Methanol-Extract/Fractions of *Dacryodes edulis* Leaves Ameliorate Hyperglycemia and Associated Oxidative Stress in Streptozotocin-Induced Diabetic Wistar Rats

Chimaobi J. Ononamadu, MSc\(^1,2\), Adamu J. Alhassan, PhD\(^2\), Aminu Ibrahim, PhD\(^2\), Abdullahi A. Imam, PhD\(^2\), Godwin O. Ihegboro, MSc\(^1,2\), Tajudeen A. Owolarafe, MSc\(^1,2\), and Mohammed S. Sule, PhD\(^2\)

Abstract

The present study evaluated the antidiabetic and antioxidant potential of the methanolic extract/solvent fractions of the leaves of *Dacryodes edulis* using a streptozotocin (STZ)-induced diabetic Albino Wistar rat model. The fasting blood glucose/insulin levels and inhibition of \( \alpha \)-amylase and \( \alpha \)-glucosidase were determined. Antioxidant activity was assessed in vitro by 2,2-diphenyl-1-picrylhydrazyl, hydroxyl, superoxide scavenging, reducing power, and total antioxidant capacity assays and in vivo by monitoring catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activities and reduced glutathione (GSH) and malondialdehyde (MDA) levels. The aqueous-methanol fraction exhibited the highest and significant \( (P < .05) \) reduction in fasting blood glucose (FBG; 54.03\%) with a concomitant inhibition of \( \alpha \)-amylase and \( \alpha \)-glucosidase activities. The ethyl acetate fraction also exhibited a significant \( (P < .05) \) reduction in FBG and an increase in insulin levels in the treated diabetic Wistar rats. A significantly \( (P < .05) \) higher reducing power and radical scavenging activity was observed in the aqueous-methanol and ethyl acetate fractions. The aqueous-methanol and ethyl acetate fractions also significantly \( (P < .05) \) reversed the alterations in oxidative stress markers (GSH, MDA, CAT, and SOD) observed in the diabetic control group. In conclusion, the study demonstrated that the methanol extract of *Dacryodes edulis* ameliorates hyperglycemia and the associated oxidative stress in STZ-induced diabetic Wistar rats. These observed activities are largely due to the compounds that partitions into the aqueous-methanol (55:45) solvent fraction. This provides scientific evidence for the use of this plant extract in folk medicine and also a baseline data for its further characterization. Further work should be carried out to characterize the aqueous-methanol solvent fractions for the active compounds.

Keywords
diabetes mellitus, oxidative stress, antioxidant, *Dacryodes edulis*

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Introduction

Diabetes mellitus is presently a major health problem globally.\(^1\) The disease is a metabolic disorder characterized by abnormally high blood glucose level as a result of an absolute or relative lack of insulin and failure of insulin to act on its targets tissues.\(^2,3\) It can cause other health complications, such as cardiovascular disease, neuropathy, high blood pressure, weakness, gangrene, retinopathy, nephropathy, and other dysfunctions.\(^2\) Basically, the disease could occur as either type 1 or type 2 diabetes, but other uncommon types have been reported.
Type 2 diabetes is adjudged the most prevalent and a result of the combination of resistance to insulin action and an inadequate compensatory insulin secretory response.⁴

Oxidative stress refers to the existence of unstable molecules possessing an unpaired electron called free radicals and reactive oxygen species (ROS) in a biological system. These unstable molecules are formed in normal physiological processes, but they become deleterious when they are not quenched by the antioxidant system. This is as the result of imbalance in the production of ROS and the activity of the antioxidants (molecules and enzymes), thereby shifting the ROS/AO balance in favor of oxidative stress.⁵ The association of oxidative stress and diabetes mellitus is well documented.⁶

In chronic type 2 diabetes, hyperglycemia gives rise to increase in ROS (majorly superoxide, hydroxyl radicals) production and consequently oxidative stress. This sets up a chain of events that culminate into diabetes complications affecting the eyes, kidney, nerves, and blood vessels.⁶⁷ Thus, suppressing the oxidative stress–mediated damages in diabetes pathophysiology using antioxidants may be potentially effective in managing diabetes.

At present, the use of insulin secretagogues and sensitizers constitute the predominant therapy used in the management of diabetes. But postprandial hyperglycemia has been reported to be a major risk factor in the development of type 2 diabetes.⁸ Thus, strict control of glucose level in the blood appears to be an effective method to prevent diabetes mellitus and hyperglycemia.⁹ The sugars in the blood originate from the hydrolysis of carbohydrates and it is catalyzed by digestive enzymes, such as α-glucosidase and α-amylase. Therefore, inhibition of these enzymes has gained prominence in the management of type 2 diabetes.¹⁰ Acarbose, Viglibiose, and Miglitol are currently the commercially available antidiabetic drugs that are inhibitors of α-amylase and α-glucosidase; however, their usage is affected by some associated side effects such as diarrhea and other intestinal disturbances such as bloating, flatulence, cramping, and abdominal pain and have been the most common reasons for noncompliance and early subject withdrawal from randomized controlled trials.¹¹¹² The strong inhibition of Acarbose on α-amylase and lack of digestion of starch have been reported as a major reason for the observed side effects. Thus, an extract that shows less inhibition on α-amylase could minimize these side effects. Plants by virtue of their diverse phytochemical constituents may provide more acceptable, cheaper, and safer lead compounds with multimodal mechanism of action in managing diabetes mellitus.

