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Inhibitory Effect of *Azadirachta indica* A. Juss Leaf Extract on the Activities of α-Amylase and α-Glucosidase

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Abstract: In recent decades, there has been a drastic increase in the incidence and prevalence of diabetic mellitus. The aim of this study was to evaluate the *in vitro* inhibitory effect of *Azadirachita indica* leaf extract on the activity of α-amylase and α-glucosidase as a means of alleviating hyperglycemia and managing diabetes mellitus. Aqueous extract of *Azadirachita indica* exhibited most potent α-amylase inhibitory activity with IC_{50} value of 9.15 mg mL^{-1} and acetone extract exhibited most potent α-glucosidase inhibitory activity with IC_{50} value of 5.00 mg mL^{-1}. Kinetic studies revealed both acetone and aqueous extract to exhibit mixed non-competitive inhibition for α-amylase and α-glucosidase. It can be concluded that the antidiabetic potential of *Azadirachta indica* may be due to its ability to inhibit both α-amylase and α-glucosidase. The presence of phytochemicals such as flavonoids, tannins and saponins in this plant may be responsible for its inhibitory activity on the two enzymes studied.

Key words: Azadirachta indica, diabetes mellitus, α-amylase, α-glucosidase, flavonoids

INTRODUCTION

Diabetes is a metabolic disorder in which insulin action is impaired or absolute insulin deficiency results in imbalance of glucose metabolism and leads to a syndrome characterized by hyperglycemia, called diabetes mellitus (Klein *et al*., 2007). It has been estimated that the world's population is in the midst of a diabetes epidemic affecting all categories of people in all continents. The number of cases of diabetes currently stand at 171 million and has been predicted to reach 366 million by 2030 (International Diabetes Federation, 2009).

Some enzymes are implicated in the onset of this disease and this includes α-amylase and α-glucosidase. Pancreatic α-amylase (E.C. 3.2.1.1) is a major enzyme in the digestive system which catalyses the initial step in the breakdown of starch to a mixture of oligosaccharides and disaccharides. α-glucosidases, on the other hand, degrade disaccharides and oligosaccharides to glucose which on absorption enters the blood-stream. Degradation of these complex carbohydrates proceeds rapidly and results to elevated postprandial hyperglycemia (Dholi *et al*., 2011).

Synthetic oral antidiabetic agents such as acarbose and miglitol elicit their effects through the inhibition of one or more of these enzymes but their administration is associated with serious side effects and failure to alleviate diabetic complications (Cheng and Fantus, 2005). Consequent upon this, several studies had been conducted on the antidiabetic potential of medicinal plants in order to find safe natural drug for the treatment of diabetes mellitus (Grover *et al*., 2002; Mukherjee *et al*., 2006). These plants include *Adansonia digitata*, *Catharanthus roseus*, *Momordica charantia*, *Murraya koenigii*, *Ocimum gratissimum*, *Piper nigrum* and *Azadirachta indica* (Bhat *et al*., 2009; Sudha *et al*., 2011).

Azadirachta indica commonly called neem and 'dongoyaro' in Nigeria is well known as one of the most versatile medicinal plants having a wide spectrum of biological activities (Siddiqui *et al*., 2004). This plant is useful in the treatment of many ailments such as malaria, neuromuscular pains, skin infections, diabetes, cancer and heart disease. Its antidiabetic potential has been validated scientifically in diabetic rats after its administration for two weeks (Sudha *et al*., 2011).

Previous studies had shown the antidiabetic potential of *Azadirachta indica* but there is dearth of information on the mechanism of action of this plant. Therefore, the aim of this study was to evaluate the inhibitory potential of *Azadirachta indica* on the activities of α-amylase and α-glucosidase, as a possible mechanism of action of this plant in the treatment of diabetes.

MATERIALS AND METHODS

Plant material: The leaf of *Azadirachta indica* was obtained from Badagry Area of Lagos in Nigeria in July

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2012. It was identified and authenticated by Dr. A. B. Kadiri of the Department of Botany, University of Lagos, Nigeria and voucher specimen (LUH 4723) was deposited in the University herbarium.

