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Inhibitory potential of *Gossypium arboreum* leaf extracts on diabetes key enzymes, α-amylase and α-glucosidase

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Abstract

One of the antidiabetic therapeutic approaches is the reduction in gastro-intestinal glucose production and absorption by inhibiting carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase. This present study evaluated the α-amylase and α-glucosidase inhibitory potential of the aqueous, ethanolic and acetone leaf extracts of *Gossypium arboreum* and to further determine the mode of inhibition of the enzymes using kinetic analysis. The aqueous and acetone extract exhibited potent α-amylase and α-glucosidase inhibitory activity with IC_{50} value of 10.1 mg/mL and 2.8 mg/mL respectively. These extracts are likely to contain non-competitive inhibitors of both enzymes with reduced Vmax values. The extracts were also found to contain phytochemicals such as tannins and steroids which may be responsible for the inhibitory effect. The results show that the aqueous and acetone extracts of *G. arboreum* leaf inhibited both α-amylase and α-glucosidase at relatively low concentrations and this could be the reason why the species is employed in the management of blood sugar related disorders by reasonable populations in Nigeria.

Introduction

Diabetes mellitus is a group of metabolic disorder in the endocrine system as a result of hyperglycemia and defect in the metabolism of glucose and lipids leading to complications like neuropathy, retinopathy and microangiopathy (Etuk et al., 2010). These occur as a result of elevation of blood glucose caused by relative or absolute deficiency of insulin. There are two main types of diabetes based on their requirement for insulin namely; Type 1 or insulin dependent diabetes mellitus (IDDM) and Type 2 or non-insulin dependent diabetes mellitus (NIDDM) (Lee et al., 2006).

Glycemic control is one of the targets for managing diabetes mellitus as it decreases the risk of developing diabetic complication (Kazeem et al., 2012). Orthodox treatment of diabetes mellitus includes a modification of life style such as exercise, good nutritional habit and administration of insulin and oral hypoglycemic drug such as biguanides and α-glucosidase inhibitors (Kelly and Mandarino, 2000). One of the strategies adopted to treat diabetes mellitus involves inhibition of carbohydrate-digesting enzymes such as α-amylase and α-glucosidase in the gastro-intestinal tract, with associated retardation of intestinal glucose absorption and lowering of postprandial blood glucose levels (Kwon et al., 2008).

Medicinal plants have contributed majorly in the treatment of many diseases and ailments including diabetes mellitus all over the world. In West Africa, particularly Nigeria, broadly identified antidiabetic plants include *Magnifera indica, Vernonia amygdalina, Calotropis procera,*

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Azadirachata indica and *Gossypium arboreum* (Gbolade, 2009). *G. arboreum* (cotton plant) has been documented as antidiabetic medicinal plant in different geo-political zones of Nigeria (Abo et al., 2007; Etuk and Mohammed, 2009). The plant is a species of cotton native to India, Pakistan and other tropical and subtropical region of the whole world. The leaves have been found to possess some antibacterial and antifungal properties (Saidu and Abdullahi, 2011). An infusion of the leaf is taken as an antedote for colds and bronchitis (Essien et al., 2011). It has also been used as oral contraceptive in humans due to the presence of gossypol contained in the leaf extract (Coutinho, 2002). Report also showed that it possesses wound healing and anti-oxidant properties (Annan and Houghton, 2008). Extracts from its leaves have further been reported to possess hypoglycemic and hypotensive effect in experimental rats (Hasrat et al., 2004) and to increase smooth muscle contraction in guinea pigs (Mans et al., 2004).

Despite the wide folkloric use of this plant in the management of several diseases and ailments, coupled with various pharmacological reports of *G. arboreum* leaf, there is dearth of information on its inhibitory effects on key enzymes linked to diabetes. For the first time, this study sought to investigate the inhibitory effect of *G. arboreum* leaf extracts on α-amylase and α-glucosidase as well as assessing its mode of inhibition in order to provide some possible mechanisms by which they elicit their antidiabetic effect.

