**Research Article**

*Alternanthera sessilis* leaf fractions possess *in vitro* inhibitory activities in mammalian α-amylase and α-glucosidase

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**ABSTRACT**

Diabetes is a metabolic disorder characterized by high blood glucose levels. With its increasing prevalence leading to a global burden, the search for new sources of antidiabetic agents are needed. Plant extracts and their fractions are essential in the management of diabetes due to numerous scientific evidences of their antidiabetic activity through various mechanisms. In this study, fractions from *Alternanthera sessilis* (Philippine variety) was investigated for their ability to inhibit key carbohydrate-degrading enzymes related to type-2 diabetes, specifically, α-amylase and α-glucosidase. The crude methanol extract of *A. sessilis* leaf was fractionated with three solvents of different polarities: water, ethyl acetate and hexane. These fractions were screened for mammalian α-amylase and α-glucosidase inhibitory activities *in vitro*, and for the presence or absence of phytochemicals. All fractions displayed inhibitory activities in porcine α-amylase and intestinal rat α-glucosidase, with the highest activity observed in the ethyl acetate fraction (IC50 amylase = 0.52 ± 0.072 mg/mL; IC50 glucosidase = 2.82 ± 0.21 mg/mL). This fraction also demonstrated no significant difference but lower inhibitory activity relative to acarbose (IC50 amylase = 0.0025 ± 0.00045 mg/mL, p=0.72, α=0.05; IC50 glucosidase = 0.36 ± 0.063 mg/mL, p=0.26, α=0.05). Phytochemical screening of this fraction showed the presence of carbohydrates, lipids, tannins, triterpenoids, flavonoids, and glycosides, as well as berberine and quercetin, which may have accounted for the observed enzyme inhibitory activity. In summary, *A. sessilis* fractions may be used as potential sources of α-amylase and α-glucosidase inhibitors, with the ethyl acetate fraction as the most potent.

1. INTRODUCTION

Diabetes is a type of metabolic, non-communicable disorder arising from impairment of insulin production and/or defects in insulin action. This results in an increased blood glucose level or hyperglycemia leading to various complications such as heart disease and stroke, kidney failure, blindness and diabetic neuropathy. Diabetes has been considered a global burden, causing more than 80% deaths in developing countries and projected to be the 7th leading cause of death within the next 25 years. In the Philippines, the estimated cases of diabetes were as high as 3.7 million in 2017, with adults aged 20 and above comprising the largest...
proportion of diabetes prevalence\(^3\), threatening the workforce and economy of one’s country.

One of the mechanisms to manage blood glucose levels is to inhibit the key enzymes involved in carbohydrate degradation: \(\alpha\)-amylase and \(\alpha\)-glucosidase. Alpha-amylase inhibitors delay starch digestion through binding with \(\alpha\)-amylase, effectively reducing the activity of the enzyme. This decreases intestinal absorption of carbohydrates and facilitates reduction of postprandial hyperglycemia. Alpha-glucosidase inhibitors also slow down absorption of carbohydrates in the intestine by retarding carbohydrate digestion, leading to decrease in postprandial blood glucose levels. Several carbohydrate enzyme inhibitors are already available in the market. However, the use of medicinal plants as sources of these inhibitors is currently explored to provide alternatives and satisfy demands.\(^4\)

The biological diversity of the Philippine ecosystem gives the opportunity to harness and utilize such abundance for drug discovery, which can potentially help in improving the status of diabetes management. One of the plants traditionally used for the treatment of diabetes in Antique, Philippines is *Alternanthera sessilis*. It has been considered as a weed in agricultural crops \(^5\) and widely utilized as vegetable in local dishes. It has also been used in folkloric medicine to treat various ailments such as headaches, hepatitis, asthma \(^6\), fever, diarrhea, dyspepsia, and liver and spleen problems \(^7\). Scientific studies of this plant include antimicrobial \(^8\), hepatoprotective \(^9\), wound-healing \(^10\), antioxidant \(^11\), and blood component (platelet and hemoglobin) augmentation\(^12\) activities. There are also previous studies of antidiabetic activities in other countries\(^13,14\). However, the mammalian \(\alpha\)-amylase and \(\alpha\)-glucosidase activities of the local variety have not been reported in literatures.

