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Evaluation of phenolic-linked bioactives of camu-camu (*Myrciaria dubia Mc. Vaugh*) for antihyperglycemia, antihypertension, antimicrobial properties and cellular rejuvenation

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ABSTRACT

Camu-camu (*Myrciaria dubia* (Kunth) Mc. Vaugh (Myrtaceae)) has shown potential for food and human health applications due to its rich bioactive functional properties linked to high antioxidant activity. The main objective of this study was to identify antioxidant-linked bioactive phenolic profiles in spray-dried and freeze dried camu-camu pulp and to evaluate potential functionality associated with anti-hyperglycemia and anti-hypertension properties using in vitro enzyme assay models. Further, antimicrobial properties and planarian-based cellular protection and regeneration were also investigated. Phenolic compounds, such as ellagitannins, ellagic acid, quercetin glycosides, syringic acid and myricetin were detected in camu-camu by LC–TOF-MS. Freeze dried camu-camu powder showed superior phenolic-linked antimicrobial properties and higher hyperglycemia relevant enzyme inhibitory activity compared to the spray dried samples. Besides that, freeze-dried powders and spray-dried (6% gum arabic at 120 °C) powders were more effective against prokaryotic *Staphylococcus aureus* and showed higher inhibition than ampicillin. Cellular regeneration of eukaryotic planarian model was stimulated with camu-camu powders when compared to control and showed potential of camu camu for redox-linked cellular protection and rejuvenation. Overall camu-camu rich in phenolic bioactive profiles showed superior antidiabetic and antimicrobial properties and has potential as part of dietary strategies in the management of early stages of type 2 diabetes and associated complications.

1. Introduction

Phenolic compounds are secondary metabolites, widely found in fruits, vegetables and grains. These phenolic secondary metabolites have shown significant human health relevant bioactive functionalities (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Some of these phenolic compounds also have antioxidant and anti-inflammatory properties and show potential to counter oxidative stress-induced chronic diseases when consumed as part of the diet. Camu-camu (*Myrciaria dubia* (Kunth) Mc. Vaugh (Myrtaceae)) from the Amazon region has high bioactive functionalities, high vitamin C, and rich phenolic profiles, such as flavonoids and ellagitannins (Fig. 1). The phenolic compounds found in camu-camu are quercetin, cyanidin-3-glucoside, ellagic acid and ellagitannins (Azevedo, Fujita, Oliveira, Genovese, & Correia, 2014; Chirinos, Galarza, Betalleluz-Pallardel, Pedreschi, & Campos, 2010; Fracassetti et al., 2013; Genovese, Pinto, Gonçalves, & Lajolo, 2008; Gonçalves, Lajolo, & Genovese, 2010; Rufino et al., 2010). This fruit has also shown high antioxidant activity and can be utilized against pathogenic bacteria responsible for human infectious diseases (Fujita, Borges, Correia, Franco, & Genovese, 2013; Gonçalves et al., 2010; Inoue, Komoda, Uchida, & Node, 2008). With such a high phenolic bioactive profile and antioxidative potential, camu camu can be incorporated with other functional ingredients and foods for diet based management of oxidative stress linked non-communicable chronic diseases (NCDs), such as type 2 diabetes.

Prevalence of NCDs, including type 2 diabetes and associated cardiovascular diseases are imposing major health care challenges in different countries and across different communities. Diabetes mellitus, especially type 2 diabetes has become an epidemic disease in different parts of the world, causing huge social and economic impacts with rising health care costs. By the year 2030, the population with diabetes cases are estimated to reach over 500 million (Wild, Roglic, Green, Sicree, & King, 2004), the 7th leading cause of death (WHO, 2015). Type 2 diabetes epidemic is a result of genetic and non-genetic factors and can be linked to unhealthy sedentary lifestyles and excess nutrition from diets comprised of refined carbohydrates and fat that cause

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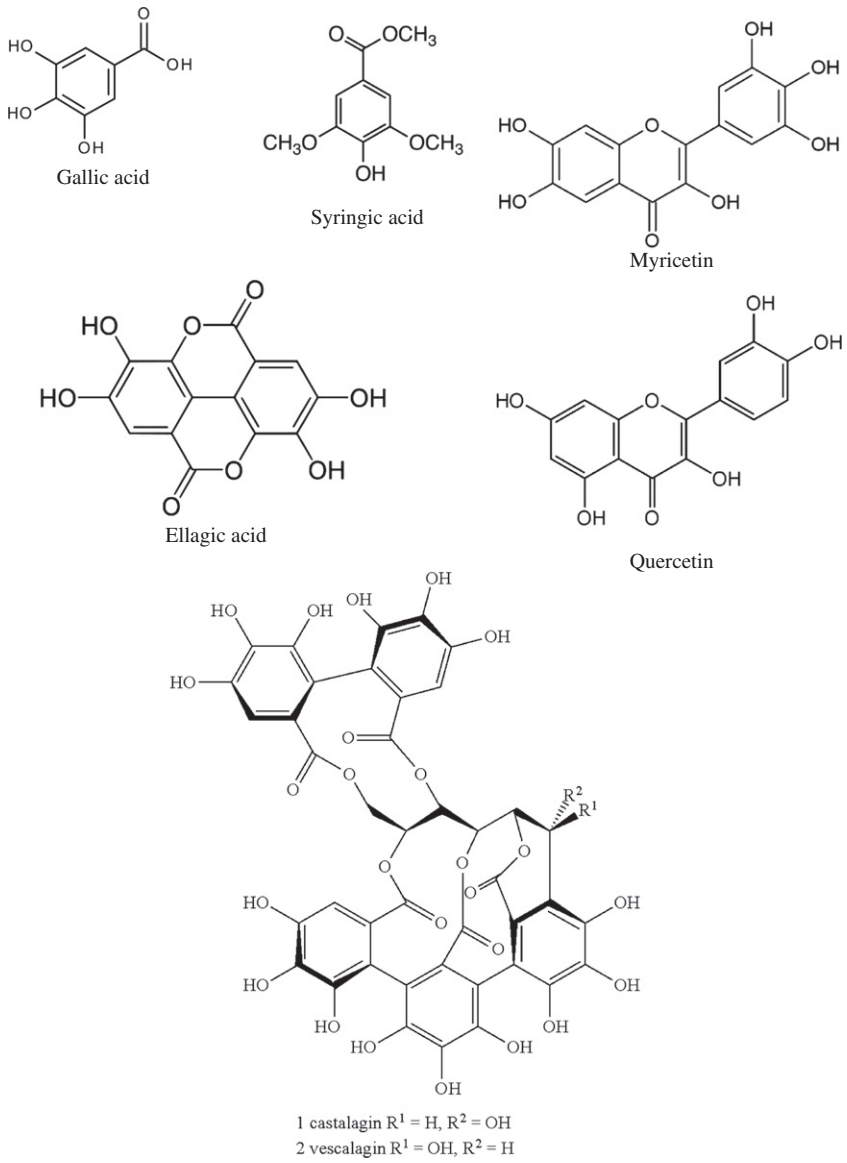


Fig. 1. Structures of gallic, syringic and ellagic acids myricetin, quercetin and ellagitannins (castalagin and vescalagin).

metabolic breakdown through chronic oxygen malfunction and concurrently induce metabolic syndrome (Oviedo & Beane, 2009). To prevent such health effects it is important to counter with bioactives from plant-based foods with high antioxidant potential that offer cost effective and safe dietary strategies to manage microvascular and macrovascular complications in humans (eukaryote). Further, they have potential to act as antimicrobials to protect against harmful bacteria (prokaryote) under diabetic and immunity breakdown.

