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Hypoglycemic Effects of the Methanolic Leaf Extract of *Tetracapidium Conophorus* In Alloxane-Induced Diabetic Rat

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ABSTRACT

Diabetes is a chronic metabolic disorder with high incidence of non-traumatic limb amputations, morbidity, and mortality. the study investigated the hypoglycemic properties of the methanolic leaf extract of *Tetracapidium conophorus* on alloxane-induced diabetic rat. Diabetes was induced in Wister rat using 150mg/kg of alloxan. Groups A and B were the test groups and received 200mg/kg and 400mg/kg of methanolic leaf extract respectively through the oral route, while Group C received glibenclamide. Group D served as the diabetic negative control. The leaf extract significantly reduced the blood sugar from 569.50±10.43 mg/dl to 368.60 ±22.93mg/dl in 2hours compared to the fasting blood sugar (FBS) level of 499.20 ± 25.4mg/dl, a positive control of 490.20 ±9.75 mg/dl and a negative control of 556.40 ± 29.69 mg/dl ($p<0.05$). In 4 hours, there was a significant reduction of blood glucose to 340.20 ±46.93mg/dl with same dose of 200mg/kg ($p<0.01$). The RBS dropped to 306.20±30.96mg/dl in 2hours and 216.00±28.58mg/dl in 4hours respectively at a dose of 400mg/kg when compared to a fasting level of 526.60 ±11.36mg/dl ($p<0.01$) and positive control of 490.20 ±7.75mg/dl. A 200mg/kg dose of the extract in sub-acute study showed a significant reduction of RBS on the 8th day with a value of 378.00 ±24.64mg/dl when compared to a negative control of 569.50 ± 10.48mg/dl. Administration of 400mg/kg caused a reduction of 262.75 ±66.58mg/dl when compared to a positive control of 462 25 ±27.51mg/dl and a negative control of 569.50 ±10.43mg/dl ($p<0.01$). The result indicated that the methanolic leaf extract of *Tetracarpidium conophorum* has hypoglycemic effects in alloxane induced diabetic rat.

Keywords: Diabetes, leaf extract, *Tetracarpidium conophorum*, rat, phytochemical constituents, blood sugar

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INTRODUCTION

Despite the global effort in the allopathic management of diabetes mellitus, this disorder still ravages humankind at an alarming rate. In developing countries, including Nigeria, most diabetic patients are finding it increasingly difficult to manage hyperglycemic condition due to high cost of synthetic anti-diabetic drugs like sulfonylurea, biguanides, and intravenous insulin injection, which have side effects [1]. This therefore calls for a biomedical research on hypoglycemic substitutes for the control of the disease from natural sources. Some plants used by the population as anti-diabetic remedies are edible plants, which have added further interest on their study because of their dual control factors as food and medicine for the management of diabetes. Some Nigeria medicinal plants have been associated with the management and control of diabetes but still, diabetes and its related complication continue to be a major medicinal problem in Nigeria [2-5]. *Tetracarpidium conophurum* also known as Ukpa (Igbo), or Asala (Yoruba) and Ekpuro (Efik and Ibibio) Karo or Ngak in Western Cameroon is a woody perennial climber commonly found in low bush especially in Africa, America, Europe, and Asia [6]. It is commonly called African walnut. It belongs to the family Euphorbiaceae. It is an economic plant widely cultivated for production of its nuts, which are used as delicacy [7]. Most of the studies on the plants have been on the nutritive value of the seeds, which is a snack and delicacy [8, 9]. The leaf extract of has been reported to possess antibacterial and antifugal activities. The leaf juice is used for treatment of prolonged and constant hiccups. The need for the development of newer hyperglycemic agents is imperative [10, 11]. This study investigated the hypglycemic effect of the crude methanolic leaf extracts of *Tetracarpidium conophorum* on alloxane induced diabetic rat.

MATERIALS AND METHOD

Animals

Forty (40) male albinos Wister rats were obtained from the Animal House of University of Nigeria Teaching Hospital (UNTH) Enugu, They were housed under standard condition of temperature ($28^oC \pm 3^oC$) with a 12 hour light/12 hour dark cycle. The animals were housed in groups and were provided with water and standard pellets (Guinea feed) at libitum. The period of acclimatization was 14 days.

Ethical considerations

The guidelines in the handling and use of animals for laboratory and experimental purposes were dully observed throughout the study 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) and the US guidelines (NIH publication #85-23, revised in

1985) [12, 29].

