A Produção do Conhecimento **nas Ciências da Saúde**

Benedito Rodrigues da Silva Neto
(Organizador)

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APRESENTAÇÃO

Com grande entusiasmo apresentamos o primeiro volume da coleção “A Produção do Conhecimento nas Ciências da Saúde”. Um trabalho relevante e sólido na área da saúde composto por atividades de pesquisa desenvolvidas em diversas regiões do Brasil.

Tendo em vista a importância dos estudos à nível microbiológico, para o avanço do conhecimento nas ciências da saúde, reunimos neste volume informações inéditas apresentadas sob forma de trabalhos científicos que transitam na interface da importância da microbiologia à nível clínico, patológico, social, ergonômico e epidemiológico.

Com enfoque direcionado às análises, avaliações, caracterização e determinantes ambientais, parasitológicos e econômicos, a obra apresenta dados substanciais de informações que ampliarão o conhecimento do leitor e que contribuirão com a formação e possíveis avanços nos estudos correlacionados às temáticas abordadas.

O interesse cada vez maior em conhecer e investigar no ambiente novos focos parasitários tem como base transformações provocadas por mudanças econômicas ou sociais, urbanização crescente, tratamentos e descartes inadequados de antibióticos, que propiciam aparecimento de novos focos. Assim, dados obtidos em diferentes locais sobre diferentes condições ambientais ou de desenvolvimento microbiano/parasitário são relevantes para atualização do conhecimento sobre mecanismos de ação do agente patológico assim como diagnóstico e tratamento eficaz.

Uma vez que a interdisciplinaridade tem sido palavra chave nas ciências da saúde observaremos aqui um fio condutor entre cada capítulo que ampliará nossos horizontes e fomentará propostas de novos trabalhos científicos.

Assim, o conteúdo de todos os volumes é significante não apenas pela teoria bem fundamentada aliada à resultados promissores, mas também pela capacidade de professores, acadêmicos, pesquisadores, cientistas e da Atena Editora em produzir conhecimento em saúde nas condições ainda inconstantes do contexto brasileiro. Desejamos que este contexto possa ser transformado a cada dia, e o trabalho aqui presente pode ser um agente transformador por gerar conhecimento em uma área fundamental do desenvolvimento como a saúde.

Dr. Benedito Rodrigues da Silva Neto

SUMÁRIO

CAPÍTULO 19

OPTIMIZATION OF HYDROALCOHOLIC EXTRACTION OF CRUDE GUARANA SEEDS: PHENOLIC CONSTITUENTS, METHYLXANTHINES AND ANTIOXIDANT CAPACITY

Ádina Lima de Santana
Bioprocesses Laboratory. School of Food Engineering. University of Campinas (Unicamp). Campinas, São Paulo, Brazil.

Gabriela Alves Macedo
Bioprocesses Laboratory. School of Food Engineering. University of Campinas (Unicamp). Campinas, São Paulo, Brazil.

Corresponding authors: adina.santana@gmail.com and macedoga@gmail.com

ABSTRACT: The obtaining of substances responsible to enhance health with the use of plant extracts is proven to be healthier than those formulated with the synthetized ones. Selective conditions on the obtaining of natural extracts with enhanced profile of phenolic constituents, methylxanthines and antioxidant capacity was investigated in this work using cold (CHE) and hot hydroalcoholic extraction (HHE) of crude guarana seeds. The solid waste seeds, obtained from each extraction condition were evaluated in terms of total phenolic content and antioxidant capacity. Results indicate that highest recovery of bioactive constituents in CHE was attributed to the hydroalcoholic solution composed of 50% ethanol and to the seeds with 1.6 8mm. Thin-layer chromatography detected positive presence of phenolic constituents and caffeine, which were confirmed quantitatively with HPLC. Establishing comparison between CHM and HHE, the highest profile of bioactive constituents in the was obtained in HHE at 60ºC. The use of temperatures higher than 60ºC resulted in thermal degradation of phenolic constituents and methylxanthines.

KEYWORDS: *Paullinia cupana*; phenolic compounds; hydroalcoholic extraction; thin-layer chromatography; antioxidant capacity

1 I INTRODUCTION

Medicinal plants, herbal drugs, and individual natural products comprise a market worth billions of dollars, in both developed and developing countries (BRAZ et al., 2012). Latin American plants are usually rich in phenolic constituents, which are important molecules in human health (LAJOLO, 2007).

Guarana (*Paullinia cupana*), or Brazilian cocoa, is known for its stimulatory properties, improvement in cognitive function and energy-expenditure increasing, which are attributed to the pharmacological action of methylxanthines, particularly caffeine (DA SILVA et al., 2017). Besides the caffeine guarana seeds contain other methylxanthines, such as theobromine and theophylline, but in smaller proportion (SANTANA e MACEDO, 2018b). In addition to methylxanthines, guarana contain catechins,

which are phenolic constituents associated with antioxidant capacity of this plant (YONEKURA et al., 2016).

The decreasing of hot flashes in breast cancer survivors (OLIVEIRA et al., 2013), and in LDL oxidation in elderly people (PORTELLA et al., 2013), besides weight stabilization (PALMA et al., 2016) were health aspects associated with catechins present in guarana.

