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ORIGINAL RESEARCH

Effect of Microwave Hydrodiffusion and Gravity on the Extraction of Phenolic Compounds and Antioxidant Properties of Blackberries (*Rubus* spp.): Scale-Up Extraction

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Abstract

The phenolic compounds of blackberries extracted with organic solvents cause environmental damage. Therefore, the objective of the present study was to verify if microwave hydrodiffusion and gravity obtain a blackberry extract with a high concentration of phenolic compounds and antioxidant capacity without the addition of any solvent. The results showed that it was possible to reach the objective with 500 W and 10 min of extraction by employing a method that meets green chemistry principles. The extract has a lower cost than the exhaustive method, is microbiologically safe, and is mainly composed of anthocyanins (85%). The presence of 5 anthocyanins and 17 non-anthocyanin phenolic compounds were identified, including hydroxyresveratrol, which was first extracted in blackberries by microwave hydrodiffusion and gravity. The phenolic compound content and antioxidant capacity were lower in the last fractions, which reduced the extraction time to 8 min. The coproduct showed phenolic, antioxidant capacity, and microbiological quality. This study presented a fast, efficient, economical, sustainable, and solvent-free method to extract phenolic compounds from blackberries.

Keywords Green extraction · Microbiological analysis · Economic analysis · Hydroxyresveratrol · Antioxidant capacity · Reactive oxygen species

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Introduction

High concentrations of phenolic compounds are found in blackberry fruit and confer high antioxidant capacity and produce beneficial effects in human health (Kaume et al. 2012). The extraction of these compounds is traditionally carried out through organic solvents by using mainly solid-liquid extraction. Solid-liquid extraction is a traditional extraction method used to remove phenolic compounds from food, both in laboratory and industrial environments, in which organic solvents such as ethanol, methanol, acetone, and dichloromethane are used and generally mixed with water. However, conventional extraction processes are expensive and require long extraction times, present toxicity, and generate large amounts of hazardous waste (Zill-e-Huma et al. 2009).

Due to the disadvantages of extracting blackberry phenolic compounds by the traditional method, research has attempted to find more sustainable, fast, and economically viable means to extract them (Garcia-Salas et al. 2010). Alternative methods such as pressurized liquid extraction (de F Machado et al. 2014), extraction with supercritical fluid (Reátegui et al. 2014), and ultrasonic-assisted extraction (Machado et al. 2018a) have already been used to extract blackberry phenolic compounds.

Another alternative to obtain these compounds is extraction by microwave hydrodiffusion and gravity (MHG), which is a sustainable method that extracts phenolic compounds without the addition of solvents and removes essential oils (Vian et al. 2008). Moreover, MHG is also used to obtain extracts of fruit by-products such as grapes (Al Bittar et al. 2013), sea buckthorns (Périno-Issartier et al. 2011), blueberries (Ravi et al. 2018), and vegetable extracts of lettuce (Périno-Issartier et al. 2016), artichokes (López-Hortas et al. 2019a), and broccoli (López-Hortas et al. 2019b) with high concentrations of bioactive compounds. Nevertheless, analyses to identify individual phenolic compounds, in vivo antioxidant activity, and economic analyses have not yet been performed on MHG-obtained extracts, in addition to there being no evaluations on whether the extraction residue (coproduct) has the bioactive and microbiological quality for potential application in other products.

This method quickly extracts phenolic compounds and provides other advantages such as low energy consumption, low levels of contaminants in the extract (Al Bittar et al. 2013), and reduced microbial content (Bozkurt-Cekmer and Davidson 2016), although the microbiological quality of MHG extracts has yet to be proven. Moreover, phenolic compounds are thermosensitive, and it may be possible to preserve these compounds after using MHG due to reduced exposure time at high temperatures.

The equipment (MHG) consists of a microwave similar to a commercial model, where extraction takes place by combining microwave heating and the gravity of the Earth at atmospheric pressure (Zill-e-Huma et al. 2009). In this system, the compounds diffused with the in situ water drop by gravity and are condensed and collected outside the oven. A study that evaluated different fractions of fruit juices extracted over time with MHG shows that the total amount of polyphenols of plum, tomato, cherry, and apricot were recovered in the first minutes of the process. In addition, a high concentration of anthocyanins was found in half of the extraction process; thus, the complete removal of the fruit extract to obtain a product rich in these bioactive compounds was not required (Cendres et al. 2014). Therefore, our objective was to verify if the MHG can achieve a blackberry extract with high phenolic compound concentrations and antioxidant capacity.

Material and Methods

Chemicals and Reagents

The acetone, formic acid (85%), Folin-Ciocalteu reagent, monobasic phosphate buffer, sodium sulfate, and chromatographic grade acetonitrile were obtained from Dinâmica (Jóia, Brazil). Bacto agar, cholesterol, ethyl alcohol, streptomycin sulfate, CM-H2DCFDA, methylcellulose (1500 cP viscosity), AAPH (2,2′-azobis-2-amidinopropane dihydrochloride), and the standards Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (≥ 97%), epicatechin (≥ 90%), cyanidin-3-glycoside (≥ 95%), and quercetin-3-glycoside (≥ 90%) were purchased from Sigma-Aldrich (Jurubatuba, Brazil). The other pure phenolic standards epicatechin (≥ 97%) and chlorogenic acid (≥ 98%) were purchased from ChromaDex (Irvine, USA) and Chem-Impex International (Wood Dale, USA). Standard gallic acid (≥ 98%), potassium chloride, sodium acetate, and dibasic phosphate buffers were obtained from Neon (Suzano, Brazil).

The fluorescein and sodium carbonate were acquired from Êxodo Científica (Sumaré, Brazil), while chromatographic grade methanol was purchased from Madrid, Spain. Double-deionized water with a conductivity below 18.2 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). The sodium chloride, calcium chloride, magnesium sulfate, dibasic sodium phosphate, and sodium hydroxide were obtained from Vetec (São Paulo, Brazil), phosphate monopotassium and dimethyl sulfoxide (DMSO) were purchased from Pró química (Canoas, Brazil), and Bacto peptone and Luria broth were obtained from Kasvi (São José dos Pinhais, Brazil). Peptone, plate count agar, lauryl sulfate tryptose broth, and deoxycholate lysine xylose agar were purchased from Himida (Mumbai, India).

Blackberry Samples

Blackberries (*Rubus* spp.) of the cultivar Tupy obtained in the year 2017 were harvested in Embrapa, located in the city of Pelotas, RS, Brazil. The fruit and extracts were kept frozen at − 18 °C until extraction or analyses.

Univariate Optimization of Extraction Power for MHG

Extraction was carried out with the MHG equipment (model NEOS-GR, Italy) equipped with an infrared temperature sensor and a glass jar (2-L capacity) with a filter network, polytetrafluoroethylene (PTFE) cap, and glass condenser, which remained connected to an ultra-thermostatic bath at 10 °C (Fig. 1). The method proposed by Ravi et al. (2018) was used to optimize the extraction, in which the tested powers were differentiated by 0.5 W/g (1, 1.5, and 2 W/g). In preliminary tests (data not reported), the lowest power (200 W corresponding to 1 W/g) was not efficient in extracting the compounds; therefore, the optimization was started with 300 W (1.5 W/g). The other powers were tested until it was possible to distinguish the highest power used without degrading the antioxidant compounds. Then, 200 g of frozen blackberry cut in half was used for each extraction. The powers of 300 (1.5 W/g), 400 (2 W/g), 500 (2.5 W/g), and 600 (3 W/g) W were evaluated until the maximum volume of extract was obtained without burning the fruit. The extracts were collected in a graduated glass. The extraction time was when the fruit was exposed to microwave radiation without burning, although it was necessary to wait until the water from the fruit was condensed and collected in the glass graduate, corresponding to the total volume of the extract obtained. The extraction time, extract volume, antioxidant capacity, total monomeric anthocyanin content, and total phenolic compounds were evaluated to determine the best power.

