From byproduct to a functional ingredient: Camu-camu (*Myrciaria dubia*) seed extract as an antioxidant agent in a yogurt model

Marina Fidelis,¹ Stephanie Maiara de Oliveira,¹ Jânio Sousa Santos,¹ Graziela Bragueto Escher,¹ Ramon Silva Rocha,² Adriano Gomes Cruz,² Mariana Araújo Vieira do Carmo,³ Luciana Azevedo,³ Tai Kaneshima,⁴ Won Young Oh,⁵ Fereidoon Shahidi,⁵ and Daniel Granato^{1,6*}

¹Department of Food Engineering, State University of Ponta Grossa (UEPG), Av. Carlos Cavalcanti, 4748, 84030-900, Ponta Grossa, Brazil

²Federal Institute of Education, Science and Technology from Rio de Janeiro (IFRJ), 20270-021, Rio de Janeiro, Brazil

³Department of Food, Faculty of Nutrition, Federal University of Alfenas, Rua Gabriel Monteiro da Silva, 714, 37130-000, Alfenas, Brazil

⁴Department of Nutritional Science and Food Safety, Faculty of Applied Biosciences, Tokyo University of Agriculture, Tokyo 156-8502, Japan

⁵Department of Biochemistry, Memorial University of Newfoundland, St. John's, NL A1B 3X9, Canada

⁶Food Processing and Quality, Innovative Food System, Production Systems Unit, Natural Resources Institute Finland (Luke), Tietotie 2, FI-02150 Espoo, Finland

ABSTRACT

This work aimed to characterize the phenolic composition and in vitro antioxidant and antiproliferative properties of lyophilized camu-camu (*Myrciaria dubia*) seed extract (LCE), and to assess the effects of LCE on the antioxidant and sensory traits of yogurt. The LCE contained 46.3% (wt/wt) total phenolic content; the main compounds quantified were vescalagin, castalagin, gallic acid, procyanidin A2, and (–)-epicatechin. The LCE had antioxidant activity, as measured by different chemical assays (2,2-diphenyl-1-picrylhydrazyl, Folin–Ciocalteu reducing capacity, total reducing capacity, ferric reducing antioxidant power, and Cu²⁺ chelating capacity), and inhibited the cell proliferation of HepG2 cells (human hepatoma carcinoma; IC₅₀ = 1,116 µg/mL) and Caco-2 cells (human colorectal adenocarcinoma epithelial cells; IC₅₀ = 608.5 µg/mL). In addition, LCE inhibited the in vitro activity of α-amylase, α-glucosidase, and angiotensin-converting enzyme, and protected DNA from peroxyl radical-induced scission. When added to yogurts, different concentrations of LCE (0, 0.25, 0.5, 0.75, and 1.0 g/100 g) increased the chemical antioxidant and reducing capacities. The camu-camu yogurt containing LCE at 0.25 g/100 g had an acceptance index of 84%, showing that camu-camu seed extract may be a potential ingredient for addition to yogurts.

Key words: food innovation, phenolic compound, ellagitannin, antioxidants, antiproliferative activity

INTRODUCTION

Camu-camu [*Myrciaria dubia* (HBK) McVaugh] is a fruit from South America that is mainly consumed on its own or as frozen pulp. The frozen pulp is exported to many European and Asian countries because of its taste and versatility in household cooking. However, its peel and seeds are discarded by frozen pulp companies, generating a large quantity of byproduct. The consumption of camu-camu pulp is related to its unique taste and high ascorbic acid content, but recently chemical and functional analyses of camu-camu byproducts have indicated in vitro and in vivo functional properties, such as antioxidant, antihyperglycemic, antihyperlipidemic, antihypertensive, antimicrobial, and neuroprotective effects (de Souza Schmidt Gonçalves et al., 2014; Azevêdo et al., 2015; Fujita et al., 2015; Miyashita et al., 2017; Fidelis et al., 2018). Camu-camu byproducts have the potential to be used in developing new bioactive-rich foods and possibly eliminating the use of synthetic chemical preservatives in processed foods. No patents are registered with the Brazilian Industrial Property Institute (INPI; search on June 3, 2019, using the key words “camu-camu yogurt” and “camu-camu dairy food”), indicating that the dairy sector should explore the use of camu-camu pulp, seeds, and peel to develop new potentially functional dairy foods. To date, yogurts manufactured with pomegranate (*Punica granatum*) and jacaranda (*Jacaranda mimosifolia*) seed flours (0.5 g of flour/100 g of yogurt) have been shown to increase antioxidant activity, as measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the degree of liking of taste (Van Nieuwenhove et al., 2019).

