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Investigation of Phytocomponents and Hypoglycaemic Effect of Hydro-methanolic Leaf Extract of *Cnidoscolus aconitifolius* (Spinach Tree) in Streptozotocin Induced - Diabetic Wistar Rats

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Authors' contributions

This work was carried out in collaboration with all authors. Author WII designed the study and collected the data. Author NA wrote the protocol and the first draft of the manuscript. Author NB performed the statistical analysis, interpreted and correlated that data. Authors NKU and DN managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: The potency of plants are largely due to the presence of phytochemicals contained in it; which establishes its efficacy in the treatment of health conditions like diabetes mellitus.
Aim: This study is aimed at investigating the phytocomponents and hypoglycaemic effect of hydro-

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methanolic leaf extract of *Cnidoscolus aconitifolius* leaf in Streptozotocin induced - diabetic Wistar rats.

Methods: Thirty six (36) wistar rats with average weight of 230 g, were randomly assigned into five groups of 6 each animals each. Group 1: Served as Negative control (Non-diabetic) and received normal rat chow and water; Group 2: Served as positive control group and received 10 mg/kg bw of glibenclamide; groups 3, 4 and 5 served as test, and received 100 mg/kg bw, 150 mg/kg bw and 200 mg/kg bw of *Cnidoscolus aconitifolius* leaf extract respectively orally for 28 days. After one week of acclimatization, diabetes was induced with a single intraperitoneal injection of Streptozotocin (STZ) at a dose of 60mg/kg bw.

Results: The phytochemical screening of *Cnidoscolus aconitifolius* revealed highly abundant levels of alkaloids and flavonoids, with moderate levels of tannins, phlobatannins, saponins, free anthraquinones, combined anthraquinones, terpenes, cardiac glycoside and cyanogenetic glycoside. 4.0% crude protein, 33% crude fibre, 7.0% crude fat, 3.0% ash and 6.1% muslin were revealed as the phytonutrients of the extract. The phytomineral screening of the extracts revealed 10 mg iron, 100 mg phosphorus, 0.01 mg sodium, 85mg magnesium, 20 mg potassium, 18 mg manganese, and 50 mg calcium. A dose dependent reduction in blood glucose level was observed after treatment when compared with glucose level before induction.

Discussion and Conclusion: This study revealed the ability of *C. aconitifolius* to lower blood glucose level; thereby suggesting that it could serve as a better therapy for diabetes mellitus and paving way for further investigation to identify the actual bioactive compounds responsible. This study provides novel information on the presence of muslin – an active phytonutrient, used in the arrest of bleeding in aneurysms. This might lead to the development of new excellent alternative natural remedy in cardiovascular studies.

Keywords: Cnidoscolus aconitifolius; phytocomponents; muslin; hypoglycaemic; streptozotocin (STZ).

1. INTRODUCTION

The potency of plants are largely due to the presence of phytochemicals contained in different parts of plants. These phytochemicals provides varied physiological effects and potential therapeutic properties of plants in the treatment of illnesses.

Herbal plants are being increasingly utilized to treat a wide variety of clinical diseases [1,2]. Herbs have been used by all cultures throughout history and thus, herbal medicine is the oldest form of health care known to mankind. It was an integral part of the development of modern civilization. Many drugs commonly used today are of herbal origin. Higher plants as source of medicinal compound continue to play a dominant role in maintenance of human health since antiquities [3]. The primary benefit of using plant derived-medicine is that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and affordable treatment [4]. Many phytochemicals have been identified as components of food and more are still being discovered [5,6]. Some of the phytochemicals of greater importance are plant steroids, flavonoids, tannins, glucosides, saponins and alkaloids.

Diabetes mellitus has become a common disease, very prevalent in many countries of the world affecting all ages in developing and developed nations [7]. Diabetes mellitus is a metabolic disorder in which the body does not produce or properly utilize insulin. It causes disturbance in carbohydrate, protein and lipid metabolism and complications such as retinopathy, microangiopathy and nephropathy. The commonly encountered acute and late diabetic complications are already responsible for major causes of morbidity, disability and premature death. The prevalence of diabetes mellitus in some countries has reached 1-2% of the total population and in Africa, especially in Nigeria, it is on the increase [8]. There are basically two main classes of diabetes mellitus: Type 1 (Insulin-dependent diabetes mellitus, IDDM). This starts early in life, common during childhood and always severe or fulminating. It results from deficiency of insulin due to pancreatic cell damage resulting in low plasma level of the hormone [9]. Insulin dependent diabetes mellitus require insulin therapy to control the hyperglycemia. Type II (Non-insulin dependent diabetes mellitus, NIDDM). This is the most common form of diabetes mellitus and referred to as “Adult onset diabetes mellitus” more prevalent in adult life. The plasma insulin level may be higher than in type 1, and sometimes may even be normal, but there may be insulin resistance; patients are less likely to develop ketosis [10].

