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Inhibition of α-glucosidase, pancreatic lipase, and antioxidant property of *Myrcia hatschbachii* D. Legrand containing gallic and ellagic acids

[Inhibición de la α-glucosidasa, la lipasa pancreática y la propiedad antioxidante de *Myrcia hatschbachii* D. Legrand que contiene ácidos gálico y elágico]

Larissa Junqueira Gatto[1], Gustavo Rezende Bellei de Oliveira[1], Katlin Suellen Rech[1], Paula Francislaine Moura[1], Caroline Gribner[1], Francis José Zortéa Merino[1], Suelen Ávila[2], Josiane de Fatima Gaspari Dias[1], Obdulio Gomes Miguel[1] & Marilis Dallarmi Miguel[1]

[1]Postgraduate Program in Pharmaceutical Science, Federal University of Paraná, Curitiba, PR, Brazil
[2]Postgraduate Program in Food and Nutrition. Federal University of Paraná, Curitiba, PR, Brazil

Reviewed by:
Suelen Pereira Ruiz
Universidad de Antioquia
Brazil

Alberto González
Universidad de Santiago de Chile
Chile

Correspondence:
Larissa Junqueira **GATTO**
lari.gatto@gmail.com

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Abstract: Several species of the Myrcia genus have been used in folk medicine to treat diabetes. Therefore, the aim of this work was to investigate the inhibitory activity of α-glucosidase and pancreatic lipase in the crude extract (EBF) and in the ethyl acetate fraction (FFA) of Myrcia hatschbachii, as well as to identify isolated phenolic compounds and to evaluate the antioxidant property and preliminary in vitro toxicity against Artemia salina. EBF (IC50: 3.21 µg/mL) and FFA (IC50: 1.14 µg/mL) showed inhibitory activity superior to acarbose (IC50: 193.65 µg/mL). In addition, they showed inhibitory effects of pancreatic lipase (IC50: 556.58 µg/mL for EBF and 532.68 µg/mL for FFA), antioxidant potential, absence of preliminary toxicity and presence of gallic and ellagic acids in FFA. The relevant results in the inhibition of α-glucosidase and pancreatic lipase motivate new studies for the development of herbal medicines that assist in the treatment of diabetic patients.

Keywords: α-Glucosidase; Ellagic acid; Gallic acid; Myrtaceae; Pancreatic lipase.

Resumen: Varias especies del género Myrcia se han utilizado en la medicina popular para tratar la diabetes. Por lo tanto, el objetivo de este trabajo fue investigar la actividad inhibitoria de la α-glucosidasa y la lipasa pancreática en el extracto crudo (EBF) y en la fracción de acetato de etilo (FFA) de Myrcia hatschbachii, así como identificar compuestos fenólicos aislados y evaluar la propiedad antioxidante y toxicidad in vitro preliminar contra Artemia salina. EBF (IC50: 3.21 µg/mL) y FFA (IC50: 1.14 µg/mL) mostraron una actividad inhibitoria superior a la acarbosa (IC50: 193.65 µg/mL). Además, mostraron efectos inhibitorios de la lipasa pancreática (IC50: 556.58 µg/mL para EBF y 532.68 µg/mL para FFA), potencial antioxidante, ausencia de toxicidad preliminar y presencia de ácidos gálico y elágico en FFA. Los resultados relevantes en la inhibición de la α-glucosidasa y la lipasa pancreática motivan nuevos estudios para el desarrollo de medicamentos a base de hierbas que ayudan en el tratamiento de pacientes diabéticos.

Palabras clave: α-Glucosidasa; Ácido elágico; Ácido gálico; Myrtaceae; Lipasa pancreática.

INTRODUCTION

Diabetes and obesity are metabolic conditions whose incidence is increasing worldwide. According to populational studies, there is a strong epidemiological association between obesity and the development of diabetes, in all age groups and ethnicities (Thingholm *et al.*, 2019). Worldwide obesity has almost tripled since 1975. In 2016, more than 1.9 billion (39%) of adults were overweight and among these more than 650 million (13%) were obese (WHO, 2020). As a direct result of the increase in obesity, it is suggested that the number of patients with diabetes will increase from 366 million in 2011 to 552 million in 2030 (Loh *et al.*, 2019).

One of the therapeutic approaches for the treatment of diabetes is acarbose, which reduces postprandial hyperglycemia by inhibiting hydrolyzing enzymes (α-glucosidase) involved in the degradation of carbohydrates (Vadivelan *et al.*, 2019). In addition, reducing glucose absorption also helps to prevent obesity (Les *et al.*, 2018). In the treatment of obesity, orlistat is used as a potent and selective inhibitor that covalently binds to the active site of gastric and pancreatic lipases, reducing the absorption of fat from ingested foods (Joyce *et al.*, 2019). However, the continuous use of these synthetic drugs is associated with side effects, such as abdominal pain, flatulence and diarrhea for acarbose (Lordan *et al.*, 2013; Peng *et al.*, 2016; Lima *et al.*, 2018) and oily spotting and severe evacuation in the orlistat (González *et al.*, 2017).

Efforts have been directed towards the discovery of medicines from natural products due to their low cost, relative safety, probability of high compliance and low incidence of undesirable side effects (González *et al.*, 2016). Natural products of great structural diversity are considered a good source for the search for enzymatic inhibitors. In this sense, phenolic compounds can inhibit some intestinal digestive enzymes, such as lipase and α-glucosidase, modulating the bioavailability of nutrients and resulting in blood glucose control and beneficial effects on obesity (Bellesia *et al.*, 2014), as an inhibition differentiation of adipocytes, decreased synthesis of fatty acids, and increased energy expenditure (Les *et al.*, 2018). In addition to helping enzymatic inhibition, phenolic compounds are also useful in reducing oxidative stress. It is known that hyperglycemia presented by diabetic patients reduces the activity of antioxidant enzymes and leads to the destruction of the antioxidant defense system (Chen *et al.*, 2019).

In this search for new active compounds, there is a highlight at the *Myrcia* genus, which the most cited traditional use is related to a group of Myrtaceae known in Brazil as "pedra-hume-caá" or "insulin plant". Infusions made from the leaves or whole plants of these species are used to treat diabetes (Cascaes *et al.*, 2015). Species of this genus demonstrated *in vitro* inhibitory activity of key diabetes targets, such as α-amylase and α-glucosidase (Oliveira & Pereira, 2015; González *et al.*, 2016; Lima *et al.*, 2018), and in addition some species showed hypoglycemic effects *in vivo* (Pepato *et al.*, 1993; Miura *et al.*, 2006; Vareda *et al.*, 2014). The genus also has other medicinal properties: *Myrcia tomentosa* showed antimicrobial activities (Sa *et al.*, 2017), *Myrcia rostrata* showed antinociceptive effects (Silva *et al.*, 2018), *Myrcia bella*, *Myrcia fallax*, and *Myrcia guianensis* presented cytotoxic and antioxidant properties (Santos *et al.*, 2018).

The species *Myrcia hatschbachii* D. Legrand is a native and endemic tree from Brazil, with confirmed occurrences in the states of the South region and Atlantic Forest domain (BFG, 2018). Considering the traditional use of the genus and the absence of studies in this species, the aim of this work was to investigate the *in vitro* inhibitory activity of α-glucosidase and pancreatic lipase, to identify isolated phenolic compounds, to evaluate the antioxidant property, and to test the preliminary *in vitro* toxicity, since substances capable of inhibiting digestive enzymes and reducing oxidative stress may be useful in the treatment of diabetic patients.

