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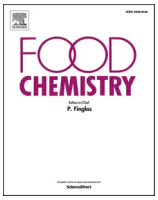

Optimization of heat and ultrasound-assisted extraction of *Eucalyptus globulus* leaves reveals strong antioxidant and antimicrobial properties

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ABSTRACT

The extraction of phenolic compounds from eucalyptus leaves was optimized using heat and ultrasound-assisted techniques, and the bioactive potential of the resulting extract was assessed. The independent variables, including time (t), solvent concentration (S), and temperature (T) or power (P), were incorporated into a five-level central composite design combined with Response Surface Methodology. Phenolic content was determined by HPLC-DAD-ESI/MS and used as response criteria. The developed models were successfully fitted to the experimental data to identify the optimal extraction conditions. Heat-assisted extraction proved to be the most efficient method for phenolic recovery, yielding 27 ± 2 mg/g extract under optimal conditions (120 min, 76.5 °C, and 25 % ethanol, *v*/v). The extracts exhibited a high concentration of phenolic glycoside derivatives, including gallotannin, quercetin, and isorhamnetin. These findings suggest that the extracts hold promise as natural additives in food technology, owing to their moderate antimicrobial activity and strong antioxidant properties.

1. Introduction

Plant-derived additives have gained significant attention in food development as natural solutions to mitigate deterioration caused by chemical and biological agents. The industrial processing of plants generates substantial byproducts, underscoring the need to explore the chemical composition of these materials for enhanced valorization (Faustino et al., 2019).

Eucalyptus globulus, a member of the Myrtaceae family, is extensively cultivated in subtropical and Mediterranean climates and has long been valued for its medicinal and therapeutic properties (Fernández-Agulló et al., 2015). *E. globulus* extract has gained attention in the food industry for its preservative potential, distinctive aromatic properties, and associated health benefits, making it a promising component for various food and beverage applications.

Recent studies have explored *E. globulus* as a food additive. For example, Manzi et al. (2022) incorporated the extract into passion fruit juice to extend shelf life by inhibiting microbial growth and slowing oxidation. Another study examined the antioxidant effects of eucalyptus leaf extract on chicken meat quality, showing that it significantly reduced lipid oxidation and improved in vitro antioxidant capacity. Furthermore, in vivo results indicated enhanced meat quality traits, such as tenderness, juiciness, and flavor, with a reduction in lipid oxidation (H. Li & Cao, 2020).

To better investigate its preservative action, some authors explored the diverse phenolic profile of *E. globulus* extracts, which include flavonoids, phenolic acids, and gallotannins. Teixeira et al. (2019) identified 18 polyphenols in aqueous extracts, with digalloyl-glucoside and 5-*O*-caffeoylquinic acid being the most abundant compounds. Gomes et al. (2018) confirmed the presence of 19 phenolic compounds, primarily flavonoids (quercetin derivatives) and phenolic acids. The study also identified critical compounds such as pentagalloyl-glucoside and tetragalloyl-glucoside. Boulekbache-Makhlouf et al. (2013) further classified phenolic compounds into gallotannins, ellagitannins, and flavonol derivatives, identifying compounds like tellimagrandin II and eucalbanin C in *E. globulus* extracts.

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Conventional extraction techniques, such as heat-assisted extraction (HAE), have been widely used to recovery phenolic compounds from plant materials. HAE typically involves the application of heat to increase the solubility and diffusion of compounds from plant matrices, but it is associated with higher energy consumption, longer extraction times, and the use of organic solvents (Gullón et al., 2017). These factors raise concerns about sustainability and environmental impact.

In contrast, ultrasound-assisted extraction (UAE) has emerged as a more sustainable alternative. UAE utilizes high-frequency sound waves to induce cavitation in the extraction medium, enhancing the release of phenolic compounds by disrupting plant cell walls. This technique offers several advantages over traditional methods, including higher extraction yields, shorter extraction times, reduced energy consumption, and the use of aqueous solvents instead of harmful organic solvents (Lavilla & Bendicho, 2017). Despite challenges in process optimization and scalability, UAE is considered a promising green technology for the extraction of phenolic compounds, aligning with the growing demand for eco-friendly solutions in the food industry (Bhuyan et al., 2017).

This study aims to optimize selected process variables to maximize the recovery of phenolic compounds from *E. globulus* leaves using heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE). Additionally, the bioactive properties of the extracts obtained under optimal conditions were evaluated. By providing a proof of concept, this study contributes to the understanding of potential applications of these extracts in food products and underscores the importance of bioactive property evaluation in developing novel food applications.

2. Material and methods

2.1. Plant material

Eucalyptus globulus Labill. leaves were collected in Águeda, Portugal, and identified by a botanist of the Instituto Politécnico de Bragança. After reaching the laboratory, the leaves were powdered to approximately 20 mesh to guarantee sample homogeneity and stored in an airtight flask, protected from light and moisture, until further analysis.

2.2. Standards and reagents

HPLC-grade formic acid and acetonitrile were supplied by Fisher Scientific (Lisbon, Portugal). Sulphorhodamine B, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2′-azobis(2-methylpropionamidine), ellipticine, trichloroacetic acid, and tris(hydroxymethyl)aminomethane were acquired from Sigma-Aldrich (St Louis, MO, USA). Standards of phenolic compounds (*p*-hydroxybenzoic acid and quercetin-3-*O*-glucoside) were obtained from Extrasynthèse (Genay, France). Dulbecco's modified Eagle's medium (DMEM) medium was obtained from Hyclone (Logan, Utah, USA). Other reagents and solvents of analytical grade were obtained from common sources. Water was treated with a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Experimental design for extraction optimization

A five-level central composite design (CCD), combined with response surface methodology (RSM), was employed to optimize the extraction of phenolic compounds from *Eucalyptus globulus* leaves. The coded and actual values for the independent variables – X_1 (extraction time, t, in minutes), X_2 (temperature, T, in °C, or power, P, in W), and X_3 (solvent proportion, S, in % ethanol, *v*/v) – are presented in Table 1. The 28 experimental points in the CCD, as shown in Table 2, were generated using MATLAB R2023a (MathWorks, Inc., Natick, MA, USA). This rotatable design incorporated six replicated center points, and a set of axial points configured to maintain rotatability, ensuring that model prediction variance remains constant for all points equidistant from the center. Experimental runs were randomized to mitigate the effects of unanticipated variability in response data.

Table 1
Natural and coded values of the independent variables used in the 5-level central composite designs (CCD) implemented for optimization of heat- and ultrasound-assisted extraction methods using the response surface methodology (RSM).

Coded values	Natural values					
	Heat-assisted extraction (HAE)			Ultrasound-assisted extraction (UAE)		
	Time X_1 (min)	Temperature X_2 (°C)	Solvent proportion X_3 (%, *v*/*v*)	Time X_1 (min)	Power X_2(W)	Solvent proportion X_3 (%, *v*/*v*)
−1.68	20	25	0	3	100	0
−1	40	37	20	11.5	180	20
0	70	55	50	24	300	50
1	100	73	80	36.5	420	80
1.68	120	85	100	45	500	100

2.4. Extraction methods

2.4.1. Heat-assisted extraction

Heat-assisted extraction (HAE) was conducted in a thermostated water bath using sealed vessels to prevent solvent evaporation. Ethanol was selected as the solvent for its low toxicity and high efficiency in recovering phenolic compounds (Boulekbache-Makhlouf et al., 2013). Powdered eucalyptus leaves (1.5 g) were combined with 50 mL of ethanol-water solvent and subjected to continuous magnetic stirring at 150 rpm, according to the conditions defined in Table 2, varying in extraction time (*t*: 20–120 min), temperature (*T*: 25–85 °C), and solvent proportion (*S*: 0–100 %). The solid-to-liquid ratio (S/L) was maintained at 30 g/L.

