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α-Glucosidase inhibitory properties of leaves and bark extracts of *Curatella americana* L.

Propriedades inibidoras de α-glucosidase de extratos de folhas e casca de *Curatella americana* L.

Propiedades inhibidoras de α-glucosidasa de extractos de hojas y cortezas de *Curatella americana* L.

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Abstract
In this article, we investigate the inhibitory activity of methanolic extracts of bark and leaves of *Curatella americana* L. against α-glucosidase. Furthermore, antioxidant activity was evaluated using DPPH and ABTS assays, and the interaction of the identified compound of the extracts and α-glucosidase was tested by molecular docking. The results show that *C. americana* has a strong inhibitory activity against α-glucosidase reaching an IC 50 value of 7.29 µg/ml and 7.26 µg/ml for the extracts of bark and leaves, respectively. The enzyme kinetics reveals a mixed competitive mechanism for the leaves extract and an uncompetitive type of inhibition for the bark extract. The results of the antioxidant activity showed that both extracts have great antioxidant capacity, with the leaves extract having a better performance. The identified compounds of the extracts have a high binding affinity to the enzyme. Therefore, the study showed that *C. americana* extracts have a great potential for the treatment of diabetes, possibly serving as a therapeutic option to treat postprandial hyperglycemia and prevent long-term pathologies associated with diabetes.
Keywords: Tannins; Postprandial hyperglycemia; Antioxidant activity; Diabetes.

Resumo
Neste artigo, investigamos a atividade inibitória de extratos metanólicos de cascas e folhas de Curatella americana L. contra α-glicosidase. Além disso, a atividade antioxidante foi avaliada por meio dos ensaios DPPH e ABTS, e a

interação do composto identificado dos extratos e a α-glicosidase foi testada por acoplamento molecular. Os resultados mostram que *C. americana* tem uma forte atividade inibitória contra a α-glicosidase atingindo um valor de IC 50 de 7,29 µg/ml e 7,26 µg/ml para os extratos de casca e folhas, respectivamente. A cinética enzimática revela um mecanismo competitivo misto para o extrato das folhas e um tipo de inibição incompetitiva para o extrato da casca. Os resultados da atividade antioxidante mostraram que ambos os extratos possuem grande capacidade antioxidante, tendo o extrato das folhas um melhor desempenho. Os compostos identificados nos extratos têm uma alta afinidade de ligação à enzima. Portanto, o estudo mostrou que os extratos de *C. americana* têm um grande potencial para o tratamento do diabetes, podendo servir como opção terapêutica para tratar a hiperglicemia pós-prandial e prevenir patologias de longo prazo associadas ao diabetes.
Palavras-chave: Taninos; Hiperglicemia pós-prandial; Atividade antioxidante; Diabetes.

Resumen
En este artículo investigamos la actividad inhibidora de extractos metanólicos de corteza y hojas de Curatella americana L. contra la α-glucosidasa. Además, la actividad antioxidante se evaluó mediante ensayos DPPH y ABTS, y la interacción del compuesto identificado de los extractos y la α-glucosidasa se probó mediante acoplamiento molecular. Los resultados muestran que *C. americana* tiene una fuerte actividad inhibitoria contra la α-glucosidasa alcanzando un valor de IC 50 de 7.29 µg/ml y 7.26 µg/ml para el extracto de corteza y hojas, respectivamente. La cinética enzimática revela un mecanismo competitivo mixto para el extracto de hojas y un tipo de inhibición no competitivo para el extracto de corteza. Los resultados de la actividad antioxidante mostraron que ambos extractos tienen una gran capacidad antioxidante, teniendo el extracto de hojas un mejor desempeño. Los compuestos identificados de los extractos tienen una alta afinidad de unión a la enzima. Por tanto, el estudio demostró que los extractos de *C. americana* tienen un gran potencial para el tratamiento de la diabetes, sirviendo posiblemente como una opción terapéutica para tratar la hiperglucemia posprandial y prevenir patologías a largo plazo asociadas a la diabetes.
Palabras clave: Taninos; Hiperglucemia posprandial; Actividad antioxidante; Diabetes.