MATERIALS AND METHODS

Plant material

Leaves of *Myrcia hatschbachii* were collected during the autumn at the Federal University of Parana, Curitiba, Brazil (25°26'52"S, 49°14'25"W; 900 m of altitude). A voucher was made and the species identification was performed by comparison with the specimen held at the herbarium (Municipal Botanical Museum of Curitiba), under registry number 72379. The species was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge under the number AEE0D2F.

Crude extract and ethyl acetate fraction

The crude extract (EBF) was obtained using the Soxhlet apparatus modified by Carvalho, (2009) patented by the National Institute of Industrial Property under nº 0601703-7 A2. The leaves were dried at room temperature, crushed, and extracted (985 g) with 96°GL ethanol. This system underwent heating and was left to reflux for 40 hours. In the end,

6 liters of extract was obtained, with a yield of 12.8%.

The crude extract was concentrated on rotary evaporator at 80°C and from this; solvents with increasing polarity (hexane, chloroform, and ethyl acetate) were used to prepare the liquid-liquid partitions and to obtain ethyl acetate fraction (FFA).

Phytochemical screening

Phytochemical screening was performed by thin-layer chromatography (TLC), using samples (EBF and FFA) on a silica gel 60 UV254 plate (Whatman® brand) and different mobile phase mixtures according to the researched metabolite. For the research of steroids and triterpenes, toluene: ethyl acetate (93:7) and 1% sulfuric vanillin developer were used. For flavonoids, tannins and polyphenols, it was used ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26) and reactive NEU (1% diphenolboryloxyethylamine in methanol) were used for the search for flavonoids and 5% ferric chloride developer in ethanol for the research of tannins and polyphenols (Wagner, 1996). For alkaloids research, chloroform: methanol (95:5) with ammonia saturation and Dragendorff developer (potassium bismuth tetraiodide) were used (Valente *et al.*, 2006) and for coumarins research, toluene: ethyl acetate (80:20) and NEU developer and 1N sodium hydroxide were used.

Purification of FFA

A chromatographic column was prepared from 3.6 g of FFA solubilized in methanol and incorporated in silica gel 60. Through this column, the sample was eluted by passing a solvent mixture initiated by hexane: ethyl acetate (40:60), to ethyl acetate (100), ethyl acetate: methanol (95:5), and ending in ethyl acetate: methanol (60:40), with variation in the proportion of the phases (increase/decrease) by 5%. For each of these gradients, flasks containing the eluted samples were collected, which were evaporated on bench at room temperature.

Chemical characterization of isolated compounds

The flasks numbered from 2 to 10, corresponding to hexane: ethyl acetate (35:65) elution, showed crystal precipitation, initially called FFA2016F2-10. Samples of these crystals were sent for identification by X-ray diffraction (XRD) and Nuclear Magnetic Resonance (NMR) methods.

Flasks 11 to 114 were combined - mobile elution phase hexane: ethyl acetate (35:65) to ethyl acetate: methanol (85:15). These were solubilized in methanol and a yellow powder was precipitated, which was decanted, collected and dried, totaling 52 mg. The precipitate called FFA2016F11-114ppt was identified by NMR.

NMR

The NMR spectra of $^{13}C\{^{1}H\}$ and ^{1}H for the sample FFA2016F2-10 were acquired at room temperature (~20°C) in MeOD-d_4 containing 0.1% (v/v) tetramethylsilane (TMS) in a Bruker DPX 200 NMR spectrometer, operated at 4.7 Tesla, observing the ^{1}H and ^{13}C nucleus at frequencies of 200.13 and 50.33 MHz, respectively.

For the sample FFA2016F11-114ppt in addition to the NMR spectra of ^{1}H and $^{13}C\{^{1}H\}$, two-dimensional experiments of direct correlation ^{1}H-^{13}C HSQC and long-distance correlation ^{1}H-^{13}C HMBC were performed, at room temperature (~20°C) in DMSO-d_6 containing 0.1% (v/v) TMS in Bruker AVANCE III 400 NMR spectrometer, operated at 9.4 Tesla, observing the ^{1}H and ^{13}C nucleus at frequencies of 400.13 and 100.6 MHz, respectively.

The chemical shifts of ^{1}H and ^{13}C were expressed in ppm and the data obtained were compared with the literature data.

XRD

Diffraction data were collected using a Bruker D8 Venture diffractometer equipped with a Photon 100 CMOS area detector, two sources of monochromatic radiation of Mo-Kα (λ = 0.7107 Å) and Cu-Kα (λ= 1.5418 Å), and Kryoflex II device, for collecting samples at low temperature. The analysis was performed at 200 K using the Cu-Kα source. For the analysis, a crystalline fragment was selected from a portion of crystals immersed in mineral oil, which was carefully transferred to a micro-mount that was fixed on the goniometer of the diffractometer. The data were processed using the APEX3 program. The unit cell parameters found were compared with literature data using the Cambridge Crystallographic Data Center (CCDC) database and are under the deposit number in the database 1451205.

Total phenolic content (TPC)

The content of phenolic compounds was evaluated according to the method described by Singleton *et al.*, (1999) with modifications. The samples (EBF and FFA) (concentrations between 2 to 18 µg/mL) and gallic acid standard (concentrations between 1 to 11 µg/mL) were prepared in methanol. The reaction was performed in triplicate. In test tubes, 2 mL of distilled

water, 200 µL of sample and 200 µL of 2N Folin-Ciocalteau were added. The tubes were stirred and left at room temperature for 10 minutes. After the time, 200 µL of 10% Na_2CO_3 and 1.2 mL of distilled water were added. The tubes were again stirred and left at room temperature for 30 minutes in the dark. The same reaction was performed for gallic acid, control, and blank. After the reaction time, the samples were read in a spectrophotometer at 760 nm. The results were expressed in grams of gallic acid equivalent (GAE)/g of sample, calculated from the line equation of the linear regression of gallic acid.

DPPH radical scavenging method

The method was based on Mensor *et al.* (2001) and Salgueiro *et al*. (2014). Sample solutions (EBF and FFA) and positive controls (gallic acid, ascorbic acid, rutin and butylated hydroxytoluene [BHT]) were prepared at the proposed concentrations (between 1 to 22 µg/mL) for the curves. The reaction was performed in a microplate, from 142 µL of a sample with the addition of 58 µL of 0.3 mM DPPH solution. A blank was prepared to account for the background color of the sample and methanol was used as a negative control. After 30 minutes of reaction in the dark and at room temperature, the absorbances of the samples were read in triplicate using a spectrophotometer at 540 nm. The percentage of antioxidant activity (AA%) was calculated using the formula: AA% = 100 - [((Abs sample - Abs blank) x 100) / Abs negative control]. By linear regression, a graph was plotted, AA% *versus* concentration in µg/mL, which served as a basis for determining the IC_{50} value (concentration required to exert 50% of the antioxidant activity).

