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Bioassay Guided Isolation and Evaluation of the Antidiabetic Principles of *Combretum dolichopetalum* Root

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Authors' contributions

Author PFU performed the plant collection, extraction, fractionation, phytochemical analysis, animal studies and the statistical analysis. Author PFU also did the literature search, participated in study design, wrote the protocol and the first draft of the manuscript. Author POO participated in study design, writing the protocol, fractionation and did the overall supervision of the work. Author EOO participated in study design, fractionation and phytochemical analysis while author MOA participated in plant extraction and fractionation. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present study was aimed at investigating the antidiabetic potentials of *Combretum dolichopetalum* root in alloxan-induced animals with the hope of isolating its antidiabetic principles.

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Study Design: Sixty four Wistar albino rats of either sexes were randomly segregated into 16 groups (n=4). Also, thirty two albino mice were segregated into 8 groups. These received various doses of the plant sample, vehicle or glibenclamide for the antidiabetic study.
Place and Duration of Study: This study was done in the laboratory of the Department of Pharmaceutical and Medicinal Chemistry, University of Nigeria, Nsukka between March and October, 2013.
Methodology: The root of *C. dolichopetalum* was extracted with methanol (ME) and fractionated successively with various solvents (n-hexane, chloroform, ethylacetate, methanol and water) to afford the respective fractions: HF, CF, EF, MF and AF. CF was further fractionated to afford six sub-fractions: C1-C6. Acute toxicity study was done using ME. Antidiabetic activity of various doses (p.o.) of ME (100, 200, 400 and 600 mg/kg body weight), its fractions (200 and 400 mg/kg) and sub-fractions (200 mg/kg), glibenclamide (0.2 mg/kg) and vehicle (control) were investigated in alloxan-induced (*i.p.*) diabetic animals for 9 h. Phytochemical analysis was also carried on ME and fractions.
Results: The extract was considered safe with LD_{50} greater than 5000 mg/kg. ME (400 mg/kg), CF (400 mg/kg) and C3 (200 mg/kg) produced maximum reduction (36.78%, 72.43% and 83.17% respectively) in fasting blood glucose of animals after 9 h which were significantly ($P < .01$, $P < .001$) different from the control and better than glibenclamide (48.18%). Phytochemical analysis showed alkaloids, flavonoids, terpens and steroids as the likely antidiabetic agent(s).
Conclusion: The root of *C. dolichopetalum* possesses potent antidiabetic activity which increases as the extract is purified. The antidiabetic effect of the plant may likely be due to the presence of alkaloids, flavonoids, terpens or steroids.

Keywords: Alloxan-induced diabetes; antidiabetic activity; combretaceae; Combretum dolichopetalum; hyperglycemia; hypoglycemia; phytochemical analysis.

1. INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by high levels of glucose in the blood due to the impaired secretion of insulin or insulin insensitivity with its attendant vascular and neurological complications [1]. The chronic hyperglycemia of diabetes is associated with long term damage, dysfunction and failure of various organs [2]. The vast majority of cases of diabetes fall into two broad etiopathogenetic categories. In one category, type 1 diabetes, the cause is an absolute deficiency of insulin secretion. In the other, which is much more prevalent, type 2 diabetes, the cause is a combination of resistance to insulin action and an inadequate compensatory insulin-secretory response [1]. The number of people suffering from the disease worldwide is increasing at an alarming rate with a projected 366 million peoples likely to be diabetic by the year 2030 as against 191 million estimated in 2000 and 285 million in 2010 [3].

The current management for DM includes diet, exercise, oral anti-diabetic and insulin therapy. However, insulin and other oral hypoglycemic drugs have characteristic profile of adverse effects [4]. This has necessitated the search for safer and effective medicines for the management of the disease and novel drugs which might act in mechanistically distinct way compared to existing drug targets [5]. WHO supports the use of effective and safe remedies and accepts traditional medicine as a valuable resource for primary health care [6]. In addition, majority of the populations in developing countries still have limited access or no

access, especially those in remote areas, to modern medicines. Instead they use traditional medicines for a range of illness including diabetic complications [7-10]. According to world ethnobotanical information reports, almost 800 plants possess antidiabetic potential [11]. Medicinal plants used to treat hypoglycemic and hyperglycemic conditions are of considerable interest to ethno botanical community as they are recognized to contain valuable medicinal properties in different parts of the plant [12]. Many of these plants have been investigated. For instance, in Africa, several plants have been screened based on their usage by the traditional healers for the treatment of diabetes. Such plants include, among others, *Anacadium occidental, Piclirima netida, Bridelia ferugina, Ginburia alypua, Vernonia amygdalina* [13]. Our literature survey did not show any report on the investigation of antidiabetic activity of *Combretum dolichopetalum* (Fam: Combretaceae).

