and antibiotic modulatory properties, as well as their effect on proton-flux rate through the cell membrane were explored on bacterial strains. The characterization of active phytochemicals which can be re-

sponsible for antioxidant, anticancer and antibiotic modulating properties of the VM extract were investigated using Liquid Chromatography-Quadrupole-Orbitrap High-Resolution Mass Spectrometry (LC-Q-Orbitrap HRMS).

Chemicals and Reagents

Folin–Ciocalteu (FC) reagent (Lot # BCBX8424, Sigma-Aldrich, Taufkirchen, Germany), ethanol (POCH S. A., Lot no. 1156/11/21, Gliwice, Poland), gallic acid (GA) (CAS 141758-74-9, Sigma-Aldrich, Taufkirchen, Germany), DPPH (#STBB0828V, Sigma-Aldrich, Taufkirchen, Germany), MTT (#MKCR0748, Sigma-Aldrich, Taufkirchen, Germany), kanamycin sulfate (#066M4019V, Sigma-Aldrich, Taufkirchen, Germany), ampicillin sodium salt (BCBW1243, Sigma-Aldrich, Taufkirchen, Germany), nutrient broth (#BCCJ4439, Sigma-Aldrich, Taufkirchen, Germany), catechin-amino-2-(hydroxymethyl)-1,3-propanediol (Trizma-Base) (Lot # BCBF0735V, Sigma-Aldrich, Taufkirchen, Germany), Sybr Green I nucleic acid gel stain (#SLCH2286, Sigma-Aldrich, Taufkirchen, Germany), Triton X-100 (#STBH6272, Sigma-Aldrich, Taufkirchen, Germany), low melting point (LMP) agarose (#SLCF3151, Sigma-Aldrich, Taufkirchen, Germany), normal melting point (NMP) agarose (#AG5-112F, Bionline, Memphis, TN, USA), phosphate-buffered saline (PBS) (#SLCL7026, Sigma-Aldrich, Taufkirchen, Germany), dimethyl sulfoxide (DMSO) (#BCCJ0028, Sigma-Aldrich, Taufkirchen, Germany), ABTS (#SLCH6886, Sigma-Aldrich, Taufkirchen, Germany). The OxiSelect™ Cellular Antioxidant Assay Kit was from Cell Biolabs, Inc. (Cat. No. STA-349, San Diego, CA, USA). The Renal Epithelial Cell Basal Media (ATCC PCS-400-030™, Manassas, VA, USA) and Renal Epithelial Cell Growth Kit (ATCC PCS-400-040, Manassas, VA, USA) were purchased from ATCC.

Plant Material Collection, Identification and Extraction

V. myrtillus aerial parts (including stem with leaves and fruits) were collected from Tavush province (Armenia, 2800–2900 m above sea level) during the fruiting period (August). The identification of the plant was carried out at the Department of Botany and Mycology, Yerevan State University (YSU), Armenia. Plant samples were deposited at the Herbarium of the same Department, where the voucher specimen serial number was given. The collected plant aerial parts were dried according to the already described protocol [27]. The dried plant materials were further finely grounded with a homogenizer and stored in hermetically sealed tubes at $-20\text{ }^{\circ}\text{C}$ until further use. VM crude extracts were prepared by maceration with ethanol (96%) at a 10:1 solvent-to-sample ratio (v/w) [27,28]. For the CAA, MTT and comet assay, 50 mg DW/mL crude ethanol extract was prepared, as described earlier [28]. The yield of extraction was quantified by performing three independent replicates of the following procedure: 500 μL of the extract

was dried at room temperature, and the dry weight was determined by weighing each sample [29]. The resulting yield was calculated to be $30.4 \pm 2.43\%$.

Determination of Antioxidant Potential in Chemical-Based Tests, Total Phenolic and Flavonoid Content

The antioxidant potential of tested extracts was evaluated by standard spectrophotometric methods, using ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (1-diphenyl-2-picrylhydrazyl) radicals according to the procedure described before [29,30]. Prior to the measurements, the stock solutions of both reagents were diluted according to the following steps: the DPPH solution was mixed with methanol until the absorbance reached a value of 0.9 ± 0.02 at a wavelength of 515 nm. Similarly, the ABTS solution was diluted with ethanol until an absorbance of 0.7 ± 0.02 at a wavelength of 734 nm was achieved. The reagents were mixed with the different dilutions of test samples and the absorbance was measured at 515 (DPPH) or 734 (ABTS) nm after 10 min of incubation at room temperature. All measurements were conducted using a TECAN Infinite M200 Spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland).

The total phenolic content of plant extracts was measured exploiting the Folin–Ciocalteu (FC) reagent employing a calibration curve of gallic acid (GA) (0–250 $\mu\text{g}/\text{mL}$) using a UV-Vis spectrophotometer (Genesys 10S, Thermo Scientific, Waltham, MA, USA) [31,32].

The total flavonoid content of *V. myrtillus* extract was determined by AlCl_3 colorimetric assay utilizing a UV-Vis spectrophotometer (Genesys 10S, Thermo Scientific, Waltham, MA, USA) [32].

Plant Material Characterization (LC-Q-Orbitrap HRMS Analysis) and Post-Column Derivatization with ABTS

The identification of the phytochemical composition of *V. myrtillus* extract was performed using a Dionex Ultimate 3000 UHPLC system (Thermo Scientific TM, Dionex, San Jose, CA, USA) equipped with Synergi TM Hydro-RP A (150 \times 4.5 mm, 4 μm , Phenomenex) column, held at a temperature of $30\text{ }^{\circ}\text{C}$ as described previously [33,34].

Raw data from high-resolution mass spectrometry was elaborated with Compound Discoverer (v. 2.1, Thermo, Waltham, MA, USA), which facilitated the peak recognition, retention times arrangement, profile assignment, and isotope pattern. Major metabolite identification was based on accurate mass and mass fragmentation pattern spectra against MS/MS spectra of compounds available on a customized database of different classes of phytochemicals created based on literature data and implemented in the software. Raw data from three experimental replicates and a blank sample were processed according to Kusznierevicz *et al.* [34].



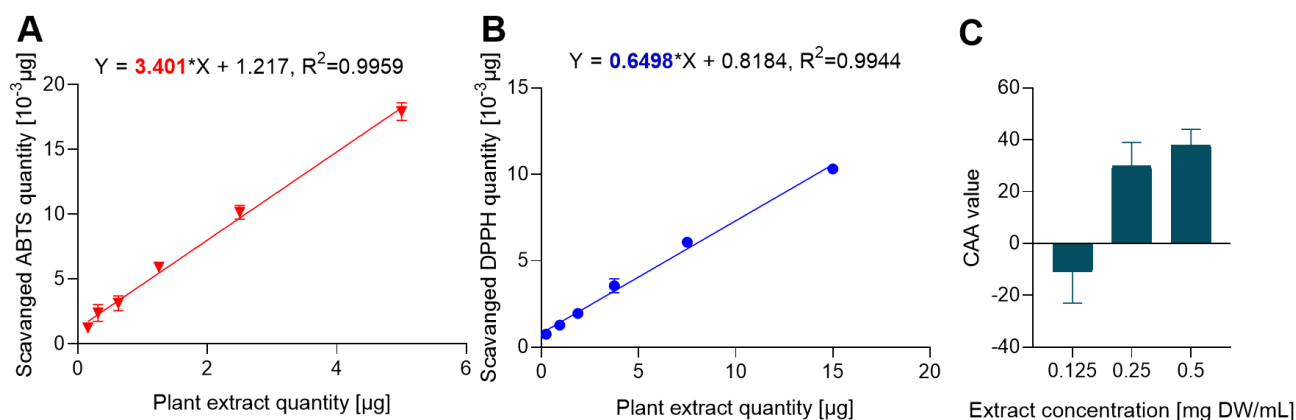


Fig. 1. Total antioxidant activity of *Vaccinium myrtillus* L. (*V. myrtillus*, VM) extract evaluated by spectrophotometric and *in vitro* cellular tests. (A) 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) reducing properties of VM extract. (B) 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of the test extract. (C) Cellular antioxidant activity (CAA) of VM extract determined in human colon adenocarcinoma (HT29) cells. The results are means \pm SD from three independent experiments, $p < 0.05$.

Profiles of phenolic compounds and antioxidants for *V. myrtillus* extract were obtained employing the HPLC-DAD system (Agilent Technologies, Wilmington, DE, USA) connected with a Pinnacle PCX Derivatization Instrument (Pickering Laboratories Inc., Mountain View, CA, USA) and UV-Vis detector (Agilent Technologies, Wilmington, DE, USA). The conditions of chromatographic separation were the same as in the case of LC-HRMS analysis.

Prior to derivatization, the chromatograms were recorded at 270 nm using a DAD detector. The eluate stream from the DAD detector was then directed to the post-column derivatization instrument. Derivatization with ABTS reagent was performed according to methods described in the literature with slight modification [35,36]. A stream of methanolic ABTS solution (1 mM) was introduced to the stream of eluate at a rate of 0.1 mL/min and then directed to the reaction loop (1 mL, 130 °C). The antioxidant profiles were recorded in a UV-Vis detector at 734 nm [31].

Cell Culture

Human colon adenocarcinoma (HT29) cells (ATCC HTB-38, Manassas, VA, USA), human breast cancer (MCF-7) cells (ATCC HTB-22, Manassas, VA, USA), human cervical carcinoma (HeLa) cells (ATCC CCL-2, Manassas, VA, USA) and human normal primary renal mixed epithelial cells (HREC) (ATCC PCS-400-012™, Manassas, VA, USA) were obtained from ATCC. HT29 and MCF-7 maintained in McCoy's 5A Medium (Sigma-Aldrich, Taufkirchen, Germany) and eagle's minimal essential medium (EMEM)(Sigma-Aldrich, Taufkirchen, Germany), respectively, both supplemented with L-glutamine (2 mmol/L), sodium pyruvate (200 mg/L), fetal bovine serum (100 mL/L), and antibiotics (100 U/mL penicillin and 100 µg/L streptomycin). HeLa cells maintained in Dulbecco's Modified Essential Medium/Nutrient

Mixture F-12 Ham (DMEM/F12, 1:1 mixture) (Sigma-Aldrich, Taufkirchen, Germany No. D6421) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Taufkirchen, Germany), 1 \times Pen/Strep. HREC were maintained in Renal Epithelial Cell Basal Media (ATCC PCS-400-030™, Manassas, VA, USA supplemented with Renal Epithelial Cell Growth Kit (ATCC PCS-400-040™, Manassas, VA, USA) components and low serum (0.5% FBS). Cultured cells were regularly examined for the presence of mycoplasma contamination using Universal Mycoplasma Detection Kit from ATCC (ATCC 30-1012K™, Manassas, VA, USA). Cell lines from passage no. 4 till passage no. 7 were used for the experiments. All cell lines have been verified by STR.

