Inhibitory activity of α-glucosidase and α-amylase by Annona stenophylla root extract as mechanism for hypoglycaemic control of DM

Tafadzwa Taderera, Lameck Shoriwa Chagonda, Gome E, Shai J.L.

Abstract

DM mellitus has become a worldwide disease. Some local TMPs in Zimbabwe are reported to have hypoglycaemic effects on blood glucose and are used as herbal medicines to treat diabetes. A. stenophylla aqueous root extracts were freeze dried and examined for inhibition of α-glucosidase and α-amylase on KAT reagents in the presence of sucrose and maltose substrates using acarbose as positive control. The IC50 values for the plant extract and acarbose for α-glucosidase in the presence of the sucrose were 0.123 ± 0.009 mg/ml and 0.101 ± 0.0176 mg/ml respectively. The IC50 values in the presence of maltose were 0.500 ± 0.128 mg/ml and 0.117 ± 0.0563 mg/ml respectively. The plant extract and acarbose showed IC50 values against amylase of 1.26 ± 0.903 mg/ml and 1.199 ± 0.0651 mg/ml respectively. The plant extract displayed mixed type inhibition kinetics for α-glucosidase with sucrose reducing Vmax value of the enzyme from 0.214 to 0.0608 mmoles. min⁻¹ whilst increasing Km from 0.0124 to 0.0580. The results suggest A. stenophylla possesses hypoglycaemic control in diabetes mellitus through inhibition of α-glucosidase and α-amylase enzymes and its standardisation could transform herbal practice in treating diabetes.

1. Introduction

Diabetes mellitus is a metabolic disorder characterised by hyperglycaemia, hyperlipidaemia and hyperaminoacidemia due to insulin hormone deficiency or resistance. Insulin is central in the metabolism of carbohydrates and lipids acting through a series of different mechanisms. Apart from using insulin and insulin analogue replacement therapies for treating Type 1 and 2 diabetes, modern drugs are known to work by stimulating insulin release, overcoming insulin resistance, promoting regeneration of pancreatic β-cells, reducing glucose production or readsoption and/or by increasing peripheral glucose uptake by adipose tissues and operating singly or in combination (Chiasson and Rabasa-Lhoret, 2004;
Krentz and Bailey, 2005; Hui et al., 2009; Wang et al., 2013). Despite this, DM remains a major worldwide health problem requiring multiple strategies to contain it and is often aggravated by acute and chronic comorbidities (Scheen, 1997; Flourescu and Kotler, 2007; Aboud et al., 2007; Levitt, 2008; WHO, 2004; 2013; Wang et al., 2013).

The apparent failure to find a one-size-fit-all drug treatment for Type 2 diabetes has led to active research both in new modern medicines and hypoglycaemic drugs from medicinal plants worldwide (Kim et al., 2006; Hui et al., 2009; Singh et al., 2010; Shojaii et al., 2011; Kavishankar et al., 2011; Tripathi et al., 2011; Wang et al., 2013). This search has been motivated through the understanding of the biological mechanisms postulated in the treatment of DM by modern drugs (oral anti-diabetic agents such as sulfonylureas, biguanides, alpha-glucosidase inhibitors and troglitazones, amylin agonists and DPP-4 inhibitors) some of which are derived from traditional medicinal plants and herbal formulations used to treat DM (Kumar et al., 2011; Wang et al., 2013). Diabetes is a chronic and progressive illness and many side effects are often observed with mono- and/or with multiple therapies of DM. Treating Type 2 diabetes with traditional Chinese and Indian medicinal herbs long used for treating T2DM has provided clinical evidence of their use and pose challenges for their incorporation into Western therapies. Many of the traditional herbs are reported to operate by multiple mechanisms, an obvious advantage in combating DM (Kim et al., 2000; Subramanian et al., 2008; Wang et al., 2013; Picot et al., 2014). The inhibition of carbohydrate metabolizing enzymes α-glucosidase and α-amylase by herbal plant extracts are evidence of the mechanisms by which some of them exert their activities in restoring normal blood glucose levels. Isolated plant extracts and active phytoconstituents: polyphenols, flavonoids, alkaloids, terpenoids, anthraquinones, saponins/steroidal glycosides, acetogenins, and polysaccharides from medicinal plants are widely reported to have hypoglycaemic effects and to inhibit carbohydrate metabolising enzymes by α-glucosidase and α-amylase (Ali et al., 2006; Hanamura et al., 2006; Hossain et al., 2009; Bhushan et al., 2010; Singh et al., 2010; Shai et al., 2011; Shojaii et al., 2011; Adesegun et al., 2013; Kumar et al., 2013; Mun’im et al., 2013; Picot et al., 2014). However, the overall search for anti-diabetic agents has not been rewarded with marketable novel agents in the last two decades (Kumar et al., 2013); attempts are still made to find such drugs.