Materials and Methods

Plant material

G. arboreum leaves were collected in February 2012 from a wild population growing at Adiyan area of Ogun state, Nigeria. The plant was identified and authenticated by Dr. A. B. Kadiri of the Department of Botany, University of Lagos, Nigeria and voucher specimen (LUH 4722) was prepared and deposited in the University herbarium.

Chemicals

α-amylase from *Aspergillus oryzae*, α-glucosidase from *Saccharomyces cerevisiae* and paranitrophenyl-glucopyranoside (PNPG) were products of Sigma-Adrich Co., St Louis, USA while starch soluble (extra pure) was obtained from J. T. Baker Inc., Phillipsburg, USA. Other chemicals and reagents were of analytical grade and water used was glass-distilled.

Preparation of plant extracts

Fresh leaves of *G. arboreum* were cut and rinsed under running water to remove all contaminants, were dried under room temperature and grounded to powder. The powdered leaves were divided into three portions of 40 g each and these were extracted in acetone, ethanol and distilled water respectively, with shaking on Labcon Platform shaker (Laboratory Consumables, PTY, South Africa) for 24 hours. All extracts were filtered using Whatman No. 1 filter paper. The filtrates from acetone, ethanol and hexane were concentrated under reduced pressure 40°C using (Cole Parmer SB 1100, Shangai, China) rotary evaporator. Acetone and ethanol used were of high analytical grade**.** The water extract was freeze-dried using Virtis Bench Top (SP Scientific Series, USA) freeze dryer. The yields were 3.1, 1.3, and 4.1 g for acetone, ethanol and water, respectively. Individual crude extracts were dissolved in 10% dimethyl-sulfoxide to yield a stock solution from which lower concentrations were prepared.

Phytochemical screening

Phytochemical compositions of the leaves were determined the methods described elsewhere (Harborne, 1973; Trease and Evans, 1996; Sofowora, 1993; Edeoga et al., 2005; Ajayi et al., 2011).

Test for tannins

In the test for tannins, 0.5 g of dried powdered sample was boiled in 20 mL of water in a test tube and filtered. Few drops of 0.1% ferric chloride was added and observed for brownish green or a blue black colouration as indication of tannins.

Test for saponin

Approximately 2 g of powdered material was boiled in 20 mL of distilled water in a water bath and filtered. Next, 10 mL of the filtrate was mixed with 5 mL of distilled water and shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously again and then observed for the formation of emulsion as indication of saponin.

Test for flavonoids

A portion of the powdered material was heated with 10 mL of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution. Development of yellow colouration is an indication of the presence of flavonoids.

Test for steroids

In this test, 2 mL of acetic anhydride was added to 0.5 g of ethanolic extract with 2 mL concentrated H_2SO_4. The colour change from violet to blue or green is indication of steroids.

Test for terpenoids (Salkowski's test)

In brief, 5 mL of extract was mixed with 2 mL chloroform and 3 mL H_2SO_4 was carefully added to

form a layer. A reddish brown colouration of the interface was indication of terpenoids.

Test for free anthraquinones

5 mL of chloroform was added to 0.5 g of the powdered plant materials of each specimen. The resulting mixture was shaken for 5 min after which it was filtered. The filtrate was then shaken with equal volume of 10% ammonia solution. The presence of a bright pink color in the aqueous layer indicated the presence of free anthraquinones.

Test for reducing sugar

To about 1 g of each sample in the test tube was added 10 mL distilled water and the mixture boiled for 5 min. The mixture was filtered while hot and the cooled filtrate made alkaline to litmus paper with 20% sodium hydroxide solution. The resulting solution was boiled with an equal volume of Benedict qualitative solution on a water bath. The formation of a brick red precipitate depicted the presence of reducing compound.