Cnidoscolus aconitifolius is a perennial shrub of the Family Euphorbiaceae commonly found in the tropics. It is commonly eaten as vegetable in soup condiment in South Western Nigeria where it is called Iyana Ipaja and Efo Jerusalem. In the Niger Delta region of Nigeria it is referred to as "Hospital too far" due to its multifaceted traditional uses. It has been demonstrated to contain phenols, saponins, cardiac glycosides and Phlobatannin [11]. Chaya consumption has also become popular among Hispanic population in southern Texas Florida. The nutritional value of Chaya is very attractive when compared with spinach other common vegetable [12,13]. The plant which is also called spinach tree, has a great potential to alleviate deficits in population of developing countries as it is rich in essential amino acid, vitamin and mineral [14,15,16].

High fiber content and antibacterial activities of this plant have been reported [15]. Apart from the antibacterial activities, the ameliorative effect of *Cnidoscolus aconitifolius* on anaemia and increased erythrocyte osmotic fragility induced by protein energy malnutrition (PEM) has been reported [17]. Anti - diabetic property of *Cnidoscolus aconitifolius* in Nigeria, have been documented by [18].

Cnidoscolus aconitifolius is primarily a food plant, it has been used therapeutically for a number of ailments such as diabetes [19], arteriosclerosis, gallstone and high cholesterol. It is also believed that *Cnidoscolus aconitifolius* cleans the circulatory system, stimulate lactation, improve eyesight, strengthens nails, improve digestion, and is a diuretic and laxative agent [20].

Despite these array of uses to which parts of *Cnidoscolus aconitifolius* are put to, scanty literature is available on its antidiabetic potential and possible phytochemical (s) portending this effect. Hence, this study therefore aims at scientifically investigate the phytochemical screening and hypoglycemic effect of hydro-methanolic leaf extract of *Cnidoscolus aconitifolius* leaf in Streptozotocin induced - diabetic Wistar rats.

2. MATERIALS AND METHODS

2.1 Experimental Plants

Fresh leaves of *Cnidoscolus aconitifolius* were obtained from a local garden at Seventh Day Adventist Church, Choba, Port Harcourt, Rivers State and were correctly identified by Dr. N. E. Edwin-Wosu of the Department of Plant Science and Biotechnology, College of Natural and Applied Sciences, University of Port Harcourt, Choba, Rivers State, Nigeria, with reference number: *UPH/PSB/015*.

2.2 Extraction of Plant

The fresh leaves were air dried and pulverized with electric grinding machine into minute pieces weighing 150 g. Hydro-methanolic (1/4, v/v) extraction was carried out with soxhlet extractor (model no. 3567, Austria). The extract obtained was filtered using Whatman No 1 filter paper. The filtrate was concentrated under reduced pressure in vacuum at 45°C using rotator evaporator (GallenKamp, UK). The resulting residues was then transferred to a hot oven where they were dried to a constant weight at 45°C. The extract was stored at 4°C.

2.3 Experimental Design

Thirty six (36) Wistar rats with average weight of 230 g, were randomly assigned into five groups of 6 each animals each. Group 1: Served as Negative control (Non-diabetic) and received normal rat chow and water; Group 2: Served as positive control group and received 10 mg/kg bw of glibenclamide; groups 3, 4 and 5 served as test, and received 100 mg/kg bw, 150 mg/kg bw and 200 mg/kg bw of *Cnidoscolus acotinifolia* leaf extract respectively orally for 28 days. The choice of the respective doses of the extract was based on the report of LD_{50} of the extract conducted by [5] "Principles of laboratory animals care" (NIH publication No. 85, revised 119 (1985), were followed as well as specific national laws where applicable [21].

At the end of extract administration, animals were anaesthesized using 25% urethane (ethyl carbamate) at the dose of 0.6 ml/100g bw ip; blood samples were collected for laboratory determination of blood glucose level.