The obtaining of extracts from guarana is traditionally performed with the use of a hydroalcoholic solution, during 24 h, followed by subsequent separation of extract from waste seeds (NAZARÉ, 1997).

Studies usually perform extraction of guarana seeds using solvents like acetone, methanol, ethyl acetate and dimethylsulfoxide (MAJHENIČ et al., 2007, MARQUES et al., 2016, ANTONELLI-USHIROBIRA et al., 2010, DALONSO e PETKOWICZ, 2012). However, these solvents may cause toxicity and the risk of low purity extracts because of the presence of residual fractions of the solvent. Residual fractions of undesirable solvents require further solvent removal processes, which may increase production costs.

Besides, recent demands for the reduction and reutilization of waste have motivated scientific research on the development of clean technologies and optimization of process parameters to achieve sustainable production (SANTANA et al., 2019).

To the best of our knowledge, detailed studies on the effect of process parameters to enhance the extraction of caffeine and catechins in guarana extracts are scarce. In this context, we investigate selective process conditions of 24h-cold and 6h-hot hydroalcoholic extractions of crude guarana for the obtaining of extracts with enhanced content of bioactive constituents. The composition of waste fraction of extractions was also evaluated in this work.

2 I MATERIAL AND METHODS

2.1 Raw material

Roasted guarana seeds were purchased from Guarana de Maués Corporation (Maués, Brazil).

The raw material was milled in a commercial blender (BL.2.201/202, Marchesoni, São Paulo, Brazil) and sieved (W.S. Tyler, Wheeling, EUA), resulting on fractions consisted of three particle diameters (1.68 mm, 125 µm and 25 µm). In the first part of this work, we investigated the performance of cold hydroalcoholic extraction for 24 h using five hydroalcoholic solvent formulations and three particle diameters of crude guarana seeds (CG). Afterwards, hot hydroalcoholic extraction (HHE) assays were carried out for 6 h using the optimized solvent formulation and particle diameter from CHE (Figure 1).

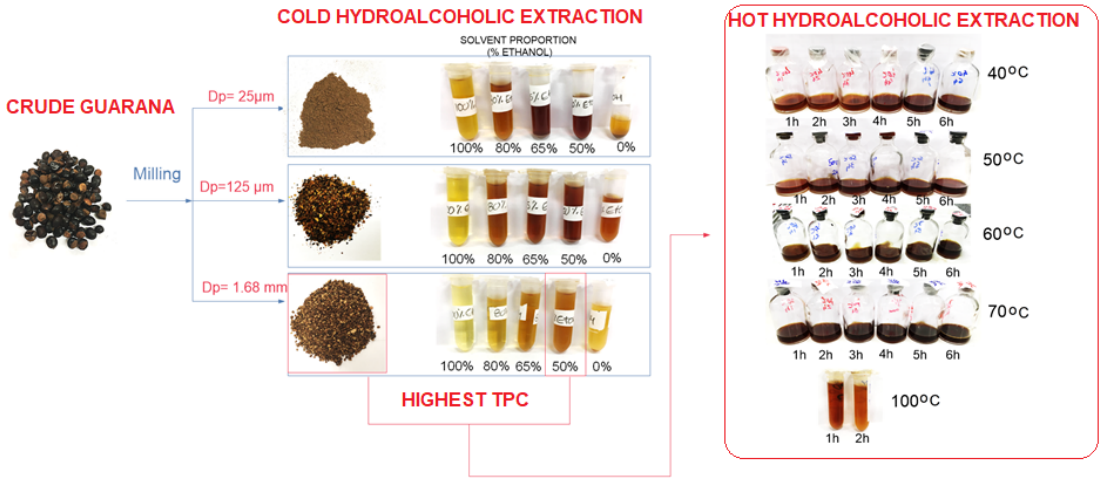

Figure 1. Selective conditions of cold hydroalcoholic extraction parameters, and subsequent hot hydroalcoholic solvent extraction.

2.1.1 Reagents

Milli-Q water (EMD Millipore Corporation, Merck, Darmstadt, Germany), gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, vanillin, linoleic acid and trolox were purchased from Sigma–Aldrich (Darmstadt, Germany). The standards (+)(-) catechin (99% pure), (-)-epicatechin (≥97%), (-)epicatechin gallate (≥98%), caffeine and theobromine (≥98%) were purchased from Sigma-Aldrich (Darmstadt, Germany). Theophylline (anhydrous, 97.50% pure) was purchased from Abbott (São Paulo, Brazil). Methanol (99.9%, HPLC grade) was obtained from JT Baker (Bridgewater, USA). Folin–Ciocalteu reagent and ethanol (99.5%), were purchased from Dinâmica (São Paulo, Brazil). Ethyl acetate, sulphuric acid and glacial acetic acid were purchased from Synth (Sao Paulo, Brazil).