1. Introduction

The search for natural antidiabetic compounds is an emergency for humanity, since synthetic products used to treat this pathology have unpleasant adverse effects with prolonged use (Junejo et al., 2017). As a result, the ethnopharmacological use of medicinal plants for the treatment of diabetes has grown, mainly due to low cost and high availability (Beidokhti & Jäger, 2017). In this context, Brazil has great potential for studies of this nature due to the great diversity of plant species.

C. americana is a plant that is present in most of the brazilian biomes, with a wide tropical dispersion (Henriques & Almeida, 2015). One of the main characteristics of this plant is the high density at the sites of occurrence, a fact that demonstrates a great potential for biotechnological application (Oestreich Filho, 2014).

It is important to note that the chemical constitution of this plant is favorable to the development of antidiabetic products, since studies with *C. americana* report the presence of important phytochemicals for this biological role, such as phenolic compounds (Cecílio et al., 2012; Ramkissoon et al., 2016).

Phenolic compounds are substances that have been reported to be capable of acting at different levels of control of diabetes mellitus, including inhibition of carbohydrate hydrolases such as α-glucosidase, controlling postprandial glycemia (Proença et al., 2017).

However, inhibition of the glycosidase enzyme is an aspect that has not yet been explored in *C. americana*, although it has a potential phytoconstitution and is easily accessible to low-income local populations. Therefore, in this work, the ability of extracts from the leaves and bark of this plant to inhibit α-glucosidase *in vitro* was investigated. Additionally, the antioxidant potential and the molecular interaction between plant phytoconstituents and the α-glucosidade enzyme *in silico* were evaluated as a strategy to find affordable alternatives to control DM.

2. Materials and Methods

2.1 Plant material

The leaves and bark of *C. americana* were collected in the city of Palmas state of Tocantins, Brazil (geographical coordinates: 10°10'42.1"S 48°21'25.8"W). The collection of plant material was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) (process number AEFD670).

2.2 Preparation of extracts

Before extraction, *C. americana* bark and leaves were dried at 50 ° C and milled using a knife mill. About 10 g of the milled material were macerated with 200 ml of an 80% methanol solution for a continuous period of 12 hours. The extraction solutions were vacuum filtered, concentrated in the rotary evaporator, and then lyophilized. The bark and leaves extract of *C. americana* were stored under vacuum in a desiccator until analysis.

2.3 Total Phenolic Content and Tannin Content

The total phenolic content was quantified using the Folin-Ciocalteu method described by Soares et al. (2014). Briefly, methanolic solutions of extracts of *C. americana* (1 mg / ml, w / v) were mixed with the Folin-Ciocalteu reagent (0.5 ml of 10%, v/v), Na_2CO_3 (1 ml of 75%, w/v) and distillated water (8.3 ml). The mixture was agitated and kept for 30 min in the dark. The absorbance was measured at 760 nm. The total phenolic content was determined by interpolation of the absorbance of the samples against a calibration curve constructed with different concentrations of gallic acid. The result was expressed as milligrams of gallic acid equivalents per gram of *C. americana* extract (mg GAE/g). The tannin content was determined by the difference between the total phenolic content and the residual phenolic content after precipitation with casein.

2.4 Total flavonoids

The total flavonoid content was determined following the methodology described by Peixoto Sobrinho et al. (2008). Briefly, reactions were carried out in triplicate by mixing the methanolic solutions of the extracts (1 mg/ml) with an aqueous solution of 0.5 ml of 60% acetic acid, 2 ml of 20% pyridine methanolic solution (v/v), 1 ml of 5% aluminum chloride (w/v) and 6 ml of distilled water. The blank was made by replacing aluminum chloride with methanol. This reaction mixture was agitated, kept in the dark for 30 min and then the absorbance was measured at 420 nm. The total flavonoid content was determined by interpolating the absorbance of the samples against a calibration curve constructed with different concentrations of the standard rutin. Results are expressed as milligrams of equivalent rutin per gram of extract (mg RE/g).

