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Full Length Research Paper

Inhibitory effects of *Anadenanthera colubrina* (Vell.) Brenan stem bark extract on *α*-glucosidase activity and oxidative stress

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In this study, we investigated the promising potential of *Anadenanthera colubrina* bark extract (BEAc) as a product to combat diabetes mellitus (DM). We evaluated the inhibitory effects of BEAc on *α*-glucosidase and the oxidation of biomolecules, as well as its main phytoconstituents. In terms of free radical scavenging, BEAc exhibited a dose-effect relationship. BEAc was more efficient than rutin and butylated hydroxytoluene and similar to ascorbic acid at the same concentrations. Evaluation of the IC_{50} confirmed the good activity of BEAc compared to positive controls and statistically determined to be equal to ascorbic acid. In *in vitro* *α*-glucosidase inhibition studies, BEAc generated 31 times more potent inhibition than acarbose and was dose-dependent at the concentrations tested. Lineweaver-Burk and Michaelis-Menten plots obtained for kinetic analysis showed that BEAc competitively inhibited the *α*-glucosidase catalyzed reaction. Chemical analysis of BEAc by HPLC revealed that the plant is rich in phenolic compounds and confirmed its capacity to inhibit *α*-glucosidase. Fourteen compounds were identified by reference to authentic standards: Gallic acid, catechin, syringic acid, chlorogenic acid, *p*-coumaric acid, naringin, vitexin, rutin, isorhamnetin, hesperidin, myricetin, morin, rosmarinic acid, and quercetin. Thus, this study provides the first evidence of the antidiabetic activity of *A. colubrina* bark and determines its possible modes of action on carbohydrate metabolism via inhibition of *α*-glucosidase and the control of biomolecule oxidation. These data support the potential use of this plant for the development of promising multi-target therapy products combining postprandial hyperglycemia control and biomolecule oxidation control.

Key words: Kinetic analysis, phenolic content, diabetes mellitus, oxidation of biomolecules.

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease resulting from the progressive disorder of the production or functioning

of the pancreatic hormone insulin, triggering impaired regulation of carbohydrate and lipid metabolism and leading to increased blood glucose levels (Ramu et al., 2014a). The main complications of this disease are chronic hyperglycemia, weight loss, polydipsia, polyuria, lethargy, and various macrovascular and microvascular complications (Kidane et al., 2018).

The influence of the *α*-glucosidase enzyme on DM has been well documented. The enzyme converts long chain carbohydrates into simpler monosaccharide units, a process that allows for rapid absorption of carbohydrates resulting in high glucose levels in the blood. This is characterized as postprandial hyperglycemia, the earliest symptom of DM. Therefore, suppression of carbohydrate absorption by enzymatic inhibition is an important approach to prevent DM (Ramu et al., 2014b). In fact, the use of inhibitors of these hydrolases is widely accepted as an efficient method to maintain normal blood glucose levels (Ramu et al., 2017); thus, several synthetic *α*-glucosidase inhibitors, such as acarbose, voglibose, and miglitol, are administered to diabetic patients (Banu et al., 2015; Ramu et al., 2017). However, these inhibitors have some restrictions of use, as they may cause adverse reactions, such as liver disorders, flatulence and abdominal cramps (Liu et al., 2014; Ramu et al., 2017). To circumvent these problems, many studies with medicinal plants are underway (Kapoor et al., 2017), especially those plants that already have a long history of use, as this favors the development of safe products and is a low-cost option (Soares et al., 2017).

The *Anadenanthera colubrina* (Vell.) Brenan plant belongs to the Mimosoideae section of the Fabaceae family, and is a botanical species with medicinal properties. It is administered orally, prepared by decoction, infusion or in the form of juice obtained after the maceration of leaves or other parts of the plant (Agra et al., 2008). Its barks are used for the treatment of inflammation, and respiratory diseases, and the leaves are used to treat anemia, inflammation and some cancers (Agra et al., 2008; Albuquerque et al., 2014). However, regarding the pharmacological potential of the secondary metabolites of *A. colubrina*, no studies have been found that evaluate the action of its main chemical constituents against DM. This demonstrates the need for further research on stem barks that are used by populations. In addition, studies on the chemical constitution of this plant have revealed a composition that is promising for the fight against DM, being rich in catechins, flavonoids, phenols, saponins, steroids, tannins, triterpenes, and xanthones (Santos et al., 2013; Melo et al., 2010).