Cellular Antioxidant Activity Test

Cellular antioxidant activity (CAA) of VM aerial part extract was tested in HT29 cells using OxiSelect Cellular Antioxidant Activity Assay Kit (green fluorescence) as described before [33]. In brief, the cells were grown until they reached 90% confluence. Subsequently, the cells were subjected to a 1-hour treatment with 50 µL of plant ethanol extracts at concentrations of 0.125, 0.25, and 0.5 mg DW/mL. Additionally, 50 µL of the fluorescent probe 2',7'-Dichlorodihydrofluorescein diacetate (H₂DCFDA) was added. The fluorescence intensity indicative of the cellular reactive oxygen species (ROS) level was measured upon the addition of the radical initiator. The fluorescence emission at 538 nm was recorded in cell cultures every 5 minutes for 1 hour following excitation at 485 nm, using the TECAN Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland).

MTT Cytotoxicity Test

The MTT test was performed as described previously [28,29] to assess the growth of test normal and cancerous

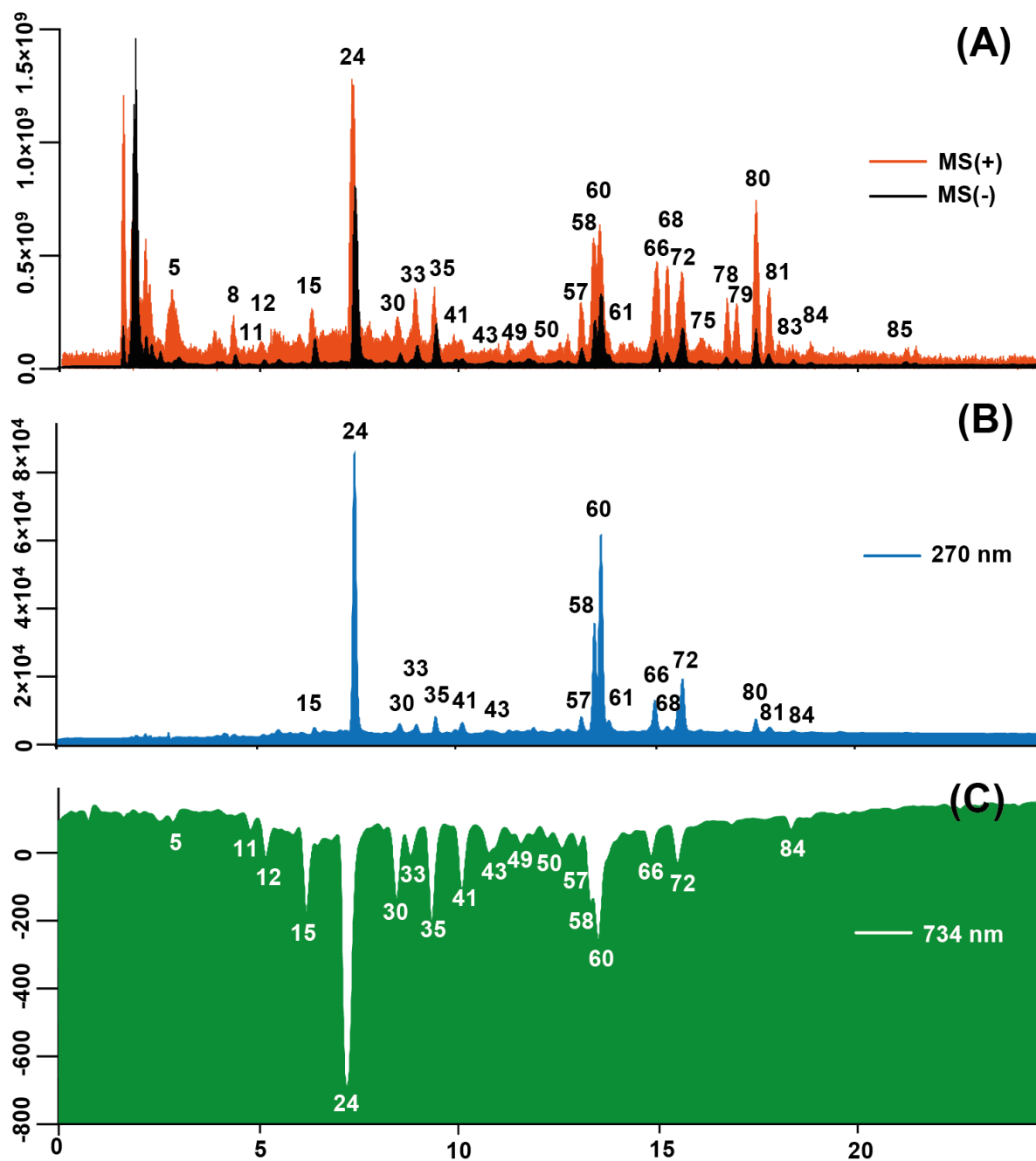


Fig. 2. Antioxidant profile and chromatograms of phenolic constituents of *V. myrtillus* extract. (A) Liquid Chromatography-Quadrupole-Orbitrap High-Resolution Mass Spectrometry chromatogram of the test extract. (B) High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) (207 nm) chromatogram of *V. myrtillus* extract. (C) The antioxidant profile of the test extract after post-column derivatization with ABTS reagent (734 nm). For identity of peaks, see also Table 1.

cells exposed to different concentrations of the VM extract for different exposure times. Cytotoxicity was expressed as the percentage of cell growth in the presence of the plant extract, normalized to the control cells treated with the corresponding volume of solvent alone (1% ethanol in the final mixture). In the control group, cell growth was considered as 100%.

Genotoxic Effects Measured by Comet Assay

Genotoxicity of VM extract was evaluated using comet assay [37]. The procedure was performed as described before [29]. Approximately 10^5 cells per well were seeded in 24-well tissue culture plates and exposed to various concentrations of plant extracts for 24 hours. The selection of plant extract concentrations was based on the results of the MTT cytotoxicity assay. The average percentage of

DNA in the comet tail served as an indicator to assess the genotoxic potential of the tested extract. Two separate measurements were conducted, each consisting of three technical replicates.

Microbial Strains and Culture Conditions

Salmonella typhimurium TA100, *Escherichia coli* BW 25113 wild type strains were used during the study. The strains were grown in Nutrient Broth (NB) media at 37 °C and pH 7.5. For proton flux studies *E. coli* BW 25113 cells were grown on solid media of the following composition: 20 g/L peptone, 5 g/L NaCl, 2 g/L K₂HPO₄, 15 g/L agar supplemented with 2 g/L glucose. pH adjusted 7.2–7.4 by 0.1 M NaOH or 0.1 M HCl. Bacterial monoclonal cells from a single colony were transferred to the new liquid medium without agar and continued to grow aerobically at 37 °C with 130 rpm rotary shaking overnight [38].

Determination of Antibacterial and Antibiotic Modulatory Activity of VM Extract

The antibacterial activity of VM extract against all tested bacterial strains was evaluated by broth microdilution assay [39]. The antibiotic modulatory activity of VM extract was tested by determining the minimal inhibitory concentrations (MICs) of antibiotics in the presence and absence of VM extract at non-inhibitory concentrations [39]. Kanamycin and ampicillin were used to explore the modulatory activity of VM extract. The decrease in MICs of antibiotics in the presence of plant crude extracts indicated antibiotic modulatory activity of plant crude extracts. Broth microdilution assay was used for the determination of MIC values, as described previously [27]. To calculate the modulation factor (MF), which indicates synergistic or modulating interactions, the following formula was used: $MF = MIC_{\text{antibiotic}} / MIC_{\text{antibiotic} + \text{extract}}$. $MF \geq 2$ indicated the presence of synergistic interaction, $n = 1$ —indifference [39].

Determination of H⁺-Fluxes through Bacterial Membrane

In order to understand the possible mechanisms underlying the antibiotic modulatory activity of VM extract, the changes in H⁺-fluxes through the membrane of *E. coli* BW25113 in the presence of *V. myrtillus* extract were investigated. Mid-logarithmic cells grown 18–20 hours in liquid media in the presence and absence of plant extract were harvested by centrifugation (Sorvall LYNX 6000 Super-speed Centrifuge, Thermo Scientific, Waltham, MA, USA) at 3500 ×g for 15 minutes after the pellet was washed with distilled water twice. Pellet was re-suspended in 150 mM Tris-HCl experimental assay containing 0.4 mM MgSO₄, 1 mM NaCl, 1 mM KCl. H⁺-flux in whole cells was determined as described [40] upon the addition of glucose (2 g/L) to the whole cell suspension, by registering the extracellular changes in H⁺-fluxes using pH-meter (Milwaukee

Instruments MW151 MAX pH/ORP/Temp meter logging bench meter, Milwaukee, USA) equipped with a selective H⁺ electrode (MA917) [41]. ΔH⁺ was determined as the negative logarithm of proton concentration in millimolar value. In whole cells incubated with or without VM extract, the same principle was applied. Results were expressed in mmol H⁺/min per 10⁸ cells in one unit of volume (mL).

Statistical Analysis

The results are presented as means ± SD or means ± SEM, as the figure legend indicates. Antioxidant activity in chemical (ABTS, DPPH) and cellular (CAA) tests were evaluated using an unpaired Student's *t*-test ($p \leq 0.05$). The statistical significance of the results was examined using one-way ANOVA followed by Dunnett's test or two-way ANOVA and Tukey's multiple comparisons tests. The statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA), and a *p*-value of less than 0.05 was considered significant.