2.4 Induction of Diabetes in Rats

After one week of acclimatization, diabetes was induced with a single intraperitoneal injection of Streptozotocin (STZ) at a dose of 60 mg/kg bw, after 18 hours fast according to the method described by [22]. The Streptozotocin (STZ) was freshly dissolved in citrate buffer (0.01 M, pH 4.5) [23].The injection volume was prepared to contain 1.0 mL/kg [24]. After 5days, blood glucose levels were measure and the animals with a concentration of more than 230 mg/dL were classified as diabetic [25].

2.5 Phytochemical Screening

Preliminary phytochemical analyses of the extracts were performed as described by [26] seeking to highlight the major groups of secondary metabolites. It was evaluated for the presence of alkaloids, anthocyanin's, coumarins, anthracene derivatives, flavonoids, lignans, mono, and diterpenes, naphthoquinones, saponins, steroids and terpenoids.

2.6 Test for Alkaloids

0.5 g of the extract was stirred with an adequate amount of aqueous hydrochloric acid and filtered. 1ml of the filtrate was treated with few drops of Mayer's reagent and a second 1ml portion was treated similarly with Dragendreffs reagent. Precipitation of these reagents was taken as preliminary evidence for the presence of alkaloids in the extract. The appearance of orange or dark-coloured spot against a pale yellow background confirmed the presence of alkaloids.

2.7 Test for Tannins

0.5 g of the extract was stirred with 20 ml of distilled water, filtered and ferric chloride added to the filtrate. A blue-black, green or blue-green precipitate indicated the presence of tannins.

2.8 Test for Phlobatannins

2 ml of the extract was boiled with 1 percent (1%) aqueous hydrochloric acid. Deposition of a red precipitate shows the presence of phlobotannins.

2.9 Test for Saponins

0.5 g of the extract was added to 5% sodium carbonate (Na_2CO_3). Fehling's solution also added and boiled for about 2 minutes. The presence of brown precipitate was taken as a positive test for saponins.

2.10 Test for Flavonoids

5ml of the extract was added to 2 ml of sodium hydroxide. Dilute sulphuric acid was then added to confirm. A yellow colour which turned creamy on addition of dilute sulphuric acid, confirms the presence of flavonoids.

2.11 Test for Anthraquinones

Borntrager's test was used for the detection of anthraquinones. 5 g of the leave extract was mixed with 10 ml benzene, filtered and 5 ml of 10% ammonia solution added to the filtrate. The mixture was shaken and the presence of a pink-red colour in the ammonical phase indicated the presence of free anthraquinones while the presence of violet colour in the lower phase indicates the presence of combined anthraquinones.

2.12 Test for Terpenes

To 2 ml of the extract solution, a few drops of glacial acetic acid were added followed by a drop of concentrated sulphuric acid. A red coloration indicates the presence of terpenes.

2.13 Test for Cardiac Glycoside

0.5 g of the extracts was dissolved in 2 ml of chloroform and concentrated sulphuric acid was carefully added to form a lower layer. A reddish brown colour at the interphase indicated the presence of steroidal ring of a glycone portion of cardiac glycoside.

3. TEST FOR PHYTONUTRIENTS

3.1 Determination of Crude Protein

5.0 g of the extract was mixed with 10 ml of 0.02 mol H_2SO_4 in a digestion flask. Selenium catalyst was added to it before heating under a fume cupboard until a clear solution was obtained. The digest was diluted in a volumetric flask and used for analysis. 10 ml of the digest was mixed with equal volume of 45% NaOH solution in Kyelkdahl distillation apparatus, the mixture was distilled into 10 ml of 4% basic acid containing 3 drops of mixed indicator (methyl red). A total of 50 ml distillates was collected and titrated against 0.02 EDTA from green to a deep end point. A reagent was also digested, distilled and titrated. The content and hence protein content was calculated using the following formula.

$$\% \text{ protein} = \% N_2 \times 6.25$$

$$\% N_2 = [100/N \times W \times 14/100 \times Vt/Va] T - B$$

Where W = Weight of extract (0.5 g)
N = Normality of titrate (0.02N H_2SO_4)
Vt = Total digest volume (100 ml)
Va = Volume of digest analyzed (10 m/s)
T = Sample
B = Blank litre value

3.2 Ash Determination

This was done by the furnaces incineration gravimetric method [27]. 5.0 g of the extract was

measured into a previously weighed porcelium crucible. The sample was burnt to ashes in a muffle furnace at 550°C. The ashes was cooled in a desiccator and weighed. The weight of ashes obtained was calculated by difference and expressed as a percentage of the weight of sample analyzed below:

