



Ultrasound and microwave-assisted extractions as green and efficient approaches to recover anthocyanin from black rice bran

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Abstract

Extraction techniques are one way to separate bioactive compounds from agro-industrial material. Emerging technologies for the extraction of bioactive compounds, such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), are preferable to conventional extraction techniques because they are more efficient, spend less time, and are environmentally friendly. This study compared ultrasound (UAE) and microwave (MAE) assisted extraction techniques to recover anthocyanins from black rice bran. By optimizing using Box Behnken design, for UAE, 83.73% of the total anthocyanins (2.44 mg C3G/g) were recovered, using as parameters temperature 50°C, a frequency 380 W, and a solvent 60:40 (v/v) citric acid 0.1 M:ethanol. For MAE, it was possible to recover 81.44% of the total anthocyanins (2.37 mg C3G/g), using parameters temperature 55°C, citric acid 0.1M:ethanol (50:50, v/v), and a mass of 0.8 g (1:24, g/ml). Both techniques were considered ecologically green by the Green Certificate (A) and excellent methods by EcoScale (scores higher than 75). Anthocyanin-rich extracts did not show cytotoxicity for L292 cells (1000 µg/mL) and showed cytoprotection (> 92% using 500 µg/mL) when subjected to oxidative stress with H₂O₂. Both extracts showed technological potential as a natural dye and as an ingredient of nutraceutical products.

Keywords Anthocyanin-rich extracts · Box-Behnken design · *Oryza sativa* L. · Stability · Cytotoxicity

1 Introduction

Some parts of plant-based products can be wasted during the food process, generating residues or by-products. These by-products have greatly increased in recent years. However, they generally present high concentrations of bioactive compounds and have been investigated as a cheap and available source of interest compounds for food and pharmaceutical applications [1, 2]. In the milling step during black rice processing, about 10% is lost, producing black rice bran, which is generally used as animal feed [3]. Anthocyanins

are considered one of the main black rice compounds with biological potential.

Anthocyanins are water-soluble natural colorants, and the intake of those compounds is related to health benefits: anti-obesity, antidiabetic, antimicrobial, anticancer, anti-cardiovascular, and neuroprotective effects [4, 5]. In the literature, anthocyanins are generally quantified as total monomeric anthocyanins by spectrophotometric methods, and the main compounds are identified through mass chromatography [6], with cyanidin-3-glucoside (C3G) being one of the majority anthocyanins in several products rich in anthocyanins, including black rice [2].

Thus, extraction techniques are one way to separate these bioactive compounds [7]. Conventional extraction techniques generally consume a lot of solvents, in addition to being time-consuming and not very efficient [3]. Therefore, new extraction methods have been developed to be more efficient, less time-consuming, and environmentally friendly [1, 8].

Among the emerging technologies for the extraction of bioactive compounds, ultrasound-assisted extraction (UAE)

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and microwave-assisted extraction (MAE) stand out. For UAE, the extraction mechanism occurs through cavitation, in which bubble formation occurs, generating microturbulence and leading to cell wall breakdown [9, 10]. In general, the generation of sound waves leads to the production of bubbles, and the growth of these bubbles is related to the expansion and compression cycles that affect the cell structure, leading to the separation of the material molecule [11]. This process consists of a strong vibration between the molecules of the sample, which causes the rupture of bubbles in the cell wall, damaging its structure and facilitating mass transfer [11, 12]. The technique's main advantage is greater penetration into the cellular material, which achieves better yields in shorter times, causing low solvent consumption. In addition, it allows the extraction of thermolabile compounds, uses less energy, and is considered an ecological technology [11, 13].

In the study by Thakur et al. [14], the authors extracted anthocyanins from black rice bran using UAE, with different concentrations of natural deep eutectic solvents (NADES), obtaining the best efficiency (anthocyanin ≈ 30 mg/L) using lactic acid:fructose as the solvent, water 12.63%, solvent-to-solid ratio 18.75 mL/g, amplitude 21.31%, and 11.25 min. In addition to the parameters already studied, the temperature and solvent concentrations (0.1 M citric acid: ethanol, v/v) will be evaluated in our study.

In the case of MAE, the generated electromagnetic radiation is absorbed by the polar molecules in the solvent and food, producing a dipolar rotation in the molecules and the migration of ions. Cell rupture occurs due to the high pressure generated by the vaporization of water from the microwaves that act on the cells [12, 15]. The main advantages of this technique are similar to UAE: shorter extraction times, higher yields, lower costs with solvents, and environmentally friendly [16].

Among the studies that carried out extraction of black rice and its by-products by MAE, in the study by Moirangthem et al. [17], microwave-assisted subcritical water extraction was used to extract anthocyanins from the straw, varying the temperature (90°C and 100°C), with a maximum efficiency of about 0.52 mg C3G/g. In the study by Abdel-Aal et al. [18], for the extraction of anthocyanins from black rice, the authors varied temperature, power frequency, and time using acetone-water (40/60, v/v) as the solvent, obtaining a maximum efficiency of 2.0 mg/g. In the study by Jha et al. [19], the authors extracted polyphenols from black rice husk UAE followed by MAE, obtaining anthocyanins equal to 0.03 mg/g. In this study, using MAE to extract anthocyanins from black rice white will be optimized by varying the temperature, solvent concentration (0.1 M citric acid:ethanol), and sample mass (g) (mass/volume ratio, g/mL).

Therefore, this study aims to optimize the extraction of anthocyanins from black rice bran by UAE and MAE

techniques as green extraction approaches. Green Certified and EcoScale analyses were carried out to verify the UAE and MAE techniques as eco-friendly approaches. Furthermore, these extracts' temperature and fluorescent light stability were also evaluated, as well as the cytotoxicity and antitumoral activity of anthocyanin-rich extracts.

2 Material and methods

2.1 Materials

The black rice bran was harvested in the February-May (2022) crop, and the bran comes mainly from the polishing/milling stage of rice processing, supplied by Ruzene (Pindamonhangaba, Brazil). Analytical standard of anthocyanin (cyanidin-3-O-glucoside), the Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid), TPTZ (2,4,6-tri(2-pyridyl)-s-triazine), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), Folin-Ciocalteu, Sodium carbonate were purchased from Sigma-Aldrich (Steinheim, Germany). Dulbecco's Modified Eagle's medium (DMEM), TrypLE Express enzyme, and phosphate-buffered saline (PBS) were purchased from Gibco. CellTiter 96@ AQueous One Solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] was purchased from Promega Biotecnologia (Brazil).

2.2 Sample preparation

The approximate composition of black rice bran (BRB) and sample preparation are described by Leonarski et al. [7] The sample used has a particle size between 20 and 32 mesh.