2.5 DPPH assay

The quantification of antioxidant activity performed by the 1,1-diphenyl-2-picrylhydrazyl acid (DPPH) assay followed the description of Sobrinho et al. (2011). Briefly, methanolic dilutions of samples or positive control (rutin and ascorbic acid) were prepared (a concentration ranging from 20 to 200 µg / ml). For the reaction, 0,5 ml of each dilution was mixed with 2,5 ml of DPPH (40 µg / ml in methanol). The blank was prepared by replacing the DPPH solution with methanol. The mixture was kept in the dark and after 30 min the absorption was measured at 517 nm. Antioxidant activity was calculated as a percentage inhibition.

2.6 ABTS assay

The radical cation solution of ABTS (ABTS + •) was prepared following the protocol of Re et al. (1990). For the analysis 200 µL of the extracts, dilute at different concentrations in methanol (20-200.00 µg/ml) were mixed with 3.0 ml of ABTS+• solution. The absorbance was measured at 734 after 6 minutes. Rutin and ascorbic acid were used as positive controls. The antioxidant activity was calculated as a percentage inhibition.

2.7 α-glucosidase inhibitory activity assay

The leaves and bark extracts of *C. americana* were tested for their ability to inhibit the yeast's α-glucosidase using the substrate pNPG according to the method described by Ramu et al. (2014) with slight modifications. In summary, 100 µl of extracts with varying concentrations diluted in dimethyl sulfoxide (DMSO) were preincubated with 100 µl of yeast α-glucosidase (0.1 U/ml) and 700 µl phosphate buffer (50 mM, pH 6.8) at 37 ° C for 10 minutes. After incubation, 100 µl of 4.0 mM pNPG solution was added and the reaction was maintained at 37 ° C for 30 min. The reaction was terminated by adding 500 µl of 0.2M Na_2CO_3. The negative control used phosphate buff instead of extracts and, for the positive control, acarbose was used. Enzyme activity was determined by measuring the absorbance of the liberated p-nitrophenol from pNPG at 405 nm. The results were expressed as a percent α-glucosidase inhibition.

2.8 α-glucosidase inhibition kinetics

The α-glucosidase inhibition kinetic studies of α-glucosidase were carried out using pNPG as substrate in a concentration range of 0.5 to 7.0 mM against the IC20 and IC60 of methanolic extracts of *C. americana.* The type of inhibition, Km, and Vmax were determined by a double reciprocal plot (Lineweaver–Burk plot) of the substrate concentration and the velocity (1/V versus 1/[pNPG]).

2.9 High performance liquid chromatography analysis (HPLC)

Extracts of *C. americana* were analyzed by HPLC using a Shimadzu® chromatograph (Shimadzu®, Kyoto, Japão) using the methodology described by Costa et al. (2019).

2.10 Molecular Docking

Protein α-glucosidase was acquired from the Protein Data Bank (PDB: 4J5T). The 3D structure of the protein was prepared using the MGLTools 1.5.6 program by removing all water molecules, adding polar hydrogens, and adding Kollman charges. The resulting structure was saved in PDBQT format.

The three-dimensional structures of the ligands (phenolics compounds) were downloaded from the NCBI PubChem database in SDF format. Structures were converted to PDB with Pymol software and optimized with MGLTools 1.5.6 with the addition of Gasteiger charges (to the molecule). The optimized structures were converted to PDBQT files with MGLTools 1.5.6 before starting molecular docking.

The docking procedure was performed using the AutoDock Vina 4.0 program (Trott; Olson, 2010). The protein structure was kept rigid throughout the process, while the ligands were allowed to be flexible. The docking parameters were the following: grid spacing of 0,500 Å; box size dimensions of 30x30x30 and exhaustiveness at 50. The coordinates were means of the coordinates of the amino acid residues at the active site of the protein (x = -7.673, y = -26.393, and z = -1.113). After the docking, Pymol and LigPlot software were used for the visualization of the tridimensional and bidimensional interactions.

