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PHYTOCHEMICAL SCREENING AND *IN VITRO* A -AMYLASE AND A-GLUCOSIDASE INHIBITORY POTENTIAL OF *CLERODENDRUM VOLUBILE* LEAF EXTRACT

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Abstract

Clerodendrum volubile leaf is one of the widely used herbs in folklore medicine for the management of diabetes. This preliminary study sought to investigate the phytochemical analysis of methanolic leaf extracts and assess its interaction with key enzymes (α-amylase and α-glucosidase) relevant to type-II diabetes *in vitro*. Methanolic extract of *C. volubile* leaf was prepared and the phytochemical analysis of *C. volubile* leaf were subsequently determined by standard methods. Finally, the effects of *C. volubile* leaf extract on α-amylase and α–glucosidase activities were assessed *in vitro*. *C. volubile* leaf extract was found to be rich in flavonoids, phenols, tannins. The leaf extract significantly inhibits α -amylase (IC_{50}= 0.40 mg/ml) and α-glucosidase (IC_{50}= 0.68 mg/ml). This study may thus suggest that *C. volubile* leaf represent a source of phenolic phytochemicals for the management of non-insulin dependent diabetes mellitus.

Keywords: *Clerodendrum volubile*; *Phytochemicals*; *α-Amylase; α-Glucosidase; Type-II diabetes*

Introduction

Clerodendrum volubile P. Beauv (Family: Lamiacea) is widely cultivated in many deciduous forests across western countries Africa [1]. It is commonly known as 'Obenetete' among the Urhobo and Itsekiri tribes of the Niger-Delta of Nigeria, as 'Marugbo or 'Ewe ata' in Ondo State, Nigeria [2, 3]. In traditional system of medicine, the plant is used for the treatment of several diseases like diabetes, rheumatism, arthritis, edema and gout [4].

The plant is known for medicinal properties like anti-inflammatory [5], hepatoprotective [6], neuroprotective [7], and antioxidant [8] potentials. The significant phytochemicals present includes saponins, terpenoids and cardiac glycosides. Experimental evidences on the plant generally have shown its potentials to reduce blood-glucose and cholesterol in diabetic conditions. [3, 9, 10]. The mechanism of action involves stimulation of glucose dependent insulin secretion from beta cells of the pancreas along with inhibition of activity of enzymes like amylase, glucosidase [3, 9]. In current situation, phytochemicals have received much attention in the treatment of diabetes for several reasons and many researchers have focused on isolation and characterization of bioactive compounds or agents from medicinal plants with hypoglycemic effect [11, 12]. Polyphenols and flavonoids from plant origins are some of the naturally occurring antidiabetic agents [3, 13] which are well documented to exhibit an inhibitory action on key enzymes relevant to carbohydrate digestion, by virtue of their ability to bind with proteins. This phenomenon is associated with the lowering of postprandial hyperglycemia in diabetes [13]. The aim of the present study was to evaluate the anti-α-amylase and anti-α-glucosidase inhibitory potential of methanolic extract of *C. volubile leaves*

Methods

Chemicals

Porcine pancreatic amylase (PPA) and α-glucosidase, dimethyl sulfoxide (DMSO), Tris-HCl buffer, and nitrophenyl glucopyranose were purchased from Sigma (St. Louis, MO, USA).. All the other chemicals used during the work were of analytical grade.

Collection and authentication

The plant was purchased from the local market of Oja Oba in Akure, Nigeria in the month of November, 2017 and was identified at Department of Biology, Federal University of Technology (FUTA), Akure, Nigeria. The leaves were cleaned, shade dried, and coarsely powdered for extraction.

Extraction

Leaves of *C. volubile* (30 g) in powdered form were extracted with 200 mL of methanol, ethanol, with the aid of a shaker in 2000r/min speed for 24 h at 37◦C. The extract were filtered with Whatman No. 1 filter paper for every 3h. Then, the collected extracts were evaporated to dryness under reduced pressure. The dried extracts were reconstituted in water and stored at 4◦C for further investigations [8].

Phytochemical Investigations

Phytochemical Qualitative Analysis

The methanolic extracts solutions were estimated for the existence of the phytochemical analysis by using the following standard methods as described [14 - 16].

Test for Combined Anthraquinones

1ml of extract was boiled with 10ml $FeCl_3$ and 5ml of 1% HCl, it was filtered while hot and add 2ml of Diethylether was added. The solution was mixed and observed for colour change viz; pink red, violet in ammonical layer.

Test for Tannins

1ml of extract boiled with 20ml of water for 5 mins and filtered, take few drops of 0.1% $FeCl_3$ was added and observed for green, blue black colour or colour change.

Test for Saponins

5.0 ml of distilled water was mixed with methanol crude plant extract in a test tube and the mixture was mixed vigorously. The frothing was mixed with few drops of olive oil and mixed vigorously and the appearance of foam showed the presence of saponins.