Ferric reducing antioxidant potential (FRAP)

The evaluation of this property was performed according to the method described by Rufino *et al.*, (2006) with modifications. Ferrous sulfate solutions were prepared in concentrations between 100 to 1800 µM in water, obtaining a calibration curve. The sample solutions (EBF and FFA) and positive controls (gallic acid, rutin, ascorbic acid, and BHT) were diluted in methanol in concentrations between 6 to 161 µM. In microplates, 30 µL of water, 10 µL of sample, and 300 µL of FRAP reagent (prepared at the time of use and composed of 25 mL of 0.3 M acetate buffer pH 3.6; 2.5 mL of a 10 mM 2,4,6-tripiridyl-s-triazine solution in 40 mM hydrochloric acid and 2.5 mL of a 20 mM aqueous solution of ferric chloride) were added. A blank was prepared the same way. The reaction was performed in triplicate. The microplate was incubated at 37°C in the spectrophotometer and after 30 minutes, the reading was performed at 570 nm. A linear regression graph was plotted for the ferrous sulfate standard, absorbance *versus* concentration in µM. From this graph, the absorbance relative to 1000 µM ferrous sulfate was calculated. For the calculation of the activity, it was replaced in the graph of each sample, absorbance *versus* concentration in mg/L, the absorbance equivalent to 1000 µM ferrous sulfate standard, obtaining the sample concentration (mg/L) equivalent to 1000 µM ferrous sulphate. Based on this concentration, it was possible to calculate the amount in mM ferrous sulfate/g of sample.

Antioxidant capacity by the radical ABTS

The described procedure was based on Re *et al.* (1999) with modifications. A trolox 50% hydromethanolic solution was prepared with concentrations between 0.0016 and 0.0002 mmol/mL, obtaining a calibration curve. The sample solutions (EBF and FFA) and positive controls (gallic acid, rutin, ascorbic acid, and BHT) were diluted in the same solvent to obtain the concentrations to be tested (0.05 to 0.71 mg/mL). The ABTS radical was prepared from 5 mL of 7 mM ABTS aqueous solution with 88 µL of 140 mM aqueous potassium persulfate solution. This mixture was kept in the dark, at room temperature for 16 hours. At the time of use, the amount of this reagent was diluted in water until the adequate absorbance was obtained at 690 nm. The reaction was performed in quadruplicate from 10 µL of the respective diluted solution and 300 µL of ABTS reagent. The microplate was left in the dark at room temperature and read at 690 nm in a spectrophotometer. The results were expressed in µmol of trolox equivalent (TE)/mg of sample, calculated from the line equation of the linear regression of the trolox.

In vitro α-glucosidase inhibitory activity assay

In vitro inhibition of the enzymatic activity of α-glucosidase was evaluated using a spectrophotometric method (Pereira *et al.*, 2012; Silva *et al.*, 2014; Tong *et al.*, 2014; Shan *et al.*, 2016), with minor modifications. The test was performed in triplicate with 6 concentrations of each sample (2.7 to 4.0 µg/mL for EBF and 0.5 to 3.0 µg/mL for FFA) in 4% DMSO. In a microtube, 150 µL of 0.1 M sodium phosphate buffer pH 6.8 (containing 0.0335 M NaCl), 150 µL of 0.0005 M reduced glutathione, 150 µL of each tested concentration, and 150 µL of α-glucosidase (0.2

units/ mL) were added. The mixture was incubated at 37°C for 15 minutes. After this period, 150 µL of 0.85 mM ρ-nitrophenyl-α-D-glucopyranoside substrate was added and incubated again at 37°C for 20 minutes. After this time, the reaction was stopped with 300 µL of 0.2 M sodium carbonate solution, stirred and read at 400 nm. The reaction control, the reaction control blank, and the sample blank were also prepared. Acarbose was used as a positive control under the same conditions as the assay. A graph of percentage inhibition *versus* log concentration in µg/mL was plotted. The percentage of inhibition (%I) was calculated according to the formula: %I = [((Abs control - Abs blank) - (Abs sample - Abs blank)) / (Abs control - Abs blank)] x 100. The results of inhibition of enzymatic activity were expressed as the average of the 50% inhibitory concentration (IC_{50}), calculated from the line equation of the linear regression of each sample.

The enzyme α-Glycosidase from *Saccharomyces cerevisiae*, ρ-nitrophenyl-α-D-glucopyranoside (ρNPG) substrate, reduced glutathione, and acarbose were obtained from Sigma-Aldrich. The reagents sodium phosphate, sodium chloride, and sodium carbonate were obtained from Vetec Química Fina Ltda.

In vitro pancreatic lipase inhibitory activity assay

In vitro inhibition of the enzymatic activity of pancreatic lipase was performed using a spectrophotometric method (Nakai *et al.*, 2005; Saifuddin & Raziah, 2008; Souza *et al.*, 2011; Oliveira *et al.*, 2015) with minor modifications. The test was performed in triplicate with 7 concentrations of each sample (300 to 900 µg/mL for EBF and 250 to 850 µg/mL for FFA) in 5% DMSO. In a microtube, 100 µL of each tested concentration and 100 µL of 20 mg/mL pancreatic lipase suspension (in 0.05 M Tris-HCl buffer pH 8.0, containing 25 mM NaCl and 10 mM $CaCl_2$) were added. The mixture was incubated at 37°C for 15 minutes. After this period, 100 µL of 8 mM ρ-nitrophenylpalmitate substrate (in Tris-HCl buffer pH 8.0 containing 10% isopropyl alcohol, 0.1% gum arabic, and 0.5% Triton-x 100) was added and again incubated at 37°C for 20 minutes. After this time, the reaction was stopped in a hot bath (85°C) for 10 minutes and 800 µL of Tris-HCl buffer solution pH 8.0 was added. The mixture cooled for 10 minutes and was centrifuged at 2500 rpm for 5 minutes. The supernatant was used for reading on a spectrophotometer at 410 nm. Reaction control and sample, control, and substrate blanks were also prepared. Orlistat was used as a positive control under the same conditions as the assay. A graph of percentage of inhibition *versus* log of the concentration in µg/mL was plotted and the percentage of inhibition (%I) was calculated according to the formula: %I = [((Abs control) - (Abs sample)) / Abs control] x 100, with Abs control = (control absorbance - enzyme blank absorbance - substrate blank absorbance), Abs sample = (sample absorbance - sample blank absorbance). The results of the inhibition of enzymatic activity were expressed as the average of the 50% inhibitory concentration (IC_{50}), calculated from the line equation of the linear regression of each sample.

The enzyme pancreatic lipase porcine type II, ρ-nitrophenylpalmitate (ρ-NPP) substrate, orlistat, and the reagents triton-X 100 and Tris (hydroxymethyl) aminomethane were obtained from Sigma-Aldrich. The reagents sodium chloride, hydrochloric acid, and isopropyl alcohol were obtained from Vetec Química Fina Ltda. Gum arabic was obtained from The Gum Arabic Company Ltda. and calcium chloride from Pro Analysis/ Isofar Ltda.

Lethality against brine shrimp (Artemia salina)

The method was based on Meyer *et al.* (1982). For the hatching of *Artemia salina* eggs, a saline solution was prepared, which was aerated for 30 minutes and, during incubation, its pH was kept between 8.0 and 10.0. The temperature was controlled between 27 and 30°C and the solution was kept under constant stirring and lighting (20 W) for 48 hours. Sample solutions (EBF and FFA) were prepared in concentrations of 10 to 1000 µg/mL in methanol, in quintuplicate. The same concentrations were prepared for the quinidine sulfate positive control. The flasks containing the sample and controls solutions were placed in a laboratory oven at 40°C for the total elimination of the dilution solvent, including the negative control flask (methanol). After evaporation, the flasks were resuspended with 1 mL of saline and the incubation of 10 nauplii (brine shrimp larva) was performed in sequence. The volume of the flasks was completed with saline solution to 5 mL and after 24 hours, the count of the live and dead nauplii was performed in the analyzed concentrations. The statistical analysis was evaluated to determine the lethal dose capable of killing 50% of brine shrimp (LC_{50}).

