

Phytochemical Study and alpha-amylase Inhibitory properties of Leaf Extracts of *Croton zambesicus* (Müll Arg.)

ABSTRACT

Aims: This study was designed to determine the phytochemical contents, percentage of alkaloids and flavonoids and also to determine the alpha-amylase inhibitory properties of the n-hexane, ethyl acetate and ethanol extracts from the leaves of *Croton zambesicus* (Müll Arg.) *in-vitro*.

Study Design: Phytochemical Screening, determination of percentage alkaloids and flavonoids and *in-vitro* evaluation of alpha-amylase inhibitory activities of the leaf extracts.

Place and Duration: This work was carried out in the Chemistry Laboratory of the Department of Science Laboratory Technology (Chemistry Unit), School of Pure and Applied Science, Lagos State Polytechnic, Ikorodu and the Nigerian Institute of Medical Research, Yaba, Lagos, Nigeria, between March and November, 2016.

Methodology: *Croton zambesicus* leaves was successively extracted with n-hexane, ethyl acetate, and ethanol by maceration to obtain the respective extracts. Phytochemical screening of the extracts for the presence of alkaloid, tannin, cardiac glycosides, steroids, saponins, flavanoids, anthraquinones and reducing sugar was carried out using standard method. The percentage of alkaloids and flavonoids was determined using Harborne and Boham and Kocipaiabyazan methods respectively. Alpha-amylase inhibitory properties of the leaf extracts was carried *in-vitro* qualitatively by starch-iodine colour assay and then quantified using dinitrosalicylic acid reagent (DNSA reagent) using acarbose as the standard.

Results: The results showed alkaloids, cardiac glycosides steroids and antraquinone derivatives were detected in all the extracts while tannins, saponins and flavonoids were detected only in the ethanol extract. The percentage of alkaloids and flavonoids in the *Croton zambesicus* leaf are $16.67 \pm 3.06\%$ and $2.67 \pm 1.16\%$ respectively. Starch iodide indicator indicated a positive Alpha-amylase inhibitory properties in the hexane and the ethanol extracts. The ethanol extract exhibited the most alpha-amylase inhibitory property ($IC_{50} = 78.7 \pm 1.7$) followed by the hexane extract ($IC_{50} = 89.2 \pm 1.1$) while the lowest was observed in the standard drug, acarbose ($IC_{50} = 114.9 \pm 11.3$).

Conclusion: The result of this study showed *Croton zambesicus* leaf extracts can effectively inhibit pancreatic alpha-amylase hence could be a source of new drugs for reducing post-prandial glucose level in diabetic patients.

Keywords: Phytochemical contents, alkaloids, flavonoids, alpha-amylase inhibitory properties, *Croton zambesicus*.

1. INTRODUCTION

Diabetes mellitus has become a global problem and the number of diabetics and the incidence of complications associated with the disease is on the increase every year [1,2]. This has necessitated the need for more research into medicinal plants which are used to manage the disease traditional. One of the methods employed in the management of Diabetes mellitus is to inhibit the activities of digestive enzymes which results in the reduction of post-prandial glucose level [3]. Pancreatic alpha-amylase (E.C.3.2.1.1) is one of the major digestive enzymes responsible for the metabolic breakdown of starch into simple sugars.

Croton zambesicus (Euphorbiaceae) is a common plant grown in most villages and towns in Nigeria especially for its medicinal properties. It is known as "bushveld" in english and among the "Yorubas", in the south-west part of Nigeria, It is known as "àṣẹ kòbàlẹ" [4,5]. In folklore medicine, several uses had been attributed to this plant: the leaf decoction is used for the treatment of hypertension, diabetics, urinary tract infections, malaria, gonorrhea arthritis, and impotent [6]. The antimicrobial and

antiplasmodial properties of the crude leaf and stem extracts was documented [7]. One of the traditional use of *Croton zambesicus* is to treat diabetes mellitus. The effects of the ethanolic leaf extract on streptozotocin-induced diabetic rats had been reported [8,9] however the alpha-amylase inhibitory property of this plant had not been investigated.

This work was aimed at determining the phytochemical content and the alpha-amylase inhibitory properties of different leaf extracts of *Croton zambesicus*.