Combretaceae is a family of plants with 20 genera and 600 species of tropical and subtropical regions of the world [14]. In West Africa, the family Combretaceae is represented by 9 genera with 72 species and the genus Combretum Loefl. is the largest genus with 48 species and 8 imperfectly known species [15]. In Nigeria, the genus Combretum Loefl. is represented by 25 species which are mainly straggling shrubs or lianes [16]. *Combretum dolichopetalum* Engl. and Diels is commonly called “achicha nza’ (food of the sun-bird) in Igboland, Nigeria. The plant is a scandent shrub or forest liane of deciduous forest and in secondary re-growth in areas receiving at least 1250 mm rainfall, usually near rivers occurring from Sierra Leone to West Cameroons. The leaves are used by Igbos for burns, and in decoction as a purgative. A root extract is also taken for dysentery, and indigestion. In northern Ghana an infusion of roots, leaves and stems is administered to cattle suffering from garli (Fula), a condition of ‘stomach staggers’ [17]. The alcoholic extract of *C. dolichopetalum* is used in folklore medicine to relieve stomach ache, blood in the stools, diarrhea, cramps and related gastrointestinal disorders [18]. The aqueous root extract of the plant is used locally in the management of diabetes. Investigation into the ethanolic extract of the plant has shown that it possess gastric antisecretory activity, increasing gastric emptying time, and acting as a smooth muscle relaxant and spasmolytic agent [16,19]. The anti-inflammatory [19] and trypanocidal [20] activities of the plant have also been demonstrated. The hepatoprotective effects of the ethanolic extract of *C. dolichopetalum* root bark were evaluated on paracetamol-induced liver intoxication in rats. Oral pre-treatment with *C. dolichopetalum* ethanolic extract significantly attenuated the elevation of serum glutamate-oxaloacetate transaminase (GOT) and glutamate- pyruvate transaminase (GPT) induced by paracetamol intoxication in rats [21]. Increasing evidence in both experimental and clinical studies suggest that oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus [22]. Thus it was thought necessary to investigate the antidiabetic potentials of the root of the plant since it has shown antioxidant effect in the previous studies; moreover, it is used locally in the management of diabetes. The present study therefore, aims to assess the antidiabetic potentials of the root of *C. dolichopetalum* and to further carry out a bio-assay guided purification with the hope of eventually isolating the antidiabetic principle(s).

2. MATERIALS AND METHODS

The workflow of the present study is summarized in Fig. 1.

2.1 Plant Materials

The plant material was collected from the forests in Nsukka area of Eastern Nigeria during April, 2013, identified and authenticated by Mr. A. Ozioko, a taxonomist with the Bioresource

Development and Conservative Centre, Nsukka. The voucher specimen (Voucher number: INTERCEDD/853) has been deposited in our research laboratory for further reference. After authentication, fresh roots were collected in bulk, washed, shade dried and pulverized in a mechanical grinder to obtain coarse powder.

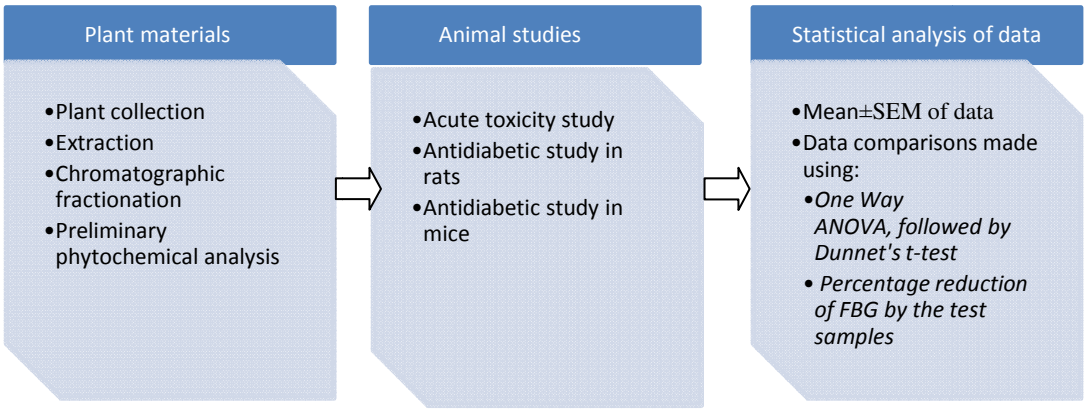

Fig. 1. A representation of the workflow of the study

2.2 Preparation of the Extract and Fractions

The powdered whole root (2000 g) was extracted with methanol by cold maceration method. This was done by soaking the dry plant material with 10 L of the solvent for 72 h at room temperature of 28±2ºC with intermittent shaking. The liquid extract was concentrated using a vacuum evaporator to afford the methanol extract (ME). 150 g of dried ME was triturated with 300 g of silica gel to increase the surface area. This was transferred into an air-tight bottle and partitioned successively with five solvents according to increasing order of polarity starting with n-hexane, chloroform, ethyl acetate, methanol to water. The collected liquid samples were concentrated to afford the corresponding fractions which were labeled HF, CF, EF, MF and AF respectively.

