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A water-based ultrasound-assisted extraction system to obtain natural blue colorant from Genipap (*Genipa americana* L.)

Laís Benvenutti [a,*], Beatriz Cesa Rovaris [a], Karina Cesca [a], Débora de Oliveira [a], Paulo Riceli Vasconcelos Ribeiro [b], Edy Sousa de Brito [b,c], Acácio Antonio Ferreira Zielinski [a,*]

[a] Department of Chemical Engineering and Food Engineering, Federal University of Santa Catarina, Florianópolis, SC, C.P. 476, Florianópolis 88040-900, Brazil
[b] Embrapa Agroindustria Tropical, Fortaleza, CE 60511-110, Brazil
[c] Embrapa Alimentos e Territórios, Maceió, AL 57020-050, Brazil

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ABSTRACT

This study aimed to optimize the extraction of a blue colorant using ultrasound-assisted technology employing water as the solvent from genipap, an Amazon fruit. Process conditions were optimized by multiresponse analysis through the antioxidant activity by the ferric reducing antioxidant potential (FRAP) assay, the total phenolic content, and the b* and C* color parameters. The optimized conditions of 70 °C, 325 W, solid: solvent ratio of 1:40 (m/v), and time of 10 min were validated externally at a level of 90 % confidence. The composition of aqueous blue extract obtained under the greatest conditions was evaluated by ultra-performance liquid chromatography (UPLC). Besides, in vitro bioactivities such as digestive enzymatic inhibitions assays, and antiproliferative activity through human cells assays were evaluated. Genipin, geniposide, and other ten iridoids were identified in the aqueous blue extract. The natural colorant obtained presented the capacity of inhibiting enzymes involved in lipid and glucose digestion, suggesting a potential control of obesity and glucose levels in blood without showing cytotoxicity. Therefore, this study presented, for the first time, the optimal operational conditions to obtain a natural blue colorant from genipap in a green approach with interesting biological properties for the food and pharmaceutical industries.

1. Introduction

Colorants are widely employed in fabrics, foods, and pharmaceuticals due to their direct impact on preference, selection, and desire by consumers (Iramaia Angélica Neri-Numa et al., 2017). It is estimated that the food color market size will reach $7.8 billion by 2032, registering a CAGR of 13.2 % from 2023 to 2032 (alliedmarketresearch.com/food-color-market).

However, synthetic colorants have been associated with an increase in health concerns, e.g., allergies, respiratory diseases, and toxicity (Sigurdson et al., 2017). Therefore, there is a trend to replace synthetic colorants with natural ones mainly in food and pharmaceutical formulations (Landim Neves et al., 2019). Among the natural colorants approved by the Food and Drug Administration (FDA), fruit and vegetable juice can be used if obtained by pressing the edible parts of the vegetables or by the water infusion of the dried vegetable (FDA, 2020). Nonetheless, the search for natural blue colorants is still an industrial challenge. Although blue colors exist in nature, it is difficult to maintain their stability by applying them to foods and beverages. Anthocyanin is the main natural molecule that can present a blue chromophore, but just in a basic medium (pH 8). Recent researches propose techniques to chelate anthocyanin molecules obtaining a blue color at a pH above 2.5 (Coultate & Blackburn, 2018). Another natural blue color is *C*-phycocyanin, a protein from the blue-green algae *Cyanobacteria* commercially known as spirulina. Technically, spirulina is an excellent colorant but presents low stability and heat sensitivity, degrading above 45 °C (Coultate & Blackburn, 2018)

Genipap (*Genipa americana* L.) is a fruit from the Amazon region that can be a natural source of blue colorant. The unripe fruit of genipap has a colorless glycoside iridoid called geniposide, which when hydrolyzed by β-glucosidase, releases genipin. Then, the primary amines react spontaneously with genipin, forming the blue pigment (Náthia-Neves et al., 2017, 2019).

Recovering the blue colorant from this underutilized plant, employing green solvents, can be an eco-friendly and economically viable approach for utilizing available resources to obtain natural

* Corresponding author.
E-mail addresses: labenvenutti@gmail.com (L. Benvenutti), acacio.zielinski@ufsc.br (A.A.F. Zielinski).

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ingredients applicable to food and pharmaceutical industries. In this sense, the use of ultrasound-assisted extraction (UAE) emerges as an efficient and industrially viable process (Kumar et al. 2021; Strieder et al. 2021). The effectiveness of UAE on the recovery of target compounds from plants is due to the high-intensity sound waves, which disrupt the plant tissue through physical forces generated during acoustic cavitation, facilitating mass transport. Consequently, this technique requires less process time and solvent consumption than conventional techniques (Kumar et al., 2021).

Strieder et al. (2021) presented UAE as an economically viable process for obtaining ethanolic genipin-rich extracts from unripe genipap. However, the extract wasn't considered a natural colorant, because genipin needs to react with amine groups to produce blue compounds. Gualberto et al. (2021) recovered bioactive compounds from agro-industrial residues, including genipap, employing UAE and solvents such as ethanol, methanol, and acetone. The highest bioactive concentrations were found in acetic extracts. However, there is a need for greener alternatives, and solvent choice is a crucial step to avoiding toxic effects on human health and the environment besides ensuring the safety and quality of extracts (Chemat et al., 2019). The use of only water as the solvent, avoiding the use of organic or chemical solvents, makes the process cost-effective and safe due to its nontoxicity, noncorrosive, nonflammability, availability, and environmental friendliness. Náthia-Neves et al. (2021) employed UAE with water or ethanol to recover natural colorants and concluded that water recovery preferentially genipin whereas ethanol enhances geniposide recovery. However, these authors did not optimize the operational conditions nor evaluate the toxicity of the obtained extract.

Process conditions such as temperature, solid-to-liquid ratio, ultrasound power, and ultrasound frequency, among others, influence the effectiveness of processes employing UAE. Therefore, response surface methodology (RSM) can be useful to optimize this process. RSM is a mathematical tool statistically designed to describe the correlation between independent variables and one or more responses, enabling process optimization with a reduced number of experimental assays (Zielinski et al., 2016).

