



Guaraná (*Paullinia cupana*) seeds: Selective supercritical extraction of phenolic compounds



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ABSTRACT

Approximately 70% of the Brazilian production of guaraná (*Paullinia cupana*) seeds is absorbed by the beverage industries. Guaraná has several pharmacological properties: energy stimulant, antimicrobial, chemoprophylactic, antigenotoxic, antidepressive, anxiolytic, and anti-amnesic effects. Supercritical carbon dioxide extraction of bioactive compounds from guaraná seeds was carried out and optimized by an orthogonal array design ($OA_9(3^4)$). The factors/levels studied were: modifier(s) (ethanol and/or methanol), extraction time (20, 40, and 60 min), temperature (40, 50, and 60 °C), and pressure (100, 200, and 300 bar). The statistical design was repeated with increasing proportions of modifiers. The percentage of modifier used was proportional to the amount of polar compounds extracted. The best conditions for the supercritical extraction, based on the content of polyphenols, epicatechin/catechin quantification, yield and operating cost, proved to be: 40% ethanol:methanol during 40 min, under 40 °C, and 100 bar. The temperature had a significant effect on the total phenolic content.

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1. Introduction

Guaraná (*Paullinia cupana* Kunth, Sapindaceae) is a Brazilian plant originally from the Amazon region, and its roasted seeds have long been used by indigenous tribes for their stimulant, aphrodisiac, and healing properties (headaches) (Henman, 1982). Due to the high potential of this plant, as well as its medicinal charac-

teristics and profitability, guaraná has become an important raw material for the cosmetics and soft drink industries. Brazil is the largest guaraná producer in the world, with a planted area of 15,182 ha and a production of 3658 t in 2015 (IBGE, 2016; Schimpl, da Silva, Goncalves, & Mazzafera, 2013). Approximately 70% of the production is used in the production of soft drinks and energy drinks (Suframa, 2013).

Guaraná has a wide variety of pharmacological properties, including anticarcinogenic (Fukumasu et al., 2008) antiproliferative (Hertz et al., 2015), antimicrobial, antioxidant (Basile et al., 2005; Yamaguti-Sasaki et al., 2007), cytoprotective (Schimpl et al., 2013), energetic, thermogenic (Andersen & Fogh, 2001), antidepressant (Audi & de Mello, 2000), and anxiolytic (Rangel, Mello, & Audi, 2013) qualities. It can also be used in the prevention of oral disease (Yamaguti-Sasaki et al., 2007), as well as in efforts to reduce oxidative effects and metabolic disorders (Portella et al., 2013), in addition to other purposes (Schimpl et al., 2013).

Abbreviations: SFE, supercritical fluid extraction; OAD, orthogonal array design; $OA_9(3^4)$, a total of nine runs per experiment, with four variables at three levels; TPC, total phenolic content; ANVISA (Portuguese acronym), National Health Surveillance Agency; mg EP g seeds⁻¹, milligrams pyrogallol equivalent per gram; MIC, minimum inhibitory concentration; ATCC, American type culture collection; MRSA, methicillin-resistant *Staphylococcus aureus*; CFU, colony forming units; MHA, Mueller Hinton-Agar; MHB, Mueller Hinton Broth; SEM, scanning electron microscopy.

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In addition to a high percentage (2.5–6%) of caffeine (1,3,7-trimethylxanthine) (Heckman, Weil, & Mejia, 2010), guaraná seeds contain smaller proportions of other purine alkaloids, theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) (Anvisa, 2010). The seeds also contain a high concentration of polyphenols, particularly proanthocyanidins (Ushirobira et al., 2007; Yamaguti-Sasaki et al., 2007). The extracts Ushirobira et al. (2007) and Yamaguti-Sasaki et al. (2007) obtained were standardised by Klein, Longhini, and Mello (2012), in addition to standardising the production in tablets (Klein, Longhini, Bruschi, & Mello, 2015).

Several studies (Castro-Vargas, Varela, Ferreira, & Alfonso, 2010; Wang et al., 2011) have used supercritical extraction to obtain bioactive compounds from natural products. However, we are not aware of any reports in the literature regarding supercritical extraction of polyphenols from guaraná seeds.

Supercritical fluid extraction (SFE) appears to be an environmentally friendly alternative to conventional extraction methods that use large amounts of organic solvents, are slower, and generate considerable waste. SFE processes are rapid and selective, and the products are free of residual solvents (Lang & Wai, 2001). One of the major characteristics of a supercritical fluid is the possibility of changing the density of the fluid by adjusting its pressure and/or temperature, and thus also changing its solubility (Herrero, Cifuentes, & Ibañez, 2006). The CO₂ used as a supercritical fluid is safe, non-toxic, non-carcinogenic, non-flammable, inert, and is easily available at a high purity level at a low cost. It also has low surface tension, low viscosity with high diffusivity, and mild critical conditions (31.1 °C, 7.38 MPa) (Huang, Shi, & Jiang, 2012). Furthermore, to improve the extraction of polar compounds due to the nonpolar character of CO₂, modifiers such as alcohols (methanol and ethanol) can be added to the system to increase its solvating power (Huang et al., 2012). The addition of modifiers often improves the extraction of solid material by cleaving bonds between the solute and plant matrices, decreasing mass transfer resistance in the extractions (Lang & Wai, 2001).

The orthogonal array design (OAD) is a fractional factorial design with a series of trials assigned in an orthogonal array. This design can considerably reduce the number of experiments because when the effect for one factor is calculated, the influence of the others is removed from consideration. Several applications of this method have been reported (Liu et al., 2013; Wang et al., 2011).

Staphylococcus spp. is part of the normal skin microflora, but may behave like the opportunistic pathogens that can cause severe infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important concerns in public health. Antimicrobial resistance has become a global problem, driving the effort to identify new sources of antimicrobial compounds, such as plant secondary metabolites (Barreca, Bellocchio, Laganà, Ginestra, & Bisignano, 2014).

This study obtained extracts from seeds of *P. cupana* by means of OAD, and investigated the effects of temperature, pressure, extraction time, and modifiers in order to develop optimal extraction conditions. As a result, the yield and composition of the phenol content were evaluated. In addition, the major compound of these extracts was quantified using HPLC.