According to Sloan (2019), 38% of US consumers are willing to pay a premium for foods that offer health benefits beyond basic nutrition, and roughly 54% of those consumers actively seek out functional foods. In

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*Corresponding author: daniel.granato@luke.fi

2018, 54% of US consumers sought out preservative-free (“natural”) foods, and within this context, dairy foods manufactured with natural extracts are suitable alternatives (Gaglio et al., 2019). Adding phenolic-rich extracts or matrices to dairy foods such as ice creams and yogurts is among a number of new approaches aimed at giving these foods a naturally healthy halo (Karnopp et al., 2017; Zikiou and Zidoune, 2019).

Yogurts and fermented milks manufactured with different natural extracts have been studied over the last 10 yr. Ramos et al. (2017) added a lyophilized clove aqueous extract to fermented milk, finding that the total phenolic and antioxidant activity increased considerably, and that the sensory acceptance of the product was enhanced. Similarly, Liu (2018) and Gaglio et al. (2019) added Fuzhuan brick tea and aqueous saffron extract, respectively, to yogurts and verified that increasing concentrations of the bioactive-rich aqueous extracts led to higher antioxidant activity in the yogurts.

In our previous work (Fidelis et al., 2018), we performed a statistical optimization of the extraction conditions of camu-camu seeds, and the most promising solvent system to extract the highest content of bioactive compounds was 40.7% water, 16% ethyl alcohol, and 43.3% propanone. Taking advantage of technological trends in the dairy sector, the present work aimed to characterize the lyophilized camu-camu seed extract (LCE) in terms of phenolic composition, antioxidant, and cytotoxic effects, and to assess the effects of different concentrations on the antioxidant activity of yogurt. We also conducted proximate analysis and a sensory evaluation of the yogurt with added LCE to characterize its potentially functional properties.

MATERIALS AND METHODS

Chemicals

We purchased gallic acid, DPPH, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, Folin–Ciocalteu reagent, 2,4,6-tripyridyl-s-triazine, acetonitrile, analytical standards (HPLC, purity >97%), ellagic acid, caffeic acid, ferulic acid, (+)-catechin, (–)-epicatechin, quercetin, quercetin-3-rutinoside, chlorogenic acid, α -amylase (A3176-500KU from porcine pancreas type VI-B), 4-nitrophenyl-D-glucopyranoside, dinitrosalicylic reagent, acarbose, and α -glucosidase (G5003-100UN from *Saccharomyces cerevisiae* type I) from Sigma-Aldrich (São Paulo, Brazil), and acquired *trans*-resveratrol from Extrasynthese (Genay, France). Ethyl alcohol 99.8%, methyl alcohol 99.8%, and FeSO_4 were purchased from Neon (São Paulo, Brazil). Hydrochloric acid, isobutanol, anhydrous $\text{CH}_3\text{CO}_2\text{Na}$, NaH_2PO_4 , $\text{K}_4[\text{Fe}(\text{CN})_6]$, and ascorbic acid were pur-

chased from Vetec (Rio de Janeiro, Brazil). Sodium hydroxide and propanone 99.5% were purchased from Synth (Diadema, Brazil). Formic acid was obtained from Reagen (Curitiba, Brazil). Aqueous solutions were prepared using ultrapure water (Millipore, São Paulo, Brazil). The HepG2, Caco-2, and IMR90 (normal lung fibroblast cells) cell lines were obtained from the Rio de Janeiro cell bank.

Camu-Camu Seeds

Samples of mature camu-camu fruit [*Myrciaria dubia* (HBK) McVaugh] were supplied by a producer from the municipality of Iguape (geographical coordinates: 24°41'51"S, 47°34'16"W at 6 m altitude) in April 2017. Fruits were sanitized (NaOCl at 100 mg/L for 15 min) and dried for 35 h at 35°C. Dried seeds were ground (model M20; Ika Werke, São Paulo, Brazil) and sieved using a 42 Tyler mesh (0.354 mm) to obtain a standardized camu-camu seed flour. This project was registered at the National Genetic Heritage Management System (SisGen) of the Brazilian Ministry of the Environment (www.sisgen.gov.br; A1848EB).

From previous work involving the extraction of phenolic compounds from camu-camu seeds (Fidelis et al., 2018), we used 3 solvents for extraction: 40.7% water, 16% ethyl alcohol, and 43.3% propanone. Extractions were performed using a 1:20 ratio (mass/vol). For this purpose, camu-camu seed flour was mixed with the solvent mixture at a ratio of 1:20 (wt/vol). We used a technique of continuous agitation with temperature control, in which the water remained at $45 \pm 1^\circ\text{C}$ for 45 min in a thermostatically controlled glass cell (Dubnoff TE-0.53; Tecnal, Ourinhos, Brazil). The extract was filtered and the solvent fully eliminated under vacuum using a rotatory evaporator. The aqueous solution was freeze-dried under vacuum at 1,200 μL of Hg (LD 1500A, Terroni, São Carlos, Brazil) to obtain the LCE.

