Food Chemistry 310 (2020) 125909

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Camu-camu seed (*Myrciaria dubia*) – From side stream to an antioxidant, antihyperglycemic, antiproliferative, antimicrobial, antihemolytic, anti-inflammatory, and antihypertensive ingredient

Marina Fidelis[a], Mariana Araújo Vieira do Carmo[b], Thiago Mendanha da Cruz[c], Luciana Azevedo[b], Takao Myoda[d], Marianna Miranda Furtado[e], Mariza Boscacci Marques[c], Anderson S. Sant'Ana[e], Maria Inês Genovese[f], Won Young Oh[g], Mingchun Wen[h], Fereidoon Shahidi[g], Liang Zhang[h], Marcelo Franchin[i,j], Severino Matias de Alencar[k], Pedro Luiz Rosalen[i,j], Daniel Granato[a,l,*]

[a] Graduate Program in Food Science and Technology, State University of Ponta Grossa, Avenida Carlos Cavalcanti, 4748, 84030-900 Ponta Grossa, PR, Brazil
[b] Faculty of Nutrition, Federal University of Alfenas, Rua Gabriel Monteiro da Silva, 714, 37130-000 Alfenas, MG, Brazil
[c] Department of Chemistry, State University of Ponta Grossa, Av. Carlos Cavalcanti, 4748, 84030-900 Ponta Grossa, PR, Brazil
[d] Department of Food Sciences and Technology, Tokyo University of Agriculture, Abashiri 099-2493, Japan
[e] Department of Food Science, Faculty of Food Engineering, University of Campinas, Campinas, SP, Brazil
[f] Laboratory of Bioactive Compounds, Food and Experimental Nutrition Department, University of São Paulo, 05508-900 São Paulo, SP, Brazil
[g] Department of Biochemistry, Memorial University of Newfoundland, St. John's, NL A1B 3X9, Canada
[h] State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, 230036 Hefei, PR China
[i] Federal University of Alfenas, MG, Brazil
[j] Pharmacology, Dept. of Physiological Sciences, Piracicaba Dental School, University of Campinas, Piracicaba, SP, Brazil
[k] Department of Agri-Food Industry, Food & Nutrition, "Luiz de Queiroz" College of Agriculture, University of São Paulo, Av. Pádua Dias 11, P.O. Box 9, 13418-900 Piracicaba, SP, Brazil
[l] Food Processing and Quality, Production Systems Unit – Natural Resources Institute Finland (Luke) – Open Innovation House, Maarintie 6, FI-02150 Espoo, Finland

ARTICLE INFO

Keywords:
Elagitannins
Desirability function
Antioxidants
Cytotoxic/cytoprotection effect
Antimicrobials
Brazilian fruits
LDL oxidation
Inflammation

ABSTRACT

Camu-camu (*Myrciaria dubia*) seeds are discarded without recovering the bioactive compounds. The main aim of the present work was to optimise the solvent mixture to extract higher total phenolic content and antioxidant capacity of camu-camu seeds. The optimised solvent system increased the extraction of phenolic compounds, in which vescalagin and castalagin were the main compounds. The optimised extract displayed antioxidant capacity measured by different chemical and biological assays, exerted antiproliferative and cytotoxic effects against A549 and HCT8 cancer cells, antimicrobial effects, protected human erythrocytes against hemolysis, inhibited α-amylase and α-glucosidase enzymes and presented *in vitro* antihypertensive effect. Additionally, the optimized extract inhibited human LDL copper-induced oxidation *in vitro* and reduced the TNF-α release and NF-κB activation in macrophages cell culture. Thus, the use of camu-camu seed showed to be a sustainable way to recover bioactive compounds with *in vitro* functional properties.

1. Introduction

Camu-camu (*Myrciaria dubia*), a fruit from the Amazon region, has received attention due to its high content of antioxidant compounds, such as ascorbic acid and polyphenols, including flavonols, anthocyanins, ellagic acid derivatives, ellagitannins, gallic acid and condensed tannins. Studies have reported higher contents of polyphenols, such as ellagic acid, quercetin and cyanidin-3-*O*-glucoside when compared to other 18 fruits from the Myrtaceae family (Gonçalves, Lajolo, & Genovese, 2010).

According to Azevedo et al. (2019), the crude extract of camu-camu represents a complex mixture of polyphenols, which presented *in vivo* synergistic effects on antimutagenesis. The extract was able to decrease oxidative stress and protect against the mutagenic effects of drugs in the bone marrow and gut micronuclei. The total phenolic content (TPC) of camu-camu seed extracts was three times higher than that of acerola,

* Corresponding author at: Natural Resources Institute Finland (Luke), Open Innovation House, Maarintie 6, FI-02150 Espoo, Finland.
E-mail address: daniel.granato@luke.fi (D. Granato).

https://doi.org/10.1016/j.foodchem.2019.125909
Received 23 May 2019; Received in revised form 12 November 2019; Accepted 13 November 2019
Available online 30 November 2019
0308-8146/

proving to be a multifunctional source for the food industry in the development of products fortified with active compounds (Myoda et al., 2010). A study performed by Yazawa, Suga, Honma, Shirosaki, and Koyama (2011) reported that the camu-camu methanolic seed extract exerted *in vivo* and *in vitro* suppressive potential against raw paw edema by inhibiting the production of nitric oxide in cells derived from macrophages.

Myoda et al. (2010) determined the TPC of camu-camu juice residue and reported a value of 369 mg GAE/100 g. On the other hand, Pereira et al. (2015), Chirinos, Galarza, Betalleluz-Pallardel, Pedreschi, and Campos (2010), Genovese, Pinto, Gonçalves, and Lajolo (2008) and Azevêdo, Fujita, Oliveira, Genovese, and Correia (2014) found TPC values of 411, 1320, 1797 and 1843 mg GAE/100 g, respectively, in fruit (pulp and/or peel) of camu-camu. However, the above-mentioned studies only investigated few bioactivities of camu-camu seed extracts, e.g., chemical antioxidant activity.

The higher TPC and antioxidant activity of camu-camu seeds and peels may be of technological interest for the food and pharmaceutical industries (Fidelis et al., 2018). Assessing the chemical compounds and functional properties of camu-camu seeds and peels and their fractions contributes to a better utilization and their economic valorization as well as to reduce carbon footprints in the fruit processing chain. The industrial use of camu-camu byproducts (e.g. seeds and peels) would reduce damages to the environment caused by waste disposal (Borges, Martins, Conceição, & Silveira, 2015).

Several process variables require individual targeting and consideration, mainly regarding the intrinsic nature and stability of chemical compounds. Since the functional properties (e.g., antioxidant, antimicrobial and antiproliferative activities) of plant extract depends on the chemical composition, the study of different solvents is a relevant and demanding factor to be considered (Dai & Mumper, 2010). Nonetheless, it is not yet known how mixtures of different solvents can affect the chemical composition and functional properties of camu-camu seed extracts.

In our previous work (Fujita et al., 2017), camu-camu pulp was spray-dried and the powders displayed chemical antioxidant activity measured by three different assays (ferric reducing antioxidant power, FRAP, scavenging activity of DPPH radical, and Folin-Ciocalteu reducing capacity) and the main antioxidants found were proanthocyanidins, ascorbic acid, and total phenolics. In another work performed by do Carmo et al. (2019), camu-camu seeds were extracted with water and ethyl alcohol at different proportions and the extract with higher chemical antioxidant activity also decreased the *in vitro* induced-cisplatin chromosomal break index. However, none of these studies reported the effects of water, ethyl alcohol, and propanone on the extraction of phenolic compounds and antioxidant activity of camu-camu seeds. Furthermore, studies related to protection of human red blood cells (erythrocytes) on camu-camu seed extracts and inhibition of lipoproteins, such as LDL, are not available.

To date, no study was found on the mathematical modeling using response surface methodology of different solvents effects on the phenolic composition and antioxidant activity of this plant extract. Considering the socioeconomic and nutritional potential of camu-camu and the importance of new natural sources of bioactive compounds, the objectives of this study were to optimise the extraction of phenolic compounds from camu-camu seeds and to assess the antioxidant, antimicrobial, antihypertensive, antihemolytic, antihyperglycemic and anti-inflammatory activities of the optimised camu-camu seed extract. Additionally, the cytotoxicity and antiproliferative effects against tumor cell lines and intracellular reactive oxygen species (ROS) generation of the optimized extract were assessed.

2. Materials and methods

2.1. Chemicals and cell lines

Gallic, 5-*O*-caffeoylquinic, syringic, *p*-coumaric, caffeic, ferulic, rosmarinic, and ellagic acids, quercetin-3-rutinoside, quercetin, (+)-catechin, (−)-epicatechin, Folin-Ciocalteu reagent, α-amylase from porcine pancreas, α-glucosidase from *Saccharomyces cerevisiae,* 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), pyrocatechol violet (3,3′,4-trihydroxyfuchsone- 2″-sulfonic acid), 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), DNA of pBR 322 (*E. coli* strain RRI), ferric chloride hexahydrate, copper sulfate pentahydrate, vanillin, angiotensin I-converting enzyme from rabbit lung (EC 3.4.15.1), and hippuryl-L-histidyl-L-leucine substrate, acetonitrile, RPMI-1640 medium, penicillin, L-glutamine, dimethyl sulfoxide (DMSO) and lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 were obtained from Sigma-Aldrich (São Paulo, Brazil). Fetal bovine serum was acquired from Gibco (Grand Island, NY, USA). *trans*-Resveratrol, malvidin-3,5-diglucoside, and cyanidin-3-*O*-glucoside were obtained from Extrasynthese (France). 2,4-Dihydroxybenzoic and 2,5-dihydroxybenzoic acids were purchased from Carl Roth (Karlsruhe, Germany). Ascorbic acid and propanone were obtained from Biotec (Paraná, Brazil). Sulfuric acid and sodium carbonate were obtained from Vetec (Rio de Janeiro, Brazil). Anhydrous sodium acetate and methyl alcohol were obtained from Anidrol (São Paulo, Brazil) while potassium hexacyanoferrate (III) was obtained from Merck (Darmstadt, Germany). Ethyl alcohol was purchased from Neon (São Paulo, Brazil). Formic acid was acquired from Reagen (Rio de Janeiro, Brazil), and Milli-Q (São Paulo, Brazil) ultrapure water was used in the experiments. A549, IMR90, HCT8, and RAW 264.7 cell lines were obtained from the Rio de Janeiro cell bank (Brazil).

2.2. Camu-camu seeds, extraction procedure and experimental design

Camu-camu fruit (*Myrciaria dubia* [H.B.K.] Mc Vaugh) was cultivated in Iguape, Brazil, at geographical coordinates 24° 41′51″ south, 47° 34′16″ west at 6 m altitude, and harvested in March 2017. The fruits were sanitized (NaClO at 200 mg/L/15 min) and the seeds removed manually. The seeds were dried (35 °C for 31 h until ~ 12% moisture) and ground to standardize the particle size (42 Tyler mesh). This project is registered at National Genetic Heritage Management System (SisGen) of the Brazilian Ministry of Environment (register# A1848EB).

The extractions were performed using a 1:20 (sample:solvent, m/v) ratio, and three different solvents were tested: ultrapure water, ethyl alcohol (EtOH), and propanone. An augmented simplex centroid mixture design containing 10 combinations of solvents was used to evaluate the isolated effects, binary and ternary mixtures of solvents (Table 1). The extraction procedure was performed at 45 °C for 45 min under continuous magnetic agitation and temperature control. The extracts were then filtered using a qualitative filter paper (Whatman 1) and immediately analyzed for their phenolic composition and functional properties.

