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Metabolome modulatory effects of *Kigelia africana* (Lam.) Benth. fruit extracts on oxidative stress, hyperlipidaemic biomarkers in STZ-induced diabetic rats and antidiabetic effects in 3T3 L1 adipocytes

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Keywords
antioxidant enzymes; avidin-biotin immunoperoxidase; diabetes mellitus; GC-TOF-MS; Kigelia africana

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Abstract

Objectives The management of diabetes is considered a global problem, and a cure is yet to be discovered. This study investigated the modulatory effect of *Kigelia africana* fruit on oxidative stress and hyperlipidaemic biomarkers in STZ-induced diabetic rats, profiled phytoconstituents using GC-TOF-MS and evaluated antidiabetic effects on 3T3 L1 adipocytes.

Methods Thirty male Wistar rats (120–150 g) were divided into six groups ($n = 5$). Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg) and treated with 100, 200 and 400 of hexane fraction of KA for 28 days. Immunohistochemical evaluation was carried out using avidin-biotin immunoperoxidase (ABI) method. Catalase and SOD activities as well as the levels of total protein, albumin, bilirubin, triglyceride, cholesterol, and high-density lipoprotein were measured.

Key findings The expressions of oxidative stress and hyperlipidaemic biomarkers alongside fasting blood glucose concentrations were remarkedly decreased in KA-treated diabetic rats. Moreover, there was a significant increase in endocrine cell distribution, area covered with increase in β-cell mass, composition and morphology of KA-treated animals. Additionally, there was constant up-regulation in 3T3 L1 adipocytes due to the presence of phytoconstituents.

Conclusion *Kigelia africana* fruit can act as a modulatory agent due to its ameliorative effects against oxidative stress.

Highlights

- *Kigelia africana* fruit increases β-cell mass within the pancreas of diabetic rats
- *K. africana* fruit consists of 376 metabolites and chemical compounds
- *K. africana* fruit exhibited an up-regulation glucose uptake in 3T3 L1 adipocytes
- *K. africana* fruit can maintain or reverse biochemical integrity of diabetic rats

Introduction

The use of decoctions and infusions from medicinal plants has gained popularity in controlling excessive high blood glucose and the resultant hyperglycaemic condition known as *diabetes mellitus* in recent times. But the complications implicated in the development of the disease is still very much the reason for organ dysfunction and death.[1] *Diabetes mellitus* (DM) which is a condition characterized by persistent hyperglycaemia is associated with various

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complications such as nephropathy, neuropathy, retinopathy and cardiovascular diseases arising potentially from the mass destruction of the β-cells of the pancreas[2] as seen in type 1 diabetes or resistance or failure of the cells to produce enough insulin as seen in type 2 diabetes. In addition, diabetes has been shown to exhibit a significant decrease in insulin immunoreactivity and number of immunoreactive β-cells in pancreas.[3,4] Furthermore, more than one third of type 2 diabetes patients actively use traditional medicine for the treatment and management of the disease every year[5,6] and phytochemicals present in these herbal preparations have been postulated to be responsible for controlling high glucose levels.[7,8] Before now, the contribution of the massive destruction in β-cell mass to the development of diabetes was debated. Several literatures have postulated the hypothesis which led to the conclusion that this singular factor remains the only aetiological factor.[9,10] Other mechanisms contributing to the failure of β-cell to produce enough insulin have been subsequently neglected.[11] Therefore, understanding the importance of this aetiological factor in the pathogenesis of diabetes gives an edge in the application of the type of antidiabetic drug which will protect the remaining β-cell from death or reverse and regenerate the β-cells of the islet of Langerhans in the pancreas. Meanwhile, protection of β-cells from death presents itself a new therapeutic target.[12–14] Recently, the use of cell lines has given studies involving metabolic diseases an advantage since it is effective and efficient with less time and energy consuming.[15,16]

