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Alpha-amylase Inhibition and Brine Shrimp Lethality Activities of Nine Medicinal Plant Extracts from South-West Nigerian Ethnomedicine

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ABSTRACT

Acanthospermum hispidum, *Alchornea laxiflora*, *Calyptrochilum christyanum*, *Heliotropium indicum*, *Holarrhena floribunda*, *Ipomoea asarifolia*, *Nauclea diderrichii*, *Piper guineense*, and *Tetracera scandens* were extracted into methanol and screened for the availability of alpha-amylase inhibitors. Plant extracts' toxicity was determined by brine shrimp lethality assay (BSLA). The most active plant, *Nauclea diderrichii*, was fractionated into four solvent systems (*n*-hexane fractions, ethyl acetate, dichloromethane, and *n*-butanol) and tested. All the extracts showed varying degrees of alpha-amylase inhibitory activity. *N. diderrichii* with $IC_{50} = 248.30 \pm 0.27 \mu\text{g.mL}^{-1}$ had the highest inhibitory activity, while its butanol fraction with $IC_{50} = 137.8 \mu\text{g.mL}^{-1}$ had activity comparable to the standard α -amylase inhibitor acarbose ($IC_{50} = 177.50 \pm 0.42 \mu\text{g.mL}^{-1}$). LC_{50} values for BSLA ranged from 11.35 to 1,127.50 $\mu\text{g.mL}^{-1}$, with *T. scandens* being the most toxic with 11.35 $\mu\text{g.mL}^{-1}$ and *N. diderrichii* the least toxic with 1,127.50 $\mu\text{g.mL}^{-1}$.

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Introduction

Health management by use of traditional herbal medicine with minimum side effects is gaining importance in the treatment of chronic disorders (2). More than 80% of the world's population rely on traditional medicine for their primary health care (9). Diabetes mellitus is a group of metabolic disorders characterized by high blood glucose (hyperglycemia) resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, and heart (5). According to the World Health Organization (WHO), the number of affected people in the world has doubled in the past few years, and it is expected to double again by the year 2025. Globally, in 2012 and 2013, diabetes resulted in 1.5–5.1 million deaths per year,

making it the eighth leading cause of death (10). Nigeria has the highest number of people with diabetes in Africa, with over 1.2 million diabetics (11, 22).

An effective approach to controlling diabetes is to inhibit the activity of alpha-amylase, an enzyme responsible for the breakdown of starch to simpler sugars such as dextrin, maltotriose, maltose, and glucose (3). Alpha-amylase catalyzes the hydrolysis of α -1,4-glucosidic and α -1,6-glucosidic bonds of polysaccharides, starch, and glycogen into oligosaccharides and thereby liberates α -maltose, α -glucose, and α -limit dextrin (12). Inhibition carried out by alpha-amylase inhibitors delays carbohydrate hydrolysis rate and maintains the serum blood glucose in hyperglycemic individuals (6).

The importance of these enzymes inhibitors has attracted interest because inhibition of the catalytic activity of α -amylase leads to retardation of the absorption of glucose and the reduction of postprandial blood glucose level. In addition, secondary metabolites that inhibit alpha-amylase play a key role in plant defense against insect pests. Some inhibitors currently in clinical use are acarbose and miglitol, which inhibit glycosidases such as α -amylase and α -glucosidase. However, many of these synthetic agents have limitations, which include non-specificity, failure to alleviate diabetic complications, and numerous side effects such as bloating, abdominal discomfort, diarrhea, and flatulence (9). The fact that the inhibitors of this enzyme are not readily available in the pharmaceutical industries underscores the continuous search for inhibitors from plants (14).

Plants have been used since antiquity as source of drugs due to their high efficacy, reduced cost, and minimal side effects, and also their safety (8). The brine shrimp lethality (BSL) assay is a simple and inexpensive bioassay developed for preliminary toxicity screening of plant extracts and compounds, although complementing tests may also be needed before drawing safety conclusions. The eggs of *Artemia salina* (brine shrimp) have been used in the BSL assay, and have yielded good results similar to the mammalian type (13, 20).