2.3. Phenolic composition

The TPC of camu-camu seed extracts was determined using a colorimetric method that uses $K_3[Fe(CN)_6]$ and $FeCl_3{\cdot}6H_2O$ at 0.5 mmol/L and results were expressed as mg of gallic acid equivalents per 100 g of seed (mg GAE/100 g). The total flavonoids content (TF) was quantified using the colorimetric method based on the complexation of aluminum with flavonoids, and results were expressed as mg of (+)-catechin equivalents per 100 g of seed (mg CE/100 g). The condensed tannins content (CT) was quantified using the vanillin-H_2SO_4 method and data were expressed as mg CE/100 g. All these methods were conducted in triplicate and are fully described and referenced in our previous works

Table 1
Chemical composition, antioxidant activity, metal chelating activity and inhibition of lipid oxidation of *Myrciaria dubia* seed extracts as affected by water, ethyl alcohol and propanone.

Assay	A (Water)	B (EtOH)	C (Propanone)	Total phenolic content (mg GAE/100 g)	Total flavonoids (mg CE/100 g)	Condensed tannins (mg CE/100 g)	DPPH (mg AAE/100 g)	Folin-Ciocalteu reducing capacity (mg GAE/100 g)	Cu^{2+} chelating activity (%)[1]	Lipid peroxidation inhibition(%)[2]
1	1	0	0	2502 ± 46^{e}	260 ± 11^{g}	171 ± 11^{i}	1963 ± 11^{e}	2114 ± 99^{f}	85 ± 1^{d}	72 ± 4^{d}
2	0	1	0	1353 ± 19^{f}	366 ± 11^{f}	512 ± 10^{g}	1190 ± 84^{h}	1502 ± 64^{g}	92 ± 1^{c}	63 ± 1^{f}
3	0	0	1	657 ± 14^{g}	265 ± 1^{g}	315 ± 2^{h}	1336 ± 50^{g}	1803 ± 163^{fg}	95 ± 1^{a}	24 ± 1^{g}
4	0.5	0.5	0	3403 ± 170^{d}	1105 ± 36^{c}	1221 ± 30^{b}	4187 ± 61^{b}	4354 ± 115^{d}	77 ± 1^{f}	86 ± 0^{a}
5	0.5	0	0.5	5420 ± 87^{b}	1332 ± 83^{b}	942 ± 18^{e}	4216 ± 34^{b}	6579 ± 658^{ab}	77 ± 1^{f}	84 ± 2^{ab}
6	0	0.5	0.5	1479 ± 17^{f}	476 ± 14^{e}	528 ± 20^{g}	1441 ± 85^{f}	3074 ± 45^{e}	93 ± 1^{b}	68 ± 1^{e}
7	0.333	0.333	0.334	5422 ± 79^{b}	1700 ± 114^{a}	1328 ± 38^{a}	4409 ± 60^{a}	6909 ± 110^{a}	77 ± 1^{f}	83 ± 0^{b}
8	0.666	0.167	0.167	5474 ± 180^{b}	1389 ± 22^{b}	1142 ± 74^{c}	4455 ± 15^{a}	6264 ± 208^{b}	77 ± 1^{f}	84 ± 0^{ab}
9	0.167	0.666	0.167	4145 ± 59^{c}	594 ± 58^{d}	725 ± 17^{f}	2873 ± 28^{d}	5201 ± 201^{c}	83 ± 0^{e}	79 ± 0^{c}
10	0.167	0.167	0.666	5619 ± 67^{a}	1384 ± 62^{b}	1052 ± 41^{d}	3424 ± 45^{c}	6274 ± 83^{b}	78 ± 1^{f}	84 ± 0^{ab}
p-value (normality)				0.8531	0.8680	0.9254	0.8361	0.8801	0.8420	0.6885
p-value(homoscedasticity)				0.8311	0.4765	0.8303	0.7521	0.3567	0.9810	0.4031
p-value (*one-way* ANOVA)				< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Note: [1]Percentage values (%) of pyrocatechol violet - Cu^{2+} complex formation. [2]Percentage values (%) of lipid peroxidation inhibition. GAE = gallic acid equivalents: CE = (+)-catechin equivalents; AAE = ascorbic acid equivalents. Different letters in the same column indicate significant statistical difference ($p \leq 0.05$).

(Fidelis et al., 2018; Santos et al., 2018).

The quantification of individual phenolic compounds was performed using a high-performance liquid chromatograph (HPLC, Shimadzu LC-20T, Tokyo, Japan) equipped with diode detector array and fluorescence detectors. The extracts were filtered through a 0.45 μm nylon membrane and injected in triplicate. Chromatographic separation was conducted on a reverse phase column (C_{18}, 150 mm × 4.6 mm, particle size 3.5 μm) according to the methodology fully described by Fidelis et al. (2018). Results were expressed as mg/100 g seed.

2.4. Antioxidant activity

The DPPH scavenging activity was analyzed using microplates and results were expressed as mg ascorbic acid equivalents per 100 g of seed (mg AAE/100 g). The Folin-Ciocalteu reducing capacity (FCRC) was quantified using a colorimetric method and results were expressed as mg GAE/100 g. Cu^{2+} chelating ability was assessed using the spectrophotometric method, that employs pyrocatechol violet as the chromogen agent and results were expressed as the percentage of formation of Cu^{2+}-pyrocatechol violet complex. Thiobarbituric acid reactive substances (TBARS), derived from Fe^{2+}-induced lipid peroxidation of egg yolk lipids, were quantified using a colorimetric assay. The reaction medium was buffered with PBS at 50 mmol/L and pH 7.4 and the reaction occurred at 37 °C to mimic human conditions and results were expressed as percentage of lipid oxidation inhibition. All these methods were conducted in triplicate and are fully described and referenced in our previous works (Santos et al., 2018; Fidelis et al., 2018).

2.5. Response surface modeling and statistical optimisation of extraction conditions

In order to evaluate the effects of the solvents on the extraction of bioactive compounds and *in vitro* antioxidant activity of the camu-camu seed extracts, response surface methodology was used (RSM) following the recommendations outlined by Granato and Calado (2014). The experimental data were adjusted to a complete cubic model – Eq. (1):

$$E(y) = b_1x_1 + b_2x_2 + b_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \quad (1)$$

where E(y) is the predicted response function, b_1, b_2, b_3 are the regression coefficients for linear effects, b_{12}, b_{13}, b_{23} represent the binary interaction coefficients (quadratic coefficients) and b_{123} is the ternary regression coefficient. Accordingly, x_1, x_2, and x_3 represent the three extraction solvents.

The proposed mathematical models included only the significant regression coefficients ($p \leq 0.05$). The statistical quality of the models was assessed by the determination coefficient (R^2), adjusted R^2 and the adherence of residuals to the normal distribution (Shapiro-Wilk test). Using RSM, regression models were obtained to describe the antioxidant capacity and chemical compounds extracted from camu-camu seeds as a function of the solvent(s). The ± 95% confidence interval was calculated for each regression coefficient and 2-dimension contour plots were used to analyze the behaviour of each response as a function of the solvent(s). To optimize the extraction conditions (e.g., solvent mixture), the desirability function was used to maximize the extraction of TPC, condensed tannins, and antioxidant activity (DPPH, Cu^{2+} chelating ability, and inhibition of lipid peroxidation). A total of 100 iterations were used to obtain the best solvent combination (Derringer & Suich, 1980). All statistical analyses were performed using the TIBCO Statistica v.13.3 software (TIBCO Statistica Inc, OK, USA).

2.6. Chemical characterization of the camu-camu seed extract

Camu-camu seed was extracted with the optimised solvent system using the same experimental conditions described in Section 2.2. The liquid camu-camu seed extract was characterized according to the TPC, individual phenolic compounds, condensed tannins, DPPH, Cu^{2+} chelating ability and lipid peroxidation inhibition using the methods described in Sections 2.3 and 2.4. Castalagin and vescalagin were quantified by HPLC-DAD and the separation was conducted using a silica-based C_{18} reversed-phase Atlantis T3 column (4.6 mm × 150 mm, particle size 3 μm, Waters, Milford, MA, USA) and water, acetonitrile and formic acid as the mobile phase according to Kaneshima et al. (2016). The mobile phase for gradient elution was as followed: solvent A was 5% acetonitrile containing 0.2% formic acid, and B was 100% acetonitrile. The gradient condition was as follows: 0 min, 0% B; 5 min, 10% B; 25 min, 15% B; 40 min, 50% B; 45–50 min, 100B; 51 min, 0% B. Analytical curves were made using six different evenly spaced concentrations ($R^2 > 0.99$) of the ellagitannins and results were expressed as mg/100 g seeds.

2.7. Bioactivity of the camu-camu seed extract: Antioxidant and antimicrobial activities

The extract obtained with the optimised solvent mixture was lyophilized under vacuum of 1200 μmL Hg (Terroni, model LD 1500A,

Brazil) and stored at −18 °C until analysis. For the biological antioxidant activity evaluation of the lyophilized optimised camu-camu seed extract (LOCSE), male Wistar rats (4 weeks old, ~400 g) were used (Ethics committee approval 047/2017). Lipid peroxidation of brain homogenates was induced by $FeSO_4$ to assess the hydrogen atom transfer capacity of LOCSE. The experimental conditions described by Fidelis et al. (2018) were used and data were expressed as percentage of lipid peroxidation inhibition. The oxygen radical absorbance capacity (ORAC) of LOCSE was determined according to the method described by Ambigaipalan, Al-Khalifa, and Shahidi (2015) using fluorescein as probe and results were expressed as mg CE/100 g.

The inhibition of LOCSE towards copper-induced low-density lipoprotein (LDL) oxidation followed the method described by Zhou, Sun, and Shahidi (2017). Human LDL (Sigma-Aldrich Canada Ltd, Oakville, Canada) was dialyzed in 10 mmol/L PBS (pH 7.4, 0.15 mol/L NaCl) using a dialysis tube (Fisher Scientific, Nepean, ON, Canada). The molecular weight cutoff was 12–14 kDa. The dialysis was conducted at 4 °C under a nitrogen blanket in the dark for 10 h. Diluted LDL (0.04 mg/mL, 0.4 mL) was mixed with (+)-catechin and LOCSE (2.5 μg/mL, 50 μL). Then the samples and $CuSO_4$ were incubated at 37 °C for 10 min. After 10 min, the $CuSO_4$ (50 μmol/L, 50 μL) was added and measured immediately at 234 nm using a diode array spectrophotometer (Agilent, Palo Alto, CA, USA). The samples were then incubated at 37 °C for 10 h. The blanks were run for each sample by replacing LDL and $CuSO_4$ with PBS for background correction. Control (LDL + $CuSO_4$ + PBS) and native (LDL + phosphate buffer) were also conducted. Results were expressed as absorbance values.

The antimicrobial activity was evaluated against *Pseudomonas aeruginosa* (IAL 1853), *Salmonella* Enteritidis (S 2887), *Salmonella* Typhimurium (IAL 2431), *Escherichia coli* (IAL 2064), *Bacillus cereus* (ATCC 14579), *Staphylococcus aureus* (ATCC 13565), *Listeria monocytogenes* (ATCC 7644), and *Saccharomyces cerevisiae* (NCYC 1006). The assays were performed according to Cleeland and Squires (1991) using the diffusion method on plate-cavity agar. The results were expressed as the inhibition halo (mm).

