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Original Article

Metanolic extract of *Malpighia emarginata* bagasse: phenolic compounds and inhibitory potential on digestive enzymes

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ABSTRACT

Adding value to fruit residues is of great interest, since they can be presented as a viable solution in search of new drugs for the treatment of obesity and related diseases, due to bioactive substances, especially phenolic compounds. Thus, the objective of this study was to prepare the methanol extract of acerola bagasse flour, in order to evaluate its potential as a source of inhibitors of the enzymes α-amylase, α-glucosidase, lipase and trypsin, and determine the content of phenolic compounds by high performance liquid chromatography. Enzymatic inhibition assays were conducted in the presence or absence of simulated gastric fluid. In the methanol extract of acerola bagasse flour, the following phenolic compounds were identified: gallic acid, syringic and *p*-coumaric acid, catechin, epigallocatechin gallate, epicatechin and quercetin; epicatechin was the major compound. In the absence of gastric fluid, simulated enzymes had a variable inhibition of the acerola bagasse flour extract, except for lipase, which was not inhibited. In the presence of simulated gastric fluid, there was an inhibition of 170.08 IEU (Inhibited Enzyme Unit in $\mu mol\ min^{-1}\ g^{-1}$) for α-amylase and 69.29 IEU for α-glucosidase, indicating that this extract shows potential as an adjuvant in the treatment of obesity and other dyslipidemia.

Introduction

Obesity is a disease resulting from the excessive accumulation of body fat, and brings multiple outcomes for health, such as the prevalence and progression of cardiovascular diseases (especially heart diseases and stroke), which were the major causes of death in 2012; Some types of cancer (endometrium, breast and colon); skeletal muscle disturbs (specially osteoarthritis – a highly incapacitating degenerative disease); hypertension and type 2 diabetes mellitus (Wanderley and Ferreira, 2010; WHO, 2015).

Between 1980 and 2014, the world's obesity prevalency doubled. Data from the World Health Organization report that, in 2014, more than 1.9 billion adults were overweight and, among them, more than 600 million were obese (WHO, 2015).

One way to fight this epidemic disease is drug treatment. Medicine to fight weight gain, which has the objective to restrict energy absorption and cause weight loss, is widely available (Boniglia et al., 2008). However, these drugs cause side effects and are prohibited by Anvisa since 2011 (Abeso, 2014). Another alternative broadly employed is the use of plant extracts. Over the last years, there was a substantial increase in its use, by the fact that the population believes its intake is harmless, with a low cost, and may inhibit digestive enzymes, leading to beneficial changes in metabolism (Simão et al., 2012). However not all natural products are beneficial and further studies are necessary to evaluate their effects on the organism.

Enzymes like α-amylase and α-glycosidase, responsible for processing dietary carbohydrates, act on starch breakdown, resulting in monosaccharide absorption by enterocytes. Therefore, their inhibition offers a promising strategy for the prevention of obesity, as well as type 2 diabetes associated to hyperglycemia, by inhibiting starch breakdown and glucose absorption in the small intestine (Kwon et al., 2008; Balasubramaniam et al., 2013).

Lipase, involved in fat metabolism, is also an important target for inhibitors, since its inhibition limits triacylglycerol absorption, leading to a decrease in caloric yield and weight loss. On the other hand, trypsin inhibition, involved in protein digestion, has a malefic effect, once it impairs the complete amino acid absorption in food, essential for the organism.

Research has been carried out for evaluating the effects of natural products on the treatment of obesity and associated comorbidities, reinforcing the need for the search of new sources of

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amylase, glycosidase and lipase inhibitors (Souza et al., 2011; Pereira et al., 2011a; Simão et al., 2012). Therefore, digestive inhibitors who assist in reducing fat and carbohydrate absorption in the small intestine may be useful helpers in the treatment of obesity.

