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Optimizing ultrasound-assisted extraction for enhanced yield of bioactive compounds from *Vaccinum myrtillus* and *Rubus idaeus* bioresidues

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ABSTRACT

Due to the high nutritional value of berries, their extensive production has led to the accumulation of bio-residues. To leverage these residues, the distinct chemical composition, and functional properties of the leaves and other aerial parts can be used to produce high-value ingredients for cosmetics. Ultrasound-assisted extraction (UAE) is considered simple, fast, efficient, and more affordable than other techniques for obtaining bioactive extracts. This study aimed to determine the optimal conditions for maximizing ultrasound-assisted extraction yields from raspberry (RB) and blueberry (BB) pruning using response surface methodology (RSM). Three factors were analyzed: the ethanol-to-water solvent ratio (ranging from 0 % to 100 % ethanol), extraction time (ranging from 5 to 30 min), and extraction power (ranging from 20 % to 100 %, with a maximum ultrasound power of 500 W). The Box-Behnken design included 17 individual randomized runs to optimize the extract's dry weight range from 22.2 to 202.8 mg/g for RB and 123.5–394.0 mg/g for BB. The optimal extraction point for RB was at 40 % ethanol-to-water ratio, 16 min, and 98 % extraction power, while for BB, it was at 59 % ethanol in water, 30 min, and 73 % extraction power. Higher levels of hydrolyzable tannins characterized the optimal point of RB, while BB showed greater contents of flavonoids. Both revealed strong antioxidant and antibacterial activities, with RB showing higher antioxidant activity than Trolox. These results suggest that the extracts from pruning are obtained at interesting compositions and yields, offering advantages to use in the cosmetic industry due to their bioactivity.

1. Introduction

Vaccinium myrtillus (blueberry) and *Rubus idaeus (raspberry)* naturally produce phenolic compounds through metabolic processes that begin with sugars derived from photosynthesis. These sugars are converted into the amino acid phenylalanine via the shikimate pathway. Phenylalanine then enters the phenylpropanoid pathway to form various phenolic compounds. Subsequently, the flavonoid pathway produces key substances such as anthocyanins (responsible for the berries' coloration), flavonols, and tannins. These compounds serve protective roles in the plant and influence the fruit's color, taste, and antioxidant capacity. Their synthesis is affected by factors such as light exposure, ripening, and environmental stress (de la Rosa et al., 2018).

Blueberry and raspberry bio-waste results from processing or harvesting and are being addressed by the fruit processing industry for environmental concerns (Ispiryan & Viškelis, 2019). Producers are now interested in obtaining high-value products from the waste biomass of berry crops, particularly for applications in cosmetics (Mirabella et al., 2014). While the fruits of blueberries and raspberries have been thoroughly researched, their bio-residues have not been extensively examined. Some findings suggest that the leaves of these plants may be a potential source of phenolic compounds. Ferlemi and Lamari (2016) studied the leaves of wild blueberries and red raspberries and found that they have varying levels of total phenolic compounds, but these levels are significantly high overall. In their research, they identified 23 phenolic compounds in 73 different species of blueberry leaves, including 13 flavonoids, most of which were quercetin derivatives. These findings underscore the potential for utilizing residues like leaves

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from cultivated plants in the sustainable development of bioactive cosmetic ingredients.

Antioxidants are essential for protecting the cells against oxidative stress. Using natural extracts and plant-based products for skin care shows a growing trend, paralleled by increased scientific knowledge about plant constituents, human molecular biology, and cell physiology (Bilirgen et al., 2021; Rojas et al., 2016). In conclusion, the study highlights the potential for using waste biomass from cultivated berry crops, mainly leaves, for high-value products such as antioxidants, phenolic compounds, and fiber for various applications in agri-food, cosmetic, and pharmaceutical products.

Different extraction procedures were tested with these materials, including water extraction, solvent extraction, and steam distillation (Cornuelle & Thaman, 2006). Studies have shown that hexane, ethyl acetate, and methanol efficiently extract polyphenols, flavonoids, and tannins. However, these methods have disadvantages, such as high solvent levels and long extraction times, making them not environmentally friendly. Advanced extraction technologies privilege the use of green solvents and sustainable extraction processes to overcome these issues (Jahromi, 1989).

Eco-friendly techniques such as Natural Deep Eutectic Solvents (NaDES), High Hydrostatic Pressure (HHP), Solid-Liquid Extraction (SLE), Microwave-Assisted Extraction (MAE), and Ultrasound-Assisted Extraction (UAE) have emerged as effective alternatives for extracting bioactive (Cádiz-Gurrea et al., 2020; Jablonský et al., 2018; Fritsch et al., 2017).

Among these, the UAE stands out for its efficiency and sustainability. It uses ultrasonic waves to generate cavitation, disrupting plant cell walls and enhancing solvent penetration, which improves extraction yields. UAE typically uses ethanol-water mixtures as solvents, and its effectiveness depends on optimized parameters such as extraction time, temperature, and solvent-to-solid ratio (González-Silva et al., 2022). This study explores the valorization of *Vaccinium myrtillus* and *Rubus idaeus* aerial parts bio-residues as sources of phenolic compounds. The objective is to optimize UAE parameters for efficient extraction and evaluate the biological activity of the resulting extracts. The novelty of this work lies in its focus on underutilized plant waste, the application of green extraction methods, and a comparative analysis of two species for their potential in cosmetic, food, and pharmaceutical industries.

2. Material and methods

2.1. Samples preparation

V. myrtillus (BB) and *R. idaeus* (RB) aerial parts, including leaves, branches and stems resulting from pruning were collected in September of 2019, after harvesting the fruits,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.

This raw material was ground to a 20 mesh (0.8 mm) particle size in a ZM200 Retsch model mill after air drying at ambient temperature (about 25 °C), within the facilities of the research centre, for 5 days. The dried sample was then stored away from light and moisture for further analysis.

2.2. Ultrasound-assisted extraction (UAE)

UAE was carried out using a Qsonica ultrasonic processor sonicator (model Q500, Newtown, USA) operating at 20 kHz. Briefly, 1.000 g of powdered sample was mixed with 150 mL of the required ethanol-water solution. The extraction was performed using 30-s cycles alternated with 10-s pauses at 20 % of a 500 W power output to prevent the samples from quenching. For higher potencies, 10-s cycles and 40-s pauses were employed. An ice bath was used for all extractions. The mixtures were then gravity filtered before being evaporated at 40 °C, 50 rpm, and 2.0 mbar under reduced pressure using a vacuum controlling system KNF (model N920), a rotary evaporator and water bath system KNF RC 600, and a condensation closed refrigeration system KNF C900 in Balterwil, Switzerland until the ethanol was completely removed. The resulting aqueous residue was lyophilized and stored in a dry chamber. The mass yield of the dried extracts was calculated and expressed as a percentage.