2.8. Effects of camu-camu seed extract on enzymes and on human erythrocyte protection

The angiotensin I-converting enzyme inhibitory (ACE-I) activity was determined following a spectrophotometric assay (Fidelis et al., 2018) and results were expressed as percentage inhibition.

The anti-hemolytic activity of the optimized camu-camu seed extract was assessed in isotonic (NaCl 0.9% w/v) and hypotonic (NaCl 0.4% w/v) conditions, according to the procedures described by Migliorini et al. (2019). The O^+ blood sample was obtained from the Clinical Laboratory of Regional University Hospital Wallace Thadeu de Mello e Silva, Ponta Grossa, Brazil. The assays were conducted with a negative control, total hemolysis (HT) – solubilization of erythrocytes in ultrapure water, causing total cell rupture. The results were compared to those obtained in the absence of the extract and were expressed as percentage of hemolysis. The biological protocol was approved by the Ethics Committee from the State University of Ponta Grossa (CAAE 94830318.1.0000.0105).

The determination of α-amylase and α-glucosidase inhibition assay followed as previously described (Johnson, Lucius, Meyer, & Gonzalez de Mejia, 2011) using different LOCSE concentrations (α-amylase: 20, 15, 10, 5, and 1 mg/mL; α-glucosidase: 500, 300, 200, 100, and 50 μg/mL). The absorbance was read at 520 nm for α-amylase and 405 nm for α-glucosidase. Results were presented as percentage inhibition of the enzymes.

2.9. In vitro cytotoxicity, proliferation, and anti-inflammatory effects of camu-camu seed extract

The *in vitro* cytotoxic effect of the optimised camu-camu seed extract was analyzed in relation to lung adenocarcinoma epithelial cell (A549), human colon carcinoma (HCT8) and noncancerous human lung fibroblast (IMR90). The cell viability was evaluated by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The parameters IC_{50}, GI_{50} and LC_{50} were calculated according to the methodology described by Escher et al. (2018). Additionally, the selectivity index (SI) was calculated by the ratio IC_{50} IMR90/IC_{50} cancer cell lines. SI value indicates selectivity of the extract to the cell lines tested, in a way that any extract with SI > 3 is considered to have high selectivity (Prayong, Barusrux, & Weerapreeyakul, 2008). For the analysis of intracellular ROS generation, A549 and IMR90 cell lines were studied using the procedures described by Escher et al. (2018). The fluorescence intensity was measured at an excitation and emission wavelength of 485 and 538 nm, respectively; the results were reported as percentage of fluorescence intensity.

For the *in vitro* anti-inflammatory activity assessment, RAW 264.7 macrophages were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 2 mmol/L L-glutamine at 37 °C in a 5% CO_2/95% atmosphere. Macrophages were cultured in 96-well plates (2×10^5 cells/well) at 37 °C, 5% CO_2 overnight. After 24 h of incubation with LOCSE (1, 3, 10, 30, 100, 300, and 1000 μg/mL), the supernatant were removed, RPMI with MTT (0.3 mg/mL) were added to the plate for 3 h incubation. The supernatant was subsequently removed again, and the cells will be resuspended in 200 μL of DMSO. The absorbance was read at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, USA) and results were expressed as % viability. Additionally, RAW 264.7 macrophages were cultured in 24-well plates (3×10^5 cells/well) and incubated overnight. After 15 min of incubation with LOCSE (3, 10, and 30 μg/mL), the LPS (10 ng/mL) added and cells incubation. After 4 h of incubation, the supernatant were removed and quantification of TNF-α was performed by ELISA assay using protocols provided by the manufacturers (R&D Systems, Minneapolis, MN, USA). Results were expressed as pg/mL. For the quantification of NF-KB activation, RAW 264.7 macrophages stably transfected with the NF-κB-pLUC gene were cultured in 24-well plates (3×10^5 cells/well) and incubated overnight. After 15 min of incubation with LOCSE (3, 10, and 30 μg/mL), the LPS (10 ng/mL) added and cells incubation. After 4 h of incubation, the cells of plate were lysed with 50 μL of Tris-NaCl-Tween buffer. The cells suspension (10 μL) was added together with 25 μL of the Luciferase Assay Reagent containing luciferin (Promega Corporation, Madison, USA). A microplate reader was used to quantify the luminescence (Franchin et al., 2016).

2.10. Statistical analysis

All measurements were conducted in triplicate and results were expressed by means followed by standard deviation. The comparison of means between the groups was performed by one-factor analysis of variances (ANOVA) and differences between treatments were identified by Fisher's test (Granato & Calado, 2014). The data set homoscedasticity was verified by the Brown-Forsythe test using the software TIBCO Statistica v.13.3 (TIBCO Statistica Inc., USA). Correlation coefficients were calculated to assess which phenolic compound/phenolic class exerted the antioxidant activity of the camu-camu seed extracts. Probability values ≤ 0.05 were regarded as significant.

3. Results and discussion

3.1. Phenolic composition of camu-camu seed extracts

Table 1 shows that there were significant differences ($p < 0.001$) between the mean values for all phenolic classes and antioxidant activity assays using both chemical (FCRC, DPPH, and Cu^{2+} chelating activity) and biological assays (inhibition of lipid peroxidation contained in egg yolk). These results demonstrate that the solvent system

directly affects the extraction of phenolic compounds and antioxidant activity of camu-camu seed extracts. The TPC varied from 657 to 5619 mg GAE/100 g of seed and the highest average concentration was obtained in the ternary mixture, composed of propanone, water and ethyl alcohol (0.666:0.167:0.167, v/v). Our values are higher than those reported by Myoda et al. (2010) −369 mg GAE/100 g – and Azevêdo et al. (2014) −1843 mg GAE/100 g. Further works should also focus on the bound phenolics from camu-camu seeds.

The TF content ranged from 260 to 1700 mg CE/100 g seed and the highest value was found in the ternary mixture - water, ethyl alcohol and propanone (0.333:0.333:0.334, v/v/v, respectively). In previous studies, quercetin, quercetin-3-rutinoside, myricetin and (+)-catechin were the main flavonoids identified in camu-camu seeds (Fracassetti, Costa, Moulay, & Tomás-Barberán, 2013; Azevêdo et al., 2014). The condensed tannins content ranged from 171 to 1328 mg CE/100 g of seed. The highest content of tannins was observed in the ternary solvent mixture (0.333:0.333:0.334, v/v/v), indicating that these compounds are more efficiently extracted when water, ethyl alcohol and propanone are used concomitantly.

External factors, such as climatic conditions, soil type, extraction solvent, extraction time and temperature influence the phenolic content and profile of fruit sections qualitatively and quantitatively. Extracting solvent and temperature reduce the solvent viscosity, facilitate cell membrane permeability, and further increase the diffusion of the phenolic compounds (Setford, Jeffery, Grbin, & Muhlack, 2017). The growing region and temperature considerably affect the endogenous synthesis of polyphenols in fruits.

Regarding the individual phenolic composition of the camu-camu seed extracts, 16 phenolic compounds were quantified (Table 2). There were significant differences ($p \leq 0.05$) between the extracts. The extract obtained with water and ethyl alcohol (0.5:0.5, v/v) presented the highest content of individual phenolic compounds (77.35 mg/100 g), followed by the extract obtained with a mixture of water and propanone (0.5:0.5 v/v −61.79 mg/100 g) or with the ternary mixture composed of 33.3% of each solvent (61.43 mg/100 g).

Gallic and ellagic acids, quercetin-3-rutinoside, malvidin-3,5-diglucoside and cyanidin-3-*O*-glucoside were the main phenolic compounds identified in most of the extracts. These compounds have already been identified and quantified in different parts (pulp and peel) of camu-camu (Genovese et al., 2008); Chirinos et al., 2010; Fracassetti et al., 2013; Kaneshima, Myoda, Toeda, Fujimori, & Nishizawa, 2013; Azevêdo et al., 2014; Fujita et al. (2015). In a study performed with camu-camu seed coat, *trans*-resveratrol was identified and its content was about ten times higher than that found in red wine (Fidelis et al., 2018). do Carmo et al. (2019) found *trans*-resveratrol contents between 0.61 and 0.93 mg/100 g of camu-camu seeds extracted with water, ethyl alcohol, or a mixture of these extracting solvents.

3.2. Antioxidant activity of camu-camu seed extracts

In the present study, three mechanisms of antioxidant action were tested in all camu-camu seed extracts: single electron transfer (DPPH and FCRC), transition metal ion chelation (Cu^{2+} chelating activity) and hydrogen atom transfer (inhibition of lipid peroxidation) and the results are shown in Table 1. The results showed that the extracts were able to eliminate the DPPH radical and the antioxidant activity was higher in the ternary solvent mixture extract (water/ethyl alcohol/propanone, 0.666:0.167:0.167 and 0.333:0.333:0.334, v/v/v). Comparatively, Fidelis et al. (2018) observed a higher DPPH value (2838 mg AAE/100 g) in camu-camu seed coat aqueous extract using the same method. Grigio et al. (2017), in a study on the antioxidant activity of camu-camu seed aqueous extract, found a DPPH value of 245 mg AAE/100 g, indicating lower values compared to those found in the present work.

The FCRC varied from 1502 to 6909 mg GAE/100 g of seed, and the highest antioxidant activity was found in a ternary mixture (0.333 water:0.333 ethyl alcohol:0.334 propanone, v/v/v). These results corroborate those obtained by Chirinos et al. (2010) and Azevêdo et al. (2014), who studied camu-camu pulp and peel and found FCRC values of 1320 and 1843 mg GAE/100 g, respectively. It is observed that the antioxidant activity found in seeds is considerably higher in comparison to other fruit fractions.

The Cu^{2+} chelating activity varied from 77 to 95% of pyrocatechol violet- Cu^{2+} complex formation. The binary combinations of water and ethyl alcohol, water and propanone (0.5:0.5, v/v) and ternary mixture of water, ethyl alcohol and propanone (0.333:0.333:0.334 and 0.666:0.167:0.167, v/v/v) presented the lowest values, thus indicating a higher antioxidant activity ($p \leq 0.05$). In this method, there are two ways to express the data: if the data is expressed as Cu^{2+}-pyrocatechol violet complex formation, it means that Cu^{2+} that was not chelated by *ortho*-diphenols present in the extract reacted with pyrocatechol violet,

Table 2
Phenolic composition of camu-camu seeds extracted with different solvent systems.