Therefore, this study aims (i) to obtain a natural blue colorant from genipap fruit employing UAE using only water as the solvent, and (ii) to present its biological activity (inhibition of digestive enzymes, and antioxidant and antiproliferative activity) and cytotoxicity. The operational conditions optimization of this green extraction approach will be presented for the first time, focusing on its potential use in industries such as food, supplement, medicine, and cosmetics.

2. Materials and methods

2.1. Chemicals

The reagents TPTZ (2,4,6-tri(2-pyridyl)-s-triazine), ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), and Folin-Ciocalteu, pNPG (p-nitrophenyl-α-D-glucopyranoside), *p*NPB (*p*-nitrophenol-butyrate), DNS (3,5-dinitro salicylic acid); standards Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (97 % purity) and gallic acid (> 97 % purity), and enzymes α-amylase from porcine pancreas, α-glucosidase from *Saccharomyces cerevisiae* and lipase from human pancreas were acquired from Sigma Aldrich (Steinheim, Germany). The aqueous solutions were prepared using distilled water (Milli-Q® Direct 8, Merck, HE, Germany).

Sodium carbonate (Sigma Aldrich), penicillin–streptomycin (pens/strep), Dulbecco's Modified Eagle's medium (DMEM) (Gibco), fetal bovine serum (FBS). TrypLE Express (Gibco) enzyme, phosphate-buffered saline (PBS) (Gibco), CellTiter 96® AQueous One Solution [3-(4,5-dimethylthiazol-2-yl)−5-(3-carboxymethoxyphenyl)−2-(4-sulfophenyl)−2H-tetrazolium, inner salt; MTS] (Promega Biotecnologia do Brasil), glutaraldehyde and formaldehyde were also used in several assays. DAPI [(4′,6-diamino-2-phenylindole, dihydrochloride) Molecular Probes Inc.], Alexa Fluor 546 conjugated to phalloidin (Molecular Probes Inc.), Triton® X100 (Sigma Aldrich) and Prolong® Gold (Molecular Probes Inc.) were used in cell culture.

2.2. Sample preparation

The unripe genipap fruits (*Genipa americana* L.) (~10 kg) were collected in Florianopolis, Santa Catarina, Brazil, at the Federal University of Santa Catarina (UFSC) (geographical coordinates: 27° 36′ 04″ S 48° 31′ 12″ W). The fruits were washed under running water, sanitized (NaCl at 200 mg/L for 15 min), and rinsed. Then, the entire epicarp (shell) and mesocarp (pulp) were removed, and the endocarp containing the seeds was used. The endocarp was fractionated and dried in an oven with air circulation at 50 °C (Lucadema, Model 82/27, SP, Brazil), ground in a knife mill (Marconi, Model MA340, SP, Brazil), and sieved to standardize particle size, from 0.60 to 0.85 mm. This project is registered in the National System of Management of Genetic Heritage (SisGen) of the Brazilian Ministry of Environment - www.sisgen.gov.br (register# A926CEA).

2.3. Ultrasound-assisted extraction (UAE)

Natural blue pigment extraction from genipap using an ultrasound probe (Eco-Sonic, SP, Brazil) was performed inside a jacketed cell controlled by a thermostatic bath (TECNAL, model TE-2005, SP, Brazil). For each extraction, 0.5 g of dry sample was added to the jacketed cell and filled with the solvent (distilled water) for a constant time of 10 min and a fixed frequency of 20 kHz. To optimize the extraction conditions, a Box–Behnken factorial design was used (Table 1). A total of fifteen assays were performed randomly. The independent variables evaluated were temperature (30, 50, and 70 °C), ultrasound power (150, 300, and 450 W), and solid-to-liquid ratio (1:20, 1:30, and 1:40 g/mL).

The extracts obtained were centrifuged (Quimis, Model Q222T, SP, Brazil) for 10 min at 412×*g* to separate all the solid content remaining in the sample, and then the liquid extract was poured into 50 mL flasks.

2.4. Extract characterization

2.4.1. Color parameters

For color determination, 300 μL of the extract was placed in a 96-well microplate. The absorbance measurement was performed using a microplate reader (Tecan, Model Infinite M200, ZH, Switzerland) with a wavelength range from 400 to 700 nm. Then, the absorbance dataset was treated using ColorBySpectra software (Ohio State Innovation Foundation, OH, United States), which compares the results at each wavelength with the CieLab scale, generating the color values using the CIE (Commission Internationale de l'éclairage) L* a* b* and L* C* h° scales according to 1964 standard observatory, using Illuminant D65 spectral distribution and 10° view angle. The color values were converted into images using an online converter (nixsensor.com/free-color-converter/).

2.4.2. Total phenolic content (TPC)

The methodology adopted for microplates described by Singleton and Rossi (1965) was used to determine the total phenolic content using the Folin-Ciocalteu reagent. The absorbances read at 765 nm were compared to the calibration curve for gallic acid [TPC = 15.6 x absorbance; R2 = 0.99), and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of sample (mg GAE/g).

2.4.3. Antioxidant activity

The antioxidant activity was determined using the ferric reducing antioxidant power (FRAP) method as proposed by Benzie and Strain (1996) with adaptations to the microplate reader (Teixeira et al., 2021) (Multileader Infinite M200 TECAN, ZH, Switzerland) and ABTS radical scavenging method, according to *Re* et al. (1999) adapted to microplate

Table 1
Box-Behnken design and results of the fifteen extraction tests regarding the concentration of total phenolics (CTF), antioxidant (FRAP) per gram of sample on a dry basis and color samples.