*Dacryodes edulis* (G. Don) H.J. Lam is the most popular specie under the genus *Dacryodes*, which derived its name from the term “edible” emphasizing its nutritional importance.¹³ The common names include African pear, Bush butter, Bush fruit, Native pear in English; Safouftier in French; Atanga in Gabon; and Ube and Eleme in Southeast and Western Nigeria, respectively.¹⁴¹⁵ In Nigeria, it is widely claimed to have originated from the southeastern part of Nigeria where it is called “ube.” Traditionally, the leaves are chewed with kola nut as an antiemetic, the leaf sap is used as ear drop to treat ear infections, while the decoction is prepared as a remedy for fever and headache.¹³¹⁵ A decoction of the leaves with other plant leaves has been claimed to ameliorate high blood sugar, hypertension, and labor pains.¹⁶ Moise et al¹⁷ and Longo-Mbenza et al¹⁸ in 2 different case-control cross-sectional studies reported that intake of the fruits reduced the odds of retinopathy and visual impairment in diabetes, respectively, in the studied populations. Uhunmwangho and Omorogie¹⁹ reported that oil extracted from the fruit exerted significant antioxidant activity in Wistar rats challenged with sodium arsenate. Oboh et al²⁰ examined comparatively the in vitro antioxidant and inhibitory activity of the fruits (roasted vs hot water treatment) against carbohydrate digesting enzymes (α-amylase and α-glucosidase), and lately, Erukainure et al²¹ reported that solvent extracts of the leaves inhibited α-glucosidase, pancreatic lipases, pancreatic ATPase, and glucose-6-phosphatase activities and exerted significant antioxidant effect on Fe²⁺ challenged (oxidatively) pancreatic and liver tissues but did not consider the antidiabetic effect in vivo in a diabetic animal model. Okolo et al²² have also reported that the hexane extract of the fruits reduced hyperglycemia in alloxan challenged Wistar rats. The studies thus far suggest that the plant has antioxidant and hypoglycemic potential; however, there are still some knowledge gaps. Till date, there is still a dearth of extensive antidiabetic experimental study on the leaves of this plant. Literature searches reveal that the few studies available were carried out using the fruits,¹⁷⁻²⁰ which is relatively limited by season and use for other purposes. The leaves are hugely available all year round with very few applications apart from ethnomedicine. There is also a need to further characterize active crude solvent extracts. Furthermore, no study has directly investigated the antioxidant and antidiabetic activities of alcohol extract/solvent fractions of the leaves of *Dacryodes edulis* in vivo in a diabetic organism model. Thus, this study evaluates the antioxidant and hypoglycemic activities of the crude methanol extract and its solvent fractions using (1) in vitro assays and (2) the streptozotocin (STZ)-induced diabetic Wistar rat model.

**Materials and Methods**

**Experimental Animals**

This research was carried out using 65 Albino Wistar rats (150-180 g), obtained from the animal facility of the Department of Biological Science, Bayero University, Kano State. They were housed and maintained at normal room temperature with standard commercial feeds and clean water ad libitum and allowed to acclimatize for 2 weeks and assigned to experimental and control groups.

**Plant Materials**

*Dacryodes edulis* leaves were obtained from a tree in the University of Nigeria, Nsukka Campus, Enugu State. The plant was identified and authenticated (Voucher No. UNNHN0601) by a taxonomist in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Enugu State.
**Chemicals and Equipment**

**Chemicals and Kits.** All the solvents (n-hexane, methanol, ethyl acetate, and chloroform) used were of analytical grade and were purchased from Loba Chemie (India); reduced glutathione (GSH) and glutathione peroxidase kit were purchased from Elabsience Biotech Ltd (China); Accu Bind ELISA Microwell Insulin Test kit was purchased from Monobind Inc (Lake Forest, CA). All other reagents were purchased from Sigma-Aldrich (China).

**Equipment.** The following instruments were used: RE2003 50 L Rotary Vacuum Evaporator (Kori Instrument, China), Binatone BLG 450 Blender/Grinder (Binatone, India), Spectra Max 340PC384 Microplate Reader (Molecular Devices, USA), SUNCOO Electric Centrifuge Machine, B0792VBNG6 (SUNCOO, China), UV_1280 UV-VIS Shimadzu Spectrophotometer (200-400 nm) (Shimadzu Corp).

**Preparation of Plant Extracts**

The crude extract and solvent fractions were prepared as described by Teke et al22 with little modifications. The leaves were shade dried and coarsely powdered using a blender. A portion (500 g) of the ground leaves was macerated in 2.5 L of methanol (95%) for 72 hours in an airtight glass cylinder. The extract was filtered with Whatman No.1 filter paper and concentrated in vacuo at 50°C using a rotary evaporator. The yield was 49.30 g of methanol extract of *Dacryodes edulis*. The solvent fractions were obtained by solvent/solvent partitioning of the crude extract in a separator funnel. In brief, a mass of 20.14 g of the crude extract was predissolved in 100 mL of methanol and then partitioned into n-hexane (350 mL x 3) in a separator funnel. The mixture was allowed to stand for 12 hours until 2 separate phases are formed (upper and lower phases). The upper (n-hexane) phase was collected and concentrated as n-hexane solvent fraction. The residual methanol phase was collected, concentrated, and then redissolved in 100 mL of aqueous-methanol (55:45 v/v) solvent and partitioned into ethyl acetate (350 mL x 3) as described above. The resulting upper (ethyl acetate) phase was collected and concentrated as the ethyl acetate fraction, while the lower (aqueous-methanol) phase was collected and concentrated as the aqueous-methanol fraction. The extract and solvent fractions were stored separately in well-labeled airtight dark bottles at 4°C until further use.

**Phytochemical Analysis**

The phytochemical analysis of the extract and fractions were carried out using the methods of Sofowora,23 Harbourne,24 and Saeed et al.25 The extracts were prepared in methanol.

**Alkaloids.** A volume of 1 mL of 1% v/v HCl was added to 3 mL solution of the extracts prepared in different test tubes. The mixture was heated for 20 minutes, cooled, and then filtered. The filtrate was used as follows.

1. Two drops of Meyer’s reagent was added to 1 mL of the extract filtrate. A creamy precipitate indicated the presence of alkaloids in the extracts.
2. Two drops of Wagner’s reagent was added to 1 mL of the extract filtrate. A reddish brown precipitate indicated the presence of alkaloids.

**Tannins.** A volume of 1 mL of freshly prepared 10% w/v KOH was added to 1 mL of the extract/fractions prepared in different test tubes. A white precipitate indicated the presence of tannins.

**Phenolics.** Two drops of 5% FeCl₃ were added to 1 mL of the extract in different test tubes. A greenish precipitate indicated the presence of phenolics.