Natural products have been gaining space and importance in the pharmaceutical industry, since they have bioactive substances capable of inspiring new phytomedicines and phytotherapic products. Phenolic compounds are among those substances. These compounds present chemical structures with hydroxyls and aromatic rings, which can be simple structures or polymers, originated from plant secondary metabolism and largely found in fruits (Angelo and Jorge, 2007). Many studies report the benefits of phenolic compounds as an adjunct in the treatment of obesity (Klaus et al., 2005; Hen et al., 2006; Alterio et al., 2007; Santiago-Mora et al., 2011; Vogel et al., 2015; Zhang et al., 2015).

Alterio et al. (2007) and Klaus et al. (2005) report that phenolic compounds act in the prevention of obesity due to their thermogenic effects, ability to oxidize body fat and by decreasing intestinal absorption of fats and carbohydrates caused by the inhibition of digestive enzymes, resulting in weight loss. Phenolic compounds, such as tannins, have the ability of combining with digestive enzymes, proteins and other polymers (such as carbohydrates), forming stable complexes, impairing absorption and, therefore, making them possible inhibitors of some of these digestive enzymes (Won et al., 2007; Gholamhoseinian et al., 2010).

In this context, the use of agro industrial residues of fruits is promising for the extraction of active principles that may be employed as an alternative to the treatment of obesity and correlated diseases. By discarding these residues, secondary metabolites of great aggregated value with possible applications in pharmaceutical and food industries, are also eliminated. For example, the acerola bagasse originated in juice processing is, according to Marques et al. (2013), rich in phenolic compounds, with record contents of 10.82 g 100 g^{-1} dry matter; however, these phenolic compounds were not yet identified.

Given the above, the objective of the present study was to prepare the methanol extract of acerola bagasse flour (ABF), evaluate its potential as a source of α-amylase, α-glycosidase, lipase and trypsin inhibitors, and determine the phenolic compounds by high performance liquid chromatography (HPLC), aiming to use it as an auxiliary in the treatment of obesity and correlated diseases, aggregating value to this residue.

Material and methods

Preparation of acerola bagasse flour

Acerola *Malpighia emarginata* DC., Malpighiaceae (BRS 238 Frutacor) bagasse was obtained from plants grown in the municipality of Perdões, MG, Brazil (21°05′27″ S; 45°05′27″ W, 848 m altitude); the local climate according to the Köppen system is classified as Cwa: mild and rainy summers with moderate temperatures, annual average temperature below 21 °C, average annual precipitation of 1529.7 mm, and relative humidity of 76% (Emater, 2002). Acerola fruits were used for pulp extraction, and the residual bagasse was provided in three batches by a fruit pulp plant firm located in Perdões, MG, Brazil.

Acerola bagasse (4 kg) was frozen at −18 °C and lyophilized in glass containers protected from light for 7 days to obtain 450 g dry bagasse. After lyophilization, acerola bagasse was homogenized using mortar and pestle, was passed in sieves and most flour particles were retained on sieves sized 40 mesh (0.425 mm) to 80 mesh (0.180 mm), thus, classified as fine and then placed in a hermetically sealed flask, protected from light in a refrigerator at 4 °C.

Obtention of the extract

To obtain the methanol extract of acerola bagasse flour (ABF), 1 g of acerola bagasse lyophilized powder was transferred to a 250 ml erlenmeyer and then added 50 ml of 50% methanol solution in three repetitions. Afterwards, it covered with a ground glass joint and put on a hot plate at 80 °C. After boiling for 15 min, the extract was filtered in filter paper and collected to a 250 ml becker. The residue was once again put on an erlenmeyer and this process repeated for two more times. After the third filtration, the becker was taken to the hot plate to evaporate the methanol until the volume reaches 16 ml (AOAC, 2012), and then submitted to enzymatic inhibition analysis.

For the chromatography process, the becker was taken to the hot plate to evaporate the methanol, posteriorly frozen and lyophilized (Free Zone® 2.5 liter Freeze Dry Sustems). Lyophilyzed extract (1 g) was solubilized in 16 ml ultrapure water obtained from a Milli-Q system (EMD Millipore, Billerica, MA, USA).

