Inhibition of digestive enzymes (α-amylase, α-glucosidase, lipase, trypsin) by aqueous *Hibiscus sabdariffa* L. (Malvaceae) extract

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Abstract

A considerable portion of the world population uses medicinal plants to treat chronic diseases. In this context, hibiscus (*Hibiscus sabdariffa* L. - Malvaceae) stands out for its supposed hypoglycemic and slimming effect. The present work aimed to carry out enzyme inhibition tests for aqueous hibiscus extracts, as a possible mechanism of action related to the supposed slimming and hypoglycemic effects. The inhibition of four digestive enzymes (α-amylase, α-glucosidase, lipase and trypsin) was tested before and after exposure to a simulated gastric fluid. In addition, a molecular anchorage study was carried out, in order to highlight possible molecular interactions between target and ligand. The results showed that the aqueous extract of hibiscus, in the proportion 1:10 (w/v), inhibits only α-glucosidase. It was observed that cyanidin-3-sambubioside, has interaction with the enzyme with properties similar to acarbose, which corroborates the possibility of the presence of an inhibitory effect in the aqueous extract of hibiscus.

Keywords: *Hibiscus sabdariffa*. Glucosidase. Diabetes. Obesity. Molecular docking.

Introduction

A considerable portion of the world population uses medicinal plants to treat chronic diseases. The use of medicinal plants in diabetes and obesity, sometimes without proper scientific proof, has been gaining ground in the treatment of these diseases. In Brazil, as in many countries, pharmacovigilance about medicinal plants is very fragile, limiting their safety and efficacy[1]. In this context, the hibiscus (*Hibiscus sabdariffa*) has gained prominence in recent years for its supposed and widespread hypoglycemic and slimming effect.

Based on the above, the present study aimed to carry out tests to inhibit digestive enzymes by aqueous hibiscus extract, as a possible mechanism of action related to the supposed slimming and hypoglycemic effects. For this, the inhibition of the enzymes α-amylase, α-glucosidase, lipase and trypsin was tested before
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and after exposure to a simulated gastric fluid. In addition, a molecular anchorage study was carried out, in order to highlight possible molecular interactions between target and ligand.

**Materials and methods**

**Obtaining samples and preparing the extract**

The hibiscus samples were collected at the Federal University of Lavras, latitude 21º 14'43'', longitude 44º 59' 59'', and altitude 3015.09ft, in October 2015, identified by Herbário ESAL and dehydrated in the greenhouse. The extract was prepared according to domestic and popular use, from the chalices of the plant, by the method of infusion for 10 minutes in the proportion of 1:10 (w/v).

**Obtaining enzymes**

α-glucosidase was obtained from the fresh porcine duodenum, the swine pancreatic enzymes were: trypsin and lipase type II (MERCK) and α-amylase type VI (SIGMA).

**Enzyme activity**

The enzyme activity was performed in a 4-stage kinetic assay. α-glucosidase according to Kwon *et al.*[2], α-amylase according to Noelting and Bernfeld[3], trypsin was determined by the methodology of Erlanger *et al.*[4] and the lipase according to Souza[5].

**Determination of inhibition**

The enzymatic activities were expressed in units (U), which corresponds to the formation of μmol of product per minute under the test conditions. For this purpose, the absorbance values are converted into μmol of product formed by means of standard curves and, in the case of trypsin, by the factor of Erlanger *et al.*[4], which is based on the substrate's molar extinction coefficient. The value of 1 EIU (enzyme inhibition unit) corresponds to the total inhibition of 1 U.

**Preparation of simulated gastric fluid**

Simulated gastric fluid was prepared, according to The United States Pharmacopeia – USP[6], with the aim of simulating gastric passage *in vitro*. The hibiscus extract was incubated with prepared according to for 1 hour in a water bath, at 37°C. After this period, it was neutralized with sodium bicarbonate and the enzyme activity tests were performed again.