Following extraction, the mixture was filtered (Filter paper, F1004 grade, ∅ = 125 mm, CHMLAB, Barcelona, Spain), and the solvent was evaporated using a rotary evaporator (Büchi RE-121 with Büchi 461 water bath, Flawil, Switzerland) at 42 °C and 100 rpm under reduced pressure. The samples were then lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) at −45 °C and 0.04 mBar until fully dried.

2.4.2. Ultrasound-assisted extraction

The ultrasound-assisted extraction (UAE) was performed using an ultrasonic system (Ultrasonic homogenizer, model CY-500, Optic Ivymen Systen, Barcelona, Spain) equipped with a titanium probe and a connector for the sample temperature control. Powdered eucalyptus leaves (1.5 g) were placed in a beaker with 50 mL of solvent and processed according to the experimental runs in Table 2, where different levels of t (3–45 min), P (100–500 W; at a frequency of 20 kHz) and S (0–100 %) are combined. The S/L was kept constant (50 g/L), as well as T (30–35 °C; a water bath was used to avoid heating the samples). After extraction, the samples were filtered, the solvent evaporated, and the residues lyophilized according to the procedure described in Section 2.4.1.

2.5. Response variable analysis for extraction process optimization

2.5.1. Evaluation of the extraction yield

The extract weight resulting from each extraction was determined gravimetrically in crucibles by evaporation of 5 mL of supernatant (extract solution) at 105 °C for 72 h. The results were expressed as percentage of crude extract per plant material (%, *w*/w).

2.5.2. Chromatographic analysis of phenolic content

Chromatographic analysis was conducted using a high-performance liquid chromatography system equipped with diode array detection and electrospray ionization mass spectrometry (HPLC-DAD-ESI/MS^2; Dionex

Table 2
Experimental results for extraction yield and the contents of phenolic acids, flavonoids, and total phenolic content under the extraction conditions defined by the central composite design (CCD) matrix, evaluated across HAE and UAE. The natural values of the independent variables X1 (time), X2 (temperature or power), and X3 (solvent proportion) are provided in Table 1.

Run	CCD design			Heat-assisted extraction (HAE)				Ultrasound-assisted extraction (UAE)			
	Coded values			Yield	TPA	TFC	TPC	Yield	TPA	TFC	TPC
	X_1	X_2	X_3	(Y_1, %)	(Y_2, mg HBA .g − 1 dw)	(Y_3, mg Q3Glc.g − 1 dw)	(Y_4, mg.g − 1 dw)	(Y_1, %)	(Y_2, mg HBA .g − 1 dw)	(Y_3, mg Q3Glc.g − 1 dw)	(Y_4, mg.g − 1 dw)
1	1	−1	1	30.37	8.48	5.33	13.81	11.38	7.36	1.94	9.3
2	1	−1	−1	22.43	9.71	5.29	15	15.74	9.84	4.01	13.85
3	−1	−1	−1	22.77	9.06	5.1	14.16	18.73	9.81	3.5	13.31
4	-1	-1	1	28.4	8.08	4.5	12.57	22.16	9.61	3.78	13.39
5	-1	1	1	32.17	13.36	5.05	18.41	18.81	9.64	3	12.63
6	-1	1	-1	24.47	13.77	5.53	19.29	19.21	10.77	3.7	14.47
7	1	1	1	35.57	13.87	5.98	19.85	19.14	9.56	3.16	12.72
8	1	1	-1	25.7	15.1	5.44	20.54	21.32	12.49	3.92	16.41
9	−1.68	−1.68	−1.68	18.77	7.07	4.92	11.98	22.3	12.07	3.67	15.74
10	−1.68	−1.68	1.68	18.6	5.29	2.39	7.68	27.17	9.47	3.96	13.44
11	1.68	−1.68	1.68	22.33	6.89	2.72	9.61	22.74	8.98	3.91	12.89
12	1.68	−1.68	−1.68	19.67	7.73	5.13	12.86	17.55	6.99	3.34	10.33
13	−1.68	1.68	1.68	25.27	9.92	3.76	13.68	15.26	9.14	3.42	12.55
14	1.68	1.68	1.68	36.03	9.11	4.34	13.44	17.31	6.21	3.33	9.54
15	1.68	1.68	−1.68	27.3	21.12	6.18	22.12	16.57	5.28	2.57	7.85
16	−1.68	1.68	−1.68	24.64	18.33	6.32	22.37	22.36	7.54	3.28	10.82
17	0	1.68	0	32.17	14.77	5.12	19.9	25.05	8.02	3.6	11.62
18	1.68	0	0	29.63	16.98	7.48	24.46	8.34	0.59	0.92	1.5
19	−1.68	0	0	27.47	16.67	6.35	23.01	13.43	1.08	1.25	2.33
20	0	0	−1.68	20.3	11.15	5.65	16.8	17.29	4.28	2.15	6.43
21	0	0	1.68	24.87	8.31	3.6	11.92	23.17	3.92	2.38	6.29
22	0	0	0	29.03	12.03	5.69	17.71	18.76	3.81	1.59	5.4
23	0	0	0	30.2	12.29	5.84	18.13	24.6	10.09	3.64	13.73
24	0	0	0	29.3	13.5	5.8	19.3	24.42	10.27	3.63	13.9
25	0	0	0	28.7	13.57	5.79	19.35	22.46	10.07	3.62	13.68
26	0	0	0	28.87	13.64	5.87	19.51	24.36	10.06	3.61	13.68
27	0	0	0	29.03	13.58	5.69	19.27	24.48	10.15	3.61	13.75
28	0	0	0	29.7	12.88	5.78	18.67	24.78	9.99	3.68	13.66

TPA, total phenolic acids (mg HBA·g^{-1} dw); TFC, total flavonoid content (mg Q3Glc·g^{-1} dw); TPC, total phenolic content (mg·g^{-1} dw); HBA, p-hydroxybenzoic acid equivalent; Q3Glc, quercetin 3-O-glucoside equivalent; dw, dry weight.

Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA). Detection was performed at 280 nm and 370 nm as the preferred wavelengths, in conjunction with a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization source operating in negative mode. Chromatographic separation utilized a Waters Spherisorb S3 ODS-2 C18 column (3 μm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) maintained at 35 °C, with gradient elution using 0.1 % formic acid in water (solvent A) and acetonitrile (solvent B) as the mobile phases. The elution gradient established was isocratic 15 % B (5 min), 15 % B to 20 % B (5 min), 20–25 % B (10 min), 25–35 % B (10 min), 35–50 % B (10 min), and re-equilibration of the column, using a flow rate of 0.5 mL/min. Data acquisition and processing were carried out with the Xcalibur software (Thermo Finnigan, San Jose, CA, USA). Phenolic acids were quantified in mg HBA/g of lyophilized extract (HBA – *p*-hydroxybenzoic acid equivalent) and flavonoids were quantified in mg Q3Glc/g of lyophilized extract (Q3Glc – quercetin 3-*O*-glucoside equivalent).