Before each bioactivity evaluation, the extracts underwent UV decontamination, by exposure to UV-C for 15 min in the laminar flow hood.

2.3. Extraction optimization

The response surface methodology (RSM) technique was used to optimize the UAE extraction. For this, three factors inherent to the extraction were varied, namely ethanol-to-water solvent ratio (X_1), which ranged from 0 % to 100 % (ethanol) extraction time (X_2), which ranged from 5 to 30 min; and ultrasound power (X_3), from 20 % to 100 % of 500 W, by applying the Box-Behnken design, 17 extractive runs in which considered the maximum, minimum and centre points of each factor.

2.4. Phenolic compounds

The phenolic compounds in each extract were identified and measured using the method described by Bessada et al. (2016). Briefly, 10 mg of the sample was dissolved in an Eppendorf microtube in 1 mL of an ethanol-water solution (20:80 *v/v*) and then filtered through a 0.22 μm disposable filter. The phenolic profile was determined using a high-performance liquid chromatography system, Dionex Ultimate 3000, which included a diode array detector and an electrospray ionization mass spectrometry detector (HPLC-DAD-ESI/MS) (Dionex Ultimate 3000 UPLC and Linear Ion Trap LQT XL, Thermo Scientific, San Jose, CA, USA). The phenolic compounds in the extracts were identified using reference standards and data from literature. Calibration curves were established for each chemical using authentic standards (Extrasynthése S.A., Genay, France) to quantify the identified molecules. The data was collected and processed using the Xcalibur® data system (Thermo Scientific, San Jose, CA, USA). The results were expressed in mg/g of extract.

2.5. Bioactive properties evaluation

2.5.1. Antibacterial activity

The antibacterial effectiveness of the extracts was assessed using various bacterial strains obtained from hospitalized patients at the Hospital Centre of Trás-os-Montes e Alto Douro in Vila Real, Portugal. The study included five Gram-negative bacteria: *Escherichia coli* (isolated from urine, VRU12881), *Proteus mirabilis* (isolated from wound exudate, VRU17684), *Klebsiella pneumoniae* (isolated from urine, VRI17214), *Pseudomonas aeruginosa* (isolated from expectoration, VRU14123), and *Morganella morganii* (isolated from urine, VRU14272); and three Gram-positive bacteria: *Enterococcus faecalis* (isolated from urine, VRU14041), *Listeria monocytogenes* (isolated from cerebrospinal fluid, VRI17214), and methicillin-resistant *Staphylococcus aureus* (MRSA) (isolated from expectoration, VRI17654). The experiment involved using the microdilution technique and adding de *p*-iodonitrotetrazolium chloride (INT) from Panreac Applichem-Barcelona, Spain, as a marker. Positive controls were used, including ampicillin (ranging from 0.15 to 20 mg/mL), imipenem, and vancomycin (ranging from 0.0078 to 1 mg/mL). A control was created using the medium applied with each bacterial strain to verify the bacterial viability. These findings were previously published by Pires et al. (2018). The extracts were tested at concentrations ranging from 0.015 to 20 mg/mL. The minimum inhibitory/bactericidal concentrations (MIC/MBC) were calculated and expressed in mg/mL.

2.5.2. Antioxidant activity

Two different tests were used to evaluate the antioxidant activity of

the extracts: oxidative hemolysis inhibition (OxHLIA) and thiobarbituric acid reactive substances (TBARS). The OxHLIA assay was carried out following the protocol described by Lockowandt et al. (2019), which evaluates the ability of the extracts to protect erythrocyte membranes from free radical-induced oxidative damage. To prepare the stock solution, the extracts were dissolved in phosphate-buffered saline (PBS) and subsequently diluted to obtain a range of concentrations. Trolox, at concentrations ranging from 7.81 to 250 μg/mL, was used as a positive control. The IC_{50} values (expressed in μg/mL) for time points Δt of 60 and 120 min represent the concentration of extract required to maintain 50 % of the erythrocyte population intact for 60 and 120 min, respectively. The TBARS assay, conducted according to Gómez-Mejía et al. (2021), measured the ability of the extracts to prevent lipid peroxidation by monitoring the inhibition of TBARS formation through the generation of malondialdehyde (MDA)-TBA adducts, which were quantified by absorbance at 532 nm using a Specord 200 spectrophotometer (Analytik Jena, Jena, Germany). Results were expressed as EC_{50} values, representing the extract concentration (mg/mL) that provided 50 % antioxidant activity, with Trolox as a positive control.

2.5.3. *Cytotoxicity*

Assays were conducted using both tumor and non-tumor cell lines. The tumor cell lines tested were MCF-7 (breast adenocarcinoma), NCI-H460 (non-small-cell lung cancer), AGS (gastric adenocarcinoma), CaCo2 (colorectal adenocarcinoma) and HeLa (Henrietta Lacks cervical cancer). The utilized non-tumor cell lines were VERO cells derived from African green monkey kidney. The potential toxicity of the extracts on non-tumor cell lines was determined using the sulphorodamine B technique. The extracts were tested in concentrations ranging from 400 to 1.56 μg/mL, and the results were presented as GI_{50} values, represented in μg/mL. Ellipticine was used as the positive control.

2.5.4. *Anti-inflammatory activity*

In this study, mice macrophage cell lines (RAW264.7) were used following the technique described by Taofiq et al. (2016). Various concentrations of the extracts, ranging from 400 to 1.56 μg/mL, were tested to suppress NO generation and showed anti-inflammatory properties. NO generation was measured using a microplate reader (Bio-Tek Instruments, ELX800), with dexamethasone used as a positive control. The absorbance at 540 nm was used to create a calibration curve.

2.6. *Statistical analysis*

All results are represented as mean ± standard deviation, and all analysis were carried out in triplicate. For the statistical analysis a Student's T Test was used to compare the means of the two extracts. The response surface methodology (RSM) was carried out using Design-Expert (Stat-Ease version 22, Minneapolis, MN, USA). Differences were considered statistically significant at the 5 % level (α = 0.05).

