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Effect of ethyl acetate fraction of *Costus afer* on glycaemic control and essential haematological and biochemical indices of streptozotocin-induced diabetic rats

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ABSTRACT

Introduction: Diabetes mellitus is a common global cause of sudden unpredictable death if undiagnosed and untreated. *Costus afer* (Costaceae) is a tropical plant with quite a lot of pharmacological properties. This study investigated the prophylactic and antidiabetic properties of ethyl acetate fraction of *C. afer* in streptozotocin-induced diabetic rats.
Methods: Acute toxicity (LD_{50}) test was done using Lorke's method. Haematological indices were determined using haematology autoanalyser. The biochemical assays were done using standard diagnostic methods.
Results: The lethal dose was 3807.9 mg/kg. There was a significant reduction ($P<0.05$) in the fasting blood glucose concentration from week one to week four in the group that was pre-treated and later post-treated with 200 mg/kg body weight (bw) of the ethyl acetate fraction of *C. afer* leaves compared with the untreated diabetic control. The result of the hematological parameters revealed a significant increase ($P<0.05$) in the hemoglobin, packed cell volume (PCV), and platelet count of the group pretreated and treated with 200 mg/kg of the fraction compared with the untreated diabetic control. The result of the biochemical assays revealed a far much better recovery from the disruptions caused by the induction of experimental diabetes as seen in the groups that were initially pretreated with 100 and 200 mg/kg before the induction of diabetes compared with the groups treated with the same dose but without pretreatment.
Conclusion: *C. afer* might be used for the prevention and management of diabetes mellitus. Its safety is evidenced by its effects on the hematological and biochemical indices.

Implication for health policy/practice/research/medical education:
Costus afer is a complementary and alternative herbal medicine that possesses antidiabetogenic and antidiabetic potentials. The mechanism of action probably involves its ability to decrease the activity of the alpha-amylase enzymes and trigger the secretion of insulin. This research reveals the promising prophylactic and antidiabetic prospects of *C. afer* in rats. Therefore, this plant might be considered as a substitute to enhance general medical care for diabetics and diabetes-prone individuals, increase life expectancy, and reduce the overall cost of treating or managing diabetes.
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Introduction

Diabetes mellitus is one of the lifelong chronic disorders affecting the populace in many parts of the world. Diabetes mellitus can be defined as a chronic disease that affects the body's ability to metabolize and utilize the energy obtained from food sources. It is an endocrine, nutritional, and metabolic disease affecting the human body. It is characterized by glucose intolerance, excess glycogen, lipoprotein abnormalities, and defect in oxidative stress moping enzymes (1). Individuals with diabetes mellitus

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are most likely to be infected with COVID-19 and are at a high risk of death from COVID-19. Several studies have reported the most common comorbidities in COVID-19 patients having diabetes mellitus and hypertension (2,3). Diabetes mellitus impairs phagocytic cells and neutrophil chemotaxis (4). It also doubles the risks of heart failure in diabetic patients compared in non-diabetic patients (5). The three major classes of diabetes mellitus are type 1 diabetes mellitus, type 2 diabetes mellitus, and gestational diabetes mellitus. Type 1 diabetes mellitus is caused by the destruction of the autoimmune beta-cells of the pancreas and consequently absolute deficiency of insulin or its insufficiency. Insulin injections are recommended for type 1 diabetes patients and their blood glucose levels are expected to be checked regularly. Some of the features include hyperglycemia, rapid utilization of fats for cholesterol synthesis by the liver, and depletion of body protein. Type 2 diabetes is caused because of insulin resistance by the organs or tissues (6); there is also associated beta-cell dysfunction (7). Treatment with insulin is the ideal treatment, especially when lifestyle modification fails in glycemic control.

According to the American Diabetes Association 2014, about 90 to 95% of diabetes mellitus cases are type 2. A procedural review and statistical analysis that combines the results of multiple scientific studies revealed that type 2 diabetes mellitus increases the risks of non-alcoholic steatohepatitis and non-alcoholic fatty liver impairment. It increases the development processes of non-alcoholic fatty liver disease (8). Gestational diabetes mellitus (GDM) occurs mostly in pregnancy in the second or third trimester, whereby the woman is unable to metabolize glucose (9). It normalizes immediately after delivery. Both the mother and the newborn are at risk of cardio-metabolic disorder. Other complications of GDM include obesity and being overweight for both mother and child (10). About one-third of women with GDM develop postpartum depression (11).

Diabetes mellitus has affected not only lives and communities but also the economy of countries (12). In 2014, $612 billion to $1099 billion was accounted for the health expenditures of diabetes mellitus annually, with North America, the Caribbean Region, and Europe responsible for over 69% of the costs, and Africa about 10% of the costs (13). About $294.6 billion was accounted for the health expenditure in the United States of America, $109 billion and 52.3 billion for China and Brazil, respectively. Diabetes-related expenditure in women and men was $382.6 and $377.6 billion, respectively, in 2019 (14). According to Dall et al (15), the economic expenditures on diabetes mellitus in 2017 were: for diagnosed diabetes – $327.2 billion, undiagnosed diabetes – $31.7 billion, prediabetes – $43.4 billion, and gestational diabetes mellitus – $1.6 billion.