2.3 Column Chromatographic Fractionation of the CF

Based on the result of the animal studies, the chloroform fraction (CF) obtained above was subjected to further fractionation by using column chromatographic technique. A known quantity (5 g) of dried CF was introduced into a chromatographic column (60 cm by 4.5 cm) parked with 250 g of silica gel (mesh size 100-400). Based on the trial TLC for the choice of the best solvent system, the column was eluted by a linear gradient with the mobile phase of chloroform: methanol starting from (10:0) to (0:10). The various eluates were collected into six sub-fractions (C1-C6) based on the similarity of their spots in the TLC plate.

2.4 Animals

Swiss albino mice (20–25 g) of either sex were used for acute toxicity study and adult Wistar albino rats of average weight (Mean ±SEM) 133.96±9.08 g and mice (15.19±0.45 g) of either sex were used for the antidiabetic evaluation. The animals were kept in standard polypropylene cages at room temperature and at 60-65% relative humidity during the experimental work with 12 h day: 12 h night cycle. They were fed with normal laboratory diet

and allowed to drink water *ad libitum*. The experimental protocols were in accordance with the guidelines of the Ethics Committee of the University of Nigeria (approved ref: NHREC/05/01/2008B). The care and handling of animals was in line with the internationally accepted principles for laboratory animal use and care as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) [23].

2.5 Acute Toxicity Study

The test was carried out in two stages according to Lorke's method [24]. For the first stage, nine mice were selected and these divided into three groups of three animals in each group. The first group received 10 mg/kg (p.o.) of ME while the second and third groups of the animals received 100 mg/kg and 1000 mg/kg of the extract (ME) respectively. After dosing, the animals were kept under observation for 24 h to find out the mortality if any. No mortality was recorded in the first stage. Following Lorke's method, the three animals for the second stage received 1600, 2900 and 5000 mg/kg of the extract respectively. LD_{50} is calculated as the geometric mean of the highest dose that produced no death and the least dose that produced 100% death in the second stage. If no death is recorded in the second stage, the LD_{50} is taken as greater than the maximum dose administered.

2.6 Antidiabetic Study of the Extract and Fractions in Rats

The acclimatized rats were kept fasting for 12 h with water *ad libitum* and injected intraperitoneally with alloxan monohydrate in distilled water at a dose of 150 mg/kg. After one hour, the animals were provided with standard laboratory diet *ad libitum*. The blood glucose level was checked with a blood glucometer (Accu-Check, Roche) before and 48 h after alloxan administration by withdrawing blood from the tip of the tail of each rat under mild anesthesia. Animals were considered diabetic when the blood glucose level was raised beyond 200 mg/dl. This condition was observed at the end of 48 h after alloxan injection. The animals were segregated into 16 groups (Groups 1 to 16) of four animals in each according to the following experimental protocols:

Group 1-4: 100 mg/kg, 200 mg/kg, 400 mg/kg and 600 mg/kg respectively of ME;
Group 5 and 6: 200/kg and 400 mg/kg respectively of HF;
Group 7 and 8: 200 mg/kg and 400 mg/kg respectively of CF;
Group 9 and 10: 200 mg/kg and 400 mg/kg respectively of EF;
Group 11 and 12: 200 mg/kg and 400 mg/kg respectively of MF;
Group 13 and 14: 200 mg/kg and 400 mg/kg respectively of AF;
Group 15: Glibenclamide (0.2 mg/kg)-standard drug;
Group 16: Vehicle only (normal saline, 2 ml/kg)-control.

The various treatments were administered (p.o.) to the overnight (12 h) fasted diabetic animals. Fasting blood glucose (FBG) level was estimated at 0, 1, 3, 6 and 9 h after administration of a single dose of the various treatments.

2.7 Antidiabetic Study of Chloroform Sub-fractions in Mice

As in the case of rats above, the acclimatized mice were fasted for 12 h and subsequently injected (i.p.) with alloxan solution at a dose of 110 mg/kg body weight. Mice with blood glucose of 200 mg/dl or more after 48 h were selected for the study. The diabetes-induced mice were segregated into eight groups. Groups 1-6 were treated orally with 200 mg/kg each

of C1 to C6 respectively. Group 7 and 8 received (p.o) glibenclamide (0.2 mg/kg) and olive oil (4 ml/kg) respectively. Blood glucose was determined as described above.