**Molecular anchoring study (docking)**

For the molecular anchoring studies, the crystallographic structure of the α-glucosidase protein complexed with the acarbose inhibitor (*FIGURE 1*) obtained from the “Protein Data Bank” (PDB - codes: 3TOP, resolution 2.88 Å) was used. The computational simulation of molecular anchoring (“docking”) is one of the most important techniques for investigating the molecular interactions between target and ligand. Molecular anchoring calculations were performed using the Molegro Virtual Docker (MVD) program, which allows determining the most likely conformation of the ligand in the enzyme. The identification of the ligand
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The conformation is done through the evaluation of several candidates (ligand conformations) estimating the energies of their interactions with the enzyme.

For the study in question, five compounds were selected, according to the literature, as possible responsible for the inhibitory effect of hibiscus extract, namely: delphinidine-3-sambubioside, cyanidin-3-sambubioside, citric acid, hydroxycitric acid and hibiscus acid. The three-dimensional structures of these selected compounds were built using the PC Spartan Pro program and their partial atomic charges were calculated using the semi-empirical method AM1. This procedure is necessary to obtain the initial conformation of the compounds, which is important for the study of molecular anchoring.

After the construction of the 3D structures of the compounds, they were transferred to the MVD program, where each ligand was anchored in the active site of the enzyme α-glucosidase. In this step, the identification of the ligand's interaction modes is interactive, evaluating a number of solutions (conformation and orientation of the ligand) and estimating the energy of its interactions with the protein.

Results and Discussion

The results of the inhibition of digestive enzymes by the aqueous hibiscus extract, before and after its exposure to the simulated gastric fluid, are shown in TABLE 1.

TABLE 1: Inhibition of digestive enzymes, in inhibited enzyme units (EIU) and percentage by aqueous extract of the chalice of Hibiscus sabdariffa, before and after exposure to the simulated gastric fluid.

| Sample² | Enzyme | w/v | Without gastric fluid | After gastric fluid | Without gastric fluid | After gastric fluid |
|---------|--------|-----|-----------------------|--------------------|-----------------------|--------------------|
|         |        |     | EIU                   | %                  | EIU                   | %                  |
| α-glucosidase | 1:10   | 128.3 ± 4.27 | 57.14 ± 2.66 | 122.2 ± 3.52 | 47.16 ± 2.62 |
| α-amylase   | 1:10   | nd ³ | nd ³                  | Nd                 | Nd                   | nd                 |
| Trypsin    | 1:10   | nd ³ | Nd                   | Nd                 | nd                   | nd                 |
| Lipase     | 1:10   | nd ³ | Nd                   | Nd                 | nd                   | nd                 |

1. Units of enzyme inhibited in μmol.min⁻¹.g⁻¹ sample; 2. Data are the mean of triplicates ± standard deviation; 3. Inhibition not detected.

As noted, there was inhibition only of the enzyme α-glucosidase, of 128.3 EIU or 57.14%, in the 1:10 dilution (w/v) of the aqueous extract of hibiscus.

From the bibliographic survey of the main molecules present in the extract and in the selection of the five most cited (delphinidine-3-sambubioside, cyanidin-3-sambubioside, citric acid, hydroxycitric acid and hibiscus acid), the study was carried out by molecular anchoring in active site of the α-glucosidase enzyme. A cavity prediction algorithm based on a 3D box was used to generate the α-glucosidase binding site using the MVD program. The cavity volume was 259.07 Å³ and is shown in FIGURE 1 together with the inhibitor acarbose (compound crystallized at the active site).
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The five compounds were anchored in the active site of α–glucosidase and compared to the active ligand (acarbose). The energies of intermolecular interaction and hydrogen ligand-protein binding were calculated to better understand what are the variations between the modes of interaction of the compounds in the active site of the enzyme and to verify which factors are responsible for the activity of the compounds. All of these interactions are highlighted in **FIGURE 2**.