Chemicals and reagents: Alpha-amylase from *Aspergillus oryzae*, α-glucosidase from *Saccharomyces cerevisiae* and paranitrophenyl-glucopyranoside 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 *Azadirachta indica* were cut and washed with water to remove all contaminants; they were dried under room temperature and grounded to powder. The powdered leaves were divided into three portions and each portion was extracted with acetone, ethanol or water. They were all left to steep in covered containers for 24 h; the resulting infusions were decanted, filtered and evaporated in a rotary evaporator (Cole Parmer SB 1100, Shangai, China). The extracts were freeze-dried using Virtis Bench Top (SP Scientific Series, USA) freeze dryer. Dried extracts were weighed and dissolved in 10% dimethylsulphoxide to yield a stock solution from which lower concentrations were prepared.

Phytochemical screening: Phytochemical compositions of the leaves were determined using the methods variously described by Trease and Evans (1996) and Sofowara (2006).

Alpha-Amylase inhibitory assay: This assay was carried using a modified procedure of McCue and Shetty (2004). A total of 250 μL of extract was placed in 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 then further incubated at 25°C for 10 min. The reaction was terminated 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 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:

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extracts}}}{\text{Abs}_{\text{control}}} \times 100$$

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 the 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^{-1}) 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 a-amylase was pre-incubated with 250 μL of phosphate buffer (pH 6.9). 250 μL of starch solution at increasing concentrations (0.30-5.0 mg mL^{-1}) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10 min at 25°C and then 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 α-amylase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot using Michaelis-Menten kinetics.

Alpha-glucosidase inhibitory assay: The effect of the plant extracts on α-glucosidase activity was determined according to the method described by Kim *et al*. (2005), using α-glucosidase from *Saccharomyces cerevisiae*. The substrate solution p-nitropheynyl glucopyranoside (pNPG) was prepared in 20 mM phosphate buffer, pH 6.9. 100 μL of α-glucosidase (E.C. 3.2.1.20) was pre-incubated with 50 μL of the different concentrations of the extracts (acetone, ethanol and water) for 10 min. Then 50 μL of 3.0 mM (pNPG) 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 activity was determined by measuring the yellow colored para-nitrophenol released from pNPG at 405 nm. The results were expressed as percentage of the blank control.

Percentage inhibition calculated as:

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extract}}}{\text{Abs}_{\text{control}}} \times 100$$

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 extracts was determined 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^{-1}) 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 a-glucosidase was pre-incubated with 50 μL of phosphate buffer (pH 6.9). Fifty microlitter of PNPG at increasing concentrations (0.63-2.0 mg mL^{-1}) 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 type (mode) of inhibition of the crude extract on α-glucosidase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot using Michaelis-Menten kinetics.

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

RESULTS AND DISCUSSION

The management of blood glucose level is an important strategy in the control of diabetes and its complications. The use of inhibitors of saccharide hydrolyzing enzymes (α-amylase and α-glucosidase) as oral hypoglycemic drugs has been of great benefit for the regulation and management of hyperglycemia especially in patients with type 2 diabetes mellitus (Bhat *et al*., 2009). Inhibition of these enzymes delay and in some cases halt carbohydrate digestion thus prolonging overall carbohydrate digestion time causing a reduction in the rate of glucose absorption and consequently reducing postprandial plasma glucose rise (Tarling *et al*., 2008).

Table 1 showed the percentage yield of different extracts of *Azadirachta indica* leaf after extraction with acetone, ethanol or water. The result showed that aqueous extract had the highest yield (4.35%). The result of phytochemical analysis of the three extracts of *Azadirachta indica* is shown in Table 2. The presence of flavonoids is common to all the extracts while tannin was present in both acetone and aqueous extract alone.