Considering the use of the plant for medicinal purposes (Agra et al., 2008; Albuquerque et al., 2014; Santos et al., 2013) and the incidence of this plant in several Brazilian states (Lorenzi, 2009), this paper forms part of an initial strategy for the development of antidiabetic herbal products in the Amazon region from *A. colubrina*. Therefore, the objective of the present work is to evaluate the potential of the extract of *A. colubrina* bark as an inhibitior of *α*-glucosidase and as an antioxidant as a prospect for the development of antidiabetic products.

MATERIALS AND METHODS

Plant material

The *A. colubrina* bark was collected from the city of Porto Nacional State of Tocantins, Brazil (Geographic coordinates: -10.182406 "S, -48.459146" W) in October 2017. The plant was authenticated by the botanist of the herbarium of the Federal University of Tocantins in Porto Nacional, and a voucher specimen was deposited in the herbarium with the number HTO 1200. The collection of the plant material was conducted under authorization National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) (process number A7EB8D7).

Preparation of extracts

To obtain the *A. colubrina* bark extracts (BEAc), the method of Soares et al. (2017) was used. Therefore the plant material (20 g of powder) was extracted 5 times for one hour each time with a mixture of methanol-acetonitrile (80/20) in an ultrasound bath (USC1600, ULTRASONIC CLEANER, UNIQUE, São Paulo, Brazil) with a frequency of 40 kHz (135 W) at room temperature. The extraction solutions obtained by ultrasound were combined, vacuum filtered, and concentrated in the rotary evaporator at -600 Hg, 70 RPM, and 50°C. BEAc extracts were lyophilized and stored under vacuum conditions in the desiccator until analysis.

DPPH antiradical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl acid (DPPH) assay followed the description of Peixoto-Sobrinho et al. (2011). Briefly, six dilutions (ranging from 10 to 250 μg/mL in methanol) of samples or positive control substances (rutin, butylated hydroxytoluene, and ascorbic acid, all from Sigma-Aldrich) were prepared. An aliquot (0.5 mL) of each dilution was mixed with DPPH (3 mL at 40 μg/mL in methanol). A blank was prepared, replacing the DPPH solution with methanol, to compare the color of the extractive solution with the reaction of the test. After 30 min the absorption was measured at 517 nm. The percentage removal of the DPPH radical was calculated from the absorption using the following equation:

$$AA (\%) = [(A_0 - (A_{sample} - A_{blank})) / A_0] \times 100$$

Where AA is the antioxidant activity, A_{sample} is the absorption of the sample, A_{blank} is the absorption of the blank and A_0 is the absorption of DPPH at 40 μg/mL without sample. By linear regression, the IC_{50} value was also calculated, determining the sample concentration

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required to decrease the absorption at 517 nm by 50%. The IC_{50} was expressed in μg/mL.

Alpha-glucosidase inhibitory assay

The *α*-glucosidase inhibitory assay for BEAc was evaluated according to a previously described method by Ramu et al. (2014). In brief, 0.20 µl of BEAc with varying concentrations (10-200 µg/mL) dissolved in dimethyl sulfoxide (DMSO) was mixed with 106 µl of sodium phosphate buffer (pH 6.8) and 54 µl of *α*-glucosidase solution (0.18 U/mL). This mixture was incubated at 37°C for 10 min, then cooled at 21°C for 5 min and mixed with 20 µL of *p*-nitrophenyl-*α*-D-glucopyranoside (pNPG) 4 µM solution in sodium phosphate buffer (pH 6.8). The reaction was maintained at 37°C for 20 min. The reaction was terminated by adding 100 µl 0.2 M Na_2CO_3. Enzyme activity was determined by measuring the absorption of the liberated *p*-nitrophenol from pNPG at 405 nm using a microplate reader (Biochrom ASYS UVM 340, Holliston, USA). The absorption was compared with the blank, containing buffer instead of a test sample. To evaluate the decrease in absorption, a positive control was prepared with all reagents without the sample. Acarbose was used as a positive control. The results were expressed as percentage *α*-glucosidase inhibition obtained using the formula given below:

$$\text{Inhibition (\%)} = [(A_{control} - (A_{sample} - A_{blank})) / A_{control}] \times 100$$

The IC_{50} value was calculated by linear regression, determining the sample concentration required to decrease the absorption at 405 nm by 50%. The IC_{50} was expressed in µg/mL.

Kinetics of alpha-glucosidase inhibition

The enzyme kinetics of the inhibition of *α*-glucosidase activity by BEAc was studied using concentrations of substrate against IC_{25} and IC_{50} inhibitory concentrations of the BEAc. The type of inhibition, Km, and Vmax were determined by a double reciprocal Lineweaver-Burk plot of the substrate concentration and the velocity (1/V versus 1/[pNPG]) (Ramu et al., 2014).