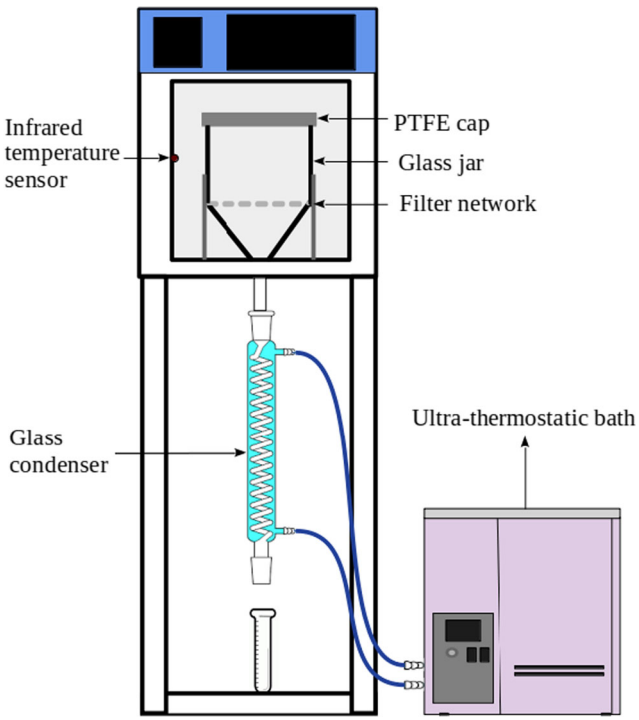

Fig. 1 Schematic figure of MHG

Phenolic Compound Extraction with Organic Solvents

The extraction with the organic solvent of the blackberry in nature and coproduct of the MHG extraction was performed according to the protocol described by Bochi et al. (2014), which uses an aqueous solution with 20% acetone and 0.35% formic acid under stirring for 18 min followed by vacuum filtration.

Obtaining Fractions of Blackberry Extract by MHG

Optimized method conditions (500 W for 10 min) were used to obtain the fractions. For this purpose, different fractions of the extract were collected during the extraction process, as described by Cendres et al. (2014). Falcon tubes (50 mL) were used to collect the samples at the bottom of the condenser. Each fraction was composed of 20 mL of extract.

Phenolic Compound Determination by UV-Visible Spectrometry

Total phenolic compound content was determined by the colorimetric method using the Folin-Ciocalteu method (Singleton et al. 1998). Total monomeric anthocyanin concentration was based on the reversible structural transformations of the chromophore of the anthocyanins, which can absorb energy as a function of the pH change in the medium, showing different absorption spectra according to the differential pH methodology (Giusti and Wrolstad 2001). The quantification of total condensed tannins was measured using methylcellulose to precipitate condensed tannins and based on the difference in the absorbance of water-soluble and ammonium sulfate (phenolic) minus methylcellulose (tannin) precipitates (Sarneckis et al. 2006).

Antioxidant Capacity Assay

Antioxidant capacity was evaluated using oxygen radical absorption capacity (ORAC) and intracellular reactive oxygen species (ROS) employing *Caenorhabditis elegans*. The ORAC method is based on exposing the extract to the action of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), which generates peroxyl radical activity and uses fluorescein as the target molecule. The non-fluorescent product of the fluorescein is monitored by spectrophotometry, measuring how much the extract protected vulnerable molecules (fluorescein) from oxidation by free radicals (peroxyl radicals) (Ou et al. 2001).

To analyze the reactive oxygen species (ROS) generation in *C. elegans*, we used wild-type (N2) animals and *Escherichia coli* OP50 strains as a food source for the worms acquired from the Caenorhabditis Genetics Center at the University of Minnesota (Minneapolis, USA). The L1 larval

stage worms were transferred to plates containing Nematode Growth Medium (NGM) and OP50 at 20 °C until the young adult stage, which took approximately 48 h (Brenner 1974). About 100 young adult worms were exposed to the pretreatment for 1 h at 20 °C, according to the concentration of total flavonoids present in the extract that was dissolved in the water, being 5, 10, and 50 μg/mL for the blackberry extract, 50 μg/mL for the coproduct, or M9 buffer as control. Then, the worms were washed three times and transferred to microtubes containing M9 buffer with 50 μM of the pro-oxidant juglone (5-hydroxy-1,4-naphthalenedione), which is a superoxide generator (Blum and Fridovich 1983) or vehicle ethanol (1% final concentration). The samples were then incubated under agitation for 1 h at 20 °C.

The ROS was measured using 2,7-dichlorodihydro-fluorescein diacetate (H2DCFDA) according to a previous protocol (da Silveira et al. 2018) with minor modifications. After the pretreatment with the blackberry extract or its coproduct and exposure to 50-μM pro-oxidant juglone, the worms were washed three times with M9 buffer and incubated with 20 μM of H2DCFDA for 2.5 h. Fluorescence intensity was measured using SpectraMax® i3x microplate reader (Molecular Devices, Sunnyvale, CA) at 20 °C with 488-nm excitation and 510-nm emission. Three experiments were performed on three different days, in quadruplicate, and the mean values calculated. The data are expressed as a percentage of control.

Identification of Non-anthocyanin Phenolic Compounds

Non-anthocyanin phenolic compounds were identified using a RRLC 1200 (Agilent Technologies, Palo Alto, CA, USA) with a degasser vacuum, autosampler, and binary pump and coupled to a time-of-flight (TOF) mass spectrometer (Bruker Daltonik, Bremen, Germany) with orthogonal electrospray interface (ESI) (model G1607, Agilent Technologies, Palo Alto, CA, USA). Calibration of the mass spectrometer was done using a 74900–00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) containing a 10-mM sodium formate cluster solution on regression mode (HPC). The ESI was operated on negative ionization mode, and the instrumental parameters of its source were established as follows: capillary voltage, + 4 kV; drying gas temperature, 210 °C; drying gas flow, 9 L/min; and nebulization gas pressure, 2.3 bar. The transfer parameters were capillary exit, − 120 V; skimmer 1, − 40 V; hexapole 1, − 23 V; RF hexapole, 80 Vpp; and skimmer 2, − 20 V. The scanning range was 50–1500 m/z (Leyva-Jiménez et al. 2018). The mass data of the molecular ions were acquired by DataAnalysis 4.0 analysis software (Bruker Daltonics).

The Zorbax Eclipse Plus C18 column, 150 mm × 4.6 mm ID (1.8 μm) (Agilent Technologies, Palo Alto, CA, USA) was used to separate the phenolic compounds. A volume of 10 μL of the sample was injected using a gradient eluting with mobile phase A (0.1% aqueous formic acid solution) and B (methanol) at a 0.4 mL/min flow rate at room temperature. The gradient used for mobile phase B was 0–42 min, 5%; 42–45 min, 95%; and 45–50 min, 5% B (Jiménez-Sánchez et al. 2015).

Anthocyanin Identification

The samples were injected at a flow rate of 5 μL/min into the HPLC-ESI-MS^n, and the anthocyanins were separated by ZORBAX Extend-C18 Rapid Resolution HT (2.1 × 100 mm, 1.8-Micron), 600 Bar column (Agilent), and mobile phases A (88.5% water, 8.5% formic acid, and 3% acetonitrile) and B (41.5% water, 8.5% formic acid, and 50% acetonitrile). Separation occurred at room temperature and with a flow level of 0.25 mL/min through the elution gradient for mobile phase B of 0–10 min, 6%; 10–20 min, 30%; 20–25 min, 50%; and 25–30 min, 6% (Barcia et al. 2014). The Agilent Technologies 6460 Triple Quadrupole 6460 (LC/MS-MS) equipment (Agilent Technologies, USA) was used to identify anthocyanins, generating electrospray ionization mass spectrum (ESI-MS). Nitrogen at 300 °C and a flow of 5 L/min were used as nebulization and collision gas. Molecular ions were generated by CID (collision-induced dissociation) using the positive mode with 15 eV collision energy, 5 V shredder, the capillary voltage at 3500 V, and the nebulizer at 45 psi.

Non-anthocyanin Phenolic Compound Separation and Quantification

Non-anthocyanin phenolic compounds were separated and quantified by carrying out the sample purification step via SPE-C18 cartridges (Giusti and Wrolstad 2001). Two mobile phases were used: mobile phase A was composed of 94.9% water, 5% methanol, and 0.1% formic acid, while mobile phase B was composed of acidified acetonitrile with 0.1% formic acid. Then, 20 μl of the purified samples were injected into the HPLC-DAD (Shimadzu). Separation occurred in a Hypersil Gold C-18 reverse phase column (5 μm particle, 150 mm, 2.1 mm) by the gradient of elution: 0–21 min, 0% B; 21–55 min, 4% B; 55–70 min, 16% B; 70–72 min, 50% B; 72–83 min, 100% B; and 83–92 min, 0% B. The oven temperature was 38 °C, and the run occurred at a flow rate of 1.0 mL/min (Quatrin et al. 2019). Calibration curves were injected with standards of flavanols (epicatechin), hydroxycinnamic acid (chlorogenic acid), and flavonols (quercetin-3-glycoside), in which the chromatograms were extracted from readings at the wavelengths of 280, 320, and 360 nm, respectively. The curves showed R^2 above 0.99 and concentrations ranging from 10.75 to 107.50 mg/L for epicatechin, 2.38 to 45.28 mg/L for chlorogenic acid, and 12.33 to 123.33 mg/L for quercetin-3-glycoside.