Dietary phenolics with high antioxidant activity have diverse health beneficial properties including antihyperglycemic, antihypertension and antimicrobial. Dietary phenolics from different plant based sources influence glucose metabolism in several ways, such as inhibition of carbohydrate digestion and glucose absorption in the intestine, stimulation of insulin secretion from the pancreatic β-cells, modulation of glucose release from the liver, activation of insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulation of hepatic glucose output (Hanhineva et al., 2010; Sarkar & Shetty, 2014). Many in vitro studies have been reported indicating that phenolics including flavonoids (anthocyanins, catechins, flavanones, flavonols, flavones and isoflavones), phenolic acids and tannins (proanthocyanidins and ellagitannins) from plant-based foods inhibit starch digesting α-amylase and α-glucosidase enzymes and have potential for the management and prevention of early stages type 2 diabetes-linked hyperglycemia and associated cardiovascular complications (Hanhineva et al., 2010).

Based on the above rationale, the primary aim of this study was to evaluate the effect of spray-drying camu-camu pulp on phenolic bioactive profiles identified by LC–TOF-MS, comparing to freeze-drying, and to study phenolic-linked bioactives functionalities using in vitro assay models for antihyperglycemic and antihypertensive properties. Such rapid in vitro assay models are health-focused metabolically driven screening of different plant based foods and provide sound biochemical evidence for further validation through clinical and epidemiological studies (Ankolekar et al., 2011; Kwon, Vattem, & Shetty, 2006; Sarkar, Ankolekar, Pinto, & Shetty, 2015). These in vitro assay models targeted key enzymes (α-amylase, α-glucosidase and angiotensin-I-converting enzyme or ACE) of glucose metabolism and hypertension and are good metabolic indicators to determine the potential role of food bioactives for dietary management of early stage type 2 diabetes-linked hyperglycemia and associated hypertension. The second objective of this study was to investigate camu camu pulp for antimicrobial activities and potential utilization in cellular protection and rejuvenation by

determining regeneration of planaria (*Dugesia* spp.) as a living in vivo model. These health-linked functional analyses would help to advance the potential of camu-camu as a functional food ingredient to incorporate in diet based preventative strategies to improve health associated with breakdown towards chronic diseases and infections.

2. Materials and methods

2.1. Materials

Two different commercial pulps of camu-camu (*M. dubia* Mc. Vaugh): one was purchased from Cupuama do Amazonas Com. Ind. Exp. Ltda. (Manaus, AM, Brazil; latitude 3°8′S and longitude 60°0′W) and another from a commercial plantation located in Registro, SP, Brazil (latitude 24°29′15″S and longitude 47°50′37″W). Both materials were kept frozen (−40 °C for one month) and defrosted prior to spray-drying.

2.2. Spray-drying

Spray-drying was performed in a pilot scale spray-dryer (Labmaq, SD 5.0, Brazil). The pulp was fed by a peristaltic pump at a fixed rate of 44 mL/min and was spray-dried at different inlet air temperatures (120, 150 and 180 °C) and with different carrier agent concentrations (6, 12 and 18%). Two carrier agents were employed: maltodextrin MOR-REX® 1910 (9 ≤ DE ≤ 12) (Corn Products, Brazil) for Amazonian pulp and gum arabic (Nexira Brazil Com. Ltd., Brazil) for Sao Paulo pulp.

2.3. Freeze-drying

Two kilograms of frozen pulp were lyophilized in a Pironi 501 freeze-drier (Thermo Electron Corporation, New York, USA) at −80 °C and 100 mTorr for 120 h.

2.4. LC–TOF-MS analysis to determine phenolic profiles

For extraction, 1 g of each powder (freeze-dried and spray-dried) was homogenized with 70% (v/v) aqueous methanol using a vortex (Vortex Genie2, G-560, Scientific Industries, Bohemia, NY, USA) and then sonicated for 10 min (Sonicator QSonica 5510R-MTH, Ultrasonic Cleaner, Newtown, CT, USA). The extracts were filtered using a 25 mm syringe filter (0.45 μm Nylon Membrane). The extracts were characterized with Waters (Milford, MA, USA) Alliance 2695 LC system equipped with a 2695 separation module unit and a 2998 PDA detector using a 100 × 2.0 mm, 2.5 μm Phenomenex Synergi Hydro-RP 100A column with 3 × 4.0 mm Phenomenex Security Guard column (Torrance, CA USA). The mobile phase consisted of solvents (A) 0.1% aqueous formic acid solution and (B) Methyl cyanide (MeCN). Gradient conditions were performed as follow: from 0% to 6% B in 5 min, from 6% to 22% B until 30 min, from 22% B to 30% B until 40 min and from 30% to 40% B to 50 min, followed by a final increase to 95% in 2 min. The flow rate and the injection volume were 0.2 mL/min and 10 μL, respectively.

High-resolution mass spectrometry was performed using an LCT premier XE TOF mass spectrometer (Waters, Milford, MA) equipped with an ESI interface and controlled by MassLynx V4.1 software. Mass spectra were acquired in both positive and negative modes over range *m/z* 100–1000. The capillary voltages were set at 3000 V (positive mode) and 2700 V (negative mode), respectively, and the cone voltage was 30 V. Nitrogen gas was used for both the nebulizer and in desolvation. The desolvation and cone gas flow rates were 600 and 20 L/h, respectively. The desolvation temperature was 400 °C, and the source temperature was 120 °C.

2.5. Extract preparation

For antimicrobial and enzyme inhibitory assays, one gram of powdered sample was added to 100 mL of distilled water and mixed using a homogenizer Tissue (Tearor MD 985370-395, Biospec Products, Mexico), at a moderate speed for 5 min, while cooled in ice. The extracts were then centrifuged at 8500 g for 30 min and stored at 10 °C until analysis. All extractions were done in duplicate, and the subsequent assays were run in triplicate.

2.5.1. Antimicrobial assay and determination of minimum inhibitory concentration (MIC)

The extracts were tested for activity against: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 8739, *Enterobacter aerogenes* ATCC 13048, *Listeria monocytogenes* ATCC 7644, *Salmonella typhimurium* ATCC 14028 and *Salmonella enteritidis* ATCC 13076, according to CLSI (CLSI, 2009).

The cultures were grown in Tryptone Soya Agar (Oxoid, Basingstoke, UK) for 18–24 h at 37 °C, and the colonies were suspended in sterile saline solution (0.85%) to reach a turbidity corresponding to 0.5 of the McFarland scale (10^8 UFC mL^{-1}). The suspensions (0.1 mL) were applied to the surface of Muller–Hinton agar plates (Oxoid, Basingstoke, UK) and wells of 13 mm in diameter were perforated using the proper perforator. The wells were filled with 100 μL of extracts, and the plates were incubated at 37 °C for 24 h, when the diameters of the inhibition zones were measured using a caliper ruler. Erythromycin (15 μg) and Vancomycin (5 μg) (Oxoid, Basingstoke, UK) were used as a positive controls. Results were evaluated according to the following scale: <16 mm, inactive; 17–19 mm, partially active; 20–25 mm, active; >25 mm, and very active.

For determination of the MIC, the microdillution method was used (CLSI, 2010). Except for the first row, the wells of sterile 96-well microplates were filled with 50 μL of Muller–Hinton broth (Oxoid, Basingstoke, UK). The wells of the first row of were filled with 100 μL of extracts, homogenized and then 50 μL were serially transferred to the subsequent wells. Finally, 50 μL of the microorganism solution (10^8 UFC mL^{-1}) were added. The microplates were incubated at 37 °C for 18 h and the MIC corresponding to the lowest concentration that inhibited the visible growth of the microorganism after 18 h. Ampicillin (0.015 to 256 μg/mL^{-1}, Oxoid, Basingstoke, UK) was used as a positive control. The tests were carried out in duplicate.

