

Inhibitory properties of combined ethanol seed extract of *P. guineense* and *T. conophorum* on α -glucosidase enzyme.Dokubo A^{1*}, Odinaka EM², Chukwu JAO³¹Department of Biochemistry, Rivers State University, Port Harcourt, Nigeria²College of Medicine and Health Sciences, Abia State University, Uturu, Nigeria³Department of Human Physiology, Alex Ekwueme Federal University, Ndufu-Alike, Ikwo, Nigeria**Abstract**

The study was aimed to evaluate the α -glucosidase inhibitory properties of combined ethanol seed extract of *Piper guineense* and *Tetracarpidium conophorum* (CEPGTC). In vitro inhibitory assay was conducted in accordance with standard methods. p-nitrophenyl-D-glucopyranoside (pNPG) was used as a substrate. Fixed amount of the enzyme was incubated with increasing concentrations of CEPGTC seed extract (20-100 μ g/ml) at 37°C for 15 min. The continuous production of p-nitrophenol was determined by measuring the absorbances at 410 nm. Acarbose at varying concentration (20-100g/ml) was used as the positive control. The median Inhibitory Concentration (IC₅₀) values were graphically determined as the half-maximal inhibitory from plots of percent inhibition against log inhibitor concentration and by non-linear regression analysis. The enzyme kinetics (pattern of Inhibition) was determined using the Michaelis Menten's equation and Lineweaver-Burk plot. The result showed that there was inhibition in all concentrations evaluated. The IC₅₀ values estimated were 2.9 μ g/ml and 2.5 μ g/ml for CEPGTC and acarbose respectively. However, The IC₅₀ value for acarbose was significantly ($p < 0.05$) different from CEPGTC which is an indication of acarbose as effective α -glucosidase inhibitor thus, used as positive control. An enzyme kinetics studies from the Lineweaver-Burke plot showed that CEPGTC seed extract demonstrated an uncompetitive inhibition on α -glucosidase while acarbose exhibited non competitive (mixed inhibition). The inhibitory response and mechanism of inhibition observed in this study showed that combined ethanol seed extract of *Piper guineense* and *Tetracarpidium conophorum* has the potential to reduce glucose turnover and improve Hyperglycemia.

Keywords: *Tetracarpidium conophorum*, *Piper guineense*, Inibitory, Kinetics, α -glucosidase, Enzyme.

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Introduction

Diabetes mellitus (T2DM) has been described as a metabolic disorder that results to hyperglycemia due to malfunctioning of insulin action and secretion [1]. Hyperglycemia can cause glucose toxicity leading to the production of free radicals via glucose autoxidation, advanced glycation end products (AGEs) [2]. Free radicals have been implicated in a number of degenerative diseases [3]. One of the mechanisms utilized in the management of hyperglycemia is inhibition of carbohydrate metabolizing enzymes such as α -glucosidase (EC 3.2.1.20) in the epithelial tissues of small intestine with the intention of delaying glucose absorption by the walls of the small intestine [4]. There are so many effective synthetic drugs used as α -glucosidase inhibitors. Some of these include e.g., acarbose voglibose however, they are frequently associated with adverse effects and toxicity [5]. Therefore, the search for newer α -glucosidase inhibitors from plant origin becomes imperative. Several classes of active phytochemicals from crude extracts of different medicinal plant products have been used to combat

hyperglycemic conditions [6]. A good number of these medicinal plants have been reported to inhibit α -glucosidase activity [6-9]. In traditional medical systems, combination of different plant species (poly therapy) has been utilized in managing hyperglycemia.

Piper guineense belongs to the Piperaceae family. It is commonly known as guinea pepper, black pepper in English, 'Uziza' in Igbo and 'Iyere' in Yoruba [3]. The leaves have been reported to exhibit anti-microbial, antifungal and antioxidant properties [10,11]. *Tetracarpidium conophorum* belongs to the Euphorbiaceae family. It is known as African walnut, 'Ukpa' in Igbo. The leaves bark and fruits of *T.conophorum* have been reported for antihyperglycemia potentials [12,3]. This study is aimed at assessing α -glucosidase inhibitory properties of combined ethanol seed extract of *Piper guineense* (*P. guineense*) and *Tetracarpidium conophorum* (*T. conophorum*) and mechanism of inhibition pattern.

