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Chemical characterization and anti-inflammatory and antioxidant potential of fruits of *Eugenia candolleana* DC

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Caracterización química y potencial antiinflamatorio y antioxidante de frutos de *Eugenia candolleana* DC

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Abstract
The aim of this study was to present a comprehensive evaluation of the chemical composition, anti-inflammatory and antioxidant activities of the pulp and seeds of *Eugenia candolleana* fruits collected at two stages of maturation. The results showed that fruits are a good source of vitamins and minerals such as thiamine and riboflavin, iron, phosphorus, magnesium, manganese, potassium and zinc, besides high amounts of protein and dietary fiber. The fruits showed a higher proportion of unsaturated fatty acids, with a predominance of linoleic acid in all samples. The seeds presented a significantly higher content of phenolic and tannins than pulp, however, anthocyanins were found exclusively in the pulp and skin of the fruits. Maturation affected the composition of phenolics where the mature stage presented significantly fewer phenolic compounds. Quercetin, pyrogallol and gallic acid were the main compounds identified in the pulp and seeds. Both pulp and seeds presented potential antioxidant and anti-inflammatory activities, however, the seeds have significantly higher activity. The seed extracts significantly reduced the production of nitric oxide and the cytokines IL-6 and TNF-α in macrophages, highlighting a potential anti-inflammatory activity. The fruits can be included in diets as a strategy to improve the diversity of food supply, in addition, the seed flour can be used as a potential food supplement, besides valuing agro-industrial by-products.
Keywords: TNF-α; Cytokines; Anti-inflammatory; Seed flour; Vitamin; Quercetin.

Resumo
O objetivo deste estudo foi apresentar uma avaliação abrangente da composição química, atividades anti-inflamatória e antioxidante da polpa e sementes de frutos de *Eugenia candolleana* coletados em dois estádios de maturação. Os resultados mostraram que as frutas são uma boa fonte de vitaminas e minerais como tiamina e riboflavina, ferro, fósforo, magnésio, manganês, potássio e zinco, além de altas quantidades de proteína e fibra alimentar. Os frutos apresentaram maior proporção de ácidos graxos insaturados, com predominância de ácido linoleico em todas as amostras. As sementes apresentaram teor de fenólicos e taninos significativamente maior do que a polpa, porém, antocianinas foram

encontradas exclusivamente na polpa e casca dos frutos. A maturação afetou a composição de fenólicos onde o estágio maduro apresentou significativamente menos compostos fenólicos. A quercetina, o pirogalol e o ácido gálico foram os principais compostos identificados na polpa e nas sementes. Tanto a polpa quanto as sementes apresentaram potencial atividade antioxidante e anti-inflamatória, porém, as sementes apresentam atividade significativamente maior. Os extratos de sementes reduziram significativamente a produção de óxido nítrico e das citocinas IL-6 e TNF-α em macrófagos, destacando uma potencial atividade anti-inflamatória. Os frutos podem ser incluídos em dietas como estratégia para melhorar a diversidade de oferta de alimentos, além disso, a farinha de sementes pode ser utilizada como um potencial suplemento alimentar, além de valorizar subprodutos agroindustriais.
Palavras-chave: TNF-α; Citocinas; Anti-inflamatório; Farinha de semente; Vitamina; Quercetina.

Resumen
El objetivo de este estudio fue presentar una evaluación integral de la composición química, actividades antiinflamatorias y antioxidantes de la pulpa y semillas de frutos de Eugenia candolleana recolectados en dos etapas de maduración. Los resultados mostraron que las frutas son una buena fuente de vitaminas y minerales como tiamina y riboflavina, hierro, fósforo, magnesio, manganeso, potasio y zinc, además de altas cantidades de proteína y fibra dietética. Los frutos presentaron mayor proporción de ácidos grasos insaturados, con predominio del ácido linoleico en todas las muestras. Las semillas presentaron un contenido significativamente mayor de fenoles y taninos que la pulpa, sin embargo, las antocianinas se encontraron exclusivamente en la pulpa y piel de los frutos. La maduración afectó la composición de fenoles donde la etapa madura presentó significativamente menos compuestos fenólicos. La quercetina, el pirogalol y el ácido gálico fueron los principales compuestos identificados en la pulpa y las semillas. Tanto la pulpa como las semillas presentaron potenciales actividades antioxidantes y antiinflamatorias, sin embargo, las semillas tienen una actividad significativamente mayor. Los extractos de semillas redujeron significativamente la producción de óxido nítrico y las citoquinas IL-6 y TNF-α en los macrófagos, destacando una potencial actividad antiinflamatoria. Los frutos pueden ser incluidos en las dietas como una estrategia para mejorar la diversidad de la oferta alimentaria, además, la harina de semilla puede ser utilizada como un potencial complemento alimenticio, además de valorizar los subproductos agroindustriales.
Palabras clave: TNF-α; Citoquinas; Antiinflamatorio; Harina de semillas; Vitamina; Quercetina.

1. Introduction

Amongst macronutrients, such as carbohydrates, proteins, and lipids, fruits have several bioactive compounds important to the body's metabolism, that can prevent various diseases due to their pharmacological activity. B-group vitamins, such as riboflavin (B2), play an important role in energy metabolism. The two flavoprotein coenzymes derived from riboflavin, FMN and FAD are crucial rate-limiting factors in most cellular enzymatic processes (Kennedy, 2016). Thiamine deficiency presents many challenges to clinicians due to the broad clinical spectrum, referred to as thiamine deficiency disorders (TDDs), affecting the metabolic, neurologic, cardiovascular, respiratory, gastrointestinal, and musculoskeletal systems (Smith et al., 2021). In addition, minerals play a significant role as enzymatic cofactors, even contributing to the reduction of reactive oxygen substances (ROS) (de Moura et al., 2022).

Inflammation usually occurs in response to a tissue injury leading to the activation of intracellular pathways for cell signaling. As a result, numerous inflammatory mediators are synthetized and secreted during inflammatory responses of different types, such as TNF-α and IL-6 cytokines (Azab et al., 2016). Free radicals such as ROS are also related to diseases associated with elevated inflammatory signaling and an altered redox balance. Therefore, the new anti-inflammatory investigation and free radical scavenging agents remain relevant. Another class of compounds abundant in fruits are phenolic compounds, to which numerous pharmacological properties are related, including antioxidant activity and anti-inflammatory activity, such as the flavonoid quercetin (Guss et al., 2017; Li et al., 2016; Milanezi et al., 2019).