Assay	Temperature (°C)	Ultrasound power (W)	Solid to liquid ratio (m/v)	TPC (mg GAE/g)	FRAP (μmol TE/g)	Cielab b*	Cielab C*	Color
1	50	150	1:20	$18.77^{fg} \pm 1.18$	$17.88^{bdc} \pm 1.63$	$-7.20^{d} \pm 0.06$	$11.37^{a} \pm 0.07$	
2	30	300	1:40	$15.71^{cde} \pm 3.90$	$23.10^{d} \pm 2.07$	$-5.71^{f} \pm 0.06$	$6.57^{i} \pm 0.09$	
3	50	450	1:40	$22.10\ g^{h} \pm 0.07$	$14.90^{abc} \pm 2.61$	$-5.49\ g \pm 0.11$	$9.49^{d} \pm 0.23$	
4	30	450	1:30	$17.94^{cd} \pm 4.09$	$25.49^{cd} \pm 2.05$	$-7.34^{c} \pm 007$	$9.73^{c} \pm 0.15$	
5	70	450	1:30	$16.13^{ef} \pm 2.49$	$21.17^{d} \pm 0.70$	$-5.65^{f} \pm 0.10$	$10.33^{b} \pm 0.11$	
6	30	150	1:30	$18.22^{b} \pm 1.95$	$31.10^{cd} \pm 1.07$	$-7.49^{b} \pm 0.05$	$9.24^{e} \pm 0.03$	
7	30	300	1:20	$18.64^{ef} \pm 3.22$	$21.44^{acd} \pm 0.92$	$-5.70^{f} \pm 0.08$	$9.68^{c} \pm 0.15$	
8	50	300	1:30	$20.60^{a} \pm 3.03$	$34.94^{abcd} \pm 3.98$	$-8.08^{a} \pm 0.06$	$9.71^{c} \pm 0.11$	
9	50	300	1:30	$23.94^{h} \pm 5.93$	$13.19^{a} \pm 2.15$	$-6.03^{e} \pm 0.12$	$7.06^{h} \pm 0.08$	
10	50	300	1:30	$21.97\ g^{h} \pm 3.05$	$15.21^{abc} \pm 1.68$	$-4.34^{i} \pm 0.04$	$6.51^{i} \pm 0.12$	
11	70	150	1:30	$20.37^{de} \pm 2.06$	$21.98^{abcd} \pm 1.34$	$-5.37^{h} \pm 0.07$	$7.65\ g \pm 0.09$	
12	50	150	1:40	$19.83^{ef} \pm 2.99$	$20.17^{abcd} \pm 0.51$	$-6.10^{e} \pm 0.62$	$8.56^{f} \pm 0.08$	
13	50	450	1:20	$22.26^{cde} \pm 0.67$	$23.12^{abc} \pm 2.39$	$-3.79^{j} \pm 0.02$	$5.05^{h} \pm 0.15$	
14	70	300	1:40	$21.46^{ef} \pm 2.24$	$20.94^{abc} \pm 1.92$	$-3.77^{j} \pm 0.04$	$5.35^{j} \pm 0.14$	
15	70	300	1:20	$23.38^{c} \pm 1.50$	$26.46^{ab} \pm 4.46$	$-3.71^{j} \pm 0.05$	$5.33^{j} \pm 0.11$	

mg GAE: milligrams of gallic acid equivalent, mg TE: milligrams of Trolox equivalent, abc: different letters indicate a significant difference between samples in the same column, $p < 0.05$.

reader. Briefly, 280 μL of ABTS+ reagent was mixed with 20 μL of sample, and after 30 min of reaction time, the absorbance was measured at 734 nm. The results were expressed in micromoles of Trolox equivalents per g of dry waste (μmol TE/g dw), which were calculated from the standard curves (FRAP = 30·x absorbance; $R^2 = 0.99$ and ABTS = ABTS = 0.36·x absorbance; $R^2 = 0.97$).

2.5. Optimization process

The mathematical modeling of the extraction of natural blue colorant from *Genipa americana* L. was performed using response surface methodology (RSM) associated with multiple regression using Statistica v.13.5 software (TIBCO Software Inc., Palo Alto, CA, USA). The data obtained by Box–Behnken design (Table 1) were applied in a second-order polynomial model to fit the dataset (Eq. (1)):

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

where y represents the predicted response, β_0, β_i, β_{ii}, and β_{ij} are regression coefficients for linear, quadratic, and interaction terms, respectively, and x_i and x_j are the independent variables. The statistical significance of the fit was verified by ANOVA for each response (TPC, FRAP, C*, and b*), and the nonsignificant terms were removed from the model. Thus, the models were refitted only with significant terms ($p < 0.05$). The adequacy of fitting was verified by the $p_{lack\ of\ fit}$, and their quality was verified by the determination coefficient (R^2) value and its adjusted R^2.

After obtaining the mathematical models, the simultaneous optimization of all responses was performed employing the desirability function according to Derringer & Suit (Derringer & Suich, 1980) aiming to maximize the responses TPC, FRAP, and C* and minimize the Cielab b*. Finally, external validation was performed to verify the adequacy of the models, comparing the predicted values to experimental data, performed under the suggested optimal conditions.

2.6. Identification of iridoids and other compounds by UPLC-Q-ToF-MS/MS

Chemical characterization was performed on an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) coupled to a quadrupole and time-of-flight (QToF) mass spectrometer (Waters, Milford, MA, USA) following the dereplication method described by Silva et al. (2017). The separation was achieved on an Acquity BEH C18 column (1.7 μm, 2.1 × 150 mm; Waters, Milford, MA, USA) maintained at 40 °C with 5 μL of sample. The mobile phase

consisted of a combination of A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile) at a flow rate of 0.4 mL/min. The elution gradient varied linearly from 5 to 95 % B (v/v) over 15 min, and then the column was washed for two min and conditioned for two min. Mass spectra were recorded in both positive and negative modes in the mass range of 110–1180 Da. The source temperature was set at 120 °C, the desolvation temperature was 350 °C, the desolvation gas flow rate was 500 L/h, and the capillary voltage was 3.2 kV. Leucine enkephalin was used as a lock mass. The spectrometer was operated in the MS^E centroid mode using a tension ramp from 20 to 40 V. The instrument was controlled using Masslynx 4.1 software (Waters Corporation). The compounds were tentatively characterized through molecular formula provided byMassLynx 4.1 software based on their accurate masses (error < 5 ppm), isotopic patterns (i-fit), MS fragmentation patterns, and chemotaxonomic survey of the occurrence of secondary metabolites in the *Rubiaceae* family.