2.5.2. α-Amylase inhibition

The α-amylase inhibitory activity was determined by an assay modified from the *Worthington Enzyme Manual* (Worthington & Ed, 1993a). A total of 500 μL of each extract and 500 μL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α-amylase solution (0.5 mg/mL) were incubated at 25 °C for 10 min. After pre incubation, 500 μL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube at timed intervals. The reaction was stopped with 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were then incubated in boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after the addition of 15 mL of distilled water, and absorbance was measured at 540 nm using a spectrophotometer (Genesys UV–visible, Milton Roy, Inc.). The readings were compared with the controls, containing buffer solution instead of sample extract. The results were calculated as percentage of α-amylase inhibition and calculated according to Eq. (1), then, expressed as mg of sample per milliliters of reaction time to inhibit 50% of enzyme (IC 50).

$$\%\text{inhibition} = \left[\left(A540^{\text{control}} - A540^{\text{extract}}\right)/A540^{\text{control}}\right] \times 100. \quad (1)$$

2.5.3. α-Glucosidase inhibition

The assay was performed according to the Worthington and Ed (1993b), with some modifications (McCue & Shetty, 2005). Alpha-glucosidase (1 unit/mL) was assayed by using 50 μL of aqueous camu-camu extracts and 100 μL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution and was incubated in 96-well plates at 25 °C for 10 min. After pre-incubation, 50 μL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 10 min. Before and after incubation, absorbance readings were recorded at 405 nm by microplate reader (Spectra Max 190, Molecular Device Co.CA) and compared to a control that contained 50 μL of buffer solution in place of the extract. The results were calculated as percent of α-glucosidase inhibition according to Eq. (2), then, expressed as mg of sample per milliliters of reaction time to IC50.

$$\%\text{inhibition} = \left[\left(\text{Abs}^{\text{control}}5\text{ min} - \text{Abs}^{\text{control}}0\text{ min}\right) - \left(\text{Abs}^{\text{extract}}5\text{ min} - \text{Abs}^{\text{extract}}0\text{ min}\right) / \left(\text{Abs}^{\text{control}}5\text{ min} - \text{Abs}^{\text{control}}0\text{ min}\right)\right] \times 100. \quad (2)$$

2.5.4. Angiotensin converting enzyme (ACE) inhibition

ACE inhibition was assayed according to a method modified by Kwon et al. (2006). The substrate hippuryl-histidylleucine (HHL) and ACE-I from rabbit lung (1 unit produces 1.0 μmol of hippuric acid from HHL per minute in 50 mM HEPES and 300 mM NaCl at pH 8.3 at 37 °C) were provided from Sigma-Aldrich (St. Louis, MO, USA) and used. Fifty microliters of water soluble supernatant was incubated with 100 μL of 1 M NaCl-borate buffer (pH 8.3) containing 2 milliunits of ACE-I solution at 37 °C for 10 min. After pre-incubation, 100 μL of a 5 milliunit substrate (HHL) solution was added to the reaction mixture. Test solutions were incubated at 37 °C for 1 h. The reaction was stopped with 150 μL of 0.5 N HCl. The hippuric acid formed was detected; the spectra were confirmed and quantified by high-performance liquid chromatography (HPLC). Five microliters of the sample was injected using an Agilent ALS 1100 autosampler into an Agilent 1260 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD1100 diode array detector. The solvents used for the gradient were (1) 10 mM phosphoric acid (pH 2.5) and (2) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for 5 min and then was decreased to 0% for the next 5 min (18 min total run time). The analytical column used was a Nucleosil 100-5 C18, 250 × 4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 mL/min at ambient temperature. During each run, the absorbance was recorded at 228 nm and the related chromatogram was integrated using Agilent ChemStation (Agilent Technologies) enhanced integrator for detection of liberated hippuric acid (A). Pure hippuric acid (purchased from Sigma Chemical Co.) was used to calibrate the standard curve and retention time. The percent inhibition was calculated according to Eq. (3):

$$\%\text{inhibition} = \left[\underline{\left(\text{A}^{\text{control}} - \text{A}^{\text{blank}}\right) - \text{A}^{\text{sample}}} / \left(\text{A}^{\text{control}} - \text{A}^{\text{blank}}\right)\right] \times 100. \quad (3)$$

2.5.5. Regeneration of black planaria (Dugesia tigrina)

The black planaria were kept and acclimatized for 30 days at room temperature in an aquarium with artificial air pump, fed once a week with fresh liver meat. For the regeneration studies, the methodology of Zhang, Tallarida, Raffa, and Rawls (2013) was applied, with the

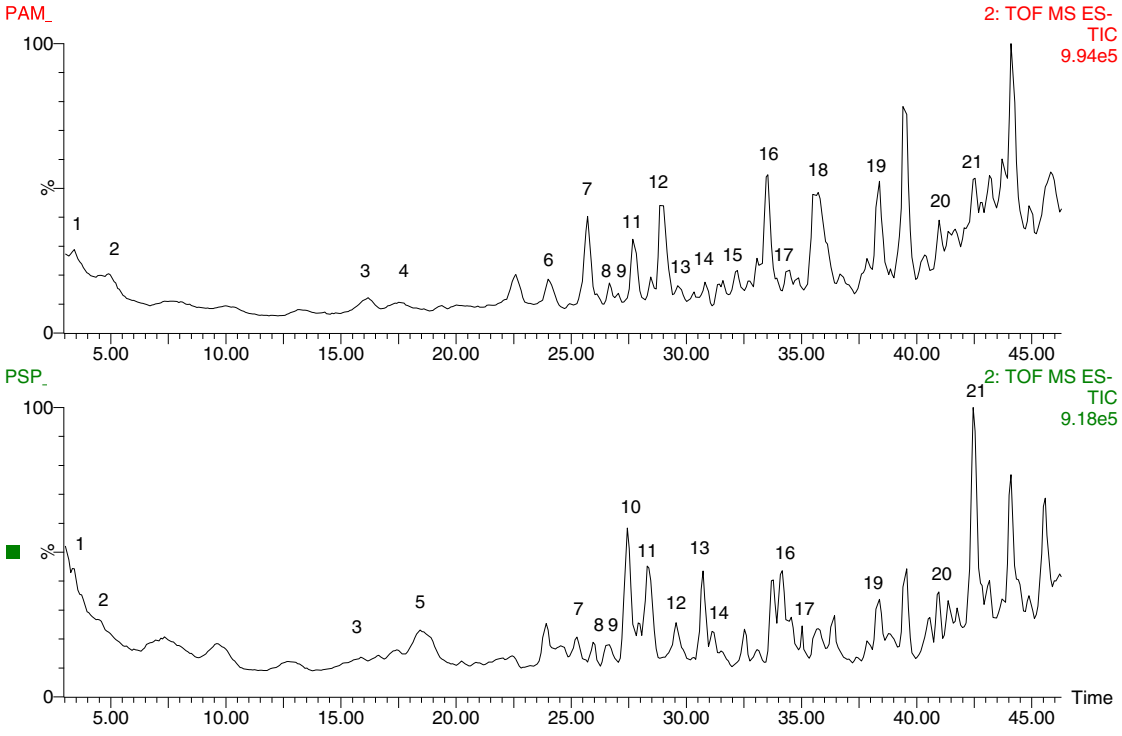


Fig. 2. Chromatogram profile of two freeze-dried powders extracted by 70% (v/v) aqueous methanol: Amazonian pulp (PAM) and Sao Paulo pulp (PSP). 1, vescalagin; 2, castalagin; 3 and 4, Di-HHDP-galloyl-glucose (casuarictin/potentillin); 5 and 10, cyanidin 3-O-glucoside; 6, 15 and 18, gallic acid derivative; 7, myricetin 3-O-hexoside; 8, myricetin 3-O-pentoside; 9, ellagic acid hexoside; 11, myricetin 3-O-pentoside; 12, ellagic acid; 13, quercetin 3-O-hexoside; 14, quercetin 3-O-pentoside; 16, syringic acid; 17, myricetin; 19, 20 and 21, ellagic acid derivatives.