Globally 9.3% of the total population suffers from diabetes; by 2030 and 2045, it has been estimated to rise to 10.2% and 10.9%, respectively. The number of cases increases more in urban settlement (10.8%) than in rural settlements (7.2%). It's prevalence in developed countries (10.4%) is higher than those in underdeveloped countries (4.0%) (16). About 14% of adults in America has either diabetes mellitus or impaired fasting glucose levels (17). A study carried out in Katsina state Nigeria, stated that the prevalence of diabetes was 3.3% and 60% of the individuals were unaware of their health status (18).

It has been reported that several herbal plants possess antidiabetic (19,20,21) and antihyperglycemic activities with experimental proofs (22). *Costus afer,* of the family Costaceae, is found in the moist and sometimes shady forests, including riverbanks of tropical West Africa. It is a tall perennial herbaceous and unbranched tropical plant with a creeping rhizome. It is usually called ginger lily or bush cane. The southeastern (Igbo) part of Nigeria calls it Okpete or Okpoto and the northern (Hausa) part of Nigeria calls it Kakizawa. The Western part (Yoruba) of Nigeria it is called Irene omode and Mbritem in Efik (23).

The high cost of conventional drugs and their attendant side effects has hindered the treatment of diabetes; thus, attention is geared towards the use of medicinal plants with reduced or no side effects at all for the treatment and management of diabetes mellitus. Although there are studies on the antidiabetic potentials of *C. afer*, there is a need to investigate the capability of the ethyl acetate fraction to prevent or delay the onset of diabetes mellitus or enhance its treatment. Also, the safety, as evidenced by its effect on hematological and biochemical indices of streptozotocin-induced diabetic rats has not been investigated. This research, therefore, primarily evaluated the use of *C. afer* for glycemic control in streptozotocin-induced diabetic rats. In addition, its safety was assessed through the assay of the associated hematological and biochemical indices. This is important because there is a need to add to the currently available modalities of management of diabetes mellitus to reduce its incidence and consequent morbidity and mortality.

Materials and Methods

Sample collection and authentication

The leaves of *C. afer* were freshly collected at 3:30 PM at Obinagu, Udi L.G.A. of Enugu State, Nigeria and identified by a taxonomist in the Faculty of Biosciences, Nnamdi Azikiwe University, Awka. The voucher number, deposited at the herbarium of the Department of Botany, Nnamdi Azikiwe University, is NAU/H/166.

Preparation of crude ethanol leaf extract of *Costus afer*

The leaves were properly washed, dried at room temperature for three weeks, and ground into a fine powder using a corona manual grinder. Two kg of the pulverized leaf powder was soaked in 5 L of 70% ethanol for 24 hours for complete extraction. The ethanol mixture was sieved using a muslin cloth and thereafter filtered

with a Whatman no 1 filter paper. The filtrate was concentrated using a water bath at 50°C. The biological yield of the extract after extraction was 118.2 g. The ethanol extract was put in universal bottles and preserved in the refrigerator.

Fractionation of crude ethanol leaf extract of *Costus afer*

The fractionation of the crude ethanol leaf extract (118.2 g) was done using the method as described by Wu et al (24). This method involved extraction by increasing polarity with n-hexane, chloroform, ethyl acetate, n-butanol, and water. Twenty-five grams of the ethanol extract was solubilized in 250 mL of Methanol/Water mixture at the ratio of 1:1 and shaken with n-hexane (2 × 250 mL). The combined mixture of the extract and solvent was left to dry on the bench producing an n-hexane fraction. The methanol (MeOH) was fractionated further with chloroform (1 × 250 mL), ethyl acetate (2 × 200 mL), and n-butanol (1 × 250 mL). Each fraction obtained was left to evaporate to dryness on the bench. After the evaporation, the yield obtained was as follows: n-hexane fraction (4.02 g), chloroform fraction (8.58 g), ethyl acetate fraction (5.77 g), n-butanol fraction (3.93 g), and water fraction (2.70 g). The fractions obtained were stoppered in a universal container and preserved in a refrigerator. The ethyl acetate fraction was thereafter prepared by solubilizing in a measured quantity of distilled water before administration.

Experimental animals

Forty-eight male rats of Wistar strain of 10-12 weeks old (weighing 150 ± 10 g), bred in the animal house of Chris Experimental Animal Farms, were purchased and used for the study. Thirteen rats were used for acute toxicity study, while the remaining 35 were used for diabetes studies. The rats were maintained in sizeable aluminum cages in the Department of Applied Biochemistry Laboratory, Nnamdi Azikiwe University, Awka, under standard environmental conditions (12/12-hour light/dark cycle) with free access to rat feed and water. The animals were acclimatized for a period of two weeks before being used for the research. The cage beddings, feed trough, and water bottles were cleaned daily throughout the period of the experiment.