2.2 Cold hydroalcoholic extraction

Extracts from guarana seeds were obtained from the respective particle diameters 1.68 mm, 125 µm and 25 µm. Hydroalcoholic solutions were obtained using five solvent formulations inserted in polyethylene tubes, according to the ratio ethanol:water (weight/weight), i.e., 100:0, 80:20, 65:35,50:50 and 0:100. Extractions at laboratory scale were carried out using a seeds:solvent proportion of 1:3 (weight/weight), established by previous assays, with subsequent vortex agitation (Model 251, Fanem, São Paulo, Brazil) of tubes for 1 minute. Afterwards, the samples were stored immobilized at 25 ºC for 24 h, in the dark.

In the next day the samples were centrifuged (Heraeus, Megafuge 16 R, Thermo Scientific, Massachusetts, USA) at 2500 rpm and 25 ºC for 10 min. The supernatant (extract) was separated from the solid fraction. The particle diameter and solvent formulation that resulted in the optimal extract, in terms of total phenolic content (TPC), and catechins, were selected for further hot hydroalcoholic extraction using 5

temperature levels (40, 50, 60, 70 and 100° C).

2.3 Hot hydroalcoholic extraction

Approximately 3 g of milled seeds were inserted into glass Erlenmeyer flasks using a seeds:solvent proportion of 1:3 (weight/weight). Extractions were performed in a stackable shaker (MaxQ 8000, Thermo Scientific, Massachusetts, USA) at 48 rpm for 6 h . Extractions at 70°C and 100°C were performed in a Dubnoff heating bath (Tecnal, TE-053, São Paulo, Brazil). Aliquots were taken during 1h interval for further analysis.

2.4 Yield

For the obtaining of yield of dried extract, the extracts were dried using a rotary evaporator (Marconi, Sao Paulo, Brazil), as showed in Figure 2. The calculation procedure was performed according to Eq.1:

$$X_0 = \frac{m_{EXT}}{F_0} \quad (1)$$

Where X_0 is the yield in extracts(%), m_{EXT} is the mass of dried extract obtained (g) and F_0 is the mass of raw material (g).

Figure 2. Drying of guarana extracts, obtained with HHE.

2.5 Characterization of guarana products

2.5.1 Sample preparing

Approximately 0.18 g of extract was diluted in 1 mL of deionized water for the analysis of total phenolic content (TPC) and antioxidant capacity with the β-carotene approach. Approximately 0.05 g of extract was diluted in 0.5 mL of an 70% methanolic solution for the analysis of antioxidant capacity with the DPPH approach.

From the solid waste seeds, approximately 0.2 g was extracted with 1 mL of ethanol. The dilutions of this products for the TPC and antioxidant capacity assays were similar to the extracts obtained directly from CHE and HHE

2.5.2 Determination of total phenolic content (TPC assay)

Total phenolic content of the extracts were measured using the Folin–Ciocalteu reagent (SINGLETON e ROSSI, 1965). A calibration curve using gallic acid was plotted in a concentration range of 16 – 300 µg /mL. The results quantified at each extraction

time were expressed as µg of gallic acid equivalent (GAE)/ mL of extract.

2.5.3 Antioxidant capacity: DPPH radical-scavenging capacity (DPPH assay)

Free radical scavenging capacity of the extracts was evaluated using the stable DPPH radical and NovoStarMicroplate reader (BMG LABTECH, Germany) at 520 nm, according to the method established (PESCHEL et al., 2006) with adaptations (MACEDO et al., 2011). Results were calculated using the linear regression equation from the plotting concentration solutions of Trolox (15 - 300 µmol/mL). The results quantified at each extraction time were expressed as µmol of Trolox equivalent/mL extract.

2.5.4 Antioxidant capacity: β-carotene-linoleic acid assay

The capacity of extracts on the inhibition of heat-oxidation of an aqueous system of β-carotene and linoleic acid was evaluated using the method of MARCO (1968), adapted by MILLER (1971) and LEAL et al. (2006). Antioxidant capacity, measured as % of inhibition of oxidation, was calculated using Eq. (2), as proposed elsewhere (ŠKERGET et al., 2005).

$$A\ (\%) = 100\left[1 - \frac{ABS^{t=0}_{SAMPLE} - ABS^{t=120}_{SAMPLE}}{ABS^{t=0}_{CONTROL} - ABS^{t=120}_{CONTROL}}\right] \quad (2)$$

Where AA is antioxidant capacity, $ABS^{t=0}_{SAMPLE}$ is the absorbance of the sample at t=0h, $ABS^{t=0}_{CONTROL}$ is the absorbance of control sample at t=0min, ABS^{t}_{SAMPLE} is the absorbance of the sample at t=120 min and $ABS^{t}_{CONTROL}$ is the absorbance of control sample at t=120 min.

2.5.5 Thin Layer Chromatography (TLC)

Ultraviolet light-sensible Silica gel plates with aluminum backs were used as the stationary phase. (UV_{254}, Alugram®, Xtra SIL G, Macherey-Nagel, Germany). on dimension 10 cm × 10 cm were used as stationary phase. The TLC plates were prepared by establishing 1 cm distance from the origin, 8 cm distance for the solvent to travel, and 1cm from the solvent front.

The liquid product of extractions and an ethanolic solution from the solid waste fraction of these procedures were spotted in TLC plates with the aid of capillary glass tubes with approximately 1 cm distance from each band. Afterwards, the plates were developed into glass chambers by elution in mobile phase constituted by ethyl acetate, glacial acetic acid, methanol and water (60:15:15:10, v/v/v/v).