One of the goals of the World Health Organization in combating the menace of *diabetes mellitus* and its complication is to screen herbal preparations from medicinal plants with β-cells regenerative or protective as well as glucose uptake mechanisms for their therapeutic potentials in the treatment of the disease since the side effects of allopathic medicines such as sulphonylureas (glimepiride and glibenclamide) and biguanides (metformin) are linked to a greater prevalence of hypoglycaemia and cardiovascular diseases particularly lactic acidosis[17,18] and whose mechanisms of action have not been fully elucidated.[19] Moreover, the cost of these drugs has sky-rocketed in recent times. In a diabetic-diseased state, there is the failure of insulin-stimulated glucose uptake which results in high glucose concentration in the blood, thereby resulting in increase in glucose uptake by insulin-independent tissue arising from the destruction of β-cells of the islets of Langerhans in the pancreas.[20] The increase in glucose flux results in improved oxidant synthesis and likewise impairs antioxidant defences by multiple interactions of mitochondrial, enzymatic and non-enzymatic pathways.[21,22] Halliwell[23] reported oxidative stress to be responsible for molecular and cellular tissue damage in humans, with DM being one of the most prominent diseases associated with it. In addition, diabetes results in lipid profile disturbances with the most profound being increased susceptibility to lipid peroxidation,[24] a major cause of atherosclerosis and complication of DM. Baynes[25] investigated increased oxidative stress resulting from over-production of free radicals, diminished antioxidant status and lipid peroxidation onset experienced by DM patients. Medicinal plants that can effectively combat the menace of DM are expected to have secondary metabolites or phytochemicals ladened with high antioxidant activities.[26]

From over 400 tradomedicinal plants that have been reported for the treatment of DM,[27,28] this study screened the hexane fraction of *Kigelia africana* (Lam.) Benth. fruit extracts for its phytochemical constituents through the use of modern gas chromatography coupled with mass spectrometer (GC-TOF-MS) and investigated the modulatory and ameliorative effects on the pancreas using avidin-biotin immunoperoxidase (ABI) method and insulin immunoreactivity image analysis alongside the hypoglycaemic, antioxidant and hypolipidaemic effects in streptozotocin-(STZ)-induced diabetic rats and finally, studied the antidiabetic activities (deoxyribose uptake assay) in 3T3 L1 adipocytes which will give a novel approach to the management of DM through the use of herbal medicine. Previous researches documented the use of *Kigelia africana* in the treatment of diabetes as an antidiabetic agent in animal models.[29,30] In addition, the pharmaceutical abilities of *K. africana* have been recognized and have witnessed a surge in research interest to even include being used as an antidiabetic agent. Akunyili and Houghton (1991) reported that the ethnomedicinal stem bark of *K. africana* is used in the treatment of diabetes.[31,32] Caffeic acid derivatives and other plant metabolites unique to this plant were attributed to the antioxidant potentials of this plant with conclusions that the plant can act as a modulatory agent in a report by Akanni et al.[33] The findings of this study will serve as a step towards the identification or discovery of a cheaper and readily available drug for the management of DM.

Materials and Methods

Chemicals and reagents used

Mouse monoclonal insulin antibody (INS05) and biotinylated goat anti-mouse immunoglobulin were purchased from Lab Vision Corp., Fremont, California, USA. Streptozocin (STZ) was procured from MedChemExpress, South Brunswick Township, NJ, USA. Diagnostic kits for the biochemical assays were obtained from Randox Laboratories Limited, UK. Pyridine and N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and all other analytically graded chemicals used were purchased from Sigma-Aldrich, St. Louis, MO, USA.

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Fruit materials

The fruits of *K. africana* (Lam.) Benth. were collected from Ayegunle-Ekiti, Ekiti State, Nigeria, on longitude 7°50′ 42.0″N and latitude 5°06′ 36.0″E. The fruit samples were identified at the Herbarium Laboratory, Obafemi Awolowo University, with a voucher specimen (IFE-17801) and were deposited at the Department of Botany, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

Preparation and fractionation of the fruit extracts

The fruits (1 kg) were air-dried and extracted three times with 7.5 l of 70% v/v ethanol to obtain 111.11 g of the ethanolic crude extract (ECE) after concentrating at 40°C with BUCHI Rotavapor R-210 rotary evaporator at the Department of Chemistry, University of South Africa, South Africa. Based on the earlier report of Fagbohun, et al.,[34] liquid–liquid partitioning was carried out using hexane from 100 g of ECE to give a yield of 60.40%. After freeze-drying with Labconco-free zone 1 L Freeze dryer system at the Department of Biochemistry, University of South Africa, South Africa, the hexane fraction (HF) obtained was stored at 4°C for further analyses.