Table 1
Phenolic compounds identified by HPLC–TOF-MS analysis of camu-camu powders.

Number	Compounds	RT (min)	$[M-H]^-$	$[M-H]^+$	λ_{max} (nm)	Formula	Samples detected	Note
1	Vescalagin	3.4	933.0527	935.0923	246	$C_{41}H_{26}O_{26}$	PAM, PSP	Reported by Fracassetti et al. (2013)
2	Castalagin	5.0	933.0656	935.0919	246	$C_{41}H_{26}O_{26}$	PAM, PSP	Reported by Fracassetti et al. (2013)
3	Di-HHDP-galloyl-glucose (casuarictin/potentillin)	16.4	935.0833	937.1047	240, 270	$C_{41}H_{28}O_{26}$	PAM, PSP	Reported by Fracassetti et al. (2013), Wu et al. (2012)
4	Di-HHDP-galloyl-glucose (casuarictin/potentillin)	17.6	935.0717	937.1036	240, 270	$C_{41}H_{28}O_{26}$	PAM	Reported by Fracassetti et al. (2013)
5	Cyanidin 3-O-glucoside	18.5	447.0938	449.1088	520	$C_{21}H_{21}O_{11}$	PSP	Reported by Fracassetti et al. (2013)
6	Gallic acid derivative	23.9	569.0947	571.1135	216, 271	$C_{27}H_{38}O_{13}$	PAM	Reported by Fracassetti et al. (2013)
7	Myricetin 3-O-hexoside	25.7	479.0838	481.0961	264, 358	$C_{21}H_{20}O_{13}$	PAM, PSP	Reported by Fracassetti et al. (2013)
8	Myricetin 3-O-pentoside	27.1	449.0743	451.0898	258, 356	$C_{20}H_{18}O_{12}$	PAM, PSP	Reported by Fracassetti et al. (2013)
9	Ellagic acid hexoside	27.7	463.2127	465.1539	255, 362	$C_{20}H_{16}O_{13}$	PAM, PSP	Reported by Fracassetti et al. (2013)
10	Cyanidin 3-O-glucoside	28.1	447.0567	449.1778	520	$C_{21}H_{21}O_{11}$	PSP	Reported by Fracassetti et al. (2013)
11	Myricetin 3-O-pentoside	28.8	449.0701	451.0866	258, 356	$C_{20}H_{18}O_{12}$	PAM, PSP	Reported by Fracassetti et al. (2013)
12	Ellagic acid	29.2	300.9941	303.0154	254, 364	$C_{14}H_6O_8$	PAM, PSP	Reported by Fracassetti et al. (2013), Wu et al. (2012)
13	Quercetin 3-O-hexoside	30.4	463.0913	465.1049	255, 362	$C_{21}H_{20}O_{12}$	PAM, PSP	Reported by Fracassetti et al. (2013)
14	Quercetin 3-O-pentoside	31.6	433.0791	435.1058	254,360	$C_{20}H_{18}O_{11}$	PAM, PSP	Reported by Fracassetti et al. (2013)
15	Gallic acid derivative	33.5	569.0941	571.1154	216, 271	$C_{27}H_{38}O_{13}$	PAM	Reported by Fracassetti et al. (2013)
16	Syringic	34.5	371.1298	373.1517	249	$C_{17}H_{24}O_9$	PAM, PSP	Reported by Wu et al. (2012)
17	Myricetin	35.7	317.0293	319.0477	252, 372	$C_{15}H_{10}O_8$	PAM, PSP	Reported by Fracassetti et al. (2013)
18	Gallic acid derivative	35.9	569.2196	571.2356	216, 271	$C_{27}H_{38}O_{13}$	PAM	Reported by Fracassetti et al. (2013)
19	Ellagic acid glycoside	39.4	719.2197	721.2391	254, 360	$C_{34}H_{40}O_{17}$	PAM, PSP	Reported by Fracassetti et al. (2013)
20	Ellagic acid glycoside	41.3	719.2149	721.2100	254,360	$C_{34}H_{40}O_{17}$	PAM, PSP	Reported by Fracassetti et al. (2013)
21	Ellagic acid glycoside	42.8	719.2187	721.2377	254,360	$C_{34}H_{40}O_{17}$	PAM, PSP	Reported by Fracassetti et al. (2013)

modifications as follows. Worms (n = 15) were fed with liver meat with aqueous extracts of different camu-camu powders (10 mg meat dipped into different concentrations of camu camu extracts, 10, 20 and 30 μL of 10 mg/mL for 5 min). After one day, worms were cut into cephalic and caudal sections and were collected in two separate fresh water Petri plates at room temperature. Samples were measured every other day for 3 weeks using compound microscope and plotting done in graphical scale. The results were calculated and reported as percentage of regeneration.

2.6. Statistical analysis

All analyses were run in triplicate and results were expressed as mean ± standard deviation (SD). For statistical analysis, the Statistic software package version 11.0 (StatSoft, Inc., Tulsa, OK) was employed. Differences between means were first analyzed by ANOVA test and then Tukey test ($p < 0.05$) (Fujita et al., 2013). Chemometric data analysis was performed according to Wu, Dastmalchi, Long, and Kennelly (2012), principal component analysis used Markerlynx v4.1 software.

3. Results and discussion

3.1. LC–TOF-MS phenolics profile analysis of camu-camu powders

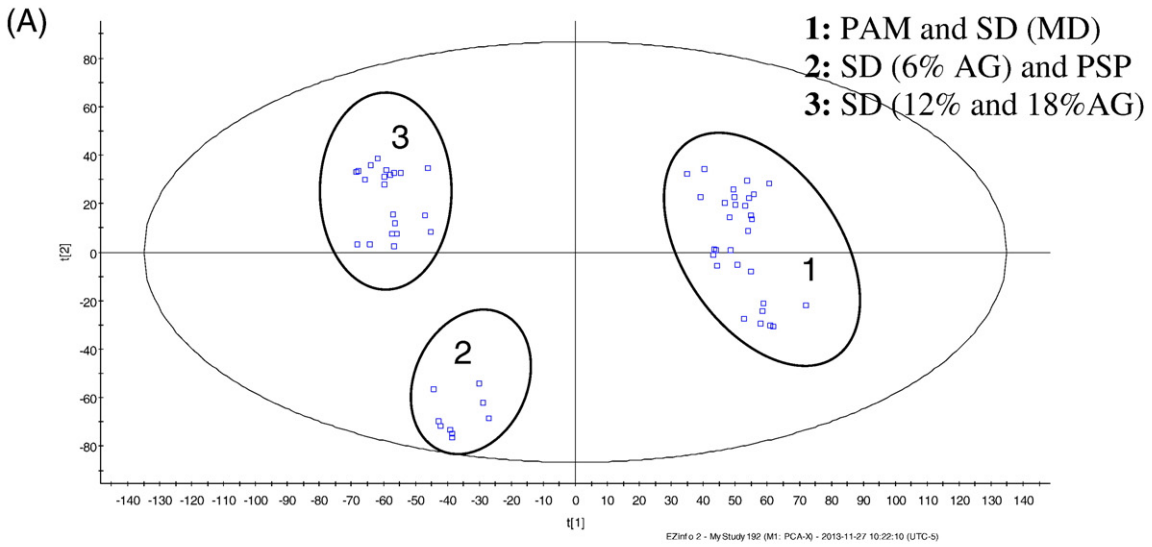
The phenolic composition of freeze-dried and spray-dried camu-camu powders were tentatively identified (Fig. 2) by their retention times, UV spectra, and exact mass spectra using fragmental ions in both positive and negative modes, and also by comparison with data found in the literature (Fracassetti, Costa, Moulay, & Tomas-Barberan, 2013; Wu et al., 2012) which were shown in Table 1. According to Table 1 and Fig. 2, it was observed that Amazonian and Sao Paulo pulps have slightly different phenolic compositions. Overall, ellagitannins, such as vescalagin, castalagin, casuarictin, potentillin, and also, ellagic acid, quercetin glycosides, myricetin, myricitin glycosides and syringic acid were detected in both of those fruit extracts. However, cyanidin-3-O-glucoside was detected only in pulp from Sao Paulo, and gallic acid derivatives were detected only in Amazonian pulp (Fig. 2). The compounds identified are similar to those reported by Fracassetti et al. (2013) and Wu et al. (2012) who analyzed camu-camu and jaboticaba (*Plinia cauliflora* (Mart.) Kausel, Myrtaceae) fruit, respectively. The reason for not finding anthocyanins in Amazonian pulp could be due to higher proportion of unripe to ripe content in the purchased sample, and/or degradation during processing, transport and storage (Pourcel, Routaboul, Cheynier, Lepiniec, & Debeaujon, 2007).