In this study, selected plant species used in South-West Nigerian ethnomedicine, *Acanthospermum hispidum*, *Alchornea laxiflora*, *Calypetrochilum christyanum*, *Heliotropicum indicum*, *Holarrhena floribunda*, *Ipomoea asarifolia*, *Nauclea diderrichii*, *Piper guineense*, and *Tetracera scandens*, were extracted into methanol and screened for the availability of alpha-amylase inhibitors. Plant extracts' toxicity was determined by brine shrimp lethality assay (BSLA).

Materials and methods

Chemicals and reagents

Alpha (α)-amylase (*Aspergillus oryzae*), sodium potassium tartrate, 3,5-dinitrosalicylic acid, potato starch, maltose, and sodium hydroxide pellets were

purchased from Sigma Aldrich USA, USA; acarbose was from (Serva Heidelberg, Germany). All other chemicals or reagents were of analytical grade.

Plant collection and authentication

Acanthospermum hispidum, *Alchornea laxiflora*, *Calyptrorchilum christyanum*, *Heliotropicum indicum*, *Holarrhena floribunda*, *Ipomoea asarifolia*, *Nauclea diderrichii*, *Piper guineense*, and *Tetracera scanden* were collected in November 2013 in South-West Nigeria, identified, and authenticated at Forest Herbarium Ibadan (FHI), Forestry Research Institute of Nigeria (FRIN), Ibadan, and voucher specimen numbers were deposited. The plant materials were air-dried for 3 weeks and milled into coarse powder.

Plant extraction

A known quantity (150–400 g) of each plant material was macerated with distilled methanol at room temperature (29°C) for 72 h. After removal of methanol *in vacuo* using a rotary evaporator, percentage yields were calculated and plant extracts were stored in the refrigerator (4°C) until needed. Thereafter, the crude methanol extract of the most active plant was subjected to liquid–liquid partitioning into hexane, dichloromethane, ethyl acetate, and butanol.

In vitro alpha-amylase inhibitory assay (21)

Plant extracts at 31.25–1,000 $\mu\text{g.mL}^{-1}$ range were prepared, 250 μL of each extract were preincubated with equal volume of α -amylase solution (50 $\mu\text{g.mL}^{-1}$ in 0.02 M sodium phosphate buffer, pH 6.9) at 25°C for 10 min. Thereafter, 250 μL of 1% starch solution was added and then incubated at 25°C for 10 min. The reaction was terminated by adding 500 μL of dinitrosalicylic acid (DNS) reagent (96 mM 3,5-dinitrosalicylic acid and 5.31 M sodium potassium tartrate in 2M sodium hydroxide). Tubes were then incubated in a water bath at 80°C for 15 min. The reaction mixture was diluted with 5 mL distilled water and the absorbance was determined at 540 nm (SPECTRA max PLUX, Analytik and Biotechnologie, Germany). An individual blank (containing 1 mL of buffer and 1 mL of 3,5-dinitrosalicylic acid solution) was prepared for correcting the background absorbance. Negative control experiments representative of 100% enzyme activity were prepared, replacing the extract with distilled water. The α -amylase inhibitory activity was calculated as percentage inhibition. Acarbose, a well-known α -amylase inhibitor, was used as positive control. The same process was repeated for the standard (maltose) at 2.0–0.05 $\mu\text{g.mL}^{-1}$ and the calibration

curve was constructed. The amount of maltose generated from the tests was extrapolated from the equation of the maltose standard curve. All measurements were carried out in triplicate. The inhibition percentage of α -amylase activity was evaluated by the following formula:

$$\text{Percent inhibition} = 100 - (\text{absorbance of test}) / (\text{absorbance of control}) \times 100$$

The net absorbance of each sample was calculated using the equation

$$A_{\text{net}} = A_{\text{sample}} - A_{\text{blank}}$$

where A_{sample} = absorbance of test sample; A_{blank} = absorbance of corresponding blank.

Brine shrimp lethality assay (13)

Brine shrimp eggs (*A. salina*) were obtained from the Department of Pharmacognosy, University of Ibadan, Ibadan, in Oyo State, Nigeria. They were hatched in a tank containing natural seawater from the Bar Beach, Lagos, Nigeria. After 48 h incubation at room temperature and under illumination, the resulting nauplii (larvae) were attracted to one side of the tank with a light source and collected with a Pasteur pipette. Ten brine shrimp (nauplii) were transferred into each extract at selected concentrations (five dilutions, $1.6\text{--}5,000\ \mu\text{g.mL}^{-1}$) in 96-well microplates. Cyclophosphamide (a reference cytotoxicity drug) was used as positive control, and 3% methanol was used as negative control. Measurements were carried out in triplicate. Crude extracts resulting in LC_{50} values of less than $100\ \mu\text{g.mL}^{-1}$ were considered toxic (19).