Phenolic compounds (mg/100 g seed)	100% H_2O	100% EtOH	100% $(CH_2)_3O$	50% H_2O + 50% EtOH	50% H_2O + 50% $(CH_2)_3O$	50% EtOH + 50% $(CH_2)_3O$
Phenolic acids						
Gallic acid	3.15 ± 0.14^{e}	2.47 ± 0.12^{ef}	ND	6.68 ± 0.32^{bc}	10.53 ± 1.71^{a}	1.54 ± 0.18^{f}
2,4-dihydroxybenzoic acid	0.27 ± 0.03^{bc}	0.14 ± 0.01^{f}	ND	0.18 ± 0.01^{ef}	0.47 ± 0.07^{a}	0.18 ± 0.01^{ef}
Syringic acid	0.63 ± 0.09^{c}	0.75 ± 0.03^{a}	0.78 ± 0.00^{a}	0.53 ± 0.01^{d}	0.75 ± 0.05^{ab}	0.67 ± 0.03^{b}
5-*O*-caffeoylquinic acid	0.43 ± 0.01 ^{g}	0.54 ± 0.01^{f}	0.47 ± 0.09 ^{fg}	0.77 ± 0.02 ^{cd}	1.22 ± 0.02^{a}	0.65 ± 0.03^{e}
Caffeic acid	1.42 ± 0.02^{c}	1.13 ± 0.00^{e}	1.10 ± 0.03^{e}	1.52 ± 0.03^{bc}	2.05 ± 0.14^{a}	1.22 ± 0.03^{de}
Ferulic acid	0.41 ± 0.01^{c}	0.54 ± 0.00^{a}	ND	0.51 ± 0.01^{b}	0.41 ± 0.01^{c}	0.36 ± 0.01^{d}
Rosmarinic acid	ND	ND	ND	ND	0.77 ± 0.02^{a}	0.74 ± 0.01^{b}
Flavonoids						
Quercetin	0.84 ± 0.01^{bc}	1.18 ± 0.13^{bc}	0.93 ± 0.01^{e}	1.95 ± 0.20^{a}	1.16 ± 0.02^{bcd}	1.02 ± 0.02^{de}
Quercetin-3-rutinoside	6.57 ± 0.06^{f}	11.27 ± 0.27^{f}	5.12 ± 0.07 ^{h}	24.89 ± 0.87^{a}	11.55 ± 0.50^{ef}	10.41 ± 0.22 ^{g}
(+)-Catechin	0.52 ± 0.01^{b}	0.29 ± 0.01^{c}	ND	0.55 ± 0.01^{a}	ND	ND
(−)-Epicatechin	3.07 ± 0.01^{a}	2.26 ± 0.13^{c}	ND	2.38 ± 0.03^{b}	ND	ND
Procyanidin A2	ND	ND	ND	ND	1.97 ± 0.54^{a}	ND
Cyanidin-3-glucoside	ND	3.51 ± 0.00^{a}	ND	3.46 ± 0.01^{bc}	4.72 ± 0.01^{bc}	3.45 ± 0.00^{c}
Malvidin-3,5-diglucoside	3.95 ± 0.02 ^{h}	4.11 ± 0.01 ^{g}	3.79 ± 0.01^{i}	4.33 ± 0.00^{d}	3.47 ± 0.01^{c}	4.28 ± 0.01^{f}
Others						
trans-Resveratrol	2.62 ± 003^{b}	0.61 ± 0.01 ^{g}	2.08 ± 0.01^{c}	0.95 ± 0.04^{ef}	2.67 ± 0.11^{ab}	0.74 ± 0.01^{d}
Ellagic acid	16.12 ± 0.04 ^{g}	16.12 ± 0.36 ^{g}	10.41 ± 0.10 ^{h}	28.65 ± 0.65^{a}	20.02 ± 0.03^{e}	18.10 ± 0.05^{f}
Total identified (mg/100 g seed)	40.00 ± 0.15^{e}	44.92 ± 0.74^{d}	24.68 ± 0.11^{f}	77.35 ± 1.96^{a}	61.79 ± 1.67^{b}	43.35 ± 0.29^{d}

Note: ND = not detected; NA = not applicable; [1]Probability values for homoscedasticity by the Brown-Forsythe test; [2]Probability values by one-way analysis of variances (ANOVA) or unpaired Student-*t* test (n = 2 treatments). Different letters in the same row represent statistically different results according to the Fisher's test ($p \leq 0.05$).

thus forming the PV-Cu^{2+} complex. However, if data is expressed as inhibition of Cu^{2+}-pyrocatechol violet complex formation, the amount of Cu^{2+} chelated by *ortho*-diphenols present in the extract can be calculated. Thus, low inhibition values mean high formation of the PV-Cu^{2+} complex which implies a low metal chelating ability. Phenolic compounds with a single hydroxyl group on the aromatic ring and without an O-CH_3 group on the benzene ring do not exhibit metal ion chelating ability. Accordingly, for flavonoids, the transition metal ions attachment sites are the 3′, 4′-dihydroxy *ortho*-diphenyl groups on ring B and the 4-keto, 3-hydroxy or 4-keto structures and 5-hydroxy on the C-ring of flavonols (Santos, Brizola, & Granato, 2017). The presence of methoxy/hydroxy groups in the *ortho* position of phenolic acids increases the chelating capacity of Cu^{2+}, while glycosylation of the OH group of the phenols prevents the binding of the metal ions (Říha et al., 2014).

Lipid peroxidation inhibition using egg homogenate ranged from 24 to 86% ($p \leq 0.05$). The highest percentage of lipid peroxidation inhibition was observed for the extract obtained with water and ethyl alcohol (0.5:0.5, v/v). This is in agreement with the data obtained by Fidelis et al. (2018) who observed greater lipid oxidation inhibition (both in egg yolk and Wistar rat brain homogenate) of camu-camu seat coat aqueous extract. It is noteworthy that this is the first work that determined lipid peroxidation inhibition of *M. dubia* seed extracts in a biological assay using Wistar rat's brain homogenate as the oxidation target. However, to have biological relevance, the capability of LOCSE to cross blood brain barrier should be further explored in future *in vivo* studies.

It is known that the solvent in which the reaction occurs has a great impact on the results, since the polarity can affect the mechanism of the reaction. In this study, a relationship between bioactive compounds and antioxidant activity showed a significant positive correlation ($p \leq 0.05$) between DPPH and TPC ($r = 0.8992$), TF ($r = 0.9156$) and CT ($r = 0.8975$). Similarly, the antioxidant activity measured by the FCRC assay correlated with TPC ($r = 0.9556$), TF ($r = 0.9153$) and CT ($r = 0.8475$). The lipid oxidation inhibition was correlated with TPC ($r = 0.7834$), TF ($r = 0.6690$) and CT ($r_{LPI} = 0.6804$). Significant ($p \leq 0.05$) correlation coefficients existed between the formation of the PV-Cu^{2+} complex and TPC ($r = -0.9204$), TF ($r = -0.8652$) and CT ($r = -0.8287$). In this case, the higher contents of phenolics, flavonoids, and condensed tannins enabled a lower formation of the PV-Cu^{2+} complex, thus indicating a greater antioxidant activity.

3.3. Response surface modeling and statistical optimization of the solvent system

Table 3 shows the mathematical equations that explain the effects of water, ethyl alcohol and propanone on the chemical composition and antioxidant activity of camu-camu seed extracts. All proposed models were significant ($p < 0.001$) and R^2 values explained > 95% the variability of the results. Residuals followed the normal distribution ($p > 0.05$, except for condensed tannins and inhibition of lipid peroxidation).

The interaction effect between water and ethyl alcohol significantly increased ($p \leq 0.05$) TPC, TF, CT, FCRC, DPPH and inhibition of lipid peroxidation. The same behavior was observed for water and propanone combinations. On the other hand, ethyl alcohol and propanone combinations significantly increased ($p \leq 0.05$) TF, CT, FCRC and inhibition of lipid peroxidation. However, the effect of ternary mixtures of ethyl alcohol, propanone and water led to an increase in TPC, TF, CT, Cu^{2+} chelating activity, FCRC, and DPPH, as shown in Fig. 1-Supplementary Material.

The simultaneous optimization suggested a solvent system containing 43.3% propanone, 40.7% water and 16% ethyl alcohol in order to maximize the values of TPC, CT, DPPH, Cu^{2+} chelating ability, and lipid peroxidation inhibition (Fig. 2-Supplementary Material). This statistical model presented a *d*-value = 0.976, indicating that approximately 98% of the desirability was satisfied. An external validation was performed with the aforementioned solvent proportions to test the models and to evaluate whether the RSM models are predictive. The predicted and observed results are shown in Table 4 and it is possible to observe that the experimental data were within the ± 95% prediction intervals proposed by the RSM models. Thus, the generated RSM models were considered predictive and can be used to have an idea about the TPC and antioxidant activity of camu-camu seed extracts obtained with different ratios of the tested solvents. In technological terms, this is the first work to report the optimization of the camu-camu seed solvent extraction system.

3.4. Chemical characterization and functional properties of optimized camu-camu seed extract

Sixteen phenolic compounds were quantified (Table 4) in the optimized camu-camu seed extract and a phenolic content of 1861 mg/100 g was found using HPLC. Two ellagitannins, namely castalagin (1036 mg/100 g) and vescalagin (785 mg/100 g) were the main phenolics in the optimized extract. Ellagic acid and ellagitannins were also the main phenolics detected by Fujita et al. (2015). Kaneshima et al. (2013) isolated gallic acid and ellagic acid in the seed extract (aqueous acetone solution, 50%, v/v).

The antimicrobial activity of LOCSE was observed for *P. aeruginosa* (8.72 ± 1.35 mm), *S.* Enteritidis (6.82 ± 1.36 mm), *S.* Typhimurium (6.42 ± 1.00 mm), *E. coli* (6.74 ± 0.80 mm), *B. cereus* (9.04 ± 1.36 mm), *S. aureus* (9.70 ± 1.92 mm), *L. monocytogenes* (8.58 ± 0.82 mm), and *S. cerevisiae* (5.74 ± 0.68 mm). Azevêdo et al. (2014) observed antimicrobial activity of camu-camu residue extracts against *S. aureus* (inhibition halo of 12 mm). Myoda et al. (2010) evaluated the antibacterial potential of methanolic extract of camu-camu seed against *S. aureus* (inhibition halo of 4.7 mm). Further studies should isolate the antimicrobial compounds from camu-camu seeds and possibly add the extracts in different foods to assess the antimicrobial effects.

LOCSE (3.5 mg/mL) inhibited 33.90 ± 0.44% of ACE-I activity, corroborating the data obtained by Fidelis et al. (2018) who investigated the potential of camu-camu seed coat extracts in inhibiting ACE-I. The authors found inhibition rates between 28 and 40% for extracts obtained with water, ethyl alcohol, and propanone. According to Fujita et al. (2015), ellagic acid present in camu-camu seed extracts can be considered one of the components responsible for inhibiting the action of ACE-I.

Considering the cancer cell viability assays, LOCSE showed cytotoxic ($LC_{50\ HCT8}$ = 802.5 μg/mL; $LC_{50\ A549}$ = 1008 μg/mL) and antiproliferative effects against both A549 and HCT8 cancer cell lines (Fig. 1A, C, and E). Interestingly, the LOCSE extract revealed a biphasic effect on cancer cell behavior, once it stimulated cell proliferation acting as pro-carcinogenic at low doses (100 μg/mL, Fig. 1A and C), but exerted antiproliferative activity at higher doses (200 – 900 μg/mL, Fig. 1A and C). Similarly, Rietjens et al. (2005) also pointed out the same biphasic behavior from quercetin. Our data corroborate the observations obtained by do Carmo et al. (2019) who analyzed camu-camu seed extracts obtained with different concentrations of water and ethyl alcohol found that the aqueous extract presented the highest cytotoxicity towards Caco-2 (IC_{50} = 204 μg/mL) and HepG2 (IC_{50} = 428 μg/mL) cells, while the extract obtained with 50% water and 50% ethyl alcohol presented the highest cytotoxicity towards A549 (IC_{50} = 278 μg/mL). The extracts also decreased ROS generation in those cells and protected chromosome breaks induced by cisplatin.

Herein, A549 cells demonstrated more resistance to the LOCSE, once they exhibited higher GI_{50} values (> 900 μg/mL), meaning low antiproliferative activity (Fig. 1C). On the other hand, HCT8 cells showed higher sensitivity to the LOCSE, since they presented lower values of GI_{50} (320.7 μg/mL), in comparison with A549 cells. LOCSE did not inhibit the growth of normal cells - IMR90 (Fig. 1B, D, and F) –

Table 3
Regression coefficients obtained by surface response methodology to evaluate the effects of solvents combination on the extraction of phenolic compounds and on the antioxidant activity of camu-camu seed extracts.