Principal component analysis (PCA) was used to determine similarities among powders in groups with same characteristics, providing "visual description" of clustering and differences or outliers groups. PCA confirmed that Sao Paulo pulp, freeze-dried and 6% of gum arabic spray-dried powders have high concentration of anthocyanins and Amazonian pulp has high ellagic acid contents (Fig. 3B).

In order to verify the influence of drying process, major phenolic compounds (myricetin, syringic, ellagic acid, casuarictin/potentilin and vescalagin/castalagin) were selected and the relative concentration of those phenolic compounds considering the values in the respective freeze-dried pulps as 100% were determined (Fig. 4A and B). The results show that Sao Paulo pulp (average of losses were 55% to 87%) was more susceptible to losses and degradation of phenolic compounds than Amazonian pulp (average of losses between 33% and 72%) during spray-drying process. However, such differences could be due to the added adjuvants or microencapsulation of the compounds. Overall, no significant differences were observed between different inlet air temperatures of drying ($p < 0.05$). However, addition of gum arabic showed statistically significant differences between 6% and other concentrations (12 and 18%, $p < 0.05$). Such differences could be confirmed in Fig. 3A, where we applied PCA to LC–TOF-MS data for all powders together and formed three respective groups: one was Amazonian freeze-dried and all their respective spray-dried Amazonian pulp with maltodextrin; another group contained Sao Paulo freeze-dried and 6% of gum arabic gum spray-dried powders and the third was spray-dried Sao Paulo pulp with 12 and 18% of gum arabic powders.

However, when orthogonal partial least squares discriminant analysis (OPLS-DA) was applied separately to each pulp, Amazonian

Fig. 3. Score (A) and loading plots (B) of freeze-dried and spray-dried powder extracts from Amazonian and Sao Paulo pulps; OPLS_DA plot (C) of freeze-dried and spray-dried powders extracts of camu-camu pulp from Sao Paulo.


(A)
1: PAM and SD (MD)
2: SD (6% AG) and PSP
3: SD (12% and 18%AG)
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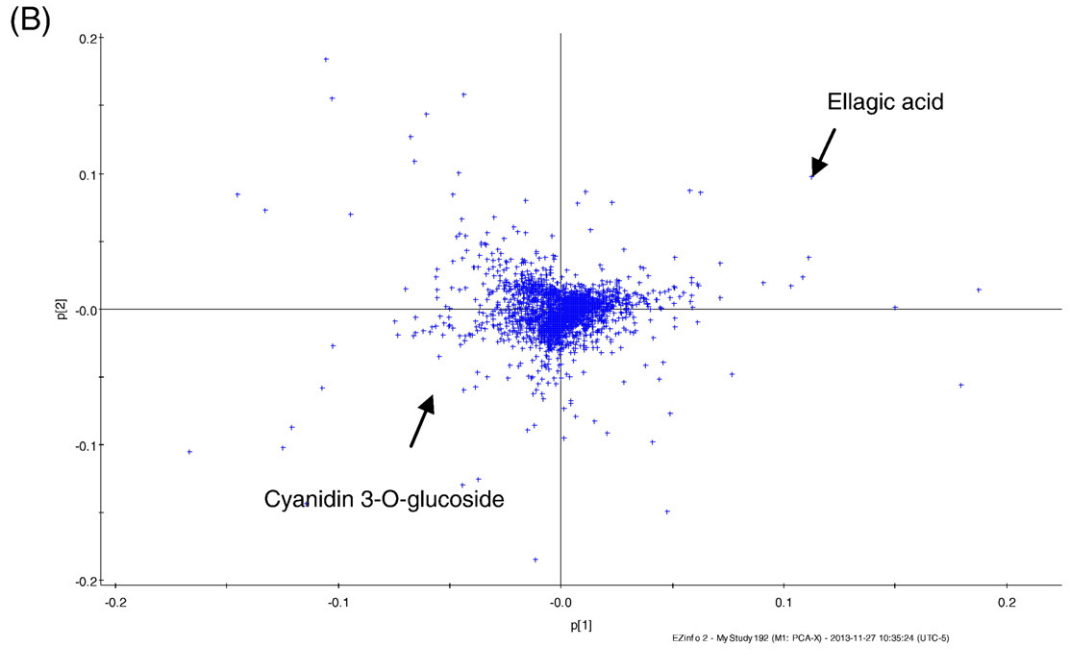
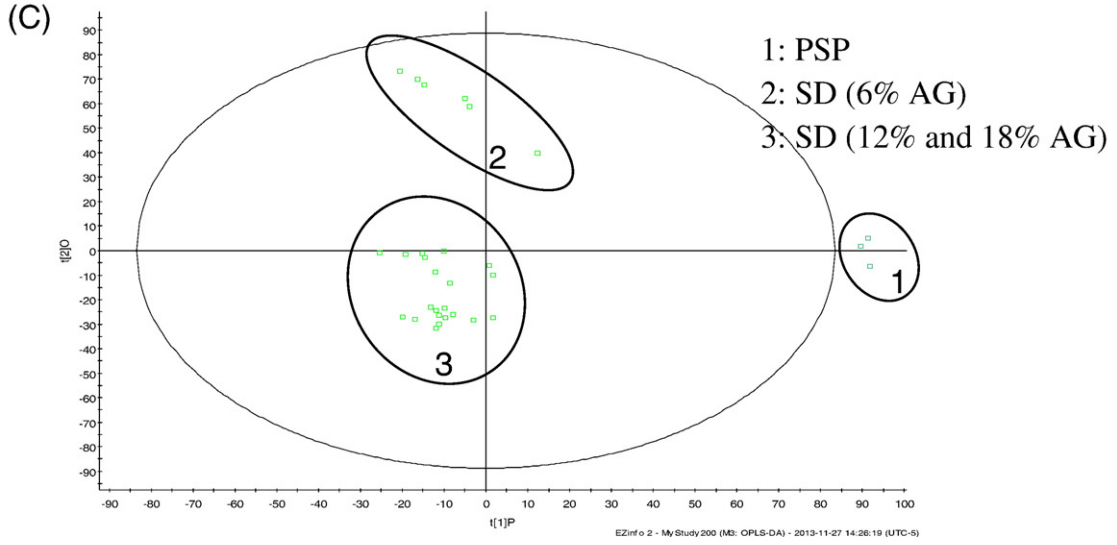