2.7. Inhibition capacity of digestive enzymes

The α-amylase inhibition was performed according to the method proposed by Ali et al. (2006). In a test tube, 40 μL of the set of samples from 4 to 20 mg/mL, 160 μL of water, 400 μL of the substrate (0.5 % starch solution, w/v), and 200 μL of the 0.5 mg/mL enzyme solution (prepared in phosphate buffer at pH 6.8) were mixed. The mixture was incubated at 25 °C for 15 min. After this period, the reaction was stopped by adding 400 μL of DNS reagent (3,5-dinitrosalicylic) followed by incubation at 90 °C for 15 min. The mixture was immediately cooled in an ice bath, and the absorbance was read in a spectrophotometer (800 XI, Femto, SP, Brazil) at 540 nm.

Pancreatic lipase inhibition was evaluated by the methodology described by Zhang et al. (2020). In the microplate, 50 μL of the set of extracts from 120 to 270 μg/mL and 50 μL of the pancreatic lipase PPL solution (1 mg/mL) in Tris–HCl pH 8 buffer was added, and the mixture was incubated at 37 °C for 10 min. Last, 50 μL of the substrate 2 mg/mL *p*-nitrophenol-butyrate (pNPB) in Tris–HCl buffer was added, followed by incubation at 37 °C for 20 min. The absorbance was read in a microplate reader at 405 nm.

The in vitro inhibitory activities for both enzymes were obtained in triplicate and expressed in% inhibition through Eq (2), and the medium inhibitory concentration (IC_{50}) was calculated in mg/mL for α-amylase and μg/mL for PL.

$$\%\ Inhibition = \left[1 - \left(\frac{C-D}{A-B}\right)\right].\ 100 \tag{2}$$

where A is the control (with enzyme and without sample), B is the blank control (with enzyme and without sample), C is the reaction (with enzyme and with sample), and D is the reaction blank (without enzyme and with sample).

2.8. In vitro cytotoxicity and antiproliferative activity

2.8.1. Cells

Human cell lines were normal fibroblast cells (L929), embryonic kidney (HEK-293), keratinocytes (HaCaT), lung adenocarcinoma (A549), human breast (MDA-MD-231), malignant skin melanoma (SK-MEL-28) and glioma (GL), in addition to cell lines of mice, melanoma (B16F10), and glioma cells (C6).

2.8.2. Culture conditions

Cell lines were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10 % (v/v) fetal bovine serum (FBS) and 1 % antibiotic (penicillin and streptomycin). Cells were kept under a moist atmosphere at 37 °C with 5 % CO_2. The cell culture medium was refreshed every 2 days until the cells reached 80–90 % confluence. Cells were detached using Tryplex, counted, and plated.

The cytotoxicity of the extracts was evaluated against cells following ISO 10993–5 (2009) using L929 cells. For both cytotoxicity and antiproliferative assays, the cells were placed in 96-well plates at a density of $1{\cdot}10^4$ cells/well. Samples of stock solutions were prepared in ultrapure water followed by successive dilutions in complete medium to give a final concentration of 500 μg/mL. After 24 h for complete adhesion, the extract was added (500 μg/mL). Doxorubicin was used as a positive control at a final concentration of 10 μg/mL. The metabolic activity was measured using MTS solution (de Sousa et al., 2020) after 24 h of contact of the extracts with the cells.

2.9. Hydrogen peroxide-induced oxidative stress in L929 fibroblast cells

The evaluation of cell viability after oxidative stress (H_2O_2) was performed according to Pitz et al. (2016). L929 fibroblast cells were seeded at a density of $1{\cdot}10^4$ cells/well into a 96-well plate containing DMEM supplemented with 10 % FBS and incubated overnight at 37 °C in a humidified 5 % CO_2 atmosphere. After incubation, DMEM with 10 % FBS containing 250 μg/mL (dry weight) anthocyanin-rich extracts (MA and PLE) was used to treat cells exposed concomitantly to 1.0 mM H_2O_2 for 24 h (dose that decreases 80 % of cell viability from L929 cells in 24 h). Cell viability was evaluated using the MTS assay (de Sousa et al., 2020).

2.10. Statistical analysis

The experimental data are presented as the mean ± standard deviation (SD). The variance homogeneity of the data set was checked by Levene's test ($p > 0.05$) followed by one-way variance analyses, and the Fisher LSD test was utilized to compare the averages. All statistical analyses were performed using Statistica v.13.5 software (TIBCO Software Inc., Palo Alto, CA, USA).

3. Results and discussion

3.1. Extraction optimization

There were significant differences ($p < 0.05$) in all responses obtained for each extraction assay, ranging from 15.71 to 23.94 mg GAE/g for TPC, from 13.19 to 26.46 for antioxidant capacity by FRAP assay, −8.08 to −3.71 for b* coordinate, and 5.05 to 11.37 for C* values (Table 1). According to the results, it was possible to observe that process conditions influenced the extraction yield. Then, the dataset was fitted by multiple regression analysis following the second-order polynomial model according to Section 2.5. Each mathematical model had its adequacy checked by the ANOVA F value to verify its statistical significance. The obtained mathematical models, Eqs. (3) to (6), were significant ($p_{model} < 0.05$) and presented high regression coefficients varying from 0.85 to 0.98 and R^2_{adj} from 0.77 to 0.96.

$$TPC = 22.75 + 0.33x_1 - 3.48x_1^2 - 2.58x_3 - 1.53x_1x_2^2 + 1.83x_1^2x_2 - 0,54x_1x_3 + 4.58x_1^2x_3 + 1.37x_2x_3 \tag{3}$$

$$FRAP = 23.46 + 5.85\ x_1 + \ 3.37\ x_1^2 - 6.16\ x_2^2 + 2.23\ x_3 + 1.34x_1x_2 - 1.91x_1x_2^2 - 1,41\ x_1^2x_3 \tag{4}$$

$$b^* = \ -3.75 - 0.57\ x_1 - 1.97\ x_1^2 - 0.71\ x_2^2 - 0.99\ x_3^4 - 0.83\ x_1x_2 \tag{5}$$

$$C^* = 5.24 - 0.70\ x_1 + 3.16\ x_1^2 + 0.87\ x_2^2 + 1.33\ x_3^2 + 1.26\ x_1x_2 \tag{6}$$

The temperature (x_1) exerted a significant influence ($p < 0.05$) on all variable responses. In general, temperature presents a positive relation to extraction yield, i.e., the temperature increase improved the yield of TPC and, consequently, the in vitro antioxidant activity. The temperature-positive effects are related to the increase in extraction rate

due to the increase in solute solubility and the decrease in solvent viscosity, improving the diffusivity in the matrix (Kumar et al. 2021). However, TPC model also presents a quadratic negative effect, which means that there is an intermediate temperature resulting in a maximum TPC value, above this point, the temperature exercises a negative effect on the TPC yield (Fig. 1). This fact can be related to the degradation of some phenolic compounds above approximately 50 °C (Benvenutti et al., 2019; Kumar et al., 2021) and also due to temperature rise weakening the cavitation phenomena of ultrasound (Kumar et al., 2021).