Model components	Regression coefficients	Standard error	*t*-Value	*p*-Value	−95% Confidence	+95% Confidence
Total phenolic content						
A (Water)	2617	278	9.43	< 0.001	2043	3192
B (Ethyl alcohol)	1569	243	6.44	< 0.001	1065	2072
C (Propanone)	955	253	3.77	< 0.001	431	1478
AB	5831	1352	4.31	< 0.001	3034	8628
AC	15,455	1345	11.49	< 0.001	12,673	18,237
ABC	57,569	8226	7.00	< 0.001	40,553	74,586
AC (A-C)	−13,161	3932	−3.35	< 0.001	−21,294	−5027
R^2	0.95					
Adjusted R^2	0.93					
p-Value (normality of residuals)	0.15					
Total flavonoids						
A (Water)	251	41	6.11	< 0.001	166	337
B (Ethyl alcohol)	357	41	8.70	< 0.001	272	443
C (Propanone)	256	41	6.22	< 0.001	170	341
AB	3133	201	15.55	< 0.001	2714	3552
AC	4242	201	21.05	< 0.001	3823	4661
BC	604	201	3.00	0.006	185	1024
ABC	12,016	1322	9.09	< 0.001	9267	14,765
AB(A-B)	7767	669	11.60	< 0.001	6375	9160
AC(A-C)	−5711	669	−8.53	< 0.001	−7103	−4318
R^2	0.99					
Adjusted R^2	0.98					
p-Value (normality of residuals)	0.88					
Condensed Tannins						
A (Water)	167	22	7.47	< 0.001	120	213
B (Ethyl alcohol)	508	22	22.75	< 0.001	461	554
C (Propanone)	311	22	13.92	< 0.001	264	357
AB	3501	109	31.98	< 0.001	3273	3729
AC	2781	109	25.40	< 0.001	2553	3009
BC	442	109	4.03	< 0.001	214	669
ABC	5848	718	8.14	< 0.001	4354	7341
AB (A-B)	5270	364	14.49	< 0.001	4514	6026
AC (A-C)	−3478	364	−9.56	< 0.001	−4234	−2722
R^2	0.99					
Adjusted R^2	0.99					
p-Value (normality of residuals)	0.001					
Cu^{2+} chelating activity						
A (Water)	85	0.67	127.02	< 0.001	83.85	86.63
B (Ethyl alcohol)	92	0.61	150.00	< 0.001	90.37	92.90
C (Propanone)	95	0.61	155.66	< 0.001	93.83	96.36
AB	−48	3.24	−14.82	< 0.001	−54.84	−41.38
AC	−53	3.24	−16.61	< 0.001	−60.66	−47.19
ABC	−99	19.73	−5.00	< 0.001	−139.66	−57.81
AB(A-B)	−31.63	10.92	−2.90	0.01	−54.27	−8.99
AC(A-C)	90.12	10.92	8.25	< 0.001	67.48	112.76
R^2	0.98					
Adjusted R^2	0.97					
p-Value (normality of residuals)	0.23					
Folin-Ciocalteu reducing capacity						
A (Water)	2090	210	9.94	< 0.001	1655	2524
B (Ethyl alcohol)	1562	210	7.43	< 0.001	1127	1997
C (Propanone)	1926	210	9.16	< 0.001	1491	2361
AB	10,255	1058	9.69	< 0.001	8066	12,444
AC	18,682	1058	17.65	< 0.001	16,493	20,871
BC	6051	1058	5.72	< 0.001	3862	8240
ABC	44,259	6976	6.34	< 0.001	29,828	58,691
R^2	0.97					
Adjusted R^2	0.97					
p-Value (normality of residuals)	0.37					
Free radical scavenging activity (DPPH)						
A (Water)	1966	32	62.15	< 0.001	1900	2031
B (Ethyl alcohol)	1193	32	37.71	< 0.001	1127	1258
C (Propanone)	1339	32	42.32	< 0.001	1273	1404
AB	10,457	155	67.43	< 0.001	10,135	10,780
AC	10,282	155	66.30	< 0.001	9960	10,605
BC	725	155	4.68	< 0.001	403	1048
ABC	14,946	1017	14.69	< 0.001	12,830	17,061
AB(A-B)	5351	515	10.39	< 0.001	4279	6422
AC(A-C)	−3519	515	−6.83	< 0.001	−4591	−2448
R^2	0.99					
Adjusted R^2	0.99					
p-Value (normality of residuals)	0.55					

(*continued on next page*)

Table 3 (*continued*)

Model components	Regression coefficients	Standard error	*t*-Value	*p*-Value	−95% Confidence	+95% Confidence
Lipid peroxidation inhibition						
A (Water)	73	1.80	40.41	< 0.001	69	77
B (Ethyl alcohol)	64	1.80	35.33	< 0.001	60	67
C (Propanone)	25	1.80	13.75	< 0.001	21	29
AB	71	8	8.80	< 0.001	54	88
AC	141	8	17.45	< 0.001	125	158
BC	97	8	11.95	< 0.001	80	114
AB(A-B)	86	29	2.91	< 0.001	25	147
AC(A-C)	−247	29	−8.38	< 0.001	−308	−186
R^2	0.97					
Adjusted R^2	0.97					
p-Value (normality of residuals)	0.002					

Table 4
Validation of the optimisation procedure: predicted and experimental values for the camu-camu seed extract obtained with the mixture of 40.7% water, 16% ethyl alcohol and 43.3% propanone and phenolic composition of the optimised camu-camu seed extract (*Myrciaria dubia*).

Responses	Predicted mean value	−95% Prediction	+95% Prediction	Experimental value
Total phenolic content (mg GAE/100 g)	5906	4254	7558	5314 ± 73
Condensed tannins (mg CE/100 g)	1233	1064	1403	1263 ± 66
DPPH (mg AAE/100 g)	4472	4077	4868	4130 ± 36
Cu^{2+} chelating activity (%)[1]	75	72	79	78 ± 2
Inhibition of lipid peroxidation (%)[2]	89	82	96	86 ± 1
Phenolic compounds of the optimised camu-camu seed extract (mg/100 g seeds)				
Phenolic acids				
Gallic acid				10.87 ± 4.00
Caffeic acid				2.15 ± 0.35
Rosmarinic acid				0.73 ± 0.00
2,5-Dihydroxybenzoic acid				3.42 ± 0.65
2,4-Dihydroxybenzoic acid				3.01 ± 0.22
Ferulic acid				0.99 ± 0.20
2-hydroxycinnamic acid				0.76 ± 0.39
Flavonoids				
Quercetin				0.45 ± 0.02
Quercetin-3-rutinoside				2.95 ± 0.36
Procyanidin A2				4.70 ± 0.70
Malvidin-3,5-diglucoside				1.27 ± 0.08
Cyanidin-3-glucoside				3.94 ± 0.03
Elagitannins				
Vescalagin				785 ± 7
Castalagin				1036 ± 16
Others				
trans-Resveratrol				0.33 ± 0.03
Ellagic acid				4.04 ± 0.26
Total identified (mg/100 g seed)				1861

Note: [1]formation of pyrocatechol violet - Cu^{2+} complex (%); [2]inhibition of lipid peroxidation (%). GAE = gallic acid equivalents: CE = (+)-catechin equivalents; AAE = ascorbic acid equivalents.

which indicates relative specificity of the extract and its safety in *in vitro* assays. Similarly, Barros et al. (2015) observed cytotoxic effects of acai seed aqueous extract (*Euterpe oleracea* Mart.) against malignant cell lines (MCF-7, NCI-H460, HeLa and HepG2), without deleterious effects on normal cells. Overall, our results revealed that LOCSE presented moderate cytotoxicity (100 μg/mL < IC_{50} in A549 and HCT8 < 1000 μg/mL) and selectivity for cancer cell lines (SI A549 > 1.55; SI HCT8 > 1.42).

The antihemolytic effect of LOCSE in relation to human erythrocytes was evaluated in isotonic (NaCl 0.9%) and hypotonic (NaCl 0.4%) conditions and the results are shown in Fig. 1G and H, respectively. In the isotonic condition, which simulates the biological conditions of osmolarity, the results demonstrated the non-toxicity of LOCSE in human erythrocytes, since the percentage of hemolysis at the LOCSE concentrations (50 to 100 mg/L) were smaller or indifferent from those present in the absence of the extract ($p \leq 0.05$). The antihemolytic effect was dose-dependent in isotonic conditions as higher LOCSE concentrations tended to present more protection against hemolysis. Similarly, in the hypotonic condition, in which there is low osmotic pressure in the medium and the erythrocytes absorb water in order to balance the osmotic pressure, LOCSE also protected the erythrocytes. However, the antihemolytic effect was not dose-dependent, at least at 50–100 mg/L. This is the first study to report the anti-hemolytic potential of *M. dubia* seeds. Phenolics present in LOCSE protect the erythrocytes against hemolysis possibly by the formation of hydrogen bonds between the –OH groups of the phenolics with the polar heads of the membrane phospholipids. This interaction increases the stiffness of the membrane, making it less susceptible to hemolysis (Sato, Yamakose, & Suzuki, 1993).

The ROS generation analysis revealed that H_2O_2 (15 μmol/L) caused oxidation of DCFH-DA indicating its irrefutable influence on ROS generation in the positive control (Fig. 2A and B). The changes of intracellular ROS generation in LOCSE-treated and untreated A549 and IMR90 cells were detected with the staining of DCFH-DA. At