3. Results and discussion

3.1. *Optimized responses and RSM conditions*

The optimizations were performed both for BB and RB using the same factors and optimized for the same response (R), namely, the final mass yield in percentage (%) of the dried extracts. (R) was determined by the mass of extract obtained ($m_{extract}$), dried through lyophilization, in grams, about the initial mass of bio residue used for the extraction (m_{sample}), in grams, after the sample preparation mentioned in section 2.1., shown in equation 1. Table 1 shows the different extraction factors (X) and the results of each extraction, both for BB and RB. X_1 represents extraction time (minutes), which varies between 5 and 30 min, while X_2 represents ultrasound power (%) between 20 and 100 % of the fixed 500 W of the ultrasound probe power. This parameter included the power output of the probe and timed cycles at low power to avoid sample quenching. Finally, X_3 is the ethanol-to-water solvent ratio used in each extraction, ranging from 0 to 100 %.

Table 1
Extraction runs for ultrasound-assisted extraction optimization using response surface methodology, including the three varying factors (X_1, X_2, and X_3) and the two responses (R_1 – blueberry (BB) and R_2 – raspberry (RB)) optimized for solid residue weight.

Run	X_1 – t (min)	X_2 – P (% W)	X_3 – EtOH (%)	R_1 - BB yield (%)	R_2 - RB yield (%)
1	5.0	60	100	17.72	4.11
2	17.5	60	50	35.23	18.14
3	30.0	60	100	36.94	3.29
4	17.5	20	100	12.35	2.22
5	17.5	60	50	36.32	18.00
6	5.0	100	50	39.40	20.16
7	17.5	100	100	30.96	5.28
8	5.0	20	50	30.82	16.71
9	17.5	60	50	35.39	16.49
10	17.5	20	0	21.81	11.67
11	30.0	60	0	28.47	15.05
12	17.5	100	0	28.00	20.18
13	5.0	60	0	24.20	13.25
14	30.0	100	50	36.47	19.39
15	17.5	60	50	31.11	16.72
16	30.0	20	50	34.26	15.90
17	17.5	60	50	33.19	19.28

$$R = \frac{m_{extract}}{m_{sample}} \times 100 \quad (1)$$

In the case of BB, the model showed a significant fit, having rendered a quadratic equation with a non-significant lack of fit. The R^2 was set at 0.9639, and the adjusted R^2 was 0.9098. The coded equation for this model was represented by equation (2).

$$R_1 = 34.25 - 0.5640X_1 + 5.31X_2 + 6.76X_3 + 3.74X_1X_2 + 3.11X_1X_3 - 6.21X_2X_3 - 7.38X_1^2 - 0.0406X_2^2 - 3.59X_3^2 \quad (2)$$

Fig. 1 shows the optimal point where the dry extract of BB is maximal, achieving 39.68 %.

The optimal point for BB was set at 59 % of ethanol, 30 min of extraction time and 73 % of ultrasound power. Fig. 2 shows the contour and 3D plots for each factor, grouping them in pairs (the missing factor in each figure is set to the optimal level). Interpreting equation (2), it is clear that the factors with higher influence in the optimal parameters are the ultrasound power and percentage of ethanol. Still, in Fig. 2-B_1, this can also be seen as the reddest contour (highest values), which ranged between 20 % and 80 % of ethanol while the ultrasound power shows higher extractability between 40 and 100 %. Regarding the 3D charts, Fig. 2-A_1 shows the highest extractability between 40 and 60 % of ethanol and the longest extraction time. Thus, longer extraction times could be considered in future work with these samples, as the inflection point was not found for this factor. In section B_2 of Fig. 2, a rounded surface was defined, meaning the limits of these two factors were correctly defined, showing a maximum extractability between 40 and 60 % ethanol and 40–80 % ultrasound power. Fig. 2-B_3 indicates once again that more extended periods of extraction could be considered, as the red zone (higher extractability of compounds) is found at the higher limit of extraction time, although in terms of ultrasonic power, the optimal zone is defined between 70 and 80 %.

Considering RB, the optimal conditions are shown in Fig. 3. The optimal points with the highest yield in dry extract were set at 40 % ethanol, 16 min of extraction time, and 98 % ultrasound power. These results were obtained through a quadratic equation with a significant model and a non-significant lack of fit. The R^2 was set at 0.9837, and the adjusted R^2 was 0.9628. The equation that defined this optimization is equation (3).

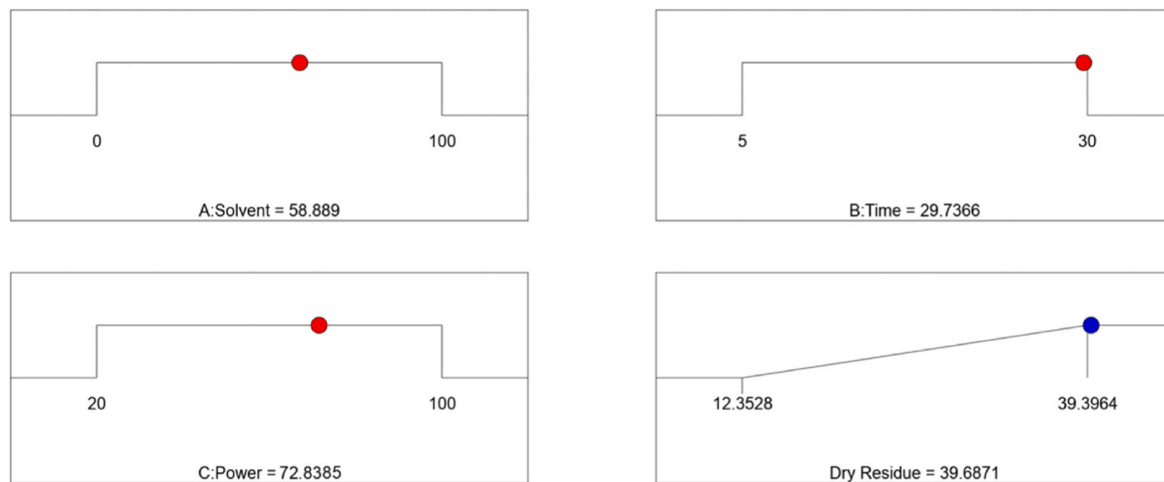

Fig. 1. Optimal extraction points and dry extract yield for blueberry (BB).