For the b* and C* value models, temperature presented a negative effect. However, this study aimed to decrease the b* and C* values since negative values are related to the blue color intensity. Probably, the increase in temperature favors the hydrolysis of geniposide on genipin besides improving the genipin solubility, which is the main phytochemical precursor com natural blue color compounds.

The ultrasound power (x_2) also presented significant effects ($p < 0.05$) for all dependent factors. The ultrasound power presented a positive effect on C* and a negative quadratic effect on b*. The increase in power increased the color intensity and blueness up to a certain point. In addition, this variable presented a negative quadratic effect on FRAP values and interactive effects with temperature and liquid-to-solid ratio in the TPC model. These effects can be explained by the influence of power on the ultrasound waves that, through compression and rarefaction process, cause cavitation bubbles, resulting in mechanical and thermal effects on the extraction system. The cavitation phenomena contribute to plant matrix cell wall breakdown and the release of target compounds into the solvent through diffusion and dissolution, improving the extraction efficiency. However, this variable can also be related to molecule breakdown effects, which probably decrease the antioxidant activity, blueness, and TPC value (Heleno et al., 2016; Kumar et al., 2021). Therefore, to obtain extracts with an intense blue color with good antioxidant activity, the intermediate ultrasound power evaluated resulted in better extraction performance.

The solid-to-liquid ratio (x_3) presented significant effects ($p < 0.05$) for all mathematical models presented. This variable positively influenced the FRAP value, color intensity, C*, and blueness (negative b* value) indicating that the maximum values of solid-to-solvent evaluated improved the extracts' antioxidant activity and blue intensity. According to the literature, this variable presents a positive correlation with the extraction yield until the process reaches equilibrium (Benvenutti et al., 2019). In UAE, the increase in the solid-to-liquid ratio can improve the cavitation effect and consequently the extraction rate due to the decrease in concentration and viscosity of the extraction medium (Kumar et al. 2021).

From these mathematical models, a multiresponse optimization was carried out aiming to maximize the TPC, FRAP, and C* values and to minimize the b* values using the desirability function (d). An optimum condition of extraction was suggested at 70 °C, 325 W, and a solid-to-liquid ratio of 1:40 (w/v). This prediction was externally validated, proving these values with experimental values obtained in the optimal conditions suggested. The antioxidant activity by the FRAP method presented a predicted value of 31.29 μmolTE/g and an experimental value of 30.67 ± 3.36 μmolTE/g. The predicted TPC value was 22.63 mg GAE/g, while the experimental value was 23.32 ± 1.04. For color parameters, the predicted values were −8 and 9.57 for b* and C*, respectively, and the experimental values were −7.86 ± 0.13 and 8.87 ± 0.14. Therefore, the optimized conditions were validated within the predicted interval of 90 % confidence.

Gualberto et al. (2021) evaluated Brazilian fruits' phenolic, flavonoid, and antioxidant activity. To genipap, they reported similar antioxidant activity by the FRAP method, with values of 26.61, 29.90, and 65.83 μmol of ferrous sulfate/g dw, using 80 % ethanol, 80 % methanol, and 80 % acetone as solvents, respectively, and lower TPC values, from 2.29 to 3.42 mg GAE/g dw using the same solvents. These authors also used UAE to recover the bioactive compounds, applying the same operating conditions to all evaluated fruit (40 °C, 30 min, and frequency of 30 kHz). Therefore, the proposed models were efficient in predicting the responses to obtain a blue extract with bioactivity from genipap using only water as the solvent. Besides the greatest yields, the optimized parameters reduce the process's energy and costs, contributing to the green character of the process (Benvenutti et al., 2022).

3.2. UPLC-Q-TOF-MS/MS characterization

Thirteen compounds were identified by UPLC-Q-TOF-MS/MS in the aqueous blue extract obtained under optimal conditions (Table 3). Galacturonic acid was identified in peak 1, besides twelve iridoids identified considering their mass fragmentations. Peak 2 presented a mass-to-charge ratio (*m/z*) value of 389.1074 for the deprotonated molecule ($[M - H]^-$) ($C_{16}H_{21}O_{11}^-$). In the MS/MS spectra, this peak produced fragment ions at *m/z* 227.0583 ($C_{10}H_{11}O_6^-$), corresponding to glucose molecule loss, and at *m/z* 209.0539 ($C_{10}H_9O_5^-$), corresponding to losses of glucose + water molecules. In addition, the fragment ions at *m/z* 183.0660 ($C_9H_{11}O_4^-$) and 165.0534 ($C_9H_9O_3^-$) probably correspond to CO_2 (44 Da) loss from ions *m/z* 227.0583 and 209.0539, respectively. According to these characteristic ions, peak 2 corresponded to the compound deacetylasperulosidic acid, which is an iridoid identified in other plants belonging to the *Rubiaceae* family (Fu et al., 2014; Zhou et al., 2010).

Both peaks 3 and 6 presented the $[M - H]^-$ ion at *m/z* 373.1135 ($C_{16}H_{21}O_{10}^-$); however, they differ in the sizes of the ion fragments. In peak 3, the major fragment is an ion *m/z* 193.0535, while in peak 6, it is at 211.0590. Where an OH group is at C-8 or the carbon is directly bonded to C-8, oxygen loss is difficult during fragmentation, resulting in a neutral loss of 162 Da, which occurred in peak 6. In the absence of this group in C-8, generally, the loss is 180 Da. Based on the molecular structure of these iridoids and the size of the ion fragment, peaks 3 and 4 correspond to compounds already identified in genipap, Gardoside, and Geniposidic acid, respectively (Bentes & Mercadante, 2014; Fu et al., 2014).

