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Decaffeination of yerba mate by supercritical fluid extraction: Improvement, mathematical modelling and infusion analysis

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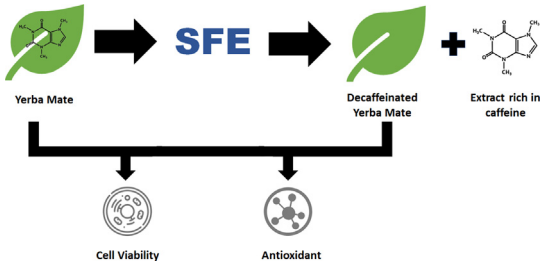
HIGHLIGHTS

- Decaffeination of yerba mate leaves by supercritical fluid extraction.
- Supercritical CO_2 extractions performed at 300 bar and 60 °C with ethanol as cosolvent.
- Box-Behnken design result: CO_2 flow (950 g/h); ethanol flow (106 g/h); time (4.25 h).
- Caffeine does not influence in the antioxidant capacity and cell viability of esophageal.
- Caffeine concentration in infusion of the decaffeinated yerba mate is 0.030% ± 0.005%

GRAPHICAL ABSTRACT



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ABSTRACT

This work aimed to improve yerba mate caffeine extraction process, considering three independent variables, carbon dioxide flow, ethanol flow and extraction time, in three levels. The following process parameters were kept constant: pressure (300 bar), temperature (60 °C), and particle size (0.428 mm). The Box-Behnken experimental design was carried out for 15 extractions and the optimized condition to obtain decaffeinated yerba mate was as follows: 950 g/h of the carbon dioxide flow; 106 g/h of the ethanol flow; 4.25 h of the time. Under these conditions, it was possible to obtain processed yerba mate with 0.16% ± 0.06% ($g_{caffeine}/g_{yerba\ mate}$). Furthermore, the removal of caffeine from yerba mate did not decrease the antioxidant capacity of extracts obtained by infusion, as well as cell viability tests showed that both extracts decreased cell viability in esophageal cancer. These results indicate that decaffeinated yerba mate maintains that properties found in traditional yerba mate.

1. Introduction

Discovered in 1820, caffeine is one of the most consumed stimulating substances worldwide [1] and is part of the everyday lives of most people in different countries [2]. Caffeine is capable of stimulating the nervous, muscular and circulatory systems [3],

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increasing mental focus; and acting against fatigue [4]. Several drinks contain caffeine, including coffee, tea, infusions such as chimarrão and tereré, guarana and cola sodas [5,6]. The consumption of these caffeine-containing products is not always recommended for everyone, since they can have undesirable health effects. The most affected groups are children, pregnant women and individuals with a higher sensibility to the effects of caffeine on the nervous system. These side effects range from insomnia, elevation of blood pressure, increasing metabolic rate, and diuresis [7]. Therefore, decaffeination of these products is important from a health perspective, while it is also economically attractive since it promotes obtaining decaffeinated products as well as natural caffeine, which is frequently used in pharmaceutical industries and in the production of energy drinks [8].

Yerba mate (*Ilex paraguariensis* St Hill) has a caffeine mass concentration of 0.5–2.0% in its dried leaves [9] and ingestion of this alkaloid usually occurs in the form of an infusion of crushed leaves and branches in hot or cold water, which are beverages called *chimarrão* or *tereré*, respectively [10]. As part of the Aquifoliaceae family, this species grows naturally in the subtropical regions of South America and is mainly found in Brazil, Argentina, Paraguay and Uruguay [11]. Several studies of yerba mate evaluate the chemical composition of the extracts, especially groups of compounds, such as flavonoids, saponins phenolic compounds and methylxanthines, including caffeine [6,12]. However, a number of factors can alter the extract chemical composition, including the planting and harvesting season, growing site and even industrial processing that the vegetable material is subjected to before being sold [6,13].

To decaffeinate leaves and seeds of different plant materials, different processes can be used: extraction with hot or cold water [14], extraction with organic solvents [15],and extraction with supercritical solvents [16–18]. In a comparison of these approaches, extraction with supercritical solvents has the advantage of being more selective than the other approaches and avoids the loss of important compounds to the final product [19]. It is important to highlight that the great majority of studies about decaffeination, using supercritical fluid extraction, usually have different types of teas and coffees as its main object of study [17,20]. Supercritical extraction of caffeine from yerba mate has been discussed in the works of Saldaña et al. [21], Cassel et al. [16], and Vieitez et al. [22].

This study aims to reduce the extraction time and solvent consumption of yerba mate decaffeination process, using ethanol as a co-solvent. Thus, the process variables CO_2 flow, ethanol flow and extraction time will be evaluated under the following process conditions: 300 bar, 60 °C. High Performance Liquid Chromatography (HPLC) was used to analyze the caffeine content on the plant before and after the extraction process yerba mate decaffeination. Furthermore, the effects of caffeine concentration in the infusions were evaluated on cell viability in different cell lines of human esophageal cancer, and on antioxidant activity. This malignancy is the eighth most common cancer in the world [23,24] and, in Southern Brazil, is a recurrent disease among consumers of yerba mate infusions. Amigo-Benavent et al. [25] reported that consumption of yerba mate does not increase proliferation of cancer cells, suggesting yerba mate might have interesting anti-proliferative potential in cancer prevention due to bioactive compounds as phenolic compounds. Therefore, we highlight the effects of phenolic compounds and methylxanthines on esophageal cancer.

2. Material and methods

2.1. Yerba mate

Yerba mate leaves were provided by Baldo S.A, located in São Mateus do Sul - Brazil. As for processing step, the plant material used in this study was recovery after the roasting or zapecado [13]. The mean particle diameter of the leaves used in the experiments was 0.428 mm, evaluated using 5 sieves from the Tyler series with mesh sizes ranging from 16/46 plus the pan, with 15 min of agitation. The moisture content of each sample was determined by the principle of thermogravimetry and the apparatus that was used was the Moisture Analyzer HB43 (Mettler Toledo) with approximately 0.65 g of yerba mate that were used. The density of the sample was determined with the gas-pycnometry technique [26], on the Quantachrome Multipycnometer The experiments were performed in duplicate.