2.3 Anthocyanin extraction

2.3.1 Ultrasound-assisted extraction

An ultrasonicator with a probe (Eco-Sonics, Ultronique Q3.0/37A, Brazil) was used to extract anthocyanin from black rice bran. Initially, was used 0.5 g of BRB and 15 mL of solvents for kinetics (using the center point parameters, temperature 45°C, frequency power 300 W, and solvent (50:50, v/v) citric acid 0.1 M:ethanol) to evaluate the best time to extract anthocyanins from BRB. The extraction assays followed a Box-Behnken design, with 15 experiments performed randomly. The factors evaluated (independent variables) were temperature (x_1 : 30, 45, and 60°C), frequency power (x_2 : 150, 300, and 450 W), and solvent

concentration (x_3 : 20:80, 50:50 and 80:20, citric acid 0.1 M:ethanol, v:v).

2.3.2 Microwave-assisted extraction

For MAE, the extractions were conducted in a microwave reactor (MonowaveTM 200, Anton Paar). The extraction time was defined through a kinetics study (temperature 45°C, solvent (50:50, v/v) citric acid 0.1 M:ethanol, and mass sample 0.67 g (1:30)). The extraction assays following a Box-Behnken design, with 15 experiments performed randomly. The factors evaluated (independent variables) were temperature (x_1 : 40, 55, and 70°C), solvent concentration (x_2 : 20:80, 50:50 and 80:20, citric acid 0.1 M:ethanol, v:v), and sample mass (x_3 : 0.33, 0.67, and 1.0 g).

2.3.3 Optimization of anthocyanin recovery

The dependent variable used in the optimization methodology by response surface methodology (RSM) was anthocyanin recovery (AR). A second-order polynomial model was used to fit the experimental data, according to Equation (1):

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{j \leq i \leq k} \beta_{ij} x_i x_j \quad (1)$$

where y is the predicted response, β_0 is the intercept, β_i , β_{ii} , and β_{ij} are the regression coefficients for linear, quadratic, and interaction terms, respectively, and x_i and x_j are the independent variables. The model was initially evaluated through the significant factors. Non-significant effects (ANOVA, $p > 0.05$) were removed, and parameters were refitted. The parameters p -lack of fit and coefficient of determination (R^2 and R^2 adjusted) were investigated to assess the adequacy and quality of the fit. The optimized values' relative error (RE) was calculated according to Benvenuti et al. [20].

2.4 Characterization of anthocyanin-rich extracts

2.4.1 Total monomeric anthocyanin, anthocyanin recovery, and polymeric color

For the analysis of total monomeric anthocyanin (TMA), 20 μ L of extract in microplates were added to 280 μ L of a buffer solution with pH 1.0 (potassium chloride 0.025 mol/L) and pH 4.5 (sodium acetate 0.4 mol/L), according to the differential pH methodology [21]. Then, the absorbances were read at 520 and 700 nm (Multileader Infinite M200 TECAN, ZH, Switzerland). The concentrations of TMA were calculated using Equations (2) and (3):

$$A = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5} \quad (2)$$

$$TMA = \frac{A \cdot MW \cdot DF \cdot 1000}{\epsilon} \quad (3)$$

where A is the calculated absorbance, MW is the molecular weight of anthocyanin standard (cyanidin-3-glucoside: 449.2 g/mol), DF is the dilution factor (15), and ϵ is the molar absorptivity of cyanidin (26900).

The anthocyanins recovery (AR) was performed according to Leonarski et al. [7]. Three sequential extractions were performed using 50 mL of 80% ethanol solution acidified with 0.1 mol/L of HCl for 0.5 g of the sample and stirring at room temperature for 30 min. Afterward, another extraction was performed using 50 mL of 70% acetone solution, also under stirring at room temperature for 30 min, for total anthocyanin content (TAC). To calculate AR (%), the TMA result (Equation 3) obtained for each assay was used and finally calculated by Equation (4):

$$\%AR = \left(\frac{TMA}{TAC} \right) \cdot 100 \quad (4)$$

The polymeric color (PC) was measured by diluting the extracts in potassium chloride (pH 1.0) and subsequently bleaching them with potassium metabisulfite (0.9 M). The metabisulfite bleached sample (PC) and the control sample (DC) were used for the calculations by Equation (5), in which A_{420} , A_{520} , and A_{700} are the absorbances at 420, 520, and 700 nm, respectively. The % PC was determined by Equation (6):

$$DC\ or\ PC = [(A_{420} - A_{700}) + (A_{520} - A_{700})] \cdot DF \quad (5)$$

$$\%PC = \left(\frac{PC}{DC} \right) \cdot 100 \quad (6)$$

Where DF is the dilution factor.

2.4.2 Total phenolic content

Adapted for microplates, the total phenolic content (TPC) was evaluated based on the colorimetric methods described by Singleton et al. [22]. TPC was expressed as mg GAE (gallic acid equivalent) per g of dry sample (mg GAE/g).

2.4.3 Antioxidant activity

The antioxidant potential was evaluated by DPPH and ABTS radical scavenging methods according to Brand-Williams et al. [23] and Re et al. [24], respectively, with

adaptations to the microplate reader. The antioxidant activity was expressed as μmol of Trolox equivalent per g of dry sample ($\mu\text{mol TE/g}$).

2.5 Individual anthocyanin by HPLC-MS

The individual anthocyanins were determined by high-performance liquid chromatography (HPLC) as described by Leonarski et al. [5]. The HPLC system (model LCMS-2020 Prominence, Shimadzu, Kyoto, Japan) consisted in a degassing unit (DGU-20A3), binary pump (LC-20AD), autosampler (SIL-20AC HT), column oven (CTO-20A), photodiode array detector (PDA, SPD-M20A) and a single-quadrupole MS detector with electrospray ionization (ESI) interface (MS 2020, Shimadzu). The system was controlled by a CBM-20A unit. Chromatographic analysis and treatment of generated data was performed with the LabSolutions® (Shimadzu, Japan) v. 5.109 software.

To achieve separation, a Kromasil® C18 column (100Å, 300 mm × 4.6 mm i.d.) was used as stationary phase, while the mobile phase consisted of aqueous 0.1 % formic acid (eluent A) and acetonitrile (eluent B). Gradient conditions: 0–25 min, 15–45% B linear; 25–30 min, 45–80% B linear; 30–35 min, 80–15% B linear. The flow rate was 0.7 mL/min and the oven was set to 25°C. Samples consisted in 10.0 μL (LC-PDA-only) or 0.1 μL (LC-PDA-MS) of the freeze-dried extracts resuspended in formic acid 0.1% and filtered through a 0.22 μm nylon syringe filter.