Acute oral toxicity assessment (LD_{50})

Acute toxicity testing was done following modified Lorke's method (25). The rats were observed and monitored continuously for ten hours and then periodically for up to 24 hours for signs of toxicity like drowsiness, restlessness, convulsions, and mortality. They were further observed for additional 13 days making a total of 14 days. The results show whether a substance is very toxic, toxic, less toxic, or only slightly toxic. The oral acute toxicity study involved two important phases. The initial phase involved the administration of low doses (10, 100, and 1000 mg/kg bw) to the rats. From the outcome of the initial phase, the second phase involved administering high doses (1600, 2900, and 5000 mg/kg bw) to the rats and observing them for mild or severe signs and symptoms of toxicity and reporting them appropriately.

First phase of the acute toxicity (LD*50*) study

The animals were weighed and sorted into 3 groups (n=3) as follows:

- Group A: 10 mg/kg bw of *C. afer* ethyl acetate fraction
- Group B: 100 mg/kg bw of *C. afer* ethyl acetate fraction
- Group C: 1000 mg/kg bw of *C. afer* ethyl acetate fraction

Second phase of the acute toxicity (LD50) study

The animals were weighed and sorted into 3 groups (n=1), as follows:

- Group D – 1600 mg/kg bw of *C. afer* ethyl acetate fraction
- Group E – 2900 mg/kg bw of *C. afer* ethyl acetate fraction
- Group F – 5000 mg/kg bw of *C. afer* ethyl acetate fraction

$$LD_{50} = \sqrt{HNLD \times LLD}$$

Where LD_{50} = Lethal dose; HNLD = Highest non-lethal dose; LLD = Least lethal dose.

Animal grouping and induction of diabetes

A total of 35 male albino rats of the Wistar strain were grouped into seven groups (A-G) of five rats, each as follows:

- Group A: Normal control rats
- Group B: Negative control rats (Diabetic untreated)
- Group C: Diabetic rats given 100 mg/kg bw metformin)
- Group D: Pretreatment + Treatment with 100 mg/kg bw ethyl acetate fraction of *C. afer*
- Group E: Pretreatment + Treatment with 200 mg/kg bw ethyl acetate fraction of *C. afer*
- Group F: Treatment only with 100 mg/kg bw ethyl acetate fraction of *C. afer*
- Group G: Treatment only with 200 mg/kg bw ethyl acetate fraction of *C. afer*

Induction of diabetes mellitus

Diabetes was induced in the rats after the pretreatment period. The rats were fasted for 16 hours but with free access to water after which they were given 50 mg/kg of streptozotocin injected intraperitoneally (26). Hypoglycemia was prevented 2 hours after the injection of streptozotocin by orally giving the rats 5 mL each of 5% glucose solution. After 48 hours of the streptozotocin injection, blood was collected *orbito-rectally,* and the fasting blood glucose concentrations were determined. The rats with fasting blood glucose concentrations greater

or equal to 200 mg/dL were labelled diabetic (27).

Group D and E were orally pretreated daily with 100 and 200 mg/kg of ethyl acetate fraction of *C. afer,* respectively, for a period of 4 weeks before the induction of diabetes. After the induction of diabetes in all groups except group A (normal control), group B was left untreated, group C was treated daily with 100 mg/kg of metformin. Treatment with the extract at varying doses of 100 and 200 mg/kg was carried out daily for groups D-G for a period of four weeks. Fasting blood glucose concentration was determined before and after induction of diabetes and was determined at weekly interval during the period of pretreatment and treatment until the completion of the experiment. At the completion of eight weeks (4 weeks pre-treatment + 4 weeks post-treatment), blood was collected by tail puncture and fasting blood glucose concentrations were again determined. Blood collection was done by ocular puncture into ethylenediaminetetraacetic acid (EDTA) bottles and plain bottles for hematological and biochemical assays respectively before sacrificing the animals.

Hematological analysis

Hematological indices were analyzed in the blood using Mindray BC-5300. The hematological parameters that were analyzed include red blood cells (RBCs), white blood cells (WBCs), hemoglobin (Hb), packed cell volume (PCV), and platelets (PLT).

Lipid profile test

Serum biochemical indices estimated for lipid profile which were analyzed include total cholesterol, triglycerides, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol (LDL-c), and very low-density lipoprotein-cholesterol. These parameters were analyzed following the procedure according to the manufacturer's instructions provided in the Randox Test Kit manual (28,29). LDL-c was calculated using a standard formula (30).

Liver function test analysis

Biochemical indices mostly estimated in the serum for liver functions were analyzed. They are alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct and total bilirubin. The procedures used were according to the manufacturer's instruction as stated in Randox diagnostic test kits.

Lipid peroxidation analysis

The thiobarbituric acid-reacting substances (TBARS) assay method of Buege and Aust (31) was used to determine the lipid peroxidation. The optical density was recorded at 532 nm against a reagent blank containing distilled water.

The lipid peroxidation activity was calculated using the formula:

$$\frac{\text{Optical density}}{\text{Time}} \times \frac{\text{Extinction co-efficient}}{\text{Amount of sample}}$$

Extinction coefficient value = $1.56 \times 10^{-5} M^{-1} CM^{-1}$

The unit is umol/MDA/mg of protein.