2.6. Extraction modelling and optimization

2.6.1. Response criteria and mathematical modelling

The response variables selected for optimization were Y_1 (extraction yield), Y_2 (total phenolic acids content, TPA), Y_3 (total flavonoid content, TFC), and Y_4 (total phenolic content, TPC). These variables were modeled to assess the recovery of phenolic compounds from eucalyptus leaves. The response surface models were fitted using a least-squares approach to the following second-order polynomial equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>1}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (1)$$

where Y represents the dependent variable to be modeled; Xi and Xj are the independent variables; $b0$ is the constant coefficient; bi is the coefficient of the linear effect; bij is the coefficient of the interaction effect; bii is the coefficient of the quadratic effect; and n is the number of variables in the model ($n = 3$).

2.6.2. Fitting procedures and statistical analysis

Coefficient estimation and statistical analysis were performed using MATLAB R2023a (MathWorks, Inc., Natick, MA, USA). Analysis of variance (ANOVA) was used to determine the regression coefficients and assess the significance of the data. Only statistically significant terms ($p < 0.05$) were included in the final models. The coefficient of determination (R^2) and the adjusted coefficient of determination (R^2_{adj}) were used to evaluate the model fit, representing the proportion of variability in the response explained by the model (Albuquerque et al., 2020). The significance of the terms was analyzed using the F-value ($p < 0.05$), and the lack of fit test was applied to assess whether the model adequately describes the relationship between the independent variables and the response. A non-significant lack of fit ($p > 0.05$) indicates an adequate model. Response surface plots were generated to visualize the relationship.

2.7. Bioactive properties

Antioxidant, cytotoxic, anti-inflammatory, and antimicrobial

activities were evaluated using the selected extracts, obtained according to the optimized parameters in Section 3.1.

2.7.1. Antioxidant activity

Extracts obtained under optimal conditions were evaluated by two different cell-based assays: inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) and cellular antioxidant activity assay (CAA).

The capacity of the extracts to prevent the thiobarbituric acid (TBA) reactive substances formation, namely malondialdehyde (MDA) produced from lipid peroxidation, was determined using porcine brain cell homogenates, following the protocol in Mandim et al. (2022). The extracts were re-dissolved in water to obtain a solution of 10 mg/mL, which was further diluted to obtain the concentrations to be tested. The color intensity of the MDA-TBA complex was read at 532 nm, and the results were presented in terms of IC_{50} values (μg/ml, half-maximal effective concentration to inhibit the lipid peroxidation). Trolox was used as positive control.

For CAA, the capacity of extracts to prevent the oxidation of intracellular 7′-dichlorodihydrofluorescein (DCFH) was determined according to a methodology previously described by de la Fuente et al. (2022), using a murine macrophage cell line (RAW 246.7). Quercetin was used as the positive control, DMEM were used as the negative control. The results are expressed as the percentage of oxidation inhibition at the maximum concentration tested (2000 μg/mL).

2.7.2. Antiproliferative activity

The antiproliferative activity of the extracts was assessed using the sulforhodamine B (SRB) colorimetric assay, following the method described by Marcelino et al. (2023). The extracts were tested against four tumor cell lines: gastric adenocarcinoma (AGS), colorectal adenocarcinoma (CaCo-2), and breast adenocarcinoma (MCF7), as well as non-tumor cell line African green monkey kidney (Vero), obtained from the Leibniz-Institut DSMZ – German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). Cytotoxicity classification was based on the National Cancer Institute (NCI) guidelines, defining extracts as highly cytotoxic with GI_{50} values $\leq$20 μg/mL, moderately active with GI_{50} values from 21 to 200 μg/mL, weakly active between 201 and 400 μg/mL, and inactive with GI_{50} values >400 μg/mL. Ellipticine (Sigma-Aldrich, St. Louis, MO, USA) served as the positive control, and untreated cell suspension as the negative control. Results were expressed as the concentration of extract required to inhibit 50 % of cell proliferation (GI_{50}, μg/mL).

2.7.3. Anti-inflammatory activity

The anti-inflammatory activity of the extracts was assessed by measuring their ability to inhibit nitric oxide (NO) production induced by lipopolysaccharide (LPS) in a murine macrophage cell line (RAW 264.7), following the procedure described by de Oliveira et al. (2023). The extracts were re-dissolved in water to a final concentration of 8 mg/mL, with concentrations ranging from 6.25 to 400 μg/mL incubated with the macrophage cells. Dexamethasone (50 mM, Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control, and untreated cells (without LPS) served as a negative control. Results were expressed as the extract concentration required to inhibit 50 % of NO production (IC_{50} values, μg/mL).

2.7.4. Antimicrobial activity

The antibacterial activity of the extracts was assessed against a range of Gram-positive and Gram-negative bacteria, following the methodology described by Pires et al. (2017). Minimum inhibitory concentrations (MICs) were determined using a colorimetric assay with p-iodonitrotetrazolium chloride (INT) in a 96-well microplate format. Stock solutions of the extracts were prepared at 20 mg/mL and serially diluted. Each dilution was added to the microplate wells along with bacterial inocula, followed by incubation at 37 °C for 24 h. The MIC was identified as the lowest concentration that inhibited bacterial growth, indicated by the absence of color change. Antifungal activity was tested against *Aspergillus fumigatus* and *Aspergillus brasiliensis* using the protocol described by Fernandes et al. (2022). Fungal spores were cultured on malt agar for 72 h, collected, and added to a 96-well microplate along with serially diluted extract samples. The plates were incubated at 28 °C for 72 h. Minimum fungicidal concentrations (MFC) were estimated by subculturing the compounds and observing growth inhibition over an additional 72 h at 26 °C. Ketoconazole served as the positive control.

3. Results and discussion

3.1. HAE and UAE optimization

3.1.1. CCD experimental data

The experimental results from the 28 runs of the central composite design (CCD) matrix for optimizing heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE) of phenolic compounds from *Eucalyptus globulus* leaves are summarized in the Table 2. The extraction yield, TPA (total phenolic acids), TFC (total flavonoid content), and TPC (total phenolic content) were evaluated under varying extraction conditions.

For HAE, extraction yields ranged from 18.6 % to 36.03 %, with the highest yield achieved in run 14, which combined a high solvent concentration (100 % ethanol) with high temperature (85 °C) and time (120 min). Conversely, the lowest yield was observed in run 10, which employed low ethanol concentration (20.3 %), low temperature (34.2 °C), and short extraction time (21.2 min). TPA values for HAE varied between 5.29 and 21.12 mg HBA/g dw, with run 15 achieving the highest phenolic acid content at high ethanol concentration and moderate time and temperature conditions. TFC and TPC also exhibited notable variations across runs, with maximum TPC (22.37 mg/g dw) observed in run 16 at a high ethanol concentration and prolonged extraction time.