Identification and quantification of phenolic compounds

HPLC was performed using a Shimadzu UHPLC chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with two LC-20AT high-pressure pumps, an SPD-M20A UV–vis detector, a CTO-20AC oven, a CBM-20A interface, and an automatic injector with an SIL-20A auto sampler. Separations were performed using a Shim-pack VP-ODS-C18 (250 mm × 4.6 mm) column, connected to a Shim-pack Column Holder (10 mm × 4.6 mm) pre-column (Shimadzu, Japan).

The mobile phase consisted of the following solutions: 2% acetic acid in water (A) and methanol:water:acetic acid (70:28:2, v/v/v) (B). Analyses were performed for a total time of 65 min at 40 °C, flux of 1 ml min^{-1}, wavelength of 280 nm, and injection volume of 20 μl in a gradient-type system (100% solvent A from 0.01 to 5 min; 70% solvent A from 5 to 25 min; 60% solvent A from 25 to 43 min; 55% solvent A from 43 to 50 min; and 0% solvent A for 10 min) until the end of the run. Solvent A was increased to 100%, seeking to maintain a balanced column. Acetic acid and methanol (HPLC grade; Sigma–Aldrich, USA) were used in the preparation of the mobile phase.

Addition of standards to the extracts was also used as an identification parameter. The phenolic standards used were gallic acid, catechin, epigallocatechin gallate, epicatechin, syringic acid, *p*-coumaric acid, ferulic acid, salicylic acid, resveratrol and quercetin all obtained from Sigma–Aldrich (St. Louis, MO, USA). The stock standard solutions were prepared in methanol (HPLC grade; Sigma–Aldrich, USA).

The ABF extract and the standards were filtered through a 0.45-μm nylon membrane (EMD Millipore, USA) and directly injected into the chromatographic system, in three replicates. The phenolic compounds in the extract were identified by comparison with retention times of standards. Quantification was performed by the construction of analytical curves obtained by linear regression using Origin 6.1 computer software (OriginLab, Northampton, MA, USA) and considering the coefficient of determination (R^2) equal to 0.99.

Enzyme obtention

Were used in the assays the enzymes: porcine pancreatic lypase (EC 3.1.1.3) type II, Sigma; porcine pancreatic α-amylase (EC 3.2.1.1) type VI B, Sigma and porcine pancreatic trypsin (EC 3.4.21.4), Merck. The α-glycosidase (EC 3.2.1.20) was obtained from fresh porcine duodenum according to Souza et al. (2011). The supernatant was collected and used as an enzymatic extract.