2.8 Preliminary Phytochemical Screening

Standard screening test of the extract (ME) and the fractions was carried out for the various plant constituents such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins etc. following standard procedures [25,26]. The procedures are briefly described below (performed on the plant extract, fractions and sub-fractions):

2.8.1 Test for alkaloids

Preliminary test*:* A 100 mg of an alcoholic extract of the plant was dissolved in dilute hydrochloric acid and filtered. The filtrate was tested with Mayer's reagent (milky precipitate), Dragendorff's reagent (brick- red precipitate), Wagner's reagent (reddish brown precipitate) and picric acid (1%) (yellow precipitate).

Confirmatory test*:* Five grams (5 g) of the alcoholic extract was treated with 40% calcium hydroxide solution until the extract was distinctly alkaline to litmus paper, and then extracted twice with 10 ml portions of chloroform. Chloroform extracts were combined and tested for the presence of alkaloids using the above reagents.

2.8.2 Test for flavonoids

Test for free flavonoids: Five milliliters (5 ml) of ethyl acetate was added to an aqueous solution of 0.5 g of the plant extract. The mixture was shaken, allowed to settle and inspected for the production of yellow color in the organic layer which is taken as positive for free flavonoids.

Lead acetate test: To an aqueous solution of 0.5 g of the extract, about 1 ml of 10% lead acetate solution was added. Production of yellow precipitate is considered as positive for flavonoids.

Reaction with sodium hydroxide: Dilute sodium hydroxide solution was added to an aqueous solution of 0.5 g of the extract. The mixture was inspected for the production of yellow color which is considered as positive test for flavonoids.

2.8.3 Test for glycosides

Fehling's solution test: About 0.1 g of the plant extract was boiled in 5 ml of distilled water bath for 5 min. The mixture was filtered and to the filtrate, 5 ml of equal volume of Fehling's solution A and B were added and the mixtures boiled for a few minutes. A brick red precipitate indicates the presence of reducing sugars.

Hydrolysis test: Dilute sulphuric acid (5 ml) was added to 0.1 g of the plant sample and boiled for 15 min on a water bath. The mixture was cooled and neutralized with 20% potassium hydroxide solution. Then 10 ml of the mixture was tested with Fehling's solutions as above.

2.8.4 Test for saponins

Distilled water (20 ml) was added to 0.2 g of the plant sample in a 100 ml beaker. It was boiled gently on a hot water bath for 2 min. The mixtures were filtered hot, allowed to cool and the filtrate used for the following tests:

Frothing test: About 5 ml of the filtrate was diluted with 20 ml of water and shaken vigorously. A stable froth indicates the presence of saponins.

Emulsion test: To the frothing solution above was added 2 drops of olive oil and the contents shaken vigorously. The formation of emulsion indicates the presence of saponins.

2.8.5 Test for tannins

Ferric chloride test: To 3 ml of the filtrate (from saponin test above), few drops of ferric chloride were added. A greenish- black precipitate indicates the presence of tannins.

Lead Sub- acetate test: A few drops of lead sub-acetate were added to 3 ml of the filtrate. Appearance of a cream precipitate indicates the presence of tannins.

2.8.6 Test for steroids

Salkowski's test: 0.5 g of the alcoholic extract was dissolved in 2 ml chloroform in a test tube. Concentrated sulfuric acid was carefully added on the wall of the test tube to form a lower layer. A reddish brown color at the interface indicates the presence of a steroid ring (i.e. the aglycone portion of the glycoside).

Lieberman's test: 0.5 g of the alcoholic extract was dissolved in 2 ml of acetic anhydride and cooled well in an ice-bath. Concentrated sulfuric acid was then carefully added. A color change from purple to blue to green indicates the presence of a steroid nucleus i.e. aglycone portion of' the cardiac glycosides.

2.8.7 Test for terpens

Another 0.5 ml portion of the chloroform extract as in the steroid test above was evaporated to dryness on the water bath and heated with 3 ml of concentrated sulphuric acid for 10 min on the water bath. A grey color indicates the presence of terpens.

2.9 Statistical Analysis

The data obtained were subjected to One Way Analysis of Variance (ANOVA) for determining the significant difference. The significance between the various groups and the control was analyzed by post hoc using Dunnet's test (2-sided). A *P*-value < .05 was considered to be significant.

3. RESULTS AND DISCUSSION

3.1 Extractive Yield

The extractive yield of the ME and various fractions are presented in Table 1. The extract (ME) had the highest yield (12. 05%) and this was higher than the yield (6.48%) obtained by

earlier workers on the plant root using methanol as the extractive solvent [27]. Among the fractions, the more polar fractions AF (yield of 38.28%) and MF (yield of 37.52%) gave higher yield suggesting that most (75.80%) of the root extract are polar in nature. The result of the yield for the sub-fractions showed that C3 had the highest yield (19.98%), followed by C4 (18.45%) and C6 (16.43%) indicating that majority of the phytoconstituents of CF reside in these sub-fractions. However, abundance of phytoconstituent is not necessarily an evidence of biological activity but could be suggestive of that.