Lyophilized Camu-Camu Seed Extract

We analyzed total phenolic content [mg of gallic acid equivalent (GAE)/100 g], *ortho*-diphenols (mg of chlorogenic acid equivalent/100 g), and condensed tannins [mg of catechin equivalent (CE)/100 g] 3 times in the LCE according to a methodology previously described (Santos et al., 2018). We determined individual phenolic compounds by liquid chromatography using a LC-20T Prominence chromatograph (Shimadzu, Tokyo, Japan) equipped with diode array and fluorescence detectors (LC-DAD). We conducted separation using a reverse phase column (C_{18} , 150 mm $\times$ 4.6 mm, particle size 3.5 μm) and the experimental conditions described by Fidelis et al. (2018). We quantified vescalagin and

castalagin by LC-DAD using the equipment and conditions described by Kaneshima et al. (2016). Results are expressed as mg/100 g of LCE.

We measured the chemical antioxidant activity of LCE using the following assays: DPPH free-radical scavenging activity [mg of ascorbic acid equivalent (**AAE**)/100 g]; Folin–Ciocalteu reducing capacity (**FCRC**; mg of GAE/100 g), ferric reducing antioxidant power (**FRAP**; mg of AAE/100 g), Cu^{2+} chelating capacity (% inhibition of pyrocatechol violet- Cu^{2+} complex formation), and total reducing capacity (mg of quercetin equivalent/100 g), following experimental conditions described by Fidelis et al. (2018) and Santos et al. (2018).

We evaluated the cytotoxicity of the camu-camu seed extract to Caco-2 and HepG2 using the MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) as previously described (Migliorini et al., 2019). Briefly, cells were seeded into 96-well plates at densities of 2×10^4 cells/well (HepG2) and 1×10^4 cells/well (Caco-2). After adhesion, the cells were treated for 48 h with serial concentrations of LCE (100, 500, 1,000, and 2,000 $\mu\text{g/mL}$). We determined cell viability at the time of treatment and after 48 h, according to the following parameters: IC_{50} represented the concentration of the agent that inhibited cell growth by 50%, $(T/C) \times 100 = 50$, where T was the number of cells at the time of treatment and C was the number of control cells at the time of treatment; GI_{50} represented the concentration of the agent that inhibited cell growth by 50% relative to untreated cells $[(T - T_0)/(C - T_0)] \times 100 = 50$, where T_0 was the number of cells at time zero; and LC_{50} represented the concentration of the agent that resulted in a net loss of 50% of cells relative to the number at the start of treatment $[(T - T_0)/T_0] \times 100 = -50$ (do Carmo et al., 2018).

We measured cellular reactive oxygen species (**ROS**) levels with fluorescence probes using 2',7'-dichlorodihydrofluorescein diacetate (**DCFH-DA**; 25 mmol/L). The cell lines IMR90 (normal human lung fibroblast) and HepG2 (6×10^4 per well) were treated for 1 h with different concentrations (10–100 $\mu\text{g/mL}$) of LCE or 15 $\mu\text{mol/L}$ H_2O_2 (positive control) or culture medium (negative control). Following treatment, H_2O_2 at 15 $\mu\text{mol/L}$ was added to all wells, and fluorescence intensity was measured at 485 nm of excitation and 538 nm of emission (Escher et al., 2018).

The inhibitory effect of LCE against peroxyl radical-induced DNA scission was assessed according to the methodology described by Alshikh et al. (2015). Supercoiled pBR 322 DNA (50 $\mu\text{g/mL}$) was dissolved in phosphate buffer (10 mmol/L, pH 7.4), and extracts (1 mg/mL) were dissolved in water. In a tube,

LCE was diluted with phosphate buffer (catechin 10 $\mu\text{g/mL}$ and extract 100 $\mu\text{g/mL}$). Then, test samples (2 μL), phosphate buffer (2 μL), DNA (2 μL), and 2,2'-azobis(2-methylpropanimidamide) dihydrochloride (11.25 mmol/L, 4 μL) were added to the Eppendorf tube. A control (DNA with peroxyl radical) and a blank (DNA only) were also prepared. To quantify the DNA scission, samples were electrophoresed using a 0.7% (wt/vol) agarose gel in Tris-acetic acid-EDTA buffer (40 mmol/L Tris acetate, 1 mmol/L EDTA, pH 8.5) at 60 V for 5 h using a B1A horizontal mini gel electrophoresis system (Owl Separation Systems Inc., Portsmouth, NH). The intensity of the bands in the photographs was analyzed using Alpha Ease standalone software (Alpha Innotech Co., San Leandro, CA). The protective effects of LCE were calculated according to the equation below and expressed the results as inhibitory percentage of DNA scission.

$$\text{DNA retention (\%)} = \left(\frac{\text{intensity of supercoiled DNA with the peroxyl radical}}{\text{intensity of supercoiled DNA in control}} \right) \times 100.$$

We also analyzed LCE for its ability to inhibit the activity of α -amylase, α -glucosidase, and angiotensin-converting enzyme (**ACE**). First, LCE (100 mg) was diluted with 1 mL of ultrapure water and stirred at 200 rpm (SL180/D, Solab, Piracicaba, SP, Brazil) for 1 h at room temperature. Then, the extract was filtered and analyzed. We assessed ACE inhibition according to Konrad et al. (2014) and expressed the results as percentage of inhibition. We assessed the inhibition of α -glucosidase and α -amylase according to the method of Ferreira et al. (2019), and expressed the results as percentage of inhibition. All analyses were performed in triplicate and results are expressed as mean and standard deviation.