Peak 5 showed an $[M - H]^-$ ion at *m/z* 391.1240 and three fragment ions with the same characteristics presented in the iridoid identified in genipap fruit as Shanzhiside by Bentes and Mercadante (2014). These authors considered the loss of hexose (162 Da) in ion *m/z* 229.0705, CO_2 (44 Da) in 185.0826, and water (18 Da) in 167.0675. Peak 9, with an $[M - H]^-$ ion at *m/z* 449.1301, was tentatively identified as gardenoside, according to Fu et al. (Fu et al., 2014). Gardenoside is an iridoid previously reported in genipap fruit (Bentes & Mercadante, 2014; Iramaia Angelica Neri-Numa, Pessôa et al., 2020). Peak 12, with an $[M - H]^-$ ion at *m/z* 549.1819, molecular form C23H33O15- and ion fragments at *m/z* 225.0751 and 207.0679, corresponded to genipin gentiobioside (Fu et al., 2014). This iridoid has already been reported in genipap fruit and is also one of the important components that present anticancer activity in *Gardenia jasminoides*, another plant of the *Rubiaceae* family (Bentes & Mercadante, 2014; Chen et al., 2017). In addition, genipin is the main responsible for the blue color (Bentes & Mercadante, 2014; Náthia-Neves et al., 2021). Geniposide, the main iridoid detected in unripe genipap fruit, was identified in peak 13, which presented an $[M - H]^-$ ion at *m/z* 433.1337 ($C_{18}H_{25}O_{12}^-$) (Bentes & Mercadante, 2014).

Peaks 4 and 8 were tentatively identified as iridoids already reported in genipap leaves. Peak 4 presented an $[M - H]^-$ ion at *m/z* 375.1291 ($C_{16}H_{12}O_{10}^-$) and a fragment ion at *m/z* 273.1150 corresponding to the compound known as asytonasioside D. In peak 8, the $[M - H]^-$ ion at *m/z* 375.1291 ($C_{16}H_{12}O_{10}^-$) and fragment ions at *m/z* 213.0696, 169.0448 and 125.0547 were compatible with loganic acid (Silva et al. 2018).

On the other hand, peak 11 was tentatively identified as ixoroside, an iridoid not yet reported in genipap extract. This compound presented an $[M - H]^-$ ion at *m/z* 405.1415 and a fragment ion at *m/z* 197.0798 due to glucose molecule loss (Fu et al., 2014). In addition, one unknown compound with an $[M - H]^-$ ion at *m/z* 387.1293 ($C_{12}H_{23}O_{10}^-$) peak 14) was identified. Probably, it is a derivative compound from geniposide, since a $[M - H]^-$ fragment at *m/z* 387 was detected in the geniposide

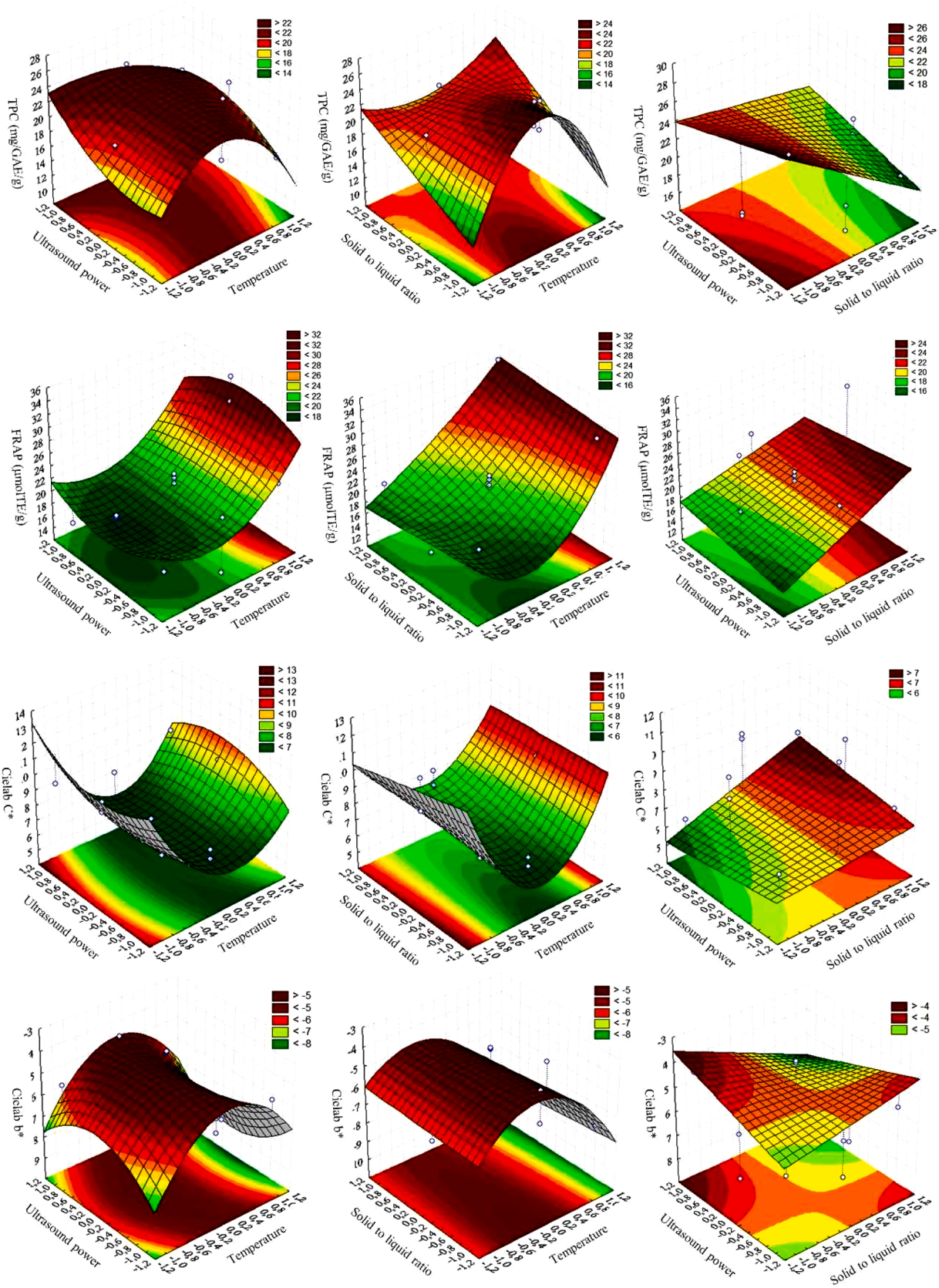

Fig. 1. Three-dimensional (3D) response surfaces showing the effect of independent variables (temperature, ultrasound power and solid to liquid ratio) on the yield of blue extract obtained evaluated by the dependent variables (TPC, FRAP, Cielab C* and Cielab b*).

peak for Bentes and Mercadante (2014) using MS/MS.