Plant collection and identification

The leaves of *Tetracarpidium conophorum* used for this work were collected from their natural habitat in and around Enugu between the months of April and May 2011. Prof. M.O. Nwosu of the Department of Botany, University of Nigeria, Nsukka, Enugu State, Nigeria identified a specimen of the plant. The plant was deposited at the herbarium of the Department of Botany, UNN for future reference (UNH/98^{D}).

Plant Preparation

The leaves were air dried under the shade to avoid decomposition of the phytochemcial constituents. They were dried for 2 weeks, after they were observed to be dry and brittle; they were ground into fine powder with a gasoline-powered blender. The dry powder was stored in dry condition until needed for the extraction process.

Extraction

The extracts were prepared by methanol solvent extraction. Powdered leaves of *Tetracarpidium conophorum* were weighed out (800g), placed in a gallon and 2.5 liters of 80% methanol was added and left for 48 hours. The mixture was filtered through muslin and then through a Whatman No. 1 filter paper and the filtrate was evaporated to dryness on a rotary evaporator (model 347/9, corning, London). The residue obtained was stored in a refrigerator at 4±2^{o}C until required 10g of methanol extract was dissolved in 3% aqueous suspension of Tween 80 and made up to 100ml with the solvent. This was stored in refrigerator at 4± 2^{o}C until required.

Experimental design

The male rats were divided into four (4) groups of (A-D) comprising of five rats each according to their body weight (x±20g).

Induction of diabetes in Rats

The rats were fasted for 24 hours but had access to water. They were injected with alloxan monohydrate (prepared freshly as a 5% solution of alloxan in saline. After 2 days the rats with fasting blood sugar >165mg/dl were selected for the study.

Acute study

Blood samples were collected from rats in all the groups for fasting blood sugar estimation via the tail vein. The fasting blood samples were labeled 0-hour samples. Rats in group A received 200mg/kg body weight of the methanolic extract. Group B received 400mg/kg body weight of methanolic extracts; group C got 10mg/kg body weight glibenclamide (Glanil®) through an oral cannula. Blood samples were collected from the rats in all the groups via the

tail vein at 2, 4 hours, and 8.0 hours, for glucose estimation.

Sub-acute study

Forty-eight (48) hours after the acute study, fasting blood glucose was measured in the entire groups (A-D) and subsequently 200mg/kg body weight and 400mg/kg body weight of the crude extract were given to the rats in group A and B respectively while those in group C received Glibenclamide (10mg/kg body weight). Group D rats served as negative control and received 5ml/kg bodyweight Tween 80. Fasting blood on the in all the groups through the median vein of the eye for renal function test (urea and creatinine), cholesterol, hemoglobin and platelet count.

Acute Toxicity Study

The Dietrich Lorke procedure for LD_{50} determination was used. It made the following assumptions; substances more toxic than 1mg/kg are also highly toxic that it is not important to calculate LD_{50}. LD_{50} greater than 5000mg/kg are of no practical interest. An approximate figure for the LD_{50} adequate to estimate the risk of acute intoxication was employed using three different doses of the extract, in an increasing order. One the first day they received 100mg/kg METC. The animals were observed for 24hrs and the number of deaths recorded. On the second day they received 1000mg/kg METC and were observed for 48hrs. On the third day, the animals received 2000mg/kg of the extract and were observed for another 48hrs.

Method of urea measurement

Method: Diacetyl monoxime method with protein precipitation [13].

Principle:

In hot acidic medium, diacetyle monoxime is hydrolyzed to diacetyle and hydrolamine. Diacetyle condenses with urea to produce a pink color product diazine; the intensity of which color is proportional to the concentration of urea in the medium. This semicarbazine and ferric iron are added to intensify and stabilize the color.

Method for measurement of creatinine

Method: Jaffe reaction [14]

Principle

Creatinine reacts with picric acid in an alkaline medium to form an orange-red color complex (creatinine-picrate complex). The absorbance of the orange-red color produced is measured in a colorimeter using a blue green filter or in a spectrophotometer at 490nm wavelength.

Method of cholesterol estimation

Method: Enzymatic colometric method [15]

Principle:

Cholestersyl ester in the serum is hydrolyzed by an enzyme cholesterol esterase to produce cholesterol and fatty acids. The hydrolyzed cholesterol in the presence of oxygen is converted to cholestenone and hydrogen peroxide formed then reacts with 4-aminoantipyrine and 3, 5 dichorohenoil in the presence of on enzyme peroxidase to produce coloured derivative and water molecule.