2.2. Supercritical fluid extraction

All experiments for extraction of nonvolatile compounds of yerba mate were conducted on a supercritical extraction pilot unit [27]. The vessel nominal volume is 500 mL with a 5.4 cm diameter and total vessel height of 21 cm. The extractions were performed using 140 g (4.6% moisture content) of yerba mate (comprising a 16.1 cm fixed bed height and 0.71 initial porosity) at 300 bar and 60 °C (maximum settings possible on the used equipment) and as independent variables were carbon dioxide flow (700, 950, and 1200 g/h ± 0.1%), ethanol (co-solvent) flow (50, 78 and 106 g/h ± 0.5%), and extraction time (2.0, 5.5, and 5.0 h). After the expansion, two 200 mL separator vessels are maintained at 3 and 1 bar, sequentially, in a way that all the extract precipitates at the first separator. These conditions were selected to maximize caffeine extraction and consequently reduce the content of caffeine in yerba mate leaves. The extraction curve, defined as the extract or caffeine mass *versus* time, was determined experimentally collecting the nonvolatile extract every 20 min. After extraction, yerba mate was removed form extraction vessel and analyzed to determinate the remaining content of caffeine.

2.3. Experimental design

The response surface methodology 2^3 with center point design was created to optimize the extraction of caffeine from yerba mate leaves, considering three independent variables, carbon dioxide flow, ethanol flow, and extraction time, in three different levels. On the other hand, the other process parameters were kept constant: pressure (300 bar), temperature (60 °C), plant mass (140 g), and particle size (0.428 mm). In total, the Box and Behnken design [28] was performed for 15 extractions (Table 1) and as response (Y) we obtained the caffeine concentration in processed yerba mate. The CO_2 mass flow accuracy of <0.1% through the cylindrical extraction vessel (Siemens® Sitrans FC Massflo® Mass 6000) and ethanol flow accuracy of 0.5% of setpoint (ISCO 260D syringe pump). An analytical scale (Mettler Toledo® ab204) was used to measure the mass of the extracts obtained with an accuracy of 0.001 g. Eq. 1 expresses the polynomial model applied to relate caffeine concentration to independent variables. In order to perform the statistical analysis of the results of the experimental design 2^3 with central point was used the software Statistica 10®.

$$Y = \beta_0 + \sum_{i=1}^{k}\beta_i X_i + \sum_{i=1}^{k}\beta_{ii} {X_i}^2 + \sum_{i=1}^{k-1}\sum_{i<j}^{k}\sum_{j=2}^{k}\beta_{ij} X_i X_j \quad (1)$$

where Y is the concentration of caffeine in yerba mate after extraction, X_1 (carbon dioxide flow), X_2 (ethanol flow), and X_3 (time) are independent variables, k is the number of variables, and β_0 is a constant, β_i is the linear coefficient, β_{ii} is the regression coefficient of quadratic term, and β_{ij} is the interaction coefficient.

Table 1
Variables and their levels used in Box-Behnken design with triplicate in central point for caffeine supercritical fluid extraction from yerba mate.

	Decoded independent variables			Codded independent variables		
Experiment	Carbon dioxide flow (g/h)	Ethanol flow (g/h) – (%)*	Time (h)	X_1	X_2	X_3
1	950	78 – 7.5	3.5	0	0	0
2	700	106 – 13.2	3.5	−1	1	0
3	1200	50 – 4.0	3.5	1	−1	0
4	700	50 – 6.7	3.5	−1	−1	0
5	950	78 – 7.6	3.5	0	0	0
6	950	106 – 10.0	2.0	0	1	−1
7	1200	78 – 6.1	2.0	1	0	−1
8	700	78 – 10.0	2.0	−1	0	−1
9	700	78 – 10.0	5.0	−1	0	1
10	1200	106 – 8.1	3.5	1	1	0
11	1200	78 – 6.1	5.0	1	0	1
12	950	50 – 5.0	5.0	0	−1	1
13	950	50 – 5.0	2.0	0	−1	−1
14	950	78 – 7.6	3.5	0	0	0
15	950	106 – 10.0	5.0	0	1	1

*Ethanol mass fraction in the total supercritical flow rate in percentage.

2.4. Caffeine analysis

The caffeine concentration in the yerba mate leaves before and after the extraction process was determined by HPLC [9]. Chromatographic analyses were conducted on an Agilent system, model 1200 with a UV detector. Compound separation was performed using a gradient mixture method with A containing MiliQ water/Acetic Acid (98:2 v/v) and B containing Methanol/Acetic Acid (98:2 v/v). The gradient went from 17% B to 20% B in 10 min with 20% isocratic in 5 min, 20% B to 23% B in 10 min and 23% B to 100% B in 5 min. The flow was 1 mL/min. The column was a C18 (4.6 × 250 mm × 5 μm) and the wave-length, λ, was 273 nm [9].

The procedure for determining the concentration of caffeine in the yerba mate leaves started with an aqueous decoction [9] of the plant material. This procedure was made using 100 mL of MiliQ water and 2 g of yerba mate leaves and the mixture was boiled for 20 min. After it cooled down, the mixture was filtered and analyzed by HPLC, which followed the previously presented method. This procedure was performed both for the yerba mate before and after the extraction process. To determine caffeine content in the extract, initially it was dried at 60 °C for 8 h to remove the ethanol remaining in the collector vessel. Thereafter, 100 mL of MiliQ water was added in the collector vessel and the extract was dissolved with the aid of ultrasound energy. This solution was collected, filtered and analyzed by method previously described (HPLC).

2.5. Infusion analyses

Traditional (ECaf) and decaffeinated (EDCaf) yerba mate infusions were prepared by 7 g of leaves in 70 mL of MiliQ water for 6 h at 60 °C to perform antioxidant and cell viability analysis. These infusions were diluted in six concentrations: 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, and 2.00 mg yerba mate/mL (three replicates for each concentration). The statistical test that was used was One-way analysis of variance (ANOVA), followed by a Tukey post-hoc test. The results are presented as the standard error of the mean. The GraphPad Prism 5.0® program was used to generate graphs.