Anthocyanin Separation and Quantification

Anthocyanins were quantified by HPLC-UV-Vis (Shimadzu). Readings were performed at 520 nm, and cyanidin-3-glycoside was used as standard for the calibration curve, which presented concentrations ranging from 0.2 to 45.2 mg/L and R^2 of 0.9998. The Zorbax column Eclipse XDB (2.1 mm × 150 mm, 3.5-μm particle size) separated the anthocyanins at an oven temperature of 38 °C and flow of 0.5 ml/min. Water acidified with 3% formic acid (mobile phase A) and acetonitrile (mobile phase B) were used in the linear gradient of 8% B in 0–25 min, 32% B in 25–27 min, 90% B in 27–39 min, and 8% B in 39–50 min (Moraes et al. 2020).

Microbiological Analyses

The fruit *in nature*, the extract, and coproduct obtained by MHG were evaluated for microbiological quality and followed the Brazilian legislation criteria for fruit extracts/juices (ANVISA 2001). Hence, the total aerobic mesophilic enumeration, *Salmonella* spp., and total and thermotolerant coliforms were analyzed (APHA 2015).

Color Analyses of the Different Fractions Collected by MHG

The instrumental analyses of the color of the fractions were performed in a colorimeter (Minolta CR-300) using the CIELAB scale.

Economic Analyses

A flowchart (Fig. 2) was developed on SuperPro Designer 9.0® software (Intelligen Inc., Scotch Plains, NJ, USA) to simulate economic parameters of MHG blackberry processing and exhaustive extraction, as the cost of manufacturing (COM) and the percentage contribution of the cost of raw material (CRM), cost of operational labor (COL), cost of utilities (CUT), and fixed capital investment (FCI).

The total costs of laboratory-scale equipment were determined based on local quotations (Table 1), $2590.14 and $20,392.54 for the exhaustive method and MHG extraction, respectively. The experimental results of laboratory-scale extraction and the economic data (Table 2) were used as input data in the simulator. An additional fixed investment of 20% was included for the extra accounting equipment not listed in this work for both extraction methods. The estimation of equipment cost for the desired capacity was predicted by the power-law equation (Eq. 1), where M is a constant depending on the equipment type, C_1 is the cost of the equipment with capacity Q_1, and C_2 is the known base cost for equipment with capacity Q_2 (Turton et al. 2012).

$$C_1 = C_2\left(\frac{Q_1}{Q_2}\right)^M$$

The yield and composition of the extracts obtained at the pilot scale were assumed to have the same behavior as those obtained at the laboratory scale. For the pilot scale, capacities were increased 100-fold for MHG extraction and 1000-fold for exhaustive extraction compared to laboratory scale. No account was considered for the difference in operational work cost due to the different labor safety standards between the individual processes, nor the cost of solvent removal and waste treatment from exhaustive extraction.

Statistical Analyses

Analysis of variance (ANOVA) followed by the Duncan test were used to choose the best power. When comparing the different extraction methods, the Student's t test was used. In the antioxidant capacity analysis with *C. elegans*, the data

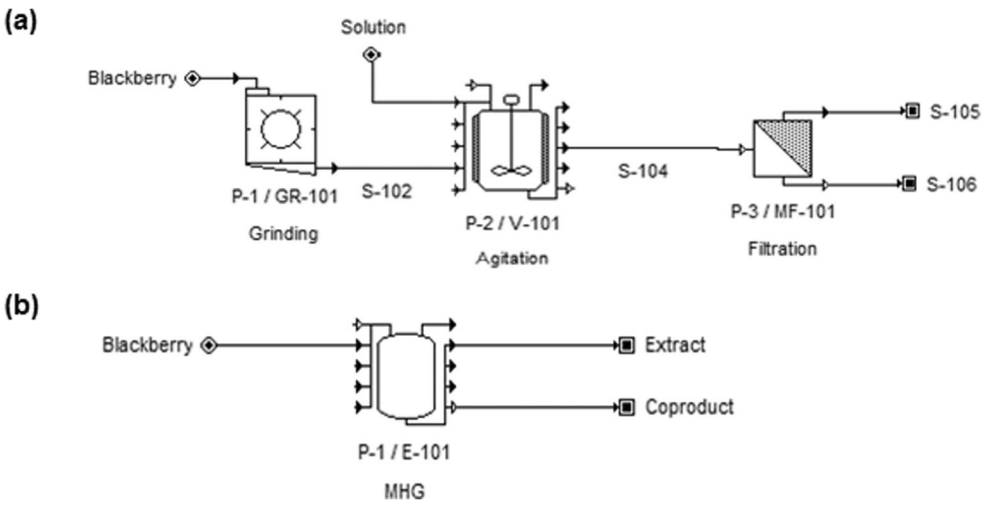

Fig. 2 Flowchart of exhaustive extraction and by MHG. (**a**) Exhaustive extraction—flowchart. (**b**) Extraction with MHG flowchart

Table 1 Base costs for equipment of MHG and exhaustive extraction

Item	M[a]	Unit base cost (US$)[b]	Quantity (un.)	Total base cost (US$)
Equipment—exhaustive extraction				
Mixer	0.59	46.77	1	46.77
Balance	0.60	1318.20	1	1318.20
Agitator	0.49	337.74	1	337.74
Kitassate	0.40	23.52	1	23.52
Vacuum pump	0.55	810.22	1	810.22
Rubber liner	0.40	5.67	1	5.67
Büchner funnel	0.40	38.02	1	38.02
Filter paper	0	0.01	1000	10.00
Total exhaustive (US$)				2590.14
Equipment—MHG				
MHG system	0.59	19,072.73	1	19,072.73
Balance	0.60	1319.81	1	1319.81
Total MHG (US$)				20,392.54

[a] M constant depending on equipment type based on Turton et al. (2012)

[b] Direct quotation for the reference year of 2018

were analyzed by ANOVA and Tukey's test. All values of $p < 0.05$ were considered significant.

Results and Discussion

Optimization of the Best Extraction Power for the MHG

The parameters evaluated (Fig. 3) show that the most suitable power for extraction was 500 W with a total run time of 10 min. Under these conditions, it was possible to quickly extract the phenolic compounds, which justifies this power choice since it was statistically equal ($p > 0.05$) to the power of 400 W in all evaluated parameters except time. Compared to 400 W extraction, the reduced extraction time led to increased extract yield per hour of work (approximately 810 and 638.30 mL/h for 500 and 400 W, respectively).

When comparing 500 W with the highest power of 600 W and the lowest of 300 W, the use of the latter revealed reduced extract volume (9 mL). This may have occurred due to the inefficiency of the hydrodiffusion process since a lower power may have made it difficult to draw water from the inside of the fruit, causing a smaller volume of extract. At 600 W, total phenolic compounds and antioxidant capacity reduce, showing that higher powers lead to phenolic compounds

Table 2 Input economic data is used to simulate the COM of blackberry extracts obtained by MHG and exhaustive extraction at laboratory and pilot scales

Input data	Laboratory scale	Pilot scale	Dimension
Fixed capital investment (FCI)			
Exhaustive plant	2590.14	133,161.5	US$
MHG plant	20,392.54	309,595.5	US$
Annual depreciation rate	10	10	%
Annual maintenance rate	6	6	%
Project lifetime	25	25	Year
Annual time worked	2640	7920	h/year
Daily time worked	8	24	h/day
Cost of raw material (CRM)			
Blackberry	1.35	1.35	US$/kg
Acetone	11.21	11.21	US$/L
Formic acid	17.12	17.12	US$/L
Distilled water	4.00	4.00	US$/$m^3$
Cost of operational labor (COL)			
Wage (with benefits and administration)	3.20	3.20	US$/h/worker
Number of workers per shift	1	2	Worker/shift
Number of daily shift	1	3	Shift
Total daily wage	24.80	24.80	US$/day
Cost of utilities (CUT)			
Water (for cleaning)	1.07	1.07	US$/$m^3$
Electricity	0.27	0.27	US$/kW/h