Tests for Flavonoids

1ml of extract was added to 2ml of NH3 diluted and small quantity of Conc H_2SO_4 added. A yellow colouration observed but also disappeared on standing showed the presence of flavonoid.

Test for Phenol

1ml of extract was added to 2ml FeCl3 solution. A deep blue or green colour observed means positive for phenol.

Tests for Glycosides

Liebermann's Test. A mixture of acetic acid (2 ml) and 2 ml of chloroform was added to the whole methanol plant crude extract. The mixture was then cooled and we added H_2SO_4 concentrated. Green color showed the entity of aglycone, steroidal part of glycosides.

Keller-Kiliani Test. A solution of glacial acetic acid (4.0 ml) with 1 drop of 2.0% $FeCl_3$ mixture was mixed with the 10 ml aqueous plant extract and 1 ml H_2SO_4 concentrated. A brown ring formed between the layers which showed the entity of cardiac steroidal glycosides.

Salkowski's Test. 2 ml H_2SO_4 concentrated was added to the whole methanol plant crude extract. A reddish brown color formed which indicated the presence of steroidal aglycone part of the glycoside.

Test for Terpenoids

Chloroform (2 ml) was added to 5 ml methanol plant extract and evaporated on the water path and then boiled with 3 ml of H_2SO_4 concentrated. A grey color formed which showed the entity of terpenoids.

Test for Steroids

2 ml of chloroform and concentrated H_2SO_4 were added with the 5 ml methanol plant crude extract. In the lower chloroform layer red color appeared that indicated the presence of steroids

Test for Phlobatannins

1ml of extract was added to 3ml of 1% HCl. The deposit of red precipitates confirms the presence. 1ml of extract was added to 10ml of DH_2O and filtered, the filtrate was boiled with 2% HCl. The presence of red precipitate shows phlobatannins are present.

Test for Alkaloids

1ml of extract was added to 4ml of 1% HCl and boiled in water bath for 5 mins and then filtered. 2ml of the filtrate was treated with 2-3 drops of dragendorff reagent. Turbidity shows present of alkaloids.

Phytochemical Quantitative Analysis

Test for Flavonoid

1.5ml of sample was added to 1.5ml of 2% Methanolic Aluminum chloride then incubated for 5mins and the absorbance was read at 510nm. Quercetin was used as standard. Quercetin preparation: 0.01g of quercetin was dissolved in 100ml of methanol.

Total phenol

1ml of sample was added to 0.4ml of Folin C reagent then it was left for 5mins. Thereafter, 7.5% of Na_2CO_3 was added to make the volume up to 10ml with DH_2O. The mixture incubated for 90mins. The absorbance was read at 750nm. Garlic acid was used as standard to calculate the phenolic content.

Alkaloids

1ml of sample was added to 20ml of 10% acetic acid in ethanol. It was covered and allow the mixture to stand for 4hrs and then filtered. The filtrate was concentrated on a water bath to reduce it to a quarter of its original volume. Then conc. NH_4OH was added and the precipitate collected through weighed filter paper. The filter paper rinsed with 1% NH_4OH. The alkaloid precipitate was dried in an oven at 60°C for 30mins and filtered papers.

It was re-weighed and the % of alkaloids calculated using the formula below:

$$\frac{\text{Final weight of Sample}}{\text{Weight of Sample}} \times 100$$

Tannin

1ml of sample was added to 10ml of distilled water and incubated for 1hr and thereafter filtered and make the volume up to 25 ml. 1ml of the filtrate was taken and added to 5ml of distilled water plus 4

drops of tannin reagent. The absorbance was taken after 10mins at 720 nm using Tannin as standard.

$$\frac{\text{Final weight of sample}}{\text{Abs of std x Conc of std}} \times 100$$

Saponin

1.0g of sample was added to 25ml of 20% ethanol, they were heated on the water bath for 2hrs and thereafter, filtered. The residue obtained, was again extracted with 50ml of 20% ethanol. The combined extract was reduced to 40ml on a hot water bath at 90°C and transferred to separating funnel. Thereafter, 2ml of Diethyl ether/petroleum ether was added and shaken very well, and recovered the layer. The ether layer was decanted and 15ml of n-butanol was added and washed twice with 3ml of 5% sodium chloride and the sample was evaporated to the dryness in an oven through beaker and reweighed and then calculate the percentage of saponin. Tannic acid was used as standard.

$$\frac{\text{Final weight of sample}}{\text{Abs of std x Conc of std}} \times 100$$

Phlobatannins

1ml of sample was added to 20ml of 50% methanol, covered with paraffin and boiled with 80% ethanol of for 1hr. It was shaken properly and filtered. The volume was made up to 50ml with DH_2O. Take 1ml out of the resultant volume was taken and added to 20ml of DH_2O, 2.5ml of folin Dennis reagent and add 10ml of 17% Na_2CO_3 was also added to the mixture and homogenized thoroughly. The absorbance was read at 550nm.