2. Material and methods

2.1. Raw material, sampling and quality control

Roasted seeds of *P. cupana* were obtained in Alta Floresta, Mato Grosso state, Brazil. A voucher specimen (HUEM No. 9065) was deposited in the Herbarium, Department of Biology, State

University of Maringá. Professor Dr. Cássia Mônica Sakuragui identified the plant. These seeds were ground in a hammer mill (Tigre, ASN5). The particles were measured and their mean diameter calculated. The moisture content was determined gravimetrically using an analytical balance (Ohaus, MB35) equipped with an infrared drying system. Quality control analyses of the seeds included the content of extractives, loss from drying, and total ash, methylxanthines, and polyphenol contents (Anvisa, 2010). All analyses were developed in quintuplicate.

2.2. Extraction procedure

The extractions were carried out, starting from 17 g of ground seeds with a standardised mean particle diameter (0.47 ± 0.02 mm). The samples were weighed and placed in stainless steel extraction vessels with a total volume of 25 mL. Automated extraction was performed in a Waters MV-10 ASFE System supercritical extractor equipped with ChromScope software. Supercritical CO₂ was used, with the addition of modifiers in a total flow of 6 mL min⁻¹. The modifiers, ethanol (Merck) and methanol (JT Baker), both of chromatography grade, were used for the extraction. The selected temperature was kept constant and the product separation was achieved using depressurisation. The extracts were freeze dried (Christ Alpha 1–4LD) and stored under refrigeration at –4 °C.

It was also performed a conventional extraction of the same lot of guaraná seeds according to Klein et al. (2012).

2.3. Experimental design

The reasons for the choice of levels in the statistical design used for this study (Table 1) were mainly based on: (1) available information in the literature (Castro-Vargas et al., 2010; Liu et al., 2013; Wang et al., 2011); (2) analyses of the operating conditions of the extraction equipment; and (3) considerations regarding the safety of extraction. Three experiments (X, Y, and Z) were conducted, each of which contained four variables at three levels, for a total of nine runs per experiment (OA₉(3⁴)). The independent variables (factors) were: modifier (ethanol and/or methanol), extraction time (20, 40, and 60 min) temperature (40, 50, and 60 °C), and pressure (100, 200, and 300 bar). The total phenolic content (TPC) and yield were investigated in three levels of each factor in experiments X, Y, and Z. The experiments differed only in the percentage of the modifier used, which was 10, 20, and 40% for experiments X, Y, and Z, respectively. These percentages were fixed for the supercritical CO₂ by adjusting the flow system. The mixture of modifiers was consistently used at a ratio of 1:1 (methanol:ethanol; v/v). All experiments were performed in triplicate.

All of the combinations of temperature and pressure were chosen to ensure that the extraction would occur at levels above the pressure saturation curves (Tochigi et al., 2010) for the binary system (CO₂:ethanol or CO₂:methanol) or ternary system (CO₂:methanol:ethanol). The operating temperature and pressure conditions chosen for the extraction solvents considered only the values of saturation pressure for the temperatures of interest.

2.4. Determination of total phenolic content (TPC) of the extracts

The TPC of the extracts obtained in Item 2.2 was performed using the Folin-Ciocalteu reagent, according Singleton et al.'s (Singleton, Orthofer, & Lamuela-Raventós, 1999) procedures, with a few modifications. Briefly, 240 µL of diluted sample was mixed with 120 µL of Folin Ciocalteu reagent. The solution was diluted to a total volume of 1560 µL using ultrapure water (Milli-Q, Millipore) before then being thoroughly mixed. After 5 min at room temperature, 1440 µL of 10.75% Na₂CO₃ solution was mixed,

Table 1
Results obtained under the experimental conditions using the OA₉(3⁴) design.

Trial	Factors/levels			Experiment X ^a			Experiment Y ^a			Experiment Z ^a		
	Modifier (%)	Extraction time (min)	Temperature (°C)	Pressure (bar)	Yield (%) ^{***}	TPC (mg EP g seeds ⁻¹) ^{***}	Yield (%) ^{***}	TPC (mg EP g seeds ⁻¹) ^{***}	Yield (%) ^{***}	TPC (mg EP g seeds ⁻¹) ^{***}	Yield (%) ^{***}	TPC (mg EP g seeds ⁻¹) ^{***}
1	Ethanol	20	40	100	1.15 ± 0.02 ^c	6.00 ± 0.09 ^g	1.35 ± 0.05 ^e	20.27 ± 0.08 ^h	1.76 ± 0.06 ^c	86.13 ± 0.24 ^c	1.76 ± 0.06 ^c	86.13 ± 0.24 ^c
2	Methanol	60	40	200	2.31 ± 0.04 ^{abc}	8.64 ± 0.04 ^d	3.42 ± 0.14 ^a	47.91 ± 0.19 ^b	3.59 ± 0.16 ^a	78.29 ± 0.41 ^d	3.59 ± 0.16 ^a	78.29 ± 0.41 ^d
3	Ethanol: methanol	40	40	300	2.14 ± 0.12 ^{bc}	8.15 ± 0.08 ^e	2.49 ± 0.14 ^{bc}	36.46 ± 0.37 ^e	3.93 ± 0.20 ^a	105.76 ± 1.09 ^a	3.93 ± 0.20 ^a	105.76 ± 1.09 ^a
4	Ethanol	40	50	200	2.35 ± 0.05 ^{ab}	16.99 ± 0.05 ^a	2.29 ± 0.03 ^c	36.30 ± 0.10 ^e	3.14 ± 0.16 ^c	84.63 ± 0.88 ^c	3.14 ± 0.16 ^c	84.63 ± 0.88 ^c
5	Methanol	20	50	300	2.12 ± 0.10 ^c	5.81 ± 0.17 ^g	2.38 ± 0.09 ^c	44.03 ± 0.46 ^c	2.86 ± 0.12 ^b	67.70 ± 1.03 ^f	2.86 ± 0.12 ^b	67.70 ± 1.03 ^f
6	Ethanol: methanol	60	50	100	1.53 ± 0.09 ^d	9.65 ± 0.17 ^c	2.73 ± 0.11 ^b	32.13 ± 0.47 ^g	3.93 ± 0.11 ^a	88.90 ± 0.03 ^b	3.93 ± 0.11 ^a	88.90 ± 0.03 ^b
7	Ethanol	60	60	300	2.17 ± 0.10 ^{bc}	10.90 ± 0.11 ^b	1.73 ± 0.08 ^d	58.04 ± 0.05 ^a	2.47 ± 0.13 ^d	68.64 ± 0.90 ^{ef}	2.47 ± 0.13 ^d	68.64 ± 0.90 ^{ef}
8	Methanol	40	60	100	1.47 ± 0.02 ^d	7.09 ± 0.08 ^f	1.39 ± 0.07 ^e	37.92 ± 0.51 ^d	3.03 ± 0.11 ^b	69.88 ± 0.88 ^e	3.03 ± 0.11 ^b	69.88 ± 0.88 ^e
9	Ethanol: methanol	20	60	200	2.46 ± 0.12 ^a	7.28 ± 0.09 ^f	3.61 ± 0.15 ^a	33.91 ± 0.07 ^f	3.78 ± 0.02 ^a	66.92 ± 0.28 ^f	3.78 ± 0.02 ^a	66.92 ± 0.28 ^f