2.5.1. Antioxidant capacity

To evaluate the impact of the process of caffeine extraction was determined the antioxidant capacity of the traditional and decaffeinated yerba mate infusions by the DPPH (2,2-difenil-1-picril-hidrazil) method as free radical [29]. This method is based on the capture of DPPH (Sigma®) by antioxidant compounds, producing a decrease in absorbance when DPPH reacts with an antioxidant compound, it is reduced, changing color. The changes in color, from deep violet to light yellow, and the absorbance was read at λ =515 nm after 2 h of reaction using a UVVIS spectrophotometer (Biospectro SP-220, Brazil). A DPPH solution 60 μM was prepared. The reaction mixture consisted of adding 2.9 mL of DPPH 60 μM and 0.1 mL of extract solution. The antioxidant capacity was calculated (Eq. 2) as a percentage of initial concentration (60 μM). Ethanol was used to zero the spectrophotometer. All determinations were performed in triplicate. To determine the control absorbance, 0.1 mL of MiliQ water was added without the presence of extract in 2.9 mL of the 60 μM DPPH solution.

$$\text{Antioxidant Capacity}\,(\%) = \frac{Abs_{sample}}{Abs_{control}} * 100 \qquad (2)$$

where $Abs_{sampçe}$ is the absorbance of infusion with different concentrations and $Abs_{control}$ is the control absorbance (without infusion).

Finally, an assay was performed using a caffeine standard (Sigma®, 99% purity) as control to ascertain if in the presence of this compound with the DPPH solution it would react. The standard was diluted to 2 mg/mL in MilliQ water and it was exposed to 60 μM DPPH solution for 2 h. Then, its absorbance was determined.

2.5.2. Cell viability

The cell lines OE33, which represented adenocarcinoma, OE21 (both donated by Dr. Luis Felipe Ribeiro Pinto - INCA) and KYSE450 (obtained commercially from Deutsche Sammlung von Mikroorganismen und Zellkulturen), which represented squamous cell carcinoma, were cultured in 10% RPMI medium and maintained under optimum culture conditions. The cells were treated for 24 h with caffeine (5, 10, 25, 50, 75, 100 and 150 μM) or yerba mate traditional infusion (ECaf) or decaffeinated infusion (EDCaf), at six infusion concentrations (0.01, 0.25, 0.50, 0.75, 1.00 and 2.00 mg/mL [25]. The entire process was performed with sterile material so that the infusion of yerba mate could be used as a treatment in cell culture. To evaluate the cell viability, the experiments were performed as described previously by Gehring et al. [30]. OE33, OE21 and KYSE450 cells were seeded at a density of 6.5×10^3 per well on a 96-well plate with 100 μL of culture medium. After the treatment, the cells were washed with PBS, and 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT) (MTT 5 mg/mL in PBS in 90% DMEM supplemented with FBS 10%) were added to the cells and incubated for 3 h. The formazan crystals were dissolved with 100 μl of dimethyl sulfoxide (DMSO). The absorbance was quantified in 96-well plates (Spectra Max M2e, Molecular Devices) at λ =540 nm. This absorbance was linearly pro-

Table 2
Caffeine concentration in yerba mate leaves processed by supercritical extraction with CO_2 + ethanol.

Experiment	Carbon dioxide flow	Ethanol flow	Time	Caffeine ($g_{caffeine}/g_{yerba\ mate}$)
1	950	78	3.5	0.381%
2	700	106	3.5	0.372%
3	1200	50	3.5	0.422%
4	700	50	3.5	0.751%
5	950	78	3.5	0.299%
6	950	106	2.0	0.483%
7	1200	78	2.0	0.710%
8	700	78	2.0	0.828%
9	700	78	5.0	0.202%
10	1200	106	3.5	0.233%
11	1200	78	5.0	0.030%
12	950	50	5.0	0.355%
13	950	50	2.0	0.576%
14	950	78	3.5	0.425%
15	950	106	5.0	0.050%

Carbon dioxide flow (g/h); ethanol flow (g/h); time (h).

portional to the number of live cells with active mitochondria. The cell viability was calculated using the Eq. 3

$$\text{Cell viability } (\%) = \frac{Abs}{Abs_{control}} * 100 \quad (3)$$

where Abs is the absorbance of cells treated with different formulations and $Abs_{control}$ is the absorbance of control cells (incubated with cell culture medium only).

2.6. Mathematical modeling

In order to fit the experimental data extract yield and caffeine *versus* time, in the optimized conditions, and obtain important mass transfer parameters, the mathematical model used in this work was based in the model developed by Reverchon [31]. The model consists of one-dimensional mass balance for the extract (pseudo-component), assuming the hypothesis that a linear behavior is adequate for the solid-fluid phase equilibrium, that the solvent density and flow rate are constant along the bed, neglecting axial dispersion and considering that the supercritical fluid extraction is mainly controlled by internal mass transfer resistance [32]. The mass balance is given below (Eqs. (4) and (5)).

Fluid phase mass balance:

$$\frac{\partial C(z,t)}{\partial t} = -\upsilon \frac{\partial C(z,t)}{\partial z} - \frac{1-\varepsilon}{\varepsilon}\rho_s \frac{\partial q(z,t)}{\partial t} \quad (4)$$

Mass balance in the solid phase:

$$\frac{\partial q(z,t)}{\partial t} = -k_{TM}\left[q(z,t) - K \cdot C(z,t)\right] \quad (5)$$

where $C(z,t)$ is the extract concentration in the vapor phase and $q(z,t)$ is its concentration in the plant; υ is the interstitial fluid velocity; ε is the porosity of the bed; k_{TM} is the internal mass transfer coefficient; ρ_s is the specific mass of the plant and where K is the equilibrium constant between the phases. The model also considers some initial and boundary conditions: $q(z,0) = q_0$ and $C(z,0) = 0$, q_0 is defined by the total amount of extract contained in the solid phase and the $C(z,0) = 0$ as a boundary condition. The linear comportment for solid-fluid phase equilibrium is expressed by $q^*(z,t) = K.C(z,t)$.

The system of partial differential equations is solved numerically using the dynamic simulator EMSO (Environment for Modeling, Simulation and Optimization), which is an equation-oriented simulator suitable for dynamic simulations [33]. The internal mass transfer coefficient k_{TM}, and the equilibrium constant K, were estimated by least squares method and the objective function minimized by the Nelder-Mead algorithm.

3. Results and discussion

The data obtained for studied properties of yerba mate leaves used in supercritical extraction process, moisture content and experimental density, are 4.6000 ± 0.0004% (m/m) = 1.3140 ± 0.0015 g/cm^3, respectively, as well as the initial caffeine concentration in yerba mate leaves for the experimental design was 2.1% ($g_{caffeine}/g_{yerba\ mate}$).