Statistical analysis

For inhibition of enzymatic activity and antioxidant property, experimental data were evaluated by

variance analysis (ANOVA) followed by the Tukey test (p<0.05), by using GraphPad Prism 6. In addition, the Pearson correlation coefficient was determined between inhibition of enzymatic activity and TPC, inhibition of enzymatic activity and antioxidants methods, and TPC and antioxidants methods.

For preliminary *in vitro* toxicity, the results were evaluated using statistical tests by the Probitos method using SPSS software version 23.0.

RESULTS

Phytochemical screening by TLC was carried out with the aim of qualitatively showing the main metabolites present in EBF and FFA (Figure No. 1). In both samples, the results were positive for flavonoids, tannins, and coumarins. Steroids/ triterpenes were positive only in EBF, while alkaloids were absent in EBF and FFA.

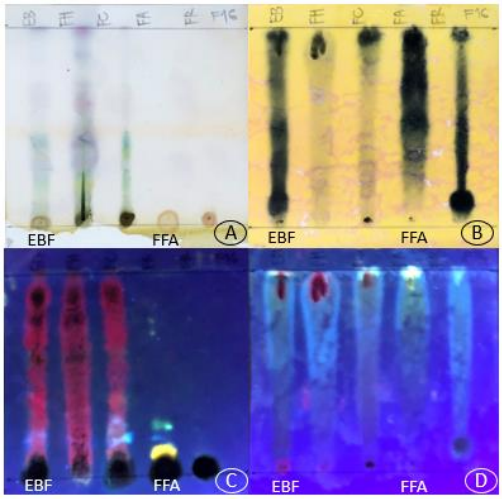

Figure No. 1
Phytochemical screening by TLC. Identification of steroids/ triterpenes (A), tannins (B), coumarins (C), and flavonoids (D) in crude extract (EBF) and ethyl acetate fraction (FFA)

The structural elucidation of the isolated compounds identified two phenolic compounds in the species. From the visualization and interpretation of the NMR spectra of the crystals of the sample FFA2016F2-10, it is noted that the signals present in the ^{1}H and $^{13}C\{^{1}H\}$ spectra coincide with the signals present in the gallic acid spectra reported in the literature (Souza *et al.*, 2006), according to Table No. 1 and Figure No. 2, and Figure No. 3.

Table No. 1
^{1}H and $^{13}C\{^{1}H\}$ NMR data for gallic acid

Position	δ_C [a]	δ_H [a]
1	121.9	
2, 6	110.4	7.07 s
3, 5	146.3	
4	139.6	
1’	170.6	

[a] NMR experiment performed at 200.13 MHz for ^{1}H and 50.33 MHz for $^{13}C\{^{1}H\}$ on MeOD-d_4.
(δ) Chemical shift in ppm

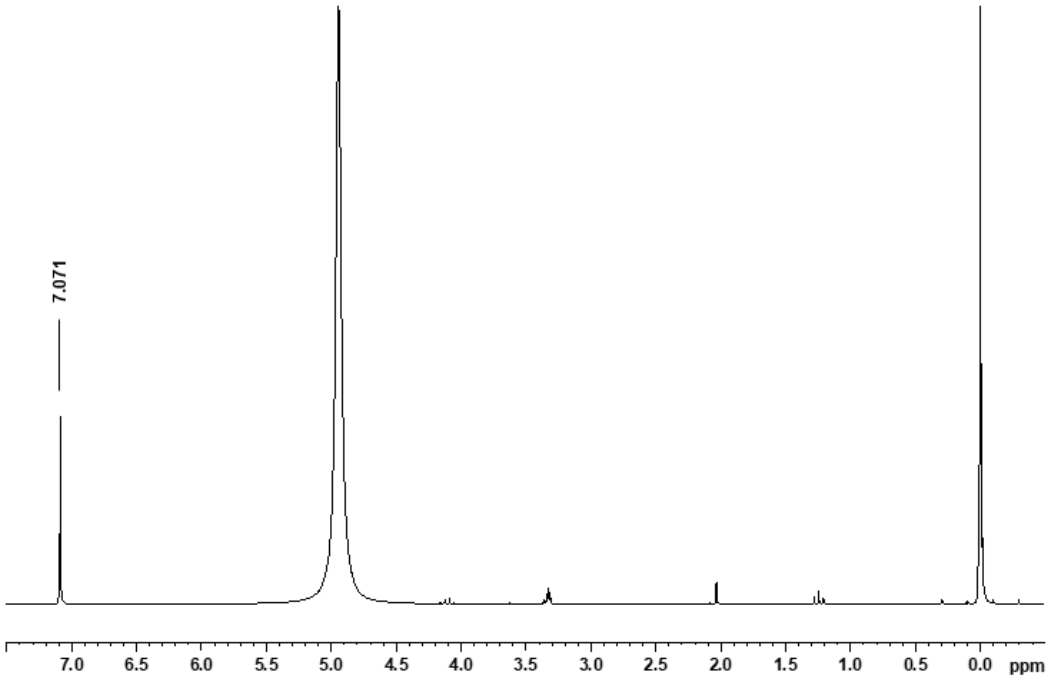

Figure No. 2
^{1}H NMR data for gallic acid (^{1}H: 200.13 MHz; MeOD-d$_4$)

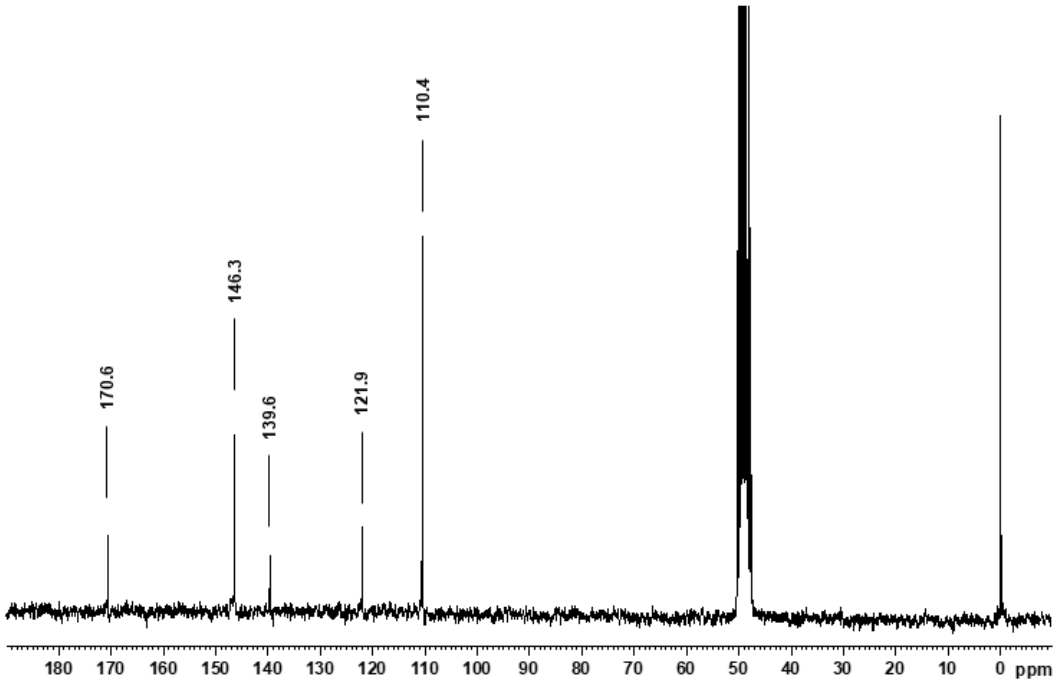

Figure No. 3
^{13}C{^{1}H} NMR data for gallic acid (^{13}C: 50.33 MHz; MeOD-d$_4$)

The crystals were also identified by X-ray diffraction. Through this analysis, it was possible to verify that the unit cell parameters obtained were following the structure of the gallic acid monohydrate, as shown in Table No. 2.