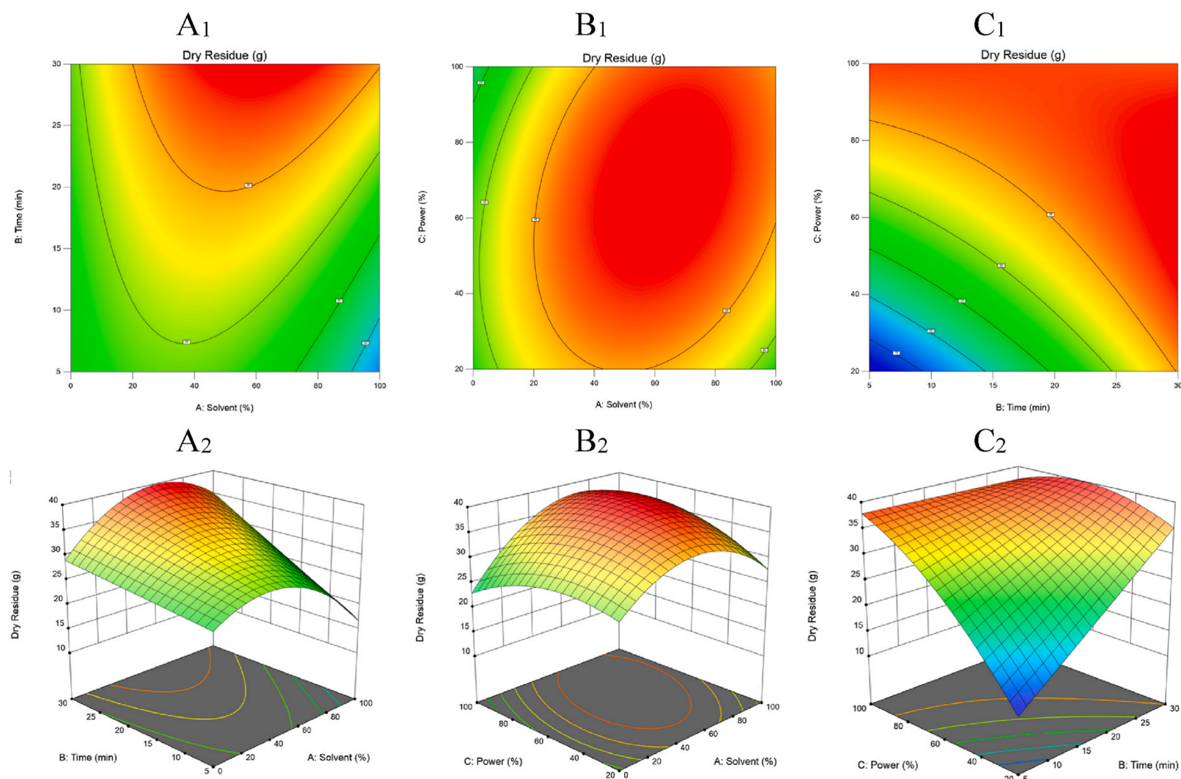

Fig. 2. Contour and 3D surface plots after optimization of the blueberry (BB) extraction.

$$R_2 = 17.73 - 5.66X_1 - 0.0740X_2 + 2.31X_3 - 0.655X_1X_2 - 1.36X_1X_3 + 0.0112X_2X_3 - 8.50X_1^2 - 0.3016X_2^2 - 0.6137X_3^2 \quad (3)$$

In the case of RB, considering the values from eq (3)., the factor with the highest importance was the ultrasound power (value of 2.31). In Fig. 3, this factor was optimal at the highest values, while extraction time was around the middle of the scale, and the ethanol-to-water solvent ratio was at 40 %. Fig. 4 shows the contour and 3D plots of RB optimization. Considering Fig. 4-A_1, it is clear that the time factor did not have a considerable impact as the red zone (higher extractability) extends from the lowest to the highest value. The same was also found in Fig. 4-C_1, where time extends over the horizontal axis with similar output. This corroborates that ultrasound power is the most important of the three towards the outcome, as there are well demarked zones of higher and lower extractability. Considering the 3D plots in Fig. 4-A_2, an inflection point is shown, meaning that the optimal point for ethanol percentage was found between 0 and 20 %. In contrast, the extraction time did not impact the optimization as its values are almost constant for all extraction times.

In Fig. 4-B_2, a similar surface was defined, in which time showed the same behavior while ultrasound power, although being almost constant, increased its extractability towards the highest intensity. This could mean that a higher-power probe could be used to increase extractability. Still, due to the meager impact of ultrasound and extraction time, Fig. 4-C_2 showed an almost flat surface with a slight increase in extractability for high ultrasound power.

The two berries showed different optimization points for maximizing the dry yield. While BB was favored by long extraction times and median

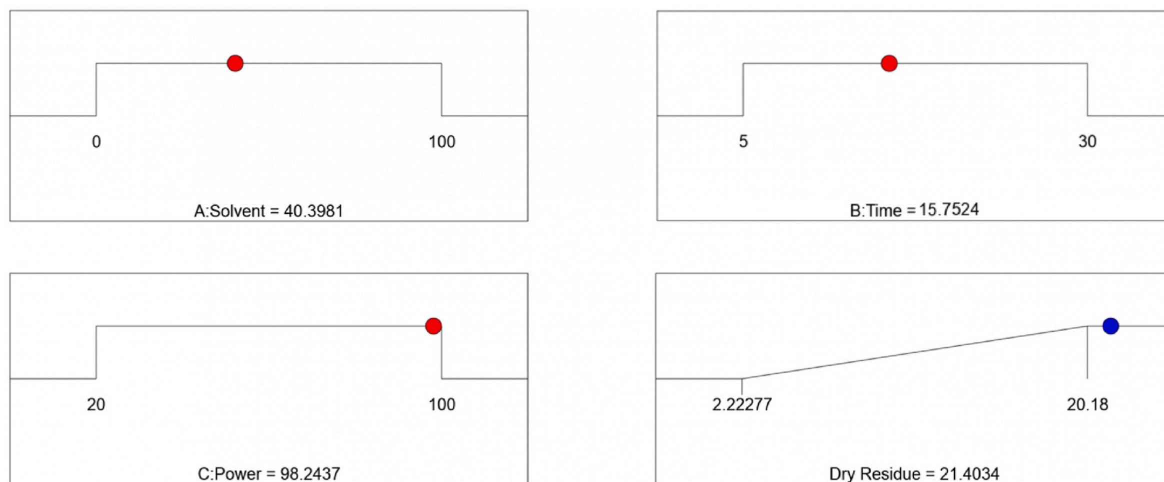

Fig. 3. Optimal extraction points and dry extract yield for raspberry (RB).