2. MATERIALS AND METHODS

2.1 Plant Collection and Identification

The fresh leaves of *Croton zambesicus* was collected in March 2016 at a garden in Ajegunle ita-oluwo, Ikorodu Local government area of Lagos State, Nigeria. It was identified and authenticated at the University of Lagos Herbarium (LUH 6959).

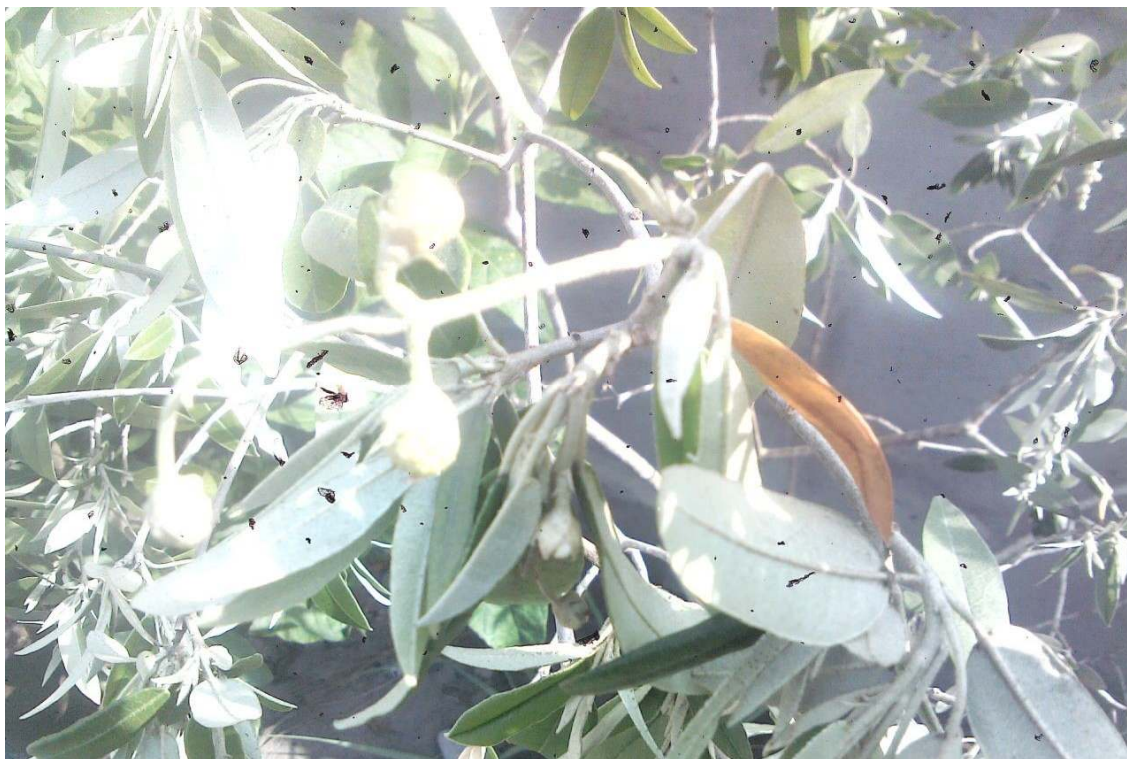


Fig.1 *Croton zambesicus*

2.2 Extraction of Plant Materials

The fresh leaves of *croton zambesicus* was dried under limited sun for three weeks. 100 g of the leaf was successively extracted by maceration using n-hexane, ethyl-acetate and ethanol (1.5 L each). The extracts were concentrated using a rotary evaporator (40 °C). This was further dried completely in an air oven at 40 °C.

2.3 Qualitative Phytochemical Screening

The various leave extracts were screened for the presence of alkaloids, tannins, cardiac glycoside, steroids, saponins, flavonoids, anthraquinones and reducing sugars using the simple and standard methods described by Evans and Sofowora [10, 11].

2.3.1 Alkaloids

72 About 0.5 g of extract was dissolved in 1% aqueous hydrochloric acid and boiled on a water bath. To
73 2 test tubes each containing 1 mL of the filtrate was added a few drops of Dragendoff's and Mayer's
74 reagent. The colour changes. reddish-brown precipitate (Dragendoff's reagent) and cream precipitate
75 (Mayer's reagent) confirms the presence of alkaloids.