**Glycosides.** A volume of 10 mL of 50% v/v H₂SO₄ was added to 1 mL of the extracts, the mix was heated in boiling water for 15 minutes, and then 10 mL of Fehling’s solution was added and the mixture was boiled. A brick red precipitate indicated the presence of glycosides.

**Saponins**

1. Frothing test: A volume of 2 mL of the extract in a test tube was shaken vigorously for 2 minutes. Frothing indicated the presence of saponins.
2. Emulsion test: Five drops of olive oil were added to 3 mL of the extract in a test tube and shaken vigorously. A stable emulsion formed indicated the presence of saponins.

**Flavonoids.** A volume of 1 mL of 10% w/v NaOH was added to 3 mL of the extract in different test tubes. A yellow coloration indicated the presence of flavonoids.

**Steroids (Salkowsti Test).** Five drops of concentrated H₂SO₄ were added to 1 mL of the extract. Red coloration indicated the presence of steroids.

**Phlobatannins.** An aliquot of 1 mL of each of the extracts was added to 1% HCl. A red precipitate indicated the presence of phlobatannins.

**Triterpenes.** Five drops of acetic anhydride were added to 1 mL of the extracts each in a different test tube. A drop of concentrated H₂SO₄ was then added and steamed for 1 hour and subsequently neutralized with NaOH followed by the addition of chloroform. A blue green color indicated the presence of triterpenes.

**Carotenoids.** A gram of each sample was extracted with 10 mL of chloroform in a test tube with vigorous shaking. The resulting mixture was filtered and 85% v/v H₂SO₄ was added. A blue color at the interface indicated the presence of carotenoids.

**Estimation of Total Phenolic Content**

The total phenolic content was determined using a spectrophotometer according to the method described by Saeed et al25 with slight modifications. To a 10 mL test tube containing 0.1 mL of extract prepared in methanol (in triplicates), 5.0 mL of distilled water and 0.5 mL of 1% v/v Folin-Ciocalteau’s phenol reagent prepared in water were added sequentially and shaken. After 5 minutes, 0.5 mL of 2% w/v Na₂CO₃ solution was added and mixed thoroughly. The mixture was kept in the dark for 30 minutes at room temperature, after which the absorbance was read against a blank (all reagents with methanol substituting the extract) at 765 nm. The standard curve for total phenolics was made using catechol standard solution (0 to 100 mg/mL) following the procedure described above for the extract. The total phenolics were determined from the calibration curve and expressed as milligrams of catechol equivalents per g of dried crude extracts or fractions.
**Estimation of Total Flavonoid Content**

Total flavonoid content was determined by the method described by Saeed et al.\(^25\) with slight modifications. To a 10 mL test tube containing 0.3 mL of extract prepared in methanol (in triplicate), 3.4 mL of 30\% v/v methanol, 0.15 mL of NaNO\(_2\) (0.5 M), and 0.15 mL of aluminum chloride (AlCl\(_3\).6H\(_2\)O [0.3 M]) were added sequentially. After 5 minutes, 1 mL of NaOH (1 M) was added. The solution was shaken to mix well and the absorbance of the resulting solutions was measured against the blank reagent (all reagents with methanol substituting extract) at 506 nm. The standard curve for total flavonoids was made using rutin standard solution (0 to 100 mg/mL) following the same procedure as described earlier for the extracts. The total flavonoids were determined from the calibration curve and expressed as milligrams of rutin equivalents per gram of dried crude extracts or fractions.

**Determination of Tannin Content**

The tannins were determined by the Folin-Ciocalteu method according to Singh et al.\(^26\) with slight modification. To a 10 mL test tube containing 0.1 mL of the sample extract prepared in methanol (in triplicate), 7.5 mL of distilled water and 0.5 mL of Folin-Ciocalteu phenol reagent were added and shaken, after 5 minutes, 1 mL of 2\% Na\(_2\)CO\(_3\) solution was added and mixed thoroughly. The mixture was kept in the dark at room temperature for 30 minutes to develop color. The absorbance for the test and standard solutions was measured against a blank (all reagents with methanol substituting the extract) at 725 nm. The standard curve for tannin was made using tannic acid standard solution (0 to 100 mg/mL) following the procedure described above for the extracts. The tannin content was expressed as milligrams of (tannic acid equivalence) TAE/gram of dried crude extract or fractions.

**Determination of Saponin Content**

Quantitative determination of saponin was carried out using the method reported by Obadoni and Ochuko.\(^27\) Exactly 100 mL of 20\% v/v aqueous ethanol was added to 5 grams of each extract in a 250 mL conical flask. The mixture was heated over a hot water bath for 4 hours with continuous stirring at a temperature of 55\(^\circ\)C. The residue of the mixture was re-extracted with another 100 mL of 20\% aqueous ethanol after filtration and heated for 4 hours at a constant temperature of 55\(^\circ\)C with constant stirring. The combined extract was evaporated to 40 mL over a water bath at 90\(^\circ\)C. A total of 20 cm\(^3\) of diethyl ether was added to the concentrate in a 250 cm\(^3\) separator funnel and agitated vigorously. The aqueous layer was recovered while the ether layer was discarded. This purification process was repeated twice. Sixty milliliters of n-butanol was added and extracted twice with 10 mL of 5\% sodium chloride. After discarding the sodium chloride layer, the remaining solution was heated in a water bath for 30 minutes, after which the solution was transferred into a crucible and was dried in an oven to a constant weight. The saponin content was calculated as a percentage:

\[
\text{Saponin (\%)} = \left(\frac{\text{weight of saponin}}{\text{weight of extract}}\right) \times 100
\]

**Determination of \(\beta\)-Carotene and Lycopene Content**

\(\beta\)-Carotene and lycopene were determined simultaneously according to the method of Nagata and Yamashita.\(^28\) A sample of extract (0.1 g) was extracted with 4 mL of acetone-hexane (4:6 by volume) at 37\(^\circ\)C for 10 minutes (in triplicate). The resulting solutions were centrifuged and the absorbance of the supernatant taken at the following wavelengths: 663 nm, 645 nm, 505 nm, and 453 nm. The \(\beta\)-carotene and lycopene content were determined per 100 mL by the following equations and subsequently expressed per gram of sample:

- Lycopene (mg/100 mL) = \(-0.0458A_{663} + 0.204A_{645} + 0.372A_{505} - 0.0806A_{453}\)
- \(\beta\)-Carotene (mg/100 mL) = \(0.216A_{663} - 1.22A_{645} - 0.304A_{505} - 0.452A_{453}\)

where \(A_{663}, A_{645}, A_{505},\) and \(A_{453}\) are absorbance at 663, 645, 505, and 452 nm, respectively.