Materials and Methods

The Seeds *Tetracarpidium conophorum* and *Piper guineense* were collected from Mile 1 and Mile 3 Markets Port Harcourt, Rivers State and were authenticated at the Plant Science and Biotechnology Department, Rivers State University, Port Harcourt, Nigeria. The seeds were sorted, cleaned and ground to fine powder using a blender. A known weight, 100 g of each powder was combined and maceration with ethanol and water (8:2) at room temperature. The mixture was intermittently shaken to facilitate extraction for 3 days. The extracts were filtered using a Buchner funnel and Whatman no. 1 filter paper. The resulting filtrate was evaporated to dryness under water bath and later oven dried at a temperature of 50°C. A stock solution of 200 mg/ml of the extract was prepared using dimethyl sulfoxide. Concentrations ranging from 20 µg/ml to 100 µg/ml were prepared for the assay.

Chemicals and reagents

pNPG (p-nitrophenyl--D-glucopyranoside), -glucosidase from *Saccharomyces cerevisiae* were purchased from Sigma Aldrich, USA. Other chemicals used were of analytical grade and obtained from the Department of Biochemistry, Rivers State, Nigeria and other reputable firms.

-Glucosidase Inhibitory assay

The effect of the combined ethanolic seed extracts of *Piper guineense* and *Tetracarpidium conophorum* on -glucosidase activity was determined according to the method with slight modification. -glucosidase from *Saccharomyces cerevisiae* was used as the enzyme source. The substrate solution p-nitrophenyl--D-glucopyranoside (pNPG) was prepared in 50 mM phosphate buffer, pH 6.9. 100 L of -glucosidase (1.0 IU/ml) was preincubated with 50 L of the different concentrations of combined ethanolic extracts of *P. guineense* and *T. conophorum* (20-100g/ml) for 10 min. Then 50 L of 3.0 mM pNPG 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 Na₂CO₃. The -glucosidase activity was determined by measuring the yellow-colored para nitrophenol released from pNPG at 410 nm. Acarbose at varying concentration (20-100g/ml) was used as the positive control. The percentage inhibition of α-glucosidase activity was calculated via the following equation:

$$\% \text{Inhibition} = [1 - \text{AE}/\text{Ac}] \times 100$$

AE=Absorbance of the Extract, Ac=Absorbance of Control

The concentration of the tested samples giving 50% inhibition of the enzyme activity (IC₅₀) values were determined from plots of percent inhibition against log inhibitor concentration and by non-linear regression analysis. All samples were assayed in triplicate.

Kinetics of inhibition

The mode of enzyme inhibition of the combined ethanolic extract of *P. guineense* and *T. conophorum* was determined

according to the method described by Ozuogwu and Akuba, 2018 with slight modifications [9]. In one set of reacting tubes, 50 l of the extract (IC₅₀ concentration) was preincubated with 100 L of -glucosidase solution for 10 min at 25°C. In another set of tubes -glucosidase was preincubated with 50 L of phosphate buffer (50 mM, pH 6.9). Then 50 L of PNPG at increasing concentrations in mM (0, 1.25, 2.5, 5.0, 10.0 and 20.0) was added to both sets to start the reaction. The mixture was then incubated for 10 min at 25°C, and 500 L of Na₂CO₃ was added to stop the reaction. Acarbose was used as a positive control. 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 extract on -glucosidase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot and Michaelis Menten kinetics.

Statistical analysis

The data obtained were expressed as the mean ± SD of three replicates. Analysis was performed using Quest Graph™ IC₅₀ Calculator, Graphpad prism 8 Software and Excel 2010. One-way analysis of variance (ANOVA) and Duncan test were used to evaluate the possible differences among the means. P values ≤ 0.05 were considered as significant differences.