Ash = 100/1x w_2 – w/wt of sample

Where W_1 = weight of empty crucible
W_2 = weight of crucible + ash

3.3 Determination of Crude Fat

The solvent extraction gravimetric method [28] was used. 5.0 g of the extract was wrapped in a porous paper (Whitman filter paper) and put in a thrimble. The thrimble now put in a soxhlet reflux and mounted into a weighted extract flask containing 200 ml of petroleum ether. The upper end of the reflux flask. Soon the sample in the thrimble was covered with the solvent, which extract the oil (fat). The sample remained in contact with the solvent until the reflux flask filled up and siphoned over, carrying its oil extract down to the boiling flask. The process went on repeatedly for 4h before the defatted sample was removed, the solvent recovered and the oil extract were left in the flask. The flask containing the oil solvent was dried in the oven at 60°C for 30 mins (to remove any residual solvent). It was cooled in a desiccator and weighed. By difference, the weight of oil (fat) extract was determined and expressed as a percentage of the weight of sample analyzed and given by the expression below:

% of fat = w2 – W1/Wt of sample x100/1

Where W1 = weight of empty extraction flask
W2 = weight of flask + (fat) extract

3.4 Determination of Hypoglycemic Properties

After two weeks period of acclimatization, thirty six (36) male and female albino Wistar rats with an average weight of 230 g were randomly divided into six groups of six rats each. Group1 (non - diabetic control) received oral administration of 1 ml of water, while diabetes was induced in the remaining groups by intraperitoneal injection of 60 mg/kg of streptozotocin (STZ) (Diabetogenic agent) after starving the rats overnight. Before and after inducing diabetes, the blood glucose level of the rats was measured using single touch Accu-chek glucometer and only established diabetic rats after 72 hours were selected for treatment. Group 2 received oral administration 10 mg/kg of glibenclamide (known antidiabetic agent), group 3 (diabetic control) received only feed and water while group 4, group 5 and group 6 were orally administered with 100 mg/kg, 150 mg/kg and 200 mg/kg of the hydro-methanolic extract of *cnidoscolus acotinifolius* respectively for 28 days.

3.5 Statistical Analysis

The results were expressed as mean of 6 replicates ± standard error of mean (SEM) and were analyzed using Statistical Package for Social Sciences (SPSS) version 20.0. One way analysis of variance (ANOVA) was performed to test the effect of each dose on the parameter under investigation at 95% level of confidence. Values were considered statistically significant at ($p<0.05$).

4. RESULTS

Phytoconstituents of leaf extracts of *Cnidoscolus aconitifolius* in Wistar rats are as shown in table 1 above. The phytochemical screening of *Cnidoscolus aconitifolius* revealed highly abundant levels of alkaloids and flavonoids, with moderate levels of tannins, phlobatannins, saponins, free anthraquinones, combined anthraquinones, terpenes, cardiac glycoside and cyanogenetic glycoside. 4.0% crude protein, 33% crude fibre, 7.0% crude fat, 3.0% ash and 6.1% muslin were obtained as the phytonutrients of the extract.

The result obtained from the phytomineral screening of the leaf extracts of *Cnidoscolus aconitifolius* revealed 10 mg iron, 100 mg phosphorus, 0.01 mg sodium, 85 mg magnesium, 20 mg potassium, 18 mg manganese, and 50 mg calcium.

A statistically significant ($p<0.05$) increase was recorded in the fasting blood glucose level of negative control animals after streptozotocin inducti on when compared with the fasting blood glucose level before streptozotocin induction (table 2). Significant ($p<0.05$) increases were observed in the glucose level of groups 2 (10 mg/kg bw Glibenclamide), 3 (100 mg/kg bw *Cnidoscolus aconitifolius*, 4 (150 mg/kg bw *Cnidoscolus aconitifolius* and 5 (200 mg/kg bw *Cnidoscolus aconitifolius*) after induction of streptozotocin when compared before streptozotocin induction respectively. However,

Table 1. Results of phytoconstituents of leaf extracts of *Cnidoscolus aconitifolius* in Wistar rats

S/No	Phytochemicals	Inference	Phytonutrients	Quantity (%)	Phytominerals	Quantity (mg/g)
1.	Alkaloids	++	Crude protein	4.0	Iron	10
2.	Tannins	+	Crude fibre	33	Phosphorus	100
3.	Phlobatannins	+	Crude fat	7.0	Sodium	0.01
4.	Saponins	+	Ash	3.0	Magnesium	85
5.	Flavonoids	++	Muslin	6.1	Potassium	20
6.	Free anthraquinones	+			Manganese	18
7.	Combined anthraquinones	+			Calcium	50
8.	Terpenes	+				
9.	Cardiac glycoside					
10.	Cyanogenetic glycoside	+				

+: Moderately abundant; ++: Highly abundant.