α-Amylase inhibitory assay

This assay was carried using a modified procedure of McCue and Shetty (2004). Briefly, 250 µL of extract was pipette into a tube and 250 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution was added. This solution was pre-incubated at 25°C for 10 min, after which 250 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at timed intervals and further incubated at 25°C for 10 min. The reaction was terminated after incubation by adding 500 µL of dinitrosalicylic acid (DNS) reagent. The tubes were then incubated in boiling water for 5 min and cooled to room temperature. The reaction mixture was diluted with 5 mL distilled water and the absorbance was measured at 540 nm using JENWAY UV-Visible spectrophotometer. A control was prepared using the same procedure replacing the extract with distilled water. The α-amylase inhibitory activity was calculated as percentage inhibition.

$$\% \text{ Inhibition} = [(Abs_{control} - Abs_{extracts})/Abs_{control}] \times 100$$

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically.

Mode of α-amylase inhibition

The mode of inhibition of *G. arboreum* leaf extract was conducted using the extract with the lowest IC_{50} according to the modified method described by Ali et al. (2006). Briefly, 250 µL of the (5 mg/mL) extract was pre-incubated with 250 µL of α-amylase solution for 10 min at 25°C in one set of tubes. In another set of tubes, α-amylase was pre-incubated with 250 µL of phosphate buffer (pH 6.9). 250 µL of starch solution at increasing concentrations (0.3-5.0 mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10 min at 25°C, and boiled for 5 min after addition of 500 µL of DNS to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically using a maltose standard curve and converted to reaction velocities. A double reciprocal plot (1/v versus 1/[S]) where v is reaction velocity and [S] is substrate concentration was plotted. The type (mode) of inhibition of the crude extract on alpha amylase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot using Michaelis-Menten kinetics.

α-Glucosidase inhibitory assay

The effect of *G. arboreum* leaf extracts on α-glucosidase activity was determined according to the method described by Kim et al. (2005). The substrate solution p-nitropheynyl glucopyranoside (pNPG) was prepared in 20 mM phosphate buffer, pH 6.9. 100 µL of alpha glucosidase (E.C. 3.2.1.20) was pre-incubated with 50 µL of the different concentrations (0.6-5.0 mg/mL) of the extracts (acetone, ethanol and water) for 10 min. Then 50 µL of 3 mM p-nitropheynyl glucopyranoside as a substrate dissolved in 20 mM phosphate buffer, pH 6.9 was then added to start the reaction. The reaction mixture was incubated at 37°C for 20 min and stopped by adding 2 mL of 0.1 M Na_2CO_3. The α-glucosidase inhibitory activity was determined by measuring the yellow colored p-nitrophenol released from pNPG at 405 nm. The results were expressed as percentage of the blank control.

Percentage inhibition calculated as

$$\%\text{inhibition} = [(Abs_{control} - Abs_{extract})/Abs_{control}] \times 100$$

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically.

Mode of α-glucosidase inhibition

The mode of inhibition of the *G. arboreum* leaf extract was conducted using the extract with the lowest IC_{50} according to the modified method described by Ali et al. (2006). Briefly, 50 µL of the (5 mg/mL) extract was pre-incubated with 100 µL of α-glucosidase solution for 10 min at 25°C in one set of tubes. In another set of tubes α-glucosidase was pre-incubated with 50 µL of phosphate buffer (pH 6.9). 50 µL of p-nitropheynyl glucopyranoside at increasing concentrations (0.6-2.0 mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10 min at 25°C, and 500 µL of Na_2CO_3 was added to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically using a paranitrophenol standard curve and converted to reaction velocities. A double reciprocal plot (1/v versus 1/[S]) where v is reaction velocity and [S] is substrate concentration was plotted. The mode of inhibition of the crude extract on α-glucosidase activity was determined by analysis of the double reciprocal (Lineweaver

Table I

Phytochemical composition of *Gossypium arbo-reum* leaf extracts

Phytochemi-cals	Extracts inference		
	Acetone	Ethanol	Water
Anthraquinones	-	-	-
Flavonoids	-	Detected	-
Reducing sugar	-	Detected	-
Saponins	-	-	-
Steroids	Detected	Detected	-
Tannins	Detected	-	Detected
Terpenoids	-	Detected	-