Results and Discussion

Glucosidases are enzymes that participate in carbohydrate metabolism. Thus, α-glucosidase inhibitors can play important role in glucose toxicity [13]. The -glucosidase inhibitory potential of combined ethanolic extract of *P. guineense* and *T. conophorum* was evaluated using *S. cerevisiae* as the source of enzyme. The substrate for the enzyme was para-nitrophenyl--D-glucopyranoside (PNPG). The inhibitory potentials on α-glucosidase activity at increasing concentrations of combined ethanolic extract of *P. guineense* and *T. conophorum* was compared to acarbose, a standard -glucosidase inhibitor (Positive control) as shown in Table 1 and Figure 1. The results showed that there was inhibition in all concentrations evaluated. However, the percentage inhibition of the combined ethanolic extract of *P. guineense* and *T. conophorum* (CEPGTC) on α-glucosidase was less than that of acarbose. The median Inhibitory Concentration (IC₅₀), which is the concentrations that produce 50% inhibition under the specific experimental conditions for the α-glucosidase activity was estimated to be 2.9 µg/ml while that of the standard, acarbose was 2.5 µg/ml. The IC₅₀ value for acarbose was significantly (p<0.05) different from the combined ethanolic extract of *P. guineense* and *T. conophorum* (CEPGTC) which is an indication of acarbose as effective -glucosidase inhibitor thus, used as positive control. Acarbose has structural resemblance to oligosaccharides. It alters enzymatic hydrolysis and absorption of glucose from dietary sources by the small intestine [14]. The inhibitory response observed in this study showed that increasing the

concentrations of the extracts (inhibitor) has the potential to reduce glucose turnover.

Table 1. The Percentage Inhibition of α -glucosidase by Acarbose and Combined Ethanolic Extract of *P. guineense* and *T. conophorum* (ratio1:1).

Substrate concentration	% Inhibition	
[S] μ g/ml	Acarbose (Positive Control)	CEPGTC
20	28.04	26.11
40	35.11	28.25
60	37.04	33.12
80	40.01	37.12
100	50.35	43.18

The values are expressed as mean \pm SD of triplicate tests.

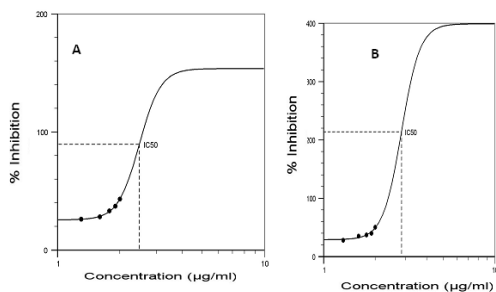


Figure 1. Inhibitory Properties of CEPGTC Extract against α -glucosidase activity. A=control (IC_{50} =2.5 μ g/ml), B=(CEPGTC) 2.5 μ g/ml. The values are expressed as mean \pm SD of triplicate tests.

The inhibition mode was elucidated using the Michaelis Menten's equation and Lineweaver - Burk plot. Results indicated a mixed and non-competitive type of inhibition as shown in Figure 2. Increasing concentrations of the inhibitors will decrease the maximum velocity of the reaction (V_{max}) and affinity between the enzyme and the substrate may.

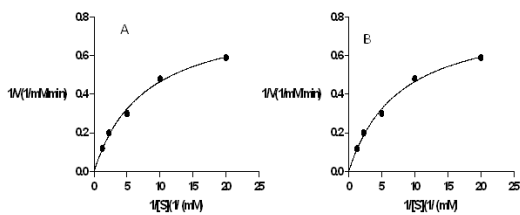


Figure 2. A graph of Substrate Concentration against Time of Inhibition. Extract (IC_{50}) and substrate (PNPG) concentrations (0, 1.25, 2.25, 5.0, 10.0, and 20.0 mM). A=Control B=CEPGTC. The values are expressed as mean \pm SD of triplicate tests.