Recently, there has been an upsurge in evidence based traditional medicinal plant research with a view to finding new hypoglycaemic drugs and establishing their action mechanisms. Half of the drugs used in the treatment of different ailments are designed as inhibitors of key enzymes and the prominent enzymes involved in carbohydrate metabolism in this case, α-glucosidase (EC 3.2.1.20) and α-amylase (EC 3.2.1.1), have been used in in vitro models for determination of inhibitory activity of potential anti-diabetic drugs from natural sources. The two enzymes cleave glycосidic bonds in complex carbohydrates into monosaccharides like fructose and glucose that can be absorbed through the small intestines into the blood stream. If these enzymes were to be inhibited, carbohydrate metabolism would be delayed resulting in lower glucose absorption rates and reduced post prandial blood glucose levels (Chiasson and Rabasa-Lhoret, 2004; Olao kun et al., 2013).

Medicinal plants are known to have multiple beneficiary pharmacological activities with less side effects when compared to some synthetic compounds, however, continuous use also may pose toxic complications (WHO, 2004; Khathi et al., 2013).

2. Materials and Methods

2.1 Objectives of the Research

In Zimbabwe, Annona stenophylla is one of the traditional medicinal plants used to treat diabetes. It has been reported to have antiparasitic, anti-infective, antiviral, antioxidant and antidiabetic activities (Gelfand et al., 1985; Shoko, 2007; Munodawafa et al., 2008). We have also reported its hypoglycaemic properties (Phiri and Chagonda, 2012). This study was conducted to establish whether its root extracts have potential to inhibit α-glucosidase and α-amylase enzymes in the presence of carbohydrate substrates and thus confirm a possible mechanism of its diabetogenic activity. It would also promote research into traditional herbs in view of their capacity to exhibit multiple mechanisms in the fight against DM (Hui et al., 2009; Tripathi et al., 2011; Wang et al., 2013) when compared to oral antidiabetic agents (Krentz and Bailey, 2005).

2.2 Plant materials and extraction

A. stenophylla Engl. & Diels (Annonaceae) (2540) roots were collected from Mazowe district (November, 2012) and identified at the Zimbabwe National Herbarium and Botanic Gardens, Harare, were voucher specimens are kept. Air-dried and powdered root bark was extracted with distilled water overnight at 37°C. The filtered extract was lyophilized in a freeze dryer and stored at -20°C until use. Lyophilized in a freeze dryer and stored at -20°C until use.
Table 1: IC$_{50}$ values for α-amylase and α-glucosidase inhibition assays

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitory concentration (IC$_{50}$) mg/ml</th>
<th>Acarbose mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-amylase</td>
<td>1.26 ± 0.903</td>
<td>1.199 ± 0.0651</td>
</tr>
<tr>
<td>α-glucosidase with sucrose substrate</td>
<td>0.123 ± 0.009</td>
<td>0.101 ± 0.0176</td>
</tr>
<tr>
<td>α-glucosidase with maltose substrate</td>
<td>0.500 ± 0.128</td>
<td>0.117 ± 0.0563</td>
</tr>
</tbody>
</table>

* p >0.05 by comparison with all pairs of columns

Graphical abstract

Inhibition of α-glucosidase activity by extract

Figure 1:

Lamnearer-Burk plot for inhibition of α-glucosidase by extract

![Graphical abstract and Figure 1]
2.3 Chemicals
Acarbose, α-glucosidase, α-amylase, sucrose, maltose and phosphate buffer were obtained from Sigma Aldrich.