The bands of compounds generated by constituents that could not be detected in the visible region were visualized with the aid of the equipment an UV (Multiband UV – 254-366 nm, UVGL-58, Mineralight® Lamp, Upland, CA, EUA) equipped with cabinet (UVP-Chromato-VUE, CC-10, Upland, CA, EUA) with short wavelength, 254 nm, and long wavelength, 366 nm.

In order to detect bioactive constituents, a sulfuric vanillin (SV) spray reagent was applied using the formulation suggested elsewhere (KRISHNASWAMY, 2003), in which 0.5 g of vanillin was diluted in 20 mL of ethanol and 80 mL of sulfuric acid, in this sequence. Before spraying with SV reagents, the plates were inserted into UV chamber to visualize the bands of compounds that could not been seen at the visible zone. The retardation factor (R_F), which is the standard measurement of substance retention, was calculated using the software ImageJ, according to the procedures described elsewhere (JOHNER e MEIRELES, 2016).

2.5.6 High Performance Liquid Chromatography

The methylxanthines (caffeine, theobromine and theophylline) and catechins (catechin, epicatechin and epicatechin gallate) in selected guarana extracts obtained in the last hour of CHE and HHE were determined by high-performance liquid chromatography coupled with diode array detector (HPLC-DAD) according to procedures determined previously (SANTANA e MACEDO, 2018a).

A Dionex UltiMate 3000 (Germany) liquid chromatographer equipped with a C-18 Acclaim® column (Dionex, 3 µm, 120 Å, 4.6 × 150 mm) maintained at 30°C was used, and the detection was performed using a UV/VIS diode array detector.

Approximately 0.09g of extracts (100 µL) were solubilized in 900 µL of a 50% methanolic solution (HPLC grade) and filtered using a 0.45 µm polyvinylidene difluoride membrane (HV PVDF, Millipore, Massachusetts, USA) before injection. The mobile phase solutions were formulated as follows: 100% water (A), 100% methanol (B), water/formic acid at 99.9:0.1, v/v (C) and methanol/formic acid at 99.9:0.1, v/v (D) .

3 I RESULTS AND DISCUSSION

The yield in dried extracts obtained in both CHE and HHE is showed in Figure 3. In CHE, high yield in extracts was attributed to the seeds with particle diameter (Dp) of 25 μm (Figure 3A). The CHE in seeds with Dp=1.68 mm using a 50% hydroalcoholic solution contributed to the obtaining of liquid extracts with the highest TPC (Figure 4B), catechins and methylxanthines (Table 2).

The yield extracts, or the global yield of an extraction, may indicate the magnitude of obtaining the compounds of interest or the possibility of obtaining other substances previously solubilized in the solvent. Besides the type of solvent, the temperature is a factor that contributes strongly to the modification of the yield of an extraction (Figure 3B).

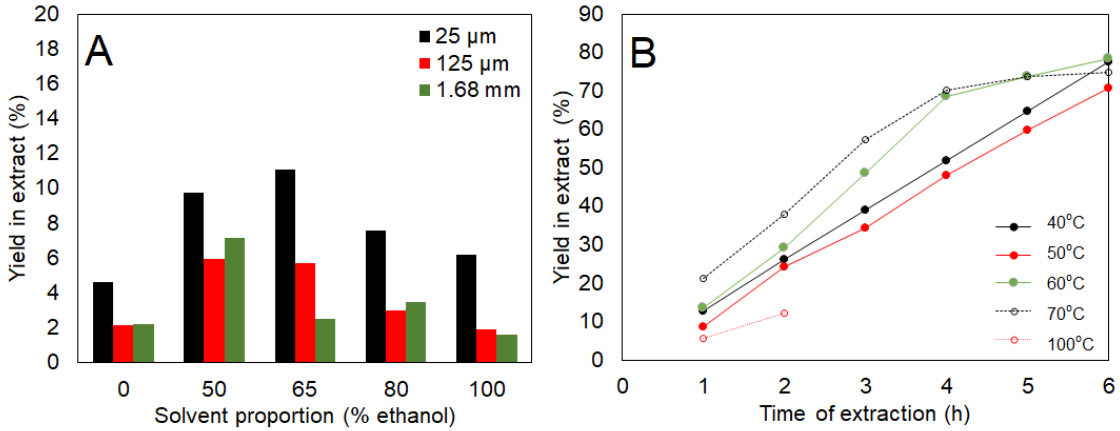

Figure 3. Yield in extracts obtained in CHE (A) and HHE (B).

The highest TPC quantified from solid waste fraction was attributed to the conditions of Dp=125 µm, and a 50% hydroalcoholic solution (Figure 4A). Calculated R_F values of guarana products eluted in TLC plates are available in Table 1. The highest TPC values in HHE were attributed in the first hour of extraction for all temperatures evaluated (Figure 5A). At 100ºC was not possible to perform extractions above 2h because of fast evaporation of solution with subsequent burning of the seeds in the equipment used.