(B)
Ellagic acid
Cyanidin 3-O-glucoside
p[2]
p[1]
EZinfo 2 - My Study 192 (M1: PCA-X) - 2013-11-27 10:35:24 (UTC-5)


(C)
1: PSP
2: SD (6% AG)
3: SD (12% and 18% AG)
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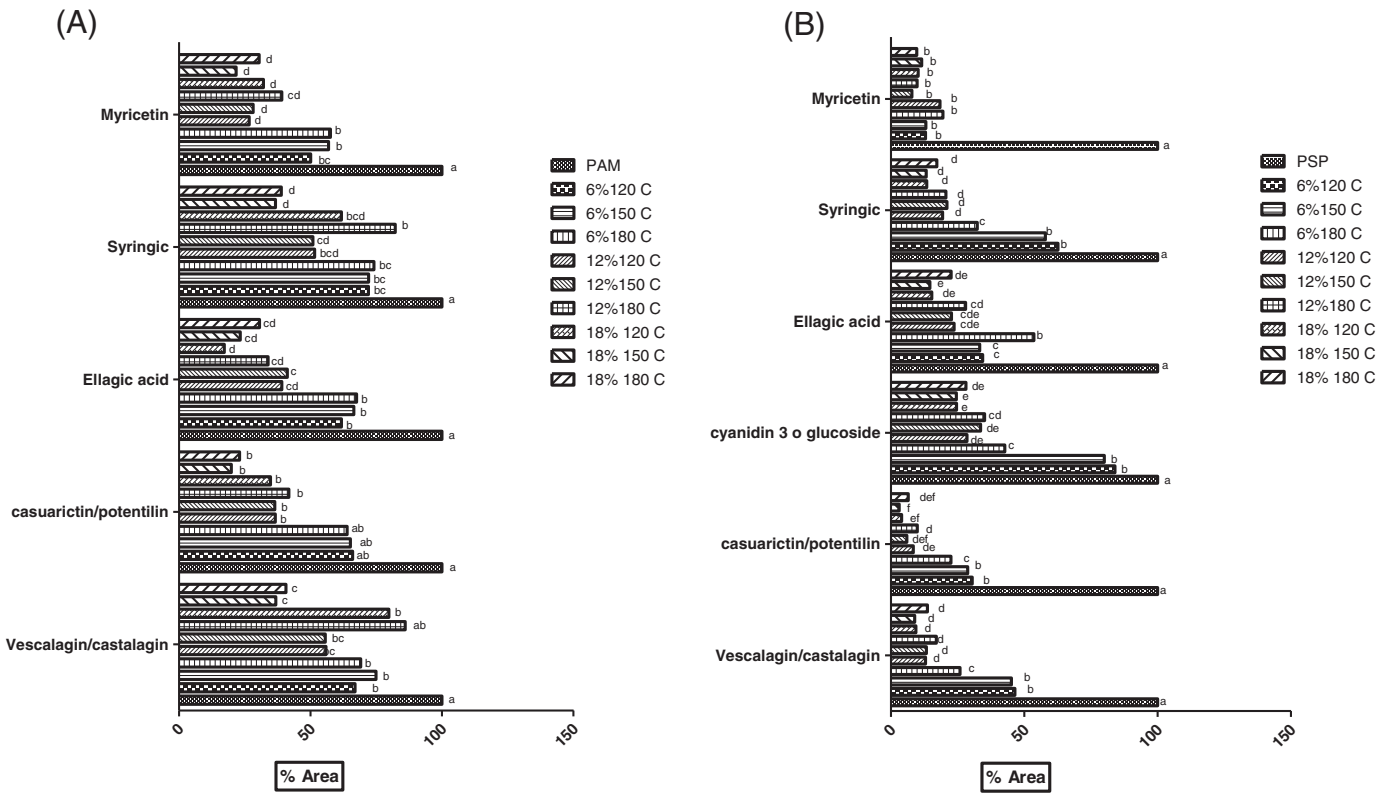

Fig. 4. Relative phenolic concentrations of camu-camu powders from Amazonian pulp with maltodextrin (A) and Sao Paulo pulp with gum arabic (B), using the respective freeze-dried powder as a control (100% peak area). Values are expressed as means ± SD (n = 3). Means denoted by different superscripts are significantly different ($p < 0.05$).

pulp showed two different groups: one was freeze-dried powder and other was all spray-dried powders (data not shown). In contrast, Sao Paulo pulp showed three groups: freeze-dried powder, 6% of gum arabic gum and finally, 12% and 18% of gum arabic together (Fig. 3C). Overall, this analysis showed Amazonian pulp did not have changes in phenolic composition under the evaluated range of inlet air temperature or concentration of maltodextrin. However, gum arabic concentrations seemed to influence the phenolic composition of camu-camu pulp from Sao Paulo.

3.2. α-Amylase and α-glucosidase inhibition

It is well-known that α-amylase and α-glucosidase are enzymes directly linked to soluble carbohydrate digestion and associated glucose metabolism, and inhibition of these enzymes helps to reduce postprandial blood glucose levels. Previous in vitro studies have shown the potential of phenolic-rich plant-based food extracts for higher inhibition of these enzymes (Anhê et al., 2013; Gonçalves et al., 2010; Hanhineva et al., 2010; Kwon et al., 2006). In this study, potential inhibitory activities of these two early stage digestive enzymes were investigated with aqueous extracts of spray-dried and freeze-dried camu-camu powders.

During carbohydrate digestion, pancreatic α-amylases hydrolyze α1-4 glucosidic linkages and also release oligosaccharides with α-1-6-oligomers (Hanhineva et al., 2010). All camu-camu powders analyzed were less efficient for α-amylase inhibition than acarbose (Table 2, IC50 = 3.05 μg/mL). Moreover, spray-dried powders showed less inhibition than freeze-dried powders, and the inhibitory activity decreased when the concentrations of carrier agents were increased. Such decrease in α-amylase inhibition might be due to losses during drying processing and/or the microencapsulation could have made phenolics more difficult to be released from the structure to act (Fujita et al., 2013).

Further, during the carbohydrate digestion, α-glucosidase hydrolyze terminal α1-4-linked glucose and release glucose in small intestine. Therefore, it is necessary to inhibit this enzyme to decrease absorption of glucose in the small intestine (Hanhineva et al., 2010). Compared to acarbose (IC50 = 152 μg/mL), all camu-camu powders are highly effective in inhibiting α-glucosidase (Table 2). No other fruits analyzed until now, such as strawberry (high inhibition at 25 mg/mL), tea (600 μg/mL), raspberry (200 μg/mL) and pear (500 mg/mL) had the same inhibitory potential as camu-camu (50 μg/mL) observed in this study (Barbosa et al., 2013; Cheplick, Kwon, Bhowmik, & Shetty, 2007; Pinto et al., 2008; Sarkar et al., 2015; Wang, Huang, Shao, Qian, & Xu, 2012).

For safe and effective management of postprandial hyperglycemia linked to type 2 diabetes, plant-based functional food should have moderate α-amylase and high α-glucosidase inhibitory activities (Pinto et al., 2008). The natural enzyme inhibitors from fruits and vegetables with the above mentioned combination of properties provide health benefits without any side effects such as flatulence, diarrhea and abdominal distention typically caused by drugs (Kotowaroo, Mahomoodally, Gurib-Fakim, & Subratty, 2006; Kwon et al., 2006; Matsui et al., 2001; McDougall et al., 2005). This result clearly showed that camu-camu has the potential to be incorporated in functional foods or in dietary strategies for safe and cost-effective management of early stage type 2 diabetes and associated complications.