In UAE, yields ranged from 8.34 % to 27.17 %, with run 10 yielding the highest extraction at a high ultrasonic power (500 W), moderate solvent concentration (53.9 % ethanol, *v*/v), and extended time (33 min). The lowest yield (8.34 %) was recorded in run 18 under conditions of low ethanol concentration and short exposure to ultrasonic power. TPA values ranged between 3.81 and 12.49 mg HBA/g dw, with the highest TPA in UAE obtained in run 8 under optimal ethanol concentration and ultrasonic power settings. TFC and TPC followed similar trends, with maximum TPC (16.41 mg/g dw) also recorded in run 8.

HAE and UAE were efficient techniques for obtaining phenolic compounds. UAE uses acoustic waves to enhance the release of compounds, while HAE utilizes high temperatures to accelerate extraction (Gullón et al., 2017; Lavilla & Bendicho, 2017). Both techniques offer advantages by optimizing the extraction process and increasing the recovery of compounds such as phenolics and flavonoids. These methods were selected for this work due to their efficiency in extracting bioactive compounds from plant matrices.

HAE demonstrated higher extraction efficiency for yield, TPA, TFC, and TPC compared to UAE. For both methods, higher ethanol concentrations generally resulted in decreased extraction efficiency, particularly for TPA and TPC. Optimal conditions were achieved by balancing solvent concentration, temperature (or ultrasonic power), and extraction time, as shown in Fig. 1.

3.1.2. Analysis of response surface models

In this study, the response values from Table 2 were fitted to a second-order polynomial model (Eq. 1) to generate mathematical models for each response variable, as shown in Eqs. (2–9). Only statistically significant parameters, identified at a 95 % confidence level ($\alpha = 0.05$), were included in the models (Table 3). The developed models effectively describe the effects of the variables on the evaluated responses, as demonstrated by their statistical robustness.

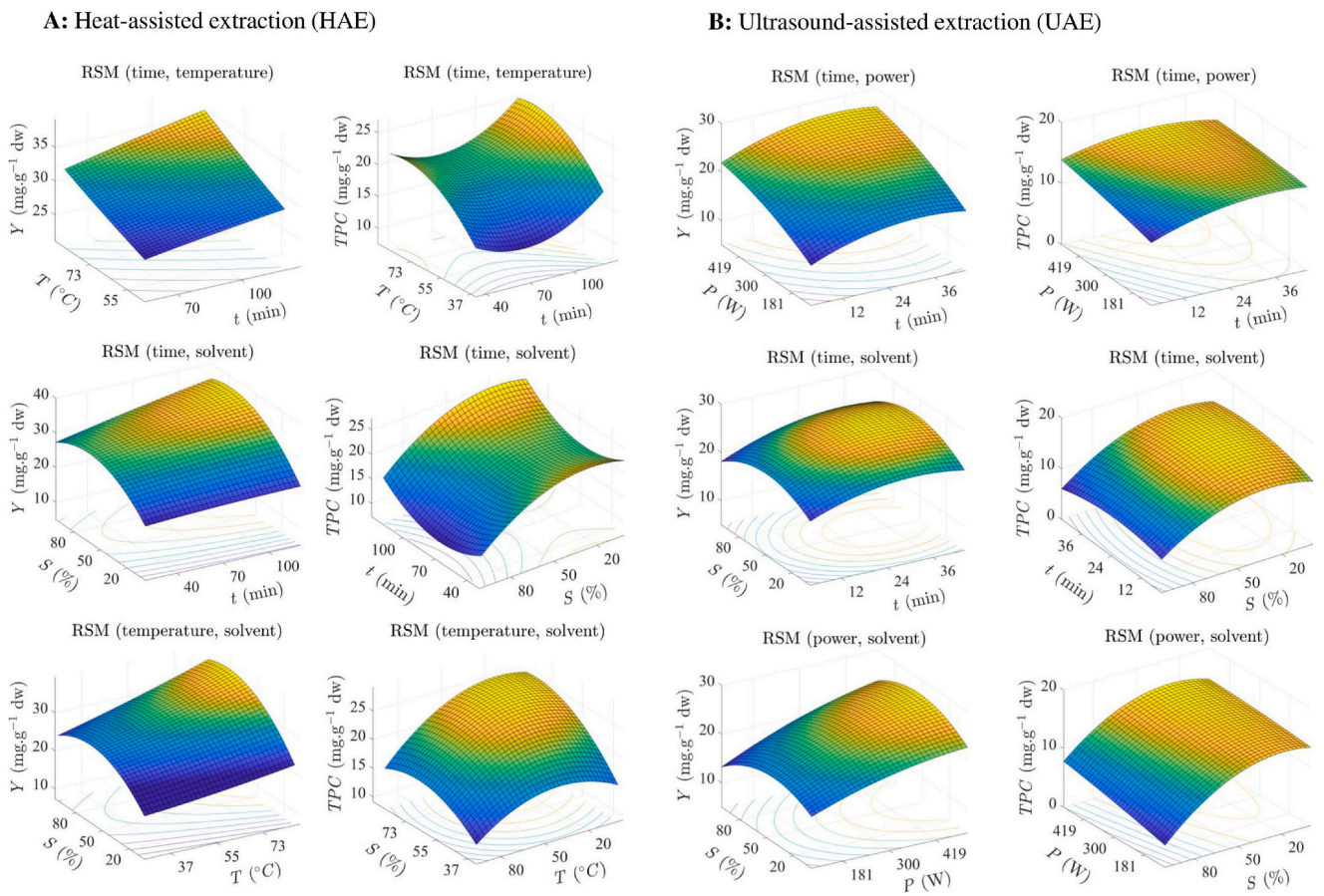

Fig. 1. Response surface plots illustrating the combined effects of time (X_1), temperature (X_2), and solvent proportion (X_3) on the extraction yield and phenolic content from eucalyptus leaves by HAE (A) and UAE (B). Each surface represents the predicted response based on the second-order polynomial model (Eq. 1). The excluded variable was fixed at its optimal value for each extraction method.

For HAE:

$$Y_1 = 29.33 + 1.88T + 1.96S - 2.21S^2 + 0.61\ tS + 0.62\ TS$$

$$Y_2 = 12.79 + 2.44T - 1.31S - 0.69TS$$

$$Y_3 = 5.77 + 0.36T - 0.54S$$

$$Y_4 = 18.56 + 2.79T - 1.85S + 1.73t^2 - 0.66TS$$

For UAE, the mathematical models revealed a distinct pattern compared to HAE:

$$Y_1 = 23.34 + 1.23t + 2.58T + 2.57S$$

$$Y_2 = 10.07 + 0.64T - 2.00S - 0.94S^2$$

$$Y_3 = 3.73 + 0.22t + 0.22T - 0.41S - 0.41S^2$$

$$Y_4 = 13.80 + 0.86T - 2.42S - 1.35S^2$$

All models demonstrated high predictive accuracy, with $R^2 \geq 0.90$ and $R^2_{adj} \geq 0.85$ for HAE, and $R^2 \geq 0.93$ and $R^2_{adj} \geq 0.90$ for UAE. The lack-of-fit tests were non-significant, indicating that the models adequately described the responses. The significant parametric values highlight the importance of solvent concentration (X_3) as the most influential variable, followed by temperature (X_2) and time (X_1).

In the HAE extraction, the total yield ranged from 18.6 to 36.0 mg/g of dried leaves. The yield was significantly influenced by extraction parameters, particularly solvent concentration (ethanol proportion), temperature, and contact time. The optimal conditions for maximum yield, predicted at 36.7 mg/g, were achieved with 77.3 % ethanol at 85 °C for 120 min. This result aligns with (Gullón et al., 2017), who observed that higher temperatures and prolonged extraction times improve the solubility and diffusivity of compounds, leading to better extraction yields.