Processing and Characterization of Yogurts with Added Camu-Camu Seed Extract

Yogurts (~1 L per formulation) were processed as described by Perina et al. (2015). Whole bovine milk (Lactobom, Toledo, Brazil; 2.5 g/100 mL of total lipids and 3.4 g/100 mL of total proteins), 11% sucrose (Union, São Paulo, Brazil), 3.5 g/100 g of whole bovine milk powder (Tyrol, São Paulo, Brazil; 27.7 g/100 g of total fat and 37 g/100 g of total carbohydrates) were mixed and pasteurized at 65°C/30 min. After pasteurization, 0.01 g/100 g of lyophilized bacterial cultures (*Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) were added when the

temperature reached 42°C. The fermentation process was stopped at pH 4.6. The curd was cooled to 6°C for 8 h, and then the LCE was added at different concentrations: 0.25, 0.50, 0.75, and 1.00 g/100 g. We also prepared a negative control yogurt (0 g of LCE/100 g) for comparison. An orange flavoring agent (Mix, São Bernardo do Campo, Brazil) was added to all samples at a concentration of 0.25 g/100 g.

To evaluate the effect of the LCE on the antioxidant activity of the yogurts, we extracted 5 g of yogurt with 10 mL of methyl alcohol, under constant stirring (vortex) for 10 min. Then, the mixture was centrifuged at $700 \times g$ for 30 min, and the supernatant was collected and immediately analyzed for FCRC (mg of GAE/100 g), FRAP (mg of AAE/100 g), and DPPH (mg of AAE/100 g) following the experimental conditions described by Fidelis et al. (2018) and Santos et al. (2018).

In a preliminary sensory evaluation of the 5 yogurt formulations (data not shown), LCE concentrations higher than 0.25 g/100 g enhanced the bitterness and astringency of the yogurts, which was an undesirable effect of the polyphenols on yogurt acceptability. Therefore, we chose the yogurt formulation containing 0.25 g of LCE/100 g for further sensory evaluation. For this purpose, a total of 75 assessors (22 men and 53 women between 18 and 60 yr of age) signed the consent form and were informed about the sensory protocol, which had been previously approved by the ethics committee (CAAE: 65493717.9.0000.0105). The yogurt (25 g) was served in coded (3 digits) tumblers at 4°C, and assessors used a 9-point hedonic structured scale (1 = extreme dislike; 5 = neither like nor dislike; 9 = extreme like) to indicate the degree of liking of taste, odor, consistency, color, and overall impression. Assessors were also asked to indicate whether they would pay (R\$0 to R\$3.00; R\$1 = US\$0.25) for a 100-g yogurt source of natural antioxidants compared with a natural yogurt without antioxidants (R\$1.00). As a supplementary analysis, we also asked assessors to indicate whether they considered yogurt to be a healthy food (yes/no) and whether they considered the consumption of yogurts with added natural extracts to be healthy (yes/no).

We determined the proximate composition of the yogurt with added 0.25 g of LCE/100 g and the negative control yogurt according to AOAC International (2006): protein ($N \times 6.38$), ash, moisture, and total lipids (Bligh-Dyer method) and results are expressed as grams per 100 g. We estimated total carbohydrates by difference, and calculated the total energy value using Atwater factors (4 kcal/g for proteins and carbohydrates and 9 kcal/g for lipids; results are expressed as kcal/100 g). The pH was also measured using a calibrated pH meter (model PHS-3B; Hanna Instruments, Barueri, Brazil), and we assessed total titratable acidity using a

0.11 mol/L NaOH solution (results are expressed as g of lactic acid/100 g of yogurt). We assessed syneresis at 5°C of the yogurt with added LCE by centrifugation ($7,000 \times g$ for 10 min); results are expressed as percentages.

In addition, the instrumental color of the yogurts was assessed by measuring lightness (L^*), red/green color (a^* coordinate), and yellow/blue color (b^* coordinate) using a handheld MiniScan EZ 4500L colorimeter (Hunter Laboratory, Reston, VA) in reflectance mode. We assessed the instrumental texture profile of the yogurts at 5°C using a TA-XT Plus Texture Analyzer (Stable Micro Systems, Godalming, UK) to assess firmness (g) and consistency (g/s). For this purpose, we used a cylindrical stainless steel probe with an acrylic disk (35 mm diameter), a penetration depth of 30 mm, and a penetration rate of 1 mm/s.