Statistical analysis

All experiments were carried out in triplicate and the data expressed as mean $\pm$ SEM. The plant extract/drug concentrations were plotted against percentage inhibitory activity of plant extract or standard drug. Fifty percent (50%) inhibitory concentration of plant extract/standard drug (IC_{50}) was determined using nonlinear regression in a commercially available statistical package (Microcal Origin). Similarly, The 50% lethal concentration (LC_{50} value) at 95% confidence interval was calculated for each plant extract in the BSLA.

Results

The percent yield of methanol extracts varied with the plant species used in the extraction (Table 1). The *in vitro* α -amylase inhibitory activity for all the extracts was higher than the standard acarbose and was the highest for *P. guineense* (Table 2). The inhibitory activities expressed as IC_{50} values ($\mu\text{g.mL}^{-1}$) of the solvent fractions of *N. diderrichii* were compared to the standard acarbose

Table 1. Details of plant species selected for the study and the percent yield of extract in methanol.

Plant	FHI ^a No.	Family	Local name (Y ^b)	Part used	Percent yield
<i>Acanthospermum hispidum</i>	110050	Asteraceae	<i>Dagunro gogoro</i>	Aerial part	6.35
<i>Alchornea laxiflora</i>	110155	Euphorbiaceae	<i>Pepe</i>	Leaf	13.44
<i>Calyptrorchilum christyanum</i>	110054	Orchidaceae	<i>Ela</i>	Whole plant	2.17
<i>Heliotropicum indicum</i>	110156	Boraginaceae	<i>Apari igun</i>	Aerial part	5.66
<i>Holarrhena floribunda</i>	110053	Apocynaceae	<i>Dagba</i>	Leaf	3.46
<i>Ipomoea asarifolia</i>	110052	Convolvulaceae	<i>Gboro ayaba</i>	Leaf	6.72
<i>Piper guineense</i>	110051	Piperaceae	<i>Iyere</i>	Leaf	6.43
<i>Tetracera scandens</i>	—	Dilleniaceae	<i>Opon</i>	Aerial part	3.82
<i>Nauclea diderrichii</i>	110049	Rubiaceae	<i>Opepe</i>	Stem bark	3.64

^aForest Herbarium Ibadan number.^bYoruba name.

(Table 3). The extracts exhibited varying degrees of IC₅₀ values, from 248.30 ± 0.27 µg.mL⁻¹ for *N. diderrichii* to >1,000 µg.mL⁻¹ for *A. hispidum*. The α-amylase inhibitory activity of the methanol extract of *N. diderrichii* was only slightly lower than that of acarbose, a known inhibitor of α-amylase with IC₅₀ of 177.50 ± 0.42 µg.mL⁻¹ (Table 2). *H. floribunda* with IC₅₀ of 249.10 ± 0.24 µg.mL⁻¹ had activity very similar to *N. diderrichii*.

Partitioning of the most active methanol extract of *N. diderrichii* into four fractions using solvents of varying polarities resulted in an *n*-butanol fraction with activity greater than acarbose with IC₅₀ value of 137.8 ± 0.37 µg.mL⁻¹; the *n*-hexane fraction had activity comparable to acarbose (188.3 ± 0.34 µg.mL⁻¹); the ethyl acetate fraction's activity was similar to that of the crude extract (Table 3), while the dichloromethane fraction was the least active, with IC₅₀ > 500.

The results of the brine shrimp lethality (BSL assay) revealed that all the plant extracts were safe, with LC₅₀ > 1,000 µg.mL⁻¹, except for the extract of *T. scandens*, which appeared to be toxic or to contain cytotoxic components,

Table 2. Alpha-amylase inhibition and brine shrimp lethal activity (BSLA) of the selected plant extracts in methanol.