PDA detection parameters were: analysis range 190 – 600 nm, with reference (correction) wavelength at 590 ± 10 nm, the cyanidin-3-glucoside was detected at 530 nm, eluting with a retention time of 7.9 min. MS ESI alternated between positive and negative modes. The interface, desolvation line and heat block temperatures were 350, 250 and 200°C, respectively. Capillary voltage were the same as the autotune (+4.5 kV and -3.5 kV in positive and negative modes, respectively). Nitrogen was used as both nebulizing (1.5 L/min) and drying gas (15 L/min). The detection program cycle consisted of four events, each with a duration of 0.55 seconds: 1. Scan (positive mode, m/z 350 to 590), 2. ion selective mode (SIM, positive mode, m/z = 450 and 491 corresponding to $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{H}+\text{MeCN}]^+$, accordingly), 3. Scan (negative mode, same range as 1), 4. SIM (negative mode, m/z = 448, $[\text{M}-\text{H}]^-$ and 490, $[\text{M}-\text{H}+\text{MeCN}]^-$). Full widths at half maximum (FWHM) were set at 0.6 per autotune procedure.

2.6 Green metric tools

The sustainability of the extraction processes proposed was evaluated by two green metric tools, Green Certified and EcoScale analyses, as described by Sajid and Płotka-Wasyłka

[25] and Van Aken et al. [26], respectively. These tools consider the process environmentally safe with a maximum score (100) and consider Penalty Points (PP) to evaluate this process according to tabulated values for different parameters. For the Green Certificate, the parameters are the amount and environmental hazard of the solvents, energy, and generated waste. The EcoScale combines the above parameters with process yield, safety, economic, and environmental aspects of the extraction.

2.7 Cell culture

The methodology for culture condition, cytotoxicity assay (L929 fibroblast cells), Hydrogen peroxide-Induced oxidative stress in L929 fibroblast cells, and antitumoral activity were described by Leonarski et al. [7]. In this study, concentrations of extracts for cytotoxicity assay used were between 10 and 1000 $\mu\text{g/mL}$ (10, 50, 100, 250, 500, 750, and 100 $\mu\text{g/mL}$), and 500 $\mu\text{g/mL}$ of extracts for hydrogen peroxide-induced oxidative stress and antitumoral activity.

2.8 Stability of the anthocyanin-rich extracts

2.8.1 Thermostability

The extracts were distributed in sealed tubes and finally subjected to temperatures (of 65, 80, and 95°C) in a thermostatic bath, removed, and immediately cooled at 0, 30, 60, 120, 180, 240, and 300 min. These points were quantified regarding TMA content to obtain their isothermal degradation. A first-order kinetic model was fitted according to Equation (7) [20, 27]:

$$\frac{C_t}{C_0} = e^{-k_d \cdot t} \quad (7)$$

where C_t is the TMA concentration with time t , C_0 is the TMA initial concentration, k_d is the degradation rate (min^{-1}), and t is the time (min).

The time of half-live ($t_{1/2}$) was determined by Equation (8), and the activation energy (E_a) was determined through the Arrhenius model by Equation (9).

$$t_{1/2} = \frac{\ln(2)}{k_d} \quad (8)$$

$$\ln(k_d) = \ln(A) - \frac{E_a}{R \cdot T} \quad (9)$$

where A is the frequency factor (min^{-1}), R is the ideal gas constant (8.314 J/mol•K), and T is the temperature (K).

For the activation enthalpy (ΔH), the free energy of activation (ΔG), and the activation entropy (ΔS), Equations (10), (11) and (12), respectively, were used for calculations:

$$\Delta H = E_a - R \cdot T \tag{10}$$

$$\Delta G = -R \cdot T \cdot \ln \left(\frac{k_d \cdot h}{k_B \cdot T} \right) \tag{11}$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \tag{12}$$

where E_a is the activation energy for the degradation reaction (J/mol), R is the ideal gas constant (8.314 J/mol•K), T is the temperature (K), k_d is the kinetic rate constant (s^{-1}), k_B is the Boltzmann constant ($1.3806 \cdot 10^{-23}$ J/K), and h is Planck's constant ($6.6262 \cdot 10^{-34}$ J/s).

2.8.2 Photostability

The effect of light was determined at room temperature ($25 \pm 2^\circ\text{C}$). The tubes were exposed to direct light from a white fluorescent lamp (20 W) in a closed wooden chamber (435 x 435 mm) at 0, 90, 180, 270, 360, 450, 540, 1440, 2880, and 4320 min (3 days). The samples were removed and quantified regarding TMA content to obtain their isothermal degradation. The constant rate for anthocyanin degradation (k) and its half-life ($t_{1/2}$) was calculated as described by Equations (7) and (8).

2.9 Statistical analysis

The results were presented as mean \pm standard deviation. The dataset was evaluated by one-way analysis of variance (ANOVA), and the significant differences were determined using Tukey's test or t -Test at a probability level of less than 5% ($p < 0.05$). The quality and adequacy of the mathematical models were assessed by the determination coefficient (R^2), adjusted R^2 , and the lack of fit (p). ANOVA also determined the statistical significance of the models used. All statistical procedures were performed using Statistica v. 13.5 software (TIBCO Software Inc., Palo Alto, CA, USA).

3 Results and discussion

3.1 Optimization of UAE and MAE extraction conditions

The results of the experimental design obtained by the Box-Behnken design for anthocyanin recovery (AR) showed a significant difference ($p < 0.05$) among all treatment conditions for both techniques, UAE (Table 1) and MAE (Table 2). The results for AR ranged from 17.56 to 77.13% for UAE and from 29.14 to 86.44% for MAE.

Table 1 Box-Behnken experimental design and UAE results of anthocyanin recovery (AR)

Assay	Independent factors			Response
	Temperature ($^\circ\text{C}$)	Frequency power (W)	Citric acid : ethanol (v/v)	AR (%)
1	0 (45)	-1 (150)	1 (80:20)	25.59 ^d \pm 1.72
2	1 (60)	1 (450)	0 (50:50)	76.77 ^{ab} \pm 4.14
3	0 (45)	-1 (150)	-1 (20:80)	18.11 ^d \pm 1.96
4	-1 (30)	0 (300)	-1 (20:80)	38.58 ^c \pm 4.17
5	1 (60)	0 (300)	1 (80:20)	59.45 ^b \pm 4.77
6	0 (45)	0 (300)	0 (50:50)	68.11 ^{ab} \pm 2.93
7	0 (45)	1 (450)	-1 (20:80)	77.56 ^{ab} \pm 4.51
8	-1 (30)	0 (300)	1 (80:20)	57.87 ^b \pm 4.89
9	0 (45)	0 (300)	0 (50:50)	69.69 ^{ab} \pm 2.78
10	1 (60)	-1 (150)	0 (50:50)	26.77 ^{cd} \pm 1.47
11	0 (45)	0 (300)	0 (50:50)	66.14 ^b \pm 2.76
12	1 (60)	0 (300)	-1 (20:80)	73.23 ^{ab} \pm 3.98
13	0 (45)	1 (450)	1 (80:20)	79.13 ^a \pm 1.90
14	-1 (30)	1 (450)	0 (50:50)	62.20 ^b \pm 4.29
15	-1 (30)	-1 (150)	0 (50:50)	27.56 ^{cd} \pm 2.93