Table 1: Percentage yield of different extracts of *Azadirachta indica*

Extracts	Initial weight	Final weight	Yield (%)
Acetone	54.17	1.99	3.67
Ethanol	67.46	1.52	2.25
Water	61.86	2.69	4.35

Table 2: Phytochemical composition of different extract of *Azadirachta indica*

	Extracts inference		
Phytochemicals	Acetone	Ethanol	Water
Anthraquinones	-	-	-
Flavonoids	+	+	+
Reducing sugar	-	+	+
Saponins	-	+	+
Steroids	+	+	-
Tannins	+	-	+
Terpenoids	-	-	-

+: Present, -: Not detected

Table 3: IC_{50} values of various extracts of *A. indica* against α-amylase and α-glucosidase

	IC_{50} (mg mL^{-1})	
Extracts	α-amylase	α-glucosidase
Acetone	10.62±0.20	5.00±0.12
Ethanol	9.50±0.32	8.70±0.24
Water	9.15±0.25	7.25±0.28

Values are Mean±SEM of three determinations

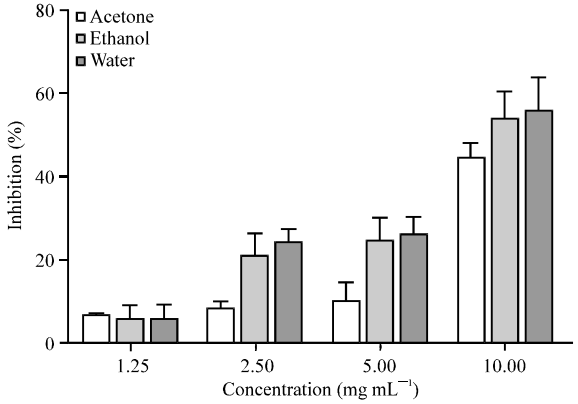

Fig. 1: Percentage inhibition of α-amylase by different extracts of *Azadirachta indica*

At each concentration tested, there were no significant differences ($p>0.05$) among the three extracts in the inhibition of α-amylase activity (Fig. 1). However, all the extracts inhibited the enzyme in a dose-dependent manner. The IC_{50} value calculated depicted that aqueous extract had the lowest IC_{50} value of 9.15 mg mL^{-1} (Table 3). This result implies that the active components (phytochemicals) responsible for the inhibitory activity of α-amylase by the extracts are mostly soluble in aqueous medium. This is in agreement with a recent work reported by Oboh *et al.* (2010) where red and white ginger inhibited α-amylase activity *in vitro*.

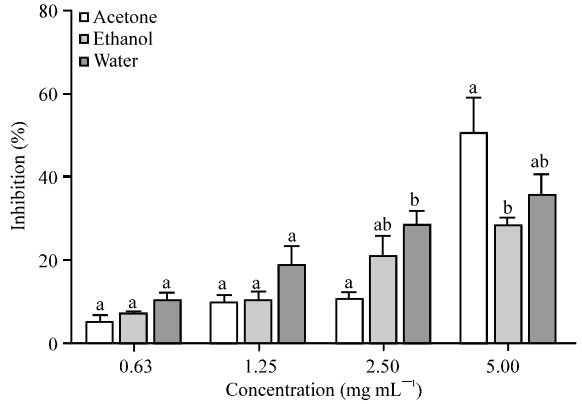

Fig. 2: Percentage inhibition of α-glucosidase by different extracts of *Azadirachta indica*, Bars carrying the different letters at the same concentration are significantly different

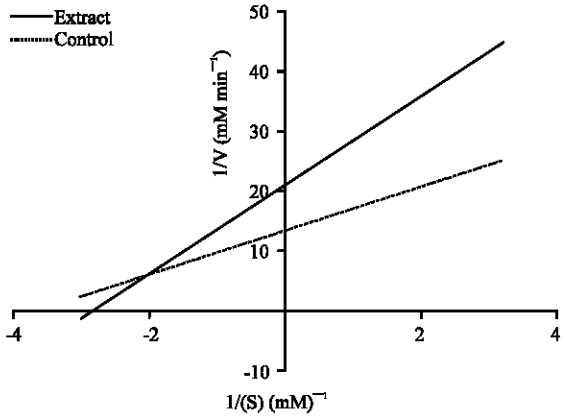

Fig. 3: Mode of inhibition of α-amylase by aqueous extract of *Azadirachta indica*