3.2 Acute Toxicity

The results of acute toxicity study on the ME showed no mortality at the various doses tested. This indicates that the LD_{50} is greater than 5900 mg/kg suggesting that the plant extract is quite safe. Investigation of the acute toxicity is the first step in the toxicological investigations of an unknown substance and the index of acute toxicity is the LD_{50} [24]. The value of the LD_{50} obtained from the present work formed the bases of the doses chosen for antidiabetic studies. However, our results contrast with the report of other authors who conducted toxicity studies in both sexes of rats (*i.p.*) and found LD_{50} to be 246.0 mg/kg [20]. Though our tests employed mice in the determination of LD_{50} as opposed to the rats used in the earlier report, no animal was found dead as a result of administration of the plant extract at the highest dose (600 mg/kg) we used for antidiabetic studies in rats. This further indicates the safety of the plant extract at high doses. Besides, various experimental studies have shown that LD_{50} should not be regarded as a biological constant as differing results are obtained on repetition or when the determinations are carried out in different laboratories [24,28,29].

3.3 Antidiabetic Studies

Administration of a single dose of alloxan induced diabetes in the experimental animals as evident from the marked elevation in the fasting blood glucose (FBG) of the animals from the basal values (Tables 2-4). Alloxan (2, 4, 5, 6-tetraoxypyrimidine) is a well-known diabetogenic agent widely used to induce diabetes in animals [30] by causing selective necrosis of the pancreatic islet β-cells. Alloxan and its reduction product, dialuric acid, establish a redox cycle with the formation of superoxide radicals which undergo dismutation to hydrogen peroxide. This leads to the formation of highly reactive hydroxyl radicals by Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of β-cells [31]. With alloxan, it is possible to produce different grades of severity of the disease by varying the dose of alloxan used: these may be classified by measuring FBG levels: e. g. in rabbits moderate diabetes has been defined as an FBG level of 180-250 mg/dl, and severe diabetes as an FBG level of above 250 mg/dl [32]. Thus the diabetes induced in the present study could be regarded as severe as the FBG of the animals were generally greater than 250 mg/dl. The studies on alloxan-induced diabetic rats showed that the ME generally produced a significant ($P < .05$) dose-dependent (at lower doses of 100-400 mg/kg) reduction in the FBG of the diabetic animals after 6 h of treatment when compared with the control group (Table 2). Maximum reduction of 17.07%, 21.10% and 36.78% in blood glucose was produced by the 100 mg/kg, 200 mg/kg and 400 mg/kg of ME respectively after 9 h of treatment. Though glibenclamide produced better reduction (48.18%) in the (FBG) than the 400 mg/kg of ME (36.78%), both effects are comparable as they differ from the control group by same level of significance ($P < .01$). Higher dose (600 mg/kg) of the extract failed to reduce the FBG of the animals to a significant ($P > .05$) extent, rather producing an increase

of 12.98% in 6 h (P =.744) and a reduction in FBG of only 22.86% in 9 h (P =.123) which is lower than the effect produced by 400 mg/kg of the extract. Thus at higher dose, the extract seems to possess less antidiabetic activity. This result suggests that the extract could contain other phytochemicals that might be antagonistic to the actions of the antidiabetic agent(s) present in the plant. The present observation further spurred our interest in the fractionation of the plant extract and testing of the fractions for antidiabetic activity.

Fractionation afforded HF, CF, EF, MF and AF which were tested for antidiabetic activity at two dose levels of 200 mg/kg and 400 mg/kg and the results shown in Table 3. HF at 400 mg/kg produced a significant (P = .008) reduction (55.91%) in FBG after 9 h while lower dose of the fraction did not produce any reduction in blood glucose rather there was an increment after 9 h. The CF produced a dose-dependent significant (P < .01) reduction in the FBG of the animals with reductions of 31.70 and 72.43% at 200 mg/kg and 400 mg/kg doses respectively. The blood glucose lowering effect by EF was comparable to that of the ME at similar doses. The antidiabetic activity of EF is however, lower than that of CF at the same doses. Similar pattern was also observed for the MF which produced dose-dependent non-significant (P < .05) reduction in the blood glucose (9.45% and 30.37% for 200 mg/kg and 400 mg/kg respectively) after 9 h. The AF, however, produced a dose-dependent significant (P < .01) reduction of 40.49 and 61.96% in blood glucose after 9 h at the two doses respectively. The results show that the various fractions possess antidiabetic activity, however, the activity resides more with the chloroform and aqueous fractions. It was based on this observation that the CF was chosen for further fractionation and purification. Interestingly, the aqueous fraction also showed antidiabetic activity which is in agreement with the folkloric usage of the plant in diabetes. Traditionally, the root is macerated in water and the infusion used for diabetes especially for severe diabetes.