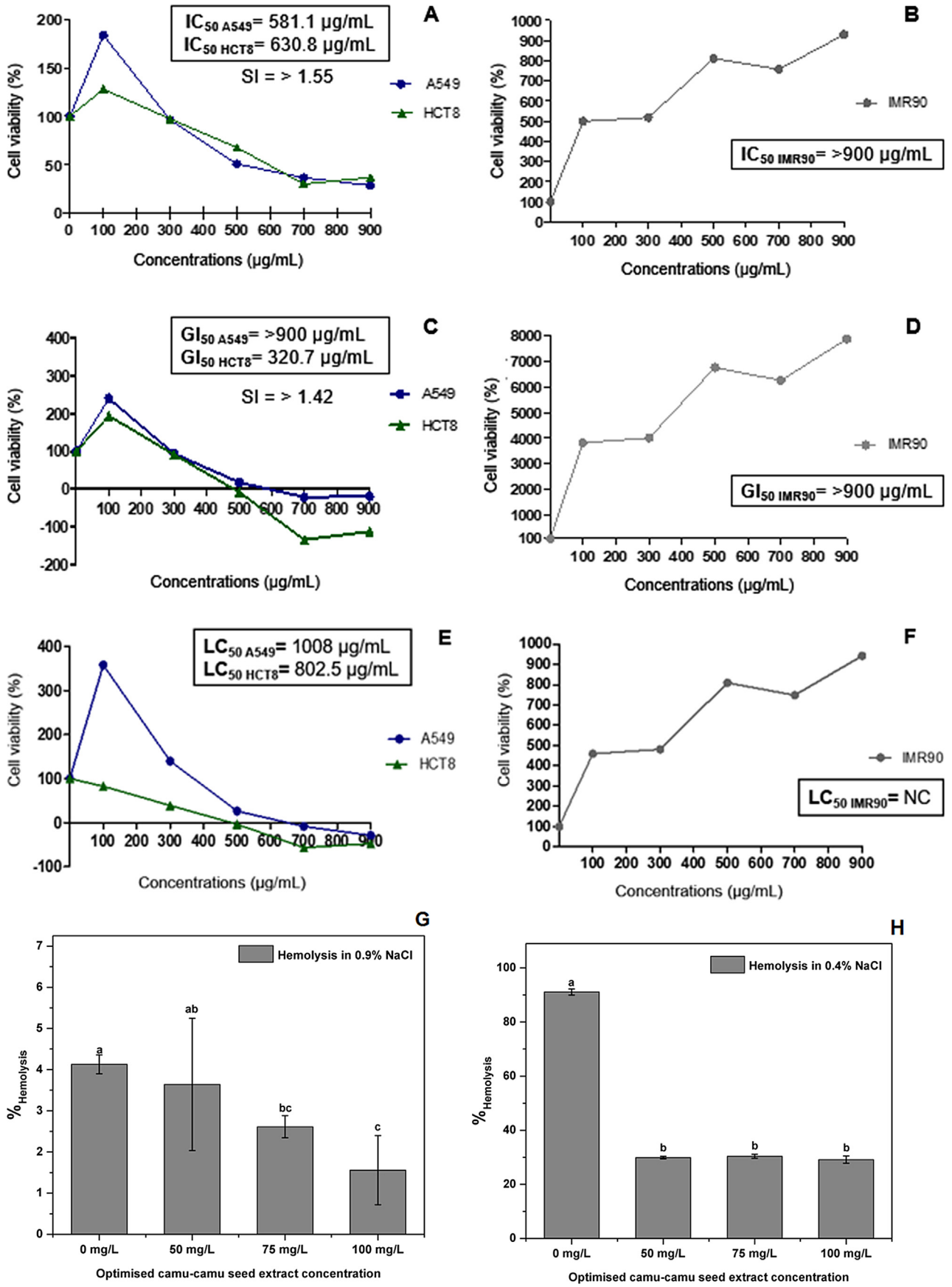

Fig. 1. Cytotoxicity and proliferation inhibition of A549, HCT8 and IMR90 cells after 48 h of exposure to the lyophilized optimised camu-camu seed extract (A–F). Antihemolytic effect of the lyophilized optimised camu-camu seed extract at different concentrations, in isotonic condition - NaCl 0.9% w/v (G), and hypotonic condition - NaCl 0.4% w/v (H). Note: NC = not convergent.

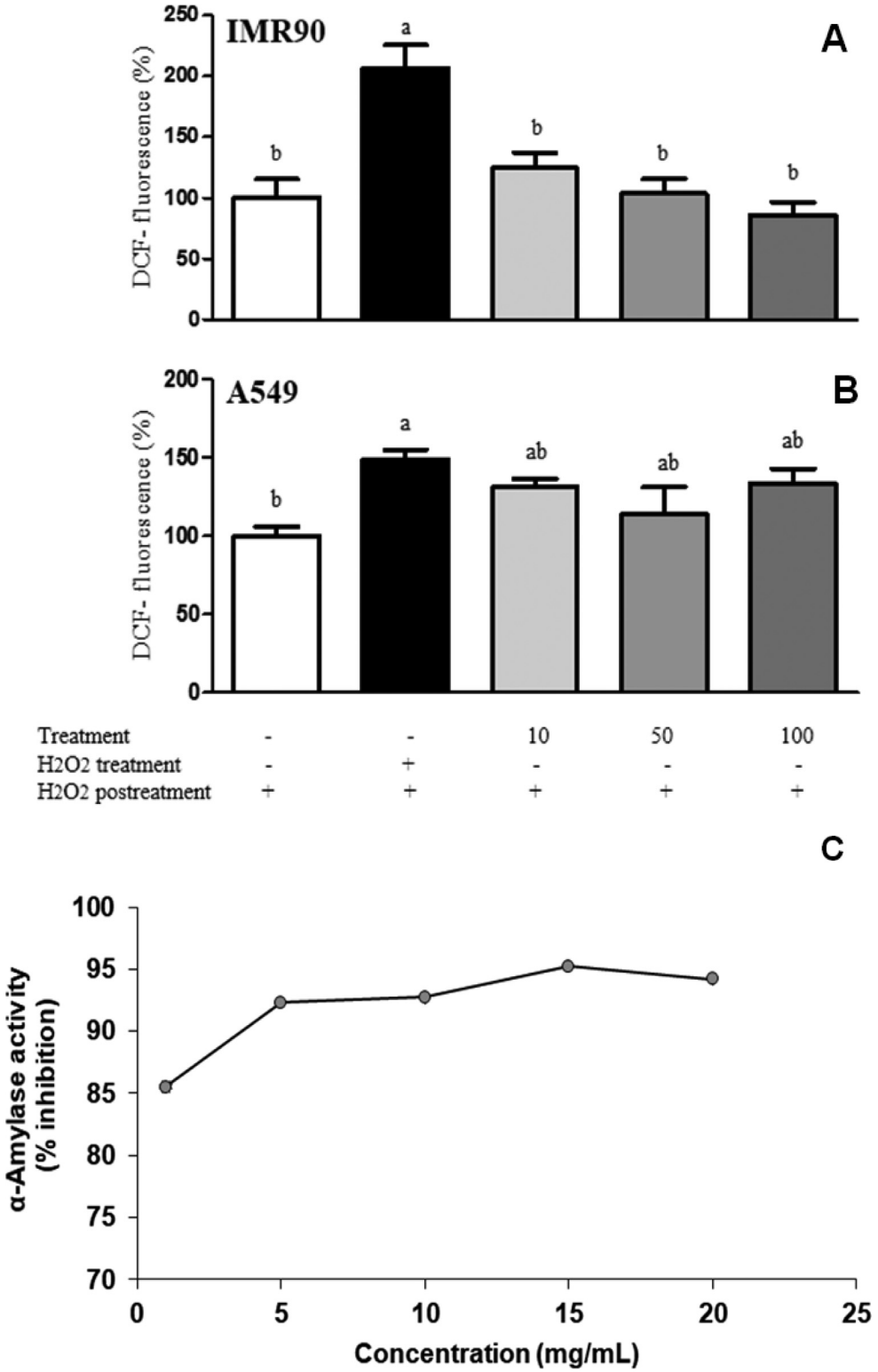

Fig. 2. Results of intracellular ROS measurement in IMR90 (A) and A549 (B) cells by spectrofluorometry. Treatment = lyophilized optimised camu-camu seed extract at 10–100 μg/mL. Inhibition of α-amylase (C), α-glucosidase (D), Wistar rat's brain lipid oxidation (E) and inhibition of copper-induced low-density lipoprotein oxidation (F) by the lyophilized optimised camu-camu seed extract. Effects of lyophilized optimised camu-camu seed extract at 1–1000 μg/mL on the viability of RAW 264.7 macrophages (G), TNF-α production (H) and NF-κB activation (I). Quantitative data are the mean ± standard deviation. Different letters comparing treatments indicate significant differences ($p \leq 0.05$). The symbol (#) indicates statistical difference compared with vehicle group and the symbol (*) indicates statistical difference compared with lipopolysaccharides (LPS) group.

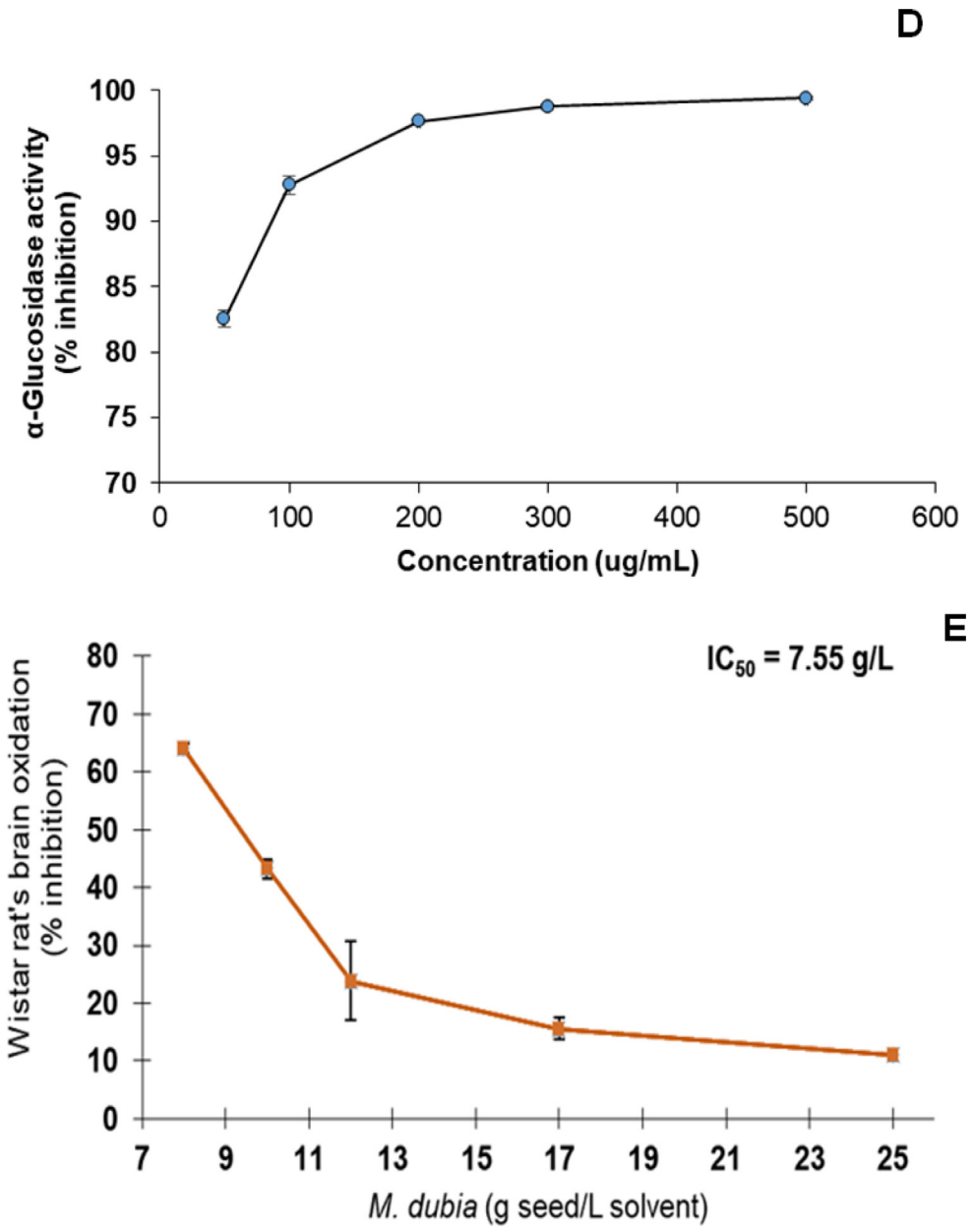

Fig. 2. (*continued*)

10–100 μg/mL LOCSE was not able to induce oxidative stress in IMR90 normal cells, corroborating with the absence of cell death in the cell viability test. Herein, these normal cells may also fully retain their ability to mount a counteracting response in the presence of increased ROS or other types of stressor agents (Cheimonidi et al., 2018). In contrast, in relation to A549 cells, all LOCSE concentrations caused a discrete ROS induction, probably insufficient to lead to cell death, once the treatments were similar to the negative and positive controls. Bearing in mind the cytotoxic effects against A549 cells, besides ROS accumulation, different physicochemical stress stimuli can initiate necrosis or apoptosis, such as cell membrane damage, mitochondrial dysfunction/destabilization (Peixoto, Galvão, & Medeiros, 2017), tumor necrosis factor α (TNFα) production, ischemia–reperfusion injury, and glutamate/calcium overload (Fulda, 2013). Taken together, our results suggest that LOCSE does not induce antiproliferative effects through ROS generation in A549 cells. Thus, the stress overload through the introduction of additional ROS may have been neutralized by LOCSE antioxidant compounds, thus preventing the cancer cell death by the ROS pathway.