**\(\alpha\)-Amylase and \(\alpha\)-Glucosidase Inhibition Assay**

The effect of the crude extract and its fractions on \(\alpha\)-amylase and \(\alpha\)-glucosidase activity was determined according to the method described by Kazeem et al.\(^29\) with modifications.

- \(\alpha\)-Amylase. To 250 mL of each extract concentration in a test tube (0-360 \(\mu\)g/mL), the following was added sequentially: buffered \(\alpha\)-amyrase (250 \(\mu\)L, 0.05 mg/mL) and starch (250 \(\mu\)L, 1\% w/v). The reaction mixture was incubated for 10 minutes at 25\(^\circ\)C. DNSA (500 \(\mu\)L) was added subsequently and then boiled for 5 minutes. It was then cooled and diluted with 5 mL of \(dH_2O\). The control was prepared in the same manner as the test samples with distilled water replacing the extract. The absorbance of each test tube content was taken at 540 nm and the percentage inhibition calculated as follows:

\[
\%\text{Inhibition} = \frac{A_c - A_t}{A_c} \times 100,
\]

where \(A_c\) and \(A_t\) are the absorbance of the control and test, respectively.

- \(\alpha\)-Glucosidase. To 50 \(\mu\)L of each extract concentration in a test tube (0-40 \(\mu\)g/mL) the following were added sequentially: buffered \(\alpha\)-glucosidase (100 \(\mu\)L, 1.0 U/mL) and incubated at 37\(^\circ\)C for 10 minutes, then pNPG (50 \(\mu\)L, 3.0 mM) and incubated at 37\(^\circ\)C for 20 minutes, and then Na\(_2\)CO\(_3\) (5\% w/v), cooled to 25\(^\circ\)C, and lastly 5 mL \(H_2O\) was added and vortexed. The absorbance of the resulting yellow \(\rho\)-nitrophenol from the different test tubes was taken at 405 nm and the percentage inhibition calculated as follows:

\[
\%\text{Inhibition} = \frac{A_c - A_t}{A_c} \times 100,
\]

where \(A_c\) and \(A_t\) are the absorbance of control and test, respectively.

The concentration of the extracts resulting in 50\% inhibition of the enzyme activity (IC\(_{50}\)) was determined graphically.

**Acute Toxicity Study**

The acute toxicity (LD\(_{50}\)) test of the methanol extract of leaves of *Dacryodes edulis* was determined using Lorke’s method as described by Chinedu et al.\(^30\) The crude extract was prepared in dimethyl sulfoxide (DMSO; 10\% v/v). The experiment was carried out using 15 Albino Wistar rats in 2 phases. In the first phase, 12 of the Wistar rats were randomly distributed into 4 groups of 3 rats each by simple...
physical randomization (by blindly selecting 12 wrapped pieces of papers denoted with the different group names [3 for each group] from a sampling box). The groups were administered 10, 100, and 1000 mg/kg body weight of the extract orally while the fourth group (control) was administered vehicle, DMSO (10% v/v). The animals were monitored for 24 hours for gross behavior and mortality. In the second phase, 3 Wistar rats were randomly distributed into 3 groups of one rat each as described above. Three higher doses, 1600, 2900, and 5000 mg/kg bw (body weight), of the extract were administered to each rat in each group and they were monitored for 24 hours for mortality. The LD50 was calculated as the geometric mean of D0 and D100 by the following formula:

$$LD_{50} = \sqrt{D_0 \times D_{100}}$$

where D0 is the highest dose that gave no mortality and D100 is the lowest dose that produced mortality.

The oral administration of the crude methanol extracts of at doses of 100 mg/kg bw to 5000 kg bw showed no lethal effect on the test animals and no death was recorded. According to Locke's method, the LD50 was determined to be ≥5000 mg/kg bw. Based on this, the following doses were used for the study: 1/50, 1/25, and 3/50 of 5000 mg/kg bw (100, 200, and 300 mg/kg bw). The lowest effective dose was used for the solvent fractions of the crude extract.

**Induction of Diabetes**

Experimental diabetes (type 2) was induced as described by Juarez-Rojo et al. with modification. Following overnight fasting, the fasting blood glucose of the test Albino Wistar rats (preinduction) was taken, and subsequently, a single intraperitoneal injection of streptozotocin (60 mg/kg bw) dissolved in sterile water for injection was administered. This was followed by an oral administration of 5% glucose solution 2 hours after administration. Control animals received sterile water as placebo. Animals were checked for successful induction of diabetes after 48 hours and 72 hours (postinduction) and animals with blood glucose above 250 mg/dL were classified as diabetic.