3.3. Biological activity of the aqueous blue extract

The antioxidant activity of the aqueous blue extract obtained under optimum conditions was evaluated by combining FRAP and ABTS methods (Table 2). The FRAP and ABTS values, 30.67 and 36.77 μgTE/g dw, respectively, are higher than the ABTS value reported by Neri-Numa et al. (2020), 24.67 μg TE/g dw, which obtained a blue extract from unripe genipap by UAE using methanol 80 % as the solvent.

The freeze-dried extracts were also evaluated in terms of inhibiting the activity of digestive enzymes, α-amylase, and pancreatic lipase (PL). α-Amylase is a hydrolytic enzyme produced by plants, fungi, and animals, including humans, that specifically hydrolyses α−−(1,4)-glycosidic linkages, leading to the formation of maltose, maltotetraose, maltodextrins, and glucose. Therefore, the inhibition ability of α-amylase and other hydrolases, such as α-glucosidase, can be associated with a decrease in the spread and progression of type 2 diabetes mellitus (T2DM) due to a reduction in the blood sugar absorption rate from the small intestine (Barik et al., 2020; Papoutsis et al., 2021). On the other hand, as PL is associated with lipid absorption, the inhibition of their activity could assist in obesity control (Xie et al., 2018).

The mean inhibitory concentration (IC_{50}) for α-amylase, calculated by linear regression of the dataset (R^2>0.96), was 9.11 ± 0.16 mg/mL. Justino et al. (2020) evaluated the α-amylase inhibition capability of *Eugenia dysenterica* fruit pulp extracts, including *Genipa americana*, which inhibited less than 40 % of the α-amylase activity at 10 mg/mL. The common positive control, acarbose, presented 66.8 % inhibition at a concentration of 0.645 mg/mL, from the same test, also using α-amylase from the porcine pancreas. Although the blue extract presents a lower inhibition potential than acarbose, it presents a potential natural anti-glycemic alternative.

For PL, the IC_{50} value determined through the linear regression of the dataset (R^2>0.95) was 128.90 ± 6.61 μg/mL; therefore, the blue extract from genipap is more effective for PL than α-amylase. Chamnansilpa et al. (2020) evaluated the inhibition of PL in anthocyanin extracts, which is a natural pigment also extracted from plants. The reported values ranged from 90.6 ± 0.4 to 181.7 ± 0.8 μg/mL. Therefore, the extract from genipap presented similar PL inhibition activity to those found for anthocyanin-rich extract, a common compound associated with the inhibition of digestive enzymes. Furthermore, the blue extract presented a higher inhibition potential than Oristatic® (IC_{50} = 180 μg/mL), a potent specific lipase inhibitor (Zhang et al., 2020).

Therefore, UAE using water as the solvent under optimized conditions was a good approach to obtain extracts with high biological potential. Beyond colorant function, the aqueous blue extract obtained from genipap can be used to prevent lipid oxidation in food besides presenting potential health benefits for foods and pharmaceutical formulations. This data supports future studies about the digestibility and bioavailability of this extract, including experimental design using in vitro and in vivo models to elucidate the biological effects, considering the potential of this underused plant matrix.

Table 2
In vitro antioxidant, antidiabetic, and antilipolytic potential of the aqueous blue extract obtained from genipap fruit.

Assay	Values
Antioxidant activity	
FRAP (μmol TE/g)	30.67 ± 3.36
ABTS (μmol TE/g)	36.77 ± 3.04
Enzymatic inhibition	
α-amylase (IC_{50} mg/mL)	9.11 ± 0.6
Lipase (IC_{50} μg/mL)	128.90 ± 6.61

3.4. In vitro cytotoxicity and antiproliferative activity

The metabolic activity of the cells after contact for 24 h with the aqueous blue extract obtained under the optimal conditions is shown in Fig. 2 and compared with the standard drug (Doxo 10 μg/mL).

For cytotoxicity analysis, the standard (ISO/EN10993-5, 2009) identifies compounds and/or materials with cytotoxic potential when cellular metabolic activity is less than 70 %. The compounds present in the genipap extract, in the best extraction condition, do not present cytotoxicity. Genipap extracts may have antitumor activity correlated to the compounds present in the extract (Neri-Numaet al., 2020). This is often influenced by the extraction method (dos Santos Faria et al., 2022) and purification procedure. In this work, we evaluated a fixed concentration of 500 μg/mL extract in different cell lines. It was not possible to observe a significant reduction in metabolic activity. The most significant expression of 30 % was observed for HEK293 cells (human embryonic kidney cell line), even though it is not considered toxic against kidney cells according to ISO 10,993–5.

To evaluate extract protection from H_2O_2-induced cellular cytotoxicity, the cells were incubated with H_2O_2, and cellular viability was analyzed in the presence of extract or vehicle. Oxidative stress induced with 1.0 mM H_2O_2 produced a reduction in cell viability of 83.5 % ± 0.5 %. Under these conditions, extract treatment improved cell viability and reduced cell viability from 77.8 % ± 0.3 % in cells exposed to H_2O_2 and treated with the extract (250 μg/mL), indicating that the extract protects against the oxidative stress-associated cell death induced by H_2O_2 in a cell.