Phytochemical analysis

Preliminary phytochemical analysis

Preliminary phytochemical analysis of the BEAc was carried out for detection of the presence of the major classes of phytochemicals, such as alkaloids, flavonoids, phenolic compounds, tannins, saponins and steroids, following standardized methods (Matos, 2009).

High performance liquid chromatography analysis (HPLC)

BEAc extracts were analyzed by HPLC using a Shimadzu® chromatograph (Shimadzu®, Kyoto, Japão) equipped with LC-10AT pump, DGU-14A degasser, UV-vis SPD-10A detector, CTO-10A column oven, Rheodyne manual injector (loop 20 µl) and a CLASS SLC-10A integrator. The column used was the Phenomenex Luna C18 5 µm (250 mm × 4.6 mm) column with direct-connect C18 Phenomenex Security Guard Cartridges ($4 \times 3.0\ mm^2$) filled with similar material as the main column at 22°C. UV detection was carried out at 280 nm. The response of the detector was recorded and integrated using Class-VP software. The mobile phase consisted of 0.1% phosphoric acid in water (phase A) and 0.1% phosphoric acid in water/acetonitrile/ methanol (54:35:11 v/v) (phase B) under the following gradient profile: 0-5 min, 0% B; 5-10 min, 30% B, 10−20 min, 40% B, 20-60 min 40% B, 60-70 min 50% B, 70-90 min 60% B, 90-100 min 80% B, 100–110 min 100% B. 110-120 min 100% B. The flow rate was 1.0 mL/min. The compounds were identified by comparing the retention times of samples and authentic standards such as gallic acid, catechin, syringic acid, chlorogenic acid, *p*-coumaric acid, naringin, vitexin, rutin, isorhamnetin, hesperidin, myricetin, morin, rosmarinic acid and quercetin (Sigma®). Before the analysis, all the extracts (at 1 mg/mL) and authentic standards (0.18 mg/mL) were filtered through 0.20 µm membrane filters of polyvinylidenedifluoride.

Statistical analysis

All experiments were carried out in triplicate and data were analyzed by Microsoft Excel 2013 using the non-linear regression analysis-aided determination of IC_{50}.The analytical data were also subjected to an analysis of variance (ANOVA) followed by Tukey's test using Prism software (GraphPad prism software version 8.0, USA).

RESULTS

Antioxidant activity

The scavenging capacity of BEAc on DPPH free radicals, shown in Figure 1, exhibited a dose-effect relationship, increasing with increasing extract concentration. BEAc was more efficient than rutin and BHT and was similar to ascorbic acid at the same concentrations. As shown in Table 1, the IC_{50} assessment confirmed the good activity of BEAc comparing with the positive controls, being statistically equal to ascorbic acid.

Alpha-glucosidase inhibitory assay

The *in vitro* *α*-glucosidase inhibitory assay showed that BEAc exhibited a potent inhibition of glucosidase, with IC_{50} values (19.04 µg/mL) 31 times more potent than acarbose (positive control, IC_{50} = 600 mg/mL). The maximum inhibition found for the BEAc to be 94.2% at a concentration of the 210 ug/mL against 88.7% for the acarbose in the concentration of 0.9 mg/mL (Figure 2).

Kinetics of alpha-glucosidase inhibition

The Lineweaver-Burk and Michaelis-Menten plots obtained for the kinetic analysis are in Figure 3A to D. The results established that BEAc competitively inhibits the reaction catalyzed by *α*-glucosidase. The enzyme kinetic curve fit calculations indicated that Km decreased with higher BEAc concentrations, but there was no effect on Vmax.