The results for supercritical extractions performed with 140 g of yerba mate at 300 bar and 60 °C and as independent variables: carbon dioxide flow (700, 950, and 1200 g/h), ethanol (co-solvent) flow (50, 78 and 106 g/h), and extraction time (2.0, 3.5, and 5.0 h) are presented in Table 2.

The three independent variables contribute to reduce caffeine content in yerba mate leaves. Comparing experiments 8 and 11, higher and lower caffeine concentration, respectively, we observe a reduction of 26.6 times. Moreover, only two conditions studied, experiments 11 and 15, were able to reach caffeine concentration lower than 0.1% ($g_{caffeine}/g_{yerba\ mate}$).

A second order polynomial equation (Eq. 6), for the codded independent variables, was adjusted from caffeine concentration results (Table 3), using a Statistica 10® software. The correlation coefficient was 0.912 and the linear coefficients presented higher values than the other coefficients, being the largest coefficient for the extraction time (2.4×10^{-3}). In addition, from Box-Behnken experimental design data, three surface plots are shown in Fig. 1. These graphic representations relate the maximum and minimum levels of two independent variables, keeping the third independent variable constant at central level ($X_i = 0$).

$$Y = 3.7 \times 10^{-3} - 9.5 \times 10^{-4}X_1 - 1.2 \times 10^{-3}X_2 - 2.4 \times 10^{-3}X_3 + 7.6 \times 10^{-4}{X_1}^2 - 2.0 \times 10^{-6}{X_2}^2 - 2.2 \times 10^{-5}{X_3}^2 + 4.7 \times 10^{-4}X_1X_2 - 1.4 \times 10^{-4}X_1X_3 - 5.3 \times 10^{-4}X_2X_3 \quad (6)$$

where Y is the caffeine concentration on processed yerba mate ($g_{caffeine}/g_{yerba\ mate}$), X_1 is the CO_2 flow, X_2 is the ethanol flow (co-solvent), and X_3 is the time. The factors and interactions influencing the caffeine concentration in the processed yerba mate were fitted from polynomial regressions using the response surface methodology. The coefficients adjusted using the coded variables (Eq. (6)) to individual significance at 90% confidence level are: $\beta_0 = 3.7 \times 10^{-3}$ ($p = 2.9 \times 10^{-3}$); $\beta_1 = -9.5 \times 10^{-4}$ ($p = 7.1 \times 10^{-2}$); $\beta_2 = -1.2 \times 10^{-3}$ ($p = 3.3 \times 10^{-2}$); $\beta_3 = -2.4 \times 10^{-3}$ ($p = 1.9 \times 10^{-3}$). The quadratic and interactions coefficients were not significant for a 90% confidence ($p < 0.1$).

It is possible to evaluate the influence of the extraction time and CO_2 flow variables, at ethanol flow constant (78 g/h) in the

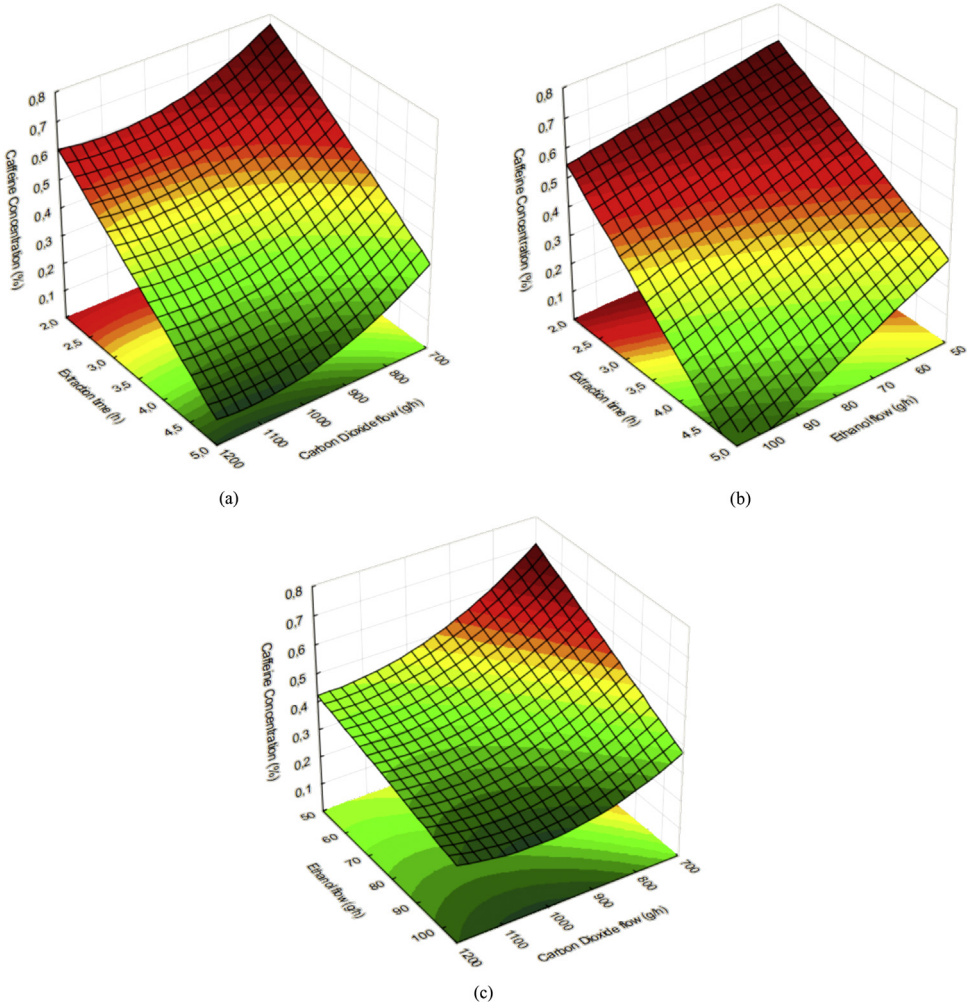

Fig. 1. Response surface plots of the caffeine concentration in processed yerba mate leaves (% $g_{caffeine}/g_{yerba\ mate}$): (a) extraction time *versus* CO_2 flow at ethanol flow constant (78 g/h); (b) extraction time *versus* ethanol flow at CO_2 flow constant (950 g/h); (c) CO_2 flow *versus* ethanol flow at extraction time constant (3.5 h).