Alpha-amylase activity

The inhibitory effect of the extract on the alpha-amylase enzyme was assayed using Alpha-amylase (Single Reagent-GALG2-CNP) Test Kit. The extinction was measured by spectrophotometry at 405 nm and the results are proportional to the activity of alpha-amylase present in the sample.

Calculation:

$\Delta E = A2 - A1$ Alpha amylase (U/L) = $\Delta E \times 765$.

Where ΔE = Change in enzyme activity; A1 = First reading of the enzyme activity; A2 = Second reading of the enzyme activity.

Insulin assay

The regeneration of the β-cells by the extract and production of insulin was assayed using an Insulin enzyme immunoassay test kit. Insulin Quantitative Test Kit is based on a solid phase enzyme-linked immunosorbent assay. The optical density was read within 15 minutes at 450 nm with a microtiter plate reader.

Statistical analysis

The data was statistically analyzed using the Statistical Package for Social Sciences software for windows, version 25 SPSS Inc., Chicago, Illinois, USA. One-way ANOVA Ttests were used to determine if a significant difference exists between the means of the test and control groups. The limit of significance was adjusted at $P < 0.05$.

Results

Results of the acute toxicity (LD_{50}) studies

The animals that were administered low doses of ethyl acetate fraction of *C. afer* survived after 24 hours and then 14 days of the administration without any sign or symptoms of toxicity such as drowsiness, restlessness, or mortality. Another set of rats was administered high doses of *C. afer*. The rat that was administered 1600 mg/kg of ethyl acetate fraction of *C. afer* was observed to be partly weak, while the rat that was administered 2900 mg/kg was observed to be very weak. Within 24 hours of administration of 5000 mg/kg of the fraction, death was recorded with observable signs of toxicity. The LD_{50} was calculated to be 3807.9 mg/kg bw.

$$LD_{50} = \sqrt{HNLD \times LLD}$$

Where LD_{50} = Lethal dose; HNLD = Highest non-lethal dose; LLD = Least lethal dose.

$$LD_{50} = \sqrt{2900 \times 5000}$$

LD_{50} = 3807.9 mg/kg bodyweight

Fasting blood glucose concentration pre- and post-induction of Diabetes mellitus and with or without treatment

The fasting blood glucose concentration of groups D and E remained normal during the four weeks pretreatment period (Table 1). After the four weeks of pretreatment, diabetes was induced, and the glucose levels were checked and recorded as week 0 before the commencement of treatment. The rats in groups B to G were treated for a period of four weeks and fasting blood glucose concentrations were recorded on a weekly basis (Table 1). Significant reduction ($P<0.05$) in the fasting blood glucose concentrations was recorded in groups D, E, and G compared with group B (diabetic-untreated control) within the first week of treatment. However, a much better reduction was noticed in group E (200 mg/kg of *C. afer* extract). In the second week of treatment a significant reduction ($P<0.05$) was observed in groups E and G compared with group B (the diabetic-untreated control) within the same week. In weeks three and four of the treatment, a significant reduction ($P<0.05$) was observed in groups C-G, although a far much better reduction was seen in group E compared with group B (diabetic-untreated control).

Results of the haematological analysis

Diabetes mellitus induced in the rats caused a significant reduction ($P<0.05$) in the haemoglobin, PCV, and platelet count of the diabetic-untreated control compared with the normal control (Table 2). The group that was pretreated with the ethyl acetate fraction of *C. afer* at a dose of 200 mg/kg showed a significant increase ($P<0.05$) in the hemoglobin concentration compared with the diabetic-untreated control (Table 2). Packed cell volume significantly increased ($P<0.05$) in groups C, D, E, and G compared with group B (diabetic-untreated control). The RBC count and white blood cell count of the pretreated-treated and the treated did not show any significant increase or decrease ($P>0.05$) compared with the untreated diabetic control rats. A significant increase ($P<0.05$) was observed in the platelet count of groups C, E, and G compared with group B (diabetic-untreated).

Results of the lipid profile test

Diabetes significantly increased ($P<0.05$) total cholesterol, triglycerides, low-density-lipoprotein cholesterol (LDL-c), and very low-density-lipoprotein (VLDL) cholesterol, while it significantly decreased ($P<0.05$) high-density-lipoprotein cholesterol in diabetic-untreated control compared with the treatment groups (Table 3). There was a significant reduction ($P<0.05$) in the total serum cholesterol of all the extract pretreated-treated and treated groups compared with the diabetic-untreated control. There was a significant increase ($P<0.05$) in the serum high-density-lipoprotein cholesterol of all the extract pretreated-treated and treated groups compared with the diabetic-untreated control. There was a significant reduction ($P<0.05$) in the triglyceride and VLDL cholesterol of groups C to E compared with group B (the diabetic-untreated control). A significant decrease ($P<0.05$) was observed in the LDL-c of groups D, E, and G compared with group B (diabetic-untreated control). In all parameters analyzed, there was an improved recovery in the group pretreated and treated with 200 mg/kg of the extract compared with the group treated with 200 mg/kg of the fraction without pretreatment (Table 3).