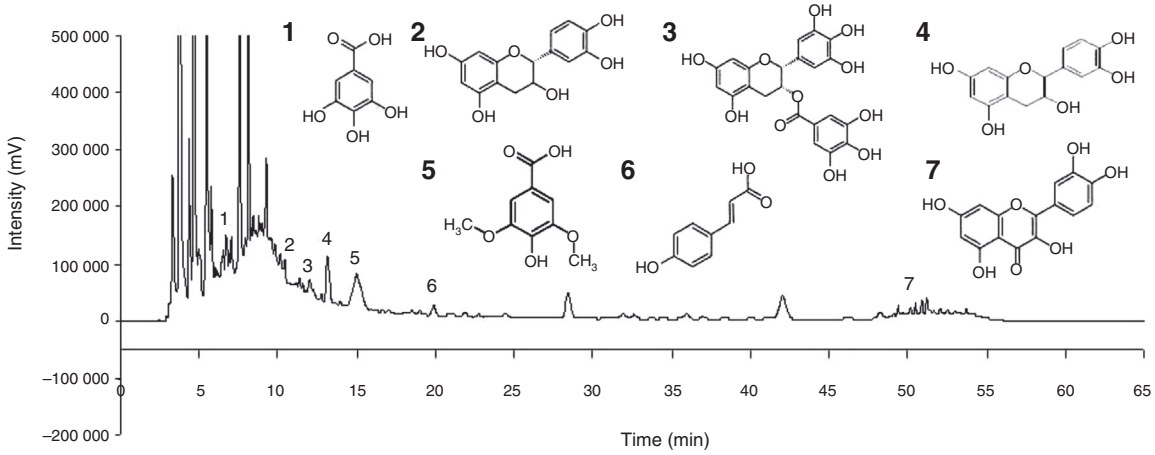

Fig. 1. Chromatogram of acerola bagasse flour extract, with peak identification: (1) Gallic acid (time = 6.541 min); (2) catechin (time = 10.419 min); (3) epigallocatechin gallate (time = 11.987 min); (4) epicatechin (time = 13.139 min); (5) syringic acid (time = 14.988 min); (6) *p*-coumaric acid (time = 19.892 min) and (7) quercetin (time = 51.185 min).

Activity of α-amylase, α-glucosidase, lipase and trypsin

The α-amylase activity was determined using the methodology proposed by Noelting and Bernfeld (1948). Thus, the extract and α-amylase enzyme were pre-incubated for 20 min, in a water bath at 37 °C. The substrate was the 1% starch prepared in Tris 0.05 mol l^{-1}, pH 7.0 buffer with 38 mmol l^{-1} NaCl and 0.1 mmol l^{-1} $CaCl_2$. After addition of 100 μl of the substrate, the mixture was incubated for four periods of time. The reaction was interrupted adding 200 μl of 3.5 dinitrosalicylic acid and the product read in spectrophotometer at 540 nm.

The α-glucosidase activity was determined according to Kwon et al. (2008), using 5 mmol l^{-1} *p*-nitrophenyl-α-D-glucopyranoside in a 0.1 mol l^{-1} pH 7.0 citrate–phosphate buffer as substrate. In the assay, extract and α-glucosidase enzyme were incubated in a water bath, at 37 °C, for four periods of time, after addition of the substrate. The reaction was interrupted adding 1.000 μl of 0.05 mol l^{-1} NaOH and the product was read in a spectrophotometer at 410 nm.

The lipase activity was determined according to Souza et al. (2011), using 8 mmol l^{-1} *p*-nitrophenylpalmitate in Tris–HCl 0.05 mmol l^{-1}, pH 8.0 buffer containing 0.5% Triton-X100 as substrate. In the assay, extract and lipase enzyme was incubated in a water bath, at 37 °C, for four periods of time, after addition of the substrate. The reaction was stopped, transferring the tubes to an ice bath and adding Tris–HCl 0.05 mmol l^{-1} pH 8.0 buffer. The *p*-nitrophenol, of yellow coloration, a product of the lipase action on *p*-nitrophenylpalmitate, was read in a spectrophotometer at 410 nm.

The trypsin activity was determined according to the methodology proposed by Erlanger et al. (1961). Thus, extract and trypsin enzyme were incubated in a water bath, at 37 °C, for four periods of time, after addition of *p*-benzoyl-DL-arginine-*p*-nitroanilide substrate (BApNA), prepared in Tris 0.05 mol l^{-1}, pH 8.2. The reaction was interrupted adding 200 μl of 30% acetic acid and the product read in a spectrophotometer at 410 nm.

For each assay of enzymatic activity, the volume of extract was different and its dilution ranged so that the enzyme inhibition ranged from 50 to 80%, according to the methodology.

The inhibition of the enzymes were obtained from the determination of the slopes of the straight lines (absorbance × time) of the control enzyme (without extract) and enzymes + inhibitor (with extract) activity assays. The slope of the straight line is due to the speed of product formation per minute of reaction and the presence of the inhibitor causes a decrease in that inclination. From that inclination, the absorbance values were converted into micromoles of product through a standard glucose curve for the amylase and of *p*-nitrophenol for glycosidase and lipase, while, for the trypsin, the of BApNA molar extinction coefficient determined by Erlanger et al. (1961) was used.