Phytochemical analysis

Preliminary phytochemical analysis

A preliminary phytochemical analysis of the bark of *A.*

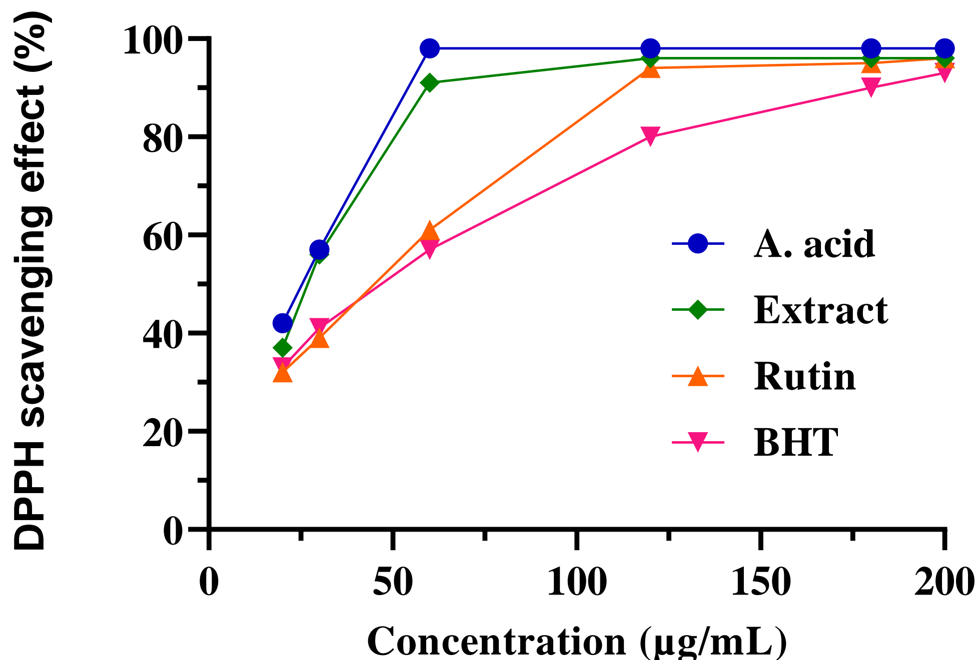

Figure 1. Percentage of inhibition of DPPH radical by *A. colubrina* bark extract and the positive controls ascorbic acid, rutin and butylated hydroxytoluene (BHT).

Table 1. IC_{50} of DPPH inhibition by *A. colubrina* barks extract and the positive controls ascorbic acid, rutin and butylated hydroxytoluene (BHT).

Sample	IC_{50} DPPH (µg/mL)
Extract	27.97 ± 0.60[a]
Rutin	44.74 ± 0.80[b]
A. Acid	25.13 ± 1.04[a]
BHT	46.79 ± 1.96[c]

Values are represented as mean ± SD of triplicates. Values followed by the same letter show no significant differences. Comparing the data of ascorbic acid, BHT and rutin with the extract, P <0.01 is verified by Tukey test.

colubrina (BEAc) revealed the presence of phenolic compounds, flavonoids, flavonols, flavones, tannins, and Phytosterols.

High performance liquid chromatography – HPLC analysis

Chemical analysis of BEAc revealed that it is rich in phenolic compounds. The chromatograms are shown in Figure 4. The diverse compounds detected had retention times (rt) ranging from 15 to 80 min. We identified fourteen compounds by referring to the standards, namely gallic acid (rt = 15.5), catechin (rt = 22.0), syringic acid (rt = 24.1), chlorogenic acid (rt = 25.2), p-coumaric acid (rt = 27.8), naringin (rt = 31.6), vitexin (rt = 37.1), rutin (rt = 42.7), isorhamnetin (rt = 56.5), hesperidin (rt = 61.6), myricetin (rt = 65.3), morin (rt = 66.9), rosmarinic acid (rt = 68.4), and quercetin (rt = 74.9).

DISCUSSION

Antioxidant activity

A. colubrina has been reported as a plant rich in antioxidant (Weber et al., 2011) compounds, mainly phenolic compounds (Melo et al., 2010). The radical scavenging activity of the extract evaluated against the stable free radical DPPH increased with increases in its