Results of the liver function test

Induction of diabetes significantly increased ($P<0.05$) all the liver function parameters assayed except the alanine

Table 1. Fasting blood glucose concentration determined during the pretreatment and treatment periods (mean ± standard error of mean [SEM])

Groups	Glucose (mg/dL)									
	Pre-induction of diabetes mellitus					Post-induction of diabetes mellitus				
	Initial	Week 1	Week 2	Week 3	Week 4	Week 0	Week 1	Week 2	Week 3	Week 4
Group A	74.7±0.51	77.3±0.21	72.5±0.52	80.5±0.79	75.1±0.44	81.3±0.34	85.5±0.14	88.7±3.26	79.6±0.17	82.6±3.20
Group B	86.1±2.36	71.0±0.46	76.5±0.50	82.6±0.91	83.0±0.62	553.3±7.16*	589.5±6.48	591.1±8.23	496.5±10.1	423.8±6.84
Group C	77.5±2.31	76.4±1.51	81.7±0.96	75.5±1.25	70.2±0.13	510.9±5.62*	416.3±4.29	452.7±6.04	309.7±3.28**	376.4±3.91**
Group D	93.1±1.64	87.2±0.65	82.5±0.93	73.1±1.18	83.7±2.86	506.4±3.14*	403.8±6.80**	418.2±5.31	315.0±8.62**	297.2±8.32**
Group E	89.7±0.32	71.3±0.82	75.6±2.92	91.6±3.01	87.5±0.35	478.6±5.73*	398.6±3.62**	250.9±8.60**	295.1±7.09**	253.6±5.41**
Group F	85.9±3.21	82.4±0.25	73.7±1.65	79.8±2.05	80.6±0.75	570.3±3.36*	413.1±3.41	496.3±5.68	338.3±6.23**	382.1±6.22**
Group G	87.3±0.91	80.1±0.41	85.2±1.40	86.3±0.73	83.9±1.12	491.7±6.23*	317.5±6.87**	307.5±6.73**	303.5±4.36**	351.7±3.81**

* Significant increase with respect to week 4 (pretreatment); ** Significant decrease with respect to week 0 (after induction of diabetes) ($P<0.05$). **Group A:** Normal control, **Group B:** Negative control (Diabetic untreated), **Group C:** Positive control (100 mg/kg metformin), **Group D:** Pretreatment + Treatment (100 mg/kg ethyl acetate fraction of *C. afer*), **Group E:** Pretreatment + Treatment (200 mg/kg ethyl acetate fraction of *C. afer*), **Group F:** Treatment only (100 mg/kg ethyl acetate fraction of *C. afer*), **Group G:** Treatment only (200 mg/kg ethyl acetate fraction of *C. afer*).

Table 2. Haematological assays determined after the pretreatment and treatment periods (mean ± standard error of mean [SEM])

Groups	Hemoglobin (g/dL)	Packed cell volume (%)	Red blood cells (×10^{12}/L)	White blood cells (×10^{9}/L)	Platelets (×10^{9}/L)
Group A	13.65 ± 0.81	39.95 ± 2.12	7.21 ± 0.37	14.52 ± 2.01	369.5 ± 11.0
Group B	9.26 ± 0.36**	27.03 ± 1.65**	5.63 ± 0.39	16.30 ± 1.47	280.3 ± 15.6**
Group C	12.76 ± 0.52	37.82 ± 3.73***	5.86 ± 0.14	18.82 ± 2.86	395.5 ± 21.9***
Group D	13.29 ± 0.61	39.11 ± 2.24***	6.38 ± 0.25	17.91 ± 1.51	308.0 ± 17.2
Group E	14.22 ± 0.33***	43.36 ± 4.82***	7.03 ± 0.91	18.23 ± 1.01	461.3 ± 12.0***
Group F	12.36 ± 0.64	35.89 ± 2.01	5.79 ± 0.27	15.85 ± 0.92	319.4 ± 18.6
Group G	13.56 ± 0.90	40.32 ± 1.25***	6.85 ± 0.61	17.43 ± 1.38	371.8 ± 13.3***

*Significant increase with respect to group A (normal control); **Significant decrease with respect to group A (normal control); ***Significant increase with respect to group B (diabetic untreated); ****Significant decrease with respect to group B (diabetic untreated) ($P < 0.05$).
Group A: Normal control, **Group B:** Negative control (Diabetic untreated), **Group C:** Positive control (100 mg/kg metformin), **Group D:** Pretreatment + Treatment (100 mg/kg ethyl acetate fraction of *C. afer*), **Group E:** Pretreatment + Treatment (200 mg/kg ethyl acetate fraction of *C. afer*), **Group F:** Treatment only (100 mg/kg ethyl acetate fraction of *C. afer*), **Group G:** Treatment only (200 mg/kg ethyl acetate fraction of *C. afer*).

