



Recovery of High Valuable Bioactive Molecules from *Vaccinium myrtillus* L. Bioresidues

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Received: 12 June 2022 / Accepted: 8 January 2023
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Abstract

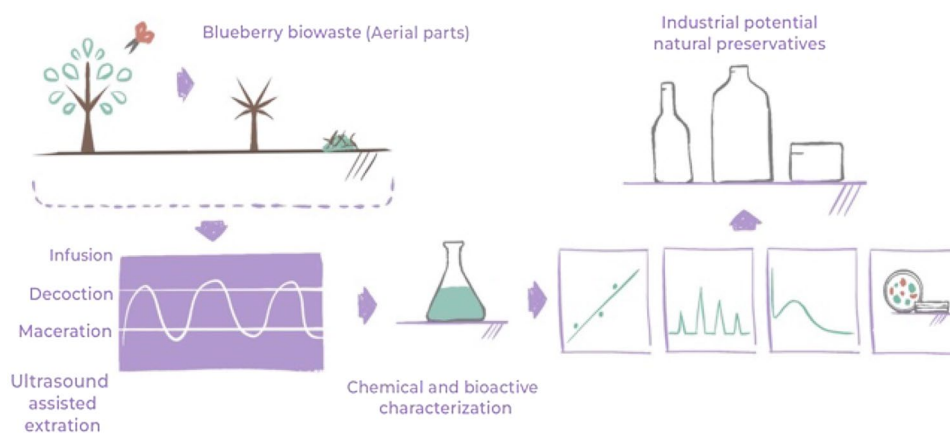
Purpose The interest in using bioresidues produced by the agri-food industry is increasingly intrinsic to the world economy. As such, researchers started to look for new ways to enhance its use, developing innovations to transform these residues into high-value raw materials with industrial application, promoting the circular economy concept.

Methods Therefore, pruning residues from the production of *Vaccinium myrtillus* L. could be an interesting exploitation field, given its position as a superfood due to its well-known antioxidant properties. The antioxidant, antibacterial, anti-inflammatory, anti-tyrosinase, and cytotoxicity potentials of aqueous and hydroethanolic extracts of *V. myrtillus* aerial parts were evaluated.

Results From the obtained results, ultrasound-assisted and maceration extracts were found to be as effective as, if not more effective than, conventional antibiotics against methicillin-resistant *Staphylococcus aureus*. Aside from antibacterial activity, the extracts also showed antioxidant and anti-tyrosinase effects, which were found to be favourably related to the level of caffeoylquinic acid derivatives.

Conclusion The obtained results highlight the bioactive potential and the importance of exploiting this bio residue as a novel candidate for industrial application, taking advantage of their biological properties.

Graphical Abstract



Keywords *Vaccinium myrtillus* L. · Chemical characterization · Biological activity · Antimicrobial activity · Agri-food biowaste

Extended author information available on the last page of the article

Statement of Novelty

A novel opportunity for exploiting blueberry pruning leftovers by recovering valuable bioactive molecules was established. The proposed strategy follows a circular bioeconomy approach by valorising a discharged agri-food bio residue, namely pruning wastes from *Vaccinium myrtillus* L. cultivation, which, contrarily to the well-known and extensively studied fruit, blueberries, are still unexplored matrices. For the first time, blueberry pruning leftovers were characterized concerning the phenolic profile and bioactivity. Among the tested properties, the extracts evidenced antioxidant properties and anti-tyrosinase capacity. Moreover, all the extracts presented an antibacterial activity similar to ampicillin. Overall, the obtained promising results highlight that blueberry aerial parts might be transformed into high-value raw materials and used as natural bioactive preservatives in food and cosmetics.

Introduction

The *Vaccinium* genus consists of woody perennial shrubs belonging to the Ericaceae family and contains, among other species, the *Vaccinium myrtillus* L. This shrub varies in size from approximately 5 to 90 cm; the flowers are bell-shaped, white, pale pink, or red, sometimes tinged greenish, producing a blue-colored berry, known as blueberry [1]. This species is well known, and its fruits are highly consumed, not only for their organoleptic characteristics but also for their medicinal properties, given their richness in bioactive molecules such as phenolic compounds [2].

The demand for these fruits is increasing in various parts of the world, with major world producers such as the United States and South American countries, finding an opportunity to increase their exportation to European retail chains that are seeking more suppliers of *V. myrtillus* [3]. Therefore, the blueberries world production has been increasing since 2003, reaching about 750 kton of blueberries in 2018, being Europe responsible for 18.2% of its distribution [4].

Because of its extensive production, the processing of these fruits generates high amounts of bioresidues, mostly from pruning and weeding or from berry processing. In response to current environmental and economic concerns regarding the sustainable (re)use of natural resources (including bioresidues), the agri-food industry is often confronted with several solutions, including the animal feed, which means investments in technology to provide a healthy and nutritive by-product. Another solution is the

spreading and composting for energy, and incineration for biogas production [5]. Beyond the bioresidues generated by the blueberries manufacture, the ones derived from berry crops are a new focus of study, since the industry is more and more interested in its valorization, namely through the sustainable obtaining of high added-value products that can find industrial application in several sectors, namely the food, cosmetic and also the pharmaceutical ones. In this context, the leaves, and other aerial parts of *V. myrtillus*, are a good example of waste biomass that can be exploited; due to their high content in antioxidants, such as phenolic compounds, and in fibers, these by-products could be a valuable raw material for the recovery of added value molecules [6].