The results of the antidiabetic studies carried out in mice with the CF sub-fractions (C1-C6) are summarized in Table 4. Mice were used for this test rather than rats (used for ME and its fractions) because the yield from these sub-fractions was low and mice generally require lower quantities of the test agents being of relatively smaller body weight. Moreover, it is thought wise to vary the type of animal used in order to further confirm the antidiabetic activity of the plant in different animals. The results clearly show that the antidiabetic activity resides mostly with C3 and possibly C6. C3 afforded the best blood glucose reduction (83.17%) after 9 h at the dose of 200 mg/kg, an effect which was significantly (P = .000) different from the control and better than 0.2 mg/kg glibenclamide (12.72%). C6 also produced a significant (P = .001) reduction (59.17%) in FBG of the mice at 200 mg/kg after 9 h. This was followed by C5 which effected a reduction of 40.91%. However, other sub-fractions did not seem to produce remarkable blood sugar reduction.

The results above have provided evidence for the antidiabetic activity of *C. dolichopetalum* root extract. Interestingly, the results indicate that partial purification of the ME increases antidiabetic activity in its various fractions. Other species in the Combretaceae family have been known to possess antidiabetic property. Chika and Bello [33] have demonstrated an antidiabetic effect for the aqueous leaf extract of *Combretum micranthum* G. Don. which produced a significant hypoglycemic and antidiabetic activity comparable to the effect of a standard drug (0.6 mg/kg glibenclamide). In addition, the antidiabetic activity of *Combretum decandrum* Roxb. (DC) in a streptozotocin-induced diabetic rats have been reported [34,35].

The effect of the active fractions and sub-fractions seem to be better than that of glibenclamide (0.2 mg/kg, a dose calculated based on maximum daily dose of 15 mg in humans). This observation provides a clue that the plant sample may be acting through

other mechanisms apart from through the release of insulin (similar to glibenclamide), since severe diabetes was induced in the animals. It has been reported that the severe diabetes produced by alloxan results in blood sugar levels equivalent to a total pancreatectomy, hence sulphonylureas, which act mainly by stimulating insulin release from β-cells, show only a small hypoglycemic effect in this instance [36]. Therefore a test plant extract producing a significant hypoglycemia (in a severely alloxan-diabetic animal) must be operating through a different mechanism [36]. Thus, it is possible that the plant, in addition to the established mechanisms of glucose-dependent insulin secretion and glucagon suppression, may be acting through other mechanisms. The delay of gastric emptying by the plant as was recorded in earlier studies [16,19] has led to the speculation that the plant may possibly have a role to play in the suppression of postprandial glucose rise. A class of the antidiabetic agents, the α-glucosidase inhibitors (such as acarbose), are known to exert their antidiabetic effect through the attenuation of the rise in postprandial glucose levels [37]. Also drugs exerting effects on postprandial glucose have been advocated as an add-on therapy to basal insulin [38]. Furthermore, reduction in hepatic gluconeogenesis and glycogenolysis (similar to the actions of metformin) are other possible mode of action of the plant. However, the detailed mechanism of the antidiabetic action of the plant was not investigated in the present work but it is necessary as a further step in understanding the actions of the plant.

3.4 Phytochemical Constituents

To determine the likely phytoconstituent(s) responsible for the observed antidiabetic activity in our present study, preliminary analysis was carried out and the results are presented in Table 5. All the tested classes of constituents, including alkaloids, saponins, glycosides, flavonoids etc are present in ME, EF and MF in medium to high concentrations. However, solvent fractionation removed many of the phytoconstituents in those of extreme non-polarity or polarity such as HF, CF and AF. In the various chloroform sub-fractions (C1-C6), steroids and terpenoids have persisted. Also CF, which showed the best reduction in blood glucose of the animals among the fractions, appears to have similar constituents with C3 which was also the best among the sub-fractions. Both contain alkaloids, flavonoids, steroids and terpenoids in a comparable concentration. The occurrence of many classes of constituents in the genus Combretum, including triterpenes, alkaloids, flavonoids, lignans and non-protein amino acids, among others have been demonstrated through phytochemical studies [34,39-41]. Several reports have shown that flavonoids, steroids, terpenoids or phenolic acids are known to be bioactive antidiabetic principles [40,42,43]. Flavonoids have been noted as one of the most numerous and widespread groups of phenolic compounds in higher plants [44]. Some of them, due to their phenolic structure, are known to be involved in the healing process of free-radical mediated diseases including diabetes [45]. Flavonoids are known to regenerate the damaged beta cells in the alloxan-induced diabetic rats and acts as insulin secretagogues [46-48]. Thus the antidiabetic activity of the *C. dolichopetalum* root may reside with any of the constituents of C3 or a combination of these constituents. However, the presence of only steroids and terpenoids in C6 (which also showed activity) suggests that a steroidal or terpenoid compound may be responsible for the observed effect. Nonetheless, further purification of the active sub-fractions could prove the type of compounds responsible for the antidiabetic effect.