Results

Antioxidant Properties of *V. myrtillus* Extract

The antioxidant capacity of VM extract was assessed using standard spectrophotometric tests employing DPPH or ABTS radicals (as shown in Fig. 1A,B) and a cellular antioxidant activity (CAA) assay (Fig. 1C). To determine the regression coefficient, which indicates the stoichiometric values, the slope of the concentration of the extract and the number of scavenged radicals' relationship was computed for both chemical-based tests. Based on the findings, the DPPH/ABTS stoichiometry values for the VM extract were 3.401 and 0.6498, respectively, meaning that 1 μg of VM crude extract resulted in the reduction of 1×10^{-3} μg of DPPH/ABTS radicals (Fig. 1A,B).

Further, the CAA test was performed to elucidate the antioxidant potential of VM aerial part extract inside the HT29 cells, taking into account that this intended to replicate biological conditions (pH—7.4 and temperature—37 °C). The results acquired from this assay demonstrated that VM extract has significant antioxidant activity in HT29 cells (Fig. 1C) relative to the reference quercetin utilized in the test. The CAA activity of the VM extract shows a direct correlation with its concentration.

Total phenolic and flavonoid contents of the VM extract were 136.2 ± 4.75 μg GAE/mg DW and 54.89 ± 3.69 μg QE/mg DW, respectively.

Identifying Phenolic Compounds within the VM Extract

The identification of phenolic constituents of VM aerial part ethanol extract was done with an advanced chromatographic technique (LC-Q-Orbitrap HRMS). The application of this method allowed the identification of 83 annotated compounds in the extract. Each compound was allo-

Table 1. Phenolic compounds identified in *Vaccinium myrtillus* L. (*V. myrtillus*, VM) extract in positive and negative polarity with retention time (RT) and relevant mass spectrometry (MS) and MS/MS information.

ID	Compound group	Name	RT [min]	Proposed formula	Positive polarity				Negative polarity			
					Adduct	Experi-mental m/z	Δm (ppm)	Main Product Ions (m/z)	Adduct	Experi-mental m/z	Δm (ppm)	Main Product Ions (m/z)
1	HCA	Quinic acid+hexose	1.82	C ₁₃ H ₂₄ O ₁₂	-	-	-	-	[M-H] ⁻	371.1193	-0.9	191.0555; 192.0589; 85.0281; 127.0388
2	HCA	Quinic acid+2hexose	1.82	C ₁₉ H ₃₄ O ₁₇	-	-	-	-	[M-H] ⁻	533.1722	-0.9	191.0555; 192.0589; 85.0280; 59.0126
3	HCA	Quinic acid	1.84	C ₇ H ₁₂ O ₆	[M+H] ⁺	193.0710	0.9	83.0497; 95.0495; 65.0393; 55.0550	[M-H] ⁻	191.0551	2.4	85.0282; 93.0333; 59.0125; 87.0074
4	HT	Galloyl glucose	2.38	C ₁₃ H ₁₆ O ₁₀	-	-	-	-	[M-H] ⁻	331.0670	-1.5	125.0233; 151.0027; 169.0134; 83.0125
5	HCA	Caffeoylquinic acid (I)	3.01	C ₁₆ H ₁₈ O ₉	[M+H] ⁺	355.1021	0.6	163.0389; 145.0284; 135.0440; 164.0423	[M-H] ⁻	353.0877	-1.2	191.0555; 192.0589; 135.0441; 93.0333
6	HBA	Gallic acid	3.38	C ₇ H ₆ O ₅	-	-	-	-	[M-H] ⁻	169.0131	3.7	69.0333; 125.0235; 124.0155; 97.0283
7	PG	Vanilloyl glucose	3.91	C ₁₄ H ₁₈ O ₉	-	-	-	-	[M-H] ⁻	329.0878	-1.5	108.0205; 152.0106; 167.0342; 123.0440
8	PG	Vanillyl alcohol glucoside	4.33	C ₁₄ H ₂₀ O ₈	[M+H] ⁺	317.1233	1.1	137.0597; 85.0290; 97.0288; 127.0391	[M-H] ⁻	315.1085	-1.5	109.0283; 153.0184; 123.0440; 153.0547
9	F-3-ol	(Epi)catechin O-hexoside	4.36	C ₂₁ H ₂₄ O ₁₁	[M+H] ⁺	453.1389	0.5	139.0389; 123.0441; 165.0545; 291.0860	-	-	-	-
10	FG	Delphinidin O-pentoside	4.44	C ₂₀ H ₁₉ O ₁₁ ⁺	[M] ⁺	435.0920	1.7	303.0497; 304.0530; 219.0286; 153.0181	-	-	-	-
11	F-3-ol	(Epi)gallocatechin	4.59	C ₁₅ H ₁₄ O ₇	[M+H] ⁺	307.0810	0.8	139.0389; 163.0388; 140.0423; 177.0544	[M-H] ⁻	305.0665	-1.4	125.0233; 137.0234; 139.0391; 165.0185
12	HCA	Caffeoylquinic acid (II)	5.05	C ₁₆ H ₁₈ O ₉	[M+H] ⁺	355.1026	0.8	163.0389; 164.0423; 499.1231; 355.1022	[M-H] ⁻	353.0876	-0.8	191.0554; 192.0588; 93.0332; 161.0235
13	FG	Cyanidin-coumaroyl-hexoside (I)	5.36	C ₃₀ H ₂₇ O ₁₃ ⁺	[M] ⁺	595.1442	1.6	127.0390; 287.0547; 139.0389; 275.0546	-	-	-	-



Table 1. Continued.

ID	Compound group	Name	RT [min]	Proposed formula	Positive polarity				Negative polarity			
					Adduct	Experimental m/z	Δm (ppm)	Main Product Ions (m/z)	Adduct	Experimental m/z	Δm (ppm)	Main Product Ions (m/z)
14	CT	B-Procyanidin dimer	5.98	C ₃₀ H ₂₆ O ₁₂	[M+H] ⁺	579.1494	0.5	127.0391; 139.0389; 163.0389; 287.0548	-	-	-	-
15	F-3-ol	(Epi)gallocatechin	6.32	C ₁₅ H ₁₄ O ₇	[M+H] ⁺	307.0810	0.6	139.0389; 163.0388; 140.0423; 177.0544	[M-H] ⁻	305.0665	-1.4	125.0233; 137.0234; 139.0391; 167.0342
16	FG	Cyanidin-coumaroyl-hexoside (II)	6.49	C ₃₀ H ₂₇ O ₁₃ ⁺	[M] ⁺	595.1444	1.3	127.0390; 139.0389; 163.0389; 287.0548	-	-	-	-
17	FG	Delphinidin <i>O</i> -pentoside	6.50	C ₂₀ H ₁₉ O ₁₁ ⁺	[M] ⁺	435.0918	1.7	303.0498; 304.0531; 165.0911; 137.0597	-	-	-	-
18	FG	Cyanidin <i>O</i> -hexoside (I)	6.69	C ₂₁ H ₂₁ O ₁₁ ⁺	[M] ⁺	449.1076	1.7	287.0548; 288.0581; 320.1809; 449.1630	[M-2H] ⁻	447.0934	-1.5	284.0328; 285.0389; 221.1179; 203.1073
19	HCA	Caffeoyl glucose	6.9	C ₁₅ H ₁₈ O ₉	-	-	-	-	[M-H] ⁻	341.0876	-1.0	135.0441; 179.0343; 180.0376; 136.0475
20	FG	Cyanidin <i>O</i> -hexoside (II)	7.07	C ₂₁ H ₂₁ O ₁₁ ⁺	[M] ⁺	449.1076	1.7	287.0548; 288.0582; 317.0660; 318.0686	-	-	-	-
21	HCA	Sinapoyl hexose	7.12	C ₁₇ H ₂₂ O ₁₀	-	-	-	-	[M-H] ⁻	385.1141	-1.5	164.0470; 208.0373; 179.0706; 223.0609
22	FG	Petunidin <i>O</i> -hexoside	7.20	C ₂₂ H ₂₃ O ₁₂ ⁺	[M] ⁺	479.1181	1.7	317.0653; 318.0687; 302.0422; 121.0650	-	-	-	-
23	HCA	Coumaroyl hexose	7.25	C ₁₅ H ₁₈ O ₈	-	-	-	-	[M-H] ⁻	325.0926	-0.9	119.0491; 163.0393; 145.0286; 120.0523
24	HCA	Caffeoylquinic acid (III)	7.33	C ₁₆ H ₁₈ O ₉	[M+H] ⁺	355.1021	0.7	163.0389; 145.0284; 135.0441; 164.0421	[M-H] ⁻	353.0876	-0.8	191.0555; 192.0588; 85.0281; 161.0235
25	F-3-ol	Catechin	7.54	C ₁₅ H ₁₄ O ₆	[M+H] ⁺	291.0860	1.0	139.0390; 123.0442; 147.0441; 161.0596	-	-	-	-
26	FG	Cyanidin <i>O</i> -pentoside	7.73	C ₂₀ H ₁₉ O ₁₀ ⁺	[M] ⁺	419.0969	2.1	287.0548; 288.0581; 71.0861; 149.0235	-	-	-	-
27	CT	B-Procyanidin trimer	8.41	C ₄₅ H ₃₈ O ₁₈	[M+H] ⁺	867.2128	0.9	289.0703; 579.1493; 291.0860; 427.1020	[M-H] ⁻	865.1989	-1.1	287.0559; 577.1354; 575.1195; 425.0882

Table 1. Continued.