**FIGURE 2**: Hydrogen interactions (green dashed) between Compound 2 and active site residues.

| Compounds                  | Intermolecular interaction energy (kcal mol⁻¹) | Hydrogen bonding energy (kcal mol⁻¹)* |
|----------------------------|-----------------------------------------------|-------------------------------------|
| delphinidine-3-sambubioside| -114.36                                       | -16.44                              |
| cyanidin-3-sambubioside    | -148.32                                       | -27.90                              |
| citric acid                | -81.14                                        | -23.48                              |
| hydroxycitric acid         | -84.90                                        | -21.48                              |
| hibiscus acid              | -84.89                                        | -13.68                              |

* Total hydrogen bonding energy between ligand and protein.

The acarbose redocking study on the α–glucosidase active site (**FIGURE 3**) was carried out with the aim of validating the calculation methodology used for the five compounds studied, and also to know the main
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Interactions that this inhibitor (already used in therapy) performs at the active site of the enzyme, comparing with the results obtained for cyanidin-3-sambubioside.

**FIGURE 3:** a) Re-docking of Acarbose at the α–glucosidase active site; b) Hydrogen interactions (green dashed) between Acarbose and active site residues.

![Redocking and Hydrogen Interactions](image)

Even after the passage of the extract through the simulated gastric fluid, α-glucosidase inhibition was maintained *in vitro*. The percentage of inhibition found, falls within the range considered ideal (40% - 85%) of good α-glucosidase inhibitors according to Kwon *et al.* The inhibition of α-glucosidase provides a decrease in caloric availability, in addition to contributing to a drop in postprandial hyperglycemia, due to the lower intestinal absorption of carbohydrates, which may suggest one of the possible mechanisms of action of hibiscus.

Buchholz and Melzig*[^16^] conducted a screening with a range of medicinal plants relating them to the inhibition of lipase and α-amylase. In the study, the methanolic extract of *H. sabdariffa* and that of several other plants were used. *H. sabdariffa* extract was the most effective lipase inhibitor (IC50: 35.8 ± 0.8 µg/mL) and good α-amylase inhibitor (IC50: 29.3 ± 0.5 µg/mL) which, as opposed to that found in our studies working with the aqueous extract, may suggest new forms of preparation, more effective and with greater therapeutic power.

In another study, Shadhan *et al.*[^17^] when evaluating the inhibition of α-glucosidase by methanolic extract of *H. Sabdariffa*, observed that the enzymatic inhibition reveals a gradual pattern with increasing concentrations of extract and methanolic fractions, which is very similar to that characterized for acarbose (positive control of the study). In addition, it was observed that the ethyl acetate fraction showed high inhibitory activity when compared to acarbose at the same concentration. In the study, a very similar inhibition was detected between the ethyl acetate fraction and acarbose, a drug used worldwide, reinforcing the relevance of research on the therapeutic potentials of hibiscus as an aid in the control of body weight and blood glucose.

The analysis of the results raises some questions. The first is the preparation of the extract, showing that different solvents extract different molecules. Thus, cultivation and the extraction method are factors that directly influence the effectiveness of the plant, requiring standardization for greater effectiveness[^18^].

On the other hand, inhibition of α-glucosidase alone is more interesting for the hypoglycemic function, given the decrease in some side effects, such as the pancreatic hypertrophy observed by Silva and Silva[^19^], caused by inhibition of trypsin, and fermentation abnormal bacterial digestion of undigested carbohydrates in the colon by α-amylase inhibition, described by Kwon *et al.*[^2^].
In another study of enzymatic inhibition, among the few produced with aqueous extract of *Hibiscus sabdariffa*, Ademiluyi and Oboh[20], observed the inhibition of α-glucosidase (IC50: 187.9 ± 10.2 µg / ml) and α-amylase (IC50: 25.2 µg / ml ± 2.4 µg / ml). With that, it is returned again to the fact of the great variation of results, either by the scarcity of researches with the extract in its most used form domestically, or by the lack of methodological standardization.