Data Analysis

The experimental data are presented as means $\pm$ standard deviations ($n = 3$ replicates). When appropriate, we compared means between groups using 1-way ANOVA. For this purpose, we verified homoscedasticity using the Brown-Forsythe test and TIBCO Statistica (version 13.3; TIBCO Ltd., Palo Alto, CA). We identified differences between means using Fisher's least significant difference (LSD) test, considering $P \leq 0.05$ to be significant. To compare sensory data between men and women and the physicochemical characteristics of the LCE and LCE-free yogurts, we used unpaired Student's t -tests, and calculated the 2-sided comparisons between acceptance indexes using the z -test (Granato et al., 2014).

RESULTS AND DISCUSSION

Phenolic Composition, Antioxidant Activity, and Cytotoxicity/Antiproliferative Evaluation of LCE

The results for phenolic composition (individual and major phenolic classes), antioxidant activity, and cytotoxic and antiproliferative effects of LCE in relation to the different cell lines are presented in Table 1.

The contents of total phenolics, *ortho*-diphenols, and condensed tannins in LCE were roughly 43.6 g of GAE/100 g of LCE, 13.2 g of CAE/100 g of LCE, and 5.8 g of CE/100 g LCE, respectively. These values were higher than those in camu-camu powder produced by spouted bed drying (8.16 g of GAE/100 g and 7.22 g of quebracho tannin equivalent/100 g; Fujita et al., 2013). Several phenolic acids, flavonoids, ellagitannins, and *trans*-resveratrol have already been identified in different parts of the camu-camu pulp, peel, or seed coat

(Genovese et al., 2008; Chirinos et al., 2010; Rufino et al., 2010; Fracassetti et al., 2013; de Azevêdo et al., 2014; Fujita et al., 2015; Kaneshima et al., 2016). In fact, Fujita et al. (2015) identified quercetin derivatives; ellagic, syringic, and gallic acids; myricetin; cyanidin-3-*o*-glucoside; castalagin; and vescalagin in camu-camu powder (peel and pulp). Fracassetti et al. (2013) also identified gallocatechin derivatives, procyanidin dimer B type, (+)-catechin, catechin gallate, and other glycosylated ellagic acid derivatives.

To determine the antioxidant activity of LCE, we used 4 chemical methods to measure the ability of LCE antioxidants to act as single-electron transfer agents: FRAP, FCRC, total reducing capacity, and DPPH. We also measured Cu^{2+} chelating capacity to

determine the capacity of LCE to chelate a transition metal ion (Table 1). The results showed strong chemical antioxidant activity using all methods, especially metal chelating capacity: LCE was diluted 100-fold to render a 17% inhibition of the pyrocatechol violet- Cu^{2+} complex formation. These antioxidant activity values were associated with the high total phenolic content in LCE. Our data were in agreement with of Kaneshima et al. (2016), who analyzed the antioxidant activity of camu-camu flour (pulp and peel) using DPPH, oxygen radical absorbance capacity, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (**ABTS**) methods. Similarly, Fujita et al. (2013) found high antioxidant activity as measured by FCRC (11 g of GAE/100 g), FRAP [65,000 mmol of Trolox equivalents (**TE**)/100

Table 1. Phenolic composition, chemical antioxidant activity, and cytotoxicity against cancerous cell lines of lyophilized camu-camu seed extract

Characteristic ¹	Value
Phenolic class	
Total phenolic content (mg of GAE/100 g)	43,598 ± 923
Total condensed tannins (mg of CE/100 g)	5,766 ± 478
Total <i>ortho</i> -diphenols (mg CAE/100 g)	13,193 ± 1,041
Phenolic composition (mg/100 g)	
Gallic acid	473.18 ± 5.03
Syringic acid	35.34 ± 0.61
Caffeic acid	42.38 ± 0.19
Rosmarinic acid	17.26 ± 0.73
Chlorogenic acid	24.91 ± 0.23
2,4-Dihydroxybenzoic acid	73.73 ± 0.72
Ferulic acid	14.96 ± 0.15
2-Hydroxycinnamic	6.07 ± 0.07
<i>p</i> -Coumaric acid	20.14 ± 0.15
Castalagin	8,771 ± 278
Vescalagin	3,967 ± 75
Quercetin	6.39 ± 0.10
Procyanidin A2	200.16 ± 3.05
(+)-Catechin	130.00 ± 0.01
(-)-Epicatechin	144.29 ± 0.14
Quercetin-3-rutinoside	136.05 ± 0.45
<i>trans</i> -Resveratrol	20.75 ± 0.09
Ellagic acid	86.61 ± 0.31
Chemical antioxidant activity	
Folin-Ciocalteu reducing capacity (mg of GAE/100 g)	21,324 ± 547
Total reducing capacity (mg of QE/100 g)	34,858 ± 1,223
Ferric reducing antioxidant power (mg of AAE/100 g)	48,436 ± 6,346
2,2-Diphenyl-1-picrylhydrazyl (mg of AAE/100 g)	49,798 ± 682
Cu^{2+} chelating capacity (% inhibition of complex formation)	17 ± 2
Cytotoxicity toward cancerous cells (μg/mL)	
Caco-2 LC ₅₀	936.8
Caco-2 IC ₅₀	608.5
Caco-2 GI ₅₀	491.8
HepG2 LC ₅₀	1,912
HepG2 IC ₅₀	1,116
HepG2 GI ₅₀	538.1
Inhibition of enzymes (% inhibition)	
α-Amylase	40.70 ± 0.40
α-Glucosidase	16.60 ± 0.46
Angiotensin-converting enzyme	34.40 ± 0.72