3. Results and discussion

3.1 Total Phenolic Content and Total Flavonoids

Polyphenols are ubiquitous in plants and this group of compounds has been a growing subject of interest due to their biological activities such as antioxidant activity (Deseo et al., 2020). Something to note is that most of the phenolic content in both extract extracts is composed of tannins, with a high ratio of tannins in the bark extract (Table 1). Tannins are a major group of bioactive compounds that have been shown to have great beneficial properties for human health in recent decades and to have a positive effect in the treatment of several pathologies (Kumari & Jain, 2012; Laddha & Kulkarni, 2019; Serrano et al., 2009).

Table 1: Total phenolic content, total tannin content **(**mg GAE / g) and total flavonoids (mg RE / g) of *C. americana methanolic extracts.*

Sample	Total phenolic content	Total flavonoids	Total Tannins
Bark extract	204,57	39,84	192,54
Leaves extract	159,69	32,24	85,16

Sources: Authors.

3.2 Antioxidant activity

Antioxidants exert protective properties on cells, either preventing the production of free radicals or neutralizing/eliminating free radicals produced in the body, preventing degenerative diseases such as diabetes and cardiovascular diseases (Kazeem et al., 2012), thus, it was perceived to be relevant to analyze these properties in *C. americana* extracts, as shown in Table 2.

Table 2: IC50 inhibition of DPPH and ABTS by *C. americana* leaves and bark extract and the positive controls ascorbic acid and rutin.

Sample	DPPH	ABTS
Bark Extract	$63,12^{a}$	$82,32^{a}$
Leaves Extract	$54,51^{b}$	$38,15^{b}$
Rutin	$17,18^{c}$	$56,03^{c}$
Ascorbic Acid	$31,36^{d}$	$35,05^{b}$

Sources: Authors.

IC50 data showed that the two extracts have excellent performance in neutralizing free radicals compared to the positive controls, rutin and ascorbic acid. This observation was more evident in the ABTS analysis, in which the IC50 of the leaves extract was better than of the rutin and did not differ statistically from ascorbic acid.

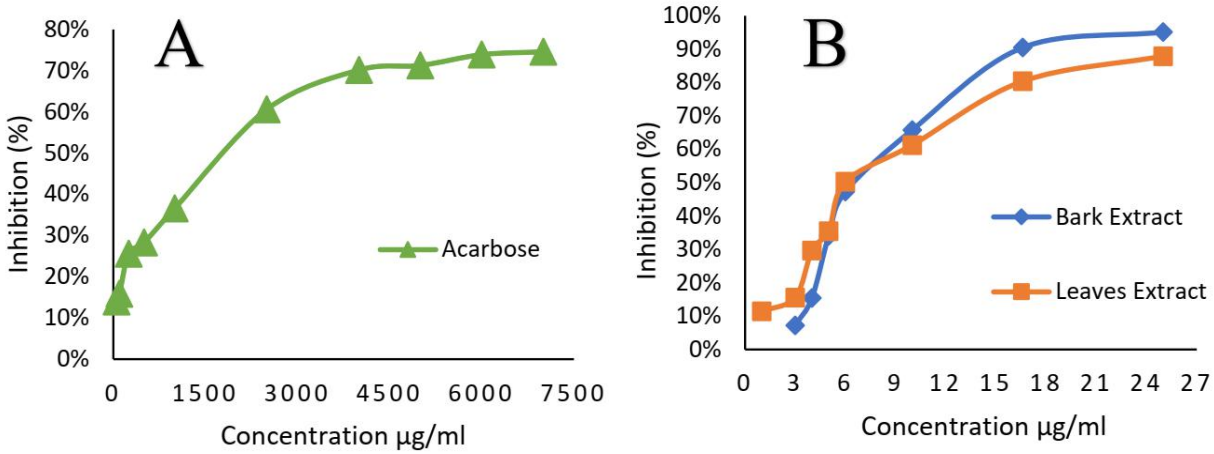
The results of the antioxidant analysis are consistent with the findings of Lopes et al. (2016) and Teles Fujishima et al. (2018), who also report great antioxidant activity for extracts of this plant.

3.3 Inhibition and inhibition kinetics of α-glucosidase

An important approach to the treatment of diabetes mellitus is the inhibition of key digestive enzymes such as α-glucosidase as a means to regulate blood glucose levels and postprandial peaks (Ghani, 2015). The search for inhibitors of this enzyme has been the subject of several studies and the results of the *in vitro* assays of this work show *C. americana* as a promising alternative for this purpose.