Plant extract	Alpha-amylase inhibitory activity, IC ₅₀ (µg.mL ⁻¹ , <i>n</i> = 3)	BSLA, LC ₅₀ (µg.mL ⁻¹ , <i>n</i> = 3)
<i>Acanthospermum hispidum</i>	>1,000	183.70
<i>Alchornea laxiflora</i>	295.60 ± 0.53	142.40
<i>Calyptrorchilum christyanum</i>	467.20 ± 0.32	294.80
<i>Heliotropicum indicum</i>	360.60 ± 0.30	391.30
<i>Holarrhena floribunda</i>	249.10 ± 0.24	595.10
<i>Ipomoea asarifolia</i>	420.70 ± 0.14	1,484
<i>Nauclea diderrichii</i>	248.30 ± 0.27	1,127.50
<i>Piper guineense</i>	580.00 ± 0.35	285.50
<i>Tetracera scandens</i>	273.20 ± 0.11	11.35
Acarbose	177.50 ± 0.42	—
Cyclophosphamide	—	16.3

n = no. of replicates.

Table 3. Alpha-amylase inhibitory activities of solvent extract fractions by liquid–liquid partitioning from the methanol extracts of *Nauclea diderrichii*.

S/N	Solvent	IC ₅₀ (μg.mL ⁻¹ , n = 3)	Percent yield*
1	Butanol	137.8 ± 0.37	40
2	Ethyl acetate	212.8 ± 0.47	6.75
3	Dichloromethane	>500	8
4	Hexane	188.3 ± 0.34	5.25
5	Acarbose	177.5 ± 0.42	—

*From liquid–liquid partitioning of methanol extract of *Nauclea diderrichii*.

with LC₅₀ of 11.35 μg.mL⁻¹, which is 1.4 times more toxic than cyclophosphamide, the control drug, with LC₅₀ of 16.3 μg.mL⁻¹ (Table 2). However, the methanol extracts of *N. diderrichii* were 69 times less active than cyclophosphamide.

Discussion

Diabetes mellitus (DM) is a metabolic disorder of multiple etiology, characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism. One of the therapeutic approaches for treating diabetes mellitus is to decrease postprandial hyperglycemia. This could be achieved by inhibiting the activity of the enzymes (α-amylase and α-glucosidase) involved in the metabolism of carbohydrate, thereby reducing the glucose level in the blood. The present study explored South-West Nigerian ethnomedicine in the search of new, safe, and potent inhibitors of carbohydrate metabolism.

Traditional plant remedies or herbal formulations have been in existence since antiquity, and their use is on the increase despite huge controversies regarding their efficacy and/or safety (18). Reports indicate that plants produce a large variety of amylase inhibitors that may also be toxic in nature, to offer protection against insects, their larvae, and microbial pathogens (7, 16). Therefore, in this study, the cytotoxicity of the plant extracts was assessed in a brine shrimp lethality (BSL) assay in addition to assessing the α-amylase inhibitory activity.

N. diderrichii is a subtropical or tropical tree known as *Opepe* in the Yoruba language in Nigeria. It is highly resistant to fungi and insects, and its resistance to termites makes it valuable in furniture, art, building, and construction industries in West Africa (1). The ability to resist insect attack may be linked to the presence of enzyme inhibitors and vice versa. A large number of proteinaceous and nonproteinaceous molecules in plants act as part of plant defense mechanism against herbivores (17, 7); α-amylase inhibitors confer resistance against herbivore attack (14, 15).

Among the assayed extract fractions from *N. diderrichii*, the *n*-butanol fraction had the highest α-amylase inhibitory activity and the

dichloromethane fraction had the lowest activity (Table 3); *n*-hexane fraction had activity comparable to acarbose, while the ethyl acetate fraction's activity was similar to that of the crude extract. Thus, the compound primarily responsible for the α -amylase inhibition activity may reside mainly in the polar component of the extract. However, the nonpolar hexane fraction also displayed significant activity. It is also noteworthy that the butanol fraction had better activity than the standard drug, while activity of both the butanol and hexane fractions was better than that of the crude extract. Hence, it is possible that the inhibitory compound(s) may be present in both the hexane and butanol fractions of *N. diderichii*. Further work is necessary to isolate, purify, and identify the inhibitory compound(s) from both fractions using chromatography.

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