Values in the same column followed by different letters are significantly different ($p < 0.05$).

^a Three percentages of modifiers were chosen for experiments X, Y, and Z: 10, 20, and 40%, respectively.

^{**} Extract yield (mass of extract divided by mass of raw material ratio, multiplied by 100) as a percentage of dry extract obtained from 17 g guaraná seeds by SFE.

^{***} The total phenolic content (TPC) was expressed in milligrams of pyrogallol equivalent per gram of guaraná seeds (mg EP g seeds⁻¹).

followed by incubation for 30 min at room temperature. The absorbance was read at 760 nm using a spectrophotometer (USB 2000+, Ocean Optics). As recommended by the Brazilian Pharmacopoeia (Anvisa, 2010), pyrogallol was used as the standard. The range of 1.6–4.8 $\mu\text{g L}^{-1}$ was used to obtain the calibration curve. All steps were performed with protection from light. TPC of the extracts was expressed in milligrams of pyrogallol equivalent per gram of guaraná seeds (mg EP g seeds⁻¹).

2.5. High performance liquid chromatography analysis

The main components of the extracts obtained in Item 2.2 were analysed using HPLC. For this analysis, the lyophilised extracts were previously defatted. First, the samples were weighed (Shimadzu, AUW 220D) and washed with 1 mL of *n*-hexane (Synth), centrifuged (Eppendorf, 5415R) for 15 min at 15,710g, after which, the supernatant was discarded. The centrifugation and washing procedures were repeated three times. After drying at room temperature in a vacuum oven (Binder), the samples were reweighed.

The Waters Alliance 2695 (Milford, MA) HPLC system was equipped with a temperature control column, in-line degasser, quaternary pump, autosampler and a Waters 2998 PDA Photodiode Array Detector. Data acquisition, analysis and reports were conducted using the software Empower 3 (Milford, MA). The chromatographic separation was performed as suggested by Klein et al. (2012) with some modifications. A Gemini C18 column (Phenomenex®, 4.6 mm × 250 mm, 5 μm) equipped with a guard column (Phenomenex C18 column, 4.6 mm × 3 mm, 4 μm) was used and maintained at 25 °C. The mobile phase used was composed of 0.05% TFA in ultrapure water (phase A) and 0.05% TFA in degassed acetonitrile (phase B), completely dissolved in an ultrasonic bath. The flow used was 0.8 mL min⁻¹. The gradient system used was: washing phase (100% A for 5 min) and 5.01–20 min–85% in phase A and 15% in phase B. The injection volume was 25 μL . The degreased samples were diluted in 30% methanol. The chromatograms of caffeine were obtained at a wavelength of 280 nm, and the chromatograms of catechin and epicatechin were obtained at 210 nm.

Analytical curves of mixed working solutions were obtained to evaluate linearity in the range of 3 $\mu\text{g mL}^{-1}$ to 100 $\mu\text{g mL}^{-1}$ for caffeine, catechin, and epicatechin (Sigma) ($n = 5$). The accuracy was determined via recovery analyses, adding measured amounts of caffeine (9, 27, and 46 $\mu\text{g mL}^{-1}$), catechin (18, 27, and 53 $\mu\text{g mL}^{-1}$), and epicatechin (15, 32, and 54 $\mu\text{g mL}^{-1}$) to samples of the extractive solution. The coefficient of variation was always lower than 5%.

The analytical method for quantification of caffeine, catechin, and epicatechin reported in this study was validated according to the guidelines established by the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH, 2005) and by Brazilian regulations (Anvisa, 2003).

Briefly, the intra- and inter-day measurements showed that the method is accurate. The selectivity was investigated by analysing the UV spectra (200–500 nm) of the samples and reference standards, acquired by PDA. The UV spectra of the samples did not differ significantly from the values for the reference standards, indicating the specificity of the method. The limits of quantification and detection for caffeine, catechin, and epicatechin were: 2.25 and 0.68 $\mu\text{g mL}^{-1}$; 0.55 and 0.16 $\mu\text{g mL}^{-1}$; and 0.52 and 0.15 $\mu\text{g mL}^{-1}$, respectively.

2.6. Bacterial strain and culture conditions

The strains methicillin-resistant *Staphylococcus aureus* (MRSA) N315 (Kuroda et al., 2001) and *Staphylococcus aureus* ATCC 29123

used in antibacterial activity experiments were deposited in the Microbial Culture Collection of the Laboratory of Basic and Applied Bacteriology, Department of Microbiology, Centre of Biological Sciences, State University of Londrina. The strain was stored in sterile Mueller Hinton Broth cation-adjusted (MHB) at -20°C , with 30% glycerol. The bacterium was routinely maintained at 4°C on Mueller Hinton-Agar (MHA) plates and subcultivated in Mueller Hinton Agar medium at 37°C prior to each assay.