In this study, the potential in vitro inhibitory activity of the leaf hexane, ethyl acetate and water fractions of *Alternanthera sessilis* in mammalian \(\alpha\)-amylase and \(\alpha\)-glucosidase was investigated. The phytochemicals responsible for these activities were also determined.

2. MATERIALS AND METHODS

2.1. Collection and preparation of *A. sessilis* leaves

The plant was obtained from San Jose, Antique every mornings of March - May and September 2016. The fresh leaves were collected and placed in a styrofoam cooler to avoid direct exposure to sunlight. The samples were garbled, washed, and air-dried. A voucher specimen, with control number: 16-04-427, was submitted to the National Museum-Philippines for proper authentication.

2.2. Preparation of *A. sessilis* leaf crude extract and fractions

The air-dried leaves of *A. sessilis* were milled into powder and macerated with methanol at room temperature for 24 h. The resulting mixture was filtered and concentrated using rotary evaporator. The residue was subjected to another two rounds of extraction at room temperature for 4 h. The extracts were pooled to produce the crude methanol extract and was used for fractionation using different solvents.

The fractionation process was based on a previous method with some modifications\(^11\). The crude methanol extract was dissolved in distilled water and extracted thrice with hexane. The hexane fraction was collected, filtered and concentrated using rotary evaporator. The aqueous layer was added with ethyl acetate and partitioned using the same procedure as above. The lower and upper layers obtained after three rounds of ethyl acetate extraction was filtered and concentrated to yield the aqueous and ethyl acetate fractions, respectively. All collected fractions were dissolved in pure dimethylsulfoxide to attain various concentrations for the in vitro inhibitory assays.

2.3. Determination of \(\alpha\)-amylase and \(\alpha\)-glucosidase inhibitory activities

The \(\alpha\)-amylase and \(\alpha\)-glucosidase inhibitory assays were performed based on a method wherein the enzyme concentration and reaction times were optimized\(^15\).

For \(\alpha\)-amylase inhibitory assay, a 10-\(\mu\)L fraction was mixed with 1 U/mL porcine \(\alpha\)-amylase and incubated for 10 min at 37°C. Starch solution was added, mixed and incubated for 30 min at 37°C. The reaction was terminated by addition of 3,5-dinitrosalicylic acid followed by incubation for 10 min at 95°C. The mixture was cooled to ambient temperature and diluted with distilled water. A 150-\(\mu\)L aliquot was added in the well and the absorbance was measured at 540 nm.

For \(\alpha\)-glucosidase inhibitory assay, a 10-\(\mu\)L fraction was mixed with phosphate buffer pH
6.8 and 50 mg/mL intestinal acetone rat powders. The mixture was incubated for 10 min at 37°C and added with p-nitrophenyl-α-glucopyranoside. Further incubation was conducted for 30 min at 37°C followed by addition of 0.1 M sodium carbonate. The absorbance was measured at 405 nm.

Control was prepared by replacing the fraction with pure dimethylsulfoxide. Sample and control blanks were prepared using the fraction and pure dimethylsulfoxide, respectively, in the absence of enzyme. Acarbose was used as positive control in both inhibitory assays.

The % inhibition was computed based on formula below:

\[
\% \text{Inhibition} = \frac{A_{\text{control}} - A_{\text{fraction/positive control}} - A_{\text{fraction/positive control blank}}}{A_{\text{control}}} \times 100
\]

2.4. Phytochemical screening and high performance liquid chromatography (HPLC) analysis

Phytochemical screening was conducted based on the previous study 16. The fractions were tested for the presence of metabolites including carbohydrates, lipids, proteins, saponins, tannins, terpenoids, steroids, flavonoids, alkaloids, and glycosides.