Fruit species belonging to the Eugenia genus have vast economic and pharmacological potential, as well as extensive commercial exploitation of their edible parts, wood, essential oils and use as ornamental plants. In Brazil, there are about 400 species belonging to the genus Eugenia, distributed throughout the country, including native species from the Atlantic Forest, such as *Eugenia brasiliensis*, *Eugenia leitonii*, *Eugenia involucrata* DC., and *Eugenia dysenterica* DC. (Gonçalves et al., 2010; Infante et al., 2016; Teixeira et al., 2015). *Eugenia candolleana*, also known as cambuí-roxo, is an endemic species from Brazil

found at open fairs as fresh fruit or in agro-industrial products, such as jellies and liqueurs. However, there are no previous studies on the nutritional composition of fruits, and biological potential, mainly from seed flour, valuing agro-industrial products. Therefore, for the first time, this study presents an extensive evaluation of the chemical composition and the antioxidant and anti-inflammatory activities of pulp and seeds of *E. candolleana* fruits collected in two maturation stages.

2. Methodology

2.1 Samples

Seven kilograms of fruit were randomly selected from specimens in the town of Domingos Martins in Espírito Santo, Brazil (-19.931572; -40.615278; 688 m). Cambuí-roxo (*E. candolleana* DC.) specimens were collected at two different stages of ripeness and classified as ripe or semi-ripe based on the color and texture of the flesh (Figure 1). The plant was identified, and one specimen was included in the UVV herbarium (no. 2621). Fruits were washed with deionized water and crushed by hand. Ripe and semi-ripe pulp samples were labeled RP and SRP, and seeds were labeled RS and SRS. Half of the samples were used for proximate composition analysis, and the other part was freeze-dried, ground in a mill, and stored at -20 ºC for further analysis. Eighty fruit units were randomly selected to evaluate the physical aspects, 40 from each stage of ripeness to assess the fruit size and average weight.

Figure 1 - Fruits of *Eugenia candolleana*.

Source: Authors (personal collection).

2.2 Proximate composition

The analyzes were performed in triplicate according to the methodology described by the Association of the Official Analytical Chemists (AOAC, 2016). Moisture content was determined in an oven at 75ºC (to avoid caramelization) until constant weight; ash content was determined by incineration at 550ºC until constant weight; total proteins were evaluated by the Kjeldahl method, and total fat was determined by the Soxhlet method. The determination of the total, soluble and insoluble dietary fiber content was performed by the enzymatic-gravimetric method, based on the official methodology of AOAC 991.43 using the total dietary fiber analysis kit. (Total dietary fiber assay kit, Sigma®). The total carbohydrate content was calculated by subtracting the sum of the remaining contents (moisture + ash + lipid + protein + fiber). Calories were calculated using the formula: [(total carbohydrate x 4) + (proteins x 4) + (total fat x 9)].

2.3 Determination of glucose, fructose and sucrose by HPLC

Extraction was performed by weighing 1 g of freeze-dried sample, adding 20 mL of water at 80°C, sonicating for 10

min, and filtering in filter paper. The procedure was repeated twice, and the volume was completed in a 50 mL volumetric flask. Each sample extract was filtered through a 0.45 µm membrane. We evaluate fructose, glucose, and sucrose in high-performance liquid chromatography (Breeze, Waters) in conjunction with a refractive index detector (Waters 2414). Sugars were separated on a Waters Carbohydrate Analysis column (3.9 x 300 mm; 10 µm) in isocratic elution mode. Acetonitrile: water (75:25) solution at a flow rate of 1.0 mL/min was used in the mobile phase. Identification was performed by retention times and co-chromatography. Quantification was performed by an external standardization curve with 5 points in triplicate (at concentrations of 1.5; 1.0; 0.75; 0.50; and 0.25 mg/mL) for each sugar. Calibration curves were also used to evaluate the linearity range. Limits of detection (LD) and quantification (LQ) were calculated by the signal-to-noise ratio, where LD was defined as the concentration of the analyte necessary to produce a signal three times higher than the noise amplitude, while LQ was defined as the analyte concentration enough to produce a signal six times higher than the noise.

2.4 Elemental composition

A Speedwave four microwave oven (Berghoff Instruments, Eningen, Germany) with a 12-tube rotor, 60 mL capacity, maximum power of 2000 W, pressure, and temperature limit of 100 bar and 230°C, respectively, was used for sample digestion. 0.5 g of the samples were weighed into the microwave tube barges with 2 mL of 70% (v/v) HNO_3 and 0.5 mL of 30% (v/v) H_2O_2. After digestion, the resulting solution was quantitatively transferred to a polypropylene tube and the volume has been filled with ultrapure water to 10 mL. The analysis was performed by inductively coupled plasma optical emission spectrometry iCAP 6000 (Thermo Fisher Scientific, Cambridge, England) with radial and axial vision. The sample introduction system consisted of a concentric nebulizer coupled to a cyclonic nebulizer chamber and peristaltic pump. ICP OES operating conditions for analysis were: 1200 W of RF power, 12 L/min plasma gas flow, 0.5 L/min auxiliary gas flow, 0.65 L nebulizer gas flow/min, 0.6 mL/min sample flow with a concentric nebulizer and cyclonic nebulization chamber. All samples were analyzed in triplicate. Quantitation was performed by an external standardization curve with 5 points in triplicate for each metal using individual standard solutions (Certified Reference Material).

2.5 Determination of organic acids by HPLC

The extraction was performed by weighing 1.0 g of lyophilized sample in a 50 mL volumetric flask and adjusted with phosphate buffer (KH_2PO_4 - 0.01M, pH 2.6). After 15 minutes in an ultrasonic bath (80 Hz), the solution was filtered on filter paper, filtered through a 0.45 µm membrane, and injected into the chromatograph. The determination of tartaric, malic, ascorbic, and citric acids was performed on a liquid chromatograph (Breeze, Waters) according to Scherer et al. (2008). Quantification was performed by an external calibration curve with 7 points. Limit of detection (LD) and quantification (LQ) limits were calculated by the signal-to-noise ratio, where LD was defined as the analyte concentration producing a signal three times higher than the noise amplitude and six times for the LQ.