While blueberry fruits have already been studied in detail, their bio residues have not been fully exploited, although some results described in the literature pointed out the leaf as a potential source of phenolic compounds [7], mainly flavonoids (quercetin derivatives), which contribute to the high antioxidant potential described for this biomass [8]. It is also well known that the regular consumption of vegetables and fruits rich in polyphenolic compounds provides protection against the harmful effects of UV radiation such as oxidative stress, inflammation, and cancer. Botanicals with a photo protective activity are gaining attention from researchers of cosmetic and pharmaceutical corporations due to the multiple health benefits and cost-effectiveness [9]. In skin applications, antioxidants are a promising tool to mitigate oxidative injury [10]. In fact, the use of natural extracts is a growing tendency since the beginning of the XXI century, mostly with those plants that have evolved a variety of photo adaptive mechanisms including the production of antioxidant and UV absorbing compounds by being exposed to intense radiation in their environmental conditions [9]. This evolutionary change in the society is reflected in a strong interest by the consumer in cosmetic care formulations having a “natural” benefit. The trend toward “nature” is paralleled by a fast-growing scientific knowledge about plant constituents, human molecular biology, and cell physiology [10]. In this sense, blueberries have already been successfully used in the cosmetic industry and were able to increase the photo protective capacity of sunscreen formulations, providing also extra benefits due to their antioxidant and anti-aging properties [11].

Bearing this in mind, the objective of the present work was to bring high added value to the pruning biowaste resulting from the production of blueberries. Plant extracts obtained from *V. myrtillus* aerial parts were characterized regarding their content in bioactive phenolic compounds. Furthermore, these extracts were also analyzed for their biological potential through antioxidant, antimicrobial, anti-inflammatory, anti-tyrosinase, and cytotoxicity, aiming at

developing a promising extract for application in the cosmetic industry.

Material and Methods

Plant Material

Vaccinium myrtillus aerial parts resulting from the pruning were collected in September of 2019 in Baião, Portugal (geographic coordinates: Latitude: 41° 09' 45" N Longitude: 8°02'04" W Elevation above sea level: 577 m = 1893 ft), by the company Hortitool Consulting Lda. After air drying at room temperature (around 25 °C) during 5 days, this raw material was grounded to a 20 mesh (~0.8 mm) particle size in a ZM200 Retsch model mill. Afterward, the dried material was kept protected from light and moisture until further analysis.

Preparation of the *V. myrtillus* Extracts

Aqueous Extracts

Infusion Approximately 1 g of powdered sample was added to 200 mL of boiling distilled water. The mixture was maintained in these conditions for 5 min to allow the extraction of the bioactive compounds.

Decoction In this case, after the addition of 200 mL of water to 1 g of powdered sample, the mixture was heated until boiling point and maintained in these conditions for 5 min.

For both the aqueous extracts, the samples were subjected to gravity filtration using a Whatman paper filter nº 4. Afterward, the aqueous extracts were frozen at – 24 °C, and lyophilized (Freeze Dryer Sacnvac, Coolsafe, 0.4 mbar, Lillerod, Denmark) at a final condensation temperature of – 100 °C and stored in a dry room.

Hydroethanolic Extracts

Maceration Approximately 1 g of the powdered sample was added to 30 mL of ethanol–water solution (80:20, v/v) and maintained with stirring for 60 min at room temperature.

Ultrasound-Assisted Extraction (UAE) This extraction was performed in a sonicator (Qsonica, Q500, ultrasonic processor sonicator, 20 kHz, Newtown, USA) following the extraction procedure described by Heleno et al. [12] with slight modifications to optimize the extraction in order to recover phenolic compounds. Briefly, about 1 g of powdered sample was mixed with 150 mL of an ethanol–water solution (80:20, v/v). The extraction was carried out for

10 min, with cycles of 30 s intercalated by pauses of 10 s, at 75% intensity of 500 W. An ice bath was also used to avoid sample overheating.

The mixtures were filtered by gravity, and evaporated under reduced pressure (Büchi R-114, rotary evaporator; Büchi B-480, waterbath, and Büchi B-721, vacuum controller system, Flawil, Switzerland) at 40 °C, 100 rpm, and ≈ 90 mbar until the ethanol was entirely removed. The aqueous residue was then frozen at – 24 °C, lyophilized, and stored in a dry room.

The extracts were subjected to UV decontamination for 15 min prior to each bioactive analysis, ensuring extracts sterility.

Chemical Characterization and Bioactive Properties

Profile in Phenolics Compounds

To identify and quantify the phenolic compounds, the followed protocol was the one of Bessada et al. [13]. Briefly, a portion of 10 mg of each different sample was dissolved in 1 mL of a methanol–water solution (20:80 v/v) and filtered through a 0.22 µm disposable filter. The phenolic compounds identification was done by ultra-performance liquid chromatography coupled to a diode array detector and an electrospray ionization mass spectrometry detector (UPLC-DAD-ESI/MS), a Dionex Ultimate 3000 (Dionex Ultimate 3000 UPLC and Linear Ion Trap LQT XL, Thermo Scientific, San Jose, CA, USA). A Waters Spherisorb S3 ODS-2C18, 3 µm (4.6 mm × 150 mm) column thermostatted at 25 °C was used. The solvents used were: (A) 2.5% acetic acid in water, (B) HPLC-grade acetonitrile. The elution gradient established was 0% B for 5 min, from 0 to 10% B for 35 min, from 10 to 14.5% B for 5 min, from 14.5 to 19% B for 10 min, from 19 to 55% B for 10 min, isocratic 80% B for 3 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280, 330, and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer. Nitrogen served as the curtain (20 psi) and collision gas (medium). The ion spray voltage was set at – 4500 V in the negative mode. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in the sample. Settings used were: declustering potential (DP) – 40 V, entrance potential (EP) – 7 V, and collision energy (CE) – 20 V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the

previous scan using the following parameters: DP – 40 V, EP – 10 V, CE – 25 V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between m/z 100 and 1800 [14]. The phenolic compounds were identified by comparing their retention time, UV–vis, and mass spectra with those obtained from standard compounds, when available. Otherwise, compounds were tentatively identified by comparing the obtained information with available data reported in the literature. To quantify the detected molecules, calibration curves were prepared for each compound using authentic standards (Extrasynthèse S.A., Genay, France). The referred standards, as also available literature data, were used to identify the phenolic compounds. The acquisition and data processing were carried out with the Xcalibur® data system (Thermo Scientific, San Jose, CA, USA). The obtained results were presented in mg/g extract.