A review paper published by Da-Costa-Rocha et al.[21], attributed to anthocyanins, flavonoids and organic acids as being the main molecules with pharmacological potential present in *Hibiscus sabdariffa*. Among the anthocyanins, delphinidin-3-sambubioside (delphinidin-3-O- (2-ObD-xylopyranosyl)-bD-glucopyranoise), named hibiscin, and cyanidin-3-sambubioside (cyanidin-3 -O- (2-ObD-xylopyranosyl)-bD-glucopyranoside), called gossypicyanin, from the calyx of the plant[7-12].

Regarding organic acids, Hida et al.[13], demonstrated that hydroxycitric acid is the main constituent of this class found in the calyx of the plant. In addition to this, two other components are present in large quantities in the goblets of *Hibiscus sabdariffa* and can be related to their enzymatic inhibition, being citric acid and hibiscus acid[9,11,12,14,15].

Considering the five molecules analyzed in the present study (delphinidine-3-sambubioside, cyanidin-3-sambubioside, citric acid, hydroxycitric acid and hibiscus acid) and as can be seen in Table 2, the compound cyanidin-3-sambubioside was the one that presented a more stable value of intermolecular interaction energy, that is, it has a greater interaction with the active site of α–glucosidase and, consequently, a greater inhibitory potential. In addition, it was the one that was best positioned inside the active site, presenting a more stable hydrogen bonding energy than the other compounds, -27.90 kcal mol-1. In other words, it presented a total of sixteen “hydrogen bonding” interactions between groups containing electronegative atoms in the amino acid residues of the active enzyme site, such as Gln1158; Pro1159; Lys1460; Asp1157; Asp1526; Arg1510 with alcoholic and phenolic hydroxyls in cyanidin-3-sambubioside.

The RMSD value between the acarbose structures superimposed on the active site was 0.99 Å (FIGURE 3A). According to the literature, an RMSD less than 2.00 Å is considered satisfactory, thus corroborating the protocol used for molecular anchoring calculations. The energies of intermolecular interaction and hydrogen bonding obtained in the redocking were -219.80 and -28.41 kcal mol-1, respectively. It was observed that acarbose performs hydrogen interactions similar to cyanidin-3-sambubioside at the active site of α–glucosidase, with the amino acid residues Asp1157; Asp1526 and Arg1510. That is, with molecular anchoring studies it is possible to suggest that cyanidin-3-sambubioside may be the molecule responsible for the in vitro inhibition of α-glucosidase observed.

Finally, after in-vitro scientific verification of the significant inhibition of alpha-glucosidase against the aqueous extract of *Hibiscus sabdariffa*, it is extremely important to raise public awareness about the range of possible adverse effects resulting from the use of the plant. More commonly, gastrointestinal effects such as abdominal pain, flatulence and diarrhea have been observed[22]. Also, due to its hypoglycemic action, dose-dependent hypoglycemic effects can be deducted from the study, which can be quite harmful. Furthermore, in another study, it was found that 14% of respondents claimed to be aware of cases of hypotension as a result of excessive use of Hibiscus sabdariffa teas[23].
In view of this, and in view of the absence of *in vivo* studies to determine adequate and safe doses, caution is essential when using the plant extract, since, even with beneficial actions found, the harmful effects of possible adverse effects can cover the expected benefits.

**Conclusion**

The results showed that the aqueous extract of hibiscus, in the proportion 1:10 (w/v), inhibits only α-gluco Bradfordase among the enzymes tested. The enzyme inhibition is maintained even after the extract passes through the simulated gastric fluid. From the molecular anchorage studies between α-glucosidase and the compounds evaluated, it is concluded that cyanidin-3-sambubioside, has interaction with the enzyme with properties similar to that of acarbose, a drug that is already widely used, which shows the inhibitory effect by the aqueous extract of hibiscus found.

The results obtained in the inhibition of α-glucosidase *in vitro* were richly elucidated by the theoretical assay, demonstrating the high therapeutic potential of hibiscus and the need for further studies with the objective of standardizing the extract, definition of possible therapeutic dose, in addition to accurate assessment efficiency and safety.

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