Terpenes

1ml of sample was added to 10ml of chloroform-methanol, shaken and incubated for 15mins and then centrifuged. The supernatant obtained was decanted, and the precipitate rewashed with chloroform-methanol and centrifuged again. The precipitate was also dissolved with 40ml of 100% sodium deodecyl sulphate. 1ml of 0.01m $FeCl_2$ was added at 30seconds interval, shaken and incubated at room temperature for 30mins and the absorbance read at 510nm.

Steroid content (Trease and Evans)

A portion of 2ml was taken from solution of 2.5g of powdered *C. volubile* plant already prepared in 50ml of distilled water and shaken for 1hr. The extract solution was washed with 3ml of 0.1M NaOH (pH 9) and later mixed with 2ml of chloroform and 3ml of ice cold acetic anhydride and two drops of Conc H_2SO_4 and the absorbance is taken at 420nm also prepare a blank.

Biochemical assays for determining the inhibition of enzymes activities

α- Amylase inhibition assay

The required methanolic extracts of *C. volubile* dilution (0–200 µl) and 500 µl of 0.02M sodium phosphate buffer (pH 6.9 with 0.006M NaCl) containing Porcine pancreatic a-amylase (EC 3.2.1.1) (0.5 mg/ml) were placed in an incubator for 10 min at 25 ⍰C. Then, 500 ll of 1% starch solution in the phosphate buffer was added to each tube. The reaction mixture was incubated at 25 °C for 10 min and the reaction was terminated with 1.0 ml of dinitrosalicylic acid color reagent. Thereafter, the mixture was incubated in a boiling water bath for 5 min, and cooled to the room temperature. 10 ml of distilled water was added to the reaction mixture to dilute it and the absorbance measured at 540 nm. The inhibition of the enzyme a-amylase activity was given as percentage inhibition [17].

α-Glucosidase inhibition assay

Briefly, appropriate dilution of the methanol *C. volubile* leaf extracts (0–200 µl) and 100 µl of α-glucosidase (EC 3.2.1.20) solution in 0.1M phosphate buffer (pH 6.9) was placed in an incubator at 25 °C for 10 min. Thereafter, 50 µl of 5mM pnitrophenyl-a-D-glucopyranoside solution in 0.1M phosphate buffer (pH 6.9) was added. The resulting mixtures were incubated at 25 ⍰C for 5 min, and the absorbance read at 405nm in the spectrophotometer. The α-glucosidase inhibitory activity was given as percentage inhibition [18].

Statistical analysis

The results are expressed as mean ± standard error of mean. Experiments were performed in triplicate. Statistical comparison was performed using analysis of variance (ANOVA) followed by

Dunnet's test. The level of statistical significance was set as $P < 0.05$.

Results

Preliminary phytochemical analysis

As shown in tables 1 and 2, the phytochemical screening reveals the presence of phenol, terpenoids, alkaloids, saponin, tannins, flavonoids, cardiac glycosides.

The inhibitory effects of C. volubile extract on α-amylase and α-glucosidase

The inhibitory activity of *C. volubile* extract on α-amylase and α-glucosidase were assessed and results shown in Figures 1 and 2. The leaf extracts had significantly higher inhibitory activity on α-glucosidase and α-amylase enzymes with IC_{50} = 0.40 and 0.68 mg/ml, respectively. A high inhibitory activity on α-glucosidase was observed when compared to its corresponding effect on α-amylase enzymes

Discussion

Diabetes mellitus is a common disorder of metabolic process which may eventually results to multiple damages to organs in the body. High level of blood glucose has been implicated as major risk factor cardiovascular disease which has been documented as the leading cause of death in diabetic patients. Hence in diabetes management, the regulation of critical enzymes linked to diabetes mellitus metabolic is a critical therapeutic approach in the management diabetes and its complications [19, 20]. These key enzymes viz: α-amylase and α-glucosidase are involved in the digestion of carbohydrates.

Carbohydrates rich diets could cause a sharp elevation in blood glucose as they are being digested by this key enzymes to monosaccharide that are easily absorbable in the body. Inhibition of these key enzymes could lead to a delay in carbohydrates digestion, extend the duration of carbohydrate digestion and consequently impede the excessive postprandial increase blood glucose level. Thus, the approach to glucose production control, especially from plant sources could serve as alternative source of oral α-amylase and α-glucosidase inhibitors which would be an efficient therapeutic management for type-2 diabetes [21].