Fig. 1a. Increasing the CO_2 flow above the central level (950 g/h) has little influence on caffeine concentration of the processed yerba mate, especially for longer extraction time. In its turn, extraction time variable presented a regular behavior within the evaluated limits, *i.e.*, we observe a decrease on caffeine concentration of the processed yerba mate as extraction time increases.

The caffeine concentration in processed yerba mate leaves (% $g_{caffeine}/g_{yerba\ mate}$) as function extraction time and ethanol flow to CO_2 flow constant at 950 g/h (central level) is shown in Fig. 1b. It is observed an inverse relationship between the independent variables (extraction time and ethanol flow) and caffeine concentration in the processed yerba mate, because at higher values of the independent variables was associated to lower caffeine concentration. It is also possible to verify that there is a region in the graph where caffeine concentration is less than 0.1% $g_{caffeine}/g_{yerba\ mate}$ for ethanol flow and time higher than 83 g/h and 4.25 h, respectively.

Finally, the influence of the CO_2 flow and ethanol flow variables at constant extraction time, 3.5 h (central level), we show in the Fig. 1c. By analyzing the response surfaces (Figs. 1a, b and c), we found that it is only possible to obtain decaffeinated yerba mate for times greater than 3.5 h (mean level), whereas this behavior was not observed for the other two variables. For CO_2 flow (950 g/h) and ethanol flow (78 g/h) at central level was possible to obtain decaffeinated yerba mate.

Statistica 10® software was used to solve the Eq. (6) and to obtain caffeine concentration less than or equal to 0.1% $g_{caffeine}/g_{yerba\ mate}$ in the in processed yerba mate leaves. The independent variable levels calculated were 0 for carbon dioxide flow, +1 for ethanol flow and +0.5 for time. Decoding the levels obtained in Eq. (6), the conditions of the independent variables were 950 g/h of the carbon dioxide flow; 106 g/h of the ethanol flow; 4.25 h of the time.

3.1. Mathematical modeling

The mass transfer parameters were fitted to the experimental data obtained at the optimized condition. The experimental data and the modeled extraction curve, mass *versus* extraction time, are presented in Fig. 2. Observing the initial behavior of the extraction curve, it can be seen that the linear phase is very fast, which leads one to believe that caffeine is found in more internal regions of the plant and not in easily accessible regions for the solvent [34,35]. The high amount of caffeine inside the yerba mate makes diffusive transport the controlling step of mass transfer and this is one of the hypotheses assumed by Reverchon [32] in the construction of the mathematical model with the exclusive presence of the mass transfer coefficient for the solid phase. The fitted mass transfer coefficient (k_{TM}) is $1.93 \pm 0.04 \times 10^{-4}$ s^{-1} and the partition coefficient (K) is $9.39 \pm 0.16 \times 10^{-4}$ m^3/kg, with a determination coefficient (R^2) of 0.998. The statistical significance of the estimated parameters was obtained from the covariance matrix and the parameters correlation is -0.402, indicating that this pair of parameters are independent and not linearly related. The partition

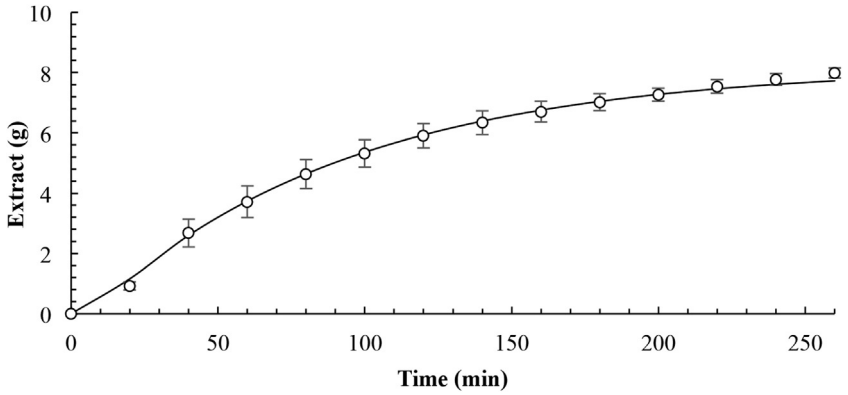

Fig. 2. Mass extract *versus* time for experimental and model curves of the *Ilex paraguariensis* processed by supercritical fluid extraction at 300 bar, 60 °C, 950 g/h CO_2 flow, and 106 g/h of the ethanol flow.

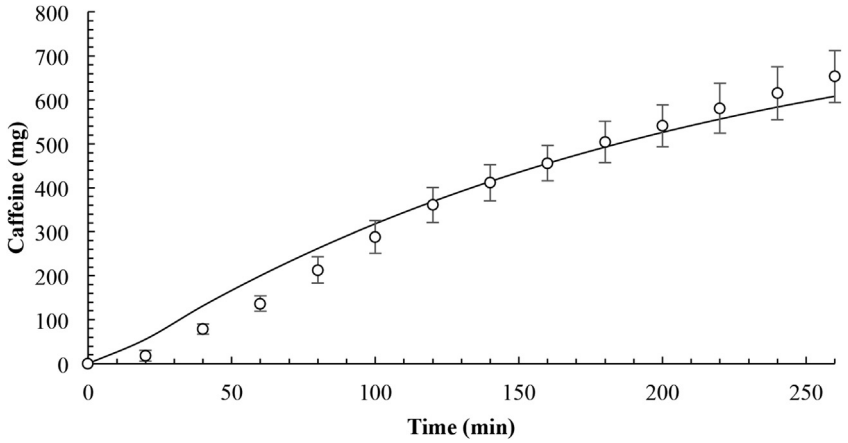

Fig. 3. Caffeine mass extracted *versus* time for experimental and model curves of the *Ilex paraguariensis* extract obtained at 300 bar, 60 °C, 950 g/h CO_2 flow, and 106 g/h of the ethanol flow by supercritical fluid extraction.