The antioxidant capacity of CHE extracts considering β-carotene assay show that highest inhibition of β-carotene oxidation were attributed to the liquid fraction from seeds with Dp= 25 µm (Figure 4C), while in HHE the inhibition of liquid fraction decreased at 70ºC and 100ºC (Figure 5C). The values obtained using the method were comparable to the volatile oils and curcuminoids extracts from turmeric (SANTANA e MEIRELES, 2017) and higher than those report to guarana methanolic extracts (MAJHENIČ et al., 2007). The DPPH radical scavenging values were comparable with the extracts obtained from citrus (NAKAJIMA et al., 2016), grape (MARTINS et al., 2016) and tea (MACEDO et al., 2011), and were higher than that obtained with guarana aqueous extracts (YAMAGUTI-SASAKI et al., 2007).

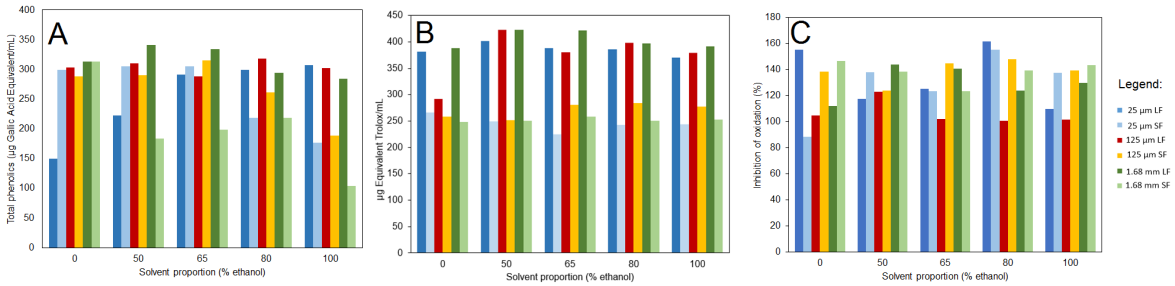

Figure 4. TPC (A), and antioxidant capacities of DPPH (B) and β-carotene (C) assays for hydroalcoholic extraction of guarana. Acronyms: LF: liquid fraction and SF: solid fraction.

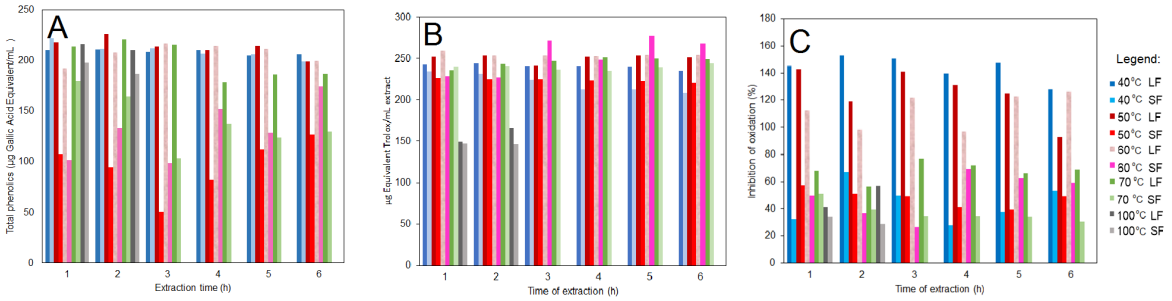

Figure 5. TPC (A), and antioxidant capacities of DPPH (B) and β-carotene (C) assays for hot hydroalcoholic extraction of guarana. Acronyms: LF: liquid fraction and SF: solid fraction.

Methods and tools used for measuring antioxidants activity or capacity have advanced remarkably during the last few decades (SHAHIDI e ZHONG, 2015). However, there is not one method that can provide unequivocal results and the best solution is to use various methods instead of a one-dimension approach (Carocho & Ferreira, 2013).

Fingerprints of guarana extracts and solid waste are characterized by a thin band surrounded by a circular spots visualized clearly at 254 nm (Figures 6-8). The circular spots indicate probable presence of caffeine, as indicated previously in the TLC plates with caffeine standard (EL SEOUD et al., 2018).

Aspects of fingerprints from CHE and HHE products were comparable to those from the extracts of aroeira and green tea (BRAZ et al., 2012) and guarana powder (EL SEOUD et al., 2018).

The spotted TLC plates prior to spraying with sulfuric vanillin reagent could be visualized only in UV. The best resolution was obtained in 254 nm (Figures 6-8) with R_F values between 0.75-0.96 for the liquid fraction and 0.84-0.96 for the solid waste fraction (Table 1) indicating the strong presence of the following phenolics catechin (R_F =0.81) and epicatechin (R_F=0.88) and weak presence of epicatechin gallate, with R_F =0.66 (BRAZ et al., 2012).

The fingerprints obtained at 366 nm showed weak visualization of developed substances (Figure 6). In this context, the TLC plates with the HHE extracts were investigated only at 254 nm and after detection with spray reagent (Figures 7 and 8). The spraying with sulfuric-vanillin resulted in appearance of dark-red spots in the thin bands of the extracts.