The results from this study (as shown in Figures 1 and 2) revealed that *C. volubile* extract showed a high α-glucosidase (IC_{50} = 0.40 mg/ml) inhibitory activity with respect to its effect on α- amylase (IC_{50} = 0.68 mg/ml) activities which portends a pharmaceutical significance. These key enzymes; α-amylase and α-glucosidase are involved in the breakdown of carbohydrates into absorbable monosaccharide in human beings. Moreover, due to the expensive nature, less efficacy and attendants sides effects of synthetic drugs like acarbose, metformin and miglitol primarily used for the treatment of type-2 diabetes, hence in recent times, a lot of focus is tailored towards plant based inhibitors of α-glucosidase sourced form plants than its α -amylase inhibitors with the research goal for an alternative to this enzyme inhibitors [22, 23].

In this study, the inhibitory effect of methanolic *C. volubile* extract on these key enzymes linked with diabetes *in vitro* was justified. The phytochemical screenings (Tables 1 and 2) revealed the presence of saponins and terpenoids which might be linked to this enzyme inhibitory activity. The polyphenols, e.g. quercetin, chlorogenic acid, rosmarinic acid and rutin found in the leaf extracts of C. *volubile* [3, 8] and also reported to be present in other vegetables, are known to have strong inhibitory action on these key enzymes. These polyphenolic compounds can also stimulate the reduction in elevated blood glucose via increased glucose uptake and glycogen synthesis [3, 13, 24] thus reducing post-prandial hyperglycemia.

Conclusion

In the current study, methanolic extract of *C. volubile* demonstrated α-amylase and α-glucosidase inhibitory potential which may serve as a drug lead compounds for isolation and identification of bioactive compounds responsible for it.

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Table 1: Phytochemical qualitative screening of methanolic extract of *C. volubile leaves*.

Phytochemicals	Composition (mg/100ml)
Flavonoids	+
Terpenoids	+
Saponin	+
Tannins	+
Total phenol	+
Cardiac glycoside	-
Alkaloids	+
Combined Anthraquinones	-
Steroids	+

+= Present, - =Absent

Table 2: Phytochemical quantitative screening of methanolic extract of *C. volubile leaves*.

Phytochemicals	Composition (mg/100 ml)
Flavonoids	50.19± 0.07
Terpenoids	+
Saponin	21.88±0.05
Tannins	63.35± 0.08
Total phenol	65.02±1.07
Cardiac glycoside	-
Alkaloids	44.40± 1.06
Combined Anthraquinones	63.57± 0.08
Steroids	11.96±0.03

Data expressed as mean ± SD (n=3)

Figure 1. α-amylase inhibitory activities of *C. volubile* leaf extract (n=3). Values represent mean ± S.D.

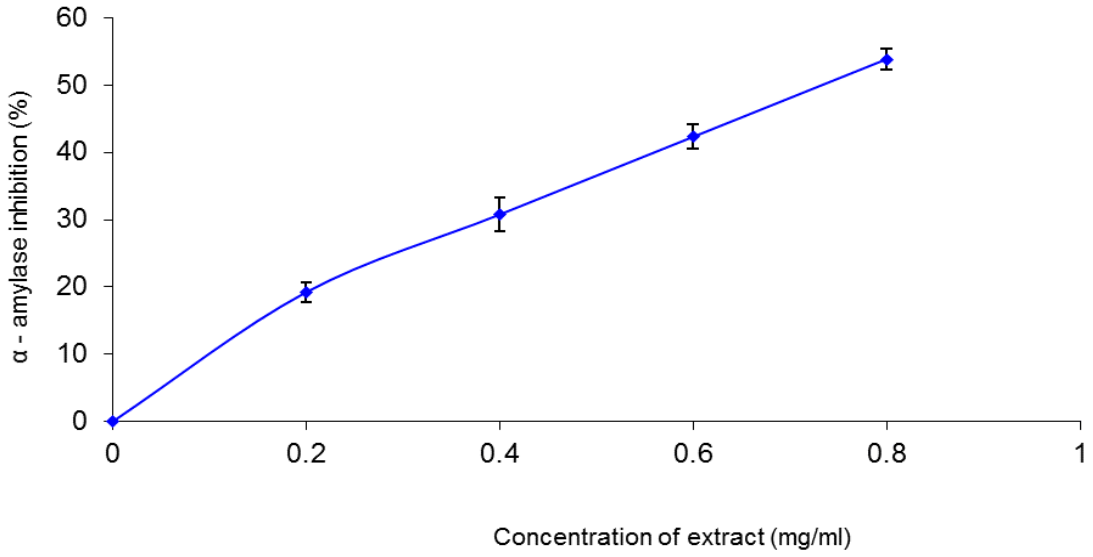

Figure 2. α-glucosidase inhibitory activities of *C. volubile* leaf extract (n=3). Values represent mean ± S.D