Different phenolic compounds have shown inhibitory activity against these important enzymes linked to glucose metabolism. McDougall et al. (2005) concluded that soluble tannins are effective in α-amylase inhibition, while anthocyanins have α-glucosidase inhibitory activity. Moreover, You, Chen, Wang, Jiang, and Lin (2012) showed

that ellagic acid (IC50 = 2.18 μg/mL) and quercetin (IC50 = 15.2 μg/mL) exhibited α-glucosidase inhibition. In this study, we found different Pearson's correlation coefficients (Table 4, $p < 0.05$) for Amazon and Sao Paulo powders. For Amazon powders, the α-amylase inhibition had good correlation with all analyzed phenolic compounds. However, Sao Paulo powders did not have good correlation for myricetin (R = 0.52). Those variations may be due to different quantity of those compounds in both powders (Fig. 4) that could affect inhibition and synergism effects between compounds, differently.

Alpha-glucosidase inhibition had good correlation to ellagitannins and ellagic acid in both powders (Pearson's correlation coefficient around 0.7, Table 4). However, syringic acid presented a higher correlation for Sao Paulo than for Amazon powders (R = 0.83 and 0.57, respectively). Since ellagic acid was the major phenolic compound, found in camu-camu fruit (Fracassetti et al., 2013) it is potentially possible that loss of ellagic acid (Fig. 4) could be responsible for the variations in enzyme inhibition with different camu camu powders, although others possibilities pointed out above, were also considered.

3.3. Angiotensin converting enzyme-I (ACE) inhibition

ACE inhibition helps manage hypertension, which is an associated risk factor in type 2 diabetes (Pinto et al., 2008). Some studies have mentioned that rich-phenolic extracts may prevent risk of developing high blood pressure (Edwards et al., 2007; Pan, Lai, & Ho, 2010). Although no ACE enzyme inhibition was observed in the 10 mg/mL aqueous extracts of camu-camu powders further analysis with more concentrated extracts may be needed to prove the findings.

Previously, Kwon et al. (2006) found that pure compounds of quercetin, ellagic acid and chlorogenic acid did not show any ACE inhibitory activity. Camu-camu has high concentrations of ellagic acid as a major phenolic compound; this could be one reason for not having ACE inhibitory activity in investigated camu-camu extracts.

3.4. Antimicrobial activity and minimum inhibitory concentration (MIC)

Antimicrobial activity is a beneficial trait in plant extracts if they can inhibit bacterial pathogens without affecting beneficial probiotic bacteria. In chronic disease, immunity is always affected and increased susceptibility to bacterial infections and antimicrobial activity can be a common problem. Antimicrobial activity related to phenolic compounds, such as tannic acid, quercetin, catechin, ellagic acid, proanthocyanidins and gallic acid, have been reported earlier (Kil et al., 2009; Rauha et al., 2000; Saraiva et al., 2012). Among all studied pathogenic microorganisms, only growth of *S. aureus* ATCC 29213 was inhibited by the crude extracts of camu-camu powders (Table 3).

Table 2
IC50 of α-amylase and α-glucosidase inhibition of extracts of camu-camu powders produced by spray drying and freeze-drying.

Drying conditions		α-Amylase (μg/mL of reaction)		α-Glucosidase (μg/mL of reaction)	
		Amazonian	Sao Paulo	Amazonian	Sao Paulo
Acarbose		3.05 ± 0.25^{a}		152 ± 47^{g}	
Freeze-dried		359 ± 105^{b}	299 ± 152^{b}	5.57 ± 1.05^{b}	2.98 ± 1.12^{a}
6%	120 °C	1129 ± 337^{cd}	825 ± 222^{c}	5.13 ± 3.39^{b}	4.28 ± 1.2^{b}
	150 °C	1045 ± 313^{cd}	940 ± 189^{c}	7.03 ± 3.83^{c}	5.78 ± 0.77^{bc}
	180 °C	1098 ± 163^{cd}	937 ± 191^{c}	4.50 ± 3.34^{b}	4.05 ± 0.87^{b}
12%	120 °C	4361 ± 205^{e}	1365 ± 363^{d}	8.92 ± 3.75^{d}	20.74 ± 0.52^{e}
	150 °C	5113 ± 850^{e}	1668 ± 86^{d}	10.83 ± 4.18^{d}	20.18 ± 1.18^{e}
	180 °C	7782 ± 191^{e}	1654 ± 160^{d}	7.86 ± 1.07^{c}	19.06 ± 2.81^{e}
18%	120 °C	ni	$11{,}563 \pm 242^{f}$	9.54 ± 3.52^{d}	26.74 ± 4.54^{f}
	150 °C	ni	$11{,}254 \pm 174^{f}$	8.31 ± 4.15^{d}	22.98 ± 5.11^{ef}
	180 °C	ni	$11{,}519 \pm 241^{f}$	6.58 ± 1.78^{bc}	26.88 ± 1.34^{f}

Values are expressed as means ± SD (n = 3). a, b, c, and d followed by different superscripts in the same column indicate significant difference ($p < 0.05$). ni = no inhibition in the evaluated concentration.

Table 3
Antimicrobial activity and minimum inhibitory concentrations (MIC) of extracts of camu-camu powders produced by spray-drying and freeze-drying against *S. aureus* strains.

Drying conditions		Maltodextrin (Amazonian pulp)		Arabic gum (Sao Paulo Pulp)	
		Inhibition (mm)	MIC of extracts (mg/mL)	Inhibition (mm)	MIC of extracts (mg/mL)
Freeze-dried		25 ± 3^{b}	0.08^{a}	29 ± 0^{b}	0.08^{a}
6%	120 °C	17 ± 2^{cdef}	0.31^{c}	19 ± 1^{c}	0.16^{b}
	150 °C	19 ± 0^{c}	0.31^{c}	19 ± 1^{c}	0.16^{b}
	180 °C	15 ± 0^{f}	0.31^{c}	18 ± 1^{cd}	0.31^{d}
12%	120 °C	17 ± 1^{de}	0.63^{d}	18 ± 1^{cd}	0.31^{d}
	150 °C	16 ± 0^{e}	0.63^{d}	16 ± 1^{de}	0.31^{d}
	180 °C	16 ± 1^{def}	0.63^{d}	17 ± 2^{cde}	0.31^{d}
18%	120 °C	18 ± 2^{cde}	0.63^{d}	16 ± 0^{e}	0.63^{e}
	150 °C	17 ± 1^{de}	0.63^{d}	17 ± 1^{cde}	0.63^{e}
	180 °C	15 ± 0^{f}	0.63^{d}	16 ± 1^{de}	0.63^{e}
Erythromycin (15 μg)		37 ± 1^{a}	ne	37 ± 1^{a}	ne
Vancomycin (5 μg)		21 ± 1^{bc}	ne	21 ± 1^{c}	ne
Ampicillin		ne	0.26^{b}	ne	0.26^{c}

Values are expressed as means ± SD (n = 3). a, b, c, and d followed by different superscripts in the same column indicate significant difference ($p < 0.05$). ne = not evaluated.

Previous studies reported inhibition against *S. aureus* with extracts of camu-camu and *P. granatum* (Azevedo et al., 2014; Fujita et al., 2013; Machado et al., 2002; Myoda et al., 2010; Pradeep, Manojbabu, & Palaniswamy, 2008). According to Caillet, Côté, Sylvain, and Lacroix (2012), cranberry phenolic compounds had significant inhibitory effects on *S. aureus*, Gram-positive bacteria where destabilization of cytoplasmic membrane, permeabilization of plasma membrane, inhibition of extracellular microbial enzymes and metal binding capacity could be reasons for the inhibition. Additionally, Silva et al. (2014) and Souza et al. (2014) reported that Gram-negative bacteria have more complex cell membranes (peptidoglycan, periplasm, and lipopolysaccharide) that making penetration difficult for antimicrobial agents.