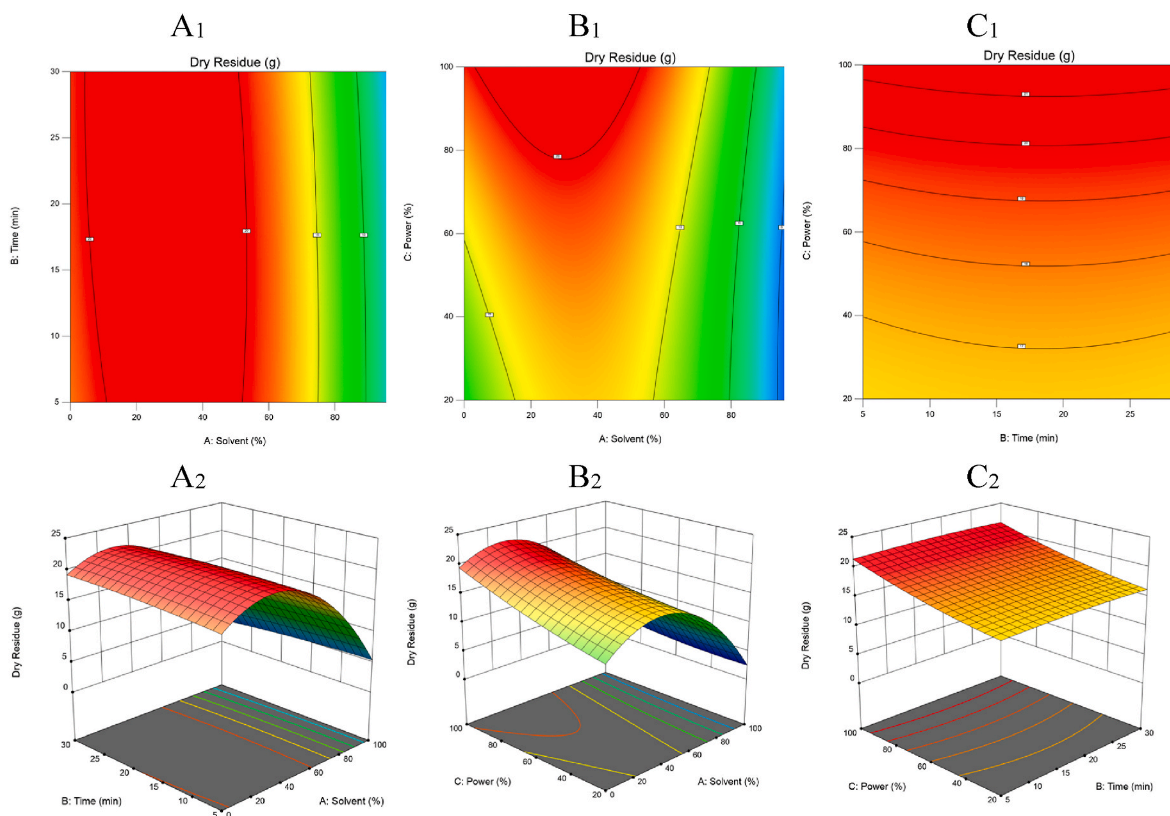

Fig. 4. Contour and 3D surface plots after optimization of the raspberry (RB) extraction.

values of ultrasound power and ethanol, RB was favored by high power extraction, with lower values of ethanol and long extraction times.

3.2. Extracts characterization

For characterization, BB and RB samples were extracted using the optimal conditions, obtained with RSM. For BB the conditions were 59 % ethanol:water ratio, 30 min and 73 % of ultrasonic power while RB were 40 % ethanol:water ratio, 98 % of ultrasonic power and 16 min. Extracts were then characterized concerning the phenolic compound profile and their antioxidant, antimicrobial, and cytotoxic properties to settle their potential for use in cosmetics.

3.2.1. Phenolics compounds profile

The extracts were then analyzed using HPLC-DAD-ESI/MS to identify their phenolic compound profiles. Tables 2 and 3 provide detailed information on the retention time (Rt), maximum absorption wavelengths in the UV-Vis region (λ_{max}), deprotonated ions, mass fragmentation patterns, and the identification of the compounds detected in BB and RB, respectively. The chromatograms of both BB and RB can be found in supplementary material (Fig. S1).

In Table 2, **peaks 1** to **11** were identified based on previous chemical characterizations of blueberry bioresidue extracts (Plasencia et al., 2023). **Peaks 1** and **4** were identified as caffeoylquinic acids, while **peaks 2** and **3** were identified as caffeoylquinic acid dimers. **Peak 9** was

Table 2
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification, and quantification (mg/g of extract) of blueberry phenolic compounds.

Peak	Rt (min)	λ_{max} (nm)	[M-H]- (*m/z*)	MS^2 (*m/z*)	Tentative Identification	Quantification (mg/g)
1	4.60	324	353	191(100), 179(55), 135(10)	3-*O*-Caffeoylquinic acid	0.49 ± 0.01
2	5.09	322	707	467(23), 353(100), 191(15)	3-*O*-Caffeoylquinic acid dimer	1.66 ± 0.01
3	6.67	322	707	467(23), 353(100), 191(15)	5-*O*-Caffeoylquinic acid dimer	1.03 ± 0.01
4	7.77	323	353	191(100), 179(8), 161(2), 135(3)	5-*O*-Caffeoylquinic acid	15.8 ± 0.3
5	8.95	283	863	711(28), 573(13), 451(15), 411(18), 289(6)	Procyanidin trimer (B- or A-type linkages)	0.73 ± 0.01
6	9.91	282	863	711(25), 573(18), 451(13), 411(31), 289(10), 285(8)	Procyanidin trimer (B- or A-type linkages)	1.5 ± 0.1
7	16.71	345	609	301(100)	Quercetin-3-*O*-rutinoside	8.9 ± 0.2
8	17.79	327	463	301(100)	Quercetin-3-*O*-glucoside	2.33 ± 0.01
9	19.35	319	515	353(100), 191(11), 179(8)	3,5-*O*-Dicaffeoylquinic acid	2.8 ± 0.2
10	19.79	340	593	285(100)	Luteolin di-*C*-hexoside	3.19 ± 0.03
11	21.03	334	447	285(100)	Luteolin 6-*C*-glucoside	2.88 ± 0.02
					TPA	21.8 ± 0.5
					TF	19.6 ± 0.3
					TPC	41.3 ± 0.8

TPA-Total phenolic acids, TF-Total flavonoids, TPC-Total phenolic compounds. calibration curves used: 1- chlorogenic acid (y = 168823x - 161172, R^2 = 0.999; LOD = 0.83 μg/mL; LOQ = 2.5 μg/mL), 2- caffeic acid (y = 388345x + 406369, R^2 = 0.993; LOD = 8.57 μg/mL; LOQ = 25.97 μg/mL), 3- catechin (y = 84950x − 23200, R^2 = 0.999; LOD = 0.17 μg/mL; LOQ = 0.68 μg/mL), 4- quercetin-3-O-glucoside (y = 34843x - 160173, R^2 = 17.01; LOD = 51.54 μg/mL; LOQ = 2.44 μg/mL), 5- apigenin-7-*O*-glucoside (y = 10683x - 45794, R^2 = 136.95; LOD = 0.80 μg/mL; LOQ = 414.98 μg/mL). nd-not detected. TPA, TF and TPC were calculated based on the identified polyphenols.