Sulfuric acid is an oxidant, and its reaction with phenolics with the presence of vanillin results in the formation of pink or dark red coloration bands against a purple background (SANTANA e MEIRELES, 2016, SANTANA et al., 2017). The reaction with sulphuric vanillin in TLC plates indicated also the absence of volatile constituents in guarana extracts, such as terpenes, which would result in appearance of purple coloration (JOHNER et al., 2017).

Cold hydroalcoholic extraction					
	Dp= 25µm	Dp= 125µm	Dp= 1.68 mm		
Liquid fraction	0.77-0.86	0.73-0.84	0.78-0.87		
Solid fraction	0.95	0.87	0.84		
Hot hydroalcoholic extraction					
	40 °C	50 °C	60 °C	70 °C	100 °C
Liquid fraction	0.75-0.96	0.8-0.96	0.79-0.96	0.77-0.88	0.77
Solid fraction	0.84-0.96	0.84	0.87	0.85	0.82

Table 1. R_F values of guarana products obtained with CHE and HHE.

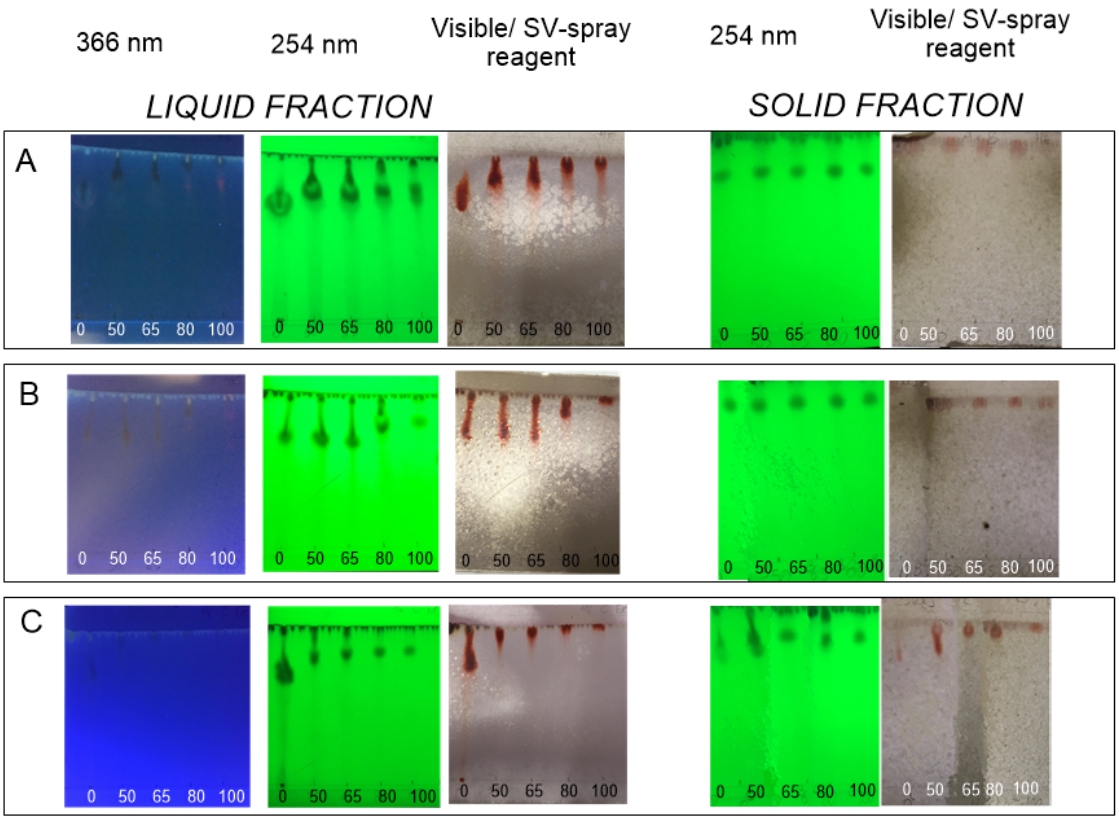

Figure 6. Thin-layer chromatography profile of guarana extracts obtained with cold hydroalcoholic extraction process using the particle diameters of 25 μm (A), 125 μm (B), and 1.68 mm (C).