ID	Compound group	Name	RT [min]	Proposed formula	Positive polarity				Negative polarity			
					Adduct	Experimental m/z	Δm (ppm)	Main Product Ions (m/z)	Adduct	Experimental m/z	Δm (ppm)	Main Product Ions (m/z)
28	FG	Malvidin <i>O</i> -hexoside (I)	8.39	C ₂₃ H ₂₅ O ₁₂ ⁺	[M] ⁺	493.1339	1.3	331.0809; 332.0843; 316.0575; 315.0490	-	-	-	-
29	FG	Peonidin <i>O</i> -hexoside	8.40	C ₂₂ H ₂₃ O ₁₁ ⁺	[M] ⁺	463.1232	1.9	301.0704; 302.0737; 286.0465; 331.0807	-	-	-	-
30	CT	B-Procyanidin dimer	8.46	C ₃₀ H ₂₆ O ₁₂	[M+H] ⁺	579.1502	0.1	127.0391; 139.0389; 287.0548; 163.0389	[M-H] ⁻	577.1354	-1.3	289.0721; 407.0773; 125.0233; 408.0809
31	CT	B-Procyanidin tetramer	8.82	C ₆₀ H ₅₀ O ₂₄	[M+H] ⁺	1155.2761	0.3	-	[M-2H] ²⁻	576.1280	-1.1	125.0233; 289.0721; 151.0392; 407.0776
32	FG	Malvidin <i>O</i> -hexoside (II)	8.82	C ₂₃ H ₂₅ O ₁₂ ⁺	[M] ⁺	493.1339	1.34	331.0809; 332.0843; 316.0572; 315.0498	-	-	-	-
33	HCA	Caffeoylquinic acid (IV)	8.89	C ₁₆ H ₁₈ O ₉	[M+H] ⁺	355.1026	0.8	163.0389; 145.0284; 135.0441; 164.0421	[M-H] ⁻	353.0876	-0.9	191.0555; 192.0589; 85.0281; 161.0234
34	FG	Peonidin <i>O</i> -pentoside	9.28	C ₂₁ H ₂₁ O ₁₀ ⁺	[M] ⁺	433.1125	2.1	301.0705; 302.0738; 286.0467; 71.08620	-	-	-	-
35	F-3-ol	(epi)Catechin	9.36	C ₁₅ H ₁₄ O ₆	[M+H] ⁺	291.0866	1.0	139.0389; 123.0442; 147.0440; 161.0596	[M-H] ⁻	289.0716	-1.3	109.0283; 123.0440; 125.0234; 137.0234
36	HCA	Caffeic acid	9.48	C ₉ H ₈ O ₄	-	-	-	-	[M-H] ⁻	179.0338	3.4	135.0443; 134.0363; 94.9917; 90.9968
37	HCA	Coumaroylquinic acid (I)	9.64	C ₁₆ H ₁₈ O ₈	-	-	-	-	[M-H] ⁻	337.0929	-1.5	173.0447; 119.0491; 191.0555; 163.0393
38	HCA	Coumaroylquinic acid (II)	9.84	C ₁₆ H ₁₈ O ₈	[M+H] ⁺	339.1077	0.7	147.0440; 119.0493; 148.0474; 91.0547	[M-H] ⁻	337.0929	-1.5	191.0555; 93.0333; 163.0393; 119.049
39	FG	Cyanidin- coumaroyl-hexoside	9.82	C ₃₀ H ₂₇ O ₁₃ ⁺	[M] ⁺	596.1520	1.7	139.0389; 127.0391; 287.0547; 163.0388	-	-	-	-
40	CT	A-Procyanidin trimer	9.97	C ₄₅ H ₃₆ O ₁₈	[M+H] ⁺	865.1974	0.6	533.1079; 287.0546; 713.1497; 695.1389	[M-H] ⁻	863.1835	-1.4	411.0725; 289.0720; 451.1044; 711.1359
41	CT	B-Procyanidin trimer	10.02	C ₄₅ H ₃₈ O ₁₈	[M+H] ⁺	867.2123	1.6	289.0703; 291.0862; 579.1494; 247.0600	[M-H] ⁻	865.1982	-0.2	287.0564; 577.1353; 289.0719; 425.0881



Table 1. Continued.

ID	Compound group	Name	RT [min]	Proposed formula	Positive polarity				Negative polarity			
					Adduct	Experi- mental m/z	Δm (ppm)	Main Product Ions (m/z)	Adduct	Experi- mental m/z	Δm (ppm)	Main Product Ions (m/z)
42	HCA	Caffeoylshikimic acid	10.18	C ₁₆ H ₁₆ O ₈	[M+H] ⁺	337.0920	1.1	163.0389; 145.0284; 135.0440; 164.0422	[M-H] ⁻	335.0771	-1.2	135.0441; 179.0342; 161.0235; 93.0333
43	CT	B-Procyanidin tetramer	10.64	C ₆₀ H ₅₀ O ₂₄	[M+H] ⁺	1155.2765	0.4	579.1495; 577.1339; 867.2120; 580.1527	[M-2H] ²⁻	576.1282	-1.6	125.0233; 289.0721; 151.0392; 407.0773
44	HCA	Feruloylquinic acid	10.75	C ₁₇ H ₂₀ O ₉	[M+H] ⁺	369.1182	0.9	177.0545; 145.0284; 178.0580; 146.0318	[M-H] ⁻	367.1032	-0.9	191.0555; 93.0333; 134.0363; 87.0074
45	FNO	Myricetin O-hexoside	11.14	C ₂₁ H ₂₀ O ₁₃	[M+H] ⁺	481.0981	0.2	319.0446; 320.0480; 85.02900; 91.03940	-	-	-	-
46	HCA	Coumaroylquinic acid (III)	11.19	C ₁₆ H ₁₈ O ₈	[M+H] ⁺	339.1077	0.8	147.0440; 119.0493; 148.0474; 91.0547	[M-H] ⁻	337.0929	-1.5	191.0555; 192.0588; 85.0281; 93.0333
47	FNO	Myricetin O-glucuronide	11.26	C ₂₁ H ₁₈ O ₁₄	[M+H] ⁺	495.0772	0.5	319.0446; 320.0481; 85.02900; 113.0236	-	-	-	-
48	CT	B-Procyanidin pentamer	11.40	C ₇₅ H ₆₂ O ₃₀	-	-	-	-	[M-2H] ²⁻	720.1602	-1.6	125.0233; 289.0721; 287.0564; 407.0775
49	HT	Galloyl-ethyl-gallate	11.65	C ₁₆ H ₁₄ O ₉	[M+H] ⁺	351.0713	0.9	153.0182; 139.0389; 154.0216; 167.0338	[M-H] ⁻	349.0564	-1.4	165.0185; 137.0234; 123.0076; 166.0219
50	HCA	Acetyl caffeoyl deoxyhexoside	11.71	C ₁₇ H ₂₀ O ₉	[M+H] ⁺	369.1183	0.7	163.0389; 153.0182; 145.0283; 167.0338	[M-H] ⁻	367.1032	-0.9	135.0441; 179.0342; 161.0235; 191.0554
51	AL	Cinchonain type II	12.42	C ₃₉ H ₃₂ O ₁₅	[M+H] ⁺	741.1816	0.5	289.0703; 451.1020; 179.0338; 433.0916	[M-H] ⁻	739.1674	-1.6	289.0721; 339.0512; 587.1201; 449.0884
52	CM	Umbelliferone glucoside	12.41	C ₁₅ H ₁₆ O ₈	[M+H] ⁺	325.0920	1.1	163.0388; 145.0284; 164.0422; 135.0440	-	-	-	-
53	HCA	Caffeoyl valeryl hexose	12.45	C ₂₀ H ₂₆ O ₁₀	-	-	-	-	[M-H] ⁻	425.1454	-1.6	161.0235; 179.0342; 162.0269; 135.0441
54	LN	Lariciresinol O-glucoside	12.46	C ₂₇ H ₃₆ O ₁₃	-	-	-	-	[M-H] ⁻	567.2084	-1.2	329.1397; 330.1431; 341.1402; 112.9846
55	CT	B-Procyanidin trimer	12.77	C ₄₅ H ₃₈ O ₁₈	[M+H] ⁺	867.2137	-0.1	289.0704; 579.1496; 291.0861; 427.1024	-	-	-	-
56	CT	B-Procyanidin dimer	12.98	C ₃₀ H ₂₆ O ₁₂	[M+H] ⁺	579.1499	0.5	127.0391; 123.0442; 287.0548; 409.0916	[M-H] ⁻	577.1353	-1.2	125.0233; 289.0721; 407.0774; 245.0821



Table 1. Continued.

ID	Compound group	Name	RT [min]	Proposed formula	Positive polarity				Negative polarity			
					Adduct	Experimental m/z	Δm (ppm)	Main Product Ions (m/z)	Adduct	Experimental m/z	Δm (ppm)	Main Product Ions (m/z)
57	HCA	Coumaroyl iridoid isomer	12.99	C ₂₅ H ₂₈ O ₁₃	[M+H] ⁺	537.1606	0.3	147.0440; 175.0389; 165.0546; 148.0474	[M-H] ⁻	535.1457	-1.0	163.0392; 147.0442; 191.0344; 119.0490
58	FO	Quercetin <i>O</i> -hexoside	13.33	C ₂₁ H ₂₀ O ₁₂	[M+H] ⁺	465.1032	0.3	303.0497; 304.0531; 91.0394; 85.0290	[M-H] ⁻	463.0882	-1.2	300.0277; 301.0341; 302.0391; 463.0884
59	HCA	Coumaroyl valeryl hexose (I)	13.40	C ₂₀ H ₂₆ O ₉	-	-	-	-	[M-H] ⁻	409.1502	-0.9	145.0285; 163.0392; 119.0490; 146.0319
60	FO	Quercetin <i>O</i> -glucuronide	13.49	C ₂₁ H ₁₈ O ₁₃	[M+H] ⁺	479.0822	0.8	303.0497; 304.053; 85.0290; 113.0236	[M-H] ⁻	477.0673	-0.7	301.0357; 302.0389; 151.0029; 178.9979
61	AL	Cinchonain type I (I)	13.65	C ₂₄ H ₂₀ O ₉	[M+H] ⁺	453.1182	0.8	191.0337; 123.0442; 137.0232; 163.0389	[M-H] ⁻	451.1036	-1.5	341.0669; 217.0139; 342.0701; 219.0295
62	F-3-ol	Kandelin B3	13.75	C ₅₄ H ₄₄ O ₂₁	[M+H] ⁺	1029.2449	0.4	451.1020; 289.0704; 409.0915; 739.1655	-	-	-	-
63	CT	Procyanidin A2	14.06	C ₃₀ H ₂₄ O ₁₂	[M+H] ⁺	577.1345	0.3	287.0548; 288.0581; 425.0866; 123.0442	-	-	-	-
64	FG	Kaempferol <i>O</i> -hexoside	14.69	C ₂₁ H ₂₀ O ₁₁	[M+H] ⁺	449.1081	0.7	287.0548; 288.0581; 85.0290; 91.0394	-	-	-	-
65	FO	Quercetin <i>O</i> -malonyl-hexoside	14.74	C ₂₄ H ₂₂ O ₁₅	[M+H] ⁺	551.1036	0.2	303.0497; 304.0531; 127.0390; 159.0287	[M-H] ⁻	549.0887	-1.2	300.0277; 301.0340; 505.0990; 302.0389
66	FO	Quercetin <i>O</i> -pentoside	14.84	C ₂₀ H ₁₈ O ₁₁	[M+H] ⁺	435.0926	0.2	303.0498; 73.0291; 304.0533; 61.0292	[M-H] ⁻	433.0773	-0.6	300.0277; 301.0333; 151.0024; 178.9978
67	HCA	Coumaric acid derivative (I)	14.89	C ₂₀ H ₂₈ O ₉	[M+H] ⁺	413.1809	0.6	147.0440; 148.0473; 119.0492; 165.0544	[M-H] ⁻	411.1659	-1.1	145.0285; 163.0392; 146.0318; 119.0490
68	HCA	Coumaroyl valeryl hexose (II)	15.14	C ₂₀ H ₂₆ O ₉	-	-	-	-	[M-H] ⁻	409.1504	-1.3	145.0285; 163.0393; 146.0319; 119.0491
69	-	Unknown	15.14	C ₁₅ H ₁₆ O ₇	[M+H] ⁺	309.0972	0.6	147.0441; 119.0494; 148.0474; 69.0343	-	-	-	-
70	FO	Quercetin xylosyl rhamnoside	15.22	C ₂₆ H ₂₈ O ₁₅	[M+H] ⁺	581.1505	0.2	303.0499; 85.0290; 73.0291; 97.0289	-	-	-	-
71	FN	Luteolin <i>O</i> -glucuronide	15.42	C ₂₁ H ₁₈ O ₁₂	[M+H] ⁺	463.0876	0.2	287.0550; 288.0583; 85.0290; 113.0236	[M-H] ⁻	461.0727	-1.4	285.0408; 286.0442; 113.0233; 85.0282