2.7. Determination of minimum inhibitory concentration (MIC)

The nine supercritical extracts from the experiment Z were evaluated for antimicrobial activity. As a control, the crude extract of guaraná seeds obtained by conventional extraction, according Klein et al. (2012), was also evaluated.

The MIC of fractions was determined according procedures of the CLSI (CLSI, 2011) at a final inoculum of 5×10^4 colony forming units (CFU) mL^{-1} . Serial 2-fold dilutions of the fractions were performed in a microdilution plate (96 wells) containing 100 μL of sterile MHB cation-adjusted. Next, the inoculum was added to each well. The microplates were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration that resulted in the inhibition of growth. The experiment was performed in triplicate and repeated three times. Stock solutions of the fractions were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich®, St. Louis, Mo., U.S.A.) and tested at concentrations from 125 to $0.24 \mu\text{g mL}^{-1}$. The solution was sterilised with a $0.22 \mu\text{m}$ membrane filter (Millipore). The DMSO was evaluated and did not affect the antibacterial activity.

2.8. Scanning electron microscopy (SEM)

The supercritical extract of guaraná seeds that originated from treatment 3 of experiment Z was chosen for this analysis. SEM was used to observe the cell morphology. Colonies from MRSA N315 culture grown in MHA (24 h, 37°C) were transferred to MHB and the cell density was adjusted to 10^8 CFU mL^{-1} . One millilitre of the cell suspension was distributed in two tubes. In the first tube, the guaraná extract was added at MIC concentration. The second tube was considered as the control, the culture in absence of the guaraná extract. The cultures were incubated at 37°C , 150 rpm for 3 h. After incubation, $10 \mu\text{L}$ of each culture and $10 \mu\text{L}$ of fixative solution (2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) were added onto poly-L-lysine-coated glass slides. After 30 min, each slide was given 500 μL of fixative solution for 4 h, following post-fixation in 1% OsO_4 for 1 h. The fixed samples were dehydrated in an ethanol gradient (70, 80, 90, and 100°GL). Samples were critical point dried with CO_2 (BALTEC CPD 030 Critical Point Dryer), coated with gold (BALTEC SDC 050 Sputter Coater) and observed under a scanning electron microscope (FEI Quanta 200).

2.9. Statistical analysis

All the analyses were carried out in triplicate, and the experimental results obtained were expressed as mean $\pm$ SD (standard deviation). The statistical analysis was performed using the Statistical Analysis System (SAS, version 9.3). After the analyses of variance, to compare the treatment means of TPC (total phenolic content), yield, and the main compounds present in the extracts, the Tukey test was used. The differences between the means of different levels were analysed using the Tukey test, and the mean values were considered significantly different when $p < 0.05$. These results were further used in the response surface analysis.

3. Results and discussion

3.1. Drug quality control

Quality control of guaraná seeds was performed to ensure that they were within the limits established by Brazilian legislation, since the seeds' chemical composition may vary with climate, genetics, soil, and storage conditions, among other factors.

The results obtained in the quality control analysis were: moisture content ($8.84 \pm 0.37\%$), particle size analysis ($0.47 \pm 0.02 \text{ mm}$), extractive content ($29.71 \pm 0.89\%$), total ash ($1.42 \pm 0.05\%$), methylxanthines content ($5.13 \pm 0.27\%$), and total polyphenol content ($9.25 \pm 0.15\%$). All quality control analyses of the plant drug confirmed that the roasted seeds were within the specifications established by the Brazilian Pharmacopoeia (Anvisa, 2010).

3.2. Yield

The yield values (mass of the extract divided by mass of the raw material, multiplied by 100) obtained in experiments X, Y, and Z are reported in Table 1. The results demonstrated that increasing the percentage of the modifier produced a higher yield, as evidenced in experiment Z, in which the four value treatments (two, three, six and nine) reached maximum values while remaining statistically similar.

3.3. Total phenolic content (TPC)

In this study, three percentages of modifiers (ethanol and/or methanol) – 10, 20, and 40% – were chosen for experiments X, Y, and Z, respectively. These percentages of modifiers, combined with the levels chosen for the factors, reached areas above the critical pressure and temperature of the mixture (modifier and CO_2).

To extract phenolics, several investigators chose to add modifiers, such as ethanol, methanol or a mixture of these, to facilitate the extraction of these compounds from different plants. In these studies, the percentages of the modifier used were 2% (Murga, Ruiz, Beltrán, & Cabezas, 2000), 10% (Castro-Vargas et al., 2010), 70%, and even 99.8% (Chang, Chiu, Chen, & Chang, 2000). Maróstica-Junior, Leite, and Dragano (2010) found that both methanol and ethanol gave qualitatively similar results.

Recently, Hsieh, Windmann, and Vrabec (2013) studied the phase behaviour at high pressure for the five mixtures CO_2 + C1–C5 alcohols (methanol, ethanol, 1-propanol, 1-butanol and 1-pentanol) and concluded that the saturated liquid lines are close to each other. Therefore, the choice to add a second solvent to the supercritical phase is based mainly on the tunable affinity between solutes and solvents. Ting, Macnaughton, Tomasko, and Foster (1993) determined the variation of the critical properties of a mixture of supercritical CO_2 with various modifiers and found that the addition of alcohols gradually increased the critical temperature of the mixture, as it also increased the percentage of these modifiers.

The TPC values increased significantly in experiments X, Y, and Z, from 5.81 to 16.99, 20.27–58.04, and 66.92–105.76 mg EP g seeds $^{-1}$, respectively (Table 1). Similarly, the TPC value was also higher when using the modifier at 40%. The values were statistically significant and the absolute values differed by up to 18 times between experiments Z and X, and by up to five times between Z and Y. These results confirm the importance of the amount of modifier used. The data in Table 1 indicate that treatment 3 in experiment Z gave the largest absolute value for TPC (mg EP g seeds $^{-1}$). Treatment 3 used the following combination: ethanol:methanol, 40 min, 40°C , and 300 bar.

The TPCs in the extract obtained in this work and those obtained by Basile et al. (2005) are different and not comparable because they are distinct lots of raw materials. Another difficulty in comparing the results obtained by this work is that the extraction of polyphenols from guaraná seeds by supercritical technology is unprecedented.