Results are presented as mean \pm standard deviation. AR anthocyanin recovery. Different letters in the same column show significant differences between the assays ($p < 0.05$) by the Tukey test

Table 2 Box-Behnken experimental design and MAE results of anthocyanin recovery (AR)

Assay	Independent factors			Response
	Temperature ($^\circ\text{C}$)	Citric acid : ethanol (v/v)	Sample mass (g)	AR (%)
1	0 (55)	0 (50:50)	0 (0.67)	79.25 ^a \pm 0.91
2	-1 (40)	0 (50:50)	-1 (0.34)	33.77 ^c \pm 1.61
3	1 (70)	1 (80:20)	0 (0.67)	57.30 ^b \pm 3.16
4	0 (55)	0 (50:50)	0 (0.67)	78.86 ^a \pm 3.42
5	0 (55)	0 (50:50)	0 (0.67)	77.58 ^a \pm 3.15
6	-1 (40)	1 (80:20)	0 (0.67)	55.03 ^b \pm 0.97
7	-1 (40)	-1 (20:80)	0 (0.67)	51.19 ^b \pm 2.86
8	1 (70)	0 (50:50)	-1 (0.34)	33.28 ^c \pm 1.47
9	-1 (40)	0 (50:50)	1 (1.0)	83.49 ^a \pm 2.79
10	1 (70)	0 (50:50)	1 (1.0)	86.44 ^a \pm 1.63
11	0 (55)	-1 (20:80)	-1 (0.34)	29.14 ^c \pm 4.38
12	1 (70)	-1 (20:80)	0 (0.67)	56.12 ^b \pm 1.69
13	0 (55)	1 (80:20)	-1 (0.34)	36.80 ^c \pm 1.00
14	0 (55)	-1 (20:80)	1 (1.0)	83.94 ^a \pm 2.46
15	0 (55)	1 (80:20)	1 (1.0)	82.60 ^a \pm 3.58

Results are presented as mean \pm standard deviation. AR anthocyanin recovery. Different letters in the same column show significant differences between the assays ($p < 0.05$) by the Tukey test

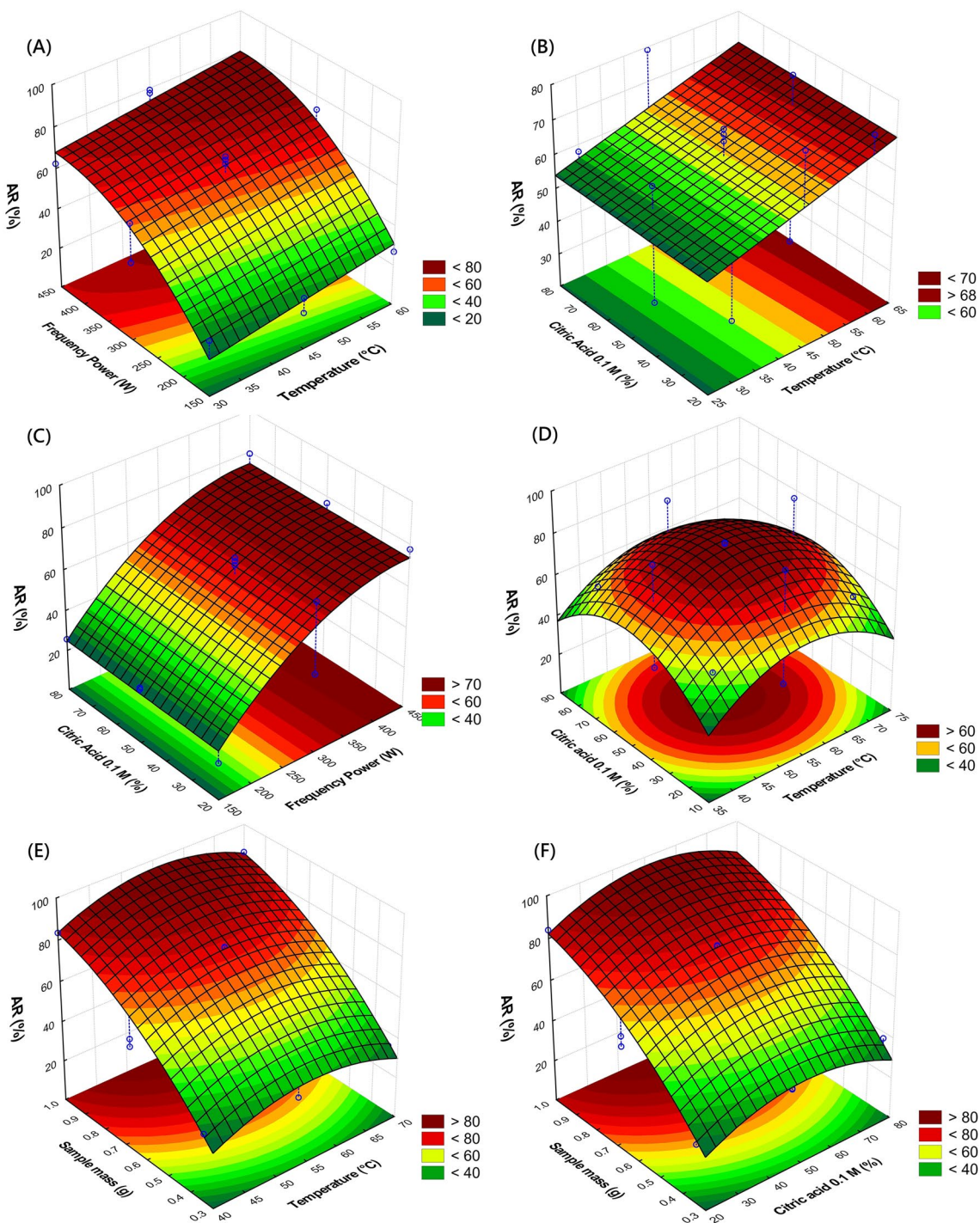


Fig. 1 Three-dimensional (3D) response surfaces show the effect of UAE independent variables (temperature, frequency power, and solvents) on the dependent variable AR (**A**, **B**, and **C**) and MAE inde-

pendent variables (temperature, solvents, and sample mass) on the dependent variable AR (**D**, **E**, and **F**)

Mathematical models were built using the data (Tables 1 and 2) to describe the behavior obtained in extracting anthocyanins from black rice bran. For the extraction by UAE, the proposed model for AR presented R^2 equal to 0.93, did not present a lack of adjustment (p

= 0.053), and was able to explain about 90% of the total variance of the experimental data (adjusted $R^2 = 0.89$). As for extraction by MAE, the model also did not show a lack of fit ($p = 0.102$), with R^2 equal to 0.99, representing 98% of the data (adjusted $R^2 = 0.98$). Furthermore, response

surfaces were generated, shown in Fig. 1(A–C) for UAE and Fig. 1(D–F) for MAE.