Table 1. Continued.

ID	Compound group	Name	RT [min]	Proposed formula	Positive polarity				Negative polarity			
					Adduct	Experi- mental m/z	Δm (ppm)	Main Product Ions (m/z)	Adduct	Experi- mental m/z	Δm (ppm)	Main Product Ions (m/z)
72	FO	Quercetin <i>O</i> -deoxyhexoside	15.52	C ₂₁ H ₂₀ O ₁₁	[M+H] ⁺	449.1083	0.1	303.0498; 73.0291; 304.0533; 61.0292	[M-H] ⁻	447.0932	-1.0	301.0349; 302.0391; 151.0026; 271.0249
73	FNN	Naringenin <i>O</i> -hexoside	15.73	C ₂₁ H ₂₂ O ₁₀	[M+H] ⁺	435.1290	0.3	273.0755; 153.0181; 147.0440; 274.0788	-	-	-	-
74	FO	Isorhamnetin <i>O</i> -glucuronide	15.91	C ₂₂ H ₂₀ O ₁₃	[M+H] ⁺	493.0981	0.2	317.0655; 318.0689; 85.0290; 113.0236	-	-	-	-
75	DFO	Dihydro-laricitrin	15.98	C ₁₆ H ₁₄ O ₈	[M+H] ⁺	335.0763	1.2	137.0234; 138.0267; 139.0390; 167.0339	[M-H] ⁻	333.0616	-1.6	165.0185; 137.0234; 121.0283; 97.0282
76	FG	Kaempferol <i>O</i> -pentoside	16.27	C ₂₀ H ₁₈ O ₁₀	[M+H] ⁺	419.0975	0.7	287.0548; 73.0291; 288.0581; 71.0862	-	-	-	-
77	FNO	Myricetin	16.56	C ₁₅ H ₁₀ O ₈	-	-	-	-	[M-H] ⁻	317.0300	-0.9	165.0184; 137.0233; 166.0217; 121.0283
78	HCA	Coumaric acid derivative (II)	16.63	C ₂₀ H ₂₈ O ₉	[M+H] ⁺	413.1808	0.9	147.0440; 148.0473; 119.0493; 165.0545	[M-H] ⁻	411.1660	-1.1	163.0392; 145.0285; 119.0490; 164.0425
79	HCA	Coumaroyl valeryl hexose (III)	16.87	C ₂₀ H ₂₆ O ₉	-	-	-	-	[M-H] ⁻	409.1504	-1.4	145.0285; 163.0392; 119.0490; 146.0319
80	HCA	Coumaric acid derivative (III)	17.36	C ₂₀ H ₂₈ O ₉	[M+H] ⁺	413.1809	0.7	147.0440; 148.0474; 119.0490; 165.0543	[M-H] ⁻	411.1660	-1.1	163.0392; 145.0285; 119.0490; 164.0425
81	HCA	Coumaric acid derivative (IV)	17.67	C ₂₀ H ₂₈ O ₉	-	-	-	-	[M-H] ⁻	411.1660	-1.1	163.0392; 145.0286; 119.0491; 164.0426
82	FG	Kaempferol <i>O</i> -deoxyhexoside	17.73	C ₂₁ H ₂₀ O ₁₀	[M+H] ⁺	433.1132	0.6	287.0548; 71.0498; 85.029; 288.0581	[M-H] ⁻	431.0982	-0.9	285.0402; 284.0329; 255.0299; 286.0443
83	-	Unknown	17.92	C ₂₁ H ₃₀ O ₁₀	[M+H] ⁺	443.1915	0.5	177.0545; 145.0284; 146.0316; 195.0653	[M-H] ⁻	441.1767	-1.4	193.0500; 175.0393; 134.0362; 149.0599
84	FLL	Cinchonain type I (II)	18.29	C ₂₄ H ₂₀ O ₉	[M+H] ⁺	453.1181	0.9	191.0337; 123.0441; 137.0232; 163.0388	[M-H] ⁻	451.1035	-1.3	341.0668; 217.0139; 219.0296; 342.0703
85	FO	Quercetin	22.18	C ₁₅ H ₁₀ O ₇	-	-	-	-	[M-H] ⁻	301.0352	-1.3	151.0027; 107.0126; 121.0283; 178.9979

HBA, hydroxybenzoic acids and derivatives; HCA, hydroxycinnamic acids and derivatives; F-3-ol, Flavan-3-ols; HT, hydrolysable tannins and derivatives; CT, condensed tannins and derivatives; FNN, flavanones; FO, flavonols; FN, flavones; LN, lignans; FNO, flavanonols; CM, coumarins and derivatives; PG, phenolic glucosides; FG, flavonoid glycoside; FLL, flavolignans; DFO, Dihydroflavonoals.



cated to specific groups after a thorough examination of the UV-visible spectrum. The identification of the compounds was achieved by analyzing the major m/z signals recorded in negative ion mode, as well as retention time and literature data, using full scan MS and MS² spectra (Table 1).

The identified compounds were classified as hydroxybenzoic acids and derivatives (only 1 compound), hydroxycinnamic acids and derivatives (26 compounds), flavan-3-ols (6 compounds), hydrolysable tannins and derivatives (2 compounds), condensed tannins and derivatives (11 compounds), flavanones (1 compound), flavonols (8 compounds), flavones (1 compound), lignans (1 compound), flavanonols (3 compounds), coumarins and derivatives (1 compound), phenolic glucosides (2 compounds), flavonoid glucosides (16 compounds), flavonolignans (1 compound) and dihydroflavonols (1 compound) (Table 1 and Fig. 2A).

Antioxidant Profiling by HPLC Coupled Post-Column Derivatization

The online post-column derivatization of compounds with ABTS reagent was carried out after the HPLC analysis of VM extract. The reduction reaction resulted in a noticeable shift in the UV-visible spectrum, causing a change (discoloration) in the absorption of ABTS reagent. The presence of antioxidants in the eluate was identified as negative peaks in the chromatogram recorded after derivatization at 734 nm (Fig. 2C).

Before derivatization at 270 nm by the DAD detector, approximately 24 major compounds were detected in the chromatograms of VM extract (Fig. 2B), out of which 16 compounds exhibited antioxidant activity after derivatization. Hydroxycinnamic acids and derivatives (compounds 5, 12, 24, 33, 50, and 57) made the greatest contribution to the overall antioxidant activity, followed by flavonols (compounds 58, 60, 67, and 72), condensed tannins and derivatives (compounds 30, 41, and 43), flavan-3-ols (compounds 15 and 35), and hydrolysable tannins and derivatives (compound 49). Antioxidant activity of most of the identified major phenolic compounds has been reported in the literature as well (Table 2, Ref. [42–57]).

Cytotoxicity of Vaccinium myrtillus L. by MTT Assay

In vitro cytotoxicity of VM extract on three cancer and one normal cell lines was evaluated with MTT assay. The human colon adenocarcinoma (HT29), human breast cancer (MCF-7), human cervical carcinoma (HeLa) and human normal primary renal mixed epithelial cells (HREC) lines were employed in the study.

Based on the obtained data the ethanol extract of VM inhibited the growth of all tested cancer cell lines (HT29, MCF-7 and HeLa cells) in a time- and dose-dependent manner (Fig. 3A,B and Fig. 4A). In all three tested cancer cell lines, considerable cell growth inhibition was observed only after 24 hours of exposure time at 0.5 mg DW/mL concentration. Shorter exposure times did not induce a notice-

able growth inhibitory effect on tested cancer or normal cell lines. The strongest inhibitory effect was observed at the longest exposure time (72 hours) for all tested cancer cell lines. However, the most important is the fact that, although VM extract exhibits remarkable cytotoxic activity on all tested cancer cell lines at 0.5 mg DW/mL or lower concentrations, it did not exhibit noticeable cytotoxicity on normal HREC lines even at much higher (10-fold) concentrations (Fig. 3C). Particularly, it brought to only the 23% growth inhibition at the highest tested concentration and longest exposure time (6.6 mg DW/mL, 72 hours).