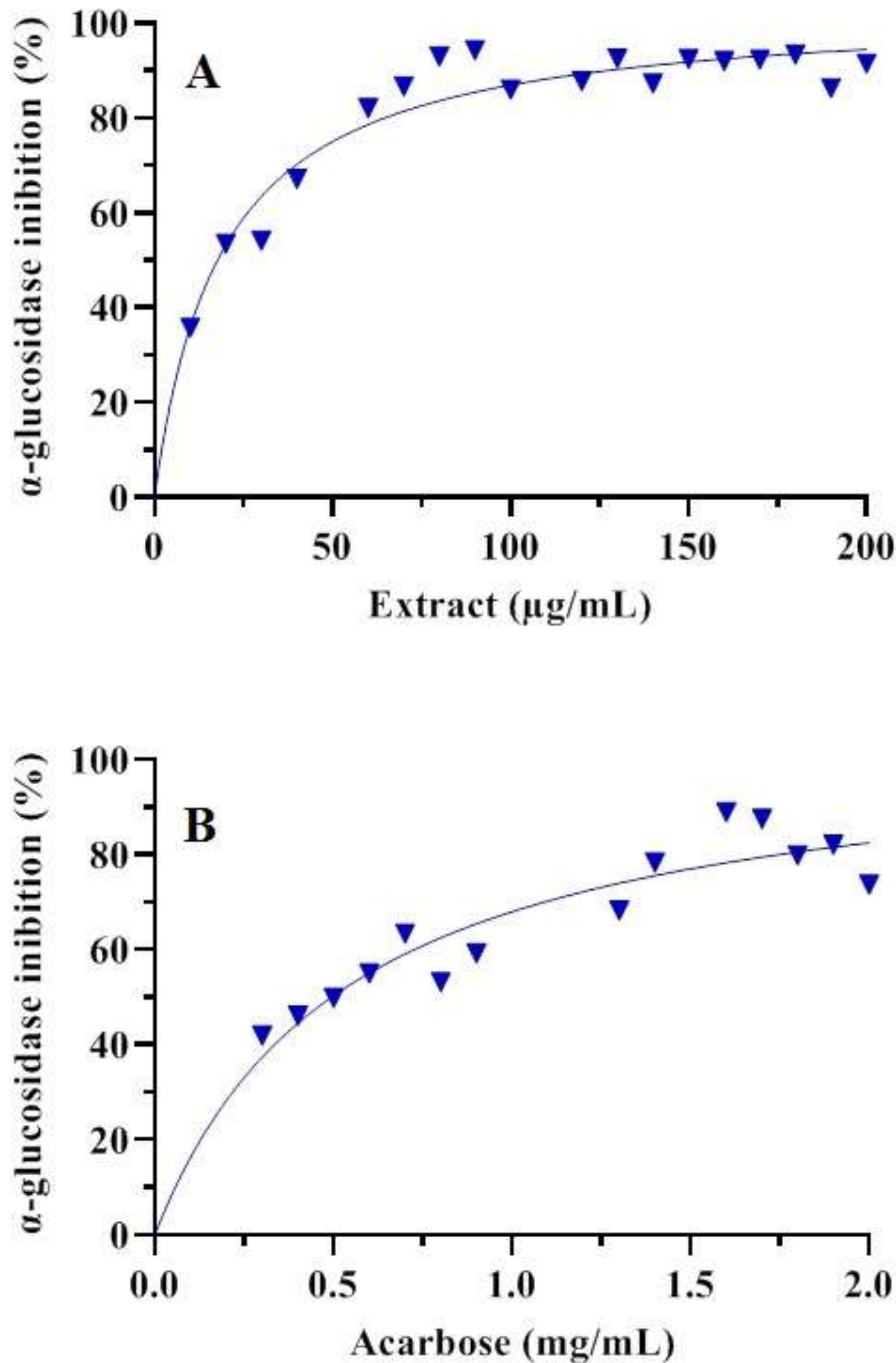

Figure 2. Inhibitory effect of *A. columbrina* (A) and Acarbose (B) bark extract (5 - 200 µg/mL) on *α*-glucosidase activity. Inhibition was calculated using pNPG (2 mM) and expressed as µg/mL.

concentration (Figure 1). Antioxidant activity of plant extracts is primarily derived from secondary metabolites, which exhibit synergistic interactions giving the plant different biological roles (Selamoglu et al., 2018). Gobbo-Neto and Lopes (2007) highlight that secondary metabolites represent a chemical interface between plants and the environment, with syntheses that are frequently affected by environmental conditions. The