The effects that were significant for AR (Eq. 13) were the positive quadratic effect of temperature (x_1^2), the positive linear effect of frequency power (x_2), the positive quadratic effect of frequency power (x_2^2), and the synergic negative effect between temperature (x_1) and solvent concentration (x_3) ($x_1 \cdot x_3$). Through the significant effects and the response surfaces, it was possible to observe that the higher temperature (Fig. 1B) and higher frequency power (Fig. 1C) provided the better anthocyanin yield. Regarding both significant effects, frequency power had a superior effect (Fig. 1A), as described by Eq. 13. However, increasing (to more than 70°C) or decreasing the temperature and solvent (0.1 M citric acid) could decrease AR yield; that is, the temperatures and solvent concentration close to the central point are the ones that presented the best results. The increase in power frequency, and temperature in our study (which causes an increase in the shock wave by repeating cycles of compression and rarefaction) led to an increase in the effectiveness of anthocyanin extraction due to the rupture of the cell wall caused by both mechanical and thermal reactions, as observed by Boateng et al. [28].

In the study by Zhang et al. [29], the increase in frequency power from 200 to 450 W did not result in a significant increase ($p < 0.05$) in the concentration of anthocyanins recovered by UAE from purple barley bran. However, in the study by Rocha and Noreña [30], in which anthocyanins were extracted from grape pomace by UAE, it was reported that at the frequency between 250 W and 450 W, there is an increase in yield, with a maximum being reached between 350 and 450 W. This result is close to related in this study, in which the highest frequencies showed the best AR yields.

$$y = 53.43 + 12.50 \cdot x_1 + 49.41 \cdot x_2 + 12.65 \cdot x_2^2 - 16.53 \cdot x_1 x_3 \quad (13)$$

For the extraction of anthocyanins, very high temperatures are generally not used. In the case of studies that extracted these compounds by UAE, the best temperatures for extraction were equal to 35°C for haskap berries [31], 47.1°C for epicarp jaboticaba [32], and 55.1°C for eggplant peel [33]. For black rice, temperatures between 30°C and 55°C are generally used [1, 7, 19]. Concerning the solvent, it was reported in the extraction of anthocyanins from *Hibiscus sabdariffa* addition of about 40% ethanol [34], while for jambolan and haskap berries, the addition reached 80% [31, 35]. In our previous study, Leonarski et al. [7], when recovering anthocyanins from black rice by pressurized liquid extraction (PLE), it was observed that the proportions close to 50% ethanol and 50% citric acid 0.1 M were the ones that presented better extraction and less degradation (measured by the polymeric color). Therefore, the optimization was carried out using a temperature of 50°C, a frequency

of 380 W, and a solvent in the ratio of 60:40 (citric acid 0.1 M:ethanol).

In the case of extraction by MAE, the significant effects were the positive quadratic effect of temperature (x_1^2), the positive quadratic effect of solvent concentration (x_2), the positive linear and (x_3) quadratic effect (x_3^2) of sample mass (Eq. 14). The response surfaces (Fig. 1D) and Equation (14) indicate that both the temperature and the solvent have less influence than sample mass (Fig. 1E–F). According to Routray et al. [36], the efficiency of microwave extraction generally increases with increasing temperature until achieving an optimal temperature (along with a solvent/solid ratio). After this temperature, efficiency tends to decrease. This fact can be observed in Fig. 1D, in which, close to 50°C, greater extraction efficiency is obtained, and after this temperature, it was observed a decrease in yield. The cell wall rupture occurs due to the high pressure generated through temperature, with heat being produced from the inside to the outside of the material in the opposite direction to what occurs in UAE extraction [12]. The conditions that resulted in the better results were similar to those reported in the extraction by UAE: temperature close to 50°C and solvent in the proportion of 50:50 of citric acid 0.1 M and ethanol.

$$y = 57.42 + 11.26 \cdot x_1^2 + 12.38 \cdot x_2^2 + 50.87 \cdot x_3 + 8.05 \cdot x_3^2 \quad (14)$$

Regarding the temperature for anthocyanin extraction, as well as for the UAE and evidenced in this study, temperatures close to 50°C have been reported for optimizations in the extraction of anthocyanins from saffron tepal's (48°C) [37], blueberry powder (47°C) [38], and *Clitoria ternatea* flowers (60°C) [39]. For solvents, generally, a proportion between 40 and 60% of ethanol [40] and acidity between 1.0 and 3.0 [41] present better yields in the extraction of anthocyanins. In this study, the 50:50 ratio of 0.1 M acetic acid and ethanol showed the best results (pH about 2.6–2.8).

Regarding the mass parameters of the sample, in some studies, such as solid/solvent ratio (g/mL), the increase in mass resulted in higher yields in the extraction of anthocyanins (Fig. 1E–F). The added solid/solvent ratio depends on the type of sample, being equal to 1:15 (g/mL) for *Clitoria ternatea* flowers [39], 1:34 (g/mL) for blueberry powder [38], and about 1:23 (g/ml) for *Hibiscus rosa-sinensis* [42]. This study obtained the best results with the solid/solvent ratio between 1:30 (1 g) and 1:20 (0.67 g). Based on these results, for the optimization of the extraction, a temperature of 55°C, citric acid 0.1M:ethanol (50:50, v/v), and a mass of 0.8 g (1:24, g/mL) were chosen.

The optimization point was externally validated for UAE and MAE, providing value within the predicted ranges (solving Eq. 13 and Eq. 14, respectively) for a 95% confidence

level. For UAE, the AR result obtained was $83.73\% \pm 3.14$ (predicted: 85.67%, RE = 2.26%), while for MAE, the AR value obtained was $81.44\% \pm 1.69$ (predicted: 79.06%, RE = 3.02%). Thus, both proposed models can predict the AR response.