The further modulating activity of sub-inhibitory concentrations of VM extract (0.125 mg DW/mL) toward fluorouracil (5-FU) on HeLa cells (Fig. 4B–D) was explored using *in vitro* MTT assay. Nevertheless, no statistically significant modulation was observed at any of the tested exposure times. In contrast, at 72 hours exposure time, VM extract decreased inhibiting properties of 5-FU on HeLa cells (Fig. 4D).

Genotoxic Effects Measured by Comet Assay

Genotoxicity of VM aerial part extract was explored by comet assay in HT29 cells. Obtained data revealed that VM aerial part extract did not induce DNA fragmentation in HT29 cells (Fig. 3D).

Antibacterial and Antibiotic Modulatory Properties of V. myrtillus Extract

The antibacterial and antibiotic modulatory properties of *V. myrtillus* extract were elucidated on two bacterial test strains: Gram-negative *E. coli* and Gram-positive *S. typhimurium* strains (for details see “Materials and Methods” section).

The obtained data indicated that *V. myrtillus* extract alone did not exhibit antibacterial properties against any of the tested bacterial strains at concentrations up to 1 mg/mL.

Although no direct inhibitory effect of the VM aerial part extract on the growth of bacteria was observed, however, it has exhibited excellent antioxidant properties in different tests. These results lead us to test the antibiotic-modulatory properties of the investigated extract through extract/antibiotic combined treatment of bacterial cells. Ampicillin and kanamycin were chosen as test antibiotics. Our study revealed synergistic interaction between VM extract and kanamycin against *E. coli* BW25113 strain (Fig. 5A). The plant extract at nontoxic concentrations for the test bacteria (0.125 and 0.25 mg/mL) combined with kanamycin decreased the MIC value of the antibiotic by two-fold against tested bacteria. However, no synergism was found in the case of *S. typhimurium* TA100 strain (Fig. 5B). On the other hand, VM extract did not increase the activity of ampicillin against any of the tested bacteria (Fig. 5C,D).

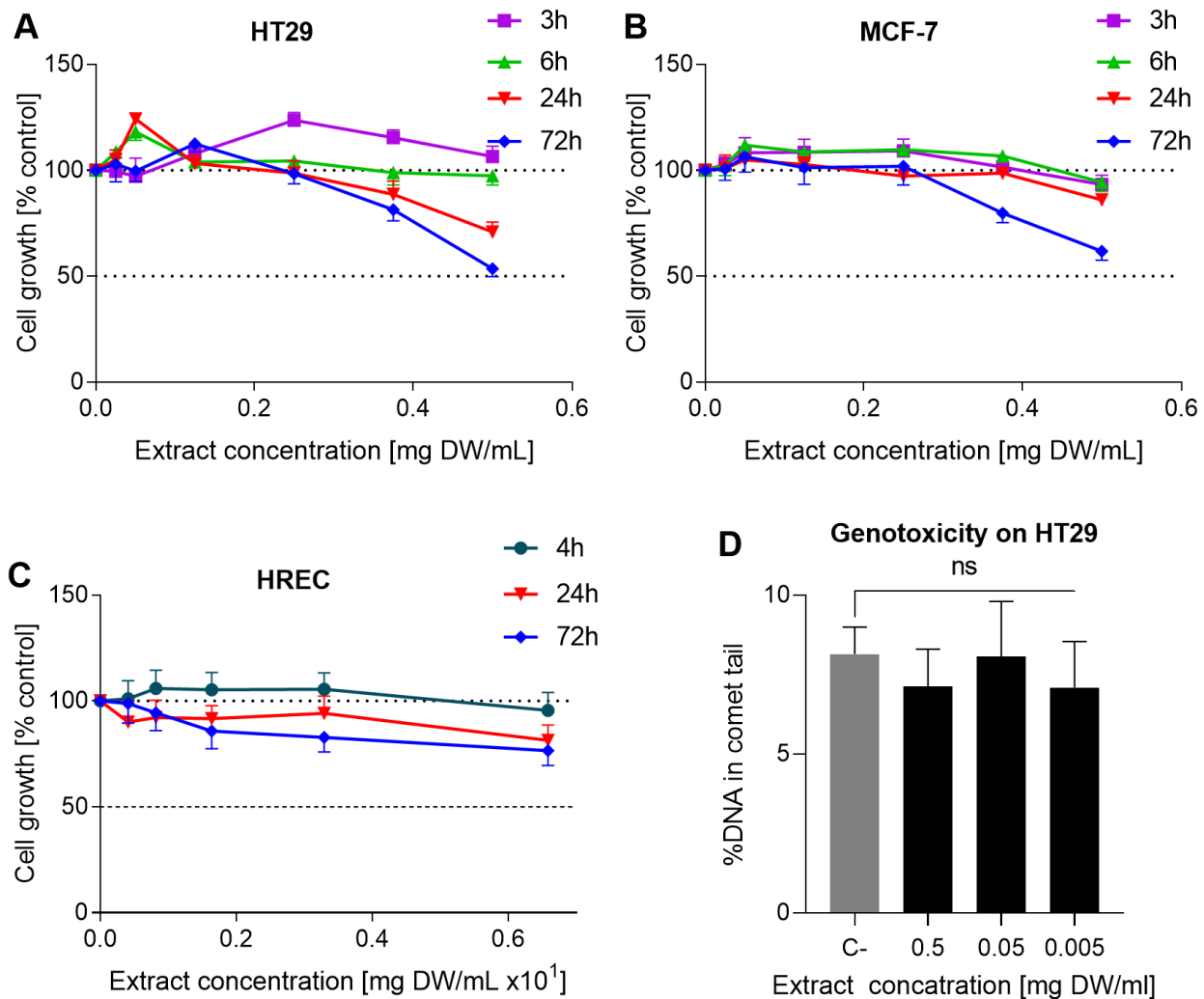


Fig. 3. Growth inhibiting properties of *V. myrtillus* extract on cancer and normal cell lines determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT test and assessment of extract's genotoxicity on HT29 cells. (A) Growth inhibitory activity of VM extract on HT29 cells. (B) Growth inhibitory activity of VM extract on human breast cancer (MCF-7) cells. (C) Growth inhibitory activity of VM extract on human normal primary renal mixed epithelial cells (HREC). (D) Evaluation of genotoxic effect of VM extract on HT-29 cells expressed as % DNA in comet tail. Results represent means \pm SD from three independent experiments; SD values did not exceed 15%. C-(Negative control) non-treated cells. No significant differences between treated and non-treated cells were observed; ns, not significant.

Determination of Proton Flux

Ion flux analysis showed that proton flux reached the 2.59 mM H⁺/min by 10⁸ cells value during glucose utilization (Fig. 6). Based on obtained data, kanamycin was not able to affect the proton flux rate in *E. coli* BW25113 cells. In contrast to this, the proton flux rate increased by 23.6% upon the addition of VM extract. This parameter remained at the same value during the combined influence of kanamycin and VM on bacterial cells.

Thus, we found that although VM aerial part ethanolic extracts do not have direct antibacterial activity, however, they increase the efficiency of the antibacterial activity of kanamycin by changing the homeostasis in bacterial cells and the permeability of bacterial membranes.

Discussion

Today, substances of plant origin are considered as one of the most promising sources of beneficial, physiologically active metabolites. The fruits of *V. myrtillus* are well known due to their abundance in health-promoting biologically active compounds and are very popular in different regions of the world as herbal medicinal products [1,2,6–10]. On the other hand, although several reports are available about the promising biological properties and presence of many active phytochemicals in VM leaves and stems. Generally, they are considered as bio-waste [15,21,25]. Taking into account this information, we decided to explore the VM aerial part extracts as a new potentially valuable source for the de-