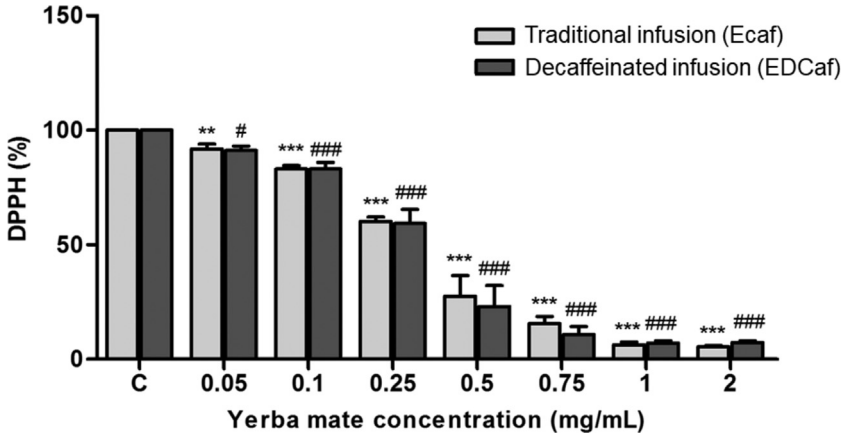

Fig. 4. Antioxidant capacity of infusions prepared from traditional (ECaf) and decaffeinated (EDCaf) yerba mate at 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2 mg/mL, and control (C).

coefficient, which represents the relationship between the concentration of the extract in the solid and fluid phases, presented a value of the order of magnitude of 10^{-4} m^3/kg, which coincides with the order of magnitude found by Rossa et al. [27], when adjusting the supercritical extraction data from *Piper hispidinervum* leaves. Bermejo et al. [20], investigating the extraction of caffeine from green tea leaves under operational conditions similar to this work, determined the mass transfer coefficient in the solid phase to be 1.9×10^{-4} s^{-1} when using ethanol as a co-solvent. This value is practically the same as that determined in our study; this can be attributed to the similarity between the type of plant material used in the extraction as well as the characteristics of the solvent and the pressure and temperature conditions of the extraction. Other reports presented in the literature for different plant species and extracts show only coincidence of order of magnitude found in this investigation [36–40]. On the other hand, Andrade et al. [41] presents the mathematical modeling for the supercritical extraction applied to coffee husks and in similar operational conditions finds a value 10 times lower for the mass transfer coefficient in the solid when compared to the value obtained in this work. This fact

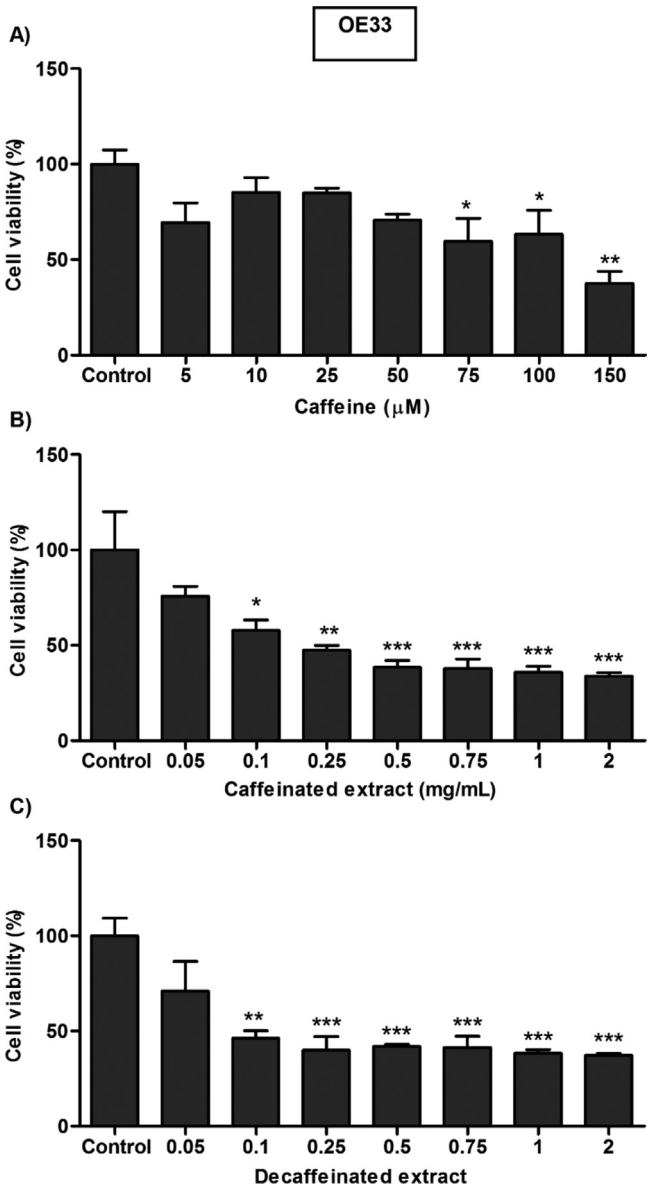

Fig. 5. OE33 cells viability for: (a) caffeine at 5, 10, 25, 50, 75, 100 and 150 μM; (b) caffeinated extract at 0.05, 0.1, 0.25, 0.5, 0.75, 1 and 2 mg/mL; (c) decaffeinated extract at 0.05, 0.1, 0.25, 0.5, 0.75, 1 and 2 mg/mL.