Antibacterial Activity

To determine the antibacterial capacity of the obtained extracts, different bacterial strains isolated from hospitalized patients (Hospital Center of Trás-os-Montes e Alto Douro (Vila Real, Portugal)) were tested, namely five Gram-negative bacteria: *Escherichia coli* (isolated from urine), *Proteus mirabilis* (isolated from wound exudate), *Klebsiella pneumoniae* (isolated from urine), *Pseudomonas aeruginosa* (isolated from expectoration) and *Morganella morganii* (isolated from urine); and three Gram-positive bacteria: *Enterococcus faecalis* (isolated from urine), *Listeria monocytogenes* (isolated from cerebrospinal fluid), and methicillin-resistant *Staphylococcus aureus* (MRSA) (isolated from expectoration). The assay was performed through the microdilution method using *p*-iodonitrotetrazolium chloride (INT) (Panreac Applichem-Barcelona, Spain) as a viability marker, as described by Pires et al. [15]. Different controls were prepared as follows: negative controls (one with Mueller–Hinton Broth (MHB)/Tryptic Soy Broth (TSB), another one with the extract, and the third with medium and antibiotic). As positive controls, for the Gram-negative bacteria, antibiotics, such as amikacin, tobramycin, amoxicillin/clavulanic acid, and gentamicin were used; for the Gram-positive bacteria, ampicillin and vancomycin were selected. Also, controls to ensure the bacterial viability were also prepared with medium and each of the bacterial strains. The extracts were analyzed in the range 0.015–20 mg/mL, being the minimal inhibitory/bactericidal concentrations (MIC/MBC) determined and expressed in mg/mL.

Antioxidant Activity

The antioxidant capacity was determined by oxidative haemolysis inhibition (OxHLIA) and thiobarbituric acid reactive substances (TBARS) formation inhibition assays. The

OxHLIA was performed according to Lockowandt et al. [16] in which the extracts (30–1000 µg/mL in PBS) were analyzed for their capacity to protect sheep erythrocytes from oxidation induced by free radicals generated by the thermal decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). Trolox (7.81–250 µg/mL) was used as a positive control. The results were expressed as IC₅₀ values (µg/mL) at Δt of 60 and 120 min, which translates to the extract concentration required to keep 50% of the erythrocyte population intact for 60 and 120 min.

Regarding the TBARS assay, the procedure was carried out according to Gómez-Mejía et al. [17] in which the capacity of the extracts to inhibit the lipidic oxidation is evaluated through the inhibition of TBARS formation, which is monitored as malondialdehyde (MDA)-thiobarbituric acid (TBA) complexes at 532 nm (Specord 200 spectrophotometer, Analytik Jena, Jena, Germany). The results were given as IC₅₀ values, meaning the extract concentration (mg/mL) providing 50% of antioxidant activity. Trolox was used as a positive control.

Cytotoxicity

In order to determine the cytotoxicity of the developed extracts in tumor and also in non-tumor cell lines, they were tested against four different human tumour cell lines (MCF-7: breast adenocarcinoma; NCI-H460: non-small-cell lung cancer; AGS gastric adenocarcinoma and CaCo-2 colorectal adenocarcinoma). The cytotoxicity for non tumor cells was evaluated in porcine liver cells (assigned as PLP₂), and kidney cells of the African green monkey (assigned as VERO). These analyses were carried out through the sulforhodamine B method [18]. The extracts were tested in a concentration range of 400 to 1.56 µg/mL. The results were expressed in GI₅₀ values (concentration that inhibits 50% of cell growth), in µg/mL. The positive control used was ellipticine.

Anti-inflammatory Activity

For this assay, mice macrophage cell lines (RAW264.7), were used, according to the procedure reported by Taofiq et al. [19]. The extracts were tested at different concentrations in the range of 400 to 1.56 µg/mL. The activity was based on the extract's capacity to inhibit the production of NO, therefore obtaining an active concentration with anti-inflammatory capacity. NO production was determined by measuring the absorbance at 540 nm in a microplate reader (Bio-Tek Instruments, ELX800) against a calibration curve and using dexamethasone as the positive control.

Anti-tyrosinase Activity

The obtained extracts were evaluated for their capacity to inhibit the tyrosinase enzyme. The assay was carried out as described by Moonrungssee et al. [20]. This approach is based on the mushroom tyrosinase oxidation of L-DOPA to *o*-dopaquinone. The extracts were dissolved in EtOH (50%) in a concentration range from 250 to 62.5 µg/mL. The percentage of tyrosinase inhibition was calculated as follows: $[(A_{\text{Blank}} - A_{\text{Sample}})/A_{\text{Blank}}] \times 100$. The EC₅₀ values, which represent the extract concentration responsible for 50% tyrosinase inhibition, were calculated based on a calibration

curve (tyrosinase inhibition percentage versus extract concentration). Kojic acid was used as the positive control.

Statistical Tools

All the assays were performed in triplicate, and the results were expressed as mean ± standard deviation (except for antimicrobial activity). Data were analyzed using an analysis of variance (ANOVA) followed by a Tukey's test for homoscedastic samples and a Tamhane T2 for non-homoscedastic samples. A significance of 0.05 was applied for all analyses throughout the whole manuscript.