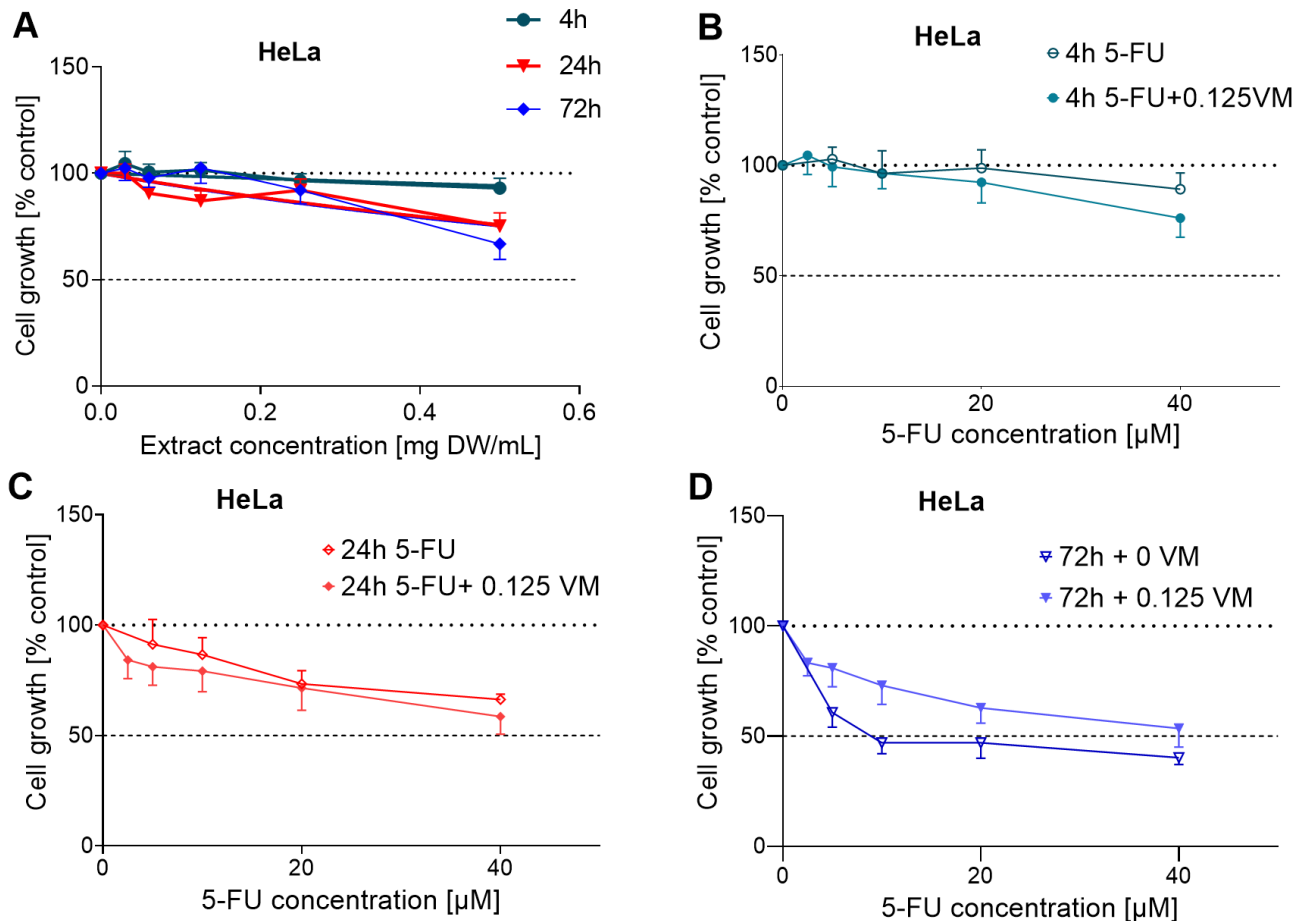


Fig. 4. Growth inhibiting properties of VM extract and 5-fluorouracil alone and in combinations against human cervical carcinoma (HeLa) cells. (A) Growth inhibition of HeLa cells treated with VM extract for 4, 24, and 72 h. (B) Growth inhibiting effect of 5-fluorouracil separately and in combination with VM extract after 4 h exposure. (C) The growth inhibiting effect of 5-fluorouracil alone and combined with VM extract after 24 h exposure. (D) The growth inhibiting effect of 5-fluorouracil alone and combined with VM extract after 72 h exposure. Results represent means \pm SD from three independent experiments; SD values did not exceed 15%.

velopment of different preparations for medicine. We have investigated the antioxidant, anticancer, antimicrobial and antibiotic modulatory properties of ethanol extract of VM aerial parts. The chemical-based tests showed its strong antioxidant potential. The strong antioxidant capacity of VM leaf or aerial parts extracts was also reported in different research works [9,15,21,25,58]. The total phenolic content of our sample was in accordance with the literature data. For example, Ștefănescu *et al.* [21] reported that the highest total phenolic content of leaf hydroalcoholic extracts of VM from different regions of Romania was 135.8 mg GAE/g DW, while it was 142.9 mg GAE/g DW in another report [15]. The total flavonoid content in our sample is twice lower compared to the abovementioned literature data, but it is also comparable to the data already published by other authors [21]. The strong antiradical capacity (including in DPPH and ABTS assays) of blueberry leaf extracts was reported in literature as well [15,21].

Further CAA test with HT29 cells was applied in order to reveal the antioxidant potential of investigated ex-

tract inside the cells. This test accounts for the bioavailability, distribution of the antioxidants within cells, and cellular metabolism of the test compounds, therefore, it is more biologically relevant compared with chemical tests [34]. Expressed cellular antioxidant activity was found in HT29 cells (Fig. 1C), which had a direct correlation to the concentration of extract. There is no literature data about the cellular antioxidant activity of VM leaf or aerial part extracts and, reported for the first time. However, Bender *et al.* [59] reported high CAA activity of blueberry juices.

Phenolic compounds are the most abundant plant secondary metabolites and have attracted more and more attention due to their wide range of biological activity including the antioxidant capacity and potential role in the prevention of different oxidative stress-associated diseases such as cancer [29,42,60,61]. The antimicrobial, antibiotic-modulatory, anti-inflammatory, anticancer and other properties of these compounds are also of great interest [27, 62]. Considering the high content of phenolics, including flavonoids, the observed antioxidant properties of the V.

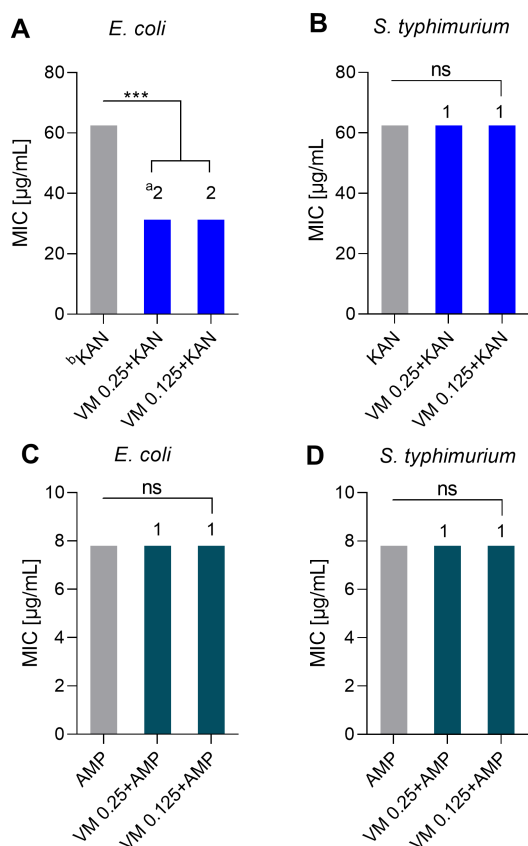


Fig. 5. Antibiotic modulatory activity of *V. myrtillus* crude ethanol extract at sub-inhibitory concentrations against some bacterial strains. (A) Modulatory activity of VM extract on kanamycin against *Escherichia coli* (*E. coli*) BW25113. (B) Modulatory activity of VM extract on kanamycin against *Salmonella typhimurium* (*S. typhimurium*) TA100 strain. (C) Modulatory activity of VM extract on ampicillin against *E. coli*. (D) Modulatory activity of VM extract on ampicillin against *S. typhimurium*. ^a (n) Modulation factor (MF) which was calculated as $\text{MF} = \text{MIC}_{\text{antibiotic alone}} / \text{MIC}_{\text{antibiotic + extract}}$ $n \geq 2$ —synergic interaction, $n = 1$ —indifference, $n \geq 2$ —synergistic interaction. ^bUsed antibiotics – KAN – kanamycin, AMP – ampicillin. *** $p < 0.001$; ns, not significant; MIC, minimal inhibitory concentration.

myrtillus extract are obviously due to the presence of redox-active constituents in this herb's metabolome. Therefore, the characterization of VM extracts' phenolic compounds is of great importance and was done using the advanced chromatographic technique (LC-Q-Orbitrap HRMS). Due to this method around 85 compounds in *V. myrtillus* extract were identified, 83 compounds of which were annotated, and only 2 were unknown. The full list of identified compounds with their molecular formula, and theoretical and experimental mass measurements are presented in Table 1.

Table 2 presents the major phenolic constituents with possible bioactive properties found in VM extract. According to existing literature, many of these major phenolics have been shown to possess antioxidant, anticancer, anti-

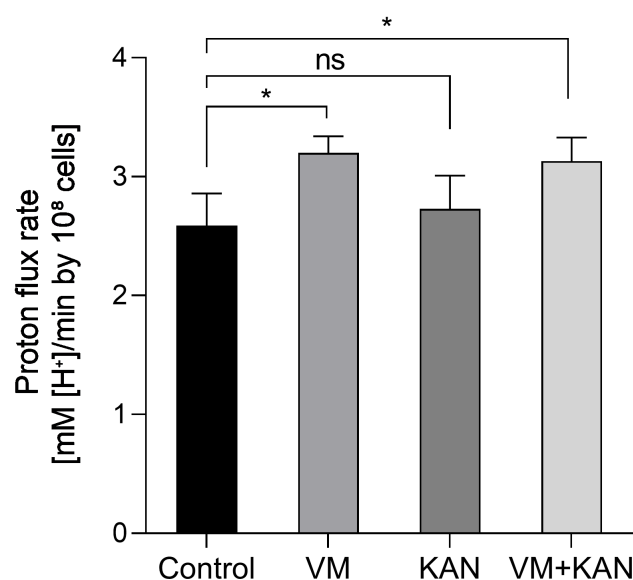


Fig. 6. VM and KAN effect on $[\text{H}^+]$ flux rate by 10^8 whole *E. coli* BW25113 cells within assimilation of glucose (2 g/L). Results represent means \pm SD from three independent experiments. * $p < 0.01$; ns, not significant.

icrobial, anti-inflammatory, or antibiotic modulatory activities. Particularly, based on our research and the available literature (Table 2), it was found that 18 out of the identified 24 major phenolic compounds exhibited antioxidant properties. Again, according to literature data, 11 of those major compounds were reported to have antibacterial activity, 16 of them possessed anti-inflammatory, and 17 have anticancer capacity. Several compounds reported to exhibit antibiotic-modulatory, antiparasitic and antiviral properties.

Growth inhibiting properties of VM aerial part extract were assessed using MTT assay on both cancer (HT29, MCF-7, HeLa) and normal HREC lines. Selection of the test cell lines was based on several factors: the HT29 cell line is widely used as a model for the human digestive tract, which is directly exposed to ingested food components, including biologically active phytochemicals, the MCF-7 and Hela cells were selected as they are among the most commonly used cancer cell lines in the *in vitro* experimental models.

It can be firmly asserted that VM extract selectively inhibited the growth of tested cancer cell lines in a time- and dose-dependent manner, whereas not affecting normal cell lines. This feature can have great importance for the consideration of the VM extract as an important source of anticancer agents. However, further more thorough investigations are required to confirm this assumption. Some literature also describes the cytotoxic properties of VM leaf or aerial part extracts against different cancer cell lines. So, Plasencia *et al.* [26] showed that hydroalcoholic extracts of VM aerial parts inhibit the growth of MCF-7, NCI-H460 (non-small cell lung carcinoma), AGS (gastric adenocarci-

noma), and CaCo-2 (colorectal adenocarcinoma) cell lines. In another study, it was reported the cytotoxic activity of VM leaf water-glycerol extracts on keratinocytes [25]. The selective targeting of cancer cells by the active phytochemicals holds a great importance, as it could serve as a key factor in the development of more effective anticancer therapeutics [29].