The fraction with the highest inhibitory activity in both assay was subjected to HPLC. The fraction was diluted with methanol to 10 mg/mL and separated on Symmetry® C18 5.0μm 3.9 x 150 mm column, connected to a pre-column of Phenomenex® C18 3 x 4 mm. The mobile phase consisted of a linear gradient of solvent A (acetonitrile) and solvent B (water/acetic acid 97:3 v/v adjusted to pH 2.8), with the following time program: 15% A (7 min), 15-20% A (5 min), 20-40% (5 min), 40% A (5 min), 40-50% A (8 min). The analysis has an applied flow rate of 0.8 mL/min, injection volume of 5 μL and UV detection of 300 nm. Standards used for the analysis were quercetin, berberine, and genistein. All the solutions were filtered using 0.45 μm PTFE syringe filter prior to injection.

2.5. Statistical analysis

The data were reported as mean ± standard deviation (n=3). Half maximal inhibitory concentration (IC₅₀) and statistical significance between the groups of in vitro inhibitory assays were computed by GraphPad Prism 7.0 software using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. Mean values were considered statistically significant when p<0.05.

3. RESULTS AND DISCUSSION

3.1. Preparation of A. sessilis leaf crude extract and fractions

The air-dried leaves of A. sessilis were extracted with methanol to yield the crude methanol extract, which was dark green, viscous and slightly water soluble. This was used for the subsequent extraction of the hexane, ethyl acetate and water fractions. The summary of the characteristics and percent yield of the extracts and fractions are shown in Table 1.

The hexane fraction gave the highest yield, followed by ethyl acetate and then water fractions, in agreement with the previous report 13. However, all three were of lower values compared to the cited literature (42.1%, 39.7%, 18.2%, respectively). This result may be due to the A. sessilis used in the mentioned study (i.e. red variety) compared to the green variety used in the present study (Figure 1).

3.2. Determination of α-amylase and α-glucosidase inhibitory activities

The hexane, ethyl acetate and water fractions from the crude methanol extract of A. sessilis leaves were analyzed for their in vitro α-amylase and α-glucosidase inhibitory activities.

<table>
<thead>
<tr>
<th>Fractions/extracts</th>
<th>Color of extract</th>
<th>Water solubility</th>
<th>Consistency</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude methanol extract</td>
<td>Dark green</td>
<td>Slightly soluble</td>
<td>Viscous</td>
<td>12.72</td>
</tr>
<tr>
<td>Hexane fraction</td>
<td>Dark green</td>
<td>Insoluble</td>
<td>Viscous</td>
<td>24.32</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>Green</td>
<td>Insoluble</td>
<td>Viscous</td>
<td>14.68</td>
</tr>
<tr>
<td>Water fraction</td>
<td>Dark brown</td>
<td>Soluble</td>
<td>Powder</td>
<td>13.32</td>
</tr>
</tbody>
</table>

Table 1. Characteristics and percent yield of A. sessilis L. leaves extract and its fractions.
Half-maximal inhibitory concentration is the concentration required to inhibit an enzymatic process by half, providing a measure of the substance’s potency in enzyme-based assays. The lower the IC$_{50}$ value, the more potent inhibitor the substance is.

For the α-amylase inhibitory assay, ethyl acetate gave the lowest IC$_{50}$ among the fractions, followed by hexane and water fractions (Table 2). Statistically, only hexane ($p=0.05$) and ethyl acetate ($p=0.72$) fractions displayed no significant difference with acarbose (positive control). However, ethyl acetate fraction had a 200-fold higher IC$_{50}$ value than acarbose, demonstrating its less effective inhibitory activity. This difference may be due to the crudeness of the fraction relative to acarbose. Thus, further purification and fractionation are necessary to utilize the potential of ethyl acetate fraction as a source of α-amylase inhibitors.

The α-amylase IC$_{50}$ value of the water fraction obtained from this study revealed an almost similar value of 11.88 mg/mL in $Aspergillus$ $oryzae$ α-amylase from the Indian variety of $A$. $sessilis$ aerial water extract from Tamil Nadu. However, another study of the same plant extract from Hyderabad, India showed no inhibitory activity against porcine α-amylase. This disparity reveals that geographical location affects the bioactivities of the same plant species, even on a country-level. No literatures have been reported for the α-amylase activity of $A$. $sessilis$ hexane and ethyl acetate fractions.