Table 1 Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification (mg/g of extract) of phenolic compounds in blueberry aerial parts

Peak	Rt (min)	λ_{max} (nm)	[M-H] <i>m/z</i>	MS ² (<i>m/z</i>)	Identification	Decoction	Infusion	Maceration	UAE
1	4.60	324	353	191(100), 179(55), 135(10)	3- <i>O</i> -Caffeoylquinic acid ¹	5.38 ± 0.05 ^b	7.00 ± 1.00 ^c	8.00 ± 0.30 ^d	3.90 ± 0.20 ^a
2	5.09	322	707	467(23), 353(100), 191(15)	Caffeoylquinic acid dimer ¹	4.02 ± 0.05 ^c	3.48 ± 0.04 ^b	nd	2.60 ± 0.20 ^a
3	6.01	310	341	179(85), 149(54), 135(100)	Caffeic acid hexoside ²	1.76 ± 0.02 ^d	1.47 ± 0.00 ^c	1.26 ± 0.02 ^b	1.10 ± 0.10 ^a
4	6.67	322	707	467(23), 353(100), 191(15)	Caffeoylquinic acid dimer ¹	40.70 ± 0.40 ^a	43.30 ± 0.30 ^b	46.00 ± 0.40 ^c	46.00 ± 0.40 ^c
5	7.77	323	353	191(100), 179(8), 161(2), 135(3)	5- <i>O</i> -Caffeoylquinic acid ¹	3.70 ± 0.10 ^a	4.14 ± 0.04 ^b	nd	nd
6	8.95	283	863	711(28), 573(13), 451(15), 411(18), 289(6)	Procyanidin trimer ³	5.80 ± 0.10 ^b	6.20 ± 0.10 ^c	4.10 ± 0.10 ^a	8.80 ± 0.10 ^d
7	9.91	282	863	711(25), 573(18), 451(13), 411(31), 289(10), 285(8)	Procyanidin trimer ³	7.70 ± 0.10 ^d	6.20 ± 0.10 ^b	4.50 ± 0.10 ^a	7.30 ± 0.10 ^c
8	12.23	282	1153	865(30), 577(17), 575(12), 561(7), 289(13)	Procyanidin tetramer ³	0.73 ± 0.00 ^b	0.92 ± 0.01 ^c	0.64 ± 0.00 ^a	nd
9	15.51	281	1153	865(37), 577(15), 575(11), 561(5), 289(10)	Procyanidin tetramer ³	5.93 ± 0.03 ^b	7.57 ± 0.02 ^c	5.20 ± 0.10 ^a	nd
10	16.71	345	609	301(100)	Quercetin-3- <i>O</i> -rutinoside ⁴	2.64 ± 0.02 ^b	2.43 ± 0.02 ^a	2.73 ± 0.02 ^d	2.47 ± 0.01 ^c
11	17.79	327	463	301(100)	Quercetin-3- <i>O</i> -glucoside ⁴	2.67 ± 0.01 ^b	2.10 ± 0.02 ^a	2.96 ± 0.02 ^d	2.90 ± 0.02 ^c
12	19.35	319	515	353(100), 191(11), 179(8)	3,5- <i>O</i> -Dicaffeoylquinic acid ¹	0.72 ± 0.04 ^d	0.58 ± 0.00 ^b	0.67 ± 0.00 ^c	0.33 ± 0.00 ^a
13	19.79	340	593	285(100)	Luteolin di-6,8- <i>C</i> -hexoside ⁵	4.87 ± 0.05 ^b	4.40 ± 0.10 ^a	4.90 ± 0.10 ^b	5.00 ± 0.20 ^b
14	21.03	334	447	285(100)	Luteolin 6- <i>C</i> -glucoside ⁵	3.50 ± 0.10 ^b	3.07 ± 0.05 ^a	4.40 ± 0.10 ^d	4.00 ± 0.20 ^c
					TPA	56.30 ± 0.10 ^b	60.00 ± 1.00 ^c	56.00 ± 0.10 ^b	54.00 ± 1.00 ^a
					TP	20.10 ± 0.10 ^c	20.90 ± 0.20 ^d	14.40 ± 0.10 ^a	16.10 ± 0.20 ^b
					TOF	13.70 ± 0.20 ^b	12.00 ± 0.20 ^a	15.10 ± 0.20 ^d	14.40 ± 0.40 ^c
					TPC	90.10 ± 0.20 ^c	93.00 ± 1.00 ^c	85.50 ± 0.40 ^a	85.00 ± 1.00 ^a

Different letters in the same row show significant difference between means of the same compounds in different extraction methods. Different letters in each row mean statistically significant differences with a significance of 0.05

Calibration curves used: 1: chlorogenic acid ($y = 168823x - 161,172$; $R^2 = 0.9999$; LOD = 0.20 µg/mL; LOQ = 0.68 µg/mL), 2: caffeic acid ($y = 388345x + 406,369$; $R^2 = 0.994$; LOD = 0.78 µg/mL; LOQ = 1.97 µg/mL), 3: catechin ($y = 84950x - 23,200$; $R^2 = 0.9999$; LOD = 0.17 µg/mL; LOQ = 0.68 µg/mL), 4: quercetin-3-*O*-glucoside ($y = 34843x - 160,173$; $R^2 = 0.9998$; LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL), 5: Apigenin-7-*O*-glucoside ($y = 10683x - 45,794$; $R^2 = 0.999$; LOD = 0.10 µg/mL; LOQ = 0.53 µg/mL)

TPA total phenolic acids, TP total procyanidin, TOF total other flavonoids, TPC total phenolic compounds, nd not detected

Results and Discussion

Profile in Phenolic Compounds

The phenolic composition of the aerial parts extracts of *V. myrtillus* is presented in Table 1. The individual phenolic compounds were tentatively identified according to their retention time (Rt), maximum absorbance wavelength (λ_{\max}), pseudomolecular ion ($[M-H]^-$), and respective fragmentation pattern (MS²). It was possible to identify fourteen phenolic compounds: six phenolic acids (caffeic acid derivatives), four flavonoids (flavones and flavonols), and four procyanidins.

Peaks 1 and 5 ($[M-H]^-$ at m/z 353), 2 and 4 ($[M-H]^-$ at m/z 707), and 12 ($[M-H]^-$ at m/z 515) were assigned as caffeoylquinic acids, caffeoylquinic acids dimers, and dicaffeoylquinic acid, respectively. These identifications were performed considering the hierarchical keys previously reported by Clifford et al. [21]. Caffeoylquinic acid derivatives were also identified in *V. myrtillus* leaves by Bujor et al. [22]. Peak 3 ($[M-H]^-$ at m/z 341) indicated that it corresponds to a caffeic acid derivative bearing one hexosyl residues (-162 u), being tentatively assigned to a caffeic acid hexoside. Both compounds, caffeic acid, and caffeoylquinic acid derivatives were also previously identified in blackberry and raspberry leaves [23].