2.4 In vitro assays
2.4.1 α-Amylase inhibition
The α-amylase enzyme inhibition assay was done using the KAT kit (Kat Medical South Africa), according to manufacturer’s instructions. Microtitre plates (96 wells) were loaded with 100 µl of 0.5 M phosphate buffer pH 6.9 followed by 100 µl of plant extract (10 mg/ml). Acarbose and phosphate buffer saline (PBS) were used as positive and negative controls. After the serial dilutions, 20 µl of the enzyme was added to each plate and the plates incubated at 37 ºC for 1 h followed by addition of 100 µl of the KAT amylase reagent. The mixtures were further incubated for 5 min. The rate of the reaction was spectrophotometrically followed at 450 nm (Anthos 2000 Spectrophotometer) and performed in triplicate for each concentration. The inhibitory activity for the negative control (without inhibitor) was considered to be 100%. Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC50 values) were determined graphically with each test repeated twice and the mean absorption used to calculate alpha amylase inhibition percentages determined by using the formula below

α-amylase inhibition (%) = \[
\left( \frac{\Delta A_{450\text{nm control}} - \Delta A_{450\text{nm plant extract}}}{\Delta A_{450\text{nm control}}} \right) \times 100
\]
2.4.2 α-Glucosidase inhibition assays

Alpha-glucosidase enzyme inhibition was assayed using rat intestinal acetone powder to a modified method described by Kim et al. (2005). To each of 100 µl of serially diluted 0.5 M phosphate buffer (pH 6.8) containing the test samples and control (PBS), 20 µL of α-glucosidase (25mg/ml) was added followed by 100 µl of either 2% sucrose or maltose substrate solutions. The mixtures were incubated at 37°C for 1 h. 100 µl of KAT glucose reagent was then added to each sample and the mixture incubated for a further 10 min. The rate of the reaction was monitored by measuring absorbance at 540 nm (Anthos 2000 Spectrophotometer). All determinations were performed in triplicate with absorbance readings considered as directly proportional to the activity of the enzyme with the test repeated twice. The results were reported as percentages of the control (without inhibitor) using the following formula.

\[
\text{α-glucosidase inhibition (%) } = \frac{(\Delta\text{Abs nm control}) - (\Delta \text{Abs nm plant extract})}{\text{ΔAbs nm control}} \times 100
\]

ΔA540 nm control = ATest–ABlank (represented 100% activity)
ΔA540 nm plant extract = ATest–ABlank

2.4.3 Kinetics of α-glucosidase inhibition

The mode of inhibition of the A. stenophylla extract against the α-glucosidase enzyme (E) from rat intestinal acetone powder was determined according to a slightly modified method previously described by Kim et al., 2006. 20 µl of 25 mg/ml of α-glucosidase was incubated at 37°C for 1 hour with increasing concentrations of sucrose substrate (S) ranging from 0.0781 mg/ml to 10 mg/ml. The determinations were done in triplicate and the test repeated twice, whilst activity was determined by measuring absorbance at 540 nm. The quantity of the product released was determined from the standard curves and converted to reaction rates according to the formula below:

Reaction rate (v) (mg.ml\(^{-1}\).min\(^{-1}\)) = [product] (mg.ml\(^{-1}\)) / incubation time (mins)

The type of inhibition, [K\(_m\)] and [V\(_{max}\)] values were determined from Lineweaver–Burk plots and values were calculated according to Michaelis-Menten kinetics.

2.5 Statistical Analysis

All data are expressed as mean ± SD. The IC\(_{50}\) value was defined as the concentration of plant extract or acarbose required to inhibit 50% of enzyme α-amylase or α-glucosidase under the assay conditions. Data manipulation and statistical analyses were performed using Graph pad prism v

5. Differences between means were considered significant when a 2-tailed value of P was less than 0.05 using one way analysis of variance (ANOVA) for multiple groups followed by Bonferroni post test or t-tests for 2 groups.

3. Results

3.1 In vitro enzyme inhibitory activities

The in vitro enzyme inhibition studies showed that A. stenophylla root extracts inhibited both α-glucosidase and α - amylase enzymes. Both plant extract and acarbose inhibited α - amylase in a dose dependent manner with maximum recorded at 64.6 ± 2.97% and 81.4 ± 1.13% respectively at 10mg/ml, the highest enzyme concentration used in this study (Fig.1). Differences between the control (acarbose) and extract treated groups using α - amylase at the different concentrations were not statistically significant (p > 0.05) except for concentration 2.5 mg/ml where p = 0.0034 (Fig 1). The IC\(_{50}\) values for plant extract and acarbose against α-amylase were 1.26 ± 0.903 and 1.199 ± 0.0651 mg/ml respectively (Table 1).