Cholesteryl ester **cholesterol esterase** Cholesterol + fatty acid (Eqn. 1)

Cholestrol + H20 + 0 **cholesterol oxidase** Cholestenone + H_20_2, (Eqn. 2)

H_20_2 + 4 amnoantipyrine + 3, 5, - Dichlorophenol **Peroxidase** coloured qunonic derivatives + $4H_20$ (Eqn. 3)

Haemoglobin Estimation

Cyanmethaemoglobin method was used

Principle

This method works on the principle that haemoglobin in the presence of reagent containing potassium ferricyanide, or potassium dihydrogen phosphate form the ferricyanide forms of met haemoglobin which is converted to cyanmethaemoglobin by the cyanide. All forms of haemoglobin except sulphaemoglobin are converted to cyanmethoaemoglobin.

Estimate of platelet count

Principle: Blood is diluted 1 in 20 in a filtrate solution for ammonium oxalate reagent which lyses red cell and thus leaving only the platelets in this solution. Platelets are then counted microscopically using an improved neubauer- ruled counting chamber and the number of platelets per litre of bloods calculated.

Phytochemcial analysis

Preliminary phytocheical tests was carried out on the crude extract of Tetracarpidium conophorum using standard reagents and procedures.

Test for carbohydrate "A"

About 0.1g of the extract was boiled with 2ml of water filtered. To the filtrate, 2 drops of napthol solution in ethanol (Molisch's reagent) was added. Concentrated sulphuric acid was gently poured down the side of the test tubes to form a lower layer. A purple interracial ring indicated the presence of carbohydrate.

A_1 Test for reducing sugar

About 0.1g of the extract was shaken vigorously with 5ml of distilled water and filtered. The filtrate was used for the following test:

Fehling test:

To 1ml portion of the filtrate was added equal volumes of feelings of solution 1 and 2 and soil on water bath far few minutes. A brick real precipitate indicates the presence of reducing sugar.

Benedict's test:

To 1ml of portion of the filtrate was added 2ml of reagent the mixture was shaken and heated a water bath for 5mins. A rusty brown precipitate indicates the presence of reducing sugar.

TEST FOR ALKALOIDS

General test

20ml of 5% sulphuric acid in 50% ethanol was added to 2g of the powered material and heated on a boiling water bath for 10 mins and was filtered. 2ml of the filtrate was tested with 2 drops of Mayer's reagent, Dragendoff's reagent, Wagner's reagent, picric acid solution (1%).

The remaining filtrate was placed in 100ml separating funnel and made alkaline with dilute ammonia solution. The aqueous alkaline solution was separated and extracted with 5mlportion of dilute sulphuric acid. The extract was tested in a few drops of Mayer's wagners. Dragendoff's reagent alkaloid gives.

(i) Milky precipitate with one drop of Wagner's

(ii) Reddish brown precipitate with one drop of Wagner's

(iii) Yellow precipitate with one drop of picric acid reagent.

(iv) Brikc and precipitate with one drop of Dragendoff's reagent

TEST FOR GLYCOSIDE

About 5ml dilute sulphuric acid were added to about 0.1kg of the extract in a test tube and asocial fun 15mins once water both then cooked and neutralized with 20% potassium hydroxide solution. 10ml of a mixture of equal parts of Fehling, solution 1 and 2 was added and boiled for 5mins. A more dense bride red precipitate indicates the presence of glycosides.

Frothing test:

About 5ml of eh filtrate was diluted with 20ml of water and shaken vigorously. As stable froth (foam) upon standing indicates the presence of saponins.

Emulsion tests:

To the frothing solution was added drop of olive oil and content shaken vigorously the formation of emulsion indicates the presence of saponins.

TEST FOR TANNINS

About 0.1g of the extract material was boiled with 50ml of water filtered and used for the

following test.

Lead subacetate test:

Few drop of lead subacetate test was added to 3ml of filtrate. A cream precipitate appearing would interfere with the presence of tannins.

TEST OF FLAVONOIDS

10ml of ethyl acetate were added to about 0.2g of the extract and leaked on a water bath for 3mins. The mixture was cooled, filtered and the filtrate sued for the following test:

Ammonium test:

About 4ml of filtrate was shaken with m1 of the dilute ammonia solution. The layer were allowed to separate and the yellow color in the ammonical layer indicates the presence of flavonoids.