Peaks 6–9 revealed a $[M-H]^-$ at m/z 863 and 1153, with a λ_{\max} around 276–280 nm, which is characteristic of proanthocyanidins, being assigned as procyanidins trimers and tetramers, respectively. According to Bujor et al. [22], epicatechin or catechin-based oligomeric flavanols involve various A-type or B-type dimeric, trimeric, and tetrameric forms that are normally present in blueberries leaves and stems. Nevertheless, these compounds were identified as procyanidins trimers and tetramers.

Among the four remaining flavonoids, peaks 10–14, they were identified as quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, luteolin di-6,8-*C*-hexoside, and luteolin-6-*C*-glucoside, respectively, after testing the coelution with their corresponding standards. Their MS data also corroborate the positive identification. These compounds have been described in blueberry leaves by Hokkanen et al. [24].

Considering the 14 phenolic compounds detected in the different extracts, their quantities varied considerably with the applied technique. Still, the most abundant phenolic compound, a dimer of caffeoylquinic acid, was present at higher statistically significant amounts in the extracts produced by maceration and UAE. This is a quite interesting finding, due to the fact that the two techniques are very different in terms of extraction time and applied energy. Maceration implies long times to be completed but

does not demand high energy input to the extractive system, apart from a gentle stirring, while the UAE requires short extraction periods, but demands significant energy input, namely in the form of ultrasonic waves. The extract similarity in this specific phenolic compound could be explained by its high amounts in the sample or also by its location in the sample, in tissues that do not need more energetic techniques to increase its extraction [25], and both long time and high energetic methods promote its extraction. Most phenolics, including caffeoylquinic acids, accumulate in vacuoles or apoplasts, suggesting that in these organs whose tissue architecture is not complete, these compounds are easier extracted. Considering maceration, it was the technique giving rise to the highest statistically significant amounts for five individual phenolic compounds: caffeoylquinic acid dimer; 3-*O*-caffeoylquinic acid; procyanidin tetramer; luteolin di-6,8-*C*-hexoside and procyanidin trimer, while the lowest amounts were found *in exequo* for the infusion, maceration and ultrasound extracts, with four individual phenolic compounds: 3,5-*O*-dicaffeoylquinic acid; procyanidin tetramer; caffeic acid hexoside and quercetin-3-*O*-rutinoside showing the lowest contents. Considering the groups of polyphenols, interestingly, the technique conducting to the highest amounts for total phenolic acids; total flavan-3-ol, and total phenolic compounds, was the infusion. This is explained by the moderate extractive capacity of the infusion (probably due to the solvent heating), which, due to the average extraction capacity for each molecule, concomitantly with the low destruction, adds up to obtain an overall high amount. The UAE might be penalized for the high amount of added energy to the system, destroying some polyphenols. On the other hand, maceration could be penalized by the long extraction time, which also promotes the destruction of some polyphenols. Decoction showed no statistical difference from infusion for the total phenolic compounds, while the highest amount of total “other” flavonoids was sought for maceration. To achieve a higher number of polyphenols, the recommended technique is infusion, while maceration is recommended to extract some of the most abundant phenols present in the sample.

Bioactive Potential

Antibacterial Activity

The antibacterial properties were studied through the microdilution method against pathogenic bacteria and the results are displayed in Table 2. All the extracts presented inhibition capacity (MIC) against most of the tested bacterial strains, but without the capacity to effectively kill these bacteria. In general, a MIC of 10 to 20 mg/mL for all the tested extracts was needed to inhibit the analyzed strains. Nevertheless, a

Table 2 Antibacterial potential of blueberry aerial parts extracts expressed in mg/mL

	UAE		Maceration		Decoction		Infusion		Ampicillin 20 mg/mL		Imipenem 1 mg/mL		Vancomycin 1 mg/mL	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria														
<i>Escherichia coli</i>	10	>20	10	>20	10	>20	10	>20	<0.15	<0.15	<0.0078	<0.0078	n.t	n.t
<i>Klebsiella pneumoniae</i>	10	>20	10	>20	10	>20	10	>20	10	20	<0.0078	<0.0078	n.t	n.t
<i>Morganella morganii</i>	10	>20	10	>20	10	>20	10	>20	20	>20	<0.0078	<0.0078	n.t	n.t
<i>Proteus mirabilis</i>	20	>20	>20	>20	20	>20	>20	>20	<0.15	<0.15	<0.0078	<0.0078	n.t	n.t
<i>Pseudomonas aeruginosa</i>	20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.5	1	n.t	n.t
Gram-positive bacteria														
<i>Enterococcus faecalis</i>	10	>20	10	>20	10	>20	10	>20	<0.15	<0.15	n.t	n.t	<0.0078	<0.0078
<i>Listeria monocytogenes</i>	10	>20	20	>20	20	>20	20	>20	<0.15	<0.15	<0.0078	<0.0078	n.t	n.t
MRSA	1.25	>20	1.25	>20	5	>20	5	>20	<0.15	<0.15	n.t	n.t	0.25	0.5

n.t. not tested, MIC minimum inhibitory concentration in mg/mL, MBC minimum bactericidal concentration in mg/mL, MRSA methicillin resistant *S. aureus*

very promising result was achieved against MRSA, a serious pathogenic bacterial strain resistant to ampicillin, for which a MIC of 1.25 mg/mL was obtained for the extracts produced by UAE and maceration, and a MIC of 5 mg/mL was achieved for the extracts of decoction and infusion methods. Therefore, maceration and UAE extracts were the most active against these microorganisms, exhibiting the lower MIC values. Compared to the positive controls, namely with ampicillin, all the extracts presented similar activity, especially against Gram-negative bacteria. For example, to inhibit *K. pneumoniae* a MIC of 10 mg/mL of ampicillin was needed, with the same MIC value presented by all the analyzed extracts. Also, a MIC of 20 mg/mL of ampicillin was obtained to inhibit *M. morganii*, while a MIC of 10 mg/mL for all the extracts was obtained, highlighting the potential of the blueberry leaf extracts in this action, given the exponential growth of bacterial resistance to common antibiotics.