Table 3
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification, and quantification (mg/g of extract) of raspberry phenolic compounds.

Peak	Rt (min)	λ_{max} (nm)	[M-H]- (*m/z*)	MS^2 (*m/z*)	Tentative Identification	Quantification (mg/g)
1	4.55	286	633	481(13), 463(12), 301(100)	Galloyl-HHDP-glucose	0.69 ± 0.001
2	5.26	298sh319	355	209(32), 191(100)	Dihydrocaffeoylquinic acid	0.73 ± 0.01
3	7.51	281	865	739(15),577(33), 575(20), 425(21), 407 (100), 289(45), 287(85)	Procyanidin trimer	2.66 ± 0.07
4	8.18	269	783	633(52), 301(100)	Pedunculagin isomer I (*bis*-HHDP-glucose)	0.93 ± 0.01
5	10.24	275	783	481(11), 301(100)	Pedunculagin isomer II (*bis*-HHDP-glucose)	0.73 ± 0.01
6	12.23	280,378	1251	1083(4), 781(13), 601(4), 301(13)	Punicalagin gallate	0.71 ± 0.03
7	13.36	276	935	633(15), 301(18)	Galloyl-*bis*-HHDP-glucose	0.96 ± 0.03
8	14.56	281	935	633(15), 301(18)	Galloyl-*bis*-HHDP-glucose isomer I	0.96 ± 0.03
9	15.01	289	935	633(15), 301(18)	Galloyl-*bis*-HHDP-glucose isomer II	0.61 ± 0.02
10	16.85	248,362	433	301(100)	Ellagic acid pentoside isomer I	1.26 ± 0.02
11	17.80	254,362	477	301(100)	Methyl ellagic acid hexoside	14.3 ± 0.1
12	19.30	253,360	301	256(6), 185(15)	Ellagic acid	2.48 ± 0.01
13	20.13	248,355	433	301(100)	Ellagic acid pentoside isomer II	0.61 ± 0.01
14	21.06	258, 357sh	461	285(100)	Luteolin-*O*-glucuronide	4.045 ± 0.005
15	22.92	350,380	1083	781(12), 601(25), 301(93)	Punicalagin	2.58 ± 0.11
					TPA	19.4 ± 0.2
					TF	6.7 ± 0.1
					THT	5.6 ± 0.1
					TPC	34.3 ± 0.5

TPA-Total phenolic acids, TF-Total flavonoids, THT-Total hydrolysable tannins, TPC-Total phenolic compounds. calibration curves used: 1- ellagic acid (y = 26719x − 317255, R^2 = 0.998; LOD = 0.41 μg/mL; LOQ = 1.24 μg/mL), 2- chlorogenic acid (y = 168823x - 161172, R^2 = 0.999; LOD = 0.83 μg/mL; LOQ = 2.5 μg/mL), 3- catechin (y = 84950x − 23200, R^2 = 0.999; LOD = 0.17 μg/mL; LOQ = 0.68 μg/mL), 4- apigenin-7-*O*-glucoside glucoside (y = 10683x - 45794, R^2 = 136.95; LOD = 0.80 μg/mL; LOQ = 414.98 μg/mL). nd-not detected. TPA, TF. THT and TPC were calculated based on the identified polyphenols.

identified as dicaffeoylquinic acid based on hierarchical keys previously reported by Clifford et al. Caffeoylquinic acid derivatives were also found in *V. myrtillus* leaves by Bujor et al. (2016).

Peaks 5 and **6** displayed characteristics of proanthocyanidins and were identified as procyanidins trimers and tetramers, respectively. These compounds are commonly present in blueberry leaves and stems, according to Bujor et al. (2016). Among the remaining flavonoids (**peaks 7**, **8**, **10** and **11**), quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, luteolin di-6,8-*C*-hexoside, and luteolin-6-*C*-glucoside were identified. Hokkanen et al. (2009) described these compounds in blueberry leaves.

Consistent with previous studies, the two most abundant phenolic compounds in the extracts obtained under optimal conditions were 5-*O*-caffeoylquinic acid (15.8 ± 0.3 mg/g) and quercetin-3-*O*-rutinoside (8.9 ± 0.2 mg/g). The total content of phenolic acids was 21.8 ± 0.5 mg/g, flavonoids 19.6 ± 0.3 mg/g, and the total phenolic compound (TPC) content reached 41.3 ± 0.8 mg/g, confirming the predominance of hydroxycinnamic acid derivatives and flavonols in this extract. These values were calculated by summing the different groups of polyphenols, and not through any biochemical assay.

Regarding the results of the RB extract, the identified peaks were assigned based on previous studies (Plasencia et al., 2024). In Table 3, **peak 1** ([M−H]$^-$ at *m/z* 633) and **peaks 7–9** ([M−H]$^-$ at *m/z* 935) were identified as hexahydroxydiphenoyl (HHDP) derivatives, specifically galloyl-HHDP-glucose and galloyl-bis-HHDP-glucose. The presence of an HHDP moiety was confirmed by the fragment at *m/z* 301 Raspberry leaf extracts have been reported to contain compounds with UV spectra consistent with galloyl and HHDP derivatives, supporting these

identifications. **Peaks 4** and **5** ([M−H]⁻ at *m/z* 783) were assigned to pedunculagin isomers, while **peak 6** ([M−H]⁻ at *m/z* 1251) was identified as punicalagin gallate.