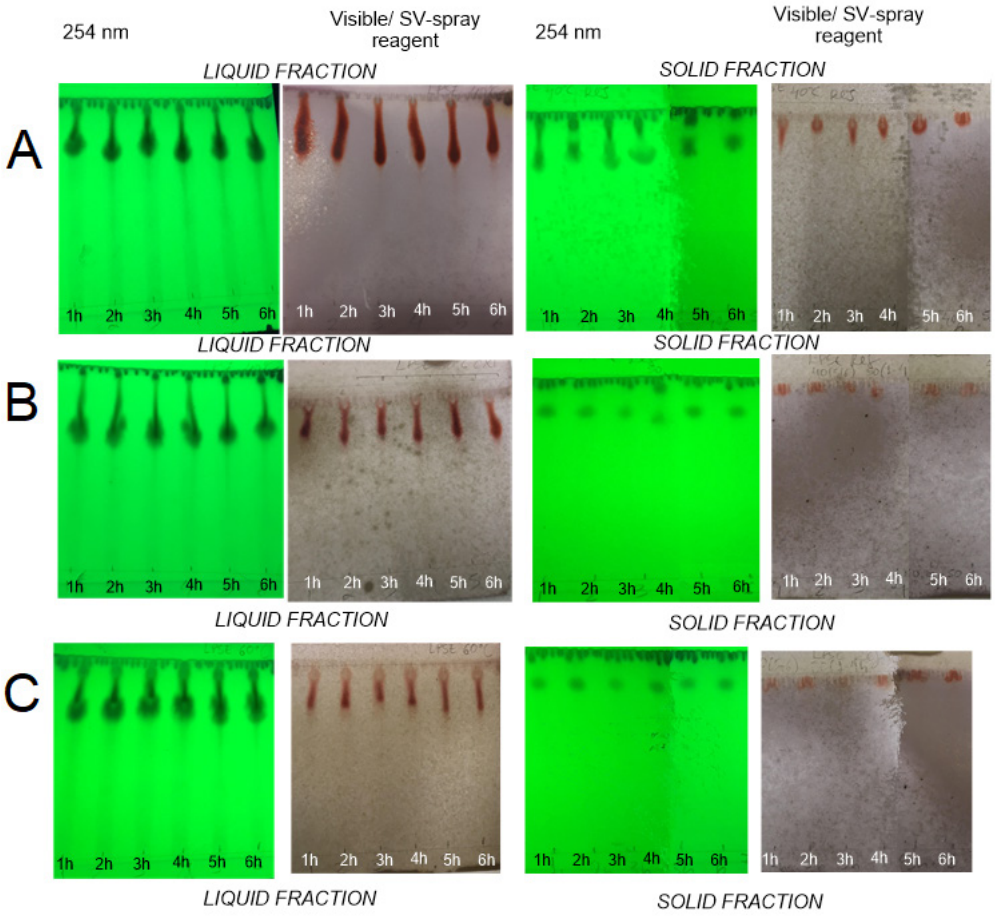

Figure 7. Thin-layer chromatography profile of guarana extracts obtained with hot hydroalcoholic extraction at 40 °C (A), 50 °C (B) and 60 °C (C).

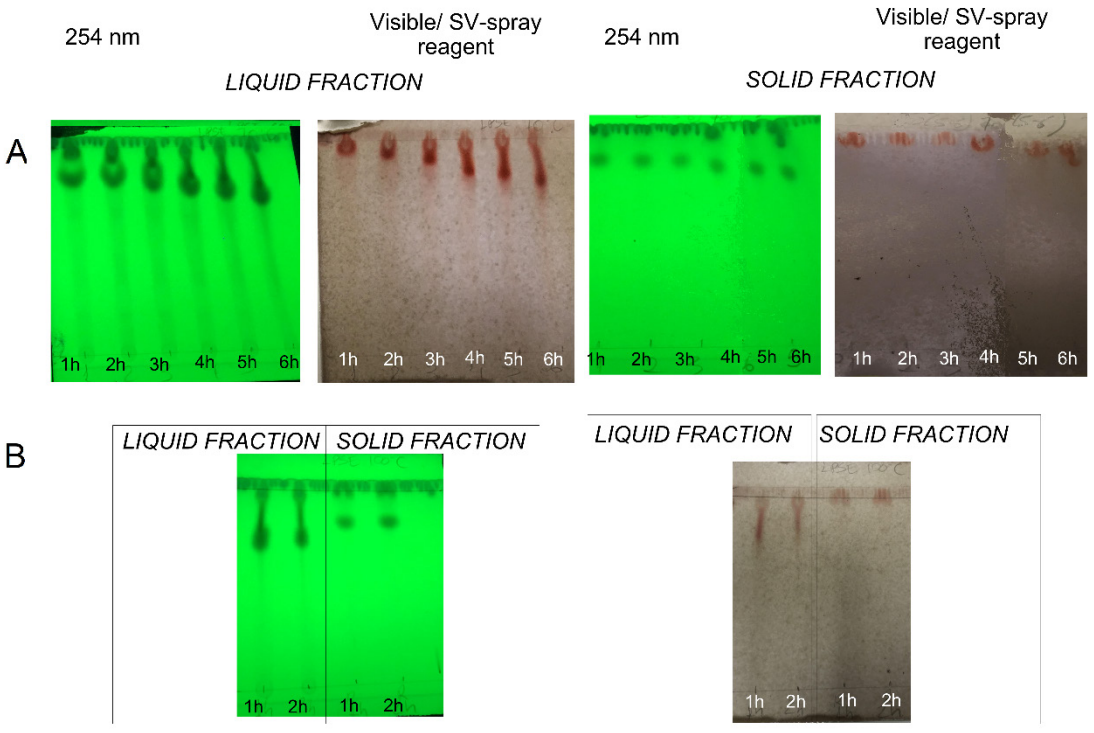

Figure 8. Thin-layer chromatography profile of guarana extracts obtained with hot hydroalcoholic extraction at 70 °C (A) and 100 °C (B).

The presence of catechins detected by TLC is confirmed by HPLC (Table 2). Extracts obtained with CHE using a 50% hydroalcoholic solution and the HHE extracts obtained in the last round of process were evaluated.