Figure 1: Inhibition of α-amylase enzyme activity by different concentrations of aqueous plant extract and acarbose. The results represent a mean and standard deviation of two triplicate experiments. p < 0.05 by comparison with respective standard (acarbose) at concentration 2.5 mg/ml

The A. stenophylla root extracts displayed effective concentration dependent inhibitory activities for α-glucosidase in the presence of either sucrose or maltose substrates. Extract inhibition of α-glucosidase for sucrose and maltose increased with increase in concentration up to 2.5mg/ml (84.9 ± 1.80% and 72.7 ± 9.34% respectively) before declining thereafter (Fig.2). Under similar conditions, acarbose displayed a concentration dependent inhibition of α-glucosidase for sucrose and maltose with values of 83.22 ± 4.39% and 70.7 ± 1.10 % at 10mg/ml respectively, the highest concentration of the test sample. The IC\(_{50}\) values for the plant extract and acarbose standard for α-glucosidase in the presence of the glucose substrate were 0.123 ± 0.009 mg/ml and 0.101±0.0176 mg/ml respectively. The corresponding IC\(_{50}\) values in the presence of maltose as substrate were 0.500 ± 0.128 mg/ml and 0.117 ± 0.0563 mg/ml respectively. Furthermore, the plant extract and acarbose showed IC\(_{50}\) values against α-amylase of 1.26 ± 0.903 mg/ml and 1.199 ± 0.0651 mg/ml respectively. Differences in enzyme inhibition values amongst the groups at all the concentrations were not statistically significant (p > 0.05) except for those highlighted on the graph (fig 2). One way ANOVA showed no significant differences amongst all the groups IC\(_{50}\) values (p > 0.05). The
A. stenophylla extract gave the lowest IC$_{50}$ value against $\alpha$-glucosidase with sucrose substrate (0.123 ± 0.009 mg/ml) comparable to acarbose (0.101 ± 0.0176 mg/ml) (Table 1) prompting further assessment through inhibition kinetics.

Figure 2: Inhibition of $\alpha$-glucosidase activity by various concentrations of aqueous extract and acarbose. The results represent a mean and standard deviation of two triplicate experiments. *p <0.05 by comparison with extract treated group using maltose substrate at concentration 0.156 mg/ml.

3.2 Kinetic analysis of $\alpha$-glucosidase inhibition by A. stenophylla

The plant extract displayed mixed type inhibition kinetics for $\alpha$-glucosidase with sucrose as the substrate reducing $V_{\text{max}}$ value of the enzyme from 0.214 to 0.0608 mmoles. min$^{-1}$ whilst $K_m$ increased from 0.0124 to 0.0580 (Fig.3).

Figure 4. Inhibitory activity of $\alpha$-glucosidase and $\alpha$-amylase by A. stenophylla root as mechanism for hypoglycaemic control in DM

Discussion:

A. stenophylla root extract displayed potent inhibition activity against carbohydrate hydrolysing enzymes $\alpha$-glucosidase and $\alpha$-amylase similar to acarbose. The inhibition of mammalian $\alpha$-glucosidase enzyme is seen as one of the best strategies for controlling postprandial glucose levels in T2DM and therefore delaying the onset of late diabetic complications. Acarbose, miglitol and voglibose are established competitive inhibitors on the market used to achieve better glycaemic control in diabetic sufferers (Krentz and Bailey, 2005; Kumar et al., 2011; Mohamed et al., 2012; Wang et al., 2013). Medicinal plants are natural sources of bioactive compounds and are also effective in reducing postprandial hyperglycaemia as (Kim et al., 2006; Matsui et al., 2007; Ayodhya et al., 2010; Bhushan et al., 2010; Mahomed et al., 2012).