76 **2.3.2 Tannins**

77 About 0.2 g of extract was stirred with 10 mL of distilled water, filtered and 15 % FeCl₃ (ferric chloride
78 reagent) was added. A deep blue or green colour indicated the presence of tannins.

79 **2.3.3 Cardiac glycosides**

80 Kedde's test for lactone ring in cardiac glycosides: 2 g of plant material was boiled with 20 mL of
81 water to obtain a water extract of the plant. The extract was concentrated to dryness and re-dissolved
82 in 10 mL of methanol. To 2 mL of this was added 1 mL of 2% of 3, 5-dinitrobenzoic acid solution in
83 methanol and 1 mL of 5.7% sodium hydroxide. The result was recorded after 5 mins.

84 Keller-Kiliani test for de-oxy sugars in cardiac glycosides: A methanol extract was obtained and the
85 extract reduced to dryness. 10 mg of this was dissolved in 1 mL chloroform. Tetraoxosulphate (VI)
86 acid was added to form a layer and the color at interphase was recorded.

87 **2.3.4 Steroidal Nucleus**

88 Liebermann-Burchard reaction for steroidal/triterpenoidal nucleus: 0.5 g of powdered sample was
89 extracted with 20 mL of methanol. The extract was filtered and the filtrate was gently concentrated to
90 dryness on a water bath. 0.2 mg of the dried extract was dissolved in 2 mL of acetic anhydride and
91 allowed to cool. With the test tube inside ice pack and slanted at an angle of about 45 deg. 2 mL
92 concentrated tetraoxosulphate (VI) acid was poured carefully by the side of the test tube.
93 Salkowski's test: 10 mg of the extract was dissolved in 2 mL of chloroform. Concentrated
94 tetraoxosulphate (VI) acid was added carefully to form a lower layer. A reddish-brown at the interface
95 indicated the presence of a steroidal ring (aglycone portion of the cardiac glycoside).

96 **2.3.5 Saponins**

97 Frothing test: About 0.2 g of the extract was shaken with water in a test tube. Frothing which persists
98 on warming indicates the presence of saponins.

99 **2.3.6 Flavonoids**

100 About 2.0 mL of dilute ammonia solution was added to a portion of the aqueous filtrate of each extract
101 followed by the addition of concentrated tetraoxosulphate (VI) acid. A yellow colouration observed in
102 each extract indicated the presence of flavonoids.

103 **2.3.7 Anthraquinone**

104 Bontrager's test: Chloroform extract of the powdered sample was obtained by boiling on a water bath.
105 To 2mL of the extract, 1 mL of dilute (10%) ammonia was added and the mixture was shaken.
106

107 **2.3.8 Reducing sugars**

108 Fehling's test: Water extract of the powdered material was obtained by boiling on a water bath. 2 mL
109 of the extract, in the test tube was added, 1 mL each of Fehling's solutions A and B. The mixture was
110 shaken and heated in a water bath for 10 mins. Reducing sugar was confirmed by the formation of a
111 brick-red precipitate.

112 **2.4 Quantitative Phytochemical Screening**

113 **2.4.1 Percentage Alkaloid**

114 5 g of the plant material was weighed and placed into a 250 mL beaker, 50 mL of 10% acetic acid in
115 ethanol was added to cover the plant material and this was allowed to stand for 4 h. This was then
116 filtered into a beaker and the extract was evaporated on water bath to one quarter of the original

volume. Concentrated Ammonium solution was added in drop wise to the extract until precipitation was complete. The whole solution was allowed to settle and precipitate for about 1 h. This was then washed with dilute ammonia solution and then filtered [12]. The residue (alkaloid) was dried and weighed. The procedure was carried out in triplicate to obtain three different results.

2.4.2 Percentage Flavonoids

5 g of the plant material was weighed into a beaker, 50 mL of 80% aqueous methanol was then added and plant-solvent mixture was left at room temperature for about 1hr. The whole mixture was filtered through Whatman filter paper No. 42 (125 mm). The filtrate was poured into a crucible and then evaporated to dryness over a water bath and weighed to a constant weight [13]. The procedure was carried out in triplicate to obtain three different results.