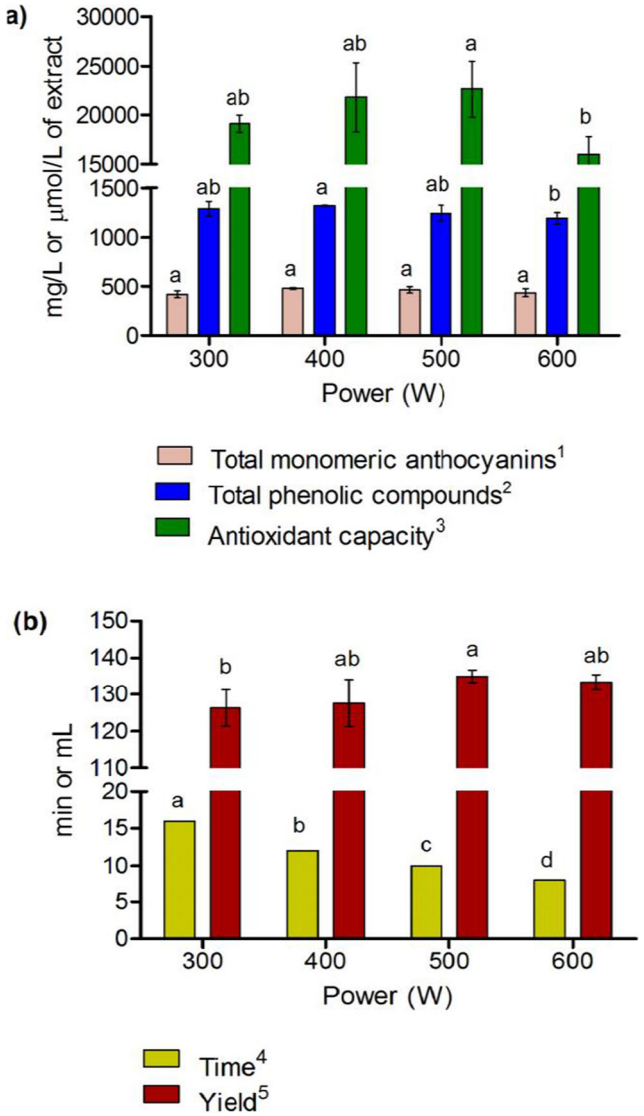

Fig. 3 Total monomeric anthocyanins, total phenolic compounds, antioxidant capacity, extraction time, and extract yield obtained from different optimized powers. [1]mg cyanidin-3-glycoside/L extract. [2]mg gallic acid/L extract. [3]µmol Trolox/L extract. [4]minutes. [5]mL

degradation and consequently reduced antioxidant capacity. Phenolic compound degradation caused by high powers in the microwave is already known, as Vinatoru et al. (2017) have already reported that the energy used in the extraction should be reduced due to excessive energy, possibly leading to the degradation of bioactive compounds, such as phenolic compounds. In addition, non-anthocyanin phenolic compounds present in blackberry, such as epicatechin (flavanols) and quercetin derivatives (flavonols), are thermosensitive and may have likely been degraded, thus reducing the antioxidant capacity of the extract. Therefore, the optimized extraction power conditions of 500 W enabled removing the highest extract volume with a high phenolic compounds concentration and antioxidant capacity in a short period (10 min).

Comparison Between Extraction Methods

The extract obtained by the MHG presented favorable characteristics compared to the exhaustive extraction with organic solvent (Fig. 4a). With MHG, it was possible to produce an extract concentrated in phenolic compounds and significantly greater antioxidant capacity (about 50 times higher than the extraction with organic solvent). Furthermore, extraction with MHG is about 3.6 times faster than conventional extraction, which takes approximately 36 min, considering sample stirring time and filtration. Although extraction by MHG did not enable the complete removal of phenolic compounds from the fruit as the exhaustive extraction (Fig. 4b), it allowed a fast extraction process without adding any solvents while only using the in situ water from fruits. This technology has advantages over extraction with organic solvents and is considered a green technology following the six principles of green extraction (Chemat et al. 2012).

Other advantages presented by MHG technology are related to better yield and phenolic content compared with organic solvent extraction, in addition to being an environmentally friendly process due to the absence of chemical contaminants. Studies that used MHG to extract phenolic compounds from fruits, such as sea buckthorn (Périno-Issartier et al. 2011) and grapes (Farias et al. 2021), also point out that the extracts obtained are more concentrated in antioxidant compounds than the extracts obtained with organic solvents, being MHG a valuable method that quickly and sustainably removes extracts rich in compounds with bioactive potential (Farias et al. 2021).

Extraction by MHG occurs by the dielectric heating of the water in situ fruit provoked by the microwaves, which causes the rupture of the vegetable cell (Chemat et al. 2019). Therefore, MHG can increase the yield of the extraction of phenolic compounds from fruits by facilitating their release because the microwaves break the cell structures, such as the membrane and cell walls where these bioactive compounds are located (Wang et al. 2020). More significant phenolic compound conservation is also responsible for the phenomena of hydrodiffusion and gravity, since after the rupture of the cell, in situ water is quickly transferred out of the plant (hydrodiffusion), and the extract is separated from the matrix by gravity and cooled and condensed by the cooling system (Chemat et al. 2017). Hence, the extract remains in contact with the heat generated by the microwaves for a short time, preserving the thermosensitive phenolic compounds.

Characterization of the MHG-Obtained Extract

The blackberry extract is mainly composed of anthocyanins, which represent about 85% of the phenolic composition. Hydroxycinnamic acids were less representative of the total extract (1% of its composition). Flavanols and flavonols

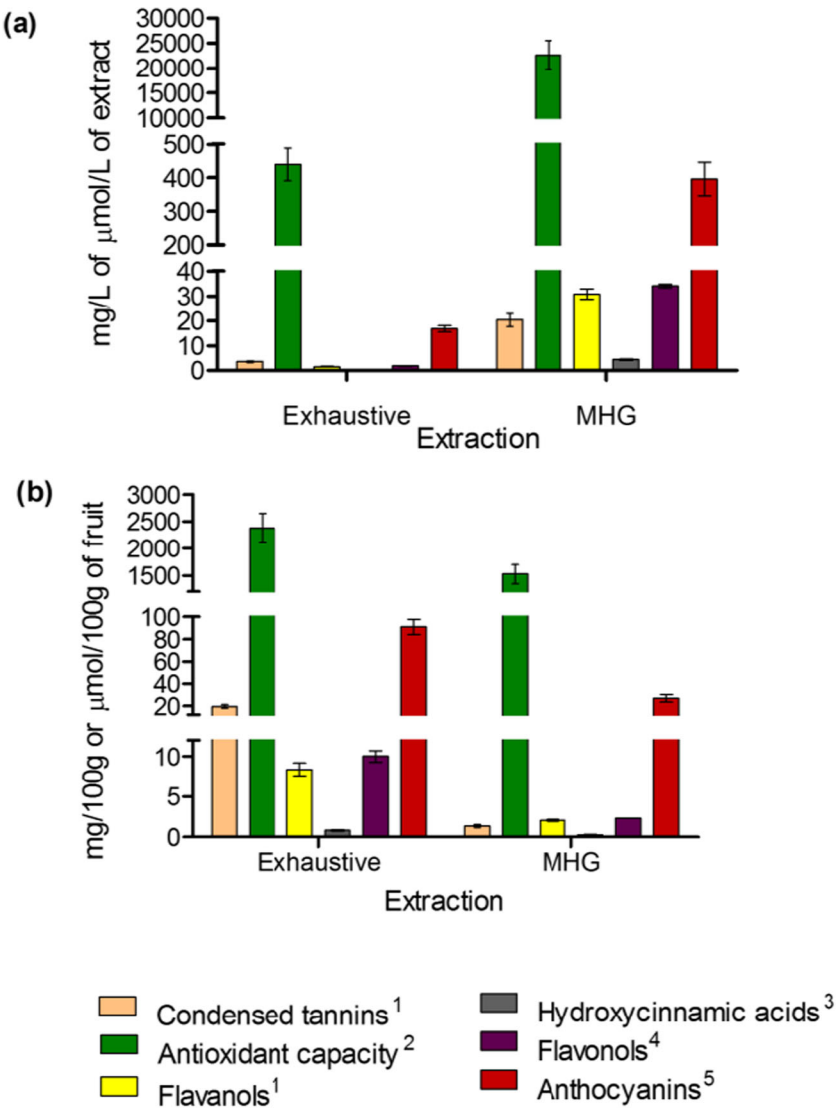

Fig. 4 Phenolic compounds and antioxidant capacity removed from blackberries by exhaustive extraction and MHG (**a**) and phenolic compounds and antioxidant capacity of blackberry extracts obtained by exhaustive extraction and MHG (**b**). [1]µg or mg of epicatechin per 100 g of fruit or per L of extract. [2]µmol Trolox per 100 g of fruit or per L of extract. [3]mg of quercetin-3-glycoside per 100 g of fruit or per L of extract. [4]mg of chlorogenic acid per 100 g of fruit or per L of extract. [5]mg of cyanidin-3-glycoside per 100 g of fruit or per L of extract. MHG and exhaustive samples differ significantly by the *t* test ($p < 0.05$) for all analyses

together account for 14% of the extract (Fig. 4b). Therefore, the blackberry extract obtained by MHG is characterized mainly by being rich in anthocyanins. Recent studies consider blackberry as a functional food because of its polyphenols, especially anthocyanins, which have high antioxidant and antidiabetic activity (Gowd et al. 2018). Just as the fruit *in nature*, the fruit extract can also provide beneficial effects to health.