Table 2. Mean fasting blood sugar level before and after extract administration in Wistar rats

Groups	Treatments	Fasting blood glucose level (mg/dl)		
		Before STZ induction	After STZ induction	After drug treatment
Group 1 (Negative Control)	Distilled water	75.33 ± 3.01	232.67 ± 5.89*	232.00 ± 6.85*
Group 2 (Positive Control)	10 mg/kg bw Glibenclamide	70.67 ± 2.19	252.17 ± 15.71*	214.33 ± 13.83*
Group 3	100 mg/kg of C.A	78.17 ± 3.04	220.83 ± 8.83*	206.83 ± 9.11*
Group 4	150 mg/kg of C.A	66.33 ± 1.33	242.00 ± 8.71*	205.50 ± 9.46*
Group 5	200 mg/kg of C.A	77.00 ± 3.39	242.33 ± 12.09*	172.83 ± 6.17*

*Values are expressed as mean ± SEM; n=6; *= Significant at p level less than 0.05 compared with glucose level before streptozotocin induction; C.A. = Cnidoscolus aconitifolius*

significant ($p<0.05$) reduction in the level of blood glucose level for the respective groups was recorded after treatment especially at the high dose of the extract administered.

5. DISCUSSION

The results of the present study indicate that the hydromethanolic extract of *Cnidoscolus aconitifolius* possess possible strong antioxidant activity, owing to the high presence of flavonoids which are known antioxidant. Flavonoids in plants are known for their scavenging ability as a result of the presence of hydroxyl groups contained in them. Other phytochemicals such as alkaloids, tannins, phlobatannins, saponins, free anthraquinones, combined anthraquinones, terpenes, cardiac glycoside and cyanogenetic glycoside were also shown. This is in agreement with previous reports [29,30].

The phytonutrient screening of the extract shows the presence of crude protein, crude fats, crude fibre, ash and muslin. The presence of muslin in the hydromethanolic leaf extract of *Cnidoscolus aconitifolius* revealed the possible use of the extract in the arrest of bleeding especially in aneurysms or intracranial vessels at risk of bleeding [31]. Muslin is used as a gauze to reinforce an artery and help prevent rupture. In emergency medicine, muslin may be used in the manufacture of bandage and tourniquets [32]. However, there is dearth of literatures in this regard. The dietary fibres or "roughages" revealed in the study, might help prevent constipation by increasing stool weight and transit time, hence reducing the risk of diseases and disorders such as diverticular disease or haemorroids, and may also have a protective effect on colon cancer [33].

The high amounts of phosphorus, calcium, and magnesium obtained from the phytomineral screening of the extract is in consonance with the report [34] of that the three minerals – calcium, phosphorus and magnesium accounts for about

98% of the body's mineral content by weight, especially in infants.

The hypoglycaemic potentials exhibited by this extract may be due to the presence of tannins and saponins revealed in this study. These compounds have since shown to have immense significance as antihypercholesterol, hypotensive and cardiac depressant properties and as blood glucose reducing agents [35]. This effect of the extract improved tremendously in a dose dependent fashion, with greater effect experienced at the high dose (200 mg/kg bw) administered. Thus, the high dose of the extract administered showed a better therapeutic effect compared to the standard antidiabetic drug (Glibenclamide) administered. However, the presence of dietary fibres in the phytonutrient screening of the extract might be implicated for the possible antidiabetic potential of this extract. Dietary fibres may slow digestion and absorption of carbohydrates and hence lower the rise in blood glucose [33].

6. CONCLUSION

This study revealed the ability of *C. aconitifolius* to lower blood glucose level; thereby suggesting that it could serve as a better therapy for diabetes mellitus and paving way for further investigation to identify the actual bioactive compounds responsible. This study provides novel information on the presence of muslin – an active phytonutrient, used in the arrest of bleeding in aneurysms. This might lead to the development of new excellent alternative natural remedy in cardiovascular studies.

CONSENT

It is 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

Authors have declared that no competing interests exist.

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