Other important analyses were the mean values of TPC, the three levels for each factor in the three experiments, and the Tukey analysis, as shown in Table 2. In these analyses, one factor was selected and the other omitted to compare the three levels without the interference of other factors.

Experiments X, Y, and Z (Table 2) varied according to the best modifier, by virtue of the percentage of modifier used. At the lowest percentage, ethanol (10%) gave better results than did methanol and the 1:1 mixture (methanol/ethanol, v/v) at the same percentage. On the other hand, methanol was best when used at a percentage of 20% (Y experiment). However, the mixture of these two compounds at 40% achieved the best mean TPC compared to ethanol and methanol used separately at the same percentage (experiment Z).

In experiments X, Y, and Z, when varying only the extraction time, the mean TPC was always lower when shorter extraction durations were used. This indicated that it is necessary to use an extraction time of at least 40 min for a higher TPC in the statistical design used here.

In experiment Z, the highest TPC values were achieved with the lowest temperature (40 °C). This low temperature decreases the likelihood of inactivation of substances in the extract (Dai & Mumper, 2010). Similarly, experiment Z, with the lowest pressure (100 bar), employed for reasons of safety and operational cost, and depending on the value of TPC, was the best choice for an extraction condition.

The analyses listed in Table 2 are also shown in Fig. 1. The response surface analysis set two factors (temperature and extraction time) at the levels that achieved the highest value of TPC and examined the results of changes in the other two factors (pressure and modifier).

Thus, by fixing the lowest temperature (40 °C) and a 40-min extraction time, the best extraction conditions were: mixture of ethanol:methanol at 40% and 100 bar. This extraction condition was defined by combining the experimental results with a statistical analysis based on the experimental design.

The response surface analysis confirmed the results of Table 3, shown in the orthogonal analysis. Table 3 analyses the raw data

of Table 1, defined for each factor. It is through Table 3 that we were able to identify the order of importance or effectiveness of the four factors, based on the R value. Temperature was found to be the most important factor, followed by modifier, and extraction time; the least important factor was found to be pressure.

3.4. Analysis of extracts using HPLC

In the analysis of the major compounds present in the guaraná extract using HPLC (Table 4), the amount of caffeine progressively decreased from experiment X to experiment Z. Therefore, the amount of caffeine extracted is inversely proportionate to the percentage of the modifier employed. Hence, treatment 7 (60 min, 60 °C, and 300 bar) of experiment X, which used 10% ethanol, appeared to be a reasonable alternative for caffeine extraction. These results are similar to others that showed caffeine solubility increasing with rising temperatures at higher pressures.

However, the opposite result occurred when the amounts of catechin and epicatechin were gradually increased, comparing experiments X, Y, and Z in order. In experiment Z, the treatments that resulted in higher levels of catechin and epicatechin combined were: 1 (ethanol 40%, 20 min, 40 °C, and 100 bar) followed by 3 (ethanol:methanol at 40%, 40 min, 40 °C, and 300 bar), which gave the highest phenolic content (Table 1).

Nonetheless, when each factor (modifier, extraction time, temperature, or pressure) was examined independently (Tables 2 and 3), that is, nullifying the effects of other factors, another scenario was observed. Analysis of the response surface of catechin and epicatechin of the extracts (data shown in Appendix) setting the extraction time at 40 min and the temperature at 40 °C, indicated that the phenolic content was higher at a low pressure using a combination of modifiers.

Finally, this set indicated that the following combination (ethanol:methanol at 40%, 40 min, 40 °C, and 100 bar) was the best extraction condition. Therefore, from this perspective, more consistent results with less variability were achieved, improving the reliability of the analysis. Another work from our group (Klein et al., 2012) extracted catechin and epicatechin from guaraná seeds of a similar lot. Despite having used a different extracting method, the results were similar.

3.5. Supercritical extraction

In supercritical extraction, the solvent flows through a bed of ground seed particles, extracting soluble substances from an insoluble matrix. The rate of internal mass transfer is directly proportional to the effective internal diffusivity and inversely proportional to the square of the particle dimension, depending on the particle shape (Sovová, 2012). The design of processes using supercritical solvents is strongly dependent on the phase equilibrium, which is highly sensitive to changes in operating conditions. Solubility is one of the thermophysical properties required for the extraction process design. The solubility of polar substances, such as polyphenols, in non-polar supercritical fluid (CO₂) is very low. However, the addition of modifiers can increase the polarity of the supercritical fluid. Thus, there is a high solubility due to the increased interaction of forces with the solute and/or by increasing the density, and consequently the solvating power. Thus, extraction of catechin and epicatechin from guaraná seeds is facilitated because these substances are more polar compared to caffeine. Furthermore, ethanol and methanol are capable of hydrogen bonding, dipole–dipole, and other polarity interactions with phenols, and may increase the extraction of them (Lang & Wai, 2001; Maróstica-Junior et al., 2010; Murga et al., 2000). Catechin and epicatechin have hydroxyl groups that are available for the formation of hydrogen bonds, but this does not occur with caffeine. Thus, the

Table 2
Mean values of TPC (mg EP g seeds⁻¹) in levels per factor (modifier, extraction time, temperature, and pressure), in experiments X, Y, and Z.

Experiments	Factors	Levels		
		1	2	3
X	Modifier	11.29 ± 4.77 ^a	7.18 ± 1.23 ^c	8.36 ± 1.05 ^b
Y		38.20 ± 16.42 ^b	43.28 ± 4.38 ^a	34.17 ± 1.91 ^c
Z		79.80 ± 8.42 ^b	71.96 ± 4.89 ^c	87.19 ± 16.87^a
X	Extraction time	6.36 ± 0.70 ^c	10.74 ± 4.70 ^a	9.73 ± 0.99 ^b
Y		32.76 ± 10.33 ^c	36.89 ± 0.83 ^b	46.02 ± 11.31 ^a
Z		73.58 ± 9.43 ^c	86.76 ± 15.64^a	78.61 ± 8.79 ^b
X	Temperature	7.59 ± 1.22 ^c	10.81 ± 4.92 ^a	8.42 ± 1.86 ^b
Y		34.88 ± 12.03 ^c	37.48 ± 5.23 ^b	43.29 ± 11.20 ^a
Z		90.06 ± 12.27^a	80.41 ± 9.73 ^b	68.48 ± 1.44 ^c
X	Pressure	7.58 ± 1.63 ^c	10.97 ± 4.55 ^a	8.29 ± 2.21 ^b
Y		30.11 ± 7.80 ^c	39.37 ± 6.49 ^b	46.18 ± 9.49 ^a
Z		81.64 ± 8.91^a	76.61 ± 7.78 ^c	80.70 ± 18.82 ^b

* Values on the same line followed by different letters are significantly different (p < 0.05).