Table No. 2
Unit cell parameters used to identify the crystalline structure

Crystal data	Experimental
$C_7H_6O_5.H_2O$	
Space group	Monoclinic, $P2_1/n$
a/ Å	7.48
b/ Å	13.87
c/ Å	14.13
α/ °	90
β/ °	96.36
γ/ °	90
Volume/ Å3	934
Analysis temperature	200 K

According to the analysis of the NMR spectra of ^{1}H and $^{13}C\{^{1}H\}$ and maps of direct correlation ^{1}H-^{13}C HSQC and long-distance correlation ^{1}H-^{13}C HMBC of the sample FFA2016F11-114ppt, it was possible to verify that they agreed with the data already reported in the literature for ellagic acid (Goriparti *et al.*, 2013), as shown in Table No. 3, Figure No. 4, Figure No. 5, and Figure No. 6.

Table No. 3
^{1}H and $^{13}C\{^{1}H\}$ NMR data for ellagic acid

Position	δ_C [ab]	δ_H [a]
1, 1’	113.0	
2, 2’	136.1	
3, 3’	139.4	
4, 4’	148.1	
5, 5’	108.6	7.35 s
6, 6’	104.4	
7, 7’	159.7	

[a] NMR experiment performed at 400.13 MHz for ^{1}H and 100.6 MHz for ^{13}C on DMSO-d_6. [b] Values determined by HSQC and HMBC NMR experiments. (δ) Chemical shift in ppm.

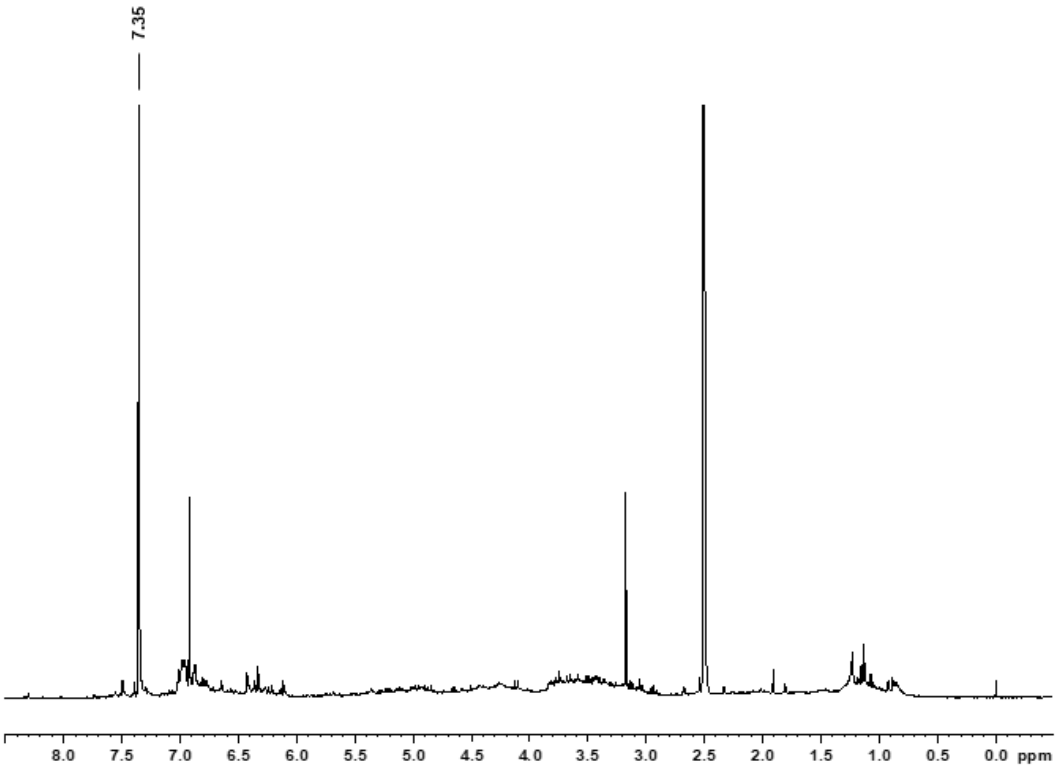

Figure No. 4
^{1}H NMR data for ellagic acid (^{1}H: 400.13 MHz; DMSO-d$_6$)

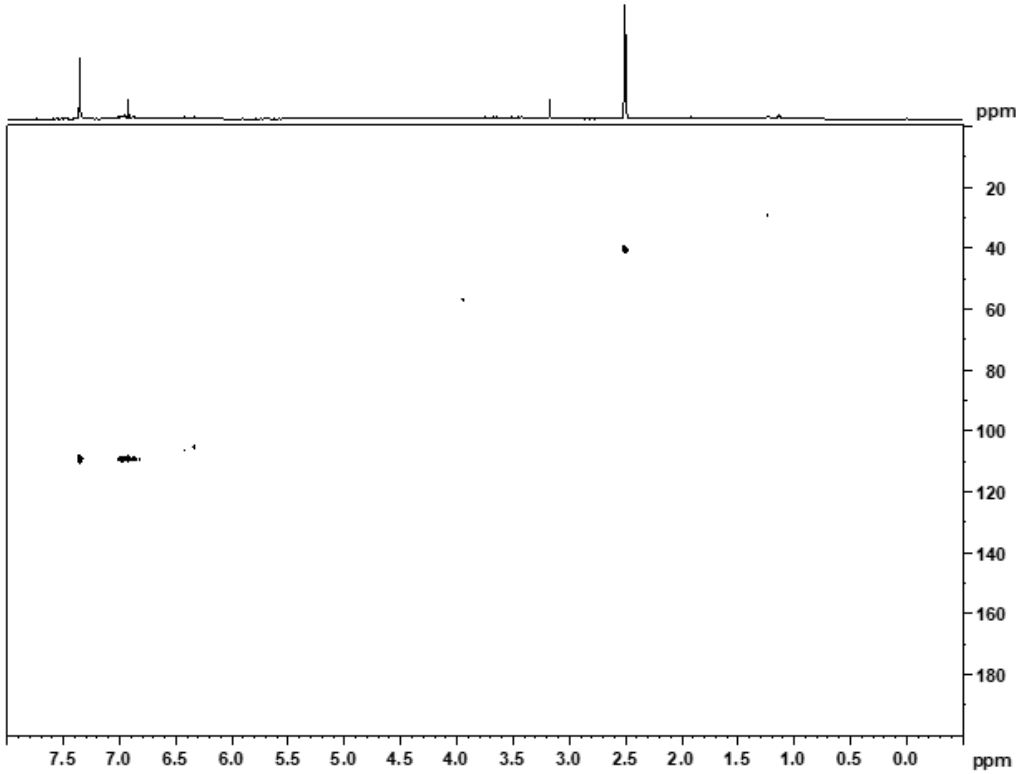

Figure No. 5
Direct correlation map ^{1}H-^{13}C HSQC of ellagic acid (^{1}H: 400.13 MHz; ^{13}C: 100.6 MHz; DMSO-d$_6$)