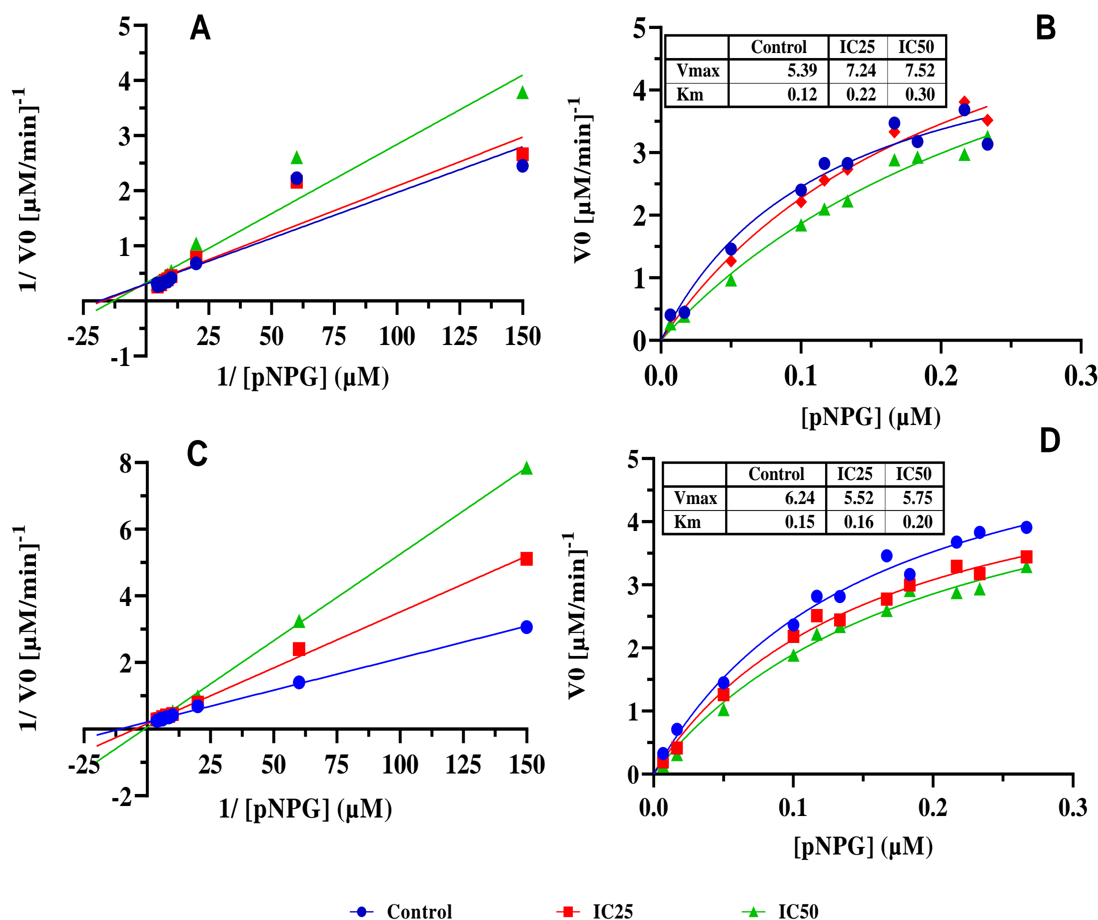

Figure 3. Modes of inhibition of [alpha]-glucosidase by acarbose (A and B) and shell extract of *A. colubrina* (C and D). (A and C) Lineweaver-Burk plots, (B and D) Michaelis-Menten plots.

results of this work clearly demonstrate this aspect. The collected sample was from a specimen from the Cerrado of Tocantins State. However, Melo et al. (2010) showed that, in contrast to our study, a different potential is observed regarding the capacity to sequester free radicals in specimens collected in the northeastern semi-arid region a different potential is observed regarding the capacity to sequester free radicals. It is worth noting that in their work, the authors demonstrated that the species *A. colubrina* is among the plants with the best antioxidant activity from the Brazilian northeastern semi-arid region and established a form of classification for the antioxidant activity, based on the performance of the crude extract of plants in sequestering free radicals *in vitro*: I - good activity (IC_{50} with values up to three times the efficient concentration of the positive control), II - moderate activity (IC_{50} ranging between three and seven times the inhibitory concentration of the positive control), III - low activity (IC_{50}, seven times the inhibitory concentration of the positive control). By this classification, *A. colubrina* had a moderate antioxidant activity when collected in the northeastern semiarid region, while in the present study, following the same criteria, the plant collected in the Cerrado of Tocantins presented good antioxidant activity.

Synthetic antioxidants have been used by the food industry to delay or prevent lipid oxidation (Anwar et al., 2018). However, consumer demand and food safety risks have suggested replacing them with alternative natural antioxidants (Berdahl et al., 2010). Natural antioxidants, including flavonoids and phenolic acids, are extracted primarily from plant sources. It is important to note that these antioxidants not only preserve food but also have the potential to protect the human body from various diseases induced by free radicals, such as cancer and diabetes (Sudhakaran et al., 2019; Sak, 2017; Nde et al., 2015). In this study, we found that *A. colubrina* peels have a great potential for application to this purpose because the IC_{50} value obtained with the extract was more efficient than the synthetic standards tested, BHT and rutin, and statistically equal to ascorbic acid.