¹GAE = gallic acid equivalent; CE = catechin equivalent; CAE = chlorogenic acid equivalent; AAE = ascorbic acid equivalent; QE = quercetin equivalent; GI₅₀ = concentration of the agent that inhibits growth by 50%, relative to untreated cells; IC₅₀ = concentration of the agent that inhibits cell growth by 50%; LC₅₀ = concentration of the agent that results in a net loss of 50% cells, relative to the number at the start of treatment.

g], and DPPH (3,800 mmol of TE/100 g) in lyophilized camu-camu powder. The in vitro chemical antioxidant activity of camu-camu frozen pulp was associated with a decrease in total cholesterol, triacylglycerols, uric acid, and thiobarbituric acid reactive substances in the liver and blood of diabetic Wistar rats (de Souza Schmidt Gonçalves et al., 2014).

Using the MTT assay, we showed that LCE decreased cell viability in a dose-dependent manner in both Caco-2 and HepG2 cancer cells. According to Figure 1, LCE exhibited pro-carcinogenic behavior at low concentrations in Caco-2 cells (100 µg/mL) but antiproliferative activity at higher concentrations (500–2,000 µg/mL). Similarly, Rietjens et al. (2005) pointed out that quercetin (a phenolic compound highly distributed in vegetables) could stimulate or inhibit cell proliferation depending on its concentration. Another study showed that *Cichorium intybus* L. extract, rich in anthocyanins and other phenolic compounds, also showed this biphasic effect (pro-carcinogenic and antiproliferative) at specific concentrations (Migliorini et al., 2019). Moreover, it is important to consider that individual bioactive compounds exist in different proportions in whole natural matrices. This explains why, in many cases, whole matrices present better bioactivity or behavior than isolated or single nutrients (Martins et al., 2016). The antiproliferative results showed that among the cell lines, HepG2 cells were more resistant to LCE, with a high GI_{50} value (538.1 µg/mL). In contrast, Caco-2 cells were more susceptible to LCE, with a lower GI_{50} value (491.8 µg/mL). Regarding cell death (cytotoxic effect), Caco-2 also showed a lower LC_{50} value (936.8 µg/mL) compared with HepG2 (LC_{50} = 1,912 µg/mL). Interestingly, Azevêdo et al. (2019) showed that camu-camu pulp extract exerted protection against 1,2-dimethylhydrazine dihydrochloride mutagenic effects on the colon in in vivo studies. Thus, according to our results, it may be that the potential bioactivity of camu-camu extracts depends on the cancer cell line and the type of study (in vivo versus in vitro).

We assessed the ROS generated in cell lines exposed to various concentrations of LCE using DCFH-DA. The levels of ROS induced by H_2O_2 was higher than in the negative control for both cells (Figure 2), indicating its influence on ROS generation. Our results revealed that LCE exhibited antioxidant activity at all concentrations against oxidative stress induced by H_2O_2 in HepG2 cancer cells and IMR90 normal cells. In the same way, Ma et al. (2019) showed that tea polyphenols could attenuate H_2O_2 -induced oxidative stress in vitro by adjusting ROS generation. We suggest that LCE induced no antiproliferative effects through ROS generation in HepG2 cells. The generation of ROS is one of the stimuli that can trigger an apoptosis cascade, lead-

Figure 1. Cell viability and evaluation of the concentration-dependent effect after 48 h exposure to camu-camu extract in Caco-2 and HepG2 cancer cells. (A) IC_{50} (concentration of the agent that inhibits cell growth by 50%); (B) GI_{50} (concentration of the agent that inhibits growth by 50%, relative to untreated cells); and (C) LC_{50} (concentration of the agent that results in a net loss of 50% cells, relative to the number at the start of treatment).