Preparation of simulated gastric fluid

With the objective of simulating the digestion process in the stomach *in vitro*, enzymatic activity assays in the presence of a simulated gastric fluid were also carried out. For such, the extract was incubated with the simulated gastric fluid prepared according to The United States and Pharmacopeia, (2005), for 1 h in a water bath at 37 °C. Subsequently, was neutralized with sodium bicarbonate salt to pH 7.2 and only then realized the activity assays.

Results and discussion

Each 100 g ABF yielded 48 g of lyophilized extract (48% yield). The following phenolic compounds were identified in the ABF extract, in mg l^{-1}: gallic acid (3.32 ± 0.23), catechin (11.33 ± 0.33), epigallocatechin gallate (9.13 ± 0.89), epicatechin (91.86 ± 1.49), syringic acid (37.16 ± 0.12), *p*-coumaric acid (2.41 ± 0.13) and quercetin (0.29 ± 0.02) (Fig. 1); gallic acid is a hydrolyzable tannin monomer, and epigallocatechin gallate, catechin and epicatechin are condensed tannin monomers. It was possible to observe that epicatechin had the highest content, followed by syringic acid. The compounds epicatechin, ferulic acid, salicylic acid and resveratrol were not identified in the ABF extract.

Lin and Lin-Shiau (2006), Alterio et al. (2007), Cho et al. (2010) and Rains et al. (2011) reported that phenolic compounds such as caffeic and chlorogenic acid, catechin, epigallocatechin gallate and quercetin have thermogenic effect, ability to oxidize fats, control appetite, regulate levels of hormones related to obesity and inhibit digestive enzymes involved in the absorption of carbohydrates and lipids. Thus, this study shows that the acerola bagasse extract has bioactive substances and can be exploited by the pharmaceutical industry in search of drugs to control obesity and related diseases.

The results for enzymatic inhibition of ABF before the exposure to gastric fluids are shown in Table 1. The ABF methanol extract inhibited the activity of α-amylase, presenting an inhibition potential of 238.96 μmol min^{-1} g^{-1} dry matter – DM. This potential exceeds the one found by Pereira et al. (2011b), who analyzed the white bean crude extract and detected an inhibition of 54.1 μmol min^{-1} g^{-1}. Simão et al. (2012), studying aqueous extracts of medicinal plants, observed an inhibition of 2907.13 μmol min^{-1} g^{-1} DM for *Tournefortia paniculata* Cham. (marmelinho), higher than that found in this study. α-Amylase is

Table 1
Inhibition of digestive enzymes by acerola bagasse powder before and after the exposure to simulated gastric fluid.

Enzyme	Inhibition (IEU[b])[a]	
	Before exposure	After exposure
α-Amylase	238.96 ± 1.64	170.08 ± 1.06
α-Glycosidase	78.51 ± 1.78	69.29 ± 0.28
Lipase	nd[c]	nd[c]
Trypsin	227.52 ± 3.59	84.73 ± 5.41

Data from three repetitions, with mean ± standard deviation.
[a] The ABF extract measured for each of the enzymes was diluted to provide an inhibition between 50% and 80%, in order to ensure result reliability.
[b] IEU = Inhibited Enzyme Unit in μmol min^{-1} g^{-1} dry matter – DM.
[c] nd = inhibition not detected.

Table 2
Content of phenolic compounds in the methanol extract of acerola bagasse flour, used in each enzymatic assay.