The microbiological quality of cosmetic products is one of the most important parameters to guarantee their safe use, and, to control the microbial growth, artificial preservatives are usually added. Since the obtained extracts can act similarly to the antibiotics, which are overused giving rise to bacteria resistance, they offer the potential to act as substitutes. Moreover, antibiotics use is very restrictive because in certain amounts they can become toxic to human health, highlighting also the benefits of developed extracts. Therefore, new challenges arise for researchers, namely the development of new products that have anti-microbial properties and at the same time, present no toxicity to the consumer [26].

Methanolic extracts of *Vaccinium myrtillus* L. berries were analyzed regarding their antimicrobial effect, with the authors describing an activity weaker than the one obtained in the present work, by presenting higher MIC

and MBC values for the Gram-positive bacteria. Concerning Gram-negative strains, a MIC of 126 mg/mL was needed against *K. pneumoniae*, and to inhibit *E. coli*, *P. mirabilis*, and *P. aeruginosa* a MIC of 31.5 mg/mL was obtained. *P. mirabilis* showed the lower MBC value of 63 mg/mL, while for *E. coli*, *K. pneumoniae*, and *P. aeruginosa* an MBC of 126 mg/mL was needed. Regarding the Gram-positive studied strains, both *E. faecalis* and MRSA presented a MIC of 63 mg/mL [27].

In other studies, [28], the berries' aqueous extracts presented MICs for Gram-negative strains, of 25 mg/mL (expressed as 2.5% (w/v)) for *E. coli* and 50 mg/mL (expressed as 5% (w/v)) for *P. aeruginosa*. These authors reported that *L. monocytogenes* was inhibited with a concentration of 12.5 mg/mL (expressed as 1.25% (w/v)) while in this work, UAE extract presented a result of 10 mg/mL against the same bacteria. Regarding MBC results, all the studied extracts presented a bacteriostatic effect. The authors Khalifa et al. [28] obtained better results against *E. coli* and *L. monocytogenes*, with 25 mg/mL (presented as 2.5% (w/v)) for both bacterial strains.

Maceration acetone–water blueberry leaf extract's anti-microbial activity were studied by the microdilution method. The authors observed a significant direct biocidal activity of the extracts from *V. myrtillus* leaves on the Gram-positive bacteria strain *S. aureus* [29]. studied. In another work, blueberry leaves were extracted by UAE with ethanol–water as a solvent and their antimicrobial activity was studied [30]. The microdilution method was applied to *S. aureus*, *E. faecalis*, *P. aeruginosa*, *Klebsiella pneumoniae*, and *E. coli*. Extracts presented high antimicrobial activity and were mostly strain-dependent.

Table 3 Antioxidant activity of blueberry aerial parts (IC₅₀ values, µg/mL)

	OxHLIA		TBARS
	Δt = 60 min	Δt = 120 min	
Decoction	20 ± 1 ^a	54 ± 2 ^c	8.0 ± 0.1 ^b
Maceration	22.0 ± 0.5 ^a	59 ± 3 ^c	8.0 ± 0.2 ^b
Infusion	25.4 ± 0.4 ^b	41 ± 2 ^a	10.0 ± 0.3 ^c
UAE	28 ± 1 ^b	65 ± 2 ^d	9.0 ± 0.3 ^b
Trolox	21.8 ± 0.2 ^a	43.5 ± 0.3 ^b	5.8 ± 0.6 ^a

Different letters in each column mean statistically significant differences between the extraction methods with a significance of 0.05

Antioxidant Activity

The antioxidant potential of the different blueberry aerial parts extracts was evaluated according to the OxHLIA and TBARS methods (Table 3). Regarding the OxHLIA assay, at 60 min, the decoction extract presented the highest capacity, with a lower IC₅₀ value (20 µg/mL). Relatively to TBARS results, the most efficient extracts were the decoction and maceration ones, with the same IC₅₀ result of 8 µg/mL. All the extracts presented low IC₅₀ values, similar to the results obtained for Trolox, the tested positive control. Previously to this study, Schiavon et al. [11] reported that blueberry extract has an antioxidant capacity of 16.71 µg/mL, equivalent to vitamin C, through the DPPH assay. Overall, at 60 min, decoction and maceration extracts showed the best IC₅₀ values, thus, the highest antioxidant activity, while the infusion and UAE ones showed statistically lower values. At 120 min, the infusion extract showed the best antioxidant capacity, followed by the decoction and maceration *in exequo*, and finally by the UAE extract, which presented the lower antioxidant activity. This antioxidant behavior over time could be explained by the protective action of the antioxidant compounds present in the extracts, including the short-term and long-term reaction kinetics and the rate at which antioxidants react with the radicals. So, while some antioxidants may react quickly and be depleted in the reaction system, others may offer prolonged antioxidant protection over time (such as what happened for the infusion). Therefore, this bioassay allowed a distinction between short-term and long-term antioxidant protection, hence the results were presented for two Δt. Once again, the high energy input of the UAE [31] seemed to not promote the extraction of the most bioactive molecules, or even decompose these compounds during the extraction process.

As observed in Table 3, the OxHLIA results were given at two Δt values since plant extracts, due to their complexity as mixtures of various antioxidant compounds, are capable of interacting with each other and offering protection during different periods of time. Interestingly, the infusion extract,

although not showing the best result for 60 min, was the most efficient in protecting the erythrocyte membranes for longer periods of exposure to the free radicals generated in the system. For the TBARS assay, which comparatively with the OxHLIA test relies on different antioxidant mechanisms, only the infusion extract was statistically different from the extracts obtained by the other extraction techniques, showing a slightly lower ability to prevent the formation of TBARS *in vitro*.