LOCSE presented *in vitro* antihyperglycemic effects by inhibiting the activity of α-amylase and α-glucosidase (Fig. 2C and D, respectively). The results ranged from 85 to 95% for α-amylase and 82 to 99% for α-glucosidase, demonstrating that LOCSE is able to modulate enzymatic activity. Azevêdo et al. (2014) observed inhibition of α-amylase (about 60%) and α-glucosidase (about 99%) of camu-camu processing residue extracts. According to Fujita et al. (2015), the inhibition of these enzymes suggests possibilities of incorporating camu-camu seed extract into foods to manage early stages of type-2 diabetes.

The hydrogen-atom transfer (antioxidant activity) of the optimized camu-camu seed extract was measured by ORAC, inhibition of Wistar rat's brain homogenate lipid peroxidation, and by the copper-induced oxidation of human LDL. LOCSE had an ORAC value of 28,838 ± 1620 mg CE/100 g and concentrations between 7 and 25 g seeds/L of liquid extract inhibited the Wistar rat's brain lipid peroxidation between 10 and 65%, exhibiting an IC_{50} value of 7.55 g seed/L of extract (Fig. 2E). Regarding the copper-induced LDL oxidation (Fig. 2F), LOCSE was able to decrease the oxidation of human LDL into conjugated dienes. The UV absorbance for the control was increased

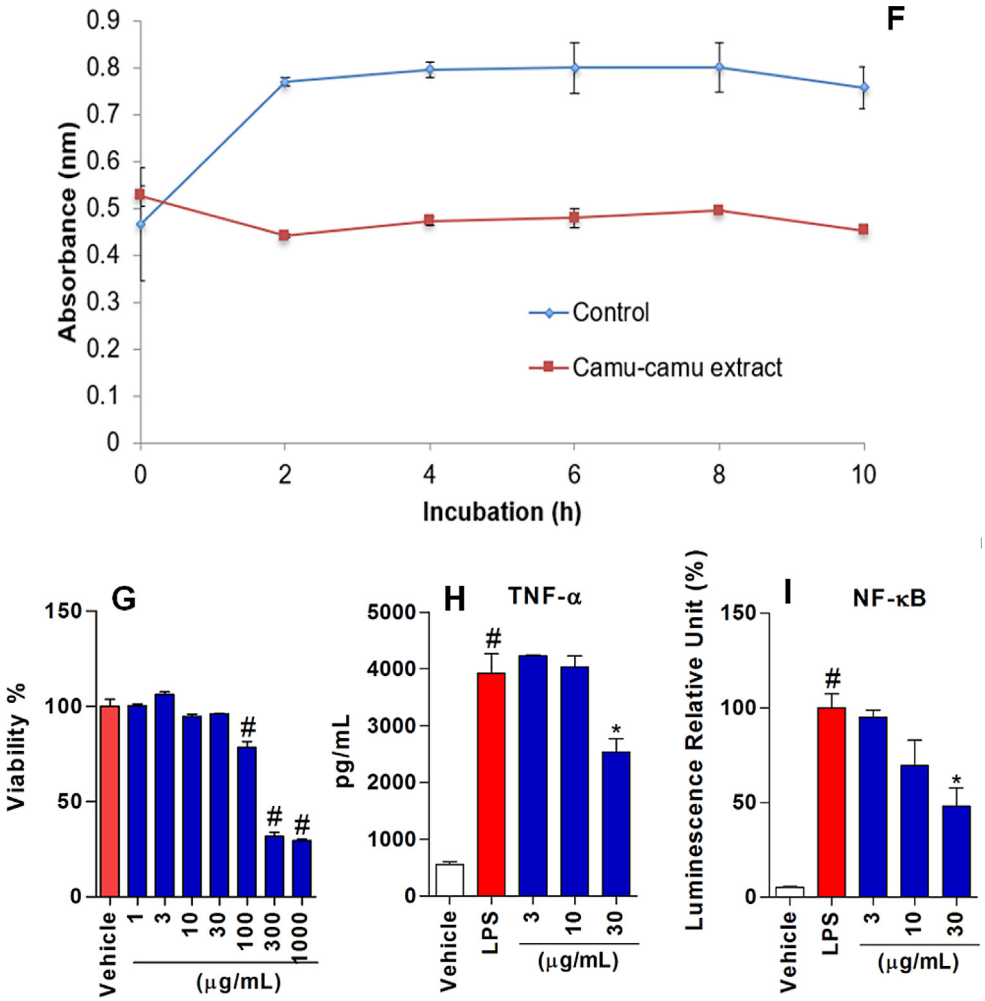

Fig. 2. (*continued*)

during the incubation time, while lower UV absorbance (lower oxidation) was observed when LOCSE was added to tubes. Thus, optimized camu-camu seed extract presented hydrogen atom transfer with considerable quenching effect on peroxyl radicals formed during LDL oxidation. Turgut, Soyer, and Işikçic (2016) found that hydrolysable and condensed tannins may also be responsible for the inhibition of lipid peroxidation *in vitro*. In previous studies, Pereira et al. (2014) reported reduced lipid peroxidation in the liver and serum of rats after consumption of tropical fruit juice containing camu-camu pulp. The results obtained can be attributed to the presence of bioactive compounds (mainly phenolics) that inhibit lipid peroxidation *in vivo* (Koleckar et al., 2008).

For the anti-inflammatory activity *in vitro*, initially we investigated the viability of RAW 264.7 macrophages incubated for 4 h with LOCSE at 1–1000 μg/mL. According to the results shown in Fig. 2G, LOCSE was found to be toxic to cells at concentrations of 100, 300 and 1000 μg/mL ($p \leq 0.05$). Based on these results, we selected the concentrations of 3, 10 and 30 μg/mL (non-toxic) to evaluate *in vitro* TNF-α release and NF-KB activation. As shown in Fig. 2H, the pretreatment with LOCSE at 30 μg/mL reduced the release of TNF-α cytokine in LPS-stimulated macrophages ($p \leq 0.05$). In addition, LOCSE at 30 μg/mL was able to reduce NF-κB activation in macrophage cultures stably transfected with the NF-κB-pLUC gene ($p \leq 0.05$; Fig. 2I), which demonstrates its mechanism of action in modulating TNF-α cytokine production. The inhibitory activity of the LOCSE on TNF-α release and NF-κB activation may be associated with the remarkable presence of phenolic compounds in its composition. Similarly, Guevara et al. (2019) observed that when RAW 264.7 macrophages were treated with LPS to induce cytotoxic damage, flavonoid-rich herbal extracts at 80 μg/mL were able to decrease ROS levels and to increase the ATP production and the enzymatic antioxidant activity (e.g. glutathione peroxidase, superoxide dismutase, and catalase) in macrophages. de Camargo et al. (2019) found that grape processed by-products from wine industry present a wide variety of stilbenoids, phenolic acids and flavonoids that reduced the NF-κB transcription factor activation. In another study, Soares et al. (2019) demonstrated that new Brazilian *superfruits* presented numerous phenolic compounds in their composition, and reduced *in vitro* NF-κB activation and neutrophil migration in inflammation *in vivo*.

4. Conclusions

The use of RSM was effective in assessing the effects of water, ethyl alcohol and propanone on the chemical composition and antioxidant activity of camu-camu seed extracts. The optimized camu-camu seed extract presented *in vitro* antioxidant activity, antimicrobial, antihypertensive, antihemolytic and antihyperglycemic effects. In addition, the optimised camu-camu seed extract displayed cytotoxic effects and antiproliferative activity against cancer cells and showed anti-inflammatory effects using an *in vitro* macrophage-based model. The results suggest that the optimized camu-camu seed extract may be possibility of technological use in the development of functional foods. In that perspective, *in vivo* studies should be performed to corroborate the functional properties of the optimised camu-camu seed extract.

Authors contributions

M. Fidelis, M.A.V. do Carmo, T.M. da Cruz, M. Wen, W.Y. Oh, M. Franchin, M.M. Furtado, and T. Myoda performed the chemical, cell-based assays and the biological activities of extracts, helped in data analysis and in the preparation of the draft. L. Azevedo, M.B. Marques, A.S. Sant'Ana, M.I. Genovese, F. Shahidi, L. Zhang, S.M. de Alencar, and P.L. Rosalen supervised the experimental activities, drafted the article and revised it critically. D. Granato conceived the idea, coordinated all activities, validated the chemical and antioxidant activity measurements, performed the statistical analyses, and drafted the paper. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Authors thank CAPES (*Coordination for the Improvement of Higher Education Personnel*) for partially funding the work, CAPES for the graduate scholarships, and C-LABMU-UEPG (Complexo de Laboratórios Multiusuários – State University of Ponta Grossa) for the infrastructure. D. Granato acknowledges CNPq (Brazilian National Council for Scientific and Technological Development) for a productivity grant (303188/2016-2) and financing (405890/2018-4). The help from Prof. Adriano G. Cruz in the analysis of ACE-I is also acknowledged. A. S. Sant'Ana thanks CNPq (Grants #302763/2014-7 and #305804/2017-0). This study was financed, in part, by the CAPES - Finance Code 001. We thank the partial financial support of Natural Resources Institute Finland (Luke project 41007-00173600).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.125909.

References

Ambigaipalan, P., Al-Khalifa, A. S., & Shahidi, F. (2015). Antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities of date seed protein hydrolysates prepared using Alcalase, Flavourzyme and Thermolysin. *Journal of Functional Foods, 18*, 1125–1137.

Azevêdo, J. C. S., Fujita, A., Oliveira, E. L., Genovese, M. I., & Correia, R. T. P. (2014). Dried camu-camu *(Myrciaria dubia* HBK McVaugh) industrial residue: A bioactive-rich Amazonian powder with functional attributes. *Food Research International, 62*, 934–940.

Azevedo, L., de Araujo Ribeiro, P. F., de Carvalho Oliveira, J. A., Correia, M. G., Ramos, F. M., de Oliveira, E. B., & Stringheta, P. C. (2019). Camu-camu *(Myrciaria dubia*) from commercial cultivation has higher levels of bioactive compounds than native cultivation (Amazon Forest) and presents antimutagenic effects *in vivo*. *Journal of the Science of Food and Agriculture, 99*(2), 624–631.

Barros, L., Calhelha, R. C., Queiroz, M. J. R., Santos-Buelga, C., Santos, E. A., Regis, W. C., & Ferreira, I. C. (2015). The powerful *in vitro* bioactivity of Euterpe oleracea Mart. seeds and related phenolic compounds. *Industrial Crops and Products, 76*, 318–322.

Borges, L. L., Martins, F. S., Conceição, E. C., & Silveira, D. (2017). Optimization of the spray-drying process for developing Jabuticaba waste powder employing response surface methodology. *Journal of Food Process Engineering, 40*(1), e12276.