As shown in Figure 1, *C. americana* methanolic extracts exhibited high α-glucosidase inhibitory activity. Bark extract reached 95% inhibition against 72% maximum inhibition of the positive control, acarbose. Under the conditions used in this study, the IC50 of the samples was 7.29 µg/ml, 7.26 µg/ml, and 1,672.85 µg/ml, for bark extract, leaves extract and acarbose, respectively. With this analysis, the potential for inhibition of α-glucosidase of this plant became more evident, demonstrating a performance 200 times better than the positive control.

Figure 1: Inhibition of α-glucosidase by acarbose and the bark and leaves methanolic extract of *C. americana*.



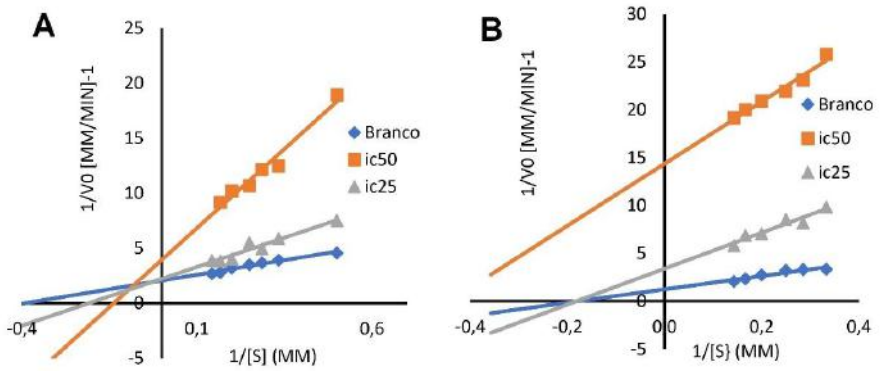
Sources: Authors.

The α-glucosidase inhibitory activity of may be correlated with the high tannin content of this plant, as some authors also report good inhibitory activity for tannins and tannin-rich extracts for other plants (Barrett et al., 2018; Lee et al., 2017; Park et al., 2018).

Lineweaver-Burk plots were used to further investigate the mode of inhibition of methanolic extracts of *C. americana* against α-glucosidase. In Figure 2A the Lineweaver-Burk graph for the extract of leaves reveals that with increasing extract concentration the Vm values (Y-axis) decreased while the Km values (X-axis) increased, implying that the inhibitory pattern of this extract against α-glucosidase was a mixed competitive type of inhibition.

The bark extract (Figure 2B) exhibits a non-competitive type of inhibition in IC25 (reduce Vmax), but with increased concentration to IC50, the Linewear-Burk plot reveals an uncompetitive inhibition behavior. Uncompetitive inhibitors bind to the enzyme and improve the binding of the substrate (thus, reducing Km), but the resultant enzyme-inhibitor-substrate complex only undergoes a reaction to slowly form the product; thus, Vmax is also reduced (Hamed et al., 2021).

Figure. 2. Lineweaver-Burk double-reciprocal plots for the inhibition of α-glucosidase in the presence of bark (A) and leaves (B) methanolic extract of *C. americana*.