Table 1. The extractive yield of the extract and various fractions of *C. dolichopetalum* root

Extract/fraction	ME	HF	CF	EF	MF	AF	C1	C2	C3	C4	C5	C6
% Yield	12.05	4.01	5.94	10.25	37.52	38.28	5.78	6.05	19.98	18.45	5.56	16.43

Table 2. Effect of the methanol extract of *C. dolichopetalum* root and glibenclamide on blood glucose of alloxan-induced diabetic rats compared with the control

Group	Treatment	Fasting Blood Glucose (FBG) concentration (mg /dl)					
		Basal	Time (h) after treatment				
		FBS (mg/dl)	0	1	3	6	9
1	ME 100 mg/kg	81.25±3.09	423.25±18.19	414.75±18.87 (2.01%)	378.75±22.33 (10.51%)	362.00±19.54* (14.47%)	351.00±19.17 (17.07%)
2	ME 200 mg/kg	66.50±8.97	415.25±40.44	390.25±38.06 (6.02%)	360.25±34.98 (13.25%)	345.25±33.33* (16.86%)	328.00±31.79 (21.01%)
3	ME 400 mg/kg	73.75±5.20	474.50±49.79	370.00±39.01* (22.02%)	362.50±38.38 (23.60%)	333.25±35.52* (29.77%)	300.00±31.97** (36.78%)
4	ME 600 mg/kg	123.25±3.15	435.25±92.27	433.25±90.72 (0.46%)	532.00±59.34 (-22.23%)	491.75±77.80 (-12.98%)	335.75±90.06 (22.86%)
5	Gliben-clamide (0.2 mg/kg)	136.00±10.53	344.00±73.24	391.25±36.87 (-13.74%)	291.00±62.19 (15.41%)	193.00±59.96*** (43.90%)	178.25±83.04** (48.18%)
6	Control	94.00±4.81	543.75±56.25	571.50±20.27	486.00±69.81	562.75±37.25	513.50±31.39

*Results expressed as Mean ±SEM (n=4). *P<0.05, **P<0.01, ***P<0.001, as compared with control group (One way, ANOVA followed by Dunnet's t-test, 2 sided). Figures in parenthesis denote percentage reduction of blood glucose from 0 h. Basal FBS=FBS before induction of diabetes.*

Table 3. Effect of the various fractions of *C. dolichopetalum* methanol root extract and glibenclamide on blood glucose of alloxan-induced diabetic rats compared with the control

Group	Treatment	Fasting Blood Glucose (FBG) concentration (mg /dl)					
		Basal	Time (h) after treatment				
		FBS (mg/dl)	0	1	3	6	9
1	HF 200 mg/kg	72.25±3.30	320.75±36.81*	389.75±36.41* (-21.51%)	333.75±40.84 (-4.05%)	543.50±56.50* (-69.68%)	346.00±84.54 (-7.87%)
2	HF 400 mg/kg	115.00±6.75	412.75±15.71	408.75±47.67* (0.97%)	390.50±47.15 (5.39%)	232.50±27.70*** (43.67%)	182.00±32.86** (55.91%)
3	CF 200 mg/kg	147.75±20.06	448.75±86.30	513.75±65.57 (-14.48%)	390.50±73.78 (12.98%)	319.25±75.80* (28.86%)	306.50±96.47 (31.70%)
4	CF 400 mg/kg	133.00±6.26	318.25±38.81	237.50±51.49*** (25.37%)	164.50±52.54* (48.31%)	114.00±22.42*** (64.18%)	87.75±8.94** (72.43%)
5	EF 200 mg/kg	95.50±2.18	420.75±60.00	419.75±36.71 (0.24%)	395.00±41.86 (6.12%)	344.50±28.05 (18.12%)	295.25±48.36 (29.83%)
6	EF 400 mg/kg	108.50±9.04	430.25±94.30	327.00±101.09* (24.00%)	300.00±104.32 (30.27%)	260.50±105.25* (39.45%)	277.00±123.14 (35.62%)
7	MF 200 mg/kg	115.75±4.27	484.25±76.56	488.75±111.25 (-0.93%)	476.75±123.25 (1.55%)	461.75±121.84 (4.65%)	438.50±114.31 (9.45%)
8	MF 400 mg/kg	96.50±4.52	535.00±38.09	426.25±57.98 (20.33%)	450.75±86.18 (15.75%)	437.00±69.08 (18.32%)	372.50±33.50 (30.37%)
9	AF 200 mg/kg	125.00±4.69	344.50±63.25	529.25±70.75 (-53.63%)	502.00±83.82 (-45.72%)	299.25±111.25 (13.13%)	205.00±56.92** (40.49%)
10	AF 400 mg/kg	131.00±6.06	403.50±75.63	446.00±90.79 (-10.53%)	327.50±78.34 (18.84%)	224.50±58.75* (44.36%)	153.50±35.53** (61.96%)
11	Gliben-clamide (0.2 mg/kg)	136.00±10.53	344.00±73.24	391.25±36.87 (-13.74%)	291.00±62.19 (15.41%)	193.00±59.96*** (43.90%)	178.25±83.04** (48.18%)
12	Control	94.00±4.81	543.75±56.25	571.50±20.27	486.00±69.81	562.75±37.25	513.50±31.39