Modulation of anticancer efficiency of chemotherapeutic agents using plant extracts or derived compounds is also a promising strategy to overcome drug resistance or decrease the high side effects of medical preparations. In recent years, the observation of the anticancer effect of herbal extracts and classical chemotherapeutic compounds combinations has become quite an effective new approach [63]. Therefore, we explored the modulating activity of sub-inhibitory concentrations of VM extract toward fluorouracil. However, modulatory activity was not observed. Moreover, at 72 hours exposure time, VM extract even decreased efficiency of 5-FU. This phenomenon is in accordance with some literature data, stating that some drugs can lose their anticancer effects as a result of herb-drug interactions [64,65]. One of the reasons for the non-modulating effect obtained by us in this work could be this type of interaction, and in such combinations, the classic chemotherapeutic compound can be replaced by compounds with a target effect on proteins involved in cancer development processes, as shown in our previous work [29]. This kind of investigation can be helpful for patients in composing the individual diet during the chemotherapeutic treatment of cancer.

It is crucial also to evaluate the genotoxicity of medicinal plants prior to their consideration in practical medicinal applications [66]. This technique enables the identification of genotoxicity by detecting the induction of cellular DNA fragmentation in response to exposure to genotoxins [67]. Based on obtained data VM aerial part extract does not exhibit genotoxic activity on HT29 cells. There are several reports about the genotoxic or anti-genotoxic properties of species within *Vaccinium* sp. For example, it is stated that hydroalcoholic extracts of *Vaccinium corymbosum* L. fruits did not induce DNA damage in mice's peripheral blood leukocytes [68]. Anti-genotoxic properties of VM fruits from cisplatin-induced toxic effect were demonstrated in *in vivo* rat models [10]. In another study, authors observed some protective effects of VM fruit powder during the modulation of tert-butylhydroperoxide induced DNA-damage in A549 (human lung adenocarcinoma) cells. In our investigations, we did not observe any genotoxic influence of VM extract on the tested cells. So, these findings are also important in terms of the practical application.

In the post-antibiotic era, alternative strategies must be employed to combat antibiotic-resistant microbes. This is because the conventional methods of discovering new antimicrobials have proven to be largely ineffective due to the swift emergence of resistance against them [39].

Plant extracts or derived compounds can possess direct antimicrobial action or act as antibiotic resistance-modifying agents and increase the efficiency of conventional antibiotics through different mechanisms including inhibition of modified targets, increasing intake of antibiotics by changing membrane permeability, inhibition of multidrug-resistant pumps, *etc.* [39]. So, antimicrobial and antibiotic modulatory properties of the VM aerial part extracts were also explored. Based on obtained data, the investigated extract did not exhibit antibacterial activity at up to 1 mg/mL concentration on the tested microorganisms. Higher concentrations were not considered for investigation as generally plant extracts are considered efficacious in antimicrobial assays only if they have MIC values below 0.1 mg/mL [69,70]. There are few reports referring antimicrobial properties of VM leaf or aerial parts extracts. Plasencia *et al.* [26] showed some antimicrobial action of VM aerial part extracts against several pathogenic bacteria at only high concentrations, where MIC values ranged between 10 and 20 mg/mL. The exception in this work was the Methicillin-resistant *Staphylococcus aureus*, against which the MIC value was reported to be 1.25 mg/mL. In contrast, the high antimicrobial activity of VM extracts was revealed in other research work from Romania, where authors documented MIC values ranging between 0.06 and 0.96 mg/mL against different Gram-negative and Gram-positive bacteria [21]. On the other hand, there are many research works providing information about the high antimicrobial activity of VM fruit extracts. For example, in another research, Satoh and Ishihara [71] showed high antimicrobial activity of VM fruit extract different fractions on several oral pathogenic bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia*, and *Streptococcus mutans* [71]. Other investigations have confirmed the presence of antimicrobial properties of this blueberry fruit extract, exhibiting effectiveness against a wide range of fungi and antibiotic-resistant and non-resistant both Gram-negative and Gram-positive bacteria, including pathogenic and non-pathogenic strains [21,26,72].

As it was mentioned above, VM extract effect on modulating of efficiency of ampicillin and kanamycin antibiotics was explored. Ampicillin, as a beta-lactam antibiotic, inhibits bacterial cell wall synthesis, whereas kanamycin is an aminoglycoside antibiotic that works by binding irreversibly to the bacterial 30S ribosomal subunit [73]. We revealed synergistic interaction between VM extract and antibiotic, but only kanamycin (Fig. 5A). There was no synergism with ampicillin against any of the tested bacteria.

Taking into account the obtained data, we hypothesized that modulation of kanamycin activity could be the result of changes in membrane-associated properties of *E. coli* BW25113 membranes after exposure to VM extract.

Table 2. The major identified compounds in *V. myrtillus* extract with possible antimicrobial, antioxidant, anticancer, anti-inflammatory and antibiotic-modulatory properties.

Compound Group	Tentative identification	Post-column derivatization with ABTS (+/-)*	Biological (antimicrobial, anticancer, antioxidant, anti-inflammatory, antibiotic modulating) activities
Hydroxycinnamic acids and derivatives	Caffeoylquinic acid (I)	+	Antioxidant, antibacterial, antiparasitic, anti-inflammatory, anticancer, antiviral, <i>etc.</i> [43,44]
	Caffeoylquinic acid (II)	+	
	Caffeoylquinic acid (III)	+	
	Caffeoylquinic acid (IV)	+	
	Acetyl caffeoyl deoxyhexoside	+	N/A
	Coumaroyl iridoid isomer	+	Anti-inflammatory, antitumor, hypoglycemic [45]
	Coumaroyl valeryl hexose (II)	-	N/A
	Coumaroyl valeryl hexose (III)	-	
Phenolic glycosides	Coumaric acid derivative (II)	-	Antimicrobial, anticancer, anticancer, anti-inflammatory [42,46,47]
	Coumaric acid derivative (III)	-	
	Coumaric acid derivative (IV)	-	
		-	
Flavan-3-ols	Vanillyl alcohol glucoside	-	Antioxidant, anticancer, anti-inflammatory [48,49]
	(Epi)gallocatechin	+	Anticancer, antioxidant, antiviral, antibiotic modulatory and antibacterial [50–53]
Condensed tannins and derivatives	(epi)Catechin	+	Antioxidant, antimicrobial, antibiotic modulatory [46,52–55]
	B-Procyanidin dimer	+	Antitumor, antioxidant, antiviral and anti-inflammatory [53]
	B-Procyanidin trimer	+	
	B-Procyanidin tetramer	+	
Hydrolysable tannins and derivatives	Galloyl-ethyl-gallate	+	N/A
	Flavonols	Quercetin <i>O</i> -hexoside	+
Quercetin <i>O</i> -glucuronide		+	
Quercetin <i>O</i> -pentoside		+	
Quercetin <i>O</i> -deoxyhexoside		+	
Flavolignans	Cinchonain type I (I)	-	Antioxidant, antibacterial [57]
Dihydroflavonols	Dihydro-laricitrin	-	N/A

*Antioxidant profiling was done by HPLC coupled post-column derivatization with ABTS reagent. “+”, presence of antioxidant activity; “-”, absence of antioxidant activity; “N/A”, not applicable.



So, it was obvious that further investigations of the effect of VM extract on proton flux changes in the bacterial cell membrane are of importance. And we did not fail: VM stimulates membrane flux. Therefore, we can conclude that the synergistic effect of the test extract with kanamycin is due to the influence of the extract components on the membrane permeability of bacteria. The fact that the investigated extract had not any synergistic activity with the beta-lactam group antibiotic indirectly proves this claim.

Moreover, there is a precise difference between the influence of VM and kanamycin on Gram-positive and Gram-negative bacteria (Fig. 5), which also indicates that the antibiotic-modulatory activity is connected with the changes in the delivery system of the bacterial cell. The antibiotic modulatory properties of VM aerial part extract were reported for the first time.

Conclusions

Based on obtained data, we confirmed that aerial parts of wild *V. myrtillus* L. plants growing in alpine zones of Armenia at altitudes of 2800 meters and above possess high antioxidant properties. We demonstrated this activity through its high total phenolic and flavonoid content, as well as with the spectrophotometric and CAA tests. It was also confirmed by the investigation of phenolic fraction with the HPLC system followed by post-column derivatization with ABTS reagent. LC-Q-Orbitrap HRMS analyses provided a reliable phytochemical characterization of *V. myrtillus* aerial part ethanolic extract. Many major compounds identified in the VM extract belonging to the hydroxycinnamic acids and derivatives, phenolic glycosides, flavan-3-ols, condensed tannins and derivatives, flavonols, flavonolignans and dihydroflavonols possessing high biological activities and can bring a great contribution to the development of new approaches in medicine. The plant extract showed remarkable selectivity in inhibiting the growth of cancer cell lines, interestingly, not affecting the growth of tested normal cells. This feature is of crucial importance for the consideration of the extract as a promising source of anticancer preparations.

The VM extract was in synergy with kanamycin possibly by enhancing the intake of antibiotics through bacterial membranes. We also found that the extracts do not have genotoxic properties, which is of great importance for considering them in practical applications. In conclusion, we propose that aerial parts of *V. myrtillus* plant, which are generally considered as bio-waste, can be suggested as a promising alternative source of bioactive natural products for the development of food supplements, nutraceuticals, or functional foods as well as in medicinal preparations.

Abbreviations

ABTS, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); CAA, cellular antioxidant activity;

DPPH, 2,2-diphenyl-1-picrylhydrazyl; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; EMEM, eagle's minimum essential medium; HT29, human colon adenocarcinoma; MCF-7, human breast cancer; HeLa, human cervical carcinoma; HREC, human normal primary renal mixed epithelial cell; DMEM, dulbecco's modified essential medium; FC, folin-cioalteau; GA, gallic acid; MF, modulation factor; FBS, fetal bovine serum; VM, *Vaccinium myrtillus* L.; PBS, phosphate-buffered saline; LMP, low melting point agarose; NMP, normal melting point agarose; YSU, Yerevan State University; DW, dry weight.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

All authors contributed to the study's conception and design. MG, MQ, AS, ABab, AM, IKM, NS and BK carried out the investigations and analyzed the outcomes. MG and NA wrote the manuscript. ABar, NA, AV and NS directed the experiments, corrected, and edited the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest. Anne Vejux is serving as one of the Editorial Board Members and Guest Editors of this journal. We declare that Anne Vejux had no involvement in the peer review of this article and has no access to information regarding its peer review.

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