2.5 Alpha-amylase Inhibitory activities of the Crude extracts

Two types of assays are used to determine the inhibition of alpha-amylase by the extracts: the starch-iodide assay and the 3,5-dinitrosalicylic acid (DNSA) assay [14].

2.5.1 Starch- iodine color assay

The total assay mixture composed of 250 μ L of the plant extract in DMSO (concentration range 100-800 μ g/mL), with the addition of 250 μ L enzyme solution (prepared by dissolving IU/mL pancreatic alpha-amylase enzyme (E.C:3.2.1.1; Sigma-Aldrich), in 0.02 M Sodium phosphate buffer with 0.006 M sodium chloride, were incubated for 10 min at 37 °C. This was followed by the addition of 250 μ L of 1% soluble starch (potato starch) in all test tubes and incubated again for 10 mins at 37 °C. 1M HCl (hydrochloric acid) of 250 μ L was added to terminate the enzymatic reaction followed by the addition of 100 μ L of iodine reagent (5Mm of I₂ and 5Mm of KI). The colour change was noted and absorbance was taken at 620 nm (Thermo Scientific GENESY 10S UV/Visible spectrophotometer). The control samples reaction representing 100% enzyme activity did not contain any plant extract. To eliminate the absorbance produced by plant extract, appropriate extract control without the enzyme were also included. Acarbose was used as a positive control at a concentration range 100-800 μ g/mL. The results were expressed as percentage of enzyme activity against concentration and the percentage inhibition was calculated according to the formula [14].

2.5.1 DNSA reagent (3,5-dinitrosalicylic acid) Assay

The total assay mixture composed of 250 μ L of the plant extract in DMSO (concentration range 100-800 μ g/mL), with the addition of 250 μ L enzyme solution (prepared by dissolving 1U/mL of pancreatic alpha-amylase enzyme (E.C:3.2.1.1; Sigma, USA) in 0.02 M Sodium phosphate buffer with 0.006 M sodium chloride) were incubated for 10 min at 37 °C. This was followed by the addition of 250 μ L of 1% soluble starch (potato starch) in all test tubes and incubated again for 10 mins at 37 °C. The reaction was terminated by adding 500 μ L of DNSA reagent (3,5-dinitrosalicylic acid:) and then boiled in the water bath at boiling point for 5 mins. The solution was cooled and diluted with 5 mL of water and absorbance was taken at 540 nm (Thermo Scientific GENESY 10S UV/Visible spectrophotometer). The control samples contain no plant extract but the vehicle, dimethyl sulfoxide (DMSO) and the same procedure was followed [14,15]. The results was expressed as percentage of enzyme activity against concentration and the percentage was calculated according to the formula.

% Relative enzyme activity = Enzyme of test/Enzyme of control $\times$ 100

% Inhibition in the α -amylase = 100 - % relative enzyme activity

1C₅₀, the concentrations of samples which will result in 50 % enzyme inhibition was calculated graphically

2.6 Statistical Analysis

All assays were carried out in triplicates and results were expressed as mean $\pm$ standard error mean. IC₅₀ was calculated using excel.

3. RESULTS AND DISCUSSIONS

3.1 Phytochemical Screening

The results showed alkaloids, cardiac glycosides, steroids and anthraquinone derivatives were detected in all the extracts while tannins, flavonoids and saponins were detected only in the ethanol extract. (Table 1). Alkaloids had been linked to several healing properties in medicinal plants [16]. Tannins, flavonoids and saponins are polyphenolic compounds known for their anti-oxidant and anti-diabetic properties [17].

Table 1. Qualitative phytochemical analysis of *Croton zambesicus* extracts

S/N	Constituent/ test	CzH	CzEA	CzEt
1.	Alkaloids			
	Dragendorff's test	+	+	+
	Wagners test	+	+	-
2.	Tannins	-	-	+
3.	Cardiac glycoside			
	Kedde	+	+	-
	Killer-killani's test	+	+	+
4.	Steroidal nucleus			
	Liebermann-burchard	+	+	+
	Salkowski test	+	+	-
5.	Saponins	-	-	+
6.	Flavonoids	-	-	+
7.	Anthraquinone	+	+	+
8.	Reducing sugar	-	-	+

Keyword: CzH= Hexane extract; CzEA = Ethyl-acetate extract; CzEt Ethanol extract

+ : Detected - : Not Detected

3.2 Percentage Alkaloids and Flavonoids

The percentage alkaloids in the plant material were peculiarly high (16.67 ± 3.06) compared with that of flavonoid (2.67 ± 1.16) (Table 2). The high percentage of alkaloids may be responsible for the various medicinal properties attributed to this plant.