In the present work, 17 non-anthocyanin phenolic compounds and 5 anthocyanins (Table 3) were identified. Among the phenolic acids and their derivatives, caffeic acid derivatives were found (chlorogenic acid and caffeine hexoside isomers). Moreover, ellagic acid was also identified as well as its added derivative of a pentose. In the literature, gallic acid in blackberry composition was also found (Pavlović et al. 2016), although only its derivative gallic acid hexoside was identified in the MHG extract. Stilbene hydroxyresveratrol was characterized in the blackberry extract for the first time. Stilbenes are relatively non-polar compounds, although the use of polar solvents such as water subjected to microwave irradiation is an excellent option to extract these compounds since water efficiently absorbs the energy in the microwave frequency (de Martins and Alvarez 2010), which may have favored the withdrawal of this compound from the fruit. Flavonoids are the family of polyphenols with the highest number of compounds identified in the blackberry. Flavanols such as (epi)catechins, flavonols including quercetin and kaempferol derivatives, and two flavones, one from apigenin and one from purascenin, were found. Regarding the anthocyanins, five cyanidins with different ligands probably linked in position 3 were identified by the data described in the literature (Lee et al. 2012).

Studies evaluating polyphenol-rich extracts highlight the importance of identifying which individual phenolic compounds are responsible for antioxidant capacity (Jiang et al. 2019; Waterhouse et al. 2017). However, few phenolic

compounds were identified in MHG-obtained fruit extracts, as only four flavonoids were observed in the sea buckthorn pomace extract (Périno-Issartier et al. 2011). On the other hand, grape (Al Bittar et al. 2013) and plum (Cendres et al. 2012) extracts exhibited ten phenolic compounds, and orange extracts (Boukroufa et al. 2015) had two phenolic compounds. This is because these compounds were determined by comparing the retention time with analytical standards, limiting the identification of the compounds present in the extract. Therefore, this work is a pioneer in the complete identification of phenolic compounds present in MHG-obtained fruit extracts.

The MHG method reduced the total aerobic mesophilic content found in the fresh fruit (4.6×10^2 CFU/g), obtaining an extract with 2.9×10^1 CFU/ml. This result is due to the effects of microbial inactivation caused by microwaves. Therefore, this technology is used in pasteurization and sterilization processes with the advantage that the pathogenic microorganisms are sensitive to microwaves (Bozkurt-Cekmer and Davidson 2016). In the extract, the absence of *Salmonella* spp. (25 mL) and coliforms (< 10 MPN/mL) were confirmed in the presumptive test, showing that it did not present total or thermotolerant coliforms in this product. Thus, low total mesophyll counts and the absence of *Salmonella*, total coliforms, and thermotolerant in the product

Table 3 Retention time and mass spectra to attempt to identify non-anthocyanin and anthocyanin phenolic compounds in blackberry extracts obtained by MHG

Non-anthocyanin phenolic compounds			
RT (min)	Molecular ion (*m/z*)	Formula molecular	Proposed compound
4.9	353.0878	$C_{16}H_{17}O_9$	Chlorogenic acid isomer 2
5.4	353.0878	$C_{16}H_{17}O_9$	Chlorogenic acid isomer 3
8.9	513.1461	$C_{19}H_{29}O_{16}$	UK
9.2	243.0617	$C_{14}H_{11}O_4$	Hydroxyresveratrol
9.5	180.0666	$C_9H_{10}NO_4$	UK
10.1	331.0671	$C_{13}H_{15}O_{10}$	Gallic acid Hexoside
14.2	368.0987	$C_{16}H_{18}NO_9$	UK
17.3	341.0878	$C_{15}H_{17}O_9$	Caffeoyl hexoside
17.9	203.0925	$C_9H_{15}O_5$	UK
18.6	289.0608	$C_{15}H_{13}O_6$	Catechin
21.0	447.0933	$C_{21}H_{19}O_{11}$	Kaempferol hexoside isomer 1
21.2	465.1038	$C_{21}H_{21}O_{12}$	Catechin hexuronide
21.8	289.0718	$C_{15}H_{13}O_6$	Epicatechin
22.8	431.1923	$C_{20}H_{31}O_{10}$	UK
23.3	401.0878	$C_{20}H_{17}O_9$	Apigenin pentoside
23.8	461.1664	$C_{23}H_{29}O_{10}$	Purpurascenin
28.1	477.0675	$C_{21}H_{17}O_{13}$	Quercetin hexuronide
28.2	433.0412	$C_{19}H_{13}O_{12}$	Ellagic acid pentoside
28.3	463.0882	$C_{21}H_{19}O_{12}$	Quercetin hexoside
28.4	609.1461	$C_{27}H_{29}O_{16}$	Rutin
29.0	607.1305	$C_{27}H_{27}O_{16}$	Quercetin 3-O-[6”-O-(3-hydroxy-3-methylglutaroyl)-β-D-galactoside]
30.0	300.9990	$C_{15}H_5O_8$	Ellagic acid
39.3	503.3378	$C_3H_{47}O_6$	UK
Anthocyanin phenolic compounds			
TR (min)	Molecular ion and ion product (*m/z*)	Proposed compound	
4.847	449-287	Cyanidin-3-glycoside	
4.848	419-287	Cyanidin-3-xyloside	
6.112	595-287	Cyanidin-3-rutinoside	
9.509	535-287	Cyanidin-3-malonylglycoside	
10.686	593-287	Cyanidin-3-dioxalylglycoside	

[a] Unidentified (UK)

were found. Although Brazilian legislation does not include the total aerobic mesophilic counts for extract/fruit juices (ANVISA 2001), most pathogenic microorganisms present in foods belong to the mesophilic group (Húngaro et al. 2014), making this analysis interesting because it is used as a food quality indicator (Lavinas et al. 2006). Compared to the limits of water (5×10^2) quantification (ANVISA 2001), the total mesophyll content of the blackberry extract was considered low since it presented a logarithmic cycle shorter than the one specified for this beverage.

Phenolic Composition of Extract Fractions

In general, the phenolic compound content and antioxidant capacity of extract fractions obtained by MHG decreased significantly in fraction 5 (80 mL of the collected extract). We observed that the last 40 mL of the blackberry extract (fraction 5 and 6) only accounted for 4% of the antioxidant capacity of the whole extract (Fig. 5). This was expected since the lower contents of all phenolic compounds were also found in these