**Experimental Design**

The animals were weighed and randomly assigned to groups (n = 5) or treatments by physical randomization (by blindly selecting wrapped pieces of papers [50] denoted with the different group names [5 for each group] from a sampling box) as follows:

- **Group 1 (NDBC):** Normal rats administered with DMSO + food and water (Nondiabetic control)
- **Group 2 (DBC):** Diabetes-induced rats administered DMSO + food and water (Diabetic control)
- **Group 3 (CRDE1):** Diabetes-induced rats administered with 100 mg/kg bw of DE + food + H2O
- **Group 4 (CRDE2):** Diabetes-induced rats administered with 200 mg/kg bw of DE + food + H2O
- **Group 5 (CRDE3):** Diabetes-induced rats administered with 300 mg/kg bw of DE + food + H2O
- **Group 6 (HXDE):** Diabetes-induced rats + hexane fraction of DE (100 mg/kg bw) + food + H2O
- **Group 7 (ETDE):** Diabetes-induced rats + ethyl acetate fraction of DE (100 mg/kg bw) + food + H2O
- **Group 8 (AMDE):** Diabetes-induced rats + aqueous-methanol fraction of DE (100 mg/kg bw) + food + H2O
- **Group 9 (GLMD):** Diabetes-induced rats + 5 mg/kg bw of Glibenclamide (standard drug) + food + H2O
- **Group 10 (ACAB):** Diabetes-induced rats + 4 mg/kg bw of Acarbose (standard drug) + food + H2O

**Determination of Blood Glucose Level**

The treatments were administered daily for 28 and 15 days with the crude extract and the solvent fractions, respectively. Blood glucose was determined on a daily basis using Accuchek glucometer; blood samples were collected via a slight incision on the lateral tail vein using a scalpel blade. The measurements were taken in duplicate to ensure consistency in the glucometer readings. After the last day of treatment, the rats were sacrificed under light anesthesia (by placing the animal in chloroform fume chamber for 10 seconds) following a 12-hour fast. Blood samples were collected, centrifuged, and the serum stored for biochemical assays.

**Determination of Serum Insulin**

Serum insulin was determined using AccuBind ELISA Microwell Insulin Test System (Product Code: 2425-300) according to the manufacturer’s manual.

**Determination of In Vitro Antioxidant Activity of the Extract**

The in vitro antioxidant activity of the methanol crude extract and hexane, ethyl acetate, and aqueous-methanol fractions of *Dacryodes edulis* were determined according to the methods described in Saeed et al.

**DPPH (2,2-diphenyl-1-picrylhydrazyl) Scavenging Assay**

The free radical scavenging activity of the fractions was measured in vitro by DPPH with slight modification. Briefly, DPPH stock solution (0.1 mM) was prepared by dissolving 4 mg of DPPH in 100 mL methanol and stored at 20°C until required. The working solution was obtained by diluting the DPPH solution with methanol to attain an absorbance of about 1.2 ± 0.09 at 517 nm using the spectrophotometer. A 3 mL aliquot of this solution was mixed with 100 μL of the various concentrations (0-100 μg/mL). The reaction mixture was shaken well and incubated in the dark for 30 minutes at room temperature. The absorbance was taken at 517 nm. The control was prepared as above without any sample. The % scavenging activity was determined by the following equation:

$$\text{DPPH scavenging activity (\%)} = \left( \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \right) \times 100$$

**Total Antioxidant Capacity**

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation as described in Saeed et al. Briefly, an aliquot of 0.1 mL of sample solution was mixed with 1
mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 minutes. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 765 nm against a blank. Ascorbic acid was used as standard. The antioxidant capacity was estimated using the following formula:

Total antioxidant capacity (%) = \left( \frac{\text{absorbance of sample} - \text{absorbance of control}}{\text{absorbance of control}} \right) \times 100

**Superoxide Scavenging Assay**

The superoxide scavenging activity of the extract was determined according to the method described in Saeed et al.25 Briefly, a 1 mL aliquot of different concentrations (0-150 µg/mL) of the extract was mixed with 0.5 mL of phosphate buffer (50 mM, pH 7.4), 0.3 mL riboflavin (50 mM), 0.25 mL PMS (20 mM), and 0.1 mL NBT (0.5 mM) sequentially. The reaction was initiated by illuminating the reaction mixture using a fluorescent lamp. After 20 minutes of incubation, the absorbance was measured at 560 nm. Ascorbic acid was used as standard. The scavenging ability of the plant extract was determined by the following equation:

Scavenging activity (%) = \left( 1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}} \right) \times 100

**Hydroxyl Radical Scavenging Assay**

The hydroxyl scavenging activity of the extract was determined according to the method described in Saeed et al.25 Briefly, the reaction mixture contained 500 µL of 2-deoxyribose (2.8 mM) in phosphate buffer (50 mM, pH 7.6), 0.25 mL of premixed ferric chloride (100 mM) and EDTA (100 mM) solution (1:1; v/v), 100 mM of H2O2 (200 mM) with 100 µL graded concentrations (0-250 µg/mL) of the extracts or without the extract for control. The reaction was triggered by adding 10 µL of 300 mM ascorbate and incubated for 1 hour at 37°C. A 0.5 mL aliquot of the reaction mixture was taken and added to 1 mL of TCA (2.8% w/v aqueous solution), then 1 mL of 1% w/v aqueous TBA was added to the reaction mixture. The mixture was heated for 15 minutes on a boiling water bath (100°C). The mixture was cooled and the absorbance at 532 nm was taken against a blank (the same solution but without the test solution). The hydroxyl scavenging activity of the extracts was calculated as follows:

Scavenging activity (%) = \left( 1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}} \right) \times 100

**Ferric Reducing Power Assay**

The reducing power was based on Fe(III) to Fe(II) transformation in the presence of the test samples that can be monitored by measuring the formation of Perl’s Prussian blue at 700 nm as described in Saeed et al.25 Briefly, various concentrations (0-200 µg/mL) of the extract/fractions (2 mL) were prepared and mixed with 2 mL of phosphate buffer (0.2 M, pH 6.6) and 2 mL of potassium ferricyanide (10 mg/mL). The mixture was incubated at 50°C for 20 minutes followed by the addition of 2 mL of trichloroacetic acid (100 mg/L). The mixture was centrifuged at 3000 rpm for 10 minutes to collect the supernatant of the solution. A volume of 2 mL from each of the mixture earlier mentioned was mixed with 2 mL of distilled water and 0.8 mL of 0.1% (w/v) fresh ferric chloride. After 10 minutes of reaction, the absorbance was measured at 700 nm. A standard curve of ferric sulfate was used to quantify the ferric reduction potential of the extract/fractions. IC50 was determined as the concentration of extract/solvent fraction that gave a reduction equivalence of 50 mM Fe2+.