2.6 Determination of B-group vitamins by LC-MS/MS

The analyses were performed according to Riches (2009). 500 mg of lyophilized sample were extracted with 10 mL of a solution of ethanol/H_2O 1:1 (v:v) with 0.2 mM HCl in an ultrasonic bath for 10 minutes. After, the samples were centrifuged for 1 minute at 12,000 rpm, and 1 mL of the solution was diluted with 1 mL H_2O and transferred to a 2 mL vial for LC/MS/MS analysis. The equipment used was a liquid chromatograph Acquity UPLC I-Class (Waters Corporation, Milford, MA, US) combined with a tandem mass quadrupole mass detector Xevo TQ-S micro (Waters Corporation, Milford, MA, US) with electrospray ionization (LC-ESI-MS/MS) in positive mode [M + H]+. An Acquity UPLC BEH C18 100 mm x 2.1 mm; 1.7 µm (Waters Corporation, Milford, MA, US) column was used at 45°C. The mobile phase consisted of an aqueous solution of formic

acid (0.1%) (A) and acetonitrile (B) using a gradient elution at 0.4 mL/min, starting with 1% B from 0 to 2 min, 1-55% B in 2-3 min, 55-99% B in 3.0-3.1min, 99% B in 3.1-4.0 min, 99-1% B in 4.0-4.1 and 1% B from 4.0 to 5.0 min for column conditioning for the next injection. The ESI ion source parameters were capillary voltage 3.0 kV, cove voltage as optimized for each compound, source temperature 130°C, desolvation temperature and flow were 650°C and 1200 L/h, respectively. The samples were diluted with HPLC ethanol (0.01 mg/mL) and filtered (0.45 µm). The analyses were performed in SRM mode (Selected Reaction Monitoring). Quantification was performed by external calibration curve with 7 points. Limit of detection (LD) and quantification (LQ) limits were calculated by the signal-to-noise ratio, where LD was defined as the analyte concentration producing a signal three times higher than the noise amplitude and six times for the LQ.

2.7 Fatty acids profile

Extraction of lipids from seeds and pulps was performed according to the methodology described by Bligh and Dyer (1959), and derivatization according to the method described by Joseph and Ackman (1992). The methyl esters were analyzed in a gas chromatograph (Shimadzu model GC-2014, Kyoto, Japan) coupled to a flame ionization detector (FID), with an HP-INNOWAX capillary column (50 m x 0.20 mm d.i x 0.20 µm) (Agilent, Santa Clara, USA) under the following chromatographic conditions: injector at 250°C, operating in split mode 1:10 for 1.0 min; nitrogen flow at 1.25 mL/min; detector temperature: 260°C; oven temperature ramp: starting at 150°C, increasing from 10°C / min to 260°C maintaining this temperature for 9 min. A fatty acid methyl ester solution (GLC-85, Nu-Check-Prep, Elysian, USA) was injected into the GC-FID under the same conditions as the sample for compound identification. Quantification of fatty acid methyl esters was performed by the internal standard method using methyl tricosanoate (C23:0Me, Nu-Check-Prep, Elysian, USA). Analyses were performed in triplicate.

2.8 Extracts

The extraction was performed using 20 g of freeze-dried material in 200 mL of 99.5% ethanol by the ultrasound-assisted maceration method (Elmasonic P 60 Hz Elma®). After 2 cycles of 30 min, the extracts were filtered through filter paper. This process was repeated twice. The organic fractions were pooled and evaporated in a rotary evaporator (Fisatom® 802 – 1200W) until ethanol was removed, with subsequent freeze drying for 24 h.

2.9 Total phenolic compounds and tannins

The procedure was performed according to the methodology described by Krepsky et al. (2012), with adaptations. For the analysis, 60 mg of the extracts were weighed and solubilized with 2 mL of water + 3 mL of ethanol. After solubilization, 150 mL of water-free carbon dioxide was added, and this solution was kept in a water bath at 60°C with reflux for 30 minutes. After cooling, the solution was transferred to a 250 mL volumetric flask and the volume was completed with water-free carbon dioxide. The entire contents of the flask were filtered, being called solution A (total polyphenols). To determine tannins, 20 mL of solution A was transferred to an Erlenmeyer flask, protected from light, with 200 mg of Polyvinylpyrrolidone and stirred for 60 minutes. After this time, the solution was filtered and called solution B (non-adsorbed polyphenols). For quantification, 25 µL of the sample solution, A or B, were transferred to a 96-well microplate, together with 10 µL of folin-ciocalteu reagent (10%) and 215 µL of sodium carbonate (10.6% w/v). After three minutes, the reading was performed in a microplate reader at a wavelength of 715 nm, using water as a blank. As standard, an analytical curve with gallic acid (0.625 – 15 µg/mL) was used, with each point analyzed in triplicate and on three different days.

2.10 Total monomeric anthocyanins

Total anthocyanins were determined using the differential pH method (Giusti & Wrolstad, 2001). The extracts (5

mg/mL) were prepared in two different buffers (potassium chloride and sodium acetate) and the absorbance was monitored according to equation 1: A = (A520nm – A700nm) pH1.0 – (A520nm – A700nm) pH4.5. The concentration (mg/g of extract) of anthocyanins was expressed as cyanidin-3-glycoside and calculated using equation 2: (A x MW × DF × 1000) / (ε × L); where A is obtained in equation 1, MW is the molecular weight for cyanidin-3glycoside, DF is the dilution factor, ε is molar absorptivity (26,900), and L is the light path (1 cm).

2.11 Phenolic compounds profile by LC-HRMS

The analysis of the phenolic compounds was performed by liquid chromatography coupled with high-resolution mass spectrometry (Orbitrap) according to Barbosa et al. (2018). Briefly, 0.1 g of the freeze-dried samples were extracted with ultrasound-assisted maceration, using 10 mL of acetone (70), water (29.9), and hydrochloric acid (0.1) solution (v/v/v). The supernatant obtained after centrifugation (3500 rpm, 15 min) was filtered (0.45 μm) and kept at −4°C until analysis. The extracts were analyzed on an Accela UHPLC system coupled to a Q-Exactive Orbitrap HRMS mass detector (Thermo Fisher Scientific, San Jose, CA, USA). Separation was performed on a porous layer (150 × 2.1 mm, 2.7 μm) Ascentis Express C18 reversed-phase column (Supelco, Bellefonte, PA, USA), with gradient elution with mobile phase A: aqueous solution of 0.1% formic acid, and mobile phase B: acetonitrile acidified with 0.1% formic acid. Conditions were as follows: 0-1 min 10% B (isocratic); 1−20 min, linear gradient from 10% to 95% B; 20−23 min 95% B (isocratic); 23−24 min 10% B; and 24 to 30 min for column rebalancing. The mobile phase flow rate was 0.3 mL/min, and the injection volume was 10 μL. The mass detector was equipped with a heated electrospray ionization source (HESI-II) operated in negative and positive ionization modes. HESI-II heater temperature at 350°C and capillary voltage at −2.5 kV were applied. The instrument capillary temperature was set at 320°C, and a Slens RF level of 50V was used. The HRMS instrument was operated in full MS scan mode with an m/z range of 100 to 1500 at a mass resolution of 70,000 full widths to half maximum (fwhm) at m/z 200. The raw HRMS data were processed by the software ExactFinder v2.0 (Thermo Fisher Scientific), applying an accurate mass database list. Parameters including chromatographic retention time, accurate mass errors, isotopic patterns and product ion spectra with scaled normalized collision energies were used for identification and confirmation purposes.