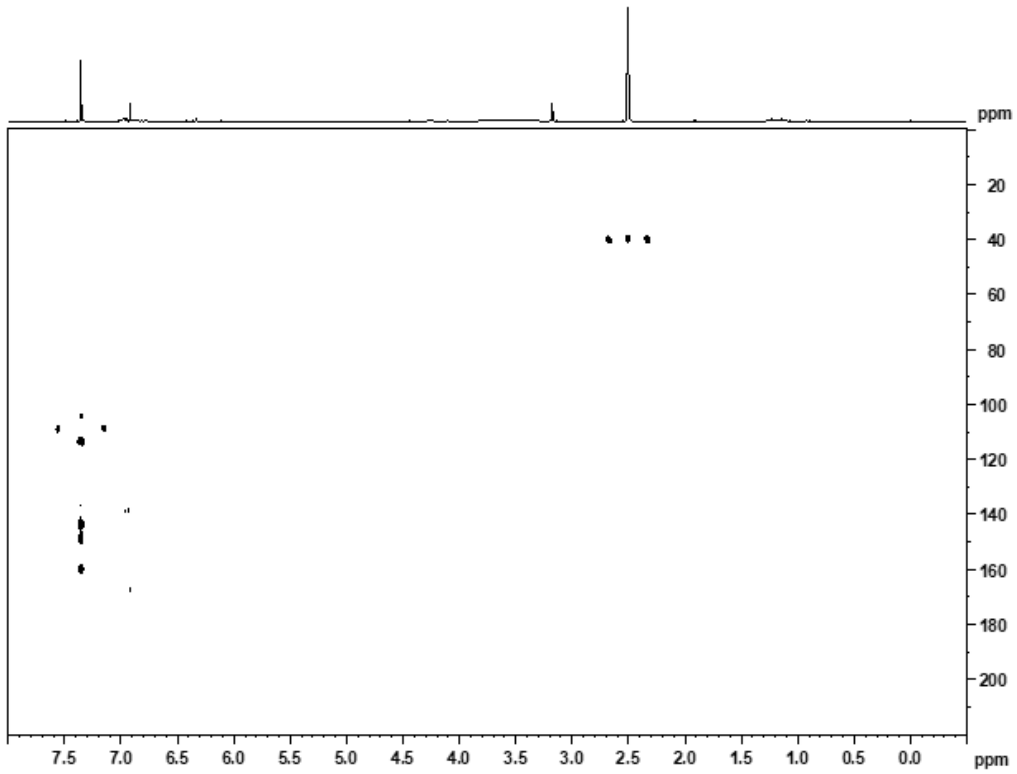

Figure No. 6
Long-distance correlation map ^{1}H-^{13}C HMBC of ellagic acid (^{1}H: 400.13 MHz; ^{13}C: 100.6 MHz; DMSO-d_6)

Phenolic compounds were quantified in 0.2538 g GAE/g (± 0.0049) for EBF and 0.7538 g GAE/g (± 0.0297) for FFA. The amount of phenolics can be classified as high (> 0.05 g GAE/g), medium high (0.03 to 0.05 g GAE/g), medium (0.01 to 0.03 g GAE/g) and low (< 0.01 g GAE/g) (Chew *et al.*, 2011). According to this classification the two samples showed a high amount of compounds.

The results of the antioxidant property, analyzed by the methods of DPPH, FRAP, and ABTS are summarized in Table No. 4. Concerning the DPPH method, FFA showed an IC_{50} result (3.81 µg/mL) lower than the ascorbic acid (1.45 times), BHT (4.00 times), and rutin (1.86 times) controls, thus showing a greater antioxidant capacity. In the FRAP and ABTS methods, FFA showed an antioxidant potential result superior to the ascorbic acid (1.95 and 1.59 times, respectively), BHT (9.36 and 5.83 times, respectively), and rutin (3.77 and 2.80 times, respectively) controls, whereas EBF did not present statistical difference compared to BHT in FRAP method and rutin in ABTS method.

Table No. 4
Determination of antioxidant property

Sample	DPPH IC_{50} µg/mL	FRAP mM ferrous sulphate/g	ABTS µmol TE/mg
EBF	11.83 ± 0.24	5.16 ± 0.10*	2.42 ± 0.14*
FFA	3.81 ± 0.13	34.71 ± 1.60	7.01 ± 0.40
Gallic acid	1.74 ± 0.06	38.51 ± 0.41	16.61 ± 1.49
Ascorbic acid	5.53 ± 0.03	17.76 ± 0.13	4.42 ± 0.23
Rutin	7.09 ± 0.15	9.21 ± 0.44	2.50 ± 0.12*
BHT	15.25 ± 0.05	3.71 ± 0.14*	1.20 ± 0.06

All averages, except those indicated with *, showed statistical difference by the Tukey test (p<0.05).
Crude extract (EBF), ethyl acetate fraction (FFA)

The results from the present study provide evidence of the inhibitory effect of EBF and FFA on α-glucosidase and pancreatic lipase activities. They were able to inhibit both enzymes in a dose-dependent response. As a measure of the inhibitory potency of the tested extracts, IC_{50} values were calculated from the enzymatic activity. Acarbose and orlistat are commercial inhibitors of α-glucosidase and pancreatic lipase, respectively, and were used as positive controls.

The *in vitro* inhibition activity of α-glucosidase showed IC_{50} of 3.2093 µg/mL (± 0.0093) for EBF, 1.1414 µg/mL (± 0.0152) for FFA, and 193.6539 µg/mL (± 5.2369) for acarbose (Figure No. 7A). EBF and FFA did not differ statistically from each other, but differed significantly from acarbose. The tested extracts were stronger inhibitors of α-glucosidase than acarbose, with 60 (EBF) and 170 (FFA) times the efficacy of the positive control.

The concentration capable of inhibiting 50% of pancreatic lipase activity was 556.5824 µg/mL (± 3.0656) for EBF, 532.6813 µg/mL (± 5.8393) for FFA, and 0.1305 µg/mL (± 0.0045) for orlistat (Figure No. 7B). In the statistical test, it was observed that orlistat differed from EBF and FFA, as well as the samples also differed from each other. At higher concentrations, EBF (900 µg/mL) showed an enzymatic inhibition of around 84%, while FFA (850 µg/mL) inhibited 80%.

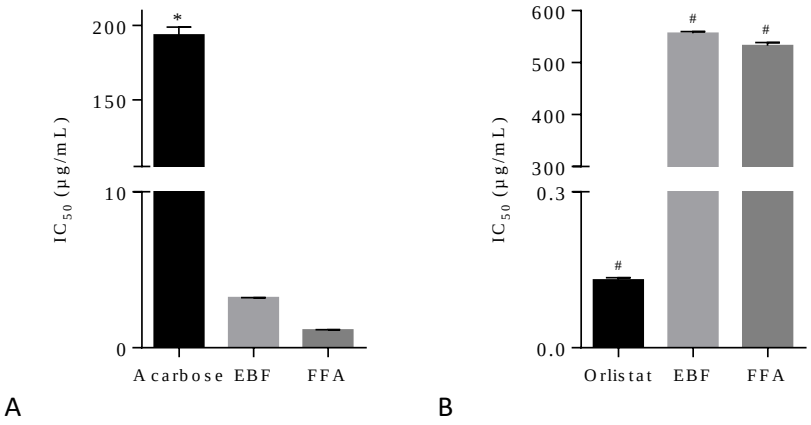

Figure No. 7
***In vitro* α-glucosidase (A) and pancreatic lipase (B) inhibitory activity. (*) indicates $p<0.05$ comparing acarbose to crude extract (EBF) and ethyl acetate fraction (FFA). (#) indicates $p<0.05$ comparing orlistat, EBF, and FFA.**

To evaluate the correlation between the inhibition of enzymatic activity and TPC, inhibition of enzymatic activity and antioxidants methods, and TPC and antioxidants methods, Pearson correlation coefficient was determined (Table No. 5). Pearson coefficient showed a strong correlation in all analyses ($r > 0.9$), demonstrating a high degree of linear statistical dependence between the variables.