**Determination of In Vivo Antioxidant Activity**

**Tissue Preparation.** Tissue homogenate (10%) was prepared by homogenizing 1 g of frozen tissue in 10 mL of 0.1 M phosphate buffer at a pH of 7.4. The homogenate was centrifuged for 10 minutes at 2500 rpm/ min. The supernatant was collected for the in vivo antioxidant assays.

**Reduced Glutathione (GSH) and Glutathione Peroxidase (GSH-PX).** Reduced glutathione and glutathione peroxidase were determined according to the method described in the Elabsciences GSH and GSH-Px assay kit (Catalog No. BC0051) manufacturer’s manual.

**Superoxide Dismutase Activity (SOD).** SOD was determined according to the method described by Gavali et al,35 with slight modification. This method is based on the competitive inhibition of the autoxidation of pyrogallol under alkaline medium by SOD. The degree of inhibition is proportioned to the SOD activity.

In brief, to an aliquot of 0.9 mL of phosphate buffered EDTA (1 mM) solution, 0.05 mL of the homogenate was added and mixed. The reaction was started by adding 0.05 mL of pyrogallol solution (20 mM). The absorbance was read at 420 nm exactly after 1 minute 30 seconds. For the control, the procedure was repeated, but 0.1 mL of buffer was added in place of the homogenate. The SOD activity was calculated as follows:

\[
\text{Inhibition ratio} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100
\]

SOD activity (U/mg tissue) = SOD inhibition ratio/50 \times \text{Conc of sample (mg/mL)} \times 0.05

One unit of SOD is defined as the amount of the enzyme required to cause 50% inhibition of pyrogallol auto oxidation under the assay condition above.

**Catalase Activity.** Catalase activity was determined according to the method described by Prabhakar et al.36 The method was based on the rate of decomposition of H2O2 (hydrogen peroxide) by catalase, which can be monitored using a spectrophotometer at 230 to 240 nm. Briefly, to 2.95 mL of H2O2 solution (0.1% v/v), 50 µL of the homogenate was added. The decrease in absorbance was monitored at 230 nm for 1 minute. The catalase activity was then calculated as expressed mmol of H2O2 decomposed per minute per milligram of tissue as shown below:

\[
\text{Catalase (U/mg tissue)} = \frac{43.6 \times \text{AAbs/minute} \times 1000}{\text{conc of tissue in sample} \times 0.05}
\]

One unit of catalase is defined as the amount of the enzyme required to decompose 1 mmol of H2O2 under the assay conditions above.

**Lipid Peroxidation.** MDA was determined according to the method described by Prabhakar et al36 with slight modification. The method
Table 1. Phytochemical Constituent of Crude Methanol Extract and Solvent Fractions of Leaves of Dacryodes edulis.

| Sample  | Saponins | Phenolics | Flavonoids | Tannins | Steroids | Carotenoids | Phlobatannins | Alkaloids | Triterpenes | Glycosides | Cardenolides |
|---------|----------|-----------|------------|---------|----------|-------------|---------------|-----------|-------------|------------|--------------|
| CRDE    | +        | +         | +          | +       | +        | +           | +             | +         | +           | +          | +            |
| HXDE    | +        | +         | +          | +       | -        | +           | +             | +         | +           | +          | +            |
| ETDE    | +        | +         | +          | +       | +        | +           | +             | +         | +           | +          | +            |
| AMDE    | +        | +         | +          | +       | +        | +           | +             | +         | +           | +          | +            |

Abbreviations: DE, Dacryodes edulis; CRDE, crude extract of DE; HXDE, hexane fraction of CRDE; ETDE, ethyl acetate fraction of CRDE; AMDE, aqueous-methanol fraction of CRDE; “+”, present; “−”, not present.

Table 2. Quantitative Phytochemical Contents of Crude Methanol Extract and the Solvent Fractions of Leaves of Dacryodes edulis*.

| Samples  | Flavonoids (mg Rutin eqv/g of Sample) | Phenolics (mg Catechol/g of Sample) | Tannins (mg TANNIC acid/g of Sample) | Saponin (%) | Lycopens (mg/g of Sample) | β-Carotene (mg/g of Sample) |
|----------|--------------------------------------|------------------------------------|-------------------------------------|-------------|--------------------------|----------------------------|
| CRDE     | 130.0 ± 6.0b                         | 331.0 ± 13.0b                      | 234.0 ± 3.0b                        | 10.3 ± 0.7c | 7.0 ± 0.2c               | 6.9 ± 0.04c                |
| HXDE     | 25.0 ± 8.0a                          | 29.0 ± 3.0a                        | 54.0 ± 2.0a                         | 2.4 ± 0.5a  | 36.1 ± 0.5d              | 7.67 ± 0.30a               |
| ETDE     | 205.0 ± 5.0c                         | 317.0 ± 3.0b                       | 232.0 ± 4.0b                        | 4.0 ± 0.7a  | 3.5 ± 0.04b              | ND                         |
| AMDE     | 295.0 ± 3.0d                         | 304.0 ± 8.0b                       | 231.0 ± 2.0b                        | 7.1 ± 0.3b  | 0.4 ± 0.02a              | ND                         |

Abbreviations: DE, Dacryodes edulis; CRDE, crude extract of DE; HXDE, hexane fraction of CRDE; ETDE, ethyl acetate fraction of CRDE; AMDE, aqueous-methanol fraction of CRDE.

*Values are mean ± SEM, n = 3. Values bearing the same superscript along a column are not statistically different (P > .05).

is based on MDA (the product of lipid peroxidation) forming a complex that absorbs maximally at 532 nm with thiobisbituric acid (TBA). Briefly, an aliquot of 0.2 mL of the tissue homogenate was mixed with 2 mL of TBA (0.375%)–trichloroacetic acid (TCA; 15%) reagent. The volume was made up to 3 mL with distilled water and then it was boiled in a water bath at 95°C for 20 minutes. The solution was then cooled and the reaction product, TBA-MDA complex, was extracted by adding 3 mL of n-butanol to the resulting solution. The absorbance of the pink colored extract was measured at 532 nm using a spectrophotometer. The amount of MDA was calculated using the molar extinction coefficient of $1.56 \times 10^5$ M$^{-1}$ cm$^{-1}$ and expressed as μmoles of MDA formed per gram of tissue as shown below.