For phenolic acids (TPA) in HAE, temperature and ethanol concentration were key factors, with solvent polarity playing a crucial role. While moderate ethanol concentrations favored higher TPA yields, excessive concentrations (above 80 %) led to lower yields due to the degradation of sensitive compounds, such as caffeic acid derivatives (Qiao et al., 2013). The maximum recovery of phenolic acids was estimated at 19 % ethanol, 120 min, and 85 °C, yielding 19.8 mg/g dw.

A similar trend was observed for flavonoid content (TFC). Temperature and solvent concentration again influenced TFC, with maximum extraction at 33.6 % ethanol, 120 min, and 62.1 °C, achieving 7.34 mg/g. Increasing ethanol concentration beyond the optimal levels caused a decline in TFC, as shown in Fig. 1.

Total phenolic content (TPC) was influenced by both temperature and ethanol concentration, with optimal yields achieved by balancing these variables. For TPC, the optimal conditions were 120 min, 76.5 °C, and 25 % ethanol (v/v), resulting in 26.6 mg/g of extract. TPC increased with temperature but decreased when ethanol concentration exceeded the optimal level.

The results demonstrated that ethanol concentration significantly influences the solubility and stability of phenolic compounds during extraction. Phenolics tend to dissolve more efficiently in organic solvents or mixtures with intermediate polarity, such as ethanol-water combinations, compared to pure water (Do et al., 2014). This occurs because water alone has limited efficiency in extracting less polar

Table 3
Parametric values of the second-order polynomial equation (Eq. 1) for each extraction method and response criteria, along with statistical information from the model fitting procedure. The superscripted parameters 1, 2, and 3 represent time (X_1), temperature or power (X_2), and solvent proportion (X_3), respectively.

Effect		Heat-assisted extraction (HAE)				Ultrasound-assisted extraction (UAE)			
		Yield (Y_1)	TPA (Y_2)	TFC (Y_3)	TPC (Y_4)	Yield (Y_1)	TPA (Y_2)	TFC (Y_3)	TPC (Y_4)
Intercept	b0	29.33 ± 1.16	12.79 ± 0.8	5.77 ± 0.29	18.56 ± 0.96	23.34 ± 0.90	10.07 ± 0.48	3.73 ± 0.18	13.80 ± 0.54
Linear	b1	n.s.	n.s.	n.s.	n.s.	1.23 ± 0.27	n.s.	0.22 ± 0.05	n.s.
	b2	1.88 ± 0.35	2.44 ± 0.24	0.36 ± 0.09	2.79 ± 0.29	2.58 ± 0.27	0.64 ± 0.15	0.22 ± 0.05	0.86 ± 0.16
	b3	1.96 ± 0.35	−1.31 ± 0.24	−0.54 ± 0.09	−1.85 ± 0.29	2.57 ± 0.27	−2.00 ± 0.15	−0.41 ± 0.05	−2.42 ± 0.16
	b11	n.s.	n.s.	n.s.	1.73 ± 0.48	n.s.	n.s.	n.s.	n.s.
Quadratic	b22	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	b33	−2.21 ± 0.58	n.s.	n.s.	n.s.	n.s.	−0.94 ± 0.24	−0.41 ± 0.09	−1.35 ± 0.27
	b12	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Interactive	b13	0.61 ± 0.18	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	b23	0.62 ± 0.18	−0.69 ± 0.12	n.s.	−0.66 ± 0.15	n.s.	n.s.	n.s.	n.s.
Statistical analysis	Model p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	Lack of fit	20.5885	30.7320	17.9468	32.2615	31.9403	52.3350	28.0703	65.9150
	R^2	0.9113	0.9392	0.9009	0.9421	0.9411	0.9632	0.9333	0.9705
	R^2_{adj}	0.867	0.9088	0.8512	0.9131	0.9116	0.9448	0.9002	0.9558
Optimal individual conditions	X_1: Time (min)	120	120	120	120	33	25	45	26.4
	X_2: Temperature (°C) or Power (W)	85	85	62.1	76.5	500	500	500	500
	X_3: Solvent (%)	77.3	18.7	33.6	25	54	22.6	34	27.2
	Optimal response	36.74 ± 0.47	19.76 ± 0.17	7.34 ± 0.15	26.62 ± 0.68	26.88 ± 0.48	11.93 ± 0.42	4.39 ± 0.06	16.03 ± 0.12

phenolics. Studies indicate that ethanol-water solvents create a more polar environment, facilitating the extraction of compounds soluble in both organic and aqueous phases (Sultana et al., 2009). Furthermore, extraction efficiency decreases when ethanol concentrations are either too low or too high, as excessively polar or non-polar environments can impair solubility (Meneses et al., 2013; Sultana et al., 2009).

UAE yields ranged from 8.34 to 27.17 mg/g, primarily influenced by ultrasonic power and extraction time. The optimized maximum yield of 26.9 mg/g was achieved with 33 min at 500 W and 53.9 % ethanol. The cavitation generated by ultrasound enhanced solvent penetration, facilitating the release of bioactive compounds. Although UAE required shorter times and lower ethanol concentrations than HAE, it provided comparable yields, making it a more sustainable approach. This result demonstrates that ultrasound enhances extraction by improving solvent-matrix contact, although prolonged exposure may degrade sensitive compounds.

Phenolic acid (TPA) extraction in UAE was also impacted by time, power, and solvent proportion. Ethanol concentrations above 40 % showed an antagonistic effect, possibly due to compound instability under such conditions. Additionally, prolonged UAE times led to slight degradation of sensitive compounds, such as caffeic acid derivatives, likely due to sonochemical effects (Tiwari, 2015).

For flavonoids (TFC), UAE reached 4.39 mg/g, approximately half the yield obtained with HAE under the same power and time conditions, using 34 % ethanol. Total phenolic content (TPC) benefited from a synergistic effect between ultrasonic power and solvent, with maximum yields achieved under high power for short durations. The ethanol-water mixture effectively extracted phenolic compounds from eucalyptus leaves, covering a wide range of polarities. However, prolonged ultrasonic exposure reduced TPC due to the degradation of sensitive components.

Ultrasound generates millions of cavitation bubbles in the solvent, and the collapse of these bubbles produces shock waves and microjets that disrupt cell walls and increase polyphenol dissolution. However, excessively high ultrasonic power can promote polyphenol degradation by increasing solvent temperature and generating hydroxyl radicals (Xue et al., 2020). This degradation can be attributed to excessive energy input and the formation of large amounts of free radicals under intense ultrasound conditions, resulting in reduced TPC and TFC (Wang et al., 2020).

(Gullón et al., 2019) and (Bhuyan et al., 2015) evaluated conditions for ultrasound-assisted extraction of *Eucalyptus* leaves, testing different temperature ranges, frequencies, and solvents. (Bhuyan et al., 2015) identified temperature as the main factor influencing yield and total phenolic content, followed by time and power. They observed a yield of approximately 163 mg of gallic acid equivalents/g with 250 W for 90 min at 60 °C using water as solvent. However, (Gullón et al., 2019) found that maintaining ultrasonic cavitation for longer periods resulted in a 15 % reduction in phenolic content and a 37 % decrease in antioxidant activity after 120 min of extraction at 40 kHz.