The same trend of IC$_{50}$ was observed for α-glucosidase inhibitory assay: ethyl acetate fraction < hexane fraction < water fraction. Moreover, only ethyl acetate fraction showed no statistical significant inhibitory activity with acarbose ($p=0.26$). However, its IC$_{50}$ value is seven times higher than acarbose, signifying a lower inhibitory activity. This trend is consistent with the results of the glucosidase inhibitory activity of $A$. $sessilis$ Red found in Malaysia, but of lower IC$_{50}$ values than those obtained in the present study. The potent inhibitory activity of the Malaysian variety may be due to the selectivity of $A$. $sessilis$ fractions to Type-1α-glucosidase $Saccharomyces$ $cerevisiae$ used in their study compared to intestinal rat powder α-glucosidase in this study. Previous studies also reported that the plant extracts which exerted significant inhibitory activity in microbial α-glucosidase failed to inhibit α-glucosidase from rat intestinal powders, suggesting that the inhibitory activity in the microbial enzyme may not necessarily apply in the mammalian form. Thus, it is more appropriate to use mammalian carbohydrate-degrading enzymes for screening of potential inhibitors before proceeding with the in vivo antidiabetic assays.

Table 2. Half maximal inhibitory concentration (IC$_{50}$) of $A$. $sessilis$ fractions against α-amylase and α-glucosidase.

<table>
<thead>
<tr>
<th>$A$. $sessilis$ fractions</th>
<th>IC$_{50}$ α-amylase, mg/mL</th>
<th>IC$_{50}$ α-glucosidase, mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>1.48 ± 0.72</td>
<td>7.18 ± 1.58*</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.52 ± 0.072</td>
<td>2.82 ± 0.21</td>
</tr>
<tr>
<td>Water</td>
<td>17.59 ± 0.34**</td>
<td>30.92 ± 1.82**</td>
</tr>
<tr>
<td>Acarbose (positive control)</td>
<td>0.0025 ± 0.00045</td>
<td>0.36 ± 0.063</td>
</tr>
</tbody>
</table>

Differences between positive control and fractions were compared by one-way ANOVA followed by Dunnett’s multiple comparison test. $P$ values < 0.05 were considered statistically significant. **, $p<0.001$, *, $p<0.01$. 

Figure 1. Green variety of $A$. $sessilis$ used in this study.
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Table 3. Phytochemical screening of hexane, ethyl acetate and water fractions of A. sessilis leaves.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Positive test</th>
<th>Hexane</th>
<th>Ethyl acetate</th>
<th>Water</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Carbohydrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molisch test</td>
<td>Purple ring</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>Maltose</td>
</tr>
<tr>
<td>Benedict’s test</td>
<td>Red, orange, green, yellow precipitate</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brown, yellow, red precipitate</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Fehling’s test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solubility test</td>
<td>H₂O: Immiscible</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol: Lower layer</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform: Miscible</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2. Lipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Corn oil</td>
</tr>
<tr>
<td>3. Proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biuret test</td>
<td>Purplish violet</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Maltose</td>
</tr>
<tr>
<td>4. Saponin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Froth test</td>
<td>Froth ≥ 2 cm for 10 min</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>5. Tannins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tannic acid</td>
</tr>
<tr>
<td>Ferric chloride test</td>
<td>Brownish green or blue green</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6. Terpenoids/Steroids</td>
<td>Liebermann-Burchard test</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deep red: Terpenoids</td>
<td>Sterol</td>
<td>-</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>Green: Sterols</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Salkowski test</td>
<td>Yellow: Triterpenoids</td>
<td>Sterol</td>
<td>Triterpenoids</td>
<td>Triterpenoids</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>Red: Sterols</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7. Flavonoids/Phenols</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Quercetin</td>
</tr>
<tr>
<td>Alkaline test</td>
<td>Formation of yellow solution which disappears with</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>addition of acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Lead acetate test</td>
<td>Yellow precipitate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8. Alkaloids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mayer’s test</td>
<td>Pale yellow precipitate</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Wagner’s test</td>
<td>Reddish brown precipitate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Hager’s test</td>
<td>Yellow precipitate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Dragendorff’s test</td>
<td>Orange or brick red precipitate</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>9. Glycosides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borntrager test</td>
<td>Pink, red or violet color at NH₄ phase</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Keller-Killiani test</td>
<td>Reddish brown/purple at junction</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>x</td>
</tr>
</tbody>
</table>

+: Presence, -: Absence. Number of + indicates degree of color/precipitate. x – no positive control.