As expected, freeze-dried powders were more effective in inhibiting *S. aureus* than spray-dried powders: the higher the carrier agent concentration, the lower the inhibition was found in spouted-bed dried camu-camu pulp (Fujita et al., 2013). For example, the extract of freeze-dried powder was classified as very active in terms of antimicrobial activity, while spray-dried powders were categorized as partially active and inactive (gum arabic and maltodextrin, respectively, Table 3).

Besides that, spray-dried extracts showed minimum inhibitory concentration (MIC) values ranging from 0.16 to 0.63 mg/mL, specially, 6% gum arabic at 120 and 150 °C compared to freeze-dried extracts (MIC values = 0.08 mg/mL), which were more effective inhibitors than ampicillin (0.26 mg/mL). Therefore, these powders have potential as functional ingredients. This result showed higher inhibition than studies for Bordo grape residues (3.1 to 25 mg/mL, Souza et al., 2014).

Table 4
Pearson's correlation coefficient between bioactive compounds and enzymatic and antimicrobial inhibitions for camu-camu powders ($p < 0.05$).

Pearson's correlation coefficient	Amazonian pulp	Sao Paulo pulp
Casuarictin × α-amylase	0.969	0.711
Ellagic acid × α-amylase	0.965	0.728
Syringic × α-amylase	0.818	0.823
Myricetin × α-amylase	0.921	0.520
Casuarictin × α-glucosidase	0.675	0.708
Ellagic acid × α-glucosidase	0.697	0.715
Syringic × α-glucosidase	0.570	0.834
Myricetin × α-glucosidase	0.679	0.491
Casuarictin × *SA* inhibition	0.726	0.985
Ellagic acid × *SA* inhibition	0.658	0.935
Syringic × *SA* inhibition	0.626	0.906
Myricetin × *SA* inhibition	0.784	0.971

SA, *Staphylococcus aureus* ATCC 29213.

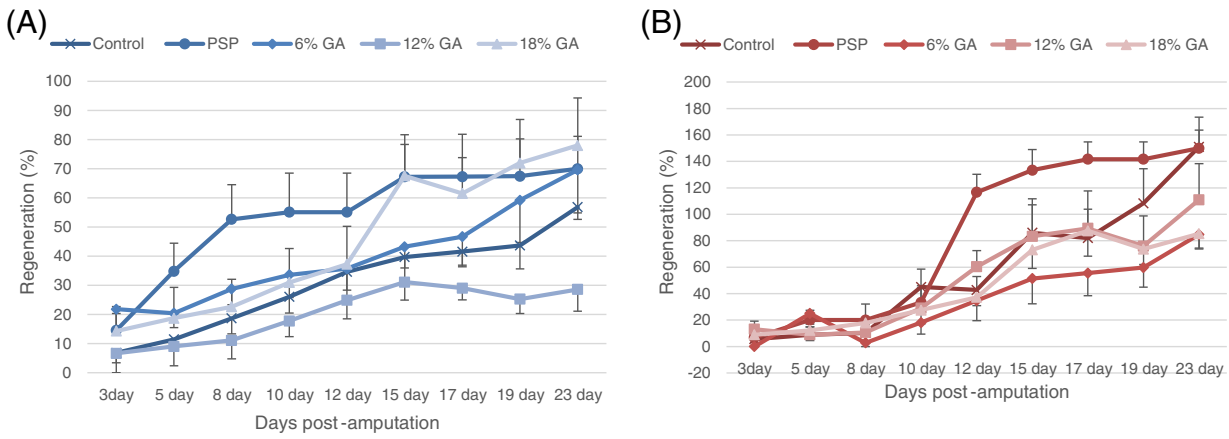

Fig. 5. Percentage of planaria (*Dugesia tigrina*) regeneration of (A) head and (B) tail after fed with different camu-camu aqueous extracts (30 μL of 10 mg/mL) and liver.

The higher the phenolic contents, the lower the MIC of the samples. The major phenolic compounds found in those powders were ellagic acid, quercetin, gallic acid, proanthocyanidins and anthocyanins (Fig. 2, Table 1), which might be responsible for antimicrobial activity against *S. aureus* (Caillet et al., 2012; Rauha et al., 2000; Saraiva et al., 2012). In this study, all analyzed phenolic compounds (ellagitannins, ellagic acid, syringic acid and myricetin) from Sao Paulo powders had better coefficient correlation with higher enzyme inhibition than for Amazonian powders (around 0.9 and 0.7, respectively Table 4). Therefore, it is reasonable to suggest that the loss and microencapsulation of phenolic compounds, and also, the presence of degradation products during the freeze-drying and spray-drying processes in this study might have reduced the antimicrobial activity of camu-camu powders but they can still be used against prokaryotic harmful bacteria like *S. aureus*.

3.5. Regeneration of planaria

Planaria animal model is considered a eukaryote regenerative model and was used to determine the potential of camu camu bioactives for cellular protection and rejuvenation (Oviedo & Beane, 2009). The potential of planaria is based on the fact that entire section of the whole organism can fully regrow from cut sections of the head or lower tail region. Percentage of regeneration was calculated for head and tail section separately (Fig. 5A and B, respectively). For head regeneration, except camu-camu spray-dried powder with 12% of gum arabic, all other extracts showed rapid regeneration and re-growth compared to the control (without camu camu extract) (Fig. 5A). However, for tail regeneration, only freeze-dried camu-camu powder showed superior re-growth (Fig. 5B). After 10 days of removal of specific cross sections in the head and tail region, the planaria cells regrew and completed the regeneration process to a fully grown and functioning organism.

This was the first study to verify planaria regeneration and cellular protection with an aqueous fruit extracts. Further studies are required to more precisely elucidate regeneration and its mechanism. Initial results however suggest that phenolic bioactive compounds (not the level of phenolic bioactive profile, rather composition, stability and bioavailability of phenolic compound might have effect on regeneration) in camu-camu powders might promote eukaryotic cell division and that the planaria model can be utilized as a cellular rejuvenation model. Besides that, oligosaccharides contained in gum arabic could stimulate planaria regeneration. Another possibility is that gum arabic gives better stability of bioactive effects, and consequently, might have stimulatory effects.

4. Conclusions

Two different analyzed pulps of camu camu from commercial sources showed different phenolic compositions and associated health-linked functionalities. Anthocyanidins were found only in Sao Paulo pulp while gallic acid derivatives were predominant in Amazonian pulp. Phenolic compounds such as ellagitannins, ellagic acid, quercetin glycosides, syringic acid and myricetin were major phenolics detected in this study and may be responsible for higher phenolic antioxidant linked functionalities.

In general, inlet air temperature had less effect on bioactive compounds losses than carrier agents concentration during spray-drying processing. Additionally, the lowest concentration of carrier agents (6%) resulted in lower losses of bioactive compounds. Besides that, the type of carrier agents (gum arabic and maltodextrin) could influence the results.

In comparison to spray drying, freeze-drying led to lower losses of phenolic compounds, which positively correlated with antidiabetic properties such as α-amylase and α-glucosidase inhibitory activity. Overall camu-camu showed low α-amylase and high α-glucosidase inhibition that is potentially ideal for incorporation into dietary strategies for management of early stages of type 2 diabetes and its associated complications. In terms of cellular associated functional studies, all the powders produced by freeze-drying and spray-dried (6% gum arabic at 120 °C) powders were more effective against bacterial pathogen *S. aureus* than Ampicillin. Further, camu-camu showed cellular regeneration in planaria model, which showed promise for cellular rejuvenation studies. This study (in vitro) suggests that camu camu with its high antioxidant activity and rich phenolic profile potentially provides protection against microvascular complications (associated with type 2 diabetes) as well as against some bacterial borne infections.

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