TEST FOR RESINS

Precipitation test

Approximately 0.2g of the extract material was extracted with 15ml 96% ethanol. The alcoholic extracted with 15ml 96% ethanol. The alcoholic extract was then poured into 29ml of distilled water in baker. Precipitate formation indicated the presence of resin.

TEST FOR PROTEINS

Millions test: To a little portion of the extract in the test tube, two drops of millions reagent were added. A white precipitate indicates the presence of protein.

Xanthoprotic reaction test: About 5ml of the extract was heated with few drops of concentrated nitric acid. A yellow colour which changes to orange on addition of an alkali indicate the presence of proteins.

TEST FOR OILS

About 0.1g of the extract was passed between filter paper and the paper observed. Translucency of the filter paper indicates the presence of oils.

About 9ml of ethanol were added to 1g of the extract and refluxing for a few minutes and then filtered. The filtrate was concentrated to 2-5ml on boiling water bath, about 5ml of hot water was added. The mixture was allowed to stand for 1 hour and the waxy master filtered off. The filtrate was extracted with 2.5ml of chloroform using separating funnel. To 0.5ml of the chloroform extract in a tube was added 1ml of concentrated sulphuric acid to form a lower layer. A reddish brown interface shows the presence of steroids.

TEST FOR TREPENOIDS

Another 0.5m of the chloroform extract was evaporated to dryness on a water bath and heated which ml of concentrated sulphuric acid for 10mins on a water bath. A grey color indicates

the presence of trepenoids.

TEST FOR ACIDIC COMPOUND

The crude extract 0.1g was placed in a clear dry test tube and sufficient water was added. The content was warmed in hot water bath and then cooled. A piece of litmus paper soaked in water was dipped into the filtrate and the colour change on the litmus paper was observed.

Statistical Analysis

Result was expressed where appropriate as mean ± Standard error of mean statistical differences between means was determined by the one way analysis of variance followed by tukey and post-hoc comparison. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Table 1: Result of preliminary photochemical analysis

Constituent	Inférence
Flavonoids	+++
Antraquinone glycosides	-
Anthracene glycosides	+++
Alkaloids	-
Tannins	++
Saponins	-
Resins	++
Proteins	+++
Carbohydrate	+
Reducing sugars	+++
Hydrolysis test for glycoside	++
Hydrolysis test for glycoside	++
Cyano-genetic glycosides	-
Fat and oils	-
Steroids	+
Acidic compounds	+++
Cardiac glycoside	-

Key: + Present, ++ moderately present, +++ abundantly present – absent

Table 2: Acute effect of crude methanol leaf extract of *Tetracarpidium conophorum* on blood glucose level of alloxane induced diabetic rats

Groups	0hrs	2hrs	4hrs	8hrs	F. ratio	P-value
A 200mg/kg	499.20±25.42	368.60±22.93[a,d,h]	340.20±36.71[b,h]	525.20±21.91	11.40	p<0.0008*
B 400mg/kg	526.60±11.36	306.20±30.96[c,e,h]	216.00±28.58[c,d,h]	530.40±26.08	38.99	p<0.0003*
C Positive control 10mg/kg	535.20±11.53	490.20±9.75	365.00[b,f]±8.63	533.00±12.56	6.23	p<0.001*
D Negative control	491.00±15.35	556.40±29.69	592.00[b,f]±8.63	533.00±12.56	5.32	p<0.05*
F-ratio	1.58	20.97	22.11	1.81		
P – value	p<0.06	p<0.00061	p<0.0042	p<0.09		

F: factor, p: level of significance, * statistical significance

Table 3: The effect of sub acute administration of crude and methanol leaf extract of *Tetracarpidium conophorum* on some biochemical and haematological parameter

Group	4th day RBS	7th day RBS	Urea mmol/l	Creatinine µmol/l	Cholesterol mmol/l	Hemoglobin g/dl	Platelet count x10/1
A 200mg/kg	561.00±16.71	378.00± 24.64[d]	17.83±2.83	34.48±2.83	1.80±0.04	13.02±0.87	278.75±20.61
B 400mg/kg	531.00±13.08	262.75±66.58 [b,e]	9.43d±0.23	43.75± 7.45	2.15±0.03[c,f]	15.17b±0.07b	405.00±102.64
C 10mg/kg Positive control	516.50±18.96	462.25±27.51	11.85±2.06[d]	27.95±5.9[d]	1.32±0.11	11.3e±0.3[e]	387.00±33.19
D Negative control	553.25±13.62	569.50±10.43	22.65±4.59	62.53±9.99 [d]	1.57±0.10	14.47±0.41b	473.50±77.51
F- ratio	1.67	11.43	5.49	4.69	19.31	10.18	1.44
P – value	p<0.035	p<0.0058	p<0.05	p<0.064	p<0.00041	p<0.01	p<0.05