The highest values obtained are shown in bold.

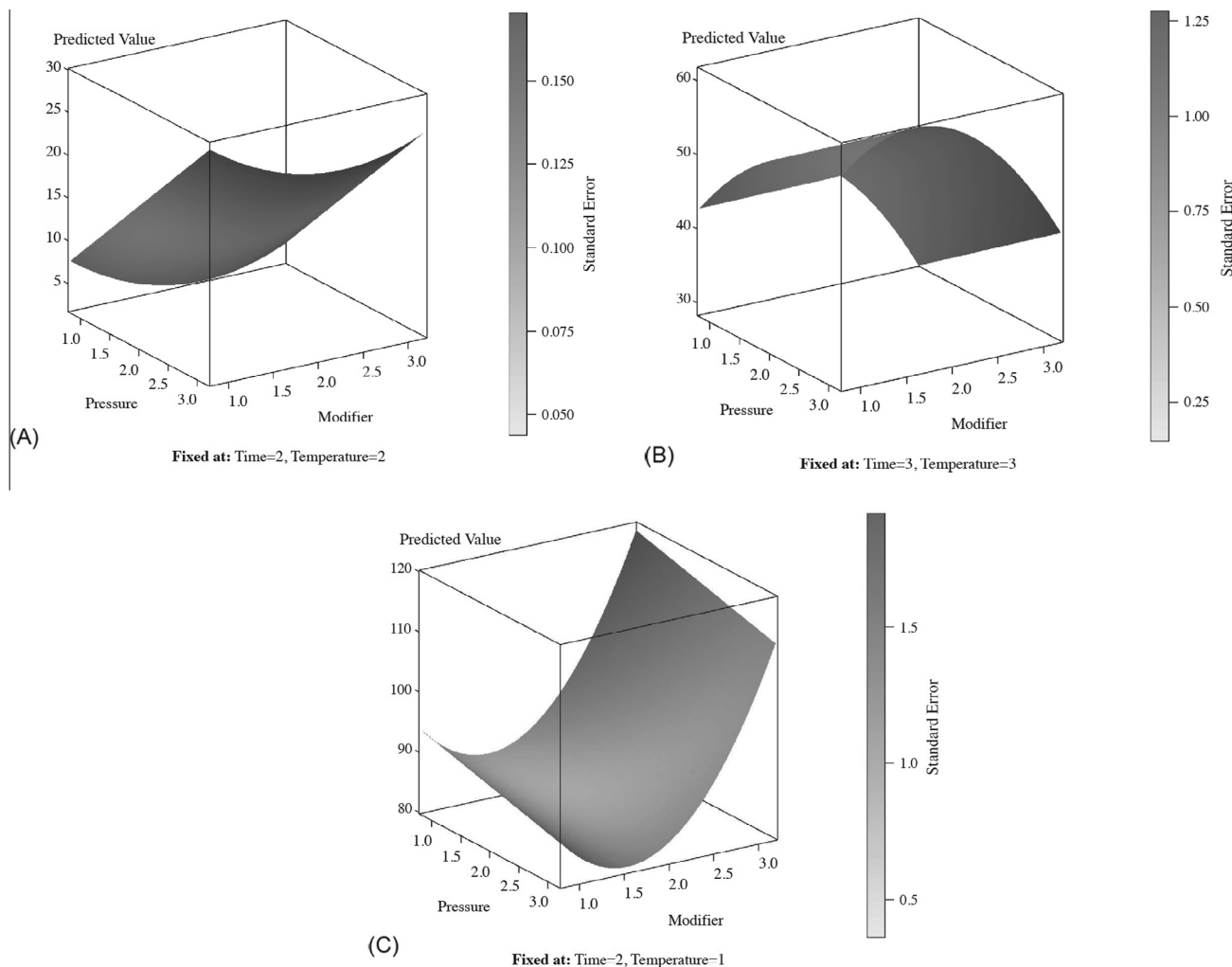


Fig. 1. Response surface analysis for the supercritical extraction of total phenolics from guaraná seeds (mg EP g seeds⁻¹), setting the following factors: (A) **Experiment X.** Extraction time = 2 (40 min) and Temperature = 2 (50 °C); (B) **Experiment Y.** Extraction time = 3 (60 min) and Temperature = 3 (60 °C); (C) **Experiment Z.** Extraction time = 2 (40 min) and Temperature = 1 (40 °C).

Table 3
Analysis of OA₉(3⁴) design results.

	TPC (mg EP g seeds ⁻¹)			
	Modifier (A)	Extraction time (B)	Temperature (C)	Pressure (D)
K_1	239.40 ^a	220.75	270.18	244.91
K_2	145.99	260.27	241.23	229.84
K_3	261.18	235.83	205.44	242.10
k_1	79.80 ^b	73.58	90.06	81.64
k_2	71.96	86.76	80.41	76.61
k_3	87.06	78.61	68.48	80.70
R	15.10 ^c	13.18	21.58	5.03
Optimal level ^d	3	2	1	1
Sequence ^e	2	3	1	4

^a $K_i^A = \sum$ the amount of target compounds at A_i .

^b $k_i^A = K_i^A/3$.

^c $R_i^A = \max\{k_i^A\} - \min\{k_i^A\}$.

^d The level at which the mean TPC was highest.