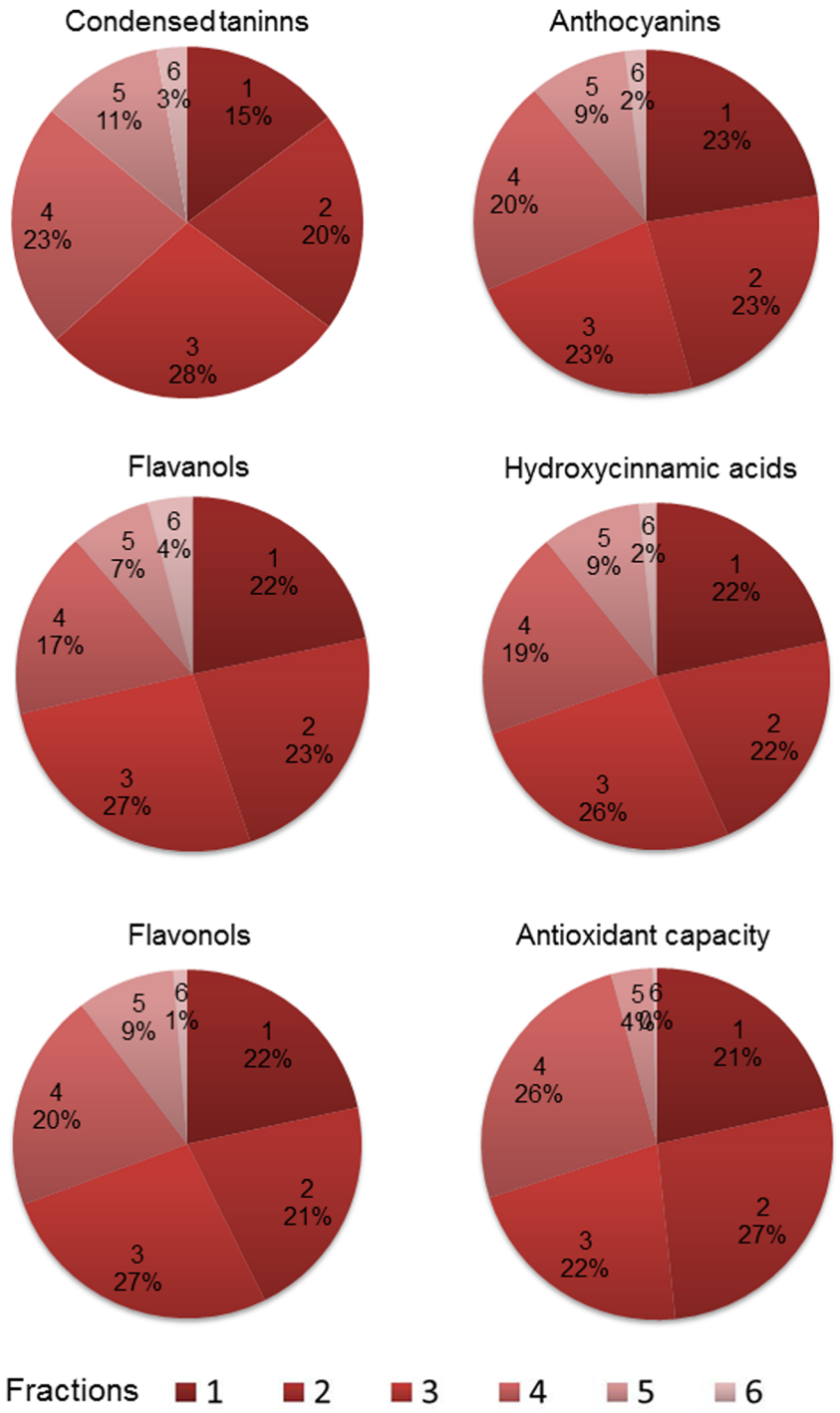

Fig. 5 Phenolic compounds and antioxidant capacity of the fractions obtained by MHG. Values expressed in % of the total extract

two fractions (Table 4). During the collection of the last two fractions, the condenser of the equipment was clear and with few red-colored drops, which is different from the first fractions (1, 2, 3, and 4). This is because, in the last fractions (5 and 6), the evaporated water condensed in the glass container lid and returned to the liquid state, falling by gravity in the last mL of extract.

The less intense color (with a higher white tendency and lower blue tendency) of fraction 6 (Fig. 6 and Table 5) is due to decreased anthocyanins, which are the main phenolic compounds of the blackberry and responsible for the red coloration and found in small proportions in this fraction. Fraction 1 was collected at 4:26 min, followed by 5:51 for fraction 2, 6:07 for fraction 3, 8:09 for fraction 4, 9:26 for fraction 5, and 10 min for fraction 6. Higher phenolic concentrations and antioxidant capacity were recovered in the first 80 mL of extract and obtained with 8:10 min of extraction, decreasing by 2 min. The results presented here corroborate Cendres et al. (2014), who reported that the most soluble molecules, such as anthocyanins, flavonoids, catechins, and phenolic acids, are extracted at higher concentrations in the first fractions of the extract. In addition, Ravi et al. (2018) also pointed out that the highest concentrations of phenolic compounds are found in the initial fractions and that the initial fractions of the blueberry extract, which have color, are the fractions that carry the phenolic compounds, while at the end of the extraction, only in situ water is obtained.

Besides compound solubility, the place (flesh or skin) where these compounds are present also interferes with their diffusion. In the case of the blackberry that has no skin, the diffusion of phenolic compounds only occurs in the initial and intermediate fractions, thus being different from grapes and cherries since the highest phenolic compound concentrations are located on the skin and therefore were found in the last fractions of the extract (Cendres et al. 2014).

Characterization of the Coproduct from MHG Extraction

At the end of the process, the fruit is shrunken and deformed in the MHG glass container due to the considerable removal of its liquid content, although the extraction coproduct may also have bioactive potential. Significant concentrations of condensed tannins (52 ± 5 mg epicatechin/100 g of coproduct), anthocyanins (120 ± 14 mg of cyanidin-3-glycoside/100 g of coproduct), flavanols (12.3 ± 0.6 mg of epicatechin/100 g of coproduct), hydroxycinnamic acids (1.7 ± 0.2 mg of chlorogenic acid/100 g of coproduct), and flavonols (21 ± 2 mg of quercetin-3-glycoside/100 g of coproduct) were detected, which may explain its high antioxidant capacity (9326 ± 1370 μmol of trolox/100 g of coproduct). Furthermore, the phenolic compounds and antioxidant capacity were superior fruit *in nature* as large amounts of water were removed and resulted in the concentration of these compounds.

Comparison of the antioxidant capacity with the fruit *in natura* shows that the coproduct has approximately 4 times more antioxidant capacity (Moraes et al. 2020). The consumption of 100 g of this coproduct may provide more antioxidant compounds than 100 g of the fruit *in natura*. Moreover, the MHG can be used for partially drying fruits in order for both products (extract and coproduct) to be used (Farias et al. 2021).

Regarding the microbiological analysis, total aerobic mesophiles (< 10 CFU/g), *Salmonella*/25 g, and total and thermotolerant coliforms (< 10 MPN/g) were not present in the material. This is due to the fruit remaining 10 min in contact with the microwave radiation that led to eliminating these microorganisms as previously described. Based on the mentioned facts, the coproduct of the extraction can be applied in other foods, incorporating a high phenolic compound content and obeying the fourth principle of green chemistry, which prioritizes using the coproducts of the extraction (Chemat et al. 2012).

Table 4 Phenolic compounds and antioxidant capacity of blackberry extract fractions obtained by MHG

Fractions	Condensed tannins[1]	Antioxidant capacity[2]	Anthocyanin[3]	Flavanols[1]	Hydroxycinnamic acids[4]	Flavonols[5]
1	20 ± 2^{bc}	$23{,}029 \pm 644^{a}$	494 ± 13^{a}	39 ± 4^{ab}	4.5 ± 0.4^{a}	39 ± 4^{a}
2	29 ± 2^{ab}	$28{,}657 \pm 1429^{a}$	507 ± 33^{a}	39.2 ± 0.4^{ab}	4.5 ± 0.4^{a}	38 ± 3^{a}
3	41 ± 3^{a}	$23{,}257 \pm 1308^{a}$	499 ± 45^{a}	48 ± 2^{a}	5.56 ± 0.09^{a}	48 ± 5^{a}
4	32 ± 4^{ab}	$27{,}437 \pm 1457^{a}$	443 ± 44^{a}	31 ± 2^{b}	4.07 ± 0.09^{a}	36 ± 2^{a}
5	15 ± 1^{bc}	4090 ± 291^{b}	201 ± 16^{b}	13 ± 1^{c}	1.9 ± 0.1^{b}	16 ± 1^{b}
6	1.7 ± 0.2^{c}	431 ± 10^{b}	44 ± 4^{b}	7.4 ± 0.7^{c}	1.3 ± 0.4^{b}	2.17 ± 0.08^{b}

[1] mg epicatechin/L extract. [2] μmol trolox/L extract. [3] mg of cyanidin-3-glycoside/L extract. [4] mg of chlorogenic acid/L extract. [5] mg of quercetin-3-glycoside/L extract. Mean value ± standard deviation ($n = 3$). The averages followed by the same lower case letter do not differ by Duncan's test ($p < 0.05$). Coefficient of variation < 15%