2.12 Antioxidant assay

2.12.1 DPPH scavenging activity

The antioxidant activities of the extracts were evaluated by the DPPH method, according to Scherer and Godoy (2009). The radical scavenging activity was calculated as follows: I (%) = [(Abs0 – Abs1) / Abs0] x 100, where Abs0 is the absorbance of the blank and Abs1 is the absorbance in the presence of the test compound at different concentrations. The IR50 (concentration capable of reducing by 50% the free radicals) was determined using a linear range calibration curve by plotting the final concentration of the extract versus the corresponding scavenging effect.

2.12.2 ABTS scavenging activity

The antioxidant activity was determined according to Re et al. (1999), with modifications. The ABTS radical cation (ABTS•+) was formed by the reaction among 7.0 mM ABTS (in 50% ethanol) and 2.45 mM potassium persulfate (in distilled water). We stored this reagent under refrigeration for at least 24 h. Before use, the reagent was diluted with 50% ethanol until the measured absorbance at 734 nm was 1.0 (± 0.02). ABTS•+ (270 μL) and the compounds (30 μL of each concentration) were added into 96-well microplates. The blank was 30 μL of ethanol. After reacting for 10 min in the dark, the reading was performed at 734 nm using a microplate reader (SpectraMax 190 Microplate Reader, Molecular Devices, California, USA). The radical scavenging activity was calculated as follows: I (%) = [(Abs0 – Abs1) / Abs0] x 100, where Abs0 is the absorbance of the blank

and Abs1 is the absorbance in the presence of the test compound at different concentrations. The results were expressed as IR50 (concentration capable of reducing 50% of the free radicals) and calculated using a calibration curve in the linear range by plotting the final concentration of the extract versus the corresponding scavenging effect.

2.12.3 Superoxide scavenging activity

The superoxide radical scavenging method was evaluated according to Suzumura et al. (1999). The preparation of the 96-well microplate consisted of adding 40 µL of extract (1000 µg/mL), 40 µL of nitroblue tetrazolium (NBT), 10 µL of phenazine metasulphate (PMS), pH 7.4 buffer solution and finally, for the formation of the superoxide radical, 10 µL of nicotinamide adenine dinucleotide (NADH) was added. The reaction was kept for 10 minutes in the dark and the reading was performed at 560 nm. In the blank was added 40 µL of 50% ethanol. The reduction index (%) of the superoxide radical was calculated through the equation: IR(%)= [(AbsC – AbsT) / AbsC] x 100], where AbsC is the average absorbance of the control and AbsT is the absorbance of the test.

2.13 Anti-inflammatory activity

2.13.1 Cell lines

RAW 264.7 murine macrophages (ATCC® TIB-71™) were purchased from the Cell Bank of Rio de Janeiro, Brazil. Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

2.13.2 Evaluation of cell viability

Cell viability was evaluated by the MTT assay, as described previously (Mosmann, 1983), with slight modifications. Briefly, the RAW 264.7 murine macrophages were seeded at a density of 6 to 8 × 104 cells/mL in 96-well plates and cultured for 24 h in the presence or absence of increasing concentrations (1.0–200 µg/mL) of extracts. After 24 h incubation, 100 µL of MTT (1 mg/mL) were added to each well. The formazan crystals that formed were dissolved with dimethyl sulfoxide. The optical density was measured at 595 nm using a microplate reader (Molecular Devises, Spectra Max 190). The experiments were carried out at least in triplicate.

2.13.3 Nitric oxide analysis

The nitrite concentration in the culture medium was measured as an indicator of nitric oxide (NO) production (Marques et al., 2019). Briefly, RAW 264.7 cells were seeded at a density of 2×105 cells/mL in 96-well plates and cultured in a humidified incubator with 5% CO_2 at 37 °C for 24 h. The cells were pre-treated with phenol red-free medium, followed by 1.0 µg/mL LPS in the presence or absence of increasing concentrations (1–100 µM) of extracts for 20h. After the incubation, NO production was spectrophotometrically evaluated by measuring nitrite concentrations via the Griess reaction. Absorbance was measured in a microplate reader at 540 nm against a calibration curve with sodium nitrite standards. Under the same experimental conditions, cellular viability was examined in parallel by the SRB assay.

2.13.4 Measurement of cytokines

TNF-α and IL-6 were quantified in the supernatant of RAW 264.7 murine macrophages unstimulated or stimulated with LPS by ELISA using specific antibodies (purified and biotinylated) and cytokine standards, according to the manufacturer's instructions (eBioscience, San Diego, California, USA). Optical densities were measured at 450 nm in a microplate reader (Mults-Mode Microplate Reader, Filter Max F5, Molecular Devices Spectra, USA). Cytokine levels were expressed in pg, and

the sensitivities were >10 pg/mL.

2.14 Statistical analysis

Results were expressed as mean ± standard deviation. Data analysis was performed by ANOVA, followed by the Tukey test when a significant difference was verified ($p < 0.05$), using the software GraphPad Prism 8.

3. Results and Discussion

3.1 Physical aspects

The ripe fruits presented dimensions of 2.4 ± 0.3 cm in diameter, 3.3 ± 0.6 cm in length, and an average weight (including seeds) of 9.9 ± 1.5 g. The fruits possess a thin black skin (edible) easily removed from the fruit, the pulp is very thick, soft, astringent, sweet, and purple in color. Also, they have a green seed easily removed from the fruit. The semi-ripe fruits displayed dimensions of 2.3 ± 0.3 cm in diameter, 3.2 ± 0.6 cm in length, and an average weight of 7.7 ± 1.3 g. The thin black skin was more adhered to the fruit and has a rough appearance. The pulp was as harder, astringent, slightly acid, and yellowish in color. With the pulping of the fruits, the ripe ones presented a yield of 90.5% of pulp with peel and 9.5% of seed, whereas, in the semi-ripe fruit, it was observed a yield of 87.7% of pulp with peel and 12.3% of seed.

3.2 Proximate composition

The results of the centesimal composition of fruits are presented in Table 1. In general, the fruit presented high content of water and low calorie, representing approximately 2% of the daily calorie intake recommendation for a healthy adult (2000 kcal). Ripe pulp presented significantly higher total sugars (11.5g/100g) than semi-ripe pulp (7.7g/100g), which was expected, as the accumulation of free sugars in fruits occurs during ripening (Ferrer-Gallego et al., 2010).