Table No. 5
Pearson correlation coefficient

Correlation		EBF	FFA
Inhibition of enzymatic activity x TPC	α-Glucosidase x Phenolics	0.9880	0.9951
	Pancreatic lipase x Phenolics	0.9933	0.9973
Inhibition of enzymatic activity x Antioxidant methods	α-Glucosidase x DPPH	0.9379	0.9823
	α-Glucosidase x FRAP	0.9930	0.9797
	α-Glucosidase x ABTS	0.9836	0.9910
	Pancreatic lipase x DPPH	0.9443	0.9868
	Pancreatic lipase x FRAP	0.9699	0.9914
	Pancreatic lipase x ABTS	0.9786	0.9966
Inhibition of enzymatic activity	α-Glucosidase x Pancreatic lipase	0.9900	0.9872
TPC x Antioxidant methods	Phenolics x DPPH	0.9710	0.9897
	Phenolics x FRAP	0.9685	0.9886
	Phenolics x ABTS	0.9590	0.9975

Crude extract (EBF), ethyl acetate fraction (FFA)

The preliminary *in vitro* toxicity test was performed against *Artemia salina*, which is a target organism used in assays focused on the detection of bioactivity of plants, particularly toxicity (Leite *et al.*, 2009; Alves *et al.*, 2010; Amarante *et al.*, 2011). According to Meyer *et al.* (1982), samples are considered toxic when the LC_{50} is less than 1000 μg/mL. Thus, the EBF and FFA samples did not show toxicity (LC_{50} > 1000 μg/mL) to the evaluated organisms, while the quinidine sulfate positive control showed a 50% lethal concentration of 101.03 μg/mL (59.52–154.92).

DISCUSSION

TLC was used as a qualitative analytical method to characterize the main metabolites present in EBF and FFA. In addition, it is a fast, efficient, low-cost test, and widely used in quality control of medicinal plants (Valente *et al.*, 2006). In both samples, the presence of flavonoids, tannins, and coumarins was noted. The Myrtaceae family is characterized by the presence of phenolic compounds (flavonoids and tannins) (Rodrigues *et al.*, 2016), as well as studies in the *Myrcia* genus also showed the presence of tannins, organic acids, and flavonoids (Cascaes *et al.*, 2015). Some flavonols glycosides such as myricitrin, mearnsitrin, quercitrin, desmanthin-1, and guaijaverin were isolated from leaves of *Myrcia multiflora*, and showed antidiabetic properties and potent inhibitory activities on aldose reductase and α-glucosidase (Yoshikawa *et al.*, 1998).

The isolations of gallic acid and ellagic acid are unpublished in *Myrcia hastchbachii* (Figure No. 8). Gallic acid has already been reported in other species of the genus, such as *Myrcia splendens* (Guldbrandsen *et al.*, 2015), *Myrcia bella* (Saldanha *et al.*, 2013), and *Myrcia guianensis* (Souza *et al.*, 2006). Ellagic acid was identified in *Myrcia bella* and *Myrcia fallax* (Santos *et al.*, 2018).

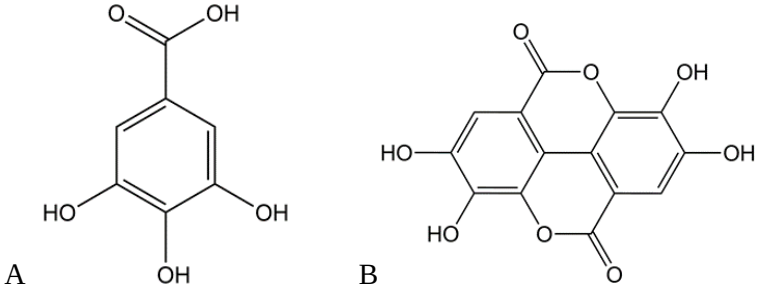

Figure No. 8
Structures of gallic acid (A) and ellagic acid (B) identified in *Myrcia hatschbachii*

The isolated phenolic compounds have antioxidant property described in the literature. The antioxidant action of gallic acid is due to the three hydroxyl groups attached to the aromatic ring in the ortho position, one in relation to the other. Gallic acid provides efficient protection against oxidative damage caused by reactive species found in biological systems, including hydroxyl, superoxide, and peroxyl groups; and non-radical species, such as hydrogen peroxide and hypochlorous acid (Badhani *et al.*, 2015).

The molecular structure of ellagic acid is composed of four fused rings with four hydroxyl groups and two lactone rings representing the hydrophilic part, thus being a dimeric form of gallic acid. The antioxidant property of this compound is related to its molecular structure, particularly the presence and number of hydroxyl groups, the effects of conjugation and resonance, and also the ability to improve the stability of its phenoxy radicals (Dávalos *et al.*, 2019). Ellagic acid directly eliminates reactive oxygen and nitrogen species, such as hydroxyl radicals, hydrogen peroxide, peroxyl, and peroxynitrite radicals. In addition, it prevents oxidative stress by activating cellular antioxidant enzyme systems and is capable of forming a complex with iron ions, which contributes to the reduction of free radical formation (Mehrzadi *et al.*, 2018).

Plants are known as sources of natural antioxidants (Suttirak *et al.*, 2014). Most of these compounds are phenolic and comprise the main group of phytochemicals found in plant species (Tohma *et al.*, 2016). Phenolic compounds are best extracted by solvents such as ethyl acetate, due to their high polarity and better solubility (Babbar *et al.*, 2014). FFA of *Myrcia hatschbachii* showed a high amount of these compounds in its composition (75%), which were extracted using a suitable solvent. As a consequence of this high content, and isolation of two phenolic acids, FFA presented relevant antioxidant potential. In addition, the strong positive correlation between phenolic compounds and the antioxidant property was demonstrated by Pearson correlation coefficient.

Previous studies with species of the *Myrcia* genus carried out the determination of phenolic compounds. The ethyl acetate fractions obtained from the crude extracts of *Myrcia splendens* and *Myrcia palustris*, showed results of 8.6% and 11.1%, respectively (Moresco *et al.*, 2014). Comparing these data with the same fraction of the researched species, the result obtained by FFA was relevant. Ethanol extracts showed results of 0.0715 g GAE/g for *Myrcia guianensis*, 0.2182 g GAE/g for *Myrcia fallax*, and 0.2154 g GAE/g for *Myrcia bella* (Santos *et al.*, 2018). The last two species mentioned presented quantification close to the EBF of *Myrcia hatschbachii* (0.2538 g GAE/ g).