RBS: Random Blood Sugar, F: factor

DISCUSSION

Plants in traditional herbal medicine are known for their hypoglycemic activities and available literature indicates that there are more than 800 plant species showing hypoglycemic activity [16]. Numerous studies demonstrated that a variety of plant extracts effectively lowered the glucose level in alloxan induced diabetic animals [17- 20.] Alloxan, a ß-cytoutoxin, induces diabetes by damaging insulin secreting ß-cell of the pancreas, resulting in decreased endogenous insulin release. Alloxan administered rats become hyperglycemia in a short period followed by hepatic glucose over production [21]. The result obtained from this study showed that methanol extract of *Tetracarpidium conophorum* had anti diabetic effect, increased a hemoglobin level, lowered blood creatinine, and urea in diabetic rats. It significantly lowered blood glucose n rats that received 200mg/kg and 400mg/kg of extract when compared with respective fasting blood glucose levels. The mechanism of action of anti-hyperglycemic effect of the extract was not explored in this study but we found its activity comparable to the anti-hyperglycemic action of glibenclamide, which acts by stimulating insulin release and inhibition of glucagon secretion [22]. Some medicinal plants with hypoglycemic properties are known to increase circulating insulin levels in normal glycemic rats [23]. The preliminary identification of alkaloids, glycosides and their co-existence with phenolic acids may explain least in part some anti-diabetic properties of the extract. The observed anti-hyperglycemic activity was in agreement with the findings of a study, which showed that ethanolic seed extract of *Tetracarpidium conophorum* decreased blood glucose level in diabetic rats [24].

Furthermore, diabetes mellitus causes renal damage due to abnormal glucose regulation, including elevated glucose, glycosylated protein tissue levels, ahemodynamic changes within the kidney tissue and increased oxidative stress [25]. The allxoan induced diabetic negative control group exhibited significant elevation in serum creatinine and urea level indicative of impaired renal function of diabetic animals [26]. Thus, it would appear that the methanol extract of *Tetracarpidium conophorum* (METC) lowered the plasma urea and creatinine level probably by reversing aetiopatholgical factor in the development of diabetic nephropathy such as tight glycemic control and consequently decreased non-enzymatic protein glycosylation; improved renal hemodynamic and attenuation of oxidative stress. The total haemoglobin level decreased in diabetic animals. During diabetes mellitus, the excess glucose present in the blood led to glycation of the tissues proteins.[27.] Consequently, renal tissues may also undergo glycosylation and lose function. The kidney play critical role in erythropoiesis through elaboration erythropoietin. Therefore, any disease of the kidney will result in failure to perform this haemopoietic activity and consequently anaemia results.

Administration of methanol extract of *Tetracarpidium conophorum* (METC) increased the level of total haemoglobin and this might be due to decreased level of blood glucose. During diabetes mellitus, there is an abnormally high concentration of serum lipids mainly due to an increase in mobilization of free acid from peripheral fat depots since insulin inhibits the hormone sensitive lipase. The marked hyperlipidemia that characterizes the diabetes state may therefore be regarded because of the uninhibited action of lipolytic hormones on fat depot [28]. Excess of fatty acid in plasma produced by alloxan promotes the liver conversion of some fatty acid to phospholipids and cholesterol. Oral administration of methaolic extract of *Tetracarpidium conophorum* (METC) increased the cholesterol levels of the diabetic rats, which might be due to increased action of hormones.

CONCLUSION

The results obtained indicated that the crude leaf extract of *Tetracarpidium conophorum* caused reduction in random blood sugar, increase in haemoglobin level and prevented hyperglycemia associated alloxan.

AUTHORS' CONTRIBUTIONS

OC, OBO, and AF, designed the work, searched for the materials, and drafted the initial manuscript, and all the authors contributed to manuscript revisions towards the final version. OBO and AF provided additional content and editorial review assistance to the final manuscript while OBO and ICC typed and formatted the final manuscript. All the authors proofread and approved the final manuscript.

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