DPPH Free-radical-scavenging activity was a method previously used by several authors to determine the antioxidant activity of blueberry leaf extracts. Stefănescu et al. [30], studied six cultivars UAE ethanol–water extracts, and described these extracts as good sources of antioxidants. Other authors using microwave citric acid aqueous solution leaf extracts observed that the antioxidant activity showed interdependency to TPC results [22]. In another report, that studied the maceration ethanol–water and acetone–water extractions, and evaluated the antioxidant activity, the authors concluded that crude extracts of blueberry leaves exhibited strong antioxidant properties regarding DPPH and TBARS methods [32]. Also, using UA acidified methanol extraction, other scientific study analysed seventy-three different leaf cultivars that presented strong *in vitro* antioxidant capacities by DPPH, ABTS, FRAP and ORAC methods [8].

Cytotoxic and Anti-inflammatory Properties

Considering the anti-inflammatory assay, the UAE showed the lowest IC₅₀ values, thus, the higher activity, followed by the infusion extract. The least effective technique to extract anti-inflammatory compounds was maceration extraction. All the extracts presented cytotoxic activity against the tumor cell lines, being the NCI-H460 cells, the most sensible ones to the tested samples, highlighting the activity of UAE with IC₅₀ values of 118 ± 5 µg/mL.

In a scientific study that investigated the cytotoxicity of acetone–water blueberry leaf extract obtained by maceration, using an *in vitro* method with mouse fibroblasts line to estimate the IC₅₀, the authors reported that plant-derived extracts exhibited lower toxicity than phytochemicals used as controls [29] (Table 4).

Appositively to the antioxidant assays, the UAE seemed to be the best technique to obtain compounds with antitumor and anti-inflammatory activity, exhibiting the best results in all assays, while decoction was the least effective technique. No cytotoxicity was sought for both the VERO and PLP2 cell lines, assuring the safety of the obtained extracts.

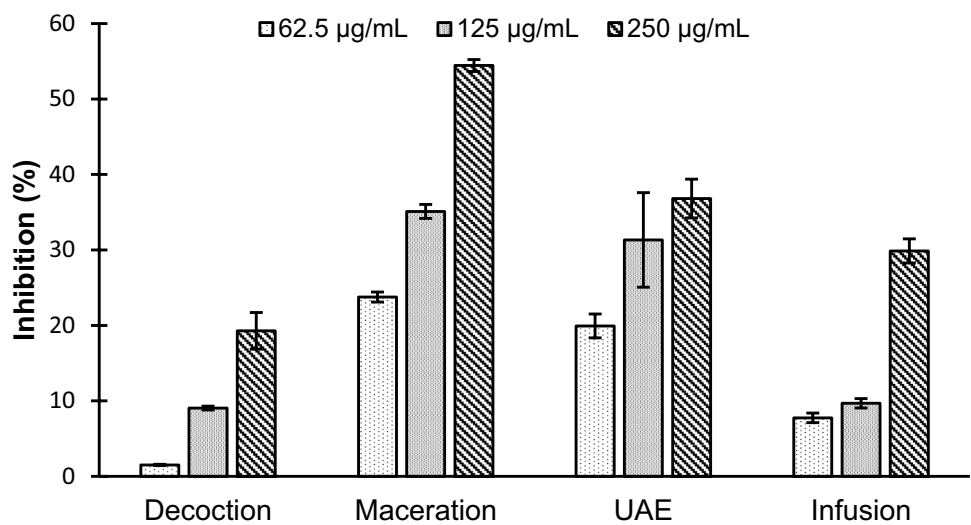
Anti-tyrosinase Activity

Tyrosinase is the rate-limiting enzyme in the pathway leading to melanin biosynthesis, and effective inhibitors of this

Table 4 Anti-inflammatory activity (IC_{50} , $\mu\text{g/mL}$) and cytotoxicity (GI_{50} , $\mu\text{g/mL}$) of blueberry aerial parts

	Decoction	Infusion	Maceration	UAE	Positive control
Anti-inflammatory activity					Dexamethasone
RAW 264.7	90 ± 2^c	70 ± 4^b	104 ± 8^d	41.4 ± 3.2^a	6.3 ± 0.4
Cytotoxicity in tumor cells					Ellipticine
MCF-7 (breast carcinoma)	257 ± 6^c	234 ± 13^b	274.4 ± 9.5^d	201 ± 1^a	1.02 ± 0.02
NCI-H460 (non-small cell lung carcinoma)	210 ± 16^c	164 ± 15^b	178 ± 7^b	118 ± 5^a	1.01 ± 0.0
AGS (gastric adenocarcinoma)	238 ± 9^c	183 ± 5^b	190 ± 12^b	158 ± 11^a	1.23 ± 0.03
CaCo-2 (colorectal adenocarcinoma)	230 ± 13^b	239 ± 6^b	305 ± 9^c	189 ± 13^a	1.21 ± 0.021
Cytotoxicity in non-tumor cells					
VERO	> 400	> 400	> 400	> 400	1.41 ± 0.06
PLP2	> 400	> 400	> 400	> 400	1.4 ± 0.1

Different letters in each row mean statistically significant differences with a significance of 0.05

Fig. 1 Anti-tyrosinase activity of blueberry aerial parts

enzyme are used in cosmeceutical formulations to suppress hyperpigmentation [33]. In the present work, the tyrosinase inhibitory effects of the different extracts from *V. myrtillus* are displayed in Fig. 1. The maceration extract presented the strongest tyrosinase inhibitory effect (more than 50% inhibition) with an IC_{50} value of $221 \pm 3 \mu\text{g/mL}$. It also appeared that the decoction, UAE, and infusion extracts presented tyrosinase inhibition up to 19%, 37%, and 30%, respectively, at the highest tested concentration (250 $\mu\text{g/mL}$) but could not be considered active enough since a 50% inhibition against the tyrosinase enzyme was not achieved.