The antioxidant property has been demonstrated in other species of the genus in the methods of DPPH and ABTS. No reports of the FRAP method were found. Studies carried out with the ethyl acetate fraction in the DPPH test showed an IC_{50} of 8.44 µg/mL for *Myrcia splendens* and 17.83 µg/mL for *Myrcia palustris* (Moresco *et al.*, 2014). In relation to these two species mentioned, FFA of *Myrcia hatschbachii* showed better antioxidant potential (IC_{50}: 3.81 µg/mL). A better potential of the extracts (2.42 – 7.01 µmol TE/ mg) was also observed in comparison to the essential oil of *Myrcia amazonica* (0.29 µmol TE/ mg) in the ABTS method (Calao, 2014). In addition, EBF and FFA presented superior antioxidant capacity compared to BHT in all tested methods, except EBF in the FRAP method, which showed similar effect. BHT is a synthetic antioxidant, which has a lipophilic character and slow reaction kinetics (Alves *et al.*, 2010). These data of present work are promising, since, although BHT is used in industry to slow food deterioration, carcinogenic effects of this compound were reported in experimental animals (Suttirak *et al.*, 2014).

α-Glucosidase is an enzyme that hydrolyzes non-reducing α-D-glucose terminal residues. Consequently, glucose is released into the blood, resulting in postprandial hyperglycemia (Chen *et al.*, 2019). The α-glucosidase inhibitory effects of *Myrcia hatschbachii* (IC_{50} = 1.1 - 3.2 µg/mL) were much stronger than those described in the literature for two species of Myrtaceae, *Backhousia citriodora* (IC_{50}: 130 µg/mL) and *Syzygium anisatum* (IC_{50} = 300 µg/mL) (Sakulnarmrat & Konczak, 2012). The effects were also similar or stronger when compared to other extracts of species of the genus, such as *Myrcia salicifolia* (IC_{50} = 1.3 -1.9 µg/mL), *Myrcia sphaerocarpa* (IC_{50} = 1.1 - 4.1 µg/mL), *Myrcia speciosa* (IC_{50} = 1.0 - 4.0 µg/mL) (González *et al.*, 2016), *Myrcia guianensis* (IC_{50} = 7.8 µg/mL), and *Myrcia torta* (IC_{50} = 5.3 µg/mL) (Lima *et al.*, 2018). All of these cited species are called "pedra-hume-caá" or "insulin plant" in Brazil and have traditional use as antidiabetic drugs (Silva *et al.*, 2015).

Lipases are interfacial active enzymes that

hydrolyze triglycerides in the gastrointestinal tract in more polar digestion products, specifically fatty acids and monoglycerides (Joyce *et al.*, 2019). The inhibitory activity of pancreatic lipase of EBF and FFA (IC_{50} = 532 - 556 µg/mL) showed less effect compared to orlistat and greater effect compared to species of the family, such as *Backhousia citriodora* (IC_{50} = 2500 µg/mL) and *Syzygium anisatum* (IC_{50} = 1550 µg/mL) (Sakulnarmrat & Konczak, 2012). *Syzygium cumini* extract (5 mg/mL) inhibited pancreatic lipase activity by 32% (Franco *et al.*, 2018), while *Myrcia hatschbachii* crude extract (0.9 mg/mL) inhibited 84%. No pancreatic lipase inhibition data were found in the *Myrcia* genus.

Ellagic acid showed an enzymatic inhibitory effect with IC_{50} of 3.6 µg/mL for α-glucosidase with a reversible and non-competitive inhibition (Yin *et al.*, 2018) and IC_{50} of 92 µg/mL for pancreatic lipase (Les *et al.*, 2018). Relating the EBF and FFA inhibitory activities and the identification of ellagic acid in the fraction, it can be inferred that the isolated compound contributed to the species activity.

The molecular interactions of phenolic compounds and digestive enzymes, such as α-glucosidase and pancreatic lipase, show that non-covalent bonding, mainly by van der Waals forces, hydrogen bonding, hydrophobic bonding, and other electrostatic forces are the key to enzymatic inhibition. A greater capacity for binding and inhibiting these enzymes is mainly related to the structure of phenolic compounds, such as number of hydroxyl groups, presence of glycosylation, position and number of glycosyl units, and structural complexity. In some cases, the presence of an extra phenolic hydroxyl group can modify the effect of phenolics on catalysis. Other aspects that influence this interaction are the composition of the enzyme (number of polar and hydrophobic amino acids, and molecular weight) and the characteristics of the reaction (pH, temperature, and incubation time) (González *et al.*, 2017).

Pearson coefficient showed that, in the studied species, the inhibition of the activity of both enzymes is strongly correlated to the content of phenolic compounds and to the antioxidant property. Previous studies with medicinal plants have also demonstrated this correlation with enzymatic activity (Sakulnarmrat & Konczak, 2012; Lordan *et al.*, 2013; González *et al.*, 2016). The association between inhibition of enzymatic activity and antioxidant capacity justifies the results obtained by the species, bringing complementary benefits, since type 2 diabetes is related to the increase in oxidative stress. Hyperglycemia causes the release of reactive oxygen species harmful to tissues, as well as disorders of antioxidant defense systems, such as alteration in antioxidant enzyme and impaired glutathione metabolism (Murugan & Pari, 2006).

The results of enzymatic inhibition of *Myrcia hatschbachii* were promising especially against α-glucosidase. Although the effect against pancreatic lipase occurs in higher concentrations, the simultaneous inhibition of both enzymes motivates further studies, especially *in vivo* tests, and a better understanding of the mechanisms by which natural inhibitors, such as phenolic compounds, act on digestive enzymes. This premise makes it possible to find alternatives to current commercial inhibitors, serving as a basis for the development of new and more effective anti-diabetic and anti-obesity agents.

The toxicity test against *Artemia salina* is used for the preliminary toxicity evaluation (Amarante *et al.*, 2011). This method demonstrates a good correlation between *in vitro* and *in vivo* toxicity methods and has been carried out in research with medicinal plants from different countries to determine acute oral toxicity, estimating the average lethal concentration (Parra *et al.*, 2001). The method is robust, easy to perform and has low cost. In addition, the development of *in vitro* tests to determine the possible toxicity of plants has been stimulated due to campaigns to reduce the use of laboratory animals (Rosa *et al.*, 2016).

The extracts of *Myrcia hatschbachii* showed absence of toxicity against *Artemia salina*, whereas the essential oil was previously tested and showed LC_{50} of 409.92 µg/mL (Gatto *et al.*, 2020). This analysis was also performed on other species of the family and genus. Regarding the Myrtaceae family, extracts of *Eugenia dysenterica* and *Eugenia pitanga* were inactive when evaluated against brine shrimp (Alves *et al.*, 2010), whereas the extract of *Pimenta dioica* showed LC_{50} of 32.78 µg/mL (Parra *et al.*, 2001). The essential oil of species of the genus showed LC_{50} results of 479.16 µg/mL for *Myrcia myrtifolia* (Cerqueira *et al.*, 2007) and 79.44 µg/mL for *Myrcia sylvatica* (Rosa *et al.*, 2016), being considered toxic (Meyer *et al.*, 1982).

CONCLUSION

In light of the proceeding discussion, the extracts of *Myrcia hatschbachii* showed relevant results in the inhibition of α-glucosidase and pancreatic lipase. Together with the antioxidant property shown in all tested methods and the isolation of phenolic compounds, such as gallic and ellagic acids, in

addition to the absence of *in vitro* toxicity in a preliminary test, new studies could be investigated for the development of herbal medicine, since substances capable of inhibiting digestive enzymes and reducing oxidative stress may be useful in the treatment of diabetic patients.

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