Table 3. Lipid profile tests determined after the pretreatment and treatment period (mean ± SEM)

Groups	Total cholesterol (mg/dL)	HDL-c (mg/dL)	Triglycerides (mg/dL)	LDL-c (mg/dL)	VLDL cholesterol (mg/dL)
Group A	42.35 ± 2.36	81.83 ± 2.31	85.35 ± 2.54	26.58 ± 3.60	18.8 ± 2.61
Group B	86.16 ± 2.99*	57.01 ± 3.26**	136.3 ± 5.68*	55.73 ± 1.98*	26.63 ± 0.23*
Group C	57.10 ± 5.72****	72.66 ± 2.06	100.8 ± 2.75****	33.18 ± 4.65	19.81 ± 0.26****
Group D	49.26 ± 7.15****	88.52 ± 2.62***	97.50 ± 2.35****	30.26 ± 2.82****	18.73 ± 3.55****
Group E	45.80 ± 4.65****	96.30 ± 1.28***	88.13 ± 3.24****	27.12 ± 5.60****	18.01 ± 1.63****
Group F	50.56 ± 3.81****	83.52 ± 4.05***	112.5 ± 3.68	35.13 ± 3.32	21.69 ± 0.31
Group G	46.21 ± 1.53****	75.02 ± 2.69***	108.1 ± 2.27	29.15 ± 2.49****	23.82 ± 1.27

HDL-c, High-density lipoprotein-cholesterol; LDL-c, Low-density lipoprotein-cholesterol; VLDL, very low-density lipoprotein-cholesterol.
*Significant increase with respect to group A (normal control); **Significant decrease with respect to group A (normal control); ***Significant increase with respect to group B (diabetic untreated); ****Significant decrease with respect to group B (diabetic untreated) ($P < 0.05$).
Group A: Normal control, **Group B:** Negative control (Diabetic untreated), **Group C:** Positive control (100 mg/kg metformin), **Group D:** Pretreatment + Treatment (100 mg/kg ethyl acetate fraction of *C. afer*), **Group E:** Pretreatment + Treatment (200 mg/kg ethyl acetate fraction of *C. afer*), **Group F:** Treatment only (100 mg/kg ethyl acetate fraction of *C. afer*), **Group G:** Treatment only (200 mg/kg ethyl acetate fraction of *C. afer*).

transaminase (Table 4). The group that was pre-treated and post-treated with a dose of 200 mg/kg of the extract showed a significant reduction ($P < 0.05$) in the aspartate transaminase enzyme level compared with the diabetic-untreated control. Also, it was observed that the ALP, direct bilirubin, and total bilirubin of the groups pre-treated before the induction of diabetes and thereafter post-treated with the same dose of the extract significantly decreased ($P < 0.05$) compared to the diabetic-untreated control (Table 4).

Results of the lipid peroxidation test, alpha-amylase activity, and insulin test

Induction of diabetes significantly ($P < 0.05$) increased the malondialdehyde (MDA) level of the rats except the normal control rats that was left uninduced (Table 5). The MDA level of the groups of rats that were pre-treated and post-treated with 100 and 200 mg/kg, respectively, decreased significantly ($P < 0.05$). Also, significant reduction in the MDA level of the group treated with 200 mg/kg without pre-treatment was also observed. There was a reduction in the MDA level of the groups treated with the standard drug (metformin) and the group treated with 100 mg/kg, although the reduction was insignificant ($P > 0.05$).

Induction of diabetes caused an observable decrease in the alpha-amylase activity of the diabetic untreated rats. However, this decrease was not statistically significant ($P > 0.05$) when compared with the normal control rats (Table 5). A significant reduction ($P < 0.05$) was observed in all the pretreatment and treatment groups when compared with the normal control group. The alpha-amylase activity of the group pretreated and treated with 200 mg/kg of the ethyl acetate fraction of *C. afer* decreased significantly ($P < 0.05$) compared with the diabetic untreated rats.

Induction of diabetes significantly decreased ($P < 0.05$) the insulin level of the diabetic untreated rats when compared with the normal control that was not induced (Table 5). The insulin level of the group treated with the standard drug (metformin) increased, although the increase was not significant ($P > 0.05$) compared with the diabetic untreated rats. The insulin level of the groups pre-treated and post-treated with 100 and 200 mg/kg of ethyl acetate fraction of *C. afer* significantly increased ($P < 0.05$) compared with the diabetic untreated rats. A similar increase was observed in the groups treated with 100 and

Table 4. Liver function test determined after the pre-treatment and post-treatment period expressed as mean ± SEM

Groups	Alanine transaminase (U/L)	Aspartate transaminase (U/L)	Alkaline phosphatase (U/L)	Direct bilirubin (mg/dL)	Total bilirubin (mg/dL)
Group A	7.23 ± 0.12	19.81 ± 0.25	42.12 ± 1.13	0.97 ± 0.65	2.47 ± 0.25
Group B	10.84 ± 0.65	33.21 ± 1.42*	53.23 ± 2.80*	2.35 ± 0.82*	5.26 ± 0.61*
Group C	8.25 ± 0.82	26.07 ± 0.30	50.72 ± 2.18	1.22 ± 0.19****	3.51 ± 1.25
Group D	7.56 ± 0.41	23.45 ± 1.38	45.25 ± 2.71****	1.56 ± 0.26****	2.72 ± 2.36****
Group E	6.73 ± 0.27	21.73 ± 1.44****	41.67 ± 1.23****	1.06 ± 0.38****	2.34 ± 0.73****
Group F	9.03 ± 0.54	26.82 ± 0.65	49.12 ± 1.74	2.25 ± 0.30	4.96 ± 1.82
Group G	7.36 ± 0.20	24.15 ± 1.21	47.52 ± 4.01****	0.92 ± 1.65****	2.77 ± 2.63****