To minimize degradation during extraction, moderate temperatures limited thermal degradation while facilitating the release of phenolic compounds through cell wall disruption. Additionally, reducing extraction time in UAE proved essential to avoid prolonged exposure to degrading conditions. Optimization of extraction parameters, such as solvent composition, temperature, and duration, was critical to achieving high recovery rates of phenolic compounds while preserving their structural integrity.

3.2. Phenolic characterization

Eleven phenolic compounds were identified in each of the experimental points and analyzed by mass spectrometry. The data obtained for all peaks, including retention times (*Rt*), UV–vis spectra, molecular ions, and fragmentations obtained by tandem mass spectrometry experiments, are presented in Table 4 and illustrated by the representative chromatogram in Fig. 2.

The quantification was estimated for the two optimal points with total phenolic content (TPC_{HAE} and TPC_{UAE}), excluding phenolic compounds that could not be identified. The extracts showed a high

Table 4
Retention time (Rt), wavelengths of maximum absorption in the UV–Vis region (λ_{max}), mass spectral data, and tentative identification of the phenolic compounds of eucalyptus leaves.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Concentration (mg/g dw)		*t*-test p-value
						TPC_{HAE}	TPC_{UAE}	
F1–1	4.12	276	483	331(20),313(18), 271(100),211(6), 169(7)	Digalloyl-glucose	8.63 ± 0.17	6.81 ± 0.00	0.060
F1–2	5.03	276	483	331(19),313(17),271(100),211(5),169 (5)	Digalloyl-glucose	4.07 ± 0.01	1.99 ± 0.00	0.004
F1–3	6.44	322	353	191(100),179(23),173(5),161(2),173 (5),161(2)	5-*O*-Caffeoylquinic acid	0.60 ± 0.00	1.00 ± 0.01	0.006
F1–4	14.16	282	499	439(12),313(25),22(111),169(100)	Gallotannin	0.32 ± 0.02	0.67 ± 0.00	0.028
F1–5	16.25	280	497	313(34),169(100)	Eucaglobulin/Globulusin B	1.48 ± 0.01	1.60 ± 0.01	0.030
F2–1	17.51	353	477	301(100)	Quercetin-3-*O*-glucuronide	3.40 ± 0.06	1.41 ± 0.01	0.015
F2–2	18.07	343	477	301(100)	Quercetin-*O*-deoxyhexoside	0.34 ± 0.00	1.33 ± 0.01	0.005
F2–3	20.93	353	447	301(100)	Quercetin-3-*O*-glucoside	0.62 ± 0.01	0.21 ± 0.00	0.011
F2–4	21.3	351	447	315(100)	Isorhamnetin-*O*-pentoside	0.29 ± 0.00	0.24 ± 0.00	0.035
F2–5	22.55	363	447	315(100)	Isorhamnetin-*O*-pentoside	0.56 ± 0.00	0.21 ± 0.00	0.000
F2–6	23.74	358	461	315(100)	Isorhamnetin-*O*-deoxyhexoside	0.23 ± 0.00	0.27 ± 0.02	0.239
					Not identified	6.08 ± 0.55	0.29 ± 0.09	0.036
					Total	26.62 ± 0.68	16.03 ± 0.12	0.020

NOTATION: TPC_{HAE} – Extract with optimal phenolic content obtained through HAE; TPC_{UAE} – Extract with optimal phenolic content obtained through UAE. Standards for quantification: *p*-hydroxybenzoic acid for phenolic acids (y = 18,424× – 109,642; R^2 = 0.9984) and quercetin-3-*O*-glucoside for flavonoids (y = 34,843× – 160,173; R^2 = 0.9990).

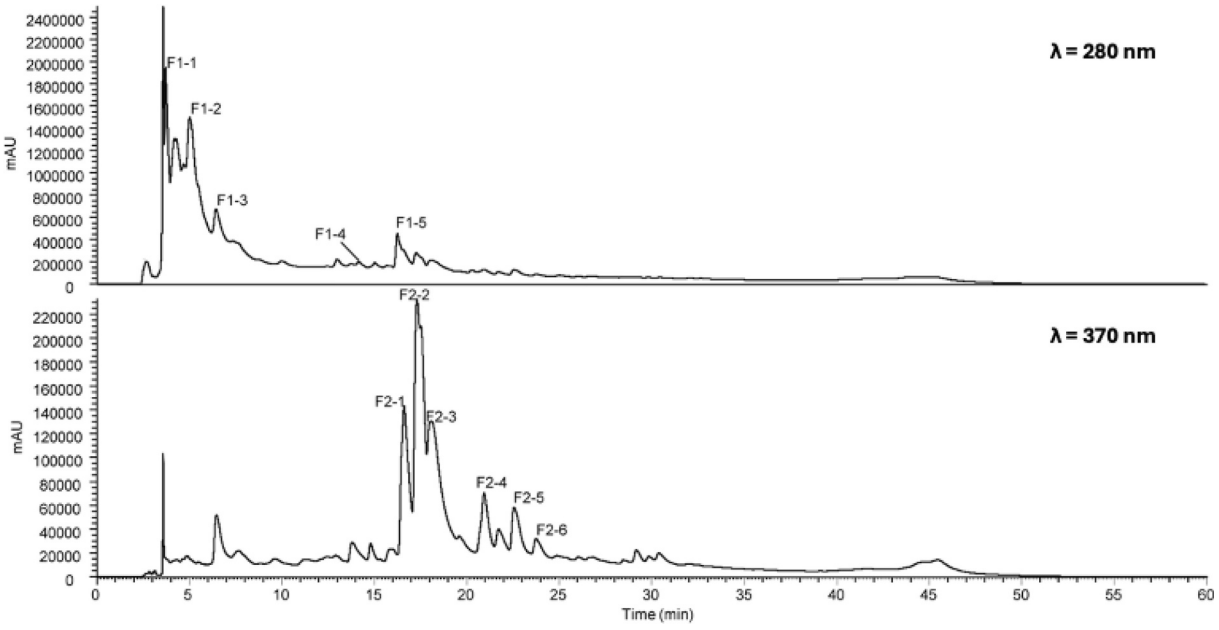

Fig. 2. Representative chromatogram showing the phenolic profile of eucalyptus leaves hydroethanolic extract recorded at 280 nm and 370 nm under 120 min, 25 °C and 0 % of ethanol (HAE). See Table 4 for peak identification.

concentration of derivatives of phenolic glycosides, gallotannin, quercetin and isorhamnetin.

In Fraction 1, the compounds F1–1 and F1–2, with λ_{max} at 276 nm and anion at *m/z* 483, revealed fragments corresponding to the loss of a galloyl (−152 u), a galloyl plus a molecule of water (−170 u), and another fragment of 212 u; this fragmentation pattern is characteristic of galloyl glucose derivatives (Boulekbache-Makhlouf et al., 2013). Peak F1–3 ($[M-H]^-$ *m/z* 353) was positively identified as 5-*O*-caffeoylquinic acid compared to the commercial standard. Peak F1–4 ($[M-H]^-$ *m/z* 499) was tentatively identified as a gallotannin based on its characteristic fragment ions, corresponding the base peak ion at *m/z* 169 to a gallic acid anion and the ion at *m/z* 313 to a galloyl glucose moiety (Boulekbache-Makhlouf et al., 2010). Compound F1–5 was tentatively identified as eucaglobulin or globulusin B based on the pseudomolecular ion at *m/z* 497 and fragmentation ions at *m/z* 313 and 169 (Boulekbache-Makhlouf et al., 2013).