Cheimonidi, C., Samara, P., Polychronopoulos, P., Tsakiri, E. N., Nikou, T., Myrianthopoulos, V., ... Argyropoulou, A. (2018). Selective cytotoxicity of the herbal substance acteoside against tumor cells and its mechanistic insights. *Redox Biology, 16*, 169–178.

Chirinos, R., Galarza, J., Betalleluz-Pallardel, I., Pedreschi, R., & Campos, D. (2010). Antioxidant compounds and antioxidant capacity of Peruvian camu camu *(Myrciaria dubia* (HBK) McVaugh) fruit at different maturity stages. *Food Chemistry, 120*(4), 1019–1024.

Cleeland, L., & Squires, E. (1991). Evaluation of new antimicrobials in vitro and experimental animal infections. In V. M. Lorian (Ed.). Antibiotics in laboratory medicine, 3, (pp. 739–788).

Dai, J., & Mumper, R. J. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules, 15*(10), 7313–7352.

Derringer, G., & Suich, R. (1980). Simultaneous optimization of several response variables. *Journal of Quality Technology, 12*(4), 214–219.

Escher, G. B., Santos, J. S., Rosso, N. D., Marques, M. B., Azevedo, L., Carmo, M. A. V., ... da Silva, M. C. (2018). Chemical study, antioxidant, anti-hypertensive, and cytotoxic/cytoprotective activities of Centaurea cyanus L. petals aqueous extract. *Food and Chemical Toxicology, 118*, 439–453.

de Camargo, A. C., Biasoto, A. C. T., Schwember, A. R., Granato, D., Rasera, G. B., Franchin, M., Rosalen, P. L., Alencar, S. M., & Shahidi, F. (2019). Should we ban total phenolics and antioxidant screening methods? The link between antioxidant potential and activation of NF-κB using phenolic compounds from grape by-products. *Food Chemistry, 290*, 229–238.

do Carmo, M. A. V., Fidelis, M., Pressete, C. G., Marques, M. J., Castro-Gamero, A. M., Myoda, T., Granato, D., & Azevedo, L. (2019). Hydroalcoholic Myrciaria dubia (camu-camu) seed extracts prevent chromosome damage and act as antioxidant and cytotoxic agents. *Food Research International, 125* ID: 108551.

Fidelis, M., Santos, J. S., Escher, G. B., do Carmo, M. V., Azevedo, L., da Silva, M. C., Putnik, P., & Granato, D. (2018). In vitro antioxidant and antihypertensive compounds from camu-camu (Myrciaria dubia Mc vaugh, Myrtaceae) seed coat: A multivariate structure-activity study. *Food and Chemical Toxicology, 120*, 479–490.

Fracassetti, D., Costa, C., Moulay, L., & Tomás-Barberán, F. A. (2013). Ellagic acid derivatives, ellagitannins, proanthocyanidins and other phenolics, vitamin C and antioxidant capacity of two powder products from camu-camu fruit (*Myrciaria dubia*). *Food Chemistry, 139*(1–4), 578–588.

Franchin, M., Rosalen, P. L., Da Cunha, M. G., Silva, R. L., Colon, D. F., Bassi, G. S., ... Cunha, T. M. (2016). Cinnamoyloxy-mammeisin isolated from geopropolis attenuates inflammatory process by inhibiting cytokine production: Involvement of MAPK, AP-1, and NF-kB. *Journal of Natural Products, 79*, 1828–1833.

Fujita, A., Sarkar, D., Wu, S., Kennelly, E., Shetty, K., & Genovese, M. I. (2015). Evaluation of phenolic-linked bioactives of camu-camu *(Myrciaria dubia* Mc. Vaugh) for anti-hyperglycemia, antihypertension, antimicrobial properties and cellular rejuvenation. *Food Research International, 77*, 194–203.

Fujita, A., Souza, V. B., Daza, L. D., Fávaro-Trindade, C. S., Granato, D., & Genovese, M. I. (2017). Effects od spray-drying parameters on in vitro functional properties of camu-camu (Myrciaria dubia Mc. Vaugh): A typical Amazonian fruit. *Journal of Food Science, 82*(5), 1083–1091.

Fulda, S. (2013). The mechanism of necroptosis in normal and cancer cells. *Cancer Biology & Therapy, 14*(11), 999–1004.

Genovese, M. I., Da Silva Pinto, M. D. S., Gonçalves, A. E. D. S., & Lajolo, F. M. (2008). Bioactive compounds and antioxidant capacity of exotic fruits and commercial frozen pulps from Brazil. *Food Science and Technology International, 14*(3), 207–214.

Gonçalves, A. E. S. S., Lajolo, F. M., & Genovese, M. I. (2010). Chemical composition and antioxidant/antidiabetic potential of Brazilian native fruits and commercial frozen pulps. *Journal of Agricultural and Food Chemistry, 58*(8), 4666–4674.

Granato, D., & de Araújo Calado, V. M. (2014). The use and importance of design of experiments (DOE) in process modelling in food science and technology. *Mathematical and Statistical Methods in Food Science and Technology, 1*, 1–18.

Grigio, M. L., Chagas, E. A., Rathinasabapathi, B., Chagas, P. C., da Silva, A. R. V., Sobral, S. T. M., & de Oliveira, R. R. (2017). Qualitative evaluation and biocompounds present in different parts of camu-camu *(Myrciaria dubi*a) fruit. *African Journal of Food Science, 11*(5), 124–129.

Guevara, M., Tejera, E., Iturralde, G. A., Jaramillo-Vivanco, T., Granda-Albuja, M. G., Granda-Albuja, S., Santos-Buelga, C., González-Paramás, A. M., & Álvarez-Suarez, J. M. (2019). Anti-inflammatory effect of the medicinal herbal mixture infusion, Horchata, from southern Ecuador against LPS-induced cytotoxic damage in RAW 264.7 macrophages. *Food and Chemical Toxicology,* 131.

Johnson, M. H., Lucius, A., Meyer, T., & Gonzalez de Mejia, E. (2011). Cultivar evaluation and effect of fermentation on antioxidant capacity and in vitro inhibition of α-amylase and α-glucosidase by highbush blueberry (*Vaccinium corombosum*). *Journal of Agricultural and Food Chemistry, 59*(16), 8923–8930.

Kaneshima, T., Myoda, T., Nakata, M., Fujimori, T., Toeda, K., & Nishizawa, M. (2016). Antioxidant activity of C-Glycosidic ellagitannins from the seeds and peel of camu-camu *(Myrciaria dubi*a). *LWT-Food Science and Technology, 69*, 76–81.

Kaneshima, T., Myoda, T., Toeda, K., Fujimori, T., & Nishizawa, M. (2013). Antioxidative constituents in camu-camu fruit juice residue. *Food Science and Technology Research, 19*(2), 223–228.

Koleckar, V., Kubikova, K., Rehakova, Z., Kuca, K., Jun, D., Jahodar, L., & Opletal, L. (2008). Condensed and hydrolysable tannins as antioxidants influencing the health. *Mini Reviews in Medicinal Chemistry, 8*(5), 436–447.

Migliorini, A. A., Piroski, C. S., Daniel, T. G., Cruz, T. M., Escher, G. B., do Carmo, M. A. V., Azevedo, L., Marques, M. B., Granato, D., Rosso, N. D., & Red (2019). Chicory (Cichorium intybus) extract rich in anthocyanins: chemical stability, antioxidant activity, and antiproliferative activity in vitro. *Journal of Food Science, 84*(5), 990–1001.

Myoda, T., Fujimura, S., Park, B., Nagashima, T., Nakagawa, J., & Nishizawa, M. (2010). Antioxidative and antimicrobial potential of residues of camu-camu juice production. *Journal of Food, Agriculture & Environment, 8*(2), 304–307.

Peixoto, M. S., Galvão, M. F. O., & Medeiros, S. R. B. (2017). Cell death pathways of particulate matter toxicity. *Chemosphere, 188*, 32–48.

Pereira, A. C. S., Wurlitzer, N. J., Dionísio, A. P., Soares, M. V. L., Bastos, M. D. S. R., Alves, R. E., & Brasil, I. M. (2015). Synergistic, additive and antagonistic effects of fruit mixtures on total antioxidant capacities and bioactive compounds in tropical fruit juices. *Archivos Latinoamericanos de Nutricion, 65*(2).

Pereira, A. C. S., Dionísio, A. P., Wurlitzer, N. J., Alves, R. E., de Brito, E. S., Silva, A. M. D. O., ... Mancini Filho, J. (2014). Effect of antioxidant potential of tropical fruit juices on antioxidant enzyme profiles and lipid peroxidation in rats. *Food Chemistry, 157*, 179–185.

Prayong, P., Barusrux, S., & Weerapreeyakul, N. (2008). Cytotoxic activity screening of some indigenous Thai plants. *Fitoterapia, 79*(7–8), 598–601.

Rietjens, I. M., Boersma, M. G., van der Woude, H., Jeurissen, S. M., Schutte, M. E., & Alink, G. M. (2005). Flavonoids and alkenylbenzenes: Mechanisms of mutagenic action and carcinogenic risk. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 574*(1–2), 124–138.

Říha, M., Karlíčková, J., Filipský, T., Macáková, K., Rocha, L., Bovicelli, P., ... Mladěnka, P. (2014). *In vitro* evaluation of copper-chelating properties of flavonoids. *Rsc Advances, 4*(62), 32628–32638.

Sato, Y., Yamakose, H., & Suzuki, Y. (1993). Mechanism of hypotonic hemolysis of human erythrocytes. *Biological and Pharmaceutical Bulletin, 16*(5), 506–512.

Setford, P. C., Jeffery, D. W., Grbin, P. R., & Muhlack, R. A. (2017). Factors affecting extraction and evolution of phenolic compounds during red wine maceration and the role of process modelling. *Trends in Food Science & Technology, 69*, 106–117.

Santos, J. S., Brizola, V. R. A., & Granato, D. (2017). High-throughput assay comparison and standardization for metal chelating capacity screening: A proposal and application. *Food Chemistry, 214*, 515–522.

Santos, J. S., Deolindo, C. T., Hoffmann, J. F., do Prado-Silva, F. C. L., Sant'Ana, A. S., Azevedo, L., do Carmo, M. A. V., & Granato, D. (2018). Optimized Camellia sinensis var. sinensis, Ilex paraguariensis, and Aspalathus linearis blend presents high antioxidant and antiproliferative activities in a beverage model. *Food Chemistry, 254*, 348–358.

Soares, J. C., Rosalen, P. L., Lazarini, J. G., Massarioli, A. P., da Silva, C. F., Nani, B. D., ... Alencar, S. M. (2019). Comprehensive characterization of bioactive phenols from new Brazilian superfruits by LC-ESI-QTOF-MS, and their ROS and RNS scavenging effects and anti-inflammatory activity. *Food Chemistry, 281*, 178–188.

Turgut, S. S., Soyer, A., & Işıkçı, F. (2016). Effect of pomegranate peel extract on lipid and protein oxidation in beef meatballs during refrigerated storage. *Meat Science, 116*, 126–132.

Yazawa, K., Suga, K., Honma, A., Shirosaki, M., & Koyama, T. (2011). Anti-inflammatory effects of seeds of the tropical fruit camu-camu *(Myrciaria dubia*). *Journal of Nutritional Science and Vitaminology, 57*(1), 104–107.

Zhou, D.-Y., Sun, Y.-X., & Shahidi, F. (2017). Preparation and antioxidant activity of tyrosol and hydroxytyrosol esters. *Journal of Functional Foods, 37*, 66–73.