4. Conclusion

The experiments show the effectiveness of extracting the blue colorant from the unripened genipap employing ultrasound-assisted extraction (UAE) using only water as the solvent. It was possible to optimize the extraction conditions (70 °C, 325 W, and a solid-to-liquid ratio of 1:40 w/v), to obtain extracts with maximum blue color intensity (b*: −7.86 and C*: 8.87) TPC yield (23.32 mg GAE/g), and antioxidant potential (FRAP: 30.67 μgTE/g and ABTS: 36.77 μgTE/g). UPLC identified the presence of fourteen iridoids in the aqueous blue extract obtained under great conditions. In addition to presenting potential use as a natural blue colorant, the recovered extract presented the capacity to inhibit digestive enzymes such as α-amylase (IC_{50} = 9.11 mg/mL) and pancreatic lipase (IC_{50} = 128.90 μg/mL). The extract did not present antiproliferative potential, however, showing no cytotoxicity against fibroblast cells. Therefore, this natural blue extract presents high potential use in the food, supplement, pharmaceutical, and cosmetic industries.

CRediT authorship contribution statement

Laís Benvenutti: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Beatriz Cesa Rovaris:** Writing – original draft, Conceptualization, Data curation, Formal analysis, Investigation. **Karina Cesca:** Data curation, Formal analysis. **Débora de Oliveira:** Writing – review & editing, Investigation, Project administration, Resources, Supervision. **Paulo Riceli Vasconcelos Ribeiro:** Formal analysis. **Edy Sousa de Brito:** Data curation, Formal analysis, Methodology. **Acácio Antonio Ferreira Zielinski:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Resources.

Declaration of competing interest

The authors declare that there are no conflicts of interest in this research.

Table 3
Identified compounds from the aqueous blue extract obtained from genipap by UPLC-QTOF-MS/MS.

Peak n°	Rt min	[M-H] $^-$ Observed	[M-H] $^-$ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm (error)	Putative Name	Ref
1	1.38	193.0345	193.0348	–	$C_6H_9O_7$	−1.6	Galacturonic acid	–
2	2.49	389.1074	389.1084	227.0583, 209.0539, 183.0660, 165.0534	$C_{16}H_{21}O_{11}$	−2.6	Deacetylasperulosidic acid	Fu et al.(2014)
3	2.64	373.1128	373.1135	193.0535, 167.0682, 149.0607	$C_{16}H_{21}O_{10}$	−1.9	Gardoside	Bentes and Mercadante (2014), Fu (2014)
4	2.72	535.1658	535.1663	373.1150	$C_{22}H_{31}O_{15}$	−0.9	Asystasioside D	Silva et al.(2018)
5	2.80	391.1242	391.1240	229.0705, 185.0826, 167.0675	$C_{16}H_{23}O_{11}$	0.5	Shanzhiside	Bentes and Mercadante (2014), Fu (2014)
6	2.87	373.1132	373.1135	211.0590, 167.0720, 149.0582	$C_{16}H_{21}O_{10}$	−0.8	Geniposidic Acid	Bentes and Mercadante (2014), Fu (2014)
7	3.00	199.0600	199.0606	181.0468, 155.0676	$C_9H_{11}O_5$	−3.0	Hydroxygenipic acid	Dickson et al.(2018)
8	3.13	375.1283	375.1291	213.0696, 169.0848, 125.0547	$C_{16}H_{23}O_{10}$	−2.0	Loganic acid	Silva et al. (2018)
9	3.20	449.1301	449.1295	403.1157, 241.0697	$C_{18}H_{25}O_{13}$	1.3	Gardenoside	Fu et al. (2014)
10	3.28	241.0715	241.0712	209.0432, 165.0559	$C_{11}H_{13}O_6$	1.2	Genipinic acid	Dickson et al. (2018)
11	3.35	405.1415	405.1397	197.0798	$C_{17}H_{25}O_{11}$	4.4	Ixoroside	Fu et al. (2014)
12	3.67	549.1821	541.1819	225.0751, 207.0679	$C_{23}H_{33}O_{15}$	0.4	Genipin Gentiobioside	Fu et al. (2014)
13	4.08	433.1337	433.1346	123.0426	$C_{18}H_{25}O_{12}$	−2.1	Geniposide	Fu et al. (2014); Bentes and Mercadante (2014)
14	4.23	387.1293	387.1291	207.0644	$C_{17}H_{23}O_{10}$	0.5	Unknown	–

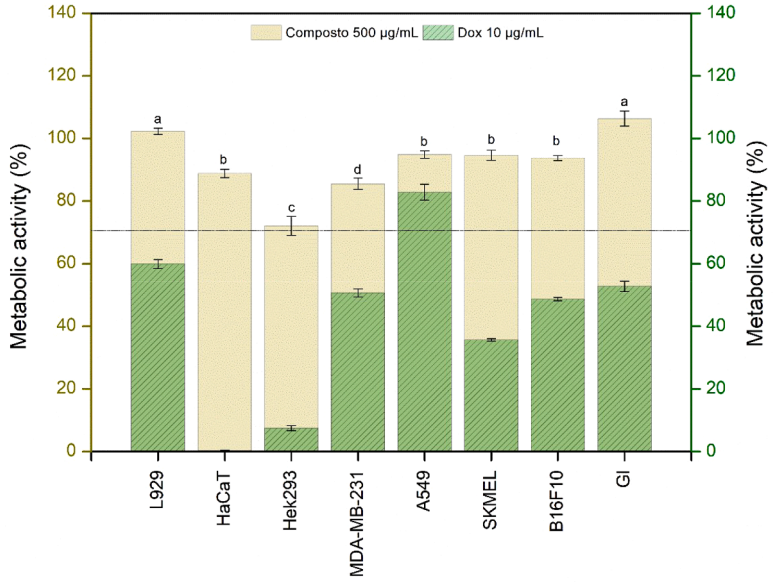

Fig. 2. Effects of aqueous blue extract (500 µg/mL) and doxorubicin (10 µg/mL) on the viability of normal fibroblast cells (L929), keratinocytes (Hacat), embryonic kidney (HEK-293), human breast cancer (MDA-MD-231), lung adenocarcinoma (A549), malignant skin melanoma (SK-MEL-28), melanoma (B16F10) and glioma cells (C6).

Data availability

No, I don't have any research data outside the submitted manuscript file.

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