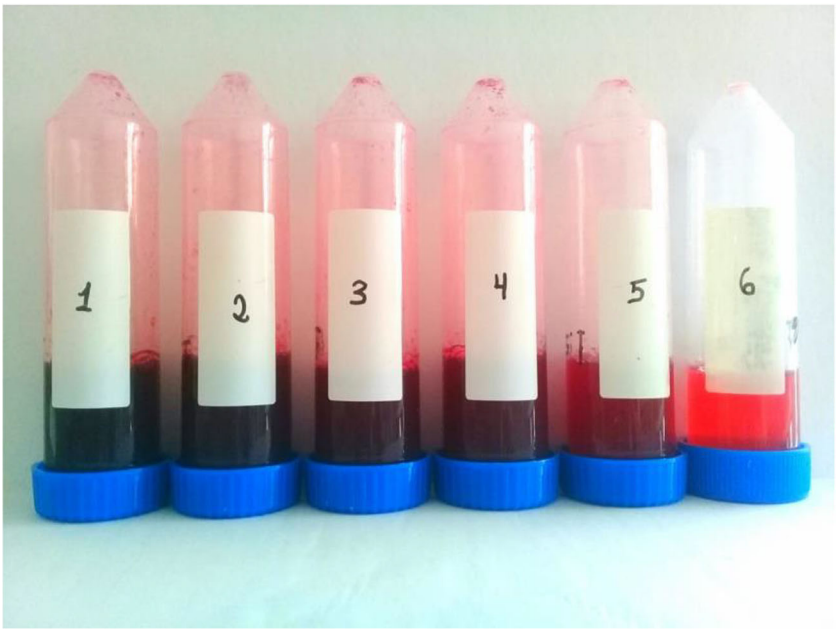

Fig. 6 Different fractions collected with MHG

Antioxidant Activity

In order to analyze if the extract or its coproduct can act directly by ROS scavengers, ROS production was measured in wild-type worms through H_2DCFDA oxidation. First, a concentration curve was made to determine the blackberry concentration capable of reducing ROS production in either basal or juglone-induced oxidative stress conditions. The concentrations analyzed were 5, 10, and 50 μg/mL for the blackberry extract.

In basal conditions, only 50 μg/mL of blackberry extracts are significantly reduced the in vivo ROS production in this system compared with the control basal group (Fig. 7a). Moreover, 5 and 10 μg/mL of the extract did not reduce ROS basal levels. Similar to other extracts with high antioxidant-rich flavonoid concentrations (Bonomo et al. 2014), the blackberry extract had antioxidant activity in basal conditions in *C. elegans*, and this activity starts at 50 μg/mL of flavonoids.

In addition to the ROS production during basic cellular processes, an imbalance between reactive and antioxidant defense systems can lead to oxidative stress and damage cellular components (Halliwell 2008). Oxidative stress has consequently been involved in several causes of cellular impairments associated with the pathophysiology of several disorders (Halliwell 2012). Therefore, to evaluate the protective effects of the extracts against excessive ROS, *C. elegans* was treated with a pro-oxidant juglone to increase *C. elegans* ROS production. The juglone per se increased ROS production by 59.3% compared to the control basal group (Fig. 7a). The worms pretreated for 1 h with different extract concentrations were protected against increased ROS production generated by the juglone. The treatments with 5, 10, and 50 μg/mL of extract reduced 32.4, 27.5, and 33.4% of ROS production, respectively, compared to the juglone control group (Fig. 7a).

Other extracts with flavonoids have demonstrated antioxidant activity against juglone-induced toxicity in conditions of exposure to oxidant stressors, such as *Ilex paraguariensis* (Machado et al. 2018b). These results suggest that the pretreatment with blackberry extract was efficient in preventing increased ROS generated by juglone. Apparently, under physiological situations, when ROS levels are normal, only higher concentrations of blackberry can reduce basal ROS production. However, during oxidative stress, lower concentrations of blackberry are enough to detoxify the increased ROS levels.

Since the coproduct showed phenolic composition and antioxidant capacity in vitro, its capacity to detoxify the *C. elegans* ROS production was also evaluated. Based on

Table 5 Color parameters of the fractions of blackberry extracts obtained by MHG

Fractions	*L**	*a**	*b**
1	37.2 ± 0.8^{b}	4.5 ± 0.4^{b}	-1.12 ± 0.09^{c}
2	37.2 ± 0.5^{b}	3.9 ± 0.3^{b}	-0.91 ± 0.09^{c}
3	37.22 ± 0.09^{b}	3.7 ± 0.3^{b}	-0.49 ± 0.03^{bc}
4	37.4 ± 0.2^{b}	4.5 ± 0.2^{b}	-0.85 ± 0.05^{c}
5	38.6 ± 0.8^{b}	8.3 ± 0.3^{b}	1.1 ± 0.1^{b}
6	49.4 ± 0.9^{a}	17.5 ± 0.4^{a}	3.6 ± 0.3^{a}

L*, luminosity; a*, positive values indicate a tendency to red and negative green; b*, positive values indicate a tendency to yellow and negative to blue

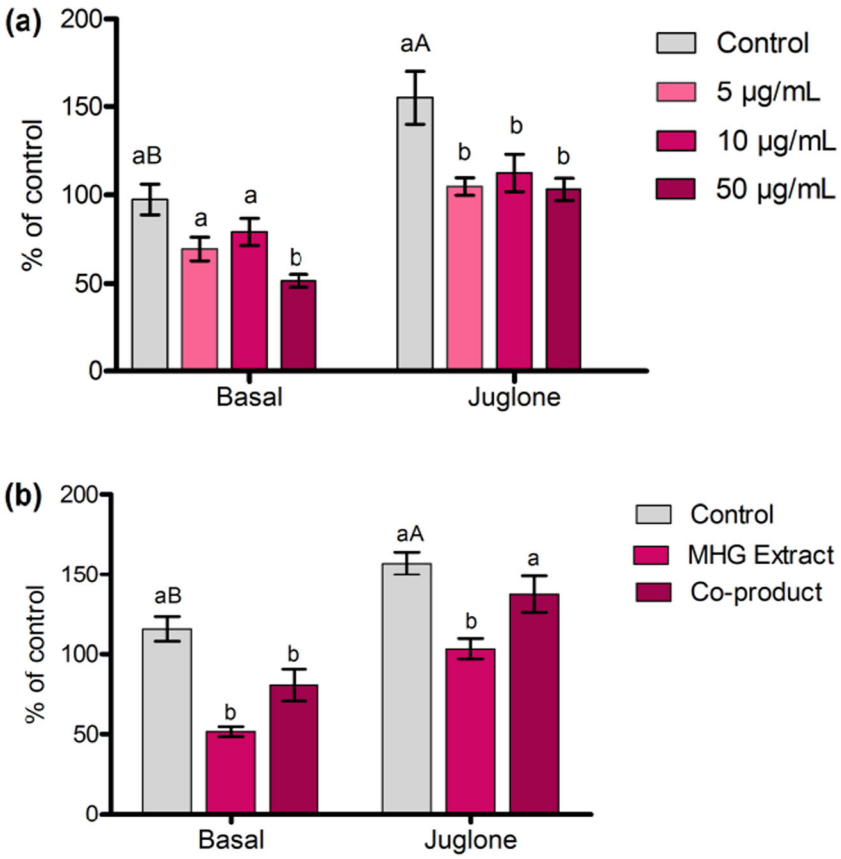

Fig. 7 Effects of blackberry (MHG extract) and its coproduct on ROS production in *Caenorhabditis elegans*. Data are expressed as a percentage of the arbitrary fluorescence unit (AFU) of control of each experiment derived from 3 independent assays ($n = 3$). Levels of ROS production in wild type worms pretreated for 1 h with (**a**) 5, 10, and 50 μg/mL for blackberry extract and (**a**) 50 μg/mL of blackberry extract or its coproduct exposed or not to 50-μM juglone. Lower case letters are compared with each control group ($p < 0.05$), and capital letters are compared between control groups ($p < 0.05$) (two-way ANOVA followed by Tukey's post test)

the previous blackberry curve concentration (Fig. 7a), only the concentration capable of detoxifying ROS production under basal and stress conditions in *C. elegans* was chosen (50 μg/mL flavonoids). Thus, we analyzed wild-type worms treated with the coproduct under basal and stress conditions at 50 μg/mL. In basal conditions, the coproduct significantly reduced the in vivo ROS production of the animals by 30.1% compared to the control basal group (Fig. 7b), while under juglone-induced oxidative stress, the coproduct significantly reduced by 18.9%. In addition to its antioxidant capacity, the effects were not as preeminent as the 50 μg/mL of extract, which reduced the ROS production by 55.3 and 34.2%, respectively, under basal and juglone-induced oxidative stress.