^e The order of importance or effectiveness of the four factors on TPC, according to the R value.

addition of greater amounts of modifiers in the system will promote the extraction of polar molecules. When comparing the amount of catechin and epicatechin extracted, experiments X, Y, and Z suggested that the solubility of these solutes increased

proportionally with the amount of modifier employed. This fact is evidenced by the increased amount of these substances extracted with increasing additions of a modifier.

The extraction of phenolic compounds by supercritical fluids from guaraná seeds is a green technology that substantially reduces the consumption of solvents in the extraction and downstream operations, and, moreover, it is faster than conventional extraction of guaraná seeds. In addition to these advantages, the extracted substances can be different in quantity and quality than those obtained from the conventional extraction, as stated by [Herrero et al. \(2006\)](#). This fact opens up a range of possibilities for potential biological activities.

3.6. Antimicrobial activity

The best extraction condition and the most important factors to achieve this result were determined in this study, and may subsequently investigate potential biological activity of these extracts. Our group ([Ushirobira et al., 2007](#)) tested guaraná seed extracts obtained by conventional extraction against several microbial strains. Both the crude extract as the semi-purified fractions have no antimicrobial activity against any of the tested strains, even against *Staphylococcus aureus* ATCC 25923. The extracts of guaraná seeds from SFE originating from experiment Z showed a higher

Table 4

Quantification of caffeine, catechin, and epicatechin in each experiment (X, Y, and Z) using HPLC.

Trial	Experiment X			Experiment Y			Experiment Z		
	Caffeine ^a	Catechin ^a	Epicatechin ^a	Caffeine ^a	Catechin ^a	Epicatechin ^a	Caffeine ^a	Catechin ^a	Epicatechin ^a
1	63.96 ± 0.54 ^{bcd}	1.03 ± 0.04 ^a	0.75 ± 0.03 ^a	53.49 ± 2.58 ^{abc}	5.02 ± 0.16 ^{ab}	4.08 ± 0.11 ^b	44.28 ± 1.48 ^{ab}	9.09 ± 0.18 ^a	7.93 ± 0.36 ^a
2	60.07 ± 1.30 ^d	0.84 ± 0.02 ^b	0.62 ± 0.02 ^b	46.36 ± 0.94 ^d	5.25 ± 0.09 ^a	4.23 ± 0.18 ^{ab}	40.09 ± 1.28 ^{bc}	8.21 ± 0.05 ^b	7.23 ± 0.20 ^{abc}
3	62.55 ± 1.17 ^{cd}	0.62 ± 0.03 ^c	0.51 ± 0.02 ^c	51.29 ± 2.12 ^c	5.19 ± 0.22 ^a	4.54 ± 0.22 ^{ab}	45.08 ± 1.03 ^{ab}	8.24 ± 0.30 ^b	7.76 ± 0.38 ^{ab}
4	66.68 ± 3.17 ^{abc}	0.33 ± 0.02 ^f	0.25 ± 0.01 ^f	51.95 ± 0.44 ^{bc}	5.05 ± 0.23 ^{ab}	4.07 ± 0.19 ^b	44.47 ± 1.37 ^{ab}	8.04 ± 0.19 ^{bc}	7.11 ± 0.33 ^{bc}
5	67.88 ± 1.72 ^{ab}	0.24 ± 0.01 ^g	0.20 ± 0.01 ^g	50.02 ± 0.41 ^{cd}	4.63 ± 0.12 ^b	3.90 ± 0.15 ^b	41.43 ± 2.02 ^{ab}	7.86 ± 0.18 ^{bc}	6.91 ± 0.19 ^c
6	66.63 ± 0.92 ^{abc}	0.42 ± 0.02 ^e	0.32 ± 0.01 ^e	51.00 ± 0.33 ^{cd}	5.25 ± 0.17 ^a	3.98 ± 0.15 ^b	44.68 ± 1.44 ^{ab}	7.59 ± 0.31 ^c	6.61 ± 0.24 ^c
7	68.49 ± 0.03 ^a	0.21 ± 0.01 ^g	0.22 ± 0.01 ^{fg}	56.44 ± 2.14 ^{ab}	2.47 ± 0.12 ^c	2.15 ± 0.09 ^c	45.90 ± 0.69 ^a	8.09 ± 0.19 ^{bc}	7.07 ± 0.31 ^{bc}
8	66.46 ± 0.40 ^{abc}	0.53 ± 0.03 ^d	0.38 ± 0.01 ^d	49.39 ± 0.46 ^{cd}	4.90 ± 0.23 ^{ab}	3.90 ± 0.01 ^b	35.66 ± 3.73 ^c	8.00 ± 0.28 ^{bc}	6.63 ± 0.11 ^c
9	66.33 ± 1.63 ^{abc}	0.22 ± 0.01 ^g	0.21 ± 0.01 ^{fg}	58.03 ± 2.83 ^a	2.21 ± 0.07 ^c	2.15 ± 0.07 ^c	42.80 ± 0.44 ^{ab}	8.99 ± 0.02 ^a	6.87 ± 0.05 ^c

Values in the **same column** followed by different letters are significantly different ($p < 0.05$).^a Percentage of defatted sample (μg of substance ($100 \mu\text{g}$ guaraná extract)⁻¹). The amount of fat from the samples in experiments X and Y ranged from 65% to 71%, and in experiment Z ranged from 56% to 64%.

TPC than those from experiments X and Y. Thus, an antimicrobial activity test was carried out from the nine extracts from experiment Z against strains of *Staphylococcus aureus* ATCC 29123 and MRSA N315. The results were promising and should be the subject of future studies for the isolation and identification of the active compounds responsible for this activity.

Other authors also claim that SFE has proven to be more effective in the extraction of substances with antimicrobial activity as compared with conventional extraction (Liu, Zhao, Wang, & Luo, 2007; Michielin et al., 2009).

The minimum inhibitory concentration (MIC) of all supercritical guaraná extracts from experiment Z against *S. aureus* ATCC 29123 was $>125.0 \mu\text{g mL}^{-1}$. However, the MIC against MRSA N315 was $31.25 \mu\text{g mL}^{-1}$ for all treatments except treatment 4 (MIC = $62.50 \mu\text{g mL}^{-1}$). There was no significant antimicrobial activity ($>2.500 \mu\text{g mL}^{-1}$) to the extract obtained from the conventional extraction. This result suggests that compounds with antimicrobial activity are found in the supercritical extracts, and are most likely absent in the crude extract from the conventional extraction.