MDA (μmoles/g tissue/ml) = Absorbance / $1.56 \times 10^5 \times$ Concentration of tissue in sample in (g/ml).

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism version 7.0 and SPSS (Statistical Package for the Social Science) version 16. All data were expressed as the mean ± SEM. Group means were compared by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test or Dunnet test (when comparing groups’ data with a control group). $P < .05$ was considered to be statistically significant.

**Results**

**Phytochemicals Analysis**

The results of the qualitative and quantitative screening of the crude methanol extract of Dacryodes edulis leaves and its solvent fractions are presented in Table 1 and 2, respectively. Table 1 shows that saponin, phenolic, flavonoids, tannins, carotenoids, triterpenes, glycosides, and alkaloids were present in all the samples (crude and fractions). Table 2 reveals that the aqueous-methanol and ethyl acetate solvent fractions possessed significantly higher composition of saponin, flavonoids, phenolic, and tannins, while the hexane solvent fraction had a relatively low composition of the 4 phytochemicals but a significantly ($P < .05$) higher levels of carotenoids (lycopenes and β-carotenes).

**In Vitro Antidiabetic Activity**

The results of the α-amylase and α-glucosidase inhibitory activity of the crude methanol extract/solvent fractions of leaves of Dacryodes edulis are presented in Table 3. The crude extract and its solvent fractions showed a dose-dependent inhibition of α-glucosidase and α-amylase activity. The aqueous-methanol solvent fraction showed the highest inhibition of (with the lowest IC$_{50}$) both enzymes, which was statistically significant ($P < .05$).
The effect of the extract/solvent fractions on blood glucose level and insulin in diabetic Wistar rats is presented in Table 4, and supplementary Figures 1 and 2. The groups treated with the crude extract and aqueous-methanol and ethyl acetate solvent fractions showed a significantly reduced the fasting blood glucose (by 73.1%, 54.03%, and 36.8%, respectively) after the respective treatment regimen. The results also revealed that the group treated with ethyl acetate solvent fraction and the standard drug glibenclamide exhibited a significantly (P < .05) higher serum insulin level when compared with the diabetic control group.

**In Vivo Antidiabetic Activity**

The effect of the extract/solvent fractions on blood glucose level and insulin in diabetic Wistar rats is presented in Table 4, and supplementary Figures 1 and 2. The groups treated with the crude extract and aqueous-methanol and ethyl acetate solvent fractions showed a significantly reduced the fasting blood glucose (by 73.1%, 54.03%, and 36.8%, respectively) after the respective treatment regimen. The results also revealed that the group treated with ethyl acetate solvent fraction and the standard drug glibenclamide exhibited a significantly (P < .05) higher serum insulin level when compared with the diabetic control group.

**In Vitro Antioxidant Activity**

The results of the in vitro antioxidant activity as determined by DPPH scavenging assay, hydroxyl scavenging assay, superoxide scavenging assay, ferric reducing power, and total antioxidant capacity assays are presented in Table 5. The crude extract and solvent fractions showed dose-dependent radical scavenging and reducing power (antioxidant) activity in the following orders: Superoxide scavenging assay: aqueous-methanol > crude > ascorbic acid > ethyl acetate > hexane; Hydroxyl scavenging assay: ascorbic acid > ethyl acetate > aqueous-methanol > crude > hexane; DPPH scavenging assay: aqueous-methanol > ethyl acetate > crude > ascorbic acid > hexane; Ferric reducing power: ethyl acetate > ascorbic acid > aqueous-methanol > crude > hexane; and Total antioxidant capacity: ascorbic acid > aqueous-methanol > ethyl acetate > crude > hexane. The hexane solvent fraction gave a significantly (P < .05) low activity (very high IC_{50}) for all 5 assays, while the aqueous-methanol and ethyl acetate solvent fractions had significant high radical scavenging activity and reducing power.
malondaldehyde (MDA) while the treated and nondiabetic control groups had relatively higher GSH and lower MDA. For the solvent fractions, the result showed that SOD (superoxide dismutase) and GSH-Px (glutathione peroxidase) increased in the diabetic control group relative to the nondiabetic control. The catalase activity was significantly (P < .05) lower in the diabetic control than in the nondiabetic control. The aqueous methanol and ethyl acetate solvent fractions showed a significantly (P < .05) lower SOD and GSH-Px when compared with the diabetic control group but higher than the nondiabetic control group. The effect of the aqueous-methanol and ethyl acetate solvent fraction on GSH and catalase activity revealed an opposite trend—higher than that of the diabetic control but lower than that observed in the nondiabetic control group.

**Discussion**

The phytochemical analysis of the crude extracts of *Dacryodes edulis* (Tables 1 and 2) revealed the presence of several phytochemicals: saponins, phenolics, flavonoids, tannins, carotenoids, alkaloids, triterpenes, glycosides, steroids, and cardenolides. These classes of compounds are similar to those reported for the previous reports with other plants.2,49-52 The aqueous-methanol and ethyl acetate solvent fractions showed significant inhibitory activity against the 2 enzymes. The aqueous-methanol solvent fraction had the highest inhibitory activity against the 2 enzymes. This result is in line with some previous reports with other plants.2,49-52 The aqueous-methanol fraction may contain compounds that are structurally analogous to the natural substrates of the enzymes like phenolics, flavonoid glycosides, and saponins and could interfere with the normal activity of the enzymes. The extracts showed a relatively higher inhibition of glucosidase, an indication that they may have a relative advantage of lesser or no side effects compared to the standard drug.