Table II

IC_{50} values of α-amylase and α-glucosidase inhibi-tion of leaf extracts of *Gossypium arboreum*

Extracts	IC_{50} (mg/mL)	
	α-amylase	α-glucosidase
Acetone	11.1 ± 0.8	2.8 ± 0.0
Ethanol	15.8 ± 1.2	11.3 ± 1.1
Water	10.1 ± 1.2	2.9 ± 0.0

-Burk) plot using Michaelis-Menten kinetics.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 statistical package (GraphPad Software, USA). The data were analyzed by one way analysis of variance (ANOVA) followed by Bonferroni test. All the results were expressed as mean ± SE for triplicate determinations.

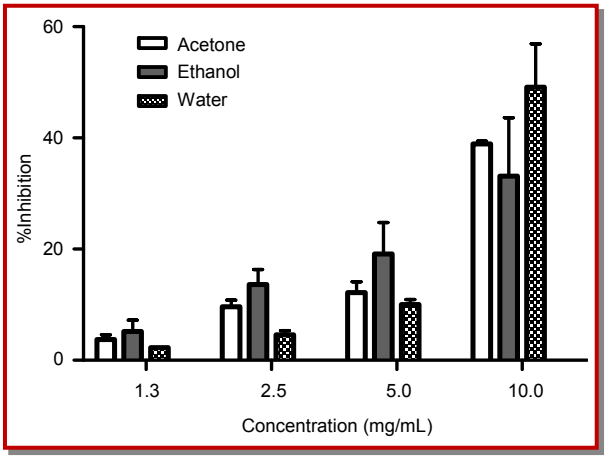

Figure 1: α-Amylase inhibitory activity of *G. arboreum* leaf extracts

Values represent mean ± SEM of triplicate experiments. Bars without superscripts at the same concentration are not significantly different

Results

The ability of *G. arboreum* leaf extract to inhibit α-amylase and α-glucosidase activity was investigated *in vitro*. The preliminary qualitative phytochemical screening testing reveals the presence of steroids and tannins in the acetone extract, the ethanol extract had flavonoids, reducing sugar, steroids and terpenoids, while the aqueous extract tested only for the presence of tannins (Table I).

Figure 1 shows the inhibitory activity of the aqueous, acetone and ethanol extract of *G. arboreum* at different concentration on α-amylase activity. All the extracts were found to be α-amylase inhibitors in a dose-dependent manner and the percentage inhibition of each extract at each concentration were not significantly ($p>0.05$) different from one another. However the IC_{50} values (Table II) revealed that the aqueous extract had the lowest IC_{50} (10.1 mg/mL) compared to the acetone and ethanol extract, thus having the highest α-amylase inhibitory activity.

To clarify the activity of the extract that showed potent α-amylase inhibition, the Lineweaver-burk plot was made from the aqueous extract inhibitory activity (Figure 2). The plot revealed that the extract inhibited α -amylase activity in a non-competitive manner.

The ability of the aqueous, ethanol and acetone leaf extract to inhibit α-glucosidase activity was investigated. The inhibitory effect at different concentrations (0.6-5.0 mg/mL) was found to be dose-dependent and not significantly different ($p<0.05$) except for the ethanolic extract at concentration of 5.0 mg/mL which was significantly different ($p<0.05$) from the acetone and water extracts (Figure 3).

The IC_{50} (extract concentration causing 50% enzyme inhibition) values of the extract against α-glucosidase activity was determined (Table II). Acetone extract had the lowest IC_{50} (2.8 mg/mL) compared to other extract indicating higher α-glucosidase effective concentration. Kinetic studies were performed to determine the mode of inhibition by Lineweaver-burk plot, using the most potent (extract with the lowest IC_{50}) inhibitor. This showed a non-competitive inhibitory activity against α-glucosidase.