Highest concentrations of catechin and epicatechin were detected in extracts. Lowest proportion was attributed to epicatechin gallate. These information are in accordance with previous studies (MAJHENIČ et al., 2007, BAUMANN et al., 1995).

Considering the methylxanthines, caffeine occupies the highest proportion in guarana. Theobromine and theophylline are usually applied for the treatment of cardiac (PINHO et al., 2018), and respiratory diseases (ALGIERI et al., 2018), respectively.

In HHE it was possible to reach high concentration of catechins and methylxanthines at 60°C. It is observed that at 70 °C and 100 °C the concentration of these molecules decreased because of thermal degradation (Table 2).

Process	Particle diameter	Temperature	CAT	EC	ECG	CAF	TBr	TPh
CHE	Dp=1.68 mm	25°C	6.95±0.14	4.04±0.16	0.02±0.01	4.70±0.02	0.04±0	0.14±0.03
	Dp=25 µm		6.39±0.03	4.04±0.04	0.03±0.01	4.70±0.03	0.04±0	0.12±0.04
	Dp=125 µm		3.99±0.01	2.48±0.10	0.02±0	3.22±0	0.03±0	0.15±0.02
HHE	Dp=1.68 mm	40°C	6.65±0.14	3.77±0.07	0.02±0.01	4.75±0.01	0.02±0	0.19±0.01
		50 °C	7.85±0.05	4.66±0.21	0.08±0.05	5.28±0.02	0.04±0	0.15±0.03
		60 °C	8.90±0.08	5.44±0.24	0.18±0.02	5.84±0.02	0.04±0	0.12±0.03
		70 °C	6.46±0.07	3.76±0.08	0.04±0	4.60±0.01	0.05±0	0.08±0.02
		100 °C	2.78±0.11	1.52±0.01	0.01±0	2.21±0.07	0.02±0	0.09±0

Table 2. The catechin and methylxanthine concentrations (mg/mL) in guarana extracts in the last hour of extraction.

CAT- catechin, EC – epicatechin, ECG – epicatechin gallate, CAF – caffeine, TBr – theobromine, TPh – theophylline.

4 I CONCLUSIONS

The effects of hydroalcoholic extraction of guarana seeds on the recovery of bioactive constituents were evaluated in this work using quantitative and qualitative approaches. In CHE, highest content of polyphenols and methylxanthines was detected in the extracts obtained from seeds with 1.68mm diameter and solvent formulation constituted of 50% ethanol. The solid wastes showed relevant antioxidant capacity in terms of inhibition of oxidation of β-carotene and the scavenging of DPPH free radical. Positive presence of phenolic compounds was detected in TLC plates with sulfuric-vanillin spray reagent, which was confirmed by quantitative evaluation with HPLC. The highest concentration of caffeine, theobromine, theophylline, catechin, epicatechin and epicatechin gallate was found in HHE extracts obtained at 60°C. The results obtained showed the potential of this ecofriendly approach, in contrast to common methods that

make use of potentially toxic organic solvents

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SOBRE O ORGANIZADOR

Dr. Benedito Rodrigues da Silva Neto

Possui graduação em Ciências Biológicas pela Universidade do Estado de Mato Grosso (2005), com especialização na modalidade médica em Análises Clínicas e Microbiologia. Em 2006 se especializou em Educação no Instituto Araguaia de Pós graduação Pesquisa e Extensão. Obteve seu Mestrado em Biologia Celular e Molecular pelo Instituto de Ciências Biológicas (2009) e o Doutorado em Medicina Tropical e Saúde Pública pelo Instituto de Patologia Tropical e Saúde Pública (2013) da Universidade Federal de Goiás. Pós-Doutorado em Genética Molecular com concentração em Proteômica e Bioinformática. Também possui seu segundo Pós doutoramento pelo Programa de Pós-Graduação Stricto Sensu em Ciências Aplicadas a Produtos para a Saúde da Universidade Estadual de Goiás (2015), trabalhando com Análise Global da Genômica Funcional e aperfeiçoamento no Institute of Transfusion Medicine at the Hospital Universitatsklinikum Essen, Germany.

Palestrante internacional nas áreas de inovações em saúde com experiência nas áreas de Microbiologia, Micologia Médica, Biotecnologia aplicada a Genômica, Engenharia Genética e Proteômica, Bioinformática Funcional, Biologia Molecular, Genética de microrganismos. É Sócio fundador da “Sociedade Brasileira de Ciências aplicadas à Saúde” (SBCSaúde) onde exerce o cargo de Diretor Executivo, e idealizador do projeto “Congresso Nacional Multidisciplinar da Saúde” (CoNMSaúde) realizado anualmente no centro-oeste do país. Atua como Pesquisador consultor da Fundação de Amparo e Pesquisa do Estado de Goiás - FAPEG. Coordenador do curso de Especialização em Medicina Genômica e do curso de Biotecnologia e Inovações em Saúde no Instituto Nacional de Cursos. Como pesquisador, ligado ao Instituto de Patologia Tropical e Saúde Pública da Universidade Federal de Goiás (IPTSP-UFG), o autor tem se dedicado à medicina tropical desenvolvendo estudos na área da micologia médica com publicações relevantes em periódicos nacionais e internacionais.

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