Peak 2 ([M−H]⁻ at *m/z* 355) was identified as dihydrocaffeoylquinic based on the parent ion and MS^2 base peak of *m/z* 191, indicating dihydrocaffeic acid with a neutral loss of 164 Da. **Peaks 10–13** were identified as ellagic acid derivatives, including ellagic acid pentoside, methyl ellagic acid hexoside, and ellagic acid. Flavan-3-ols were also detected in raspberry plant aerial parts, with **peak 3** ([M−H]⁻ at *m/z* 865) assigned to procyanidin trimer. The only flavone detected (**peak 14**) was identified as luteolin-*O*-glucuronide based on its deprotonated ion at *m/z* 461 and a characteristic fragment at *m/z* 285, corresponding to the luteolin aglycone. **Peak 15** was y identified as punicalagin, according to its deprotonated molecular ion [M-H]⁻ at m/z 1083 and MS^2 fragment at m/z 301, characteristic of ellagic acid, a marker for ellagitannins. Hydrolysable tannins were predominant phenolic compounds in the RB extract, aligning with previous reports on *R. idaeus* aerial parts. The total content of hydrolysable tannins was 5.6 ± 0.1 mg/g, while phenolic acids totaled 19.4 ± 0.2 mg/g and flavonoids 6.7 ± 0.1 mg/g. The total phenolic content (TPC) was 34.3 ± 0.5 mg/g, slightly lower than that of BB. As with RB, these values were calculated by summing the different groups of polyphenols, and not through any biochemical assay. The results reinforce the variation in phenolic composition across different plant parts, as previously demonstrated by Subbiah et al. (2021).

The predominance of hydrolysable tannins in RB and caffeoylquinic acid derivatives and flavonols in BB suggests that, as expected, the phenolic composition of the aerial parts of these plants differs significantly from that of their fruits due to the different functionality and environmental exposure of each plant component. These findings highlight the potential of these residues as a source of bioactive compounds, suggesting new applications in pharmaceutical and food industries. Further research should explore the bioactivity and stability of these compounds in different formulations.

3.2.2. Antibacterial activity

The antibacterial properties of BB and RB, at the optimal extracts, were studied using the microdilution method against pathogenic bacteria. The results are displayed in Table 4. The BB extracts showed inhibition capacity (MIC) against most tested bacterial strains but could not effectively kill them. Generally, a MIC of 1.25–5 mg/mL for all the tested extracts was needed to inhibit the analyzed strains. However, an auspicious result was achieved against MRSA, a severe pathogenic bacterial strain resistant to ampicillin, for which a MIC of 1.25 mg/mL was obtained. The extracts presented the most potent activity compared to positive controls, such as ampicillin, particularly against Gram-negative bacteria. For instance, a MIC of >10 mg/mL of ampicillin was needed to inhibit *M. morganii* and *P. aeruginosa*. In contrast, a MIC of 5 mg/mL for the extract was obtained, highlighting the potential of the blueberry aerial parts extracts in this action, given the exponential growth of bacterial resistance to common antibiotics.

Similarly, RB extract inhibited most tested bacterial strains, with MIC values varying from 1.25 to 10 mg/mL. It inhibited the Gram-negative bacteria *E. coli*, *M. morganii*, and *P. mirabilis* with a 1.25 mg/mL MIC. Regarding the Gram-positive bacteria, a MIC of 2.5 mg/mL against *E. faecalis* and the methicillin-resistant *S. aureus* was achieved for RB. Compared with the positive control ampicillin, the antimicrobial activity was stronger. In the case of *M. morganii* and *P. aeruginosa*, ampicillin needed >10 mg/mL, while RB responded with 1.25 and 2.5 mg/mL, respectively. The extract also showed promising results against Gram-positive strains, especially against *E. faecalis* and MRSA, with a MIC of 1.25 mg/mL.

The antibacterial effects of berry extracts against Gram-negative bacteria, including *E. coli*, were studied (Puupponen-Pimiä et al., 2001), and an activity decrease from raspberry to blueberry was observed. Denev et al. (2014) investigated the antimicrobial activity of extracts obtained from the leaves of blueberry and raspberry plants obtained with a mixture of 80 % acetone and an aqueous formic acid solution. In their study, raspberry extract presented a MIC of 0.031 mg/mL against the methicillin-resistant *S. aureus* strain, while blueberry extract reached a value of 0.13 mg/mL. Concerning *K. pneumonia* strain, 25 and 0.63 mg/mL values were found for blueberry and raspberry extracts, respectively. In previous studies by Plasencia et al. (2023, 2024), MICs of 1.25 and 2.5 mg/mL were recorded for extracts of the aerial parts of raspberry and blueberry plants, respectively, against methicillin-resistant *S. aureus*. For *K. pneumoniae* strain, these authors reported 10 and 5 mg/mL, while in the present work, studied extracts resulted in >10 and 10 mg/mL of MIC. Differences in antimicrobial activity responses can be explained by differences in extraction conditions, which allow the extraction of different compounds or differences in their quantities, influencing the chemical composition of the extract and, consequently, its antimicrobial activity.

In our current study, RB generally exhibited stronger antibacterial activity across a broader range of bacteria, especially Gram-negative strains, than BB. Both extracts were highly effective against MRSA, with identical MIC values, indicating equivalent potential in combating this resistant strain. While BB showed strong effects against MRSA, RB demonstrated superior potency against Gram-negative bacteria, suggesting it may be more versatile. In summary, although BB offers significant inhibition capabilities, especially against MRSA, RB presents more potent and broader antibacterial activity, making it potentially more effective as an antimicrobial agent, particularly against Gram-negative bacteria.

3.2.3. Antioxidant activity

The antioxidant activity of the two optimal extracts was studied

Table 4
Antibacterial potential of raspberry and blueberry and aerial parts extracts, expressed in mg/mL.

	RB		BB		Positive Controls					
					Ampicillin (10 mg/mL)		Imipenem (1 mg/mL)		Vancomycin (1 mg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria										
Escherichia coli	1.25	>10	5	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	10	>10	>10	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	1.25	>10	5	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	1.25	>10	5	>10	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	2.5	>10	5	10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria										
Enterococcus faecalis	1.25	>10	2.5	>10	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	5	>10	2.5	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	1.25	>10	1.25	>10	<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 *Staphylococcus aureus*.

using the OxHLIA and TBARS methods, and are shown in Table 5. According to the results from the TBARS method, both the BB and RB extracts showed similar antioxidant activity, although they were less efficient than the Trolox reference standard. Between them, BB showed a statistically significant higher activity (showed by a lower EC_{50}, concentration that quenches 50 %of free radicals). These are similar to previous research (Plasencia et al., 2023, 2024), in which the antioxidant activity (EC_{50}, μg/mL) of extracts from remains of blueberry (9.0 ± 0.3) and raspberry (8.0 ± 0.3) plants, respectively, resulting from pruning, were studied, the antioxidant activities of the optimized extracts, in the present study was closer to standard. Furthermore, the comparative results were inverted; that is, the antioxidant activity of the blueberry sample was slightly better than that of the raspberry, which was different from previous results. In the OxHLIA analysis, both extracts showed more effective antioxidant activity than Trolox at 60 and 120 min. Specifically, the RB extract with significantly lower activity, showed promising results, with IC_{50} values at 60 and 120 min being less than half of those obtained for the standard. The BB extract, although weaker than RB, also demonstrated strong antioxidant activity, achieving approximately half of the standard Trolox.