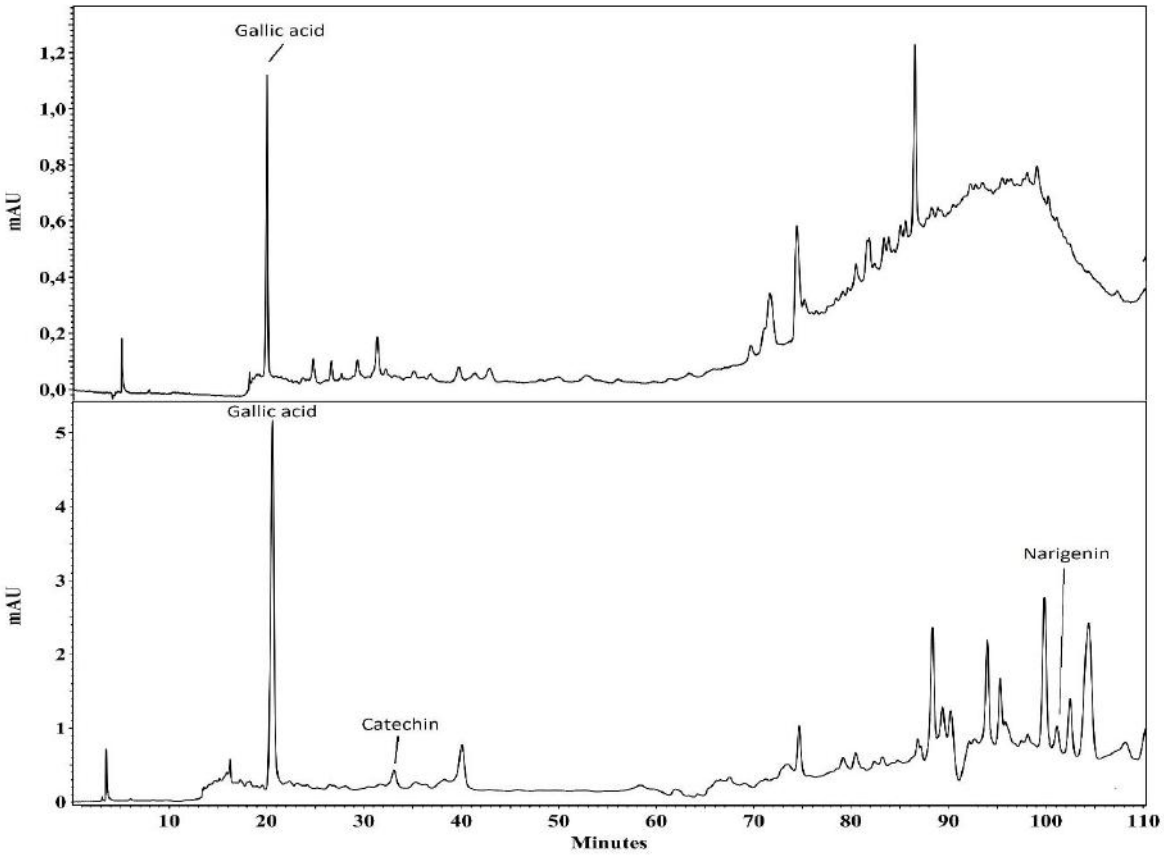


Sources: Authors.

3.4 HPLC analyzes and Molecular docking

The HPLC analyzes revealed the presence of gallic acid in both the extract of *C. americana* and the flavonoids catechin and narigenin in the extract of the leaves. The chromatogram also shows the characteristic humps found in sample with high tannins content (Habib et al., 2014; Ky & Teissedre, 2015; Ma et al., 2018). The data obtained in the HPLC analysis agree with the spectrophotometric quantifications, considering that gallic acid and catechin are precursors to tannins.

Figure 3: High-performance liquid chromatography (HPLC) fingerprint of the bark (A) and leaves extract (B) of *C. americana* detected at 280 nm.

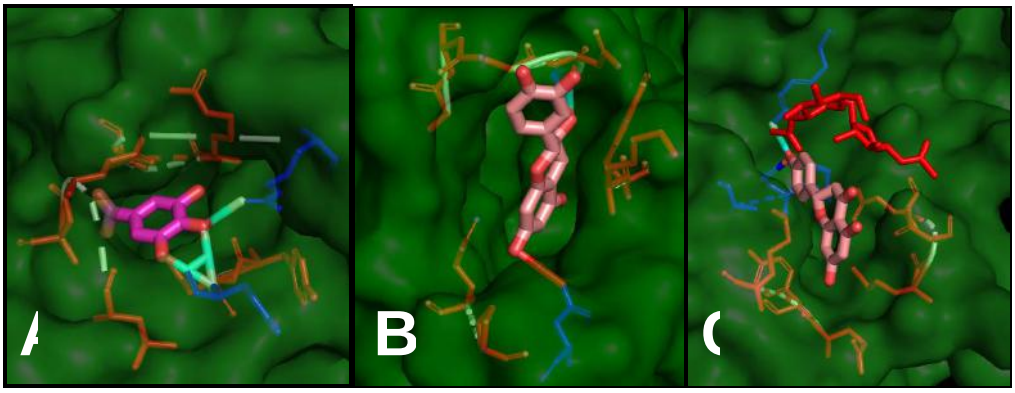


Sources: Authors.