Economic Analysis

Economic evaluation is a critical analysis that confers valuable information to support process decisions and encourage further business application advancement. In this study, the economic evaluation provides COM results and detailed costs (Fig. 8) to process blackberries at laboratory or pilot scales. These parameters are the primary responses evaluated in this area (Hatami et al. 2019). In this case, the promising extraction of MHG was compared with the exhaustive extraction. The COM was presented based on the extract volume, gallic acid (GAE) mass in the extract, or mass of cyanidin-3-glycoside (CGE) in the extract (Fig. 8). These scenarios were evaluated because the composition of the extracts was different in the exhaustive and MHG extractions.

The COM was lower for MHG extraction and accounted for only US$ 0.25 per 100 mL of extract or US$ 1.99 for each gram of GAE in the extract. These financial results corroborate the experimental results, in which the antioxidant capacity was higher and the phenolic compounds more concentrated in MHG extracts. Lower COM for the MHG extracts is also attributed to the processing time, which was shorter than the exhaustive extraction. Consequently, a more concentrated extract can be obtained by this promising method during a daily shift.

When comparing the scales, the pilot scale presented lower COM than the laboratory scale, which was expected as some extra expenses may be optimized. For example, the same worker can operate equipment that process 1 kg/h or 100 kg/h. Naturally, an additional worker may be required to perform additional activities (pretreatment, storage, among others) for more extensive capabilities. However, a 100-fold increase in capacity does not require a 100-fold increase in the number of workers. Therefore, the percentage contribution of COL on COM is reduced. Here, the COL was in the range of 54.62 to 79.35% for the laboratory scale and 0.95 to 3.19% for the pilot scale (Fig. 8). Despite the economic advantages of MHG due to reduced energy and time consumption (Périno-Issartier

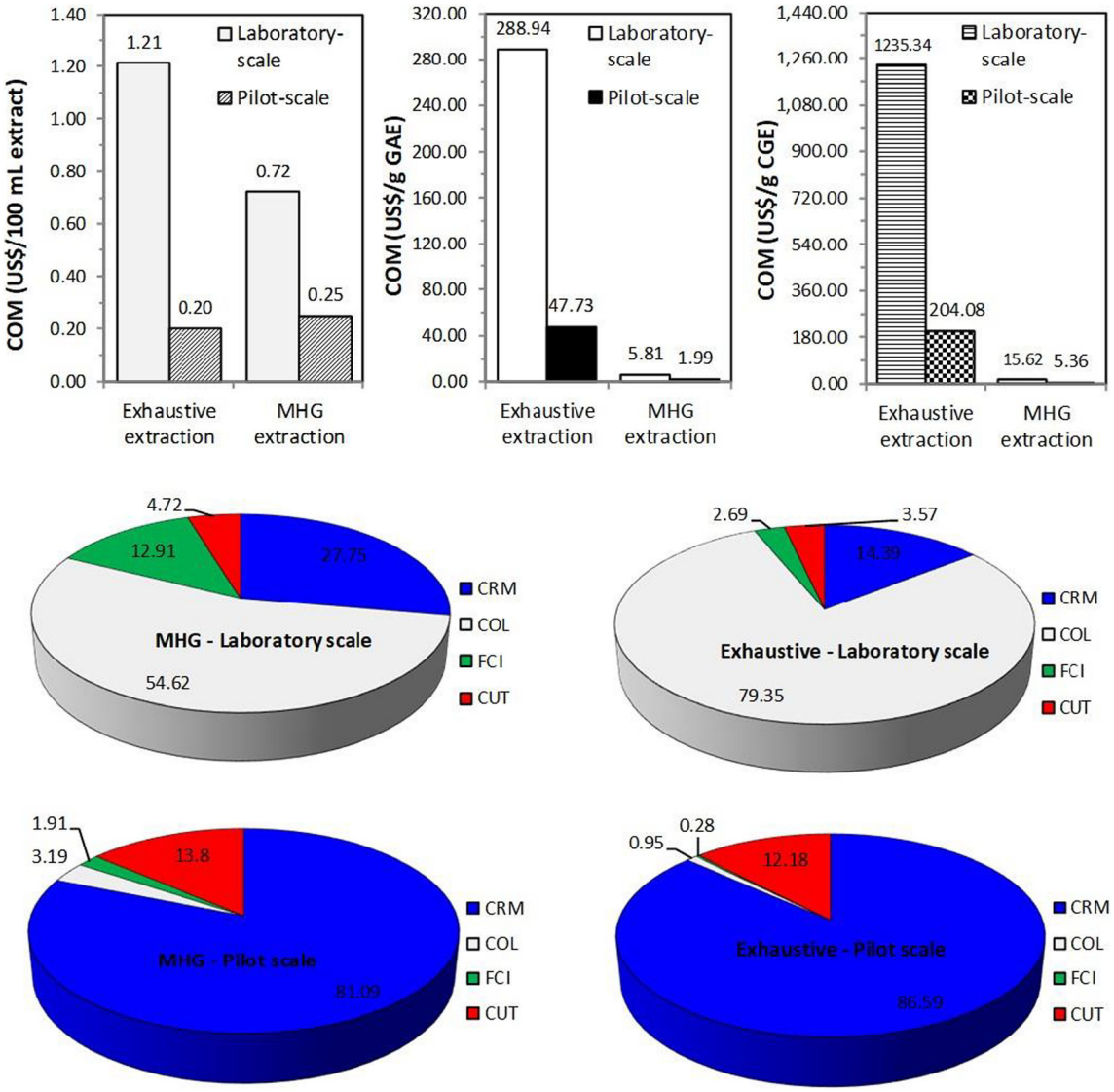

Fig. 8 COM and percent contribution (%) of itemized costs (CRM, COL, FCI, and CUT) of extracts obtained by exhaustive extraction and MHG extraction at laboratory or pilot scales; GAE, gallic acid equivalent; CGE, cyanidin-3-glycoside equivalent

et al. 2011), it was impossible to find any economic assessment made for the extraction of phenolic compounds by MHG to compare to our results. Nevertheless, the use of other promising methods to obtain fruit phenolic compounds (such as extraction with supercritical fluids) for application in food-related areas demonstrates similar behaviors in terms of COM reduction as obtained in this study (Zabot et al. 2017).

The influence of scaling on the percentages of itemized costs is shown in Fig. 8. The CRM increased in the pilot scale (up to 86.59%) because a 100-fold increase in capacity needs 100 times more blackberry. The CUT also increased in the pilot scale due to the consumption of electricity, water, and other utilities directly related to the number of processed blackberries. Although MHG equipment is more expensive than conventional equipment for exhaustive extraction, the FCI did not exceed 1.91% of the COM at a pilot scale. Hence, the main point to be negotiated is the cost of buying the fruits. If significantly reduced, the COM is also significantly reduced, making the process even more attractive.

Conclusion

This study proved the efficiency of MHG to obtain a blackberry extract with high phenolic compounds concentration and antioxidant capacity, where the power of 500 W was used to obtain the extract in approximately 10 min. We also noticed that phenolic compound content and antioxidant capacity reduced in the last fractions obtained in the process; thus, it is possible to reduce the extraction time to 8 min. The MHG extract had low total mesophyll counts and the absence of *Salmonella* spp., total coliforms and thermotolerant coliforms.

In addition, the extract presents high antioxidant capacity and is mainly composed of five anthocyanins and seventeen non-anthocyanin phenolic compounds, among which the stilbene hydroxyresveratrol was identified for the first time in blackberry extracts. Compared to the exhaustive extraction, the MHG presented low cost in obtaining the extract, manufacturing cost, and higher concentration of phenolic compounds and antioxidant capacity. Furthermore, the technique allows obtaining a coproduct with potential for application in foods because it contains a high concentration of phenolic compounds and the absence of mesophilic, *Salmonella* spp., and total coliform microorganisms and thermotolerant coliforms. The MHG is a sustainable extraction process in which no type of solvent is added and no unnecessary generation of residues occurs as the blackberry fruits are harvested in their entirety. These findings present a fast and green method to extract phenolic compounds from blackberries in which the extract and coproduct obtained in this process may present potential application for the food industry, being consumed as juice or applied directly to other foods as a functional ingredient.

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Data Availability Not applicable.

Compliance with ethical standards

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

Code Availability Not applicable.

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