* Significant increase with respect to group A (normal control); ** Significant decrease with respect to group A (normal control); ***Significant increase with respect to group B (diabetic untreated); **** Significant decrease with respect to group B (diabetic untreated) ($P < 0.05$).
Group A: Normal control, **Group B:** Negative control (Diabetic untreated), **Group C:** Positive control (100 mg/kg metformin), **Group D:** Pretreatment + Treatment (100 mg/kg ethyl acetate fraction of *C. afer*), **Group E:** Pretreatment + Treatment (200 mg/kg ethyl acetate fraction of *C. afer*), **Group F:** Treatment only (100 mg/kg ethyl acetate fraction of *C. afer*), **Group G:** Treatment only (200 mg/kg ethyl acetate fraction of *C. afer*).

Table 5. Malondialdehyde (MDA), alpha-amylase activity, and insulin test determined after pre-treatment and post-treatment periods (mean ± SEM)

Groups	Description of groups	MDA level (µmol/L)	α-Amylase activity (U/L)	Insulin level (ng/mL)
Group A	Normal control	0.135 ± 0.01	51.28 ± 0.37	33.20 ± 0.22
Group B	Negative control (Diabetic untreated)	0.352 ± 0.02*	45.32 ± 0.21	21.51 ± 0.05
Group C	Positive control (100 mg/kg metformin)	0.263 ± 0.01	36.61 ± 1.15	35.08 ± 0.03
Group D	Pretreatment + Treatment (100 mg/kg ethyl acetate fraction of *C. afer*)	0.238 ± 0.01****	39.27 ± 1.62	51.20 ± 0.14***
Group E	Pretreatment + Treatment (200 mg/kg ethyl acetate fraction of *C. afer*)	0.156 ± 0.03****	27.12 ± 1.22****	55.31 ± 0.16***
Group F	Treatment only (100 mg/kg ethyl acetate fraction of *C. afer*)	0.251 ± 0.01	30.57 ± 0.26	47.85 ± 0.55***
Group G	Treatment only (200 mg/kg ethyl acetate fraction of *C. afer*)	0.203 ± 0.01****	33.50 ± 0.60	53.92 ± 0.43***

* Significant increase with respect to group A (normal control); ** Significant decrease with respect to group A (normal control); ***Significant increase with respect to group B (diabetic untreated); **** Significant decrease with respect to group B (diabetic untreated) ($P < 0.05$).
Group A: Normal control, **Group B:** Negative control (Diabetic untreated), **Group C:** Positive control (100 mg/kg metformin), **Group D:** Pretreatment + Treatment (100 mg/kg ethyl acetate fraction of *C. afer*), **Group E:** Pretreatment + Treatment (200 mg/kg ethyl acetate fraction of *C. afer*), **Group F:** Treatment only (100 mg/kg ethyl acetate fraction of *C. afer*), **Group G:** Treatment only (200 mg/kg ethyl acetate fraction of *C. afer*).

200 mg/kg of the fraction without pre-treatment.

Discussion

The result of the acute toxicity (LD_{50}) study of the ethyl acetate fraction of *C. afer* suggests that the fraction is not very toxic. The fasting blood glucose concentration of groups D and E remained normal during the four weeks pretreatment period (Table 1). After four weeks pretreatment, diabetes was induced, and the fasting blood glucose concentrations were checked and recorded as week 0 before the commencement of treatment. The rats in groups B to G were treated for a period of four weeks and the fasting blood glucose concentrations were recorded weekly (Table 1). A significant decrease ($P < 0.05$) in the fasting blood glucose concentrations was recorded in groups D, E, and G compared with group B (the untreated diabetic control) within the first week of treatment. However, a much better reduction was noticed in group E (200 mg/kg of *C. afer* fraction), which was initially pretreated for one month before the induction of diabetes. In the second week of treatment a significant reduction ($P < 0.05$) was observed in groups E and G compared with group B (the diabetic-untreated control) within the same week. In the weeks three and four of the treatment, a significant reduction ($P < 0.05$) was observed in groups C, D, E, F, and G, although a much better reduction was observed in the rats in group E. This suggests that pre-treatment with the ethyl acetate fraction of *C. afer* has the capability of boosting the immune system of the rats helping them withstand the effect of streptozotocin-induced diabetes. Also, during the research, it was observed that the pretreated groups did not severely show the symptoms of diabetes as seen in the other test groups that were not pretreated. The pretreated groups were more active and responded to treatment better compared to the groups that were only treated after the induction of diabetes. This agrees with the results of our earlier study on medicinal plants using neem leaves (32).