In Fraction 2, the main compounds in all samples studied were flavonols, especially those derived from quercetin (λ_{max} around 353 nm and MS^2 fragment at *m/z* 301), were particularly abundant. Two glycoside quercetin derivatives (F2–1 and F2–3) were positively identified according to their retention time, mass, and UV–*vis* characteristics compared to commercial standards, while F2–2 was tentatively identified as quercetin-*O*-deoxyhexoside. Another group of flavanols detected was isorhamnetin glycoside derivatives (F2–4, F2–5, and F2–6)

according to their UV–Vis and mass spectra (all of them released an MS^2 ion at *m/z* 315), differing in their λ_{max} and retention time (Barros et al., 2012; Boulekbache-Makhlouf et al., 2013).

A *t*-test was performed to verify significant differences in obtaining each phenolic compounds using HAE and UAE. The techniques showed statistically significant differences in terms of the content of phenolic compounds with a confidence interval of 90 %.

In general, the phenolic profile of the extracts obtained is similar to those reported in the literature. Teixeira et al. (2019) identified 18 polyphenols in aqueous extracts, the majority being galotannins, followed by phenolic acids and flavonoids, with a focus on digalloyl-glucoside and 5-*O*-caffeoylquinic acid. Gomes et al. (2018) detected 19 phenolic compounds in methanol/water extracts, predominantly flavonoids (derived from quercetin) and phenolic acids (gallate and ellagic acid), identifying compounds such as digalloyl-glucoside and pentagalloyl-glucoside. Boulekbache-Makhlouf et al. (2013) fractionated *E. globulus* extracts, identifying compounds such as gallic acid, eucaglobulin, globulusin B, and derivatives of ellagic acid and flavonoids, as well as ellagitannins as pedunculagin and pentagalloyl-glucoside. Quantitative differences can be ascribed to different techniques and solvents, regional varieties, and collection conditions. Some authors suggest that, for studies involving a complete characterization of plant extracts, analyzes should be carried out using samples collected periodically in the same region, under the same conditions of collection and treatment, considering that there may be variability in the chemical profile over time (Caleja et al., 2017; Mahumane et al., 2016).

3.2.1. Antibacterial activity

Gram-negative bacteria were more resistant to the tested extracts than Gram-positive bacteria, according to the results in Table 5. This may be due to the layer of lipopolysaccharides present in the membrane of Gram-negative cells, which makes access to the membrane more restricted. The Y_1 extract obtained by UAE showed a higher growth inhibition of gram-negative bacteria, mainly for *E. coli* and *S. enterica*. Among the gram-negative strains, *Y. enterocolitica* showed greater sensitivity considering all extracts. Regarding gram-positive bacteria, *S. aureus* was the most susceptible. All extracts did not present bactericide capacity under the maximum concentration tested (10 mg/mL). In most cases, UAE extracts showed antibacterial capacity superior to their equivalent obtained by HAE. The results are according to values previously reported in the literature (Dezsi et al., 2015; Fernández-Agulló et al., 2015; Gullón et al., 2017), except for *E. cloacae*.

3.2.2. Antifungal activity

Antifungal activity was tested on filamentous fungi *Aspergillus* with positive results related to growth inhibition, as shown in Table 5. Maximum efficiency was reported for Y1 for HAE, and Y_3 and Y_4 for UAE for growth inhibition of *A. fumigatus*, with a MIC of 1.25 mg/mL. There is scientific evidence that the antifungal effect of extracts is a result of the interaction of phenolic compounds with fungi membranes, resulting in their destabilization and eventual inhibition of the growth of *Aspergillus* species (Pizzolitto et al., 2015). However, the extract that showed the most activity against the strains tested was the one corresponding to the total yield, indicating that other constituents have a positive combined effect due to synergism/antagonism dynamics in the samples. Further studies should be conducted to confirm this hypothesis.

3.2.3. Antioxidant activity

Extracts from plants and herbs have a wide variety of antioxidants compounds and mechanisms; thus, more than one test is recommended to analyze the antioxidant activity. Two assays were used to determine the influence of the extraction conditions of *E. globulus* leaves, using as a parameter the composition of the four optimal extracts evaluated. Lyophilized extracts obtained at the optimal extraction conditions were used in both tests.

According to the values in Table 6, the results showed maximum cell-based antioxidant activity for the extracts Y_4 (HAE and UAE) rich in phenolic compounds, which may suggest that this group of compounds plays an important role in the secondary metabolism of the plant and may be responsible for inhibiting lipid peroxidation in porcine brain cells. The antioxidant capacity might directly be affected by the ethanol concentration in the solvent during extraction, where there was an increase in the antioxidant capacity with the increase in ethanol content up to approximately 50 %.

For CAA, the effectiveness of the cell lineage antioxidant treatments was quantified by examining the percent reduction in fluorescence. The highest antioxidant capacity was found for the extract Y_1 obtained by HAE, with a 40 % reduction. These data indicate that other classes of compounds can act directly on the plant's antioxidant potential and might work synergistically with phenolic compounds for this mechanism. The remaining samples did not return significant values at the concentration tested, including all extracts of UAE. Results of cellular antioxidant activity were not found in the literature for *E. globulus* leaves extracts tested with RAW 246.7 cell line using fluorescent methods and further studies should be conducted to evaluate the absence of

Table 5
Values of minimal inhibitory concentration (MIC) of eucalyptus leaves extract obtained through HAE and UAE under optimized conditions.

	E. globulus extracts								Positive control			
	HAE Yield (Y_1)	TPA (Y_2)	TFC (Y_3)	TPC (Y_4)	UAE Yield (Y_1)	TPA (Y_2)	TFC (Y_3)	TPC (Y_4)	Streptomycin	Methicillin	Ampicillin	Ketoconazole
Gram-negative bacteria												
E. cloacae	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.007	*n.a.*	0.15	*n.a.*
E. coli	5	5	2.5	2.5	0.6	2.5	2.5	5	0.010	*n.a.*	0.15	*n.a.*
P. aeruginosa	5	2.5	2.5	2.5	1.25	2.5	2.5	2.5	0.060	*n.a.*	0.63	*n.a.*
S. enterica	5	2.5	5	5	0.6	2.5	2.5	2.5	0.007	*n.a.*	0.15	*n.a.*
Y. enterocolitica	2.5	1.25	1.25	0.6	1.25	2.5	2.5	2.5	0.007	*n.a.*	0.15	*n.a.*
Gram-positive bacteria												
B. cereus	0.15	1.25	1.25	0.6	1.25	1.25	1.25	1.25	0.007	*n.a.*	*n.a.*	*n.a.*
L. monocytogenes	1.25	2.5	0.6	0.6	0.6	2.5	0.6	2.5	0.007	*n.a.*	0.15	*n.a.*
S. aureus	0.6	0.3	0.15	0.3	0.3	2.5	2.5	2.5	0.007	0.007	0.15	*n.a.*
Fungi												
A. brasiliensis	2.5	2.5	5	5	10	10	10	10	*n.a.*	*n.a.*	*n.a.*	0.06
A. fumigatus	1.25	2.5	5	10	2.5	2.5	1.25	1.25	*n.a.*	*n.a.*	*n.a.*	0.5

Maximum extract concentration tested of 10 mg/mL. Concentrations of 1.0 mg/mL for streptomycin, 1.0 mg/mL for methicillin, 20.0 mg/mL for ampicillin and 1.0 mg/mL for ketoconazole applied for positive control. *n.a.*: not applicable.