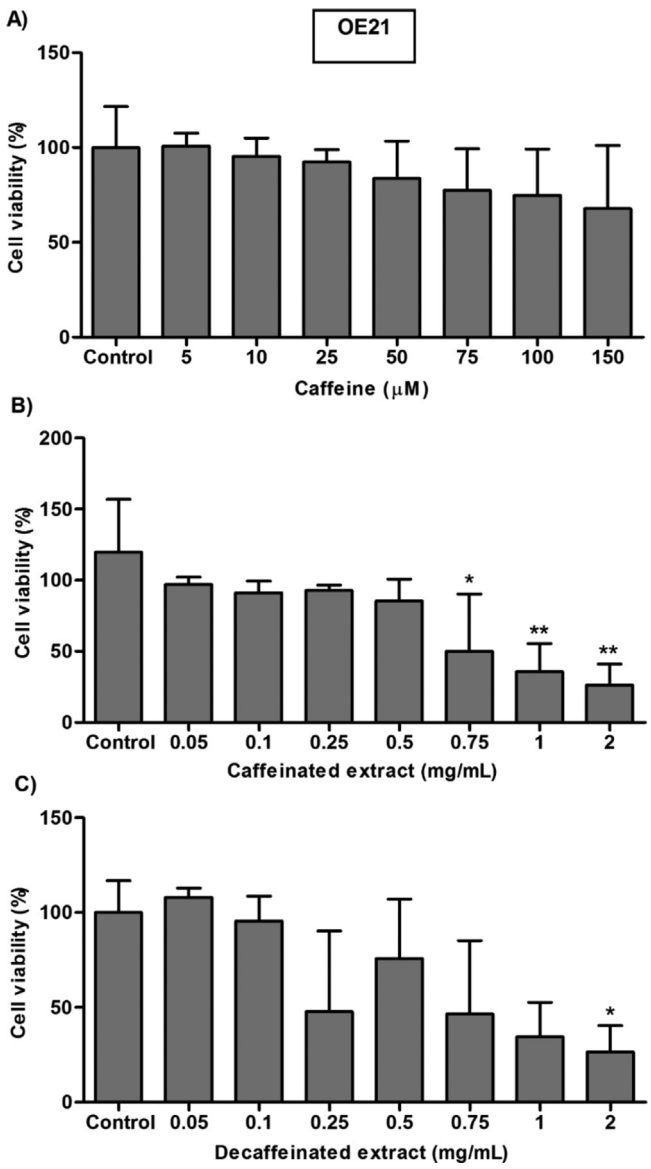

Fig. 6. OE21 cells viability for: (a) caffeine at 5, 10, 25, 50, 75, 100 and 150 μM; (b) caffeinated extract at 0.05, 0.1, 0.25, 0.5, 0.75, 1 and 2 mg/mL; (c) decaffeinated extract at 0.05, 0.1, 0.25, 0.5, 0.75, 1 and 2 mg/mL.

must be associated with the type of structure of the plant material; the husks must have structures where caffeine has more difficult to diffuse than in green tea leaves and yerba mate. The optimization of extraction processes requires well-adjusted mathematical models to be carried out; this fact shows the relevance of determining the parameters of these models. In addition, the knowledge of the adjustable parameters of the models is decisive for the process simulation procedure as well as for the scale-up procedure [42,43].

The amount of caffeine present in the extract was also evaluated throughout the extraction process and its experimental data fitted with the same model. The experimental values and the fitted model are presented in Fig. 3. The specific caffeine mass transfer coefficient (k_{TM}^{caf}) is 8.65 ± 0.14 × 10^{-5} s^{-1} and its partition coefficient (K^{caf}) is 8.04 ± 0.11 × 10^{-4} m^3/kg, with a determination coefficient (R^2) of 0.989 and parameter correlation of 0.699 (low degree of linear dependence). The caffeine concentration of the extract obtained at 300 bar, 60 °C, 950 g/h CO_2 flow, 106 g/h of the ethanol flow, and 4.5 h (optimized condition) was 8.18% ± 0.8% ($g_{caffeine}/g_{extract}$). However, after a simple process of filtering the resins it is possible to reach concentrations 47.8% ± 2.8% ($g_{caffeine}/g_{extract}$).

In the experiments performed to obtain the extraction curves (Figs. 2 and 3) were used blanching yerba mate samples provided by Baldo S.A., but with different leaves in relation to batch used in the experimental design by Box Behnken. The purpose of using a different sample of yerba mate leaves was to evaluate the feasibility of applying the result of experimental design in the production of decaffeinated yerba mate, taking into account the variability of the raw material, a characteristic of natural products. The initial caffeine concentration in the yerba mate used for optimized condition was 1.29% ($g_{caffeine}/g_{yerba\ mate}$) and the caffeine concentration in the processed yerba mate was 0.16% ± 0.06% ($g_{caffeine}/g_{yerba\ mate}$). The value obtained was slightly higher than predicted by the model, which would be 0.1% ($g_{caffeine}/g_{yerba\ mate}$).

To reach the final caffeine concentration of 0.16% ± 0.06% ($g_{caffeine}/g_{yerba\ mate}$) the experiment consumed 2.89 kg of CO_2 for each 100 g of processed yerba mate. This value is lower than that used by Saldaña et al. [21], where for a supercritical fluid extraction

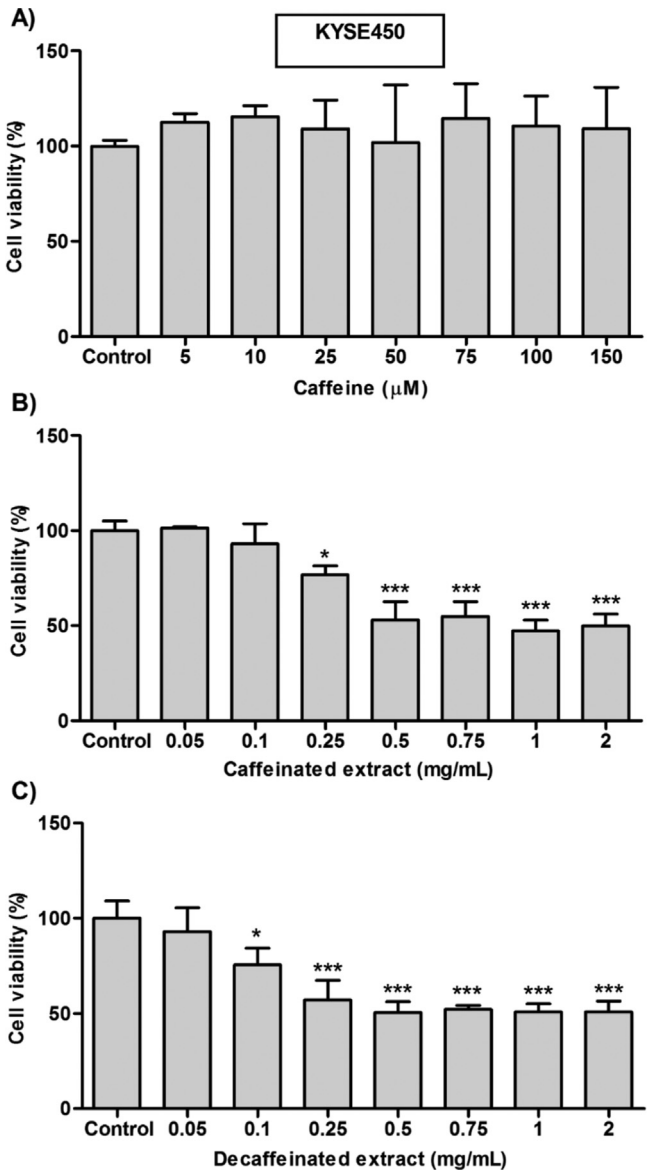

Fig. 7. KYSE450 cells viability for: (a) caffeine at 5, 10, 25, 50, 75, 100 and 150 μM; (b) caffeinated extract at 0.05, 0.1, 0.25, 0.5, 0.75, 1 and 2 mg/mL; (c) decaffeinated extract at 0.05, 0.1, 0.25, 0.5, 0.75, 1 and 2 mg/mL.