3.3. Phytochemical screening and HPLC analysis of ethyl acetate fraction

Phytochemical screening is used to provide a preliminary data on the possible class of metabolites present in plant extracts or fractions. Table 3 shows the phytochemical screening conducted on hexane, ethyl acetate and water fractions of A. sessilis leaves.

Hexane fraction showed the presence of lipids, sterols, flavonoids, and glycosides. Both ethyl acetate and water fractions were positive for carbohydrates, triterpenoids, and flavonoids. However, only ethyl acetate contains tannins, lipids and glycosides compared to water fractions. The result of the phytochemical screening is slightly different compared to previous study wherein the phytochemicals present in the ethyl acetate fraction of the Malaysian red variety are phenols, terpenoids, alkaloids and secondary amines. Aqueous extracts of A. sessilis from India confirmed the presence of alkaloids, flavonoids, tannins, saponins, terpenoids, phenols and carbohydrates, having more phytochemicals compared to the present study. This signifies that the variety of A. sessilis obtained in Antique is not the same as that from Malaysia or from India. This also confirms that geographical differences affect the biochemical compounds present in the plants of the same species.

The potent inhibitory activity of the ethyl acetate fraction may be attributed to the tannins and glycosides that is present in this fraction.
Glycosides, particularly deoxy-difluoroglycosides, have been reported to inactivate α-amylase and α-glucosidase via formation of a stable glycosyl enzyme intermediate, resulting in slow product turnover. Moreover, flavonoid glycosides have been shown to inhibit α-amylase through formation of hydrogen bonds between the hydroxyl groups of individual ligand and carboxylic acid side chains of the bound cleft residues. Flavonoid glycosides also interact with α-glucosidase through hydrogen bond, arene-arene and arene-cation formation. These interactions result in complexation of the enzymes leading to their decreased activity.

Tannins also influence the activity of carbohydrate-degrading enzymes. It has been reported that increasing the tannin concentration increases the inhibitory activity of α-amylase in vitro, with the inhibitory activity dependent on the size and complexity of the tannins in the extract/fractions. Another study also displayed effective inhibition of α-amylase in tannin-rich extracts, while removal of tannins resulted to a decrease in inhibition. Ellagitannins and their related polygalloylglucoses are also observed to effectively inhibit rat intestinal α-glucosidase complexes, with higher galloylglucoses units contributing to their potency. The ability of tannins to precipitate proteins through binding of its phenolic group results to conformational changes in the carbohydrate-degrading enzymes, leading to reduced activity by association and precipitation.

To check for specific compounds present in the ethyl acetate fraction, HPLC analysis was conducted. The chromatograms of the standards (quercetin, berberine and genistein), ethyl acetate fraction and diluent (methanol) are shown in Figure 2. Chromatogram of ethyl acetate fraction showed several peaks and two were identified as berberine and quercetin at retention times 11.542 and 13.374 min based on the standard chromatograms.

Quercetin and berberine were identified as natural products that can inhibit α-glucosidase and α-amylase in silico. When compared with acarbose at IC_{50} = 3000 μM, quercetin (IC_{50} = 280 μM) and berberine (IC_{50} = 313 μM) demonstrated a 10.7- and 9.6-fold increase, respectively, in vivo yeast α-glucosidase inhibitory activity. Similarly, quercetin (IC_{50} = 500 μM) and berberine (IC_{50} = 170 μM) displayed 2- to 5.9-fold increase in in vivo bacterial α-amylase inhibitory activity compared to acarbose at IC_{50} = 1000 μM. Berberine has also showed attenuation of fasting blood glucose and HbA1c levels of patients with diabetes comparable to the effect of metformin. They also improved body mass index and leptin/adiponectin ratio of diagnosed metabolic patients by increasing insulin sensitivity and adipogenesis. Likewise,
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Conflict of interest (If any)
The authors declare no conflict of interest in this study.

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Ethical approval
This study was registered in the University of the Philippines Manila Research Grants Administration Office. No ethical approval was sought as the study did not use human subjects and/or animals.

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