3.2 Comparison between extraction methods

Regarding the comparison between the methods, as observed previously by the optimization, the anthocyanins recovery (AR) obtained by both extraction methods (UAE and MAE) was similar, equal to 83.73% and 81.44% (Table 3). In terms of total anthocyanins, UAE presented a value of 2.44 mg C3GE/g, and MAE showed 2.37 mg C3GE/g, not differing from each other ($p < 0.05$). Concerning studies that performed extraction by UAE of anthocyanins from black rice bran, Das et al. [1] presented a result of 31.9 mg/L, using 23.78% (v/v) ethanol concentration, 35.97°C temperature, pH 2.52, and 22.84 min. According to the authors, an ultrasonic bath was used, reaching a maximum value of 81.19 mg/L of anthocyanins. In addition, the authors used time as an optimization parameter, which resulted in a longer time than in this study. Generally, using an ultrasonic probe has better results than using an ultrasonic bath to extract polyphenols [43, 44]. However, the frequency and power parameters are important factors to be considered in this evaluation. To extract anthocyanins by MAE from black rice, Abdel-Aal et al. [18] used 50°C, 1200 W, and 20 min reported values of 2.0 mg C3GE/g, close to those reported in this study.

The main anthocyanin of black rice is cyanidin-3-glucoside (C3G), corresponding to about 90% of total anthocyanins [7, 45]. In this study, it was identified that for UAE, 97.3% of the total anthocyanins correspond to C3G (2.37

mg/g), while for MAE, 89.1% of the total anthocyanins correspond to C3G (2.11 mg/g). In the study by Laokuldilok et al. [46], was identified after extracting anthocyanins from black rice with methanol acidified with 1 N HCl (85:15, v/v) at 4°C for 24 h, C3G values between 58% and 95%. In the study by Abdel-Aal et al. [18], by extracting anthocyanins by MAE, the authors reported 88.1% of C3G, a value close to that reported in this study.

Concerning polymeric color (PC), it can be observed that both samples presented close results, with these values comparable to those reported by Chañi-Paucar et al. [47] on the extraction of polyphenols from grape waste (46.6%). The higher the PC values, the greater the degradation of anthocyanins by polymerization [48]. Generally, this degradation of anthocyanins occurs by increasing temperature and leads to the production of chalcones that are subsequently degraded into brown products (such as melanoidins) [49, 50]. However, for Jiang et al. [50], it is more plausible that the increase in polymer coloration during exposure to high temperatures is due to the reaction between the anthocyanins or anthocyanins with phenolic compounds. Furthermore, the degradation of anthocyanins by polymerization depends on the conditions used in extraction [51], in the case of UAE and MAE, mainly frequency power and temperature.

For total phenolic compounds (TPC), both samples showed values close to 11.5 mg GA/g, not differing from each other ($p < 0.05$). These phenolic compounds come from anthocyanins and phenolic acids, mainly protocatechuic acid, vanillic acid, 4-hydroxybenzoic acid, and syringic acid [1, 52]. In the study by Leonarski et al. [7], when extracting anthocyanins from black rice bran by PLE, TPC equal to 10.7 mg GAE/g was reported, while in the study by Das et al. [53], when extracting phenolic compounds from black rice bran by conventional technique (water bath), a value equal to 8.5 mg GAE/g was reported, both values close to those reported in this study.

Regarding the antioxidant activity, there was no difference between the results obtained by the DPPH radical ($p < 0.05$) for the samples extracted by both techniques. Both samples showed values close to 62 $\mu\text{mol/g}$, close to that reported by Leonarski et al. [7] for black rice bran extracted by PLE (65.2 $\mu\text{mol/g}$). For ABTS, the sample extracted by UAE showed a higher value than MAE, differing from each other ($p < 0.05$). Finocchiaro et al. [54] reported values of antioxidant activity by ABTS for two species of black rice, 42.01 $\mu\text{mol/g}$ and 94.81 $\mu\text{mol/g}$, values close to those reported in this study.

UAE and MAE extractions are considered eco-friendly approaches. Considering this, the green metric tools Green Certificate and EcoScale (Section 2.6) were applied to assess how eco-friendly these methods are. For using the green certificate, penalties related to the solvent, energy, and residue generated in the extraction are assessed (Fig. 2A). The

Table 3 Characterization of the extracts obtained by optimized UAE and MAE

Analysis	UAE	MAE
AR (%)	83.73 ± 3.14^a	81.44 ± 1.69^a
TMA (mg C3GE/g DW)	2.44 ± 0.09^a	2.37 ± 0.04^a
C3G (mg/g DW)	2.41 ± 0.01^a	2.11 ± 0.01^b
PC (%)	44.35 ± 1.44^a	48.47 ± 2.37^a
TPC (mg GA/g DW)	11.80 ± 0.14^b	11.45 ± 0.35^a
DPPH ($\mu\text{mol TE/g DW}$)	61.55 ± 1.38^a	63.05 ± 1.45^a
ABTS ($\mu\text{mol TE/g DW}$)	72.86 ± 3.13^a	60.80 ± 3.77^b

Results showed a mean \pm standard deviation. TMA total monomeric anthocyanins, AR anthocyanin recovery, C3GE cyanidin-3-glucoside equivalents, TPC total phenolic compounds, GAE gallic acid equivalents, C3G cyanidin-3-glucoside, TE Trolox equivalents, UAE ultrasound-assisted extraction, MAE microwave-assisted extraction. Different letters on the same line indicate significant differences ($p < 0.05$) between the experiments using by t test