These results of the HPLC analysis also give consistency to the two biological activities evaluated *in vitro* in this work, as gallic acid, catechin, and naringenin are compounds with reported strong antioxidant and antihyperglycemic activity (Ahmed et al., 2017; Fu et al., 2021; Oboh et al., 2016).

The molecular docking simulations revealed that the identified compounds are capable of binding to various amino acid residues of the α-glucosidase enzyme. In Figure 4 it is possible to see the three-dimensional interaction between phenolic compounds with various amino acid residues forming a binding pocket in the protein with hydrophobic interactions and hydrogen bonds.

Figure 4: Tridimensional view of the complex of α-glucosidase (PDB: 4J5T) with A: Gallic acid, B: Catechin and C: Naringenin. Residues in blue interact with hydrogen bonds, and residues in red interact hydrophobically.



Sources: Authors.

Table 3 shows the amino acid residues involved in molecular interactions (hydrogen bonding and hydrophobic interaction) with compounds identified in *C. americana* extracts and the docking affinity score. The TYR and GLU residues were shown to interact the most with the compounds of *C. americana* extracts, both by hydrogen bonds and hydrophobic interaction. The binding score appears to indicate that tannins that have catechin as a precursor may have a higher affinity for the protein (-8.1) than tannins derived from gallic acid. These data are consistent with those found by Ou-Yang et al. (2020) who found strong inhibition of tannin-rich extracts of Clausena lansium against α-glucosidase.

Table 3: Compounds identified in the *C. americana* ligands and their interaction with amino acids from α-glucosidase (PDB ID: 4J5T) and the binding affinity score.

Name	Hydrogen bonds	Hydrophobic interaction	Docking Affinity score (kcal/mol)
Gallic acid	TYR343, ARG26	PHE342, TYR341, GLU435, ASN20, ARG18, ALA436, LEU432	-6,9
Catechin	ASN129, GLU435	TRP206, ARG26, PRO34, GLY433, ASN434, LYS203, GLY344, ASN345	-8,2
Naringenin	TYR29, GLU410	GLU506, TYR507, LYS510, MET1, ALA413, GLN5	-8,1

Sources: Authors.

It is worth mentioning that plant extracts consist of complex mixtures of substances with peculiar combinations capable of favoring one or another biological role, and for the activities evaluated here, the leaves extract was more bioactive regarding the ability to scavenge free radicals and both act with similar efficiency in the inhibition of α-glucosidase.

The combination of antioxidant and antihyperglycemic effects detected here, associated with other beneficial biological properties that plant extracts can provide to human health, demonstrate that *C. americana* can act on DM at different stages of diabetic complications. The diversity of peaks detected in HPLC reinforces the idea that this plant has great potential for the development of antidiabetic products and further research needs to be carried out in order to elucidate its chemical constitution.

4. Conclusion

The extracts of *C. americana* showed a high phenolic content with a preponderance of tannins. The α-glucosidase inhibitory activity and kinetics assays show that the leaves extract acts as a mixed competitive inhibitor with an IC50 7,29 µg/ml and the bark extract acts as an uncompetitive inhibitor with an IC50 of 7,26 µg/ml. With HPLC analysis, gallic acid was identified in both extracts, while catechin and naringenin were only identified in the leaves extract. These compounds show high affinity do α-glucosidase in molecular docking simulations. The study demonstrated that *C. americana* extracts have great potential for the treatment of diabetes therefore, we hope that this research inspires future *in vivo* studies to improve the knowledge of C. americana as a possible therapeutic option to treat postprandial hyperglycemia and prevent long-term pathologies associated with diabetes.

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