According to Choi et al. (2015) the evaluation of antioxidant activity is an integral part of the screening of potential antidiabetic resources. This aspect was clearly observed in the present work. The extract of *A. colubrina*

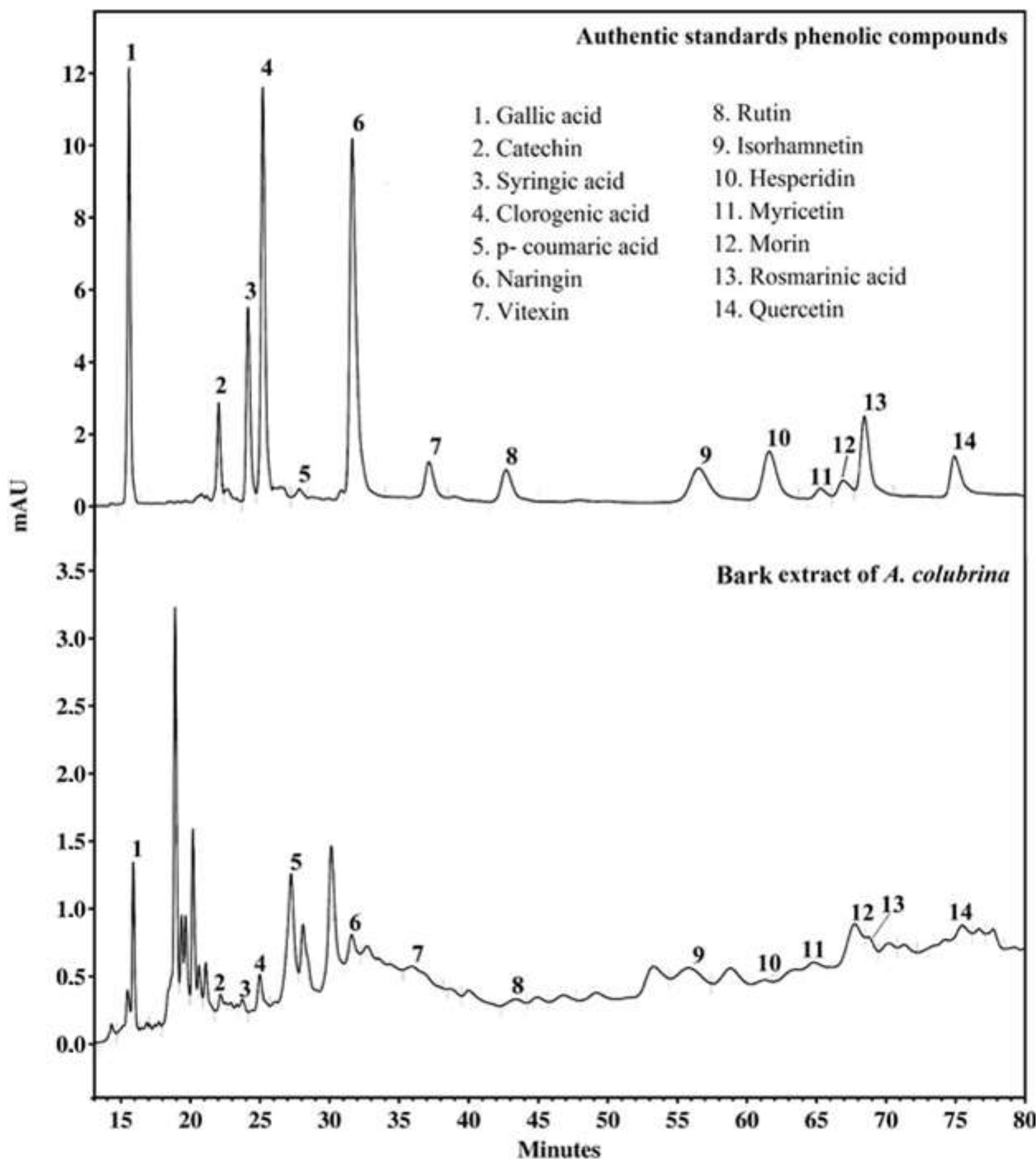

Figure 4. High-performance liquid chromatography (HPLC) fingerprint of the shell extract of *A. colubrina* detected at 280 nm.

had a remarkable inhibitory effect on *α*-glucosidase and this was dose-dependent, being more efficient than acarbose, a known *α*-glucosidase inhibitor currently used for the treatment of diabetic patients. The IC_{50} data indicated that the extract is 31 times more potent than this synthetic standard. When we compare the data obtained in this work with those of Doan et al. (2018), developed with the stem bark extract of *Chrysophyllum cainite* L., we find that the strong antioxidant activity exhibited by plant extracts is strongly related to the potent ability to inhibit the action of *α*-glucosidase. In fact, both the work of Doan et al. (2018) and our current study have demonstrated a good strong inhibition of *α*-glucosidase which occurred parallel to a good antioxidant activity, regardless of the differences in the plants.