*Results expressed as Mean ±SEM (n=4). *P<0.05, **P<0.01, ***P<0.001, as compared with control group (One way, ANOVA followed by Dunnet's t-test, 2 sided). Figures in parenthesis denote percentage reduction of blood glucose from 0 h. Basal FBS=FBS before induction of diabetes.*

Table 4. Effect of the chloroform sub-fractions of *C. dolichopetalum* methanol root extract and glibenclamide on blood glucose of alloxan-induced diabetic mice compared with the control

Group	Treatment	Fasting Blood Glucose (FBG) concentration (mg /dl)					
		Basal FBS (mg/dl)	Time (h) after treatment				
			0	1	3	6	9
1	C1 200 mg/kg	112.50±11.24	580.00±17.44	600.00±0.00 (-3.45%)	512.75±37.79 (11.59%)	443.00±43.04 (23.62%)	361.00±11.07 (37.76%)
2	C2 200 mg/kg	115.75±10.89	597.50±2.50	589.75±10.25 (1.30%)	587.50±12.50 (1.67%)	570.75±25.34 (4.48%)	486.75±37.84 (18.54%)
3	C3 200 mg/kg	95.50±21.24	600.00±0.00	595.50±4.50 (0.75%)	558.75±23.82 (6.88%)	379.25±39.23** (36.79%)	101.00±17.48*** (83.17%)
4	C4 200 mg/kg	48.50±6.40	600.00±0.00	568.00±24.18 (5.33%)	554.75±42.63 (7.54%)	504.25±43.78 (15.95%)	433.50±21.40 (27.75%)
5	C5 200 mg/kg	52.75±16.51	572.00±25.40	536.75±37.13 (6.16%)	481.50±25.67** (15.82%)	441.50±14.27** (22.81%)	338.00±40.64 (40.91%)
6	C6 200 mg/kg	50.75±7.92	600.00±0.00	591.75±4.97 (1.38%)	558.75±23.92 (6.88%)	507.50±31.83 (15.42%)	245.00±22.66** (59.17%)
7	Glibenclamide 0.2 mg/kg	98.75±5.66	597.50±1.89	590.50±6.60 (1.17%)	584.00±11.31 (2.26%)	554.00±25.78 (7.28%)	521.50±30.54** (12.72%)
8	Control (olive oil 0.4 ml/kg)	66.00±16.47	600.00±0.00	594.00±4.24	590.00±4.08	546.50±18.90	404.75±1.65

*Results expressed as Mean ±SEM (n=4). *P<0.05, **P<0.01, ***P<0.001, as compared with control group (One way, ANOVA followed by Dunnet's t-test, 2 sided). Figures in parenthesis denote percentage reduction of blood glucose from 0 h. Basal FBS=FBS before induction of diabetes.*

Table 5. Results of the phytochemical analysis of the extract and fractions of *C. dolichopetalum* root

Phytochemical	ME	HF	CF	EF	MF	AF	C1	C2	C3	C4	C5	C6
Alkaloids	+++	+	++	+++	+++	-	+	-	++	+	-	-
Glycosides	++	-	-	++	+	+	-	-	-	-	-	-
Saponins	++	-	-	+	+	+	-	-	-	-	-	-
Flavonoids	+++	+	++	+++	+	+	-	+	+	-	+	-
Tannins	++	-	-	++	++	+	-	-	-	-	-	-
Steroids	++	+	++	++	++	-	+	++	++	+	+	+
Terpenoids	+++	+	++	+++	++	-	+	+	+	+	-	+

Key: +++: High concentration, ++: Medium concentration, +: Low concentration, -: Absent

4. CONCLUSION

The current investigation has provided evidence for the safety and high antidiabetic potential of the root extract of *C. dolichopetalum*. As the extract is further purified, the antidiabetic activity increased. Through bioactivity guided fractionation, this study has demonstrated that the most active sub-fraction was C3 which was obtained from chloroform fraction of methanol root extract. The observed activity could be due to the presence of alkaloids, flavonoids, steroids or terpenoids or a combination of these. Meanwhile, research is ongoing in our laboratory to isolate and characterize the individual components of the active sub-fractions.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "principles of laboratory animal care" (NIH publication no. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this article.

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