Table 2. Percentage composition of the Alkaloids and Flavonoids in *Croton zambesicus*

Phytochemical	Mean Percentage Constituent $\pm$ SEM
Alkaloids	16.67 ± 3.06
Flavonoids	2.67 ± 1.16

3.3 Alpha-amylase Inhibitory Activities

The alpha-amylase inhibitory properties of the leave extracts of *Croton zambesicus* was studied using the starch-iodine colour complex formation assay and the DNSA (3,5-dinitrosalicylic acid) chromogenic reagent assay.

3.3.1 Starch-Iodide Complex Assay

In the starch-iodine colour complex assay, the presence of starch is indicated by a dark-blue colour while a yellow and brownish colour indicate the absence of starch and partially degraded starch in the reaction mixtures respectively. When the starch added to the enzyme assay mixture is not degraded, a blue colour complex is formed which is the case when the extract contain enzyme inhibitors. However, in the absence of an inhibitor no complex is formed [14]. The preliminary starch-iodine complex assay gave positive results for the hexane and ethanol extracts (Table 3) while a partial degradation of starch (brown colour) was obtained for the ethyl acetate extract (Table 3).

Table 3. Colour of complex formed from the starch-iodine Colour Reaction Assay

Extract	Colour in Iodine Reagent	Result
CzH	Dark-blue	+
CzEA	Brown	±
CzEt	Dark-blue	+

Key: CzH= *Croton zambesicus* hexane extract; CzEt= *Croton zambesicus* ethanol extract
+: positive; ± partial positive

3.3.2 DNSA reagent (3,5-dinitrosalicylic acid) Assay

The extent of inhibition of alpha-amylase enzyme by the extracts which exhibited complete positive results (hexane and ethanol) from the starch-iodide assay was quantified using the DNSA (3,5-dinitrosalicylic acid) reagent method and acarbose as the standard drug. The assay results showed that both extracts exhibited alpha-amylase inhibitory properties which increased in a concentration dependent manner (Fig.2). At the lowest concentration of extracts (100 µg/mL), the ethanol extract exhibited the highest inhibitory activity which was higher than that of acarbose. However, at the concentration of 800 µg/mL, the highest inhibition was observed in the hexane extract with acarbose having the lowest alpha-amylase inhibitory property. The overall results show that the highest alpha-amylase inhibitory activities were observed in the ethanol and hexane extracts with IC_{50} of 78.7 ± 1.7 and 89.2 ± 1.1 (Fig.3) respectively while the lowest effect was observed in the standard drug, acarbose (IC_{50} = 114.9 ± 11.3). This result indicated that the hexane and ethanol leave extracts of *croton zambesicus* could inhibit alpha-amylase enzyme activity hence suppressing the hydrolysis of starch leading to reduction in post-prandial glucose level and hence reducing the complication of diabetes mellitus. The results obtained also supported the preliminary starch-iodine complex formation assay result.