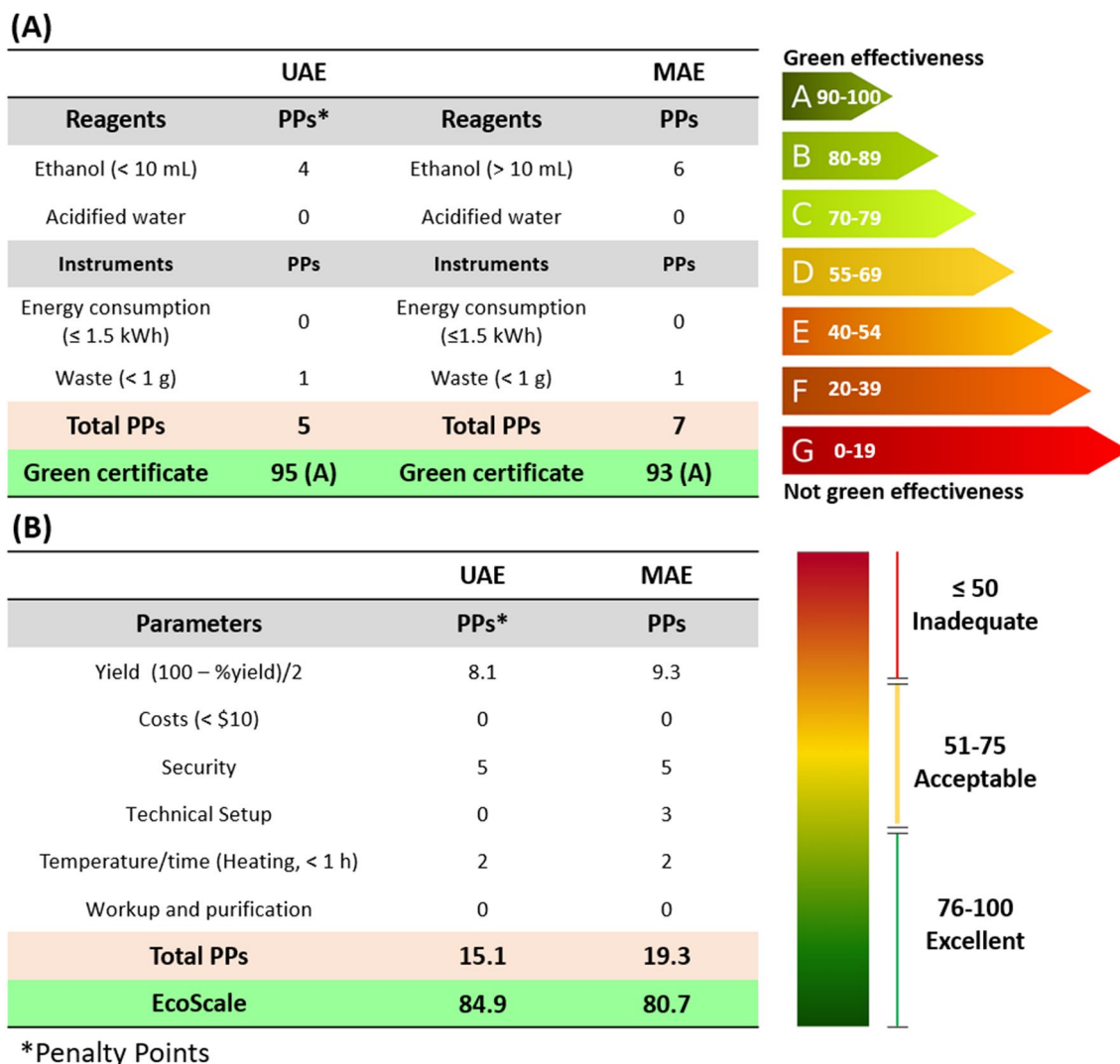


Fig. 2 A The Green Certificate and the B EcoScale of the UAE and MAE techniques

methods showed close values (95 for UAE and 93 for MAE), and both methods were considered highly effective green (Green certificate), in which the scale varies from A to G (Fig. 2A), with A being considered the greenest process [55, 56]. This difference in green certificate values was due to the higher addition of ethanol in MAE extraction since both techniques have a low energy cost. The study by Espino et al. [57], when extracting phenolic compounds from *Larrea cuneifolia* by UAE and using NADES as solvent (mixture of lactic acid with dextrose), presented a green certificate of 95.99. In the study by Abd-Elsalam et al. [58] using the MAE extraction of quercetin from red onion, the authors reported a value of 85.

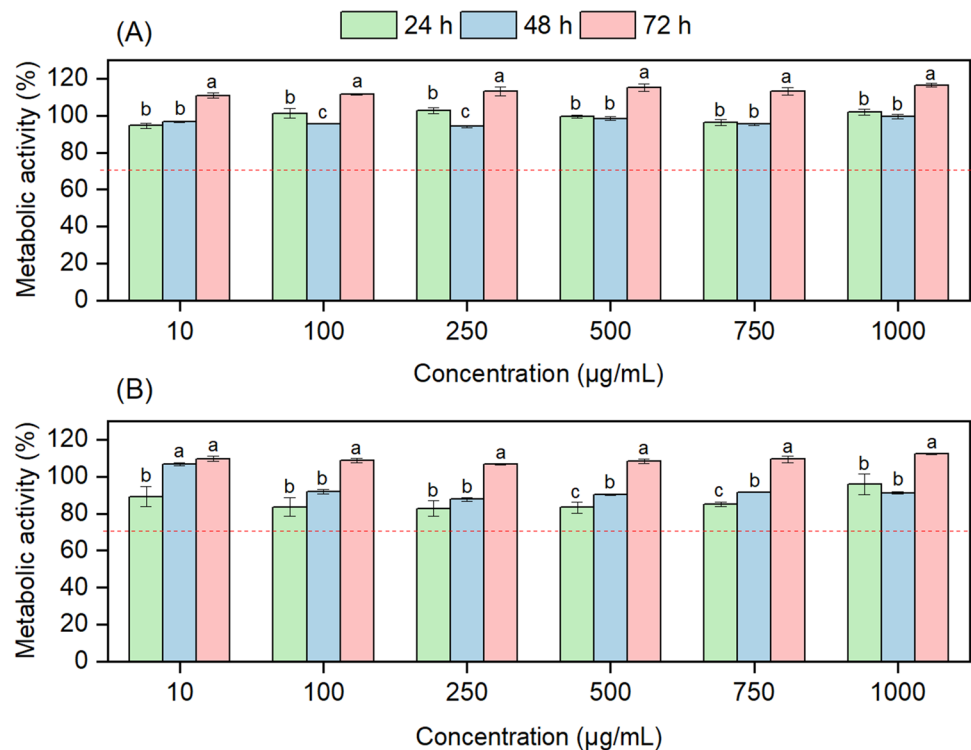
Regarding the EcoScale, six parameters presented in Fig. 2B are considered. It can be observed that both samples showed similar values (84.9 for UAE and 80.7 for MAE), being higher for the UAE. However, values above 75–80 are

considered excellent, while values below 50 are considered inadequate [55, 59]. Benvenuti et al. [20], when performing anthocyanin extraction from jaboticaba by-product by PLE, reported higher EcoScale values (69.4–72.5) when using NADES as solvent (mixture of choline chloride and propylene glycol and mixtures of choline chloride and malic acid).

3.3 Cell culture

Fig. 3 shows the cytotoxicity results for L929 cells (normal tissue of the mouse) at 24 h and 48 h for both extracts (UAE and MAE). According to ISO 10993-5:2009 [60], the minimum cell viability must be 70% for the extracts not to present cytotoxicity. In Fig. 3, none of the extracts showed cell viability lower than 80%, showing no cytotoxicity. Furthermore, it can be observed that in 72 h, there was a significant increase ($p < 0.05$) in cell viability for both extracts. In the

Fig. 3 **A** Metabolic activity of L929 cells treated with the UAE anthocyanin-rich extract and **B** MAE anthocyanin-rich for 24, 48 and 72 h



study by Aprodu et al. [61], microencapsulated black rice extract did not show cytotoxicity (10–1000 µg/mL), similar to those reported in this study. Thus, the highest concentrations (750 and 1000 µg/mL) were tested for the analysis of antitumor activity.