The antioxidant activity observed in BB and RB extracts aligns with the presence of their most abundant phenolic compounds. In BB, 5-*O*-caffeoylquinic acid (15.8 ± 0.3 mg/g), quercetin-3-*O*-rutinoside, and luteolin di-*C*-hexoside are well-known for their strong radical-scavenging effects. In RB, methyl ellagic acid hexoside (14.3 ± 0.1 mg/g), luteolin-*O*-glucuronide, and ellagic acid likely play a major role in the extract's antioxidant capacity. Although less concentrated, compounds such as pedunculagin isomers in RB (Hardman, 2014) and other caffeoylquinic acid derivatives in BB (H. J. Park, 2010) may provide complementary activity. Since free radicals contribute to cellular damage and related diseases, the phenolic compounds in these pruning waste extracts may offer protective effects. These findings highlight the potential of upcycling berry pruning residues into valuable antioxidant sources for food and cosmetic applications.

An asterisk in each column represents a statistically significant difference among the two samples, using a significance of 0.05. BB – blueberry; RB – raspberry.

Table 5
Antioxidant activity at the optimal conditions for each extract (μg/mL).

Samples	TBARS (EC_{50})	OxHLIA (IC_{50})	
		Δ*t* 60 min	Δ*t* 120 min
BB	6.01 ± 0.07*	10.5 ± 0.20*	21.7 ± 0.20*
RB	6.86 ± 0.08	6.70 ± 0.30	20.6 ± 0.40
Trolox	9.1 ± 0.3	21.5 ± 0.10	43.5 ± 0.20

3.2.4. *Cytotoxicity and anti-inflammatory activity*

The cytotoxicity analysis was evaluated on tumor and primary cell lines, as shown in Table 6. The cytotoxicity values of both BB and RB across various tumor cell lines are significantly higher than those of the positive control (ellipticine). As with the antioxidant activity, higher values indicate lower activity. This indicates that both extracts BB and RB are much less effective in killing tumor cells compared to ellipticine, but in terms of toxicity to primary cell lines, the toxicity is much lower. In terms of tumor cell lines, comparing both berry types, there were statistically significant differences between them for AGS and CaCo, in which BB showed better activity, while no statistical difference was found for MCF-7, NCI-H460. For the primary cell lines VERO, no differences were recorded, showing a lack of toxicity to these cells.

As with cytotoxicity, both extracts show low anti-inflammatory activity when compared to the positive control, although between them, RB showed a statistically higher activity. Previous studies by Plasencia et al., in 2023 and 2024 have validated the anti-inflammatory advantage of raspberry plant extracts over blueberry.

Overall, both extracts show anti-inflammatory activity, explained by their phenolic composition in galloyl-bis-HHDP-glucose (Lachowicz et al., 2020; Pinheiro et al., 2019) for RB, and in quercetin (Septembre-Malaterre et al., 2020) for BB, which has potential cosmetic interest.

An asterisk in each line represents a statistically significant difference among the two samples, using a significance of 0.05.

Table 6
Anti-inflammatory activity (IC_{50}, μg/mL) and cytotoxicity (GI_{50}, μg/mL) of blueberry and raspberry extracts.

	BB	RB	Positive control
Anti-inflammatory activity			**Dexamethasone**
RAW 264.7	219 ± 8*	184 ± 12	6.3 ± 0.4
Cytotoxicity in tumour cells			**Ellipticine**
MCF-7 (breast carcinoma)	269 ± 18	272 ± 17	1.02 ± 0.02
NCI-H460 (non-small cell lung carcinoma)	209 ± 3	211 ± 18	1.01 ± 0.01
AGS (gastric adenocarcinoma)	153 ± 7*	179 ± 9	1.23 ± 0.03
CaCo2 (colorectal adenocarcinoma)	220 ± 9*	244 ± 7	1.21 ± 0.02
HELA	204 ± 9	215 ± 10	–
Cytotoxicity in non-tumour cells			
VERO	303 ± 13	298 ± 16	1.41 ± 0.06

4. Conclusions

Optimizing extraction conditions by ultrasound-assisted methodology and ethanol-to-water solvent ratio for RB and BB revealed distinct optimal parameters for maximizing the solid yield. BB extraction was optimized at 59 % ethanol, 30 min, and 73 % ultrasound power, while RB extraction was optimal at 40 % ethanol, 16 min, and 98 % ultrasound power. Both extracts demonstrated notable bioactive properties, including antimicrobial and antioxidant activities, with the RB showing slightly stronger effects for the antimicrobial activity and BB for the antioxidant. The phenolic profiles of the extracts were consistent with previous studies, confirming the presence of critical bioactive compounds. Both extracts exhibited anti-inflammatory activity, with RB slightly more potent and minimal cytotoxic effects on non-tumor cells, indicating potential for cosmetic and therapeutic applications. However, the extracts were less effective in cytotoxicity assays against tumor cells, suggesting limited potential as anticancer agents. These findings highlight the different applications of RB and BB extracts in health-related industries, particularly in antimicrobial, antioxidant, and anti-inflammatory products.

The RB is the best candidate for cosmetic applications. It demonstrated slightly more vigorous anti-inflammatory activity than the BB, which is particularly valuable in cosmetics for soothing and reducing skin inflammation.

CRediT authorship contribution statement

Paula Plasencia: Writing – original draft, Investigation, Formal analysis. **Márcio Carocho:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Tiane C. Finimundy:** Formal analysis. **Ricardo C. Calhelha:** Formal analysis. **Adriana K. Molina:** Formal analysis. **Tânia C.S.P. Pires:** Formal analysis. **Maria Filomena Barreiro:** Writing – review & editing, Supervision, Conceptualization. **Pablo A. Garcia:** Writing – review & editing, Supervision. **Lillian Barros:** Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have not known competing financial

interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2025.107407.

Data availability

Data will be made available on request.

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