ing to cell death. Thus, the activation of apoptosis may be independent of an extract's antioxidant capacity (Curti et al., 2017). In the present study, the cell death observed in the cell viability test could have occurred as a result of different types of physicochemical stress stimuli, such as cell membrane damage; mitochondrial dysfunction and destabilization (Peixoto et al., 2017); tumor necrosis factor- α production; ischemia-reperfusion injury; glutamate; and calcium overload (Fulda, 2013). Moreover, plant extracts rich in polyphenols, as well as pure polyphenols alone or in combination, can exert many pro-apoptotic effects, often selectively

Figure 2. Results of intracellular reactive oxygen species measurement in (A) IMR90, and (B) HepG2 cell lines. Treatment = lyophilized camu-camu seed extract at concentrations of 10, 50, or 100 µg/mL. DCF = 2',7'-dichlorofluorescein. Quantitative data are mean $\pm$ SD. Bars with different letters (a, b) differ ($P \leq 0.05$).

targeting cancer cells, through extrinsic and intrinsic mechanisms of action, tested in vitro and at concentrations generally higher than those found in food matrices or in herbal medicine (Curti et al., 2017). For instance, *trans*-resveratrol, a phenolic compound in our camu-camu extract, was found to possess apoptotic effects due to the formation of acidic vesicular organelles, autophagic vacuoles, and DNA fragmentation in oral cancer cells (Chang et al., 2017).

We hypothesize that the antioxidants contained in LCE neutralized additional ROS production, preventing cancer cell death by the ROS pathway. Because we added LCE to a food consumed by all age groups on all continents, animal-based experiments and clinical trials should be undertaken to assess the effects of yogurt with added camu-camu seed extract on the biomarkers of oxidative and nitrosative stress, such as lipid peroxidation, low-density lipoprotein oxidation, and induction of proinflammatory cytokines (Da Silva and Rudkowska, 2016).

In addition to its high chemical antioxidant activity, LCE protected DNA against peroxyl radical-induced scission. Indeed, LCE (100 µg/mL) had an inhibition rate of $89.3 \pm 2.7\%$, which was comparable ($P = 0.713$) to the protection offered by (+)-catechin (10 µg/mL; $88.6 \pm 1.4\%$; Figure 3). These results show that both LCE and (+)-catechin protected DNA from oxidative reactions and scission. In fact, peroxyl radicals may

Figure 3. Gel electrophoresis showing inhibition of lyophilized camu-camu seed extract (LCE at 100 µg/mL) against peroxyl radical-induced DNA scission compared with blank (DNA only), control (DNA with peroxyl radical), and (+)-catechin as a standard (10 µg/mL).

cause oxidative stress in cells that can lead to DNA damage, causing mutagenesis and carcinogenesis in vivo (Perera et al., 2018). This is the first report indicating the DNA protection of camu-camu seed extract against ROS.

Characterization of Yogurts with LCE

The effects of LCE [0 (negative control), 0.25, 0.50, 0.75, and 1.00 g/100 g] on the antioxidant activity of yogurt (FRAP, DPPH, and FCRC) are shown in Figure 4. All antioxidant assays showed dose-dependent effects: that is, the higher the concentration of LCE, the greater the in vitro antioxidant activity of the yogurts. Similar observations have been obtained for yogurts and fermented milks manufactured with added herbal and other natural extracts rich in bioactive compounds. Zhang et al. (2019) showed that the addition of moringa aqueous extract (*Moringa oleifera*; 0–0.20 g/100 g) to yogurts enhanced FCRC (6.5–12.5 mg of GAE/100 mL) and antioxidant activity as measured by the DPPH (10–72% radical inhibition) and ABTS (15–83% radical inhibition) assays in a dose-dependent manner. Similarly, Liu (2018) observed that when Fuzhuan brick tea leaves were added to yogurts (1–3 g/100 mL), FCRC ranged from 15 to 65 mg/100 mL, and antioxidant activity as measured by the ABTS assay ranged from 20 to 85% radical inhibition. Gaglio et al. (2019) added saffron (5 g/40 kg of yogurt) and found a mean FCRC of 18.5 mg of GAE/100 g and FRAP of 40.3 mmol of TE/100 g. Kwon et al. (2019) used a chia extract obtained with ethanol or water to prepare yogurts and observed dose-dependent total phenolic content and antioxidant activity as measured by the DPPH and ABTS assays when extracts were added at 0.05 to 0.10 g/100 g. These results emphasize the remarks made by Granato et al. (2018) that dairy foods should be the target of research regarding the

Figure 4. Effects of different concentrations (0 to 1 g/100 g) of lyophilized camu-camu seed extract (LCE) on (A) Folin–Ciocalteu reducing capacity, (B) 2,2-diphenyl-1-picrylhydrazyl (DPPH), and (C) ferric reducing antioxidant power (FRAP) of yogurts. GAE = gallic acid equivalent; AAE = ascorbic acid equivalent. Data are expressed as means followed by SD ($n = 3$). Different letters for the same method represent statistically significant differences between samples ($P < 0.05$).

addition of natural bioactive-rich extracts, aimed at decreasing the use of synthetic compounds.