without co-solvent at 255 bar and 70 °C the consumption of CO_2 per 100 g of processed yerba mate was 29.5 kg. In turn, Cassel et al. [16] obtained yerba mate with a final concentration of caffeine in the leaves of yerba mate 0.69% with a consumption of 2 kg of CO_2 per 100 g of yerba mate for a supercritical fluid extraction without co-solvent at 170 bar and 60 °C. When comparing the values found in this work with those obtained by Cassel et al. [16], it is possible to observe that there is a reduction in the concentration of caffeine in processed yerba mate by 77%.

3.2. Effects of the extracts on antioxidant capacity

The results for antioxidant capacity of infusions prepared from yerba mate by the DPPH method are presented in Fig. 4. Regarding caffeine masses determined on infusions prepared with traditional (ECaf) and decaffeinated (EDCaf) yerba mate, respectively, the following values were obtained: 0.468% ± 0.003% ($g_{caffeine}/g_{yerba\ mate}$) and 0.030% ± 0.005% ($g_{caffeine}/g_{yerba\ mate}$). These results show that supercritical extraction at optimized condition was able to reduce the caffeine amount without significantly altering antioxidant capacity in the infusions.

According to antioxidant capacity of infusions, to the same concentration, we observed that was not found significant difference between the results for both samples of yerba mate, traditional and decaffeinated. By increasing the yerba mate concentration (Fig. 4), the antioxidant capacity was also increased. However, as from 0.75 $mg_{yerba\ mate}$/mL a growth in antioxidant capacity was not observed. This behavior may be because the DPPH consumption available on the system. Possibly, the results not been greater than 95.3% because the DPPH-extract reaction products and the excess extract had absorbance at wavelength used [44].

The antioxidant capacity assay of caffeine, using same methodology as the infusion analyses, indicated that the caffeine standard did not react with DPPH, because after 2 h of reaction the absorbance for the caffeine standard and for the control sample were the same. From this, we can infer that caffeine extraction from yerba mate will not interfere with antioxidant capacity of the infusions. This behavior is in agreement with the results found by Anesini et al. [15] who describe caffeine as a pro-oxidant compound.

3.3. Effects of the extracts on cell viability

In order to investigate the action of caffeine on *I. paraguariensis* infusions, different esophageal cell lines OE33, OE21 and KYSE450 were treated with caffeine, yerba mate traditional infusions (ECaf) or decaffeinated infusions (EDCaf), and cell viability was evaluated. Here, we tested a curve of concentration (50–2000 μg/mL) in order to study the effect of physiological and supra-physiological doses of yerba mate during 24 h to mimic chronic consumption. We observed that 75 μM caffeine exerted a significant decrease in the viability of the adenocarcinoma OE33 cells when compared to the control, and the same occurred after treatment with ECaf at 0.05 mg / mL and with EDCaf at 0.1 mg/mL (Fig. 5). For the ESCC OE21 strain, the results showed that caffeine treatment did not alter the cell viability. In addition, both mate extracts led to a reduction in cell viability at the highest concentrations (0.75, 1 and 2 mg/mL) (Fig. 6). Likewise, treatment with caffeine had no effects on cell viability in the squamous cells KYSE450, whereas ECaf led to a significant decrease at the concentration of 0.25 mg/mL, and similar results were obtained with EDCaf at 0.1 mg /mL (Fig. 7).

The set of results presented herein showed that caffeine at high concentrations was able to decrease the viability of adenocarcinoma OE-33 lineage. On the other hand, we observed, for the first time a decrease in the viable cells treated with traditional yerba mate with caffeine or decaffeinated in both types of cancer esophageal cell lines (ESCC and EAC), indicating that this effect was related to yerba mate. Yerba mate contains high concentrations of flavonoids and chlorogenic acids among other substances, such as alkaloids, phenolic compounds, and mineral, as well as glycerides and lipids [45]. Other studies have demonstrated that some compounds present in yerba mate infusions inhibit the growth of diverse cancer cells *in vitro* as well as in different *in vivo* cancer models [25,46,47]. Dietary intake of total flavonoids, anthocyanidins, flavanones, and flavones was associated with a decreased of esophageal cancer risk, which correlates with our data [48]. Additionally, diets high in flavonoids may improve survival for esophageal and gastric cancer [49]. Furthermore, a study in a Swedish population showed that a dietary pattern characterized by the intake of lignans, quercetin and resveratrol may have a protective role in the development of esophageal cancer [50].

4. Conclusion

The study on process variables of yerba mate decaffeination by supercritical fluid extraction at 300 bar and 60 °C concluded that the caffeine extraction was more influenced to variables time extraction and ethanol flow (co-solvent) than CO2 flow. As well as the use of a yerba mate from another source than that used at experimental design, resulted in a caffeine concentration of 0.163% ± 0.06% ($g_{caffeine}/g_{yerba\ mate}$), validating the results of the Box-Behnken experimental design. In relation to mathematical model used to represent the extraction curve, this adhered to experimental data for both total extract (R^2 = 0.9998) and caffeine (R^2 = 0.9898).

Associated to extraction process studies, the analysis of infusions obtained from traditional and decaffeinated yerba mate indicated that caffeine removal does not influence the antioxidant capacity and cell viability of esophageal cells. It was also observed that the caffeine concentration in infusion prepared with unprocessed yerba mate is greater than 0.1%, while caffeine concentration in infusion prepared with decaffeinated yerba mate is 0.030% ± 0.005% ($g_{caffeine}/g_{yerba\ mate}$). The results of this work, both in relation to extraction process and to decaffeinated yerba mate properties, come to support the agro-industrial sector of yerba mate that seeks to make a decaf product.

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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