Discussion

Diabetes mellitus is a progressive metabolic disorder affecting majority of the population across the world. It is associated with hyperglycemia, a condition characterized by abnormal postprandial increase of blood glucose. Its management is critical and various measures have been used to manage this condition which includes lowering the blood glucose level with

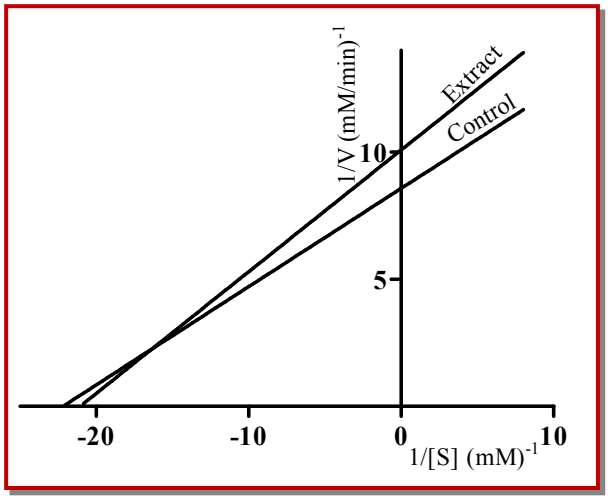

Figure 2: Mode of inhibition of α-amylase by aqueous extract of *G. aeboretum*

insulin and other oral hypoglycemic agents like sulfonylureas and biguanides (Schwab and Diem, 2009).

Alpha-amylase and alpha-glucosidase inhibitors delay the action of α-amylase and α-glucosidase to digest carbohydrate and prolong overall carbohydrate digestion time thereby lowering the absorption of glucose and consequently reducing the postprandial plasma glucose. This has been used as an oral hypoglycemic drug especially in patient with type 2 diabetes mellitus (Oboh et al., 2012). In individuals with normal or impaired glucose tolerance with hyperinsulinemia, these inhibitors decrease this condition and improve insulin sensitivity (Lebovittz, 2010).

α-Glucosidase inhibitors like acarbose, miglitol and voglibose are used in conjunction with other antidiabetic drugs, but these inhibitors have been found to possess side effects like flatulence and diarrhoea (Oboh et al., 2012). As a result of this, researchers have grown interest in discovering new and effective α-amylase and α-glucosidase inhibitors with minimal side effects from medicinal plant with known and scientifically proven antidiabetic properties (Onal et al., 2005; Kwon et al., 2008; Shai et al., 2010).

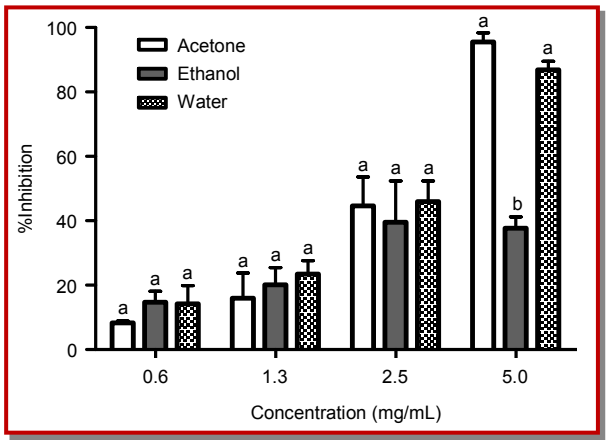

Figure 3: α-Glucosidase inhibitory activity of *G. arboreum* leaf extract

Values represent mean ± SEM of triplicate experiments. Bars with different superscripts at the same concentration are significantly ($p<0.05$) different

The analysis of the α-amylase inhibitory activity of the extracts showed there was no significant difference in the percentage inhibition of all the extracts at the same concentration. This could be an indication that the α-amylase inhibitory phytoconstituent(s) is/are soluble in the extracting solvents. Earlier report has shown that certain active ingredients from plant origin are extracted almost at the same rate by different solvents (Eloff, 1998). The results from the present study also showed that the inhibitions were mild culminating in high IC_{50} values. This is in agreement with earlier reports that showed that plant phytochemicals are mild inhibitors of α-amylase (Kwon et al., 2007). Though, the IC_{50} values suggested that the aqueous and acetone extracts were potent inhibitors of α-amylase. These solvents have previously been reported to possess the capability of extracting a near full complement of both polar and non-polar compounds (Eloff, 1998) in plant materials.