Table 6
Antioxidant, antiproliferative and anti-inflammatory activities of optimizes eucalyptus leaves extract using HAE and UAE.

	HAE				UAE			
	Yield (Y_1)	TPA (Y_2)	TFC (Y_3)	TPC (Y_4)	Yield (Y_1)	TPA (Y_2)	TFC (Y_3)	TPC (Y_4)
Antioxidant activity								
TBARS (EC_{50}, mg/mL)	6.47 ± 0.08	5.67 ± 0.26	6.55 ± 0.18	5.59 ± 0.08	7.35 ± 0.55	5.12 ± 0.33	5.81 ± 0.09	4.87 ± 0.32
CAA (% inhibition)	0.4	0.15	–	–	–	–	–	–
Cytotoxicity (GI_{50}, μg/mL)								
AGS	192.9 ± 8.8	124.7 ± 8.5	208.3 ± 12.1	108.6 ± 10.6	58.6 ± 0.8	96.4 ± 1.7	125.3 ± 5.8	86.5 ± 6.2
CaCo2	192.2 ± 8.8	128.0 ± 8.5	204.3 ± 12.1	156.1 ± 10.6	65.0 ± 5.8	67.3 ± 3.6	110.4 ± 7.4	88.1 ± 7.3
MCF-7	170.6 ± 15.8	117.8 ± 11.4	161.7 ± 8.6	87.6 ± 4.7	48.1 ± 2.53	145.5 ± 14.13	122.7 ± 3.14	76.2 ± 5.14
Vero	8.2 ± 0.2	68.9 ± 6.1	41.0 ± 3.9	21.9 ± 1.7	54.4 ± 0.62	65.2 ± 1.45	74.9 ± 1.37	73.3 ± 1.80
Anti-inflammatory activity (IC50, μg/mL)								
RAW 264.7	164.6 ± 8.1	156.3 ± 13.7	130.0 ± 3.2	87.9 ± 4.1	>400	>400	>400	>400

EC_{50} values for Trolox: 9.3 ± 0.02 μg/mL. Quercetin: 95 ± 5 % oxidation inhibition at 0.3 μg/mL. GI_{50} values for Ellipticine: 1.23 ± 0.03 μg/mL (AGS); 1.21 ± 0.02 μg/mL (Caco2); 1.02 ± 0.02 μg/mL (MCF-7); 1.41 ± 0.06 μg/mL (VERO). IC_{50} values for Dexamethasone 6.3 ± 0.4 μg/mL (RAW 264.7). Quercetin: 95 ± 5 % oxidation inhibition at 0.3 μg/mL. AGS (gastric adenocarcinoma), CaCo-2 (colorectal adenocarcinoma), MCF-7 (breast carcinoma), VERO (a renal epithelial cell line from an African green monkey), RAW 264.7 (murine macrophage cell line).

antioxidant capacity for UAE extracts in the applied conditions.

3.2.4. Cytotoxicity

This colorimetric assay for the evaluation of cytotoxicity was based on Sulforhodamine B binding to the amino acids of cellular proteins, providing an estimate of the total protein mass and, by correspondence, the number of viable cells. All extracts tested showed growth inhibition potential for tumor cell lines; however, the samples obtained by UAE showed a more significant toxicity. Among the tumor cell lines, colorectal adenocarcinoma (CaCo-2) cells showed greater sensitivity to the extracts, and the Y_1 and Y_4 extracts obtained by UAE returned the lowest GI_{50} values. Primary non-tumor cells were tested to assess toxicity in standard strains to ensure future applications of the extracts in reliance on this study. In this work, the cytotoxic approach only determines the GI_{50} and does not consider the interaction mechanism between cells and extract components. In-depth studies are recommended for a better understanding of the effect of these extracts on these and other cell lines.

3.2.5. Anti-inflammatory activity

As shown in Table 6, all extracts obtained by HAE showed the ability to inhibit nitric oxide production at the concentrations tested, with IC_{50} values between 88 and 165 μg/mL. Among the extracts, TPC showed the highest efficiency in inhibiting NO production. This could be related to its high concentration in phenolic compounds. In contrast, UAE extracts had no impact on inhibiting the production of mediators of the inflammatory process. Although information on the anti-inflammatory potential of eucalyptus extracts remains limited, some authors have demonstrated positive inhibitory activity in other cell lines and in vivo models (Brezáni et al., 2018; Sugimoto et al., 2011; Vigo et al., 2010).

4. Conclusions

This study provides a comprehensive evaluation of HAE and UAE for the recovery of phenolic compounds from *Eucalyptus globulus* leaves, with insights into their phenolic profiles and bioactivities. A total of 11 phenolic compounds were identified, including derivatives of gallotannin, quercetin, and isorhamnetin, where UAE extracts exhibited higher concentrations of phenolic compounds, particularly quercetin-3-O-glucuronide, quercetin-O-deoxyhexoside, and isorhamnetin-O-pentoside.

Bioactivity assessments revealed distinct advantages for each extraction method. UAE extracts showed superior antibacterial and antifungal activities, with significant inhibition of *Y. enterocolitica* and *S. aureus*, as well as antifungal activity against *A. fumigatus*. UAE extracts also exhibited potent cytotoxicity, particularly against colorectal adenocarcinoma (CaCo-2) cells, while maintaining low toxicity in non-tumor primary cells, indicating their selective therapeutic potential.

In contrast, HAE extracts were efficient in antioxidant and anti-inflammatory activities. Phenolic-rich HAE extracts inhibited lipid peroxidation and demonstrated a 40 % inhibition in cell-based antioxidant assay (CAA), highlighting their potential as natural antioxidants. Additionally, HAE extracts significantly inhibited nitric oxide production, suggesting anti-inflammatory properties with IC_{50} values ranging from 88 to 165 μg/mL.

The comparative analysis underscores the versatility of both extraction methods. While UAE provides higher phenolic yields and enhanced antimicrobial and cytotoxic activities, HAE offers superior antioxidant and anti-inflammatory effects. These findings suggest that eucalyptus leaves extract can be applied to target specific bioactivities in food technology, nutraceuticals, and pharmaceuticals.

CRediT authorship contribution statement

Laíres Lima: Writing – original draft, Methodology, Investigation, Formal analysis. **Ana I. Pereira:** Writing – review & editing, Supervision, Data curation. **Clara B. Vaz:** Writing – review & editing, Data curation, Conceptualization. **Olga Ferreira:** Writing – original draft, Data curation. **Maria Inês Dias:** Writing – review & editing, Formal analysis. **Sandrina A. Heleno:** Writing – review & editing, Formal analysis, Conceptualization. **Ricardo C. Calhelha:** Methodology, Formal analysis. **Lillian Barros:** Writing – review & editing, Validation. **Marcio Carocho:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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