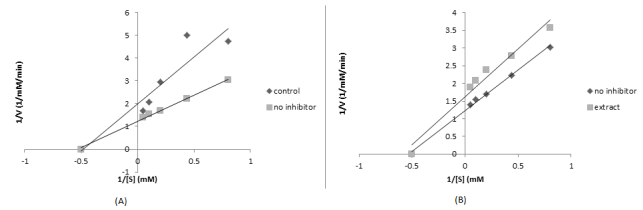


Figure 3. (A) Lineweaver-Burk Plot of Inhibition Mode of Acarbose (IC_{50}) and Concentrations of Substrate (PNPG). The values are expressed as mean \pm SD of triplicate tests (B) Lineweaver-Burk Plot of Inhibition Mode of CEPGTC extract (IC_{50}) and concentrations of substrate (PNPG). The values are expressed as Mean \pm SD of triplicate tests.

The enzyme kinetics showed the mechanism of inhibition according to Michaelis Menten's and Lineweaver-Burk equation. It gives a visual representation of the types of enzyme inhibition. The features of mixed inhibition include the lines for different concentrations of inhibitors in the Lineweaver Burk plots intersecting to the left of the $1/v_0$ axis. Furthermore increasing concentrations of the inhibitors result to decrease in the maximum velocity (V_{max}) while the K_m which is a measure of binding affinity between the enzyme and the substrate may decrease or increase. The inhibitors bind to both the free enzyme (E) where substrates also bind and enzyme-substrate complex (ES) forming enzyme-substrate-inhibitor complexes (ESI) that alters catalysis and product formation. This type of inhibition was observed for acarbose with decrease in V_{max} (0.816-0.502 mM/min) and Increase in K_m (1.87-2.08 mM) as shown in Figure 2. This is an indication that there was no completion with the substrate for binding to the active site but binds to a separate site (allosteric site) on the enzyme to inhibit the conversion glucose. Other studies conducted in *in vitro* inhibition of carbohydrate metabolizing enzymes and *in vivo* anti-hyperglycaemic potential of methanol extract of *Desmodium velutinum* leaves reported similar inhibition pattern [9]. This mechanism may be used by the extract to suppress the production of glucose during hydrolysis of dietary carbohydrates. The features of uncompetitive inhibitors results to both decrease in V_{max} and K_m [15,16]. in this type inhibition, the inhibitor binds to the enzyme substrate complex only forming an inactive enzyme substrate inhibitor complex. In this case, increase in availability of substrate facilitates binding of the inhibitor to ES complex [17,15]. This mode of inhibition was observed for the extract with decrease in V_{max} (0.816-0.616 mM/min) and decrease in K_m (1.87-1.67 mM) as shown in Figure 3. This is an indication that the extract had the potential to alter catalysis with substrate binding decreasing production of glucose. The type of inhibition pattern is an indication that both the extract and acarbose are not structural analog with the substrate [9]. Their presence decreased the affinity of the substrate for the enzyme and reduced glucose turn over. Furthermore, increased concentration of substrate enhance this type of inhibition, This could be a reason of the increased percentage inhibition observed for α -glucosidase activity as the concentration of extract increase, delaying glucose production. This study is in

agreement with previous reports which have shown that plant products exhibit strong inhibition against -glucosidase [18-20].

Conclusion

The inhibitory response observed in this study show that increasing the concentrations of the extracts (inhibitor) has the potential to reduce glucose turnover. The type of inhibition pattern is an indication that both the extract and acarbose are not structural analog with the substrate. The presence of the extract can affect decrease in the affinity of the enzyme for the substrate which may cause increase or decrease in K_m but always decrease V_{max} due to the formation of inactive enzyme-substrate-inhibitor complex. The potency of this type of inhibition cannot be affected by increase in substrate concentration. Thus, these spices can be utilized as effective inhibitors of α -glucosidase in managing NIDDM.

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