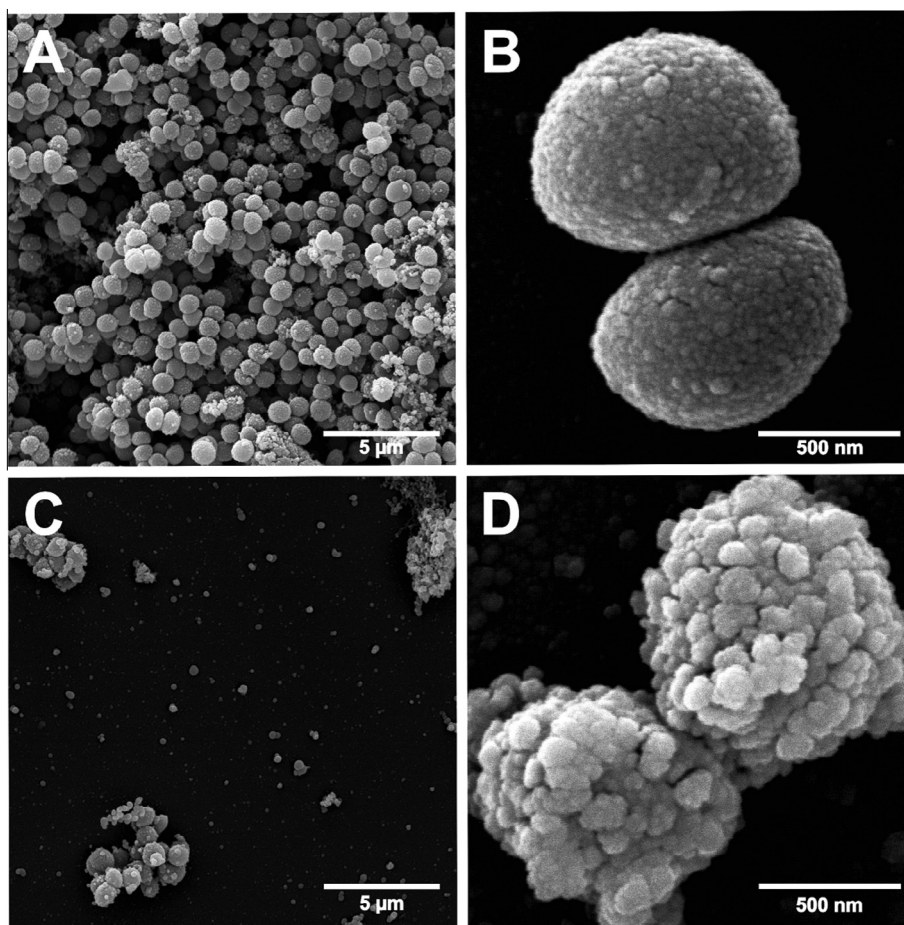


Fig. 2. Scanning electron microscopy of strains methicillin-resistant *Staphylococcus aureus* N315 treated with extract from seeds of *Paullinia cupana* by from supercritical technology. Treatment 3 used for this assay has the following combination: ethanol:methanol, 40 min, 40 °C, and 300 bar. A: untreated MRSA N315 (control) at 10,000× magnification; B: untreated MRSA N315 (control) at 80,000× magnification; C: MRSA N315 treated with guaraná extract at 10,000× magnification; D: MRSA N315 treated with guaraná extract at 80,000× magnification.

The greatest antimicrobial activity of supercritical extracts of guaraná against the MRSA N315 strain could be explained by the collateral sensitivity effect in resistant bacterial strains. Collateral drug sensitivity networks may further serve as a basis for designing treatments in which multiple antibiotics are cycled over time. Imamovic and Sommer (2013) proposed a new treatment—collateral sensitivity cycling—in which drugs with compatible collateral sensitivity profiles are used sequentially to treat infection and avoid resistance development. The authors predict that collateral sensitivity cycling will contribute to the sustainable use of drugs in the clinic for the management of diseases where drug resistance is a concern.

3.7. Scanning electron microscopy

The supercritical extract of guaraná seeds that originated from treatment 3 (ethanol:methanol, 40 min, 40 °C, and 300 bar) from experiment Z had a higher TPC of the extracts evaluated in this study. Thus, this extract was chosen to evaluate the morphology of bacterial cells by scanning electron microscopy (SEM). In SEM evaluation, untreated MRSA N315 prepared in standard MHB medium exhibited a large number of smooth cells, spherical in grape-like clusters, and their average size unaltered (Fig. 2A). At high magnification, the intact surface of MRSA N315 was observed (Fig. 2B). For MRSA N315 treated with extract of guaraná seeds, it was possible to identify a reduction in the number of cells, the cell surface protrusions, amorphous mass and cell debris (Fig. 2C). At high magnification, the morphology of the cells changed completely, introducing small bubbles (Fig. 2D). These results suggest that the cells were lysed by the action of the extract of guaraná seeds.

4. Conclusions

Pressurising the modifiers improved the yields and concentrations of the extracts compared to other conventional extraction methods. The caffeine content in the extract was similar to that in other published studies, but the TPC was higher compared to the level obtained using ethanol extraction. Thus, the inclusion of modifiers in the supercritical extraction process is well worth considering.

The OAD statistical strategy proved to be an efficient method that provided instructive data, using minimum resources. In experiment Z, the extraction conditions of 40% ethanol:methanol at 40 min, 40 °C, and 100 bar proved to be the condition of choice, based on the combination of several factors, including operating cost, safety, yield and value of TPC obtained. Generally, experiment Z showed promise compared to experiments X and Y, relative to the amount of bioactive compounds. The HPLC corroborated this. The percentage of modifier used is proportionate to the polarity of the extracted substances.

The supercritical extracts of guaraná seeds from experiment Z showed a promising antimicrobial activity against MRSA N315. Thus, this extract has the potential for treating nosocomial infections or as adjuvant, in this case.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.06.028>.

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