Antidiabetic activity

Glucosidase has been recognized as a therapeutic target for the modulation of postprandial hyperglycemia (Banu et al., 2015), a primary risk factor in the development of

type 2 diabetes mellitus (Liu et al., 2014). At the same time, the fight against oxidative stress is closely related to the control of both DM and various medical conditions, including aging, atherosclerosis, cancer, and neurodegenerative disorders (Chen et al., 2018; Sudhakaran et al., 2019). As the data obtained indicate that *A. colubrina* bark has an efficient action on both oxidative stress and carbohydrate metabolism, it is a promising product for multi-target therapy combining control of postprandial hyperglycemia and control of oxidation of biomolecules among other functions.

Several methods of glucose inhibition have been proposed for extracts of medicinal plants used in the treatment of diabetes mellitus (Ramu et al., 2014; Sheliya et al., 2016; Wang et al., 2019; Bhatia et al., 2019). However, none was previously postulated for *A. colubrina*. In this study, the Lineweaver-Burk plot plotted from the kinetic data has generated new information about this new biological role of this plant. The plot indicated that the extract is a competitive inhibitor of *α*-glucosidase competing directly with the substrate of the *α*-glucosidase, thus inhibiting the reaction. This is a strong biochemical reason for *A. colubrina* bark to be used in diabetes treatment in the future. Furthermore, *A. colubrina* bark has the potential to be used as an antidiabetic product both in the direct version and in components with synthetic products for deleterious effects.

Phytochemicals from *A. colubrina* extract have been the subject of several studies. The main classes of compounds already described for the species are flavonoids, phytosterols, and tannins (Weber et al., 2011; Santos et al., 2013). The preliminary results of the phytochemical analysis of BEAc in our study are consistent with the literature description for this plant. Information on the chemical composition of BEAc obtained by HPLC analysis confirmed its capacity as an inhibitor of *α*-glucosidase. The classes of compounds found in the bark of *A. colubrina* contained substances capable of acting synergistically in different biological roles. For the activities evaluated here, the extract of *A. colubrina* proved to be very efficient, indicating that it is a source of a wide range of substances that act simultaneously on the oxidation of biomolecules and the metabolism of carbohydrates, with a significant contribution from phenolic compounds.

In HPLC analysis, comparison of the retention time of the detected peaks with that of the authentic standards led to the identification of tannins, phenolic acids, catechins, and flavonoids. Scientific evidence on the performance of the compounds identified (gallic acid, catechin, syringic acid, chlorogenic acid, naringin, vitexin, rutin, isorhamnetin, hesperidin, myricetin, morin, rosmarinic acid and quercetin) in human metabolism are consistent with the results found on the biological activities evaluated in the present study. In addition, it has been shown that the use of aortic stenosis in the treatment of postprandial hyperglycemia has been shown to inhibit *α*-glucosidase (Srinivasan et al., 2014; Zhu et al., 2014; Zeng et al., 2016; Arumugam et al., 2016; Ahangarpour et al., 2019).

In continuation of the effort to investigate the biological effects of the phytoconstituents of *A. colubrina* bark extract, we verified that the compounds identified in the HPLC analysis can act on several factors related to diabetes mellitus. Gallic acid and *p*-coumaric acid are believed to be effective against diabetes mellitus, both in early stages and in disease progression. Abdel-Moneim et al. (2017) have shown that these compounds can significantly improve glucose tolerance and reduce cerebral oxidative stress, being able to act against neurodegeneration in diabetics. Quercetin is able to protect the body against oxidative stress and glycemic control (Carrasco-Pozo et al., 2016). Rutin can inhibit *α*-glucosidase and reduce glucose uptake in the small intestine, as well as inhibiting intracellular pathways responsible for diabetic complications (Ghorbani, 2017), while catechins, in addition to inhibiting *α*-glucosidase in intestinal microvilli (Matsui, 2015), can also be transformed by bacteria that colonize the human intestine into metabolites of remarkable antioxidant activity and high bioavailability (Santangelo et al., 2019). Hesperidin and myricetin have an inhibitory effect on the development of neurodegenerative diseases (Hajialyani, 2019; Ben-Azu et al., 2019). It is important to note that other biological activities have already been demonstrated for this plant; the compounds identified may provide benefits other than those reported here, and not all peaks have been identified by the method applied. So, further research may expand the pharmacological spectrum of this plant.

Conclusions

This study provides the first evidence of the activity of *A. colubrina* bark, showing a possible mode of action on carbohydrate metabolism via inhibition *α*-glucosidase and the control of biomolecule oxidation. These data support the potential use of this plant for medical conditions related to diabetic complications and the development of promising products for multi-target therapy combining the control of postprandial hyperglycemia and the control of the oxidation of biomolecules.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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