Kojic acid is a well-known tyrosinase inhibitor used in many topical formulations. Even though there are many challenges regarding its long-term clinical effectiveness, it is still commonly used as a reference compound in the tyrosinase inhibition assay [34]. In the present work, kojic acid presented a tyrosinase inhibition effect characterized

by a very low IC_{50} value ($5.84 \pm 0.1 \mu\text{g/mL}$). Nevertheless, these results showed that the type of used extraction method, besides significantly influencing the bioactive composition of the extracts, also impacted the bioactive properties. There is enormous evidence reporting a positive correlation between tyrosinase inhibition and the total phenolic content. There is a longstanding interest in naturally occurring caffeoylquinic acid derivatives due to their widely reported biological properties. In vitro studies have shown that caffeoylquinic acid derivatives, including 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, and 5-*O*-caffeoylquinic acid at lower concentrations, effectively inhibited tyrosinase activity and melanin biosynthesis [35]. Considering the tested extracts, which were abundant in phenolic acids and related compounds, the observed anti-tyrosinase effect might be attributed to the contribution of this class of compounds (Fig. 2).

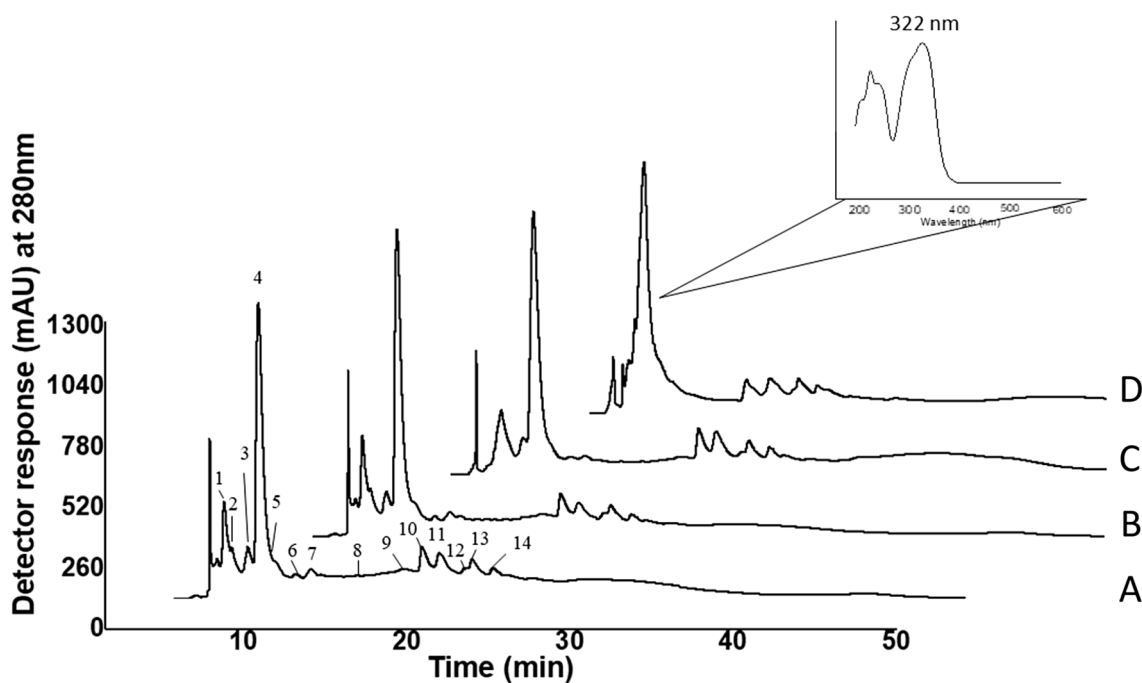


Fig. 2 Phenolic profile of *V. myrtillus* recorded at 280 nm. (A) Decoction; (B) Infusion; (C) Maceration; (D) UAE. For peak, numbers refer to Table 1

Conclusions

The huge amounts of biowastes generated by the agri-food industries, which are constantly discarded in land fields, are considered a serious environmental threat. In line with well-established priorities by several governmental entities, this work intended to transform discarded biowastes from the production of blueberries, transforming them into valuable raw materials to be explored as sources of bio-based bioactive molecules, which are in constant demand by the industry. According to the obtained results crossing the different types of extractions, all the applied technologies allowed to obtain the target molecules, namely phenolic compounds, which revealed bioactive potential according to the performed assays. It should be highlighted that, e.g., the infusion technique can be used to obtain high levels of phenolics, but maceration allowed the highest yields in some of the major compounds. Regarding the bioactivity, all the extracts were able to exert biological function, highlighting the UAE and maceration extracts that revealed a strong capacity to inhibit MRSA, a multiresistant bacterial strain. Also notable is the performance of all the extracts that presented an antibacterial activity similar to the common antibiotic ampicillin. The extracts also presented antioxidant properties and anti-tyrosinase capacity, positively correlated with the presence of caffeoylquinic acid derivatives. This work resulted in important scientific evidence about the potential of blueberry aerial parts as raw materials for bioactive molecules

obtainment, which can provide the industry sectors with natural agents, compliant with the constant demand of, e.g., natural preservatives, by the cosmetic and food industries.

Acknowledgements Foundation for Science and Technology (FCT, Portugal) for financial support through national funds FCT/MCTES to CIMO (UIDB/00690/2020); the contract of L. Barros through the institutional scientific employment program-contract, the contracts of M. Caroch and S.A. Heleno (CEEC-IND/00831/2018 and CEEC-IND/03040/2017) through the individual scientific employment program-contract, and the PhD studentship granted to M. Añibarro-Ortega (2020.06297.BD). To the “La Caixa” Foundation and to FCT for the financial support through Project “Aquae Vitae — Thermal water as a source of life and health”, Promove Mobilizer programme. This work is also supported by MICINN for the Juan de la Cierva Formación contract for T. Oludemi (FJC2019-042549-I).

Funding Open access funding provided by FCTIFCCN (b-on).

Data Availability Enquiries about data availability should be directed to the authors.

Declarations

Conflict of interest The authors declare they have no conflict of interest.

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