Induction of diabetes in the rats significantly reduced ($P < 0.05$) the haemoglobin concentration, PCV, and

platelet count but treatment with 200 mg/kg bodyweight of ethyl acetate fraction of *C. afer* reversed the changes; however, it had no significant effect on red and white blood cell count (Table 2). Previous research has reported significant alteration in haemoglobin parameters in diabetic rats (33). This is in line with the present study. Reactive oxygen species are being produced when diabetes is induced (34), which causes cell death (apoptosis) leading to RBC damage (35). The increase of haemoglobin and PCV of the pre-treated group may be because of the reduction of oxidative stress generated on the cell membrane due to the antioxidants in the plant extract. The extract not only prevented cell death of the RBCs due to reactive oxygen species but boosted the immune system, as seen in the group that was pre-treated. The groups that were pretreated before the induction of diabetes were agile after the induction of diabetes, showing that the extract can boost their immunity and protect the rats from the severity of diabetes.

Pretreatment and treatment with 200 mg/kg of the extract also caused a significant reduction ($P < 0.05$) in the aspartate transaminase enzyme, ALP, direct bilirubin, and total bilirubin level compared with the diabetic-untreated control. Ezejiofor et al (36) reported an increase in ALT, AST, and ALP in diabetic rats and the restoration of these enzymes after the administration of *C. afer* extract. Gupta et al (37) also reported an increase in the levels of these enzymes in diabetic patients, especially in type 2 diabetic patients. The liver plays a critical role in the body (it filters blood, detoxifies chemicals, and metabolizes drugs); therefore, a protective agent should be a priority (38). Oxidative stress can damage the liver cells modifying nucleic acids, lipids, and proteins and leading to cell death (39). Xenobiotics, such as drugs, produce free radicals that increase the oxidative stress in the cells leading to the release of these enzymes to the circulation; however, this is reversed by the extract of *C. afer.*

Elevated bilirubin level is associated with an increased risk of type 1 diabetes in childhood (40). Therefore, *C. afer* may reduce the complications that come with diabetes mellitus by reducing its glucose concentration. Its antidiabetic effects are attributed to its phytochemical components. Experimental studies have reported that plants and fruit extracts containing flavonoids, saponin, and polyphenols possess antioxidant activities (41,42).

Diabetes significantly increased total serum cholesterol, triglycerides, LDL-c, and VLDL cholesterol while it significantly decreased high-density-lipoprotein cholesterol in diabetic-untreated control compared to the treatment groups. This agrees with previous research carried out in Indonesia (43). Diabetes is frequently associated with undiagnosed dyslipidemia, which causes arteriosclerosis cardiovascular disease, one of the complications of diabetes and the major cause of death in diabetic patients (44). *C. afer* extract significantly reduced the concentrations of these unhealthy lipids. It has a high concentration of flavonoid myricetin, which lowers LDL cholesterol (45) and diosgenin, a saponin with anti-inflammatory and antioxidant properties, which reduces cholesterol levels (46). Diosgenin also improves insulin resistance by blocking IKKβ and insulin resistance syndrome-1 pathway (IRS-1 pathway), thereby accelerating glucose uptake.

Administration of ethyl acetate fraction of the ethanol extract of *C. afer* decreased alpha-amylase activity. However, this decrease was only significant in the pretreated and treated group with 200 mg/kg of the fraction. This suggests that the antidiabetic property of the ethyl acetate fraction might be because of its capability to inhibit the alpha-amylase enzyme activity. This agrees with the findings of Dai et al (47), who stated that type 2 diabetic patients have lower alpha amylase levels, and that a high level is protective. In our earlier studies involving the antidiabetic properties of *A. indica*, it was observed that the aqueous and ethanol extracts of *A. indica* significantly ($P < 0.05$) decreased alpha-amylase activity (26,48).

The insulin levels were increased in the groups administered with the ethyl acetate fraction of *C. afer* leaf. The group of rats left untreated showed a noticeable decrease in their insulin levels, which might be because of the destruction of the β-cells of the pancreas by streptozotocin. Many of medicinal plants may act directly on the pancreas, thereby stimulating insulin release (49). The antidiabetic property of *C. afer* might also be through the regeneration of destroyed β-cells of the pancreas. Diabetes mellitus can be effectively managed by any substance that can act directly on the pancreas, thereby stimulating the release of insulin which have the capability of lowering the blood glucose concentration in the blood (26).

Conclusion

The findings of this research reveal that ethyl acetate fraction of *C. afer* might be used for the prevention, treatment, and management of diabetes mellitus. The fraction also has good safety profiles, as evidenced by their favorable effects on the hematological and biochemical indices, thereby capable of ameliorating the symptoms and complications of diabetes mellitus.

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Authors' contributions

OCE and DNO designed the research, OCE and CSA analyzed the data, OCE, COO, and MEO wrote the paper,

OCE, DNO, CSA, COO, and MEO edited the paper. All authors read and approved the final paper.

Conflict of interests

The authors hereby declare that no conflict of interest exists in this research.

Ethical considerations

The experimental protocol was examined and approved by the ethics committee of Nnamdi Azikiwe University, Awka, Anambra State, Nigeria in accordance with the Institutional Animal Care and Use policy in Research, Education and Testing. The ethical approval code is NAU/AREC/0002A. The experiment was carried out in strict compliance with the principle for the use and handling of laboratory animals.

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