The mode of inhibition of α-amylase by the aqueous extract was determined using Lineweaver-burke plot revealed non-competitive inhibition exhibited by this extract which may suggest that the active component of the extract binds to a site other than the active site of the enzyme and combine with either free enzyme or enzyme-substrate complex possibly interfering with the action of both (Mayur et al., 2010).

The inhibition of α-glucosidase was in a dose-dependent manner and that the percentage inhibition of ethanol extract at 5.0 mg/mL was significantly different ($p<0.05$) having shown potency lesser than that of acetone and aqueous extract with higher inhibitory effect at the same concentration. The high inhibition displayed by acetone and aqueous extracts of this plant is in agreement with earlier reports which showed that plant phytochemicals are strong inhibitors of α-glucosidase (Kwon et al., 2007).

However, the extract with the lowest IC_{50} values is acetone (2.8 mg/mL) and so was the most potent inhibitor of α-glucosidase. The non-competitive mode of inhibition revealed by the Lineweaver-Burke plot of the acetone extract also suggests that the active component in the acetone extract does not compete with the substrate for its binding site rather; it binds to a separate site on the enzyme to retard the conversion of substrate to product (Shai et al., 2010).

The inhibitory effect of aqueous and acetone extract of

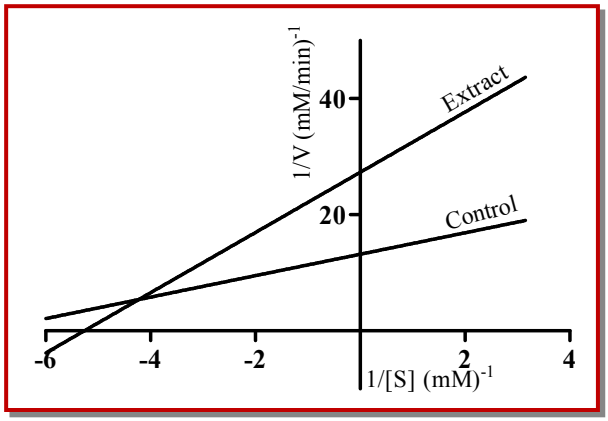

Figure 4: Mode of inhibition of α-glucosidase by acetone extract of *G. arboreum*

Values represent mean ± SEM of triplicate experiments. Bars with different superscripts at the same concentration are significantly ($p<0.05$) different

G. arboreum on α-amylase and α-glucosidase respectively could be as a result of the phytochemicals present especially tannins. Tannins are naturally occurring polyphenolics found in many plants and foods such as fruits and legumes. They possess antioxidant and free-radical scavenging properties as well as anticarcinogenic effect (Min et al., 2008). It has also been used traditionally as astringent, antibacterial, and anti-enzymatic agents. Recent studies also indicated that tannins may have health benefits on Alzheimer and diabetes (Ono et al., 2004). Tannins induced phosphorrylation of the insulin receptors as well as translocation of glucose transporters 4, the protein factor involved in the signaling pathway of insulin-mediated glucose transport and further demonstrated the inhibition of the expression of key gene for adipogenesis thereby helping to reduce blood glucose level without increase adiposity (Liu et al., 2005).

Conclusion

The aqueous and acetone extract of *G. arboreum* exhibited most effective α-amylase and α-glucosidase inhibition respectively. It may also be suggested that these inhibitory activities may be attributed to the presence of phytochemicals such as tannins in this extracts.

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