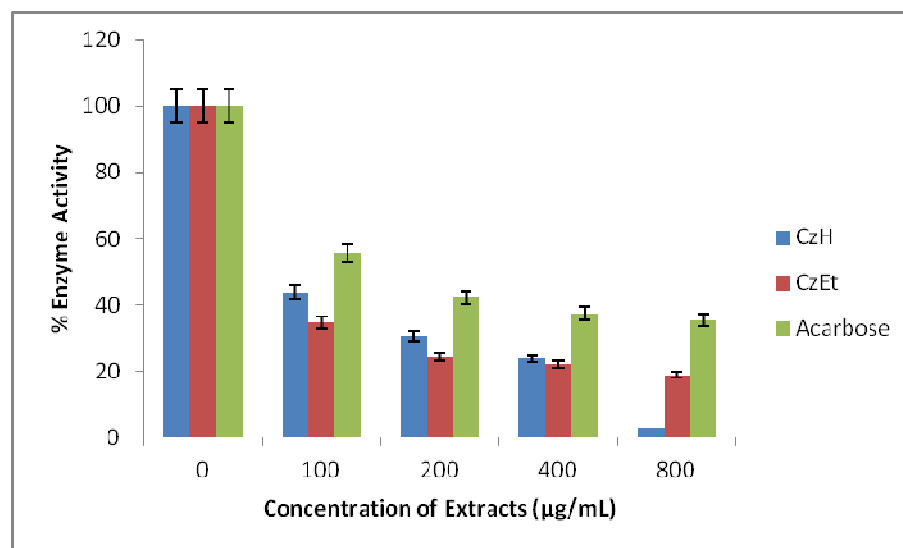


Fig.2. Percentage Enzyme activity against concentration of Extracts (100, 200, 400, 800 µg/mL)

Mean ± S.E.M = Mean values ± Standard error of means of three experiments

Key: CzH= *Croton zambesicus* hexane extract; CzEt= *Croton zambesicus* ethanol extract

Conc(µg/ml)	CzH	CzEt	Acarbose
0	0.0±0.00	0.0±0.00	0.0±0.00
100	56.06±0.57	65.23±1.22	44.38±3.78
200	69.51±0.38	75.70±1.72	57.84±1.32
400	76.18±2.23	77.90±0.39	62.60±4.21
800	97.27±0.48	81.23±0.65	64.57±1.13

IC ₅₀	89.2 ± 1.06	78.72 ± 1.68	114.89 ± 11.28
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Table 4. Percentage inhibition of *Croton zambesicus* hexane and ethanol extracts at different concentrations with IC₅₀ values

Mean ± S.E.M = Mean values ± Standard error of means of three experiments

Key: CzH= *Croton zambesicus* hexane extract; CzEt= *Croton zambesicus* ethanol extract

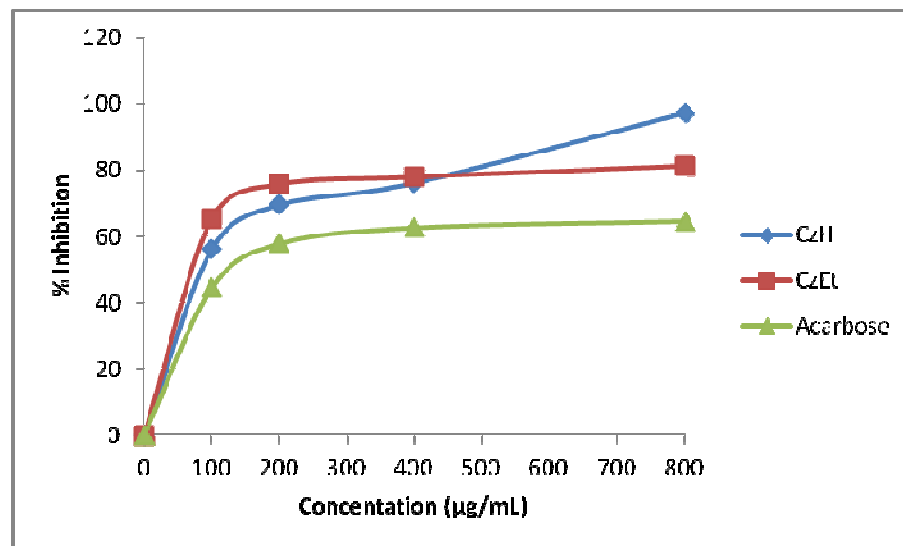


Fig.3. Percentage Inhibition against concentration of Extracts (100, 200, 400, 800 µg/mL)

Mean ± S.E.M = Mean values ± Standard error of means of three experiments

Key: CzH= *Croton zambesicus* hexane extract; CzEt= *Croton zambesicus* ethanol extract

4. CONCLUSION

This study confirmed the traditional uses of *Croton zambesicus* leaf in the management of diabetes mellitus as it can effectively suppress the hydrolysis of starch by pancreatic alpha-amylase hence reducing post-prandial glucose level.

CONSENT (WHERE EVER APPLICABLE)

Not applicable

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

Not Applicable

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