According to results presented in the supplementary material (Figure S1), no antitumor activity (500 µg/mL) was observed for the cells: lung adenocarcinoma cells (A549), rat glioma cells (C6) and mouse glioma cells (GL261). Antitumor activity was also not verified for A549 cells using anthocyanin-rich extract (500 µg/mL) from pressurized liquid extraction (PLE) and heat-stirring extraction (HSE) [7]. The results on H₂O₂-induced oxidative stress in L292 cells showed that when using 500 µg/mL of sample, both extracts showed a cytoprotective effect close to 93%, equal to 94.5% for UAE and 92.9% for MAE. In contrast, the sample without extract showed a cytoprotection of only 4.6%. In the

study by Leonarski et al. [7], the authors reported about 82% for 250 µg/mL extracts rich in anthocyanins.

3.4 Stability of UAE and MAE anthocyanin-rich extracts

The stability of anthocyanins is one of the factors most affected by thermal processing [62]. Tables 4 and 5 show the kinetic and thermodynamic parameters, obtained through the thermal degradation curves. It can be observed that the extract obtained by MAE (pH ≈ 2.8) presented slightly higher $t_{1/2}$ than the extract obtained by UAE (pH ≈ 2.6). In the study by Guimarães et al. [63], the author reported a $t_{1/2}$ value for C3G (pH 3.0) extracted from frozen blackberries of 9.23, 3.30, and 2.0 h at 65, 80, and 95 °C, respectively, values close to those reported in this study. According to Oancea [64], the increase in temperature shifts the balance

Table 4 Kinetics parameters of thermal and light stability of anthocyanin-rich extracts obtained by UAE and MAE

	T (°C)	k_d (min ⁻¹)	$t_{1/2}$ (h)	R^2	Light	k_d (min ⁻¹)	$t_{1/2}$ (days)	R^2
UAE	65	0.0014	8.13	0.986				
	80	0.0027	4.23	0.974	Fluorescent	0.013	2.22	0.876
	95	0.0066	1.75	0.991				
MAE	65	0.0012	9.39	0.987				
	80	0.0025	4.62	0.978	Fluorescent	0.0015	1.93	0.982
	95	0.0044	2.62	0.943				

UAE ultrasound-assisted extraction, MAE microwave-assisted extraction, k_d degradation rate, R^2 determination coefficient, $t_{1/2}$ time of half-live

Table 5 Thermodynamic parameters of the anthocyanin degradation in the extracts obtained by UAE and MAE

	E_a (kJ/mol)	R^2	Temperature (°C)	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (kJ/mol K)
UAE	52.85	0.986	65	50.04	88.38	-0.11
			80	49.92	90.54	-0.11
			95	49.79	91.78	-0.11
MAE	44.02	0.998	65	41.21	88.78	-0.14
			80	41.08	90.77	-0.14
			95	40.96	93.02	-0.14

UAE ultrasound-assisted extraction, MAE microwave-assisted extraction, E_a activation energy, ΔH activation enthalpy, ΔG free energy of activation, ΔS activation entropy

of anthocyanins to the tautomeric open-form; that is, the colorless chalcone prevails in monoglycosidic anthocyanins and does not have a substituent in the C3 position.

Regarding the thermodynamic parameters (Table 5), it can be seen that the activation energy (E_a) is higher for the extract obtained by UAE. Teixeira et al. [27] presented an E_a value of 40.56 kJ/mol for an anthocyanin-rich bean peel extract obtained by PLE. In the study by Guimarães et al. [63], the authors presented E_a for C3G of 52.5 kJ/mol. The higher E_a values, the lower the susceptibility to anthocyanin degradation.

A positive activation enthalpy (ΔH) indicates an endothermic reaction for both extracts (UAE and MAE). For an anthocyanin-rich extract from hibiscus (*Hibiscus sabdariffa* L.), Cheok and Ragnathan [65] presented ΔH values close to 51 kJ/mol, results close to the extract obtained by UAE. The ΔH is a measure of the energy barrier that must be overcome by the reacting molecules, representing the strength of the bonds that are broken and rebuilt during the transition state; therefore, higher values indicate that these are more likely to transform into other forms (degradation) [20, 66].

Positive ΔG values indicate that the degradation is non-spontaneous, and negative ΔS means that the reaction is irreversible and the molecules are more organized than in the initial reaction [20, 65, 66]. In studies by Leonarski et al. [7] and Benvenuti et al. [20], both authors presented ΔG values close to 90 kJ/mol for anthocyanin-rich extracts from black rice bran and jaborcaba extracted from PLE, results close to those reported in this study.

Regarding the stability of fluorescent light, both samples showed a $t_{1/2}$ of approximately 2 days. These values were close to those reported in the study by Junior et al. [67] for anthocyanin-rich extract from grumixama berries, which showed a $t_{1/2}$ of 1.9 days (45.61 h).

4 Conclusion

Using UAE and MAE techniques with the Box-Behnken design tool efficiently optimized the recovery of anthocyanins from black rice bran. Both extraction methods showed

similar anthocyanin recovery when optimized, equal to 83.73% for UAE and 81.44% for MAE. UAE showed a higher amount of cyanidin-3-glucoside (2.41 mg/g) when compared to MAE (2.11 mg/g). Both methods were considered eco-friendly, presenting a green certificate above 90 (A), and EcoScale was considered excellent (above 75). There was no evidence of cytotoxicity in the UAE, and MAE extracts were evaluated by L929 cells at concentrations of 10 $\mu\text{g/mL}$ to 1000 $\mu\text{g/mL}$ of extract. A slight advantage is observed for the UAE method due to the ease of the technique compared to MAE, in which high pressure is required. The extracts obtained by both methods have interesting characteristics for use in several areas, such as natural coloring, cosmetics, and biomedical industries. Future studies should be carried out to highlight these promising characteristics, as in the application in wound healing due to the cytoprotection evidenced.

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Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions E.L.: conceptualization, methodology, formal analysis, data curation, writing—review and editing. M.K.: conceptualization, methodology, formal analysis, data curation, writing—review and editing. E.H.S.: conceptualization, methodology, formal analysis. L.B.: formal analysis. P.A.D.M.: formal analysis. K.C.: writing—original draft, investigation, visualization, supervision. D.O.: investigation, resources, writing—original draft, visualization, supervision, project administration. A.A.F.Z.: investigation, resources, writing—original draft, visualization, supervision, project administration

Declarations

Ethical approval Not applicable

Conflict of interest The authors declare no competing interests.

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