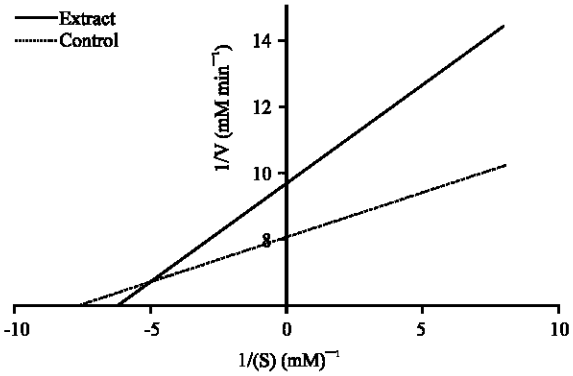

Fig. 4: Mode of inhibition of α-glucosidase by acetone extract of *Azadirachta indica*

Figure 2 showed the inhibition of α-glucosidase by acetone, ethanol and aqueous extracts of *Azadirachta indica*. At lower concentrations (0.63-1.25 mg mL^{-1}), there were no significant differences ($p>0.05$) in the inhibition among all the extracts. However, there were significant differences ($p<0.05$) in this enzyme inhibition among all the extracts at higher concentrations. It is worthy of note that acetone displayed the highest percentage inhibition and this attested to by the IC_{50} value which was the lowest (5.00 mg mL^{-1}) when compared to other extracts (Table 3). The lowest IC_{50} exhibited by acetone extract suggests that the active constituent inhibiting the enzyme is most soluble in acetone (Eloff, 1998). The inhibition of α-glucosidase was found to be strong as evidenced in the low IC_{50} value. This is in agreement with earlier reports that showed that plant phytochemicals are strong inhibitors of α-glucosidase and mild inhibitors of α-amylase (Kwon *et al*., 2007).

In order to determine the mode of inhibition of these enzymes by aqueous and acetone extracts of *Azadirachta indica*, Lineweaver-Burke plot was made. This plot showed that both aqueous and acetone extracts of this plant inhibited α-amylase and α-glucosidase, respectively in a mixed non-competitive manner (Fig. 3, 4). The non-competitive inhibition exhibited by this extract suggests that the active component of the extract binds to a site other than the active site of the enzymes and combine with either free enzymes or enzyme-substrate complex possibly interfering with the action of both (Mayur *et al*., 2010). This also implies that the extracts had different affinities for both the free enzymes and the enzyme-substrate complexes resulting in mixed non-competitive inhibition.

The inhibitory effect of both the aqueous and acetone extract of *Azadirachta indica* on the activities of α-amylase and α-glucosidase may not be unconnected to the presence of phytochemicals such as flavonoids and tannins in the extracts. Flavonoids is one of the most diverse and widespread group of natural compounds, having hydroxyl group present in it which confers scavenging ability and also plays an important role in preventing lipid peroxidation (Mayur *et al*., 2010). Tannin which is composed of a central glucose molecule derivatized at its hydroxyl groups with one or more galloyl residue and in the presence of copper ions act as an antioxidant suppressing hydroxyl radical formation (Andrade *et al*., 2005). Both flavonoid and tannin may preserve beta-cell function by reducing oxidative stress-induced tissue damage and therefore protect against the progression of insulin resistance or insulin dysfunction in diabetes (Song *et al*., 2005).

CONCLUSION

It can be concluded that aqueous and acetone extracts of *Azadirachta indica* leaf inhibited the activities of α-amylase and α-glucosidase, respectively and that the mode of inhibition of these enzymes by the extracts is mixed non-competitive inhibition. Therefore, part of the mechanism by which *Azadirachta indica* produced its antidiabetic effect is by inhibiting α-amylase and α-glucosidase activities. The inhibitory effect of these extracts on the enzymes studied may be attributed to the presence of phytochemicals such as flavonoids and tannins in these plant extracts. Further study is required to isolate the specific compound that may be responsible for this effect.

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