Streptozotocin has been shown to cause direct irreversible damage (necrosis) to β-cells of pancreatic islets of Langerhans. The inhibition of carbohydrate digesting enzymes (α-amylase and α-glucosidase) as presented in Table 3 showed that the crude *Dacryodes edulis* extract and the solvent fractions showed significant inhibitory activity against the 2 enzymes. The aqueous-methanol solvent fraction had the highest inhibitory activity against the 2 enzymes. This result is in line with some previous reports with other plants.2,49-52 The aqueous-methanol fraction may contain compounds that are structurally analogous to the natural substrates of the enzymes like phenolics, flavonoid glycosides, and saponins and could interfere with the normal activity of the enzymes. The extracts showed a relatively higher inhibition of glucosidase, an indication that they may have a relative advantage of lesser or no side effects compared to the standard drug.

**Table 7. Effect of Solvent Fractions of Methanol Extract of *Dacryodes edulis* on Oxidative Stress Markers in Diabetic Wistar Rats**

| Groups (Dose) | GSH (mg/g of Tissue) | MDA (μmol/g of Tissue) | CAT (U/g of Tissue) | SOD (U/mg of Tissue) | GSH-Px (nmol GSH/min/mg of Tissue) |
|--------------|----------------------|------------------------|--------------------|----------------------|----------------------------------|
| AMDE         | 205.8 ± 3.0bc        | 66.33 ± 3.91b          | 10.24 ± 0.55b      | 0.141 ± 0.010b       | 0.305 ± 0.004c                   |
| ETDE         | 203.6 ± 5.1b         | 67.61 ± 1.39b          | 10.15 ± 1.91b      | 0.138 ± 0.018b       | 0.310 ± 0.010b                   |
| HXDE         | 184.6 ± 10.6ab       | 82.59 ± 4.76b          | 9.68 ± 1.40b       | 0.169 ± 0.04b        | 0.313 ± 0.020b                   |
| ACAB         | 186.8 ± 6.9bc        | 80.36 ± 6.50b          | 9.23 ± 0.63b       | 0.165 ± 0.020b       | 0.300 ± 0.005a                   |
| DBC          | 171.2 ± 6.8b         | 96.63 ± 6.10b          | 7.5 ± 1.90b        | 0.22 ± 0.040a        | 0.329 ± 0.005b                   |
| NDBC         | 232.5 ± 9.7c         | 57.72 ± 1.39b          | 12.8 ± 1.90b       | 0.12 ± 0.010b        | 0.284 ± 0.010a                   |

Abbreviations: DE, *Dacryodes edulis*; ETDE, administered with ethyl acetate fraction of DE (100 mg/kg bw); AMDE, aqueous-methanol fraction of DE (100 mg/kg bw); HXDE, administered with residual hexane fraction of DE (100 mg/kg bw); ACAB, administered with acarbose (4 mg/kg bw); DBC, diabetic control administered 10% DMSO; NDBC, nondiabetic control administered 10% DMSO; GSH, reduced glutathione; MDA, malonaldehyde; CAT, catalase; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase.

*Values are mean ± SEM, n = 5. Values bearing the same superscript along a column are not statistically different (P > .05).
resulting in the loss (of) or impaired insulin secretions and, consequently, hyperglycemia. Hence, it is used as a model for studying diabetes mellitus. In this present study, the induction consequently, hyperglycemia. Hence, it is used as a model for studying diabetes mellitus. Studies have shown that diabetes mellitus can lead to hyperglycemia, which results in the loss or impaired insulin secretions and, consequently, hyperglycemia. In this present study, the induction of diabetes with streptozotocin significantly increased the fasting blood glucose beyond 300 mg/dL within 48 hours. The administration of the crude extract and solvent fractions (aqueous-methanol and ethyl acetate) showed a significant potential to reverse the hyperglycemic condition in the diabetic Wistar rats. This was most significant in the aqueous-methanol fraction. This ability to reduce fasting blood glucose may be connected to the ability to stimulate the secretion of insulin as observed in the ethyl acetate solvent fraction and the marked inhibition of amylase and glucosidase enzymes observed with the aqueous-methanol fraction. This study also provided evidence that the aqueous-methanol solvent fraction may have improved insulin sensitivity when compared with the ethyl acetate fraction. This is apparent from Table 4. The aqueous-methanol fraction caused a higher percentage reduction in blood glucose level but with a low level of insulin, while the ethyl acetate, with higher level of insulin, produced a marginal percentage reduction in blood glucose level.

Antioxidant enzymes as well as non-enzymatic antioxidants are the first line of defense against reactive oxygen species–induced oxidative damages in a living organism. In this study, malonaldehyde (MDA), superoxide dismutase, and GSH-Px activity increased significantly while GSH and CAT activity decreased in the diabetic control group compared to the normal control. The increased MDA and decreased level of GSH are indicative of oxidative stress in the diabetic control group. The increased SOD and GSH-PX activity may be a compensatory mechanism to counter the high-level oxidative stress precursors such as superoxide, hydroxyl radicals, and hydrogen peroxides, which are common free radicals associated with hyperglycemia. Treatment with the crude extract and aqueous-methanol and ethyl acetate solvent fractions significantly reversed these trends of antioxidant status in the diabetic rats. This could be due to the ability of the crude extract and its fractions to assist in mopping up some of the free radicals and thereby reducing the oxidative stress burden on the antioxidant system of the diabetic rats. The improved outcomes recorded with Acarbose may be attributed to its ability to significantly ameliorate oxidative stress status. This was possibly mediated via consistent inhibition of carbohydrate digesting enzymes, marginal stimulation of insulin secretion, improved insulin sensitivity, and free radical scavenging. Further work would be required to characterize the aqueous-methanol and ethyl acetate solvent fractions of the active compounds responsible for the activities observed in this study.

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Author Contributions
MSS and CJ conceived the work. MSS and AJA supervised the work. AI and AAI validated the designs and provided relevant interpretations. CJO carried out the bench work. GOI and TAT prepared the manuscript with the assistance of all the other authors.

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Ethical Approval
The experimental protocols and procedures used in this study were approved by the Ethics Committee for the Care and Use of Laboratory Animals of Nigeria Police Academy, Wudil, Kano State, Nigeria.

Supplemental Material
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