The fruit pulps proved to be good sources of dietary fiber (Table 1), which corresponds to approximately 13% of the dietary reference intake (RDI) (Institute of Medicine., 2006). The seeds also presented high amounts of fibers, reaching more than 70% of RDI. As expected, due to the cell wall structure, in both pulp and seed samples, higher insoluble fiber content was observed. Grumixama, a fruit of the same genus, can also be considered a fiber source fruit with 4.6g/100g (Teixeira et al., 2015). Currently, it is known that fibers have other important functions in the body besides improving intestinal function, such as reducing risk factors for cardiovascular disease, weight control, and immune function (Evans, 2020).

Table 1 - Centesimal (g/100g) and elemental (mg/100g) composition of cambuí-roxo fruits (*Eugenia candolleana*).

Component	Semi-ripe pulp	Ripe pulp	Semi-ripe seed	Ripe seed
Moisture	86.3 ± 0.03a	83.2 ± 0.1b	50.5 ± 0.25c	50.2 ± 0.34c
Ash	0.46 ± 0.02b	0.46 ± 0.01b	0.86 ± 0.10a	0.83 ± 0.07a
Lipids	0.54 ± 0.01c	0.33 ± 0.01d	1.64 ± 0.09b	2.03 ± 0.04a
Proteins	1.01 ± 0.08c	0.94 ± 0.07c	3.74 ± 0.14b	4.25 ± 0.32a
Total Carbohydrates	7.73	11.5	21.0	19.6
Fructose	3.33 ± 0.44b	4.82 ± 0.17a	0.17 ± 0.02c	0.26 ± 0.01c
Glucose	3.34 ± 0.44b	4.69 ± 0.10a	0.30 ± 0.03c	0.33 ± 0.02c
Sucrose	0.77 ± 0.09b	ND	1.26 ± 0.43a	1.23 ± 0.13a
Dietary fiber	4.10 ± 0.3b	3.6 ± 0.2b	22.2 ± 1.7a	24.1 ± 1.4a
Insoluble fiber	3.3 ± 0.2	2.3 ± 0.3	19.3 ± 3.8	20.9 ± 4.4
Soluble fiber	0.8 ± 0.1	1.3 ± 0.1	2.9 ± 0.4	3.2 ± 0.5
Kcal (kcal/100 g)	38.9 ± 4.03a	52.6 ± 1.32a	113 ± 2.3b	104 ± 1.8a
Calcium	8.66 ± 0.2	< 0.5	37.6 ± 1.1	< 0.5
Copper	0.04 ± 0.0	0.04 ± 0.0	0.4 ± 0.0	0.30 ± 0.0
Iron	0.17 ± 0.0	0.12 ± 0.01	1.4 ± 0.1	1.56 ± 0.1
Phosphorous	12.66 ± 0.7	9.63 ± 0.8	72.3 ± 2.1	68.4 ± 1.8
Magnesium	6.89 ± 0.1	3.98 ± 0.1	20.3 ± 0.9	12.3 ± 0.4
Manganese	0.07 ± 0.0	0.04 ± 0.0	0.22 ± 0.0	0.13 ± 0.0
Potassium	107.7 ± 6.1	79.0 ± 1.1	288 ± 16	288 ± 14
Selenium	< 0.05	< 0.05	< 0.05	< 0.05
Sodium	< 0.5	< 0.5	4.15 ± 0.1	< 0.5
Zinc	0.13 ± 0.0	0.09 ± 0.0	0.72 ± 0.0	0.6 ± 0.0

Different lowercase letters between columns represent significant differences between samples ($p < 0.05$).
Source: Authors.

Regarding proteins and lipids, the pulps presented amounts below 5g/100g. The seed flour (Ripe seed) has 8.5 g/100g of protein, a significant value when considering populations with food restrictions in underdeveloped countries. Further studies evaluating the composition and bioavailability of this protein could indicate the potential use of this seed to produce a dietary supplement, for example. In the ash analysis, the seeds presented significantly higher values than the pulps, but the ripeness did not influence the ash contents found in both parts of the fruit (Table 1).

3.3 Elemental composition

Seeds can be considered an excellent source of copper (Table 1), with ripe seeds contributing 33% of the RDI, and semi-ripe seeds contributing 40% (Institute of Medicine., 2006). Copper is an essential micronutrient required for fundamental biological processes in all organisms. It can donate and accept electrons and switch between reduced and oxidized states. This property allows it to play a relevant role in oxy-reduction reactions, acting as a catalytic cofactor for the function of numerous enzymes (Arredondo & Núñez, 2005).

Seed flour has an average of 3.0 mg/100g of iron, a value that represents 37% of the RDI (Institute of Medicine., 2006). Iron performs important cellular functions, such as oxygen transport, DNA synthesis, energy production and cell proliferation. It mediates electron transfer by acting as an electron donor in the ferrous state and an acceptor in the ferric state (DeLoughery, 2017). Even considering that iron in vegetables is not equally bioavailable as iron in animal-based foods (Gibson, 2007), cambuí-roxo seeds can be included in the human diet as a more accessible supplementation strategy than flour.

Seed flour is still considered a good source of phosphorus, magnesium, manganese, potassium and zinc, with values around 20%, 12%, 24%, 12%, and 18% of the DRI (Institute of Medicine., 2006). Phosphorus is an abundant element in the human body that plays a role in several physiological processes. We can mention cell signaling, nucleic acid synthesis, neurological activities, electrolyte transport, bone mineralization, and the usual white and red blood cells generation (Shaman &

Kowalski, 2016). Manganese is essential for immune functions, bone growth, blood coagulation and defense against reactive oxygen species (Aschner & Erikson, 2017). Zinc is essential to the structure and function of several enzymes regulating cellular processes and cellular signaling pathways. The mineral modulates immune response and exhibits antioxidant and anti-inflammatory activity (Jarosz et al., 2017). No significant content of selenium and sodium was found.

Table 2 - B-group vitamins and organic acids in cambuí-roxo fruits (*Eugenia candolleana*).