Phenolic compound (μg)	Enzymatic assays			
	α-Amylase[a]	α-Glycosidase[b]	Lipase[c]	Trypsin[c]
Gallic acid	0.02	0.06	0.17	0.66
Catechin	0.07	0.19	0.57	2.26
Epigallocatechin-gallate	0.05	0.15	0.46	1.83
Epicatechin	0.54	1.53	4.60	18.4
Siringic acid	0.22	0.62	1.86	7.44
p-Cumaric acid	0.01	0.04	0.12	0.48
Quercetin	1.71×10^{-3}	4.83×10^{-3}	0.01	0.06
$\sum$ Phenolic compounds	0.91	2.59	7.79	31.13

[a] Extract dilution 1:7.5.
[b] Extract dilution 1:2.
[c] Crude extract.

related with the digestion of carbohydrates and, consequently, with the elevation in glycemic levels after a meal. High glycemic levels lead to serious health problems in the population, such as type 2 diabetes. The intake food rich in α-amylase poses as an interesting strategy in the prevention and treatment of hyperglycemia, by slowing postprandial glucose levels in blood after the ingestion of carbohydrates (Vadivel et al., 2011).

The inhibition of α-glucosidase by the ABF extract was about 78.51 μmol min^{-1} g^{-1} DM. The inhibitory potential of ABP found in this paper surpasses the ones verified by Simão et al. (2012) who, studying aqueous extracts of medicinal plants like *Aloe vera* (L.) Burm. (Aloe), *Simaba ferruginea* St. Hil. (calunga), *Baccharis trimera* (Less.) DC (carqueja), *Garcinia cambogia* Desr. (garcinia), *T. paniculata* Cham. (marmelinho), found inhibitions of 0.58 and 35.46 μmol min^{-1} g^{-1} DM, as well as those from Pereira et al. (2011a), who analyzed commercial samples of *Hoodia gordonni*, used as an auxiliary in the treatment of obesity, and found inhibitions of 10.40 e 16.70 μmol min^{-1} g^{-1} DM.

The inhibition of α-glucosidase extends gastric emptying, leads to satiety and weight loss, effects which can be useful in the treatment of obesity (Chen et al., 2008).

Therefore, the inhibition of α-amylase and α-glycosidase by natural products can provide an alternative for the treatment of obesity in substitution to synthetic drugs now available on the market, besides controlling glucose levels in blood in type 2 diabetes patients (McDougall et al., 2005a).

The ABF extract was not able to inhibit lipase, an enzyme involved in lipid metabolism, neither before nor after the exposure to simulated gastric fluid. However, for trypsin, an inhibition of 227.52 μmol min^{-1} g^{-1} DM was observed. When trypsin inhibitors are present in the diet, these may lead to a reduction in growth rate in animals, followed by a decrease in protein digestibility, leading to weight loss and endogenous protein catabolism (McDougall et al., 2005a). Therefore, the trypsin inhibitor is considered an antinutritional factor.

The passage of the ABF extract through the gastrointestinal cavity may lead to structural modifications on the inhibitors because of the pH of the gastric acid. Considering the expressive inhibition of α-amylase, α-glycosidase and trypsin in the presence of the ABF extract, this extract was submitted to a gastric fluid assay (Table 1).

In the presence of simulated gastric fluid, the ABF methanol extract still maintained 71% of its inhibitory activity over α-amylase and 88% of inhibitory activity over α-glycosidase. Therefore, the extract did not show a considerable inhibitory activity over these two enzymes after they were submitted to simulated gastric fluid.

The ABF extract decreased the inhibition of trypsin by 63% in the presence of simulated gastric fluid. This reduction in trypsin inhibition is considered positive since, when inhibition occurs, it is considered antinutritional, impairing protein digestion, which is the main source of essential amino acids. However, a residual inhibitory activity of 37% was still observed. It is noted that the resistance of the inhibitor to pass through the simulated gastric fluid is a strong indicative that these results will repeat in *in vivo* assays.

In this study, the inhibition of digestive enzymes can probably be explained by the presence of phenolic compounds in the methanol ABF extract, whose levels were different for each enzymatic assay assessed (Table 2). α-Amylase was the one that had the smallest content of phenolic compounds, which led to an inhibition of 0.91 μg. On the other hand, the content of phenolic compounds was higher (31.13 μg) for the trypsin assay, that is, 34 times superior to that found for α-amylase. Therefore, this suggests that smaller contents of phenolic compounds may not lead to trypsin inhibition, which would be beneficial, since it could reduce the absorption of carbohydrates and allow protein digestion.