The proximate composition results of the yogurt with 0.25 g of LCE/100 g and the negative control yogurt were, respectively, as follows: moisture, 76.30 ± 5.91 and 72.61 ± 0.04 g/100 g; total carbohydrates 16.79 ± 6.16 and 18.20 ± 0.10 g/100 g; proteins 4.03 ± 0.31 and 4.20 ± 0.10 g/100 g; total lipids 1.97 ± 0.10 and $2.05 \pm$

0.10 g/100 g; ashes 0.91 ± 0.01 and 1.03 ± 0.01 g/100 g; pH 4.02 ± 0.01 and 4.01 ± 0.01 ; total titratable acidity 0.99 ± 0.01 and 1.38 ± 0.01 g/100 g (as lactic acid); and mean caloric value 101 and 108 kcal/100 g. These values were in accordance with the proximate composition of yogurts with added extracts rich in bioactive compounds (Karaaslan et al., 2011; Karnopp et al., 2017; Pires et al., 2018). In a study relating the effects of added grape peel flour and grape juice on the proximate composition of an organic yogurt with added grape pomace, Karnopp et al. (2017) observed similar values for moisture (76.81 g/100 g), total carbohydrates (7.16 g/100 g), protein (3.39 g/100 g), total lipids (6.81 g/100 g), and ash (0.72 g/100 g).

Regarding instrumental color attributes, the yogurt with added LCE had a higher ($P < 0.001$) a^* color (2.21 ± 0.07) value than the LCE-free yogurt (-1.26 ± 0.02). Conversely, the LCE-free yogurt had higher ($P < 0.001$) lightness (88.89 ± 0.15) value than the yogurt with added LCE (72.91 ± 0.48). We observed no significant difference ($P = 0.053$) for the b^* coordinate between the LCE-free yogurt (13.11 ± 0.06) and the yogurt with added LCE (13.47 ± 0.22). The yogurt with added LCE had $28.4 \pm 1.2\%$ syneresis, a consistency of $1,451 \pm 219$ g/s, and a firmness of 59.33 ± 8.50 g.

Sensory analysis of the yogurt formulation containing 0.25 g of LCE/100 g was conducted with 75 untrained consumers (22 men and 53 women; 18 to 60 years of age) who consumed yogurt or fermented milk at least twice a week. The mean scores of liking for taste (7.6 ± 1.1), odor (7.5 ± 1.4), consistency (7.1 ± 1.5), color (7.7 ± 1.4), and overall acceptance (7.6 ± 0.1) were between “moderately liked” and “liked very much.” The global acceptance index of the yogurt formulation containing 0.25 g of LCE/100 g was 84.3%, indicating a high sensory acceptance of the yogurt. Comparing the sensory data between men and women we found no statistically significant difference in the degree of liking of taste (men vs. women: 7.73 ± 0.93 vs. 7.53 ± 1.22 ; $P = 0.495$), consistency (6.68 ± 1.72 vs. 7.28 ± 1.31 ; $P = 0.104$), odor (7.54 ± 1.37 vs. 7.41 ± 1.46 ; $P = 0.721$), color (7.45 ± 1.33 vs. 7.79 ± 1.47 ; $P = 0.356$), overall acceptance (7.50 ± 0.96 vs. 7.62 ± 0.92 ; $P = 0.607$), and acceptance index (83.3 vs. 84.4%; $P = 0.906$). These results showed consensus in the scores attributed to the sensorial parameters evaluated. Of the 75 assessors, 73 considered yogurt to be a healthy food, and 74 considered the consumption of yogurts with added natural antioxidants to be healthy. In addition, 56 assessors indicated that they would pay from R\$2.00 to R\$3.00 for yogurts (100 g) that were sources of natural antioxidants compared with a conventional antioxidant-free yogurt (R\$1.00).

CONCLUSIONS

Camu-camu seed extract is a rich source of phenolic compounds, especially vescalagin, castalagin, gallic acid, and procyanidin A2. The bioactive compounds in the extract promoted high chemical antioxidant activity as measured by 5 different assays; showed antiproliferative and cytotoxic effects in cancerous cell lines (HepG2 and Caco-2); and decreased ROS generation in a normal human cell line (IMR90). When added to yogurts, different concentrations of camu-camu seed extract increased the yogurts' antioxidant capacity. The camu-camu yogurt containing 0.25 g of LCE/100 g was sensorially accepted, indicating that camu-camu seed extract may be a sustainable ingredient for addition to potentially functional yogurts. Future studies should assess the shelf life and the effects of simulated digestion on the bioactive compounds of yogurt with added camu-camu seed extract.

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