Vitamins / Organic Acids *	Linearity (r2)	LD (µg/mL)	LQ (µg/mL)	Semi-Ripe Pulp	Ripe pulp	Semi-Ripe Seed	Ripe seed
Thiamine (B1)	0.9903	1.4	4.3	5.43 ± 0.33	6.54 ± 0.58	28.8 ± 4.87	33.0 ± 4.0
Riboflavin (B2)	0.996	1.3	3.8	28.9 ± 0.96	68.3 ± 5.05	14.7 ± 0.41	23.8 ± 2.0
Nicotinic acid (B3)	0.9927	0.4	1.1	ND	ND	ND	ND
Pantothenic acid (B5)	0.984	0.3	0.8	1.05 ± 0.03	1.16 ± 0.05	43.3 ± 0.03	53.4 ± 0.2
Pyridoxine (B6)	0.994	0.1	0.4	0.12 ± 0.03	0.21 ± 0.03	2.36 ± 0.13	2.22 ± 0.2
Biotin (B7)	0.9955	1.5	4.7	0.04 ± 0.00	0.07 ± 0.00	0.20 ± 0.01	0.25 ± 0.01
Cyanocobalamin (B12)	0.9995	0.3	0.9	0.02 ± 0.01	ND	0.06 ± 0.00	ND
Ascorbic acid (C)	0.9983	0.2	0.5	2.65 ± 0.12	2.58 ± 0.14	ND	ND
Citric acid	0.9997	0.5	1.1	1,124 ± 56	1,105 ± 52	1,864 ± 124	1,891 ± 35
Malic acid	0.9997	2.7	5.4	ND	ND	653 ± 22	638 ± 76
Tartaric acid	0.9995	1.3	2.7	ND	ND	593 ± 20	693 ± 69

*Results expressed in:
B vitamins: µg/100g of fresh Fruit
Organic Acids: mg/100g of fresh Fruit
ND – Not Detected
Source: Authors

3.4 B-group vitamins and organic acids composition

All B vitamins were found in fruits except for B3 (Table 2). The ripe pulp can be considered a source of riboflavin (5.3% DRI), displaying higher amounts of these components than those found in *Eugenia involucrata* (15.1 µg/100g), *Eugenia uniflora* (24.7 µg/100g), and *Eugenia pyriformis* (2.9 µg/100g) (Schmidt et al., 2019). Riboflavin has been related through direct or indirect mechanisms to the prevention of oxidative stress, in addition to various diseases such as migraine, anemia, cancer, hypertension, and diabetes mellitus (Thakur et al., 2017). The seed flour, besides riboflavin, can be considered a source of thiamine with values around 5% of the DRI (Institute of Medicine., 2006). Citric acid was found in all samples as major organic acid, with levels ranging from 1.1 to 1.9 g/100g of fresh fruit. Tartaric and malic acids were found only in seed (Table 2), and ascorbic acid (vitamin C) was found only in pulp samples, but in quantities that do not impact the daily intake recommendation.

3.5 Fatty acids composition

The predominant fatty acids were linoleic (C18:2 n-6), followed by palmitic acid (C16:0) in the pulp, and oleic acid (C18:1 n-9) in the seeds (Table 3). Similar values were reported for pitanga, where palmitic, oleic and linoleic acids were the predominant (Bagetti et al., 2011). The fruits of cambuí-roxo presented a higher proportion of unsaturated fatty acids (59.4-70.9%) compared to saturated fatty acids (29.1-40.6%). Linoleic and linolenic acids are essential for humans. Omega 6 increases the production of cytokines, with vasoconstrictor action, that promote platelet aggregation, i.e., presents pro-inflammatory action. This activity is related to the occurrence of cardiovascular, autoimmune and inflammatory diseases, such as arthritis, asthma, psoriasis, lupus, and ulcerative colitis. Omega 3 displays antagonistic anti-inflammatory action, promoting vasodilation and inhibition of platelet aggregation, thus being related to the prevention of hypertension, atherosclerosis, hypercholesterolemia, arthritis and other autoimmune and inflammatory diseases, as well as the most diverse cancer. Therefore, diets with an omega 6/omega 3 ratios between 5:1 and 8:1 is recommended (Martin et al., 2006). Cambuí-roxo pulps presented ratio of 6:1 (ripe) and

5:1 (semi-ripe).

Table 3 - Fatty acid composition in cambuí-roxo fruits (*Eugenia candolleana*).

Fatty Acid		Semi-Ripe Pulp	Ripe pulp	Semi-Ripe Seed	Ripe seed
Palmitic Acid	C16:0	32.5 ± 0.28	45.9 ± 1.14	227 ± 5.19	189 ± 4.08
Stearic Acid	C18:0	16.1 ± 0.33	19.5 ± 0.82	68.0 ± 3.80	60.7 ± 2.55
Oleic Acid	C18:1 n-9	15.5 ± 0.87	21.8 ± 0.52	296 ± 2.59	240 ± 5.21
Linoleic Acid	C18:2 n-6	55.3 ± 0.87	85.0 ± 3.29	342 ± 4.93	270 ± 2.86
Linolenic Acid	C18:3 n-3	9.2 ± 0.26	12.7 ± 0.68	137 ± 1.47	106 ± 3.98
Araquidic Acid	C20:0	4.81 ± 0.07	5.74 ± 0.48	27.1 ± 1.12	26.7 ± 0.41
Gadoleic Acid	C20:1 n-9	1.14 ± 0.03	1.43 ± 0.06	36.7 ± 6.48	24.9 ± 1.33
Docosanoic Acid	C22:0	1.93 ± 4.39	2.93 ± 0.18	11.9 ± 0.61	10.9 ± 0.51
Total Saturated (%)		40.6	38.0	29.1	30.9
Total Unsaturated (%)		59.4	62.0	70.9	69.1

Results are expressed in mg/100g of fresh fruit.
Source: Authors.

3.6 Phenolic compounds

Table 4 shows the results of total phenolic compounds, anthocyanins, and tannins, and Table 5 shows the main phenolics identified. The seeds presented phenolic and tannin contents significantly ($p < 0.05$) higher than the pulp. On the other hand, the seeds did not present anthocyanins, exclusively present in the pulp and skin of the fruits. Maturation affected the composition of phenolics, especially in seeds, where the mature stage presented significantly ($p < 0.05$) fewer phenolic compounds (Table 4). It is interesting to note that with ripening, although the ripe fruits presented a purple coloration of the pulp (Figure 1), the anthocyanin content decreased ripening (Table 4), probably there was a significant reduction of anthocyanins in the skin.