Gallic acid is considered a hydrolyzable tannin, when found in the form of gallic acid esters, while catechin, epicatechin gallate and epicatechin, when found in the form of flavonoids, are considered condensed tannins. These compounds have strong interactions with metal ions and macromolecules such as polysaccharides, besides the ability to form soluble complexes with several proteins, as digestives enzymes (Won et al., 2007; Gholamhoseinian et al., 2010). This ability that tannins exhibit to interact with proteins makes this class of substances powerful digestive enzyme inhibitors.

McDougall et al. (2005b) report that red fruit extracts in phenolic compounds inhibit two main enzymes involved in starch digestion, α-amylase and α-glycosidase, *in vitro*. In a similar way, recent studies with red fruits reported inhibition of α-amylase and α-glycosidase, and mentioned that tannins were the most effective compounds in inhibiting these enzymes (Boath et al., 2012). Kam et al. (2013), studying the effects of extracts from different parts of pomegranate (pulp, peels, seeds and flower) over the digestive enzymes α-amylase and α-glycosidase, showed that the methanol extract from the pomegranate flower, where the phenolic compounds gallic acid and ellagic acid are found, exhibits a potent inhibitor effect on these enzymes.

Klaus et al. (2005) reported that rats fed epigallocatechin gallate, purified from green tea, had an obesity decrease induced by the diet, due to a reduction in energy absorption and an increase in lipid oxidation. On the other hand, Bryans et al. (2007) reported that black tea is efficient in reducing postprandial blood glucose levels and related this fact to the presence of phenolic compounds such as epigallocatechin, epigallocatechin gallate, epicatechin and epicatechin gallate.

Flavonoids like quercetin comprise a heterogeneous class of phenolic compounds present in plants, which can also act in the organism, inhibiting digestive enzymes. Wenzel (2013) reported that quercetin is a promising enzyme inhibitor, limiting

carbohydrate digestion and controlling postprandial glucose levels in blood, thus confirming the result obtained by Tadera et al. (2006), who reported the inhibitory activity of quercetin in the presence of α-amylase.

Lin and Lin-Shiau (2006), report that flavonoids have the ability to act on the sympathetic nervous system through the modulation of noradrenaline, thus increasing thermogenesis and fat oxidation. It also prevents the increase in the size and number of adipocytes, therefore preventing the deposition of fat in the body and regulating body weight.

Phenolic extracts of lentils containing *p*-hydroxybenzoic acid, syringic acid, trans-*p*-coumaric acid, epicatechin gallate, quercetin and kaempferol were shown to be good inhibitors of lipase and α-glycosidase, contributing to control glucose levels in blood, as well as obesity (Zhang et al., 2015).

Most phenols previously mentioned were found in the ABF extract, which could have led to a complexation with digestive enzymes and, probably, contributed to its inhibition. The inhibition of digestive enzymes is a promising alternative for the treatment of obesity and type 2 diabetes, especially by the fact they act in the small intestine, without acting in the central nervous system, where prescribed anorexigenic drugs usually act.

Conclusion

The ABF methanol extract that contains the phenolic compounds gallic acid, catechin, epicatechin gallate, epicatechin, siringic acid, p-cumaric acid and quercetin, was able to inhibit *in vitro* digestive enzymes α-amylase and α-glucosidase. This shows that the ABF extract may represent a good source of inhibitors, and can be used as an auxiliary in the treatment of obesity, associated comorbidities and in the control of type 2 diabetes.

Authors' contributions

TRM contributed in running the laboratory work, analysis of the data and drafted the paper. AAC contributed in running the laboratory work. AAS participated of enzyme activity assays. FCOC contributed to chromatographic analysis. VOR participated of enzyme activity assays. ADC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

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