Alpha-glucosidase inhibitors from microorganisms like acarbose are reported to have some side effects: abdominal distension, flatulence and possibly diarrhoea due to increased $\alpha$-amylase activity. Speculation is that plant derived sources may have the desired higher $\alpha$-glucosidase and reduced $\alpha$-amylase activities thus lowering toxicities/side effects (Carrascosa et al., 2001; Krentz and Bailey, 2005; Kwon et al., 2006; Jaiswal et al., 2012; Mohamed et al., 2012). In this study, A. stenophylla displayed higher inhibition activity for $\alpha$-glucosidase (85% at 2.5 mg/ml) compared to $\alpha$-amylase (65% at 10 mg/ml). Earlier, we reported A. stenophylla to have remarkable glycaemic effects comparable to glibenclamide in a mice model (Phiri and Chagonda, 2012) and to have anti-infective, phytochemical, antioxidant and toxic activities (Munodawafa, 2008; Munodawafa et al., 2010; Munodawafa et al., 2013). Its antiparasitic, anti-infective and antiviral activities have also been reported (Gelfand et al., 1985; Shoko, 2007).

This study has demonstrated the potential of A. stenophylla root extract ability to reduce postprandial hyperglycaemia. The low IC$_{50}$ values of less than 1.5 mg/ml for all the inhibitions tested which were comparable to acarbose is motivation for additional studies on further antidiabetic activities, active phytoconstituent profiling, sub-acute and acute-toxicities, herbal formulations and pre-clinical studies as is the case in CAM Chinese and Indian traditional medicines (Chawla et al., 2013; Wang et al., 2013; Talreja and Kaur, 2014). A. stenophylla plant extract displayed mixed type inhibition kinetics for $\alpha$-glucosidase on sucrose substrate reducing $V_{\text{max}}$ value of the enzyme by about 4x and increasing the $K_m$ value by about 5x reflecting binding to both substrate and enzyme-substrate complex with different affinities. Depending on the magnitude of binding affinities for either S or ES, competitive and uncompetitive mechanisms could also be involved.

Research Highlights

1. A. stenophylla root extracts inhibited $\alpha$-glucosidase and $\alpha$-amylase enzymes in vitro in the presence of carbohydrate substrates.

2. A. stenophylla inhibition for the enzymes was comparable to acarbose, a drug used for the hypoglycaemic control in T2DM.

3. A. stenophylla exhibited mixed inhibition in exerting its antihyperglycaemic activity.

4. The results confirms the use of A. stenophylla in treating DM in traditional medicine and further research should be carried out to standardise its herbal extracts for clinical trials.

Limitations

The results were carried out using plant materials gathered in the wild from a single geographical location in summer. There may be geographical site and seasonal variations in the results.
Recommendations

1. The study should be carried using plants from different geographical locations to determine any variations.

2. The plants should be cultivated to avoid variations in geographical and environmental conditions.

3. Studies should be carried out to determine seasonal effects on the results.

4. The plant extracts should be standardised and clinically tested.

5. International finance/collaboration should be encouraged to expand the scope of such promising results.

Justification of Research

Modern hypoglycaemic drugs used to treat diabetes work by different mechanisms whilst traditional herbs often operate by multiple mechanisms in combating DM. Many developing countries still use traditional herbs alongside Western medicines.

We have investigated the hypoglycaemic properties of A. stenophylla as it is used to treat diabetes in our traditional practice. In the present work, we examined its potential to inhibit key metabolic enzymes to determine possible mechanisms of action. Such work would promote its use, standardisation and clinical evaluation of its herbal extracts.

Conclusion

The present work demonstrated that A. stenophylla inhibited both α-glucosidase and α-amylase enzymes in order to achieve glycaemic control and confirming its use in traditional medicine to treat diabetes. The extract achieved comparable inhibitory activity for sucrose with acarbose, an effective α-glucosidase used as an antidiabetic drug. It displayed mixed enzyme inhibition. These findings indicate the potential to develop A. stenophylla products for the control of DM. Further studies are underway to explore other relevant multiple mechanisms consistent with findings from traditional practices in India and China on hypoglycaemic herbs.

Funding and policy aspects

Governments in developing countries should take an active role in exploiting their unique natural products through providing generous research funds. International donors, financiers, collaborating institutions, organisations and interest groups could play a crucial role in providing funds to achieve the goal of ‘Health for All’ in the long run.

Author’s Contribution and Competing Interests

The experimental work was carried out by Tafadzwa Taderera and is part of ongoing Ph.D research. Professor Lameck Chagonda is the lead supervisor and corresponding author. I declare there is no competing interest amongst the authors.

Acknowledgements

The authors are grateful for the research funds from Southern African Consortium for Research Excellence (SACORE), the University of Zimbabwe Research Board and to the Biomedical Department of Tshwane University of Technology for technical assistance.

References