Table 4 - Total phenolics (TPC), tannins (TAN) and anthocyanins (ACY), and antioxidant activity of extracts of pulp and seeds

Compound*	Semi-Ripe Pulp	Ripe pulp	Semi-Ripe Seed	Ripe seed
TPC	22,0 ± 2,2 c	14,2 ± 4,3 c	79,8 ± 3,3 a	61,5 ± 5,2 b
TAN	6,60 ± 0,5 c	5,40 ± 0,7 c	41,5 ± 3,5 a	31,5 ± 5,7 b
ACY	19,7 ± 0,2 a	13,2 ± 0,9 b	--	--
DPPH (IC_{50})	363 ± 8,5 b	527 ± 13,8 c	49,7 ± 3,1 a	48,6 ± 2,6 a
ABTS (IC_{50})	310 ± 6,5 b	458 ± 6,6 c	58,3 ± 1,7 a	59,5 ± 1,0 a
Superoxide (I%)	< 10%	< 10%	53,3 ± 4,5 a	58,6 ± 4,5 a

* Results expressed in:
Total phenolic compounds (TPC): mg of gallic acid equivalents/g of extract
Tannins (TAN): mg of gallic acid equivalents/g of extract
Total Anthocyanins (ACY): mg of cyanidin-3-glycoside equivalents/g of extract
ABTS and DPPH: µg/mL.
Superoxide: percentage of inhibition of the extract at a concentration of 500 µg/mL.
Different lowercase letters between columns represent significant differences between samples ($p < 0.05$).
Source: Authors

Quercetin, pyrogallol and gallic acid were the main compounds identified in the pulp and seeds, in addition to homovanillic acid in the seeds (Table 5). With the ripening of the fruits, there was a significant reduction ($p < 0.05$) in the quercetin content, probably due to the appearance of anthocyanins with a prevalence of purple coloration, since in the semi-ripe

fruit, the pulp color is slightly yellow. Another significant change was the reduction of pyrogallol and the appearance of gallic acid with maturation, evidencing the carboxylation reaction of pyrogallol in gallic acid. Quercetin, pyrogallol and gallic acid are substances with recognized antioxidant and anti-inflammatory activity (Guss et al., 2017; Li et al., 2016; Milanezi et al., 2019; Scherer & Godoy, 2009).

3.7 Antioxidant and anti-inflammatory activities

Table 4 shows the results of antioxidant activity. It was observed that the seeds have significantly higher antioxidant activity than the pulp, and there was no influence of maturation on the seeds. On the other hand, there was a significant reduction in the antioxidant activity of the pulp with ripening. Similar results were found in grapes, where a higher content of phenolic compounds was found in the seeds than in the pulp and skin, and the seeds also showed higher antioxidant activity (Freire et al., 2022). It is important to highlight the ability scavenge superoxide radicals presented by the seeds (Table 4). Reactive oxygen species (ROS), such as the superoxide anion (O2•-), are normally produced in aerobic metabolism. ROS are highly reactive molecules that chemically alter cellular structures, such as proteins, lipids, and nucleic acids, and can cause damage to cells and tissues (Scherer et al., 2019).

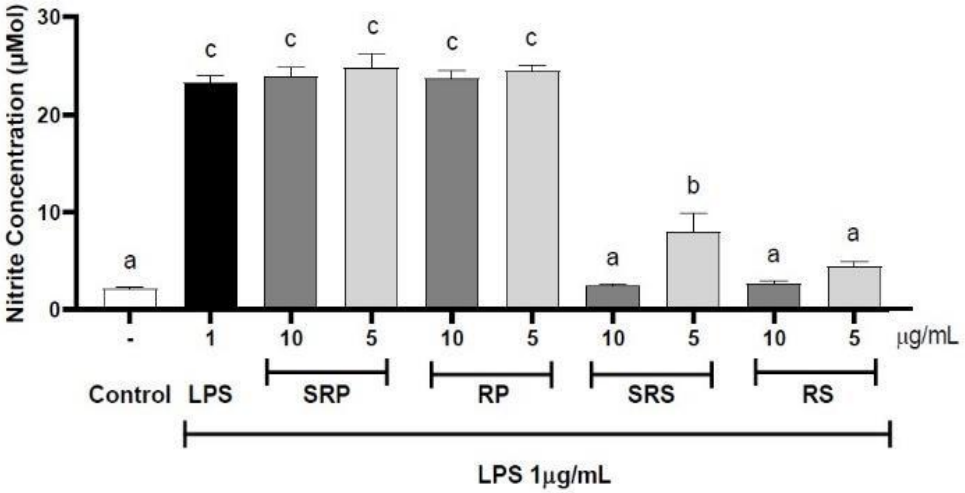
Table 5 - Phenolic compounds in pulp and seed obtained by LC-MS/MS (µg/100g fresh fruit).

Compound	$[M-H]^-$	Exact mass (*m/z*)	Experimental mass (*m/z*)	*Error (ppm)	Semi-Ripe Pulp	Ripe pulp	Semi-Ripe Seed	Ripe seed
(-)-Epicatechin	C15H13O6	289.07066	289.0716	-0,697	-	14.9 ± 0.7	-	13.8 ± 0.2
Chlorogenic acid	C16H17O9	353.08671	353.0875	-0,836	13.4 ± 0.1	38.1 ± 0.8	-	-
Ethyl gallate	C9H9O5	197.04444	197.0455	0,017	-	-	-	50.7 ± 0.1
Gallic acid	C7H5O5	169.01215	169.0138	-2,346	-	694 ± 0.1	990 ± 20.0	973 ± 1.0
Homovanillic acid	C9H9O4	181.04953	181.0506	-0,067	-	-	718 ± 6.0	374 ± 4.0
Polydatin	C20H21O8	389.12309	389.1234	-1,93	11.9 ± 0.1	4.0 ± 0.3	19 ± 0.1	-
Pyrogallol	C6H5O3	125.02332	125.0242	-1,658	808 ± 1.0	Nd	857 ± 5.0	-
Quercetin	C15H9O7	301.03428	301.0351	-0,883	1706 ± 7.0	500 ± 10	115 ± 1.0	102 ± 2.0

* The biggest error was considered.
Source: Authors

None of the pulp and seed extracts showed cytotoxic activity at concentrations of 5 and 10 µg/mL in the MTT assay. Therefore, these concentrations were used for the anti-inflammatory activity studies. Figures 2, 3 and 4 show the results of inhibition of nitric oxide production and proinflammatory cytokines IL-6 and TNF-α. It was found that stimulation of macrophages with bacterial lipopolysaccharide (LPS) triggered the inflammatory process in the cells, which was visible by the significant increase ($p < 0.05$) in the production of nitric oxide, IL-6 and TNF-α compared to the non-stimulated cells (control).

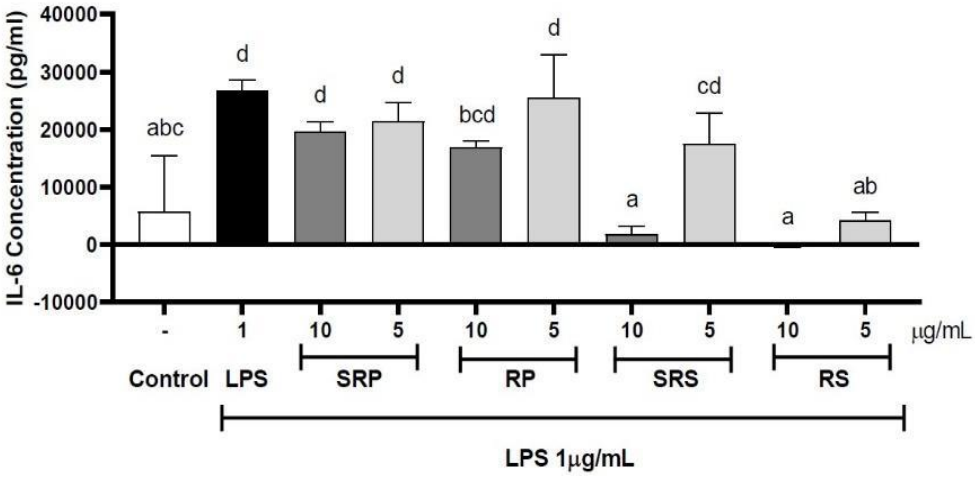
Figure 2 - Effect of the ethanolic extracts of cambui-roxo (*Eugenia candolleana*) (10 and 5 µg/mL) on the production of NO in macrophages (RAW 264.7) stimulated by LPS. Results are expressed as mean ± SD. Different lowercase letters represent significant differences between samples ($p < 0.05$). SRP: semi-ripe pulp; RP: ripe pulp; SRS: semi-ripe seed; RS: ripe seed.



Source: Authors.

It was found that stimulation of macrophages with bacterial lipopolysaccharide (LPS) triggered the inflammatory process in the cells, which was visible by the significant increase ($p < 0.05$) in the production of nitric oxide, IL -6 and TNF-α compared to the non-stimulated cells (control). Figure 2 shows that the seeds were able to significantly reduce the production of nitric oxide in macrophages. The pulp, on the other hand, showed no activity. A similar result can be observed in the inhibition of IL-6 cytokines, where seeds also showed a significant reduction in cytokine production and reached baseline levels (Figure 3).

Figure 3 - Effect of the ethanolic extracts of cambuí-roxo (*Eugenia candolleana*) (10 and 5 µg/mL) on the production of IL-6 in macrophages (RAW 264.7) stimulated by LPS. Results are expressed as mean ± SD. Different lowercase letters represent significant differences between samples ($p < 0.05$). SRP: semi-ripe pulp; RP: ripe pulp; SRS: semi-ripe seed; RS: ripe seed.

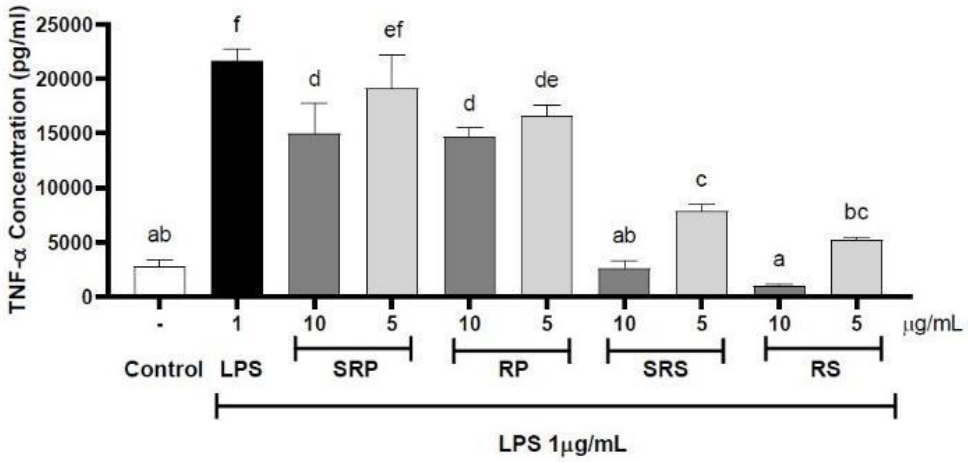


Source: Authors.

In tumor necrosis factor (TNF-α) production, both seeds and pulp showed the ability to inhibit production, but seeds

showed significantly higher activity than pulp (Figure 4). IL-6 and TNF-α are cytokines involved in the pathogenesis of a number of physiological processes that control inflammation, anti-tumor responses, and immune system homeostasis (Mehta et al., 2018). In this study, it was shown that the production of IL-6 and TNF-α by LPS-simulated macrophages was significantly suppressed by seed extracts. Therefore, suppressing the overproduction and activity of these proinflammatory cytokines may be successful in treating inflammatory processes in chronic diseases.

Figure 4 - Effect of the ethanolic extracts of cambuí-roxo (*Eugenia candolleana*) (10 and 5 µg/mL) on the production of TNF-α in macrophages (RAW 264.7) stimulated by LPS. Results are expressed as mean ± SD. Different lowercase letters represent significant differences between samples ($p < 0.05$). SRP: semi-ripe pulp; RP: ripe pulp; SRS: semi-ripe seed; RS: ripe seed.



Source: Authors.

Nitric oxide is a signaling molecule that plays a relevant role in inflammation pathogenesis and is considered a pro-inflammatory mediator that induces inflammation due to overproduction in abnormal situations. Increased nitric oxide production is related to some diseases, such as neurodegenerative diseases and cancer (Man et al., 2022). Thus, reducing the production of this molecule can contribute to preventing these diseases. In the study, we could observe that small doses (5 µg/mL) can considerably reduce nitric oxide production.

Inadequate micronutrient intake also called hidden hunger, is a current health problem that affects more than 2 billion people worldwide, mainly in developing countries. We noticed a source of minerals and vitamins, such as iron, copper, phosphorus, magnesium, manganese, potassium and zinc, vitamin B1 and B2, dietary fiber, proteins and carbohydrates, and bioactive compounds such as quercetin, considering 100 g of cambuí-roxo seed flour. That shows the potential to be used as a food supplement, besides valuing agro-industrial by-products. We also verified a robust antioxidant and anti-inflammatory activity.

4. Conclusion

The present study presented for the first time the composition of Cambuí-roxo (*Eugenia candolleana*). From these results, it can be concluded that the cambuí-roxo fruit has a good nutritional profile and can be included in people's diets as a strategy to improve the diversity of food supply and appreciation of native Brazilian fruits. The fruit has vitamins and minerals that classify it as sources of these nutrients. In addition, the extracts have high antioxidant and anti-inflammatory activity. The seed has potential as a food supplement, however, future studies can evaluate the bioavailability of these nutrients present in the

seed flour, with the objective of evaluating the use of this flour as a product for food enrichment in the industry or as a food supplement.

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