Cell culture

In the study, mouse adipocyte (3T3 L1) cell line was cultured using Dulbecco's modified Eagles medium (DMEM) (GIBCO, Pittsburgh, PA, USA) supplemented with 10% heat-inactivated foetal bovine serum (BioWest, Nuaillé, France) and 1% antibiotics (100 µg/ml penicillin, 100 µg/ml streptomycin and 250 µg/l fungizone; GIBCO). The cells were grown at 37°C with 5% CO_2 and 95% humidity. The cells were sub-cultured every two-to-three days after the cells had formed a confluent monolayer.

Animals

Male Wistar rats with weight between 120 and 130 g from Animal House, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria, were obtained. The experimental animals were kept under standard conditions (12-h dark/light cycle, 25 ± 2°C) and satiated with standard pellet diet (Ladokun feeds, Ibadan, Oyo State, Nigeria) and water *ad libitum*. The experimental protocol was according to the guidelines of Laboratory Animal Care adopted from NIH Publication No. 85-23 principles (NIH Publication Revised, 1985), and ethical approval was obtained from the Institute of Public Health, Obafemi Awolowo University, Ile-Ife, Nigeria, with approval number IPH/OAU/12/1263. The experimental rats were fasted without food after acclimatization but had access to water for 12 h. The fasting blood glucose level was determined in the blood drawn from the retro-orbital plexus of the animals using fine-test glucometer as described by Mohammadi Sartang, et al.[35]

Induction of diabetes

The rats were induced by a single intraperitoneal injection with freshly prepared streptozotocin (STZ) dissolved in 0.01 M citrate buffer (pH 4.5, 60 mg/kg BW). The animals were immediately maintained on 5% glucose solution with the aid of feeding bottles to overcome the initial hypoglycaemic phase normally associated with the induction of chemical diabetes and allowed to stabilize for 2 weeks.[36] Rat feeds were returned to the animals after the administration of streptozotocin. The determination of fasting blood glucose was repeated 48 h after streptozotocin administration, and only animals with blood glucose level higher than 200 mg/dl were assigned to various groups and used for the study.

Experimental design

Thirty (30) male Wistar rats were assigned into six groups consisting of five animals each. Twenty-five (25) male Wistar rats in the experimental group were induced with streptozotocin. Group A was non-diabetic rats which received 0.5 ml of citrate buffer. Group B was diabetic rats which received 0.5 ml of citrate buffer, while groups C–E were diabetic rats which received 0.5 ml corresponding to 100, 200 and 400 mg/kg body weight of the hexane fraction of *K. africana* fruit. Group F served as the control group and constituted diabetic rats which received 0.5 ml corresponding to 10 mg/kg body weight of glibenclamide (reference drug) as adopted from Adam.[37] All animals were given their respective intervention and treatment for 28 days. Fasting blood glucose (FBG) levels were determined on day (s) 1, 7, 14, 21 and 28 in fasting condition. The experimental animals were sacrificed at the end of the study, while the liver was rinsed in ice-cold saline. Tissue homogenate was prepared with PBS buffer; pH 7.4. After centrifugation at 1552 *g* for 10 min, the supernatant was collected and stored at −80°C until use for biochemical investigations. The blood samples were centrifuged using Bench Centrifuge at 1552 *g* for 10 min. A dry Pasteur pipette was used in collecting the supernatant (plasma) and stored at −4°C for further biochemical analyses.

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Analyses

Oxidative stress biomarkers

Catalase (CAT) activity was measured by the method of Aebi[38] based on the decomposition of H_2O_2 and expressed in micromoles of H_2O_2 separated within one min with 1 g of wet weight cells, while superoxide dismutase (SOD) activity was assayed using the method of Misra and Fridovich[39] based on the inhibition of oxidation of adrenaline to adrenochrome per min.

Total protein, albumin and bilirubin concentrations

Total protein concentration was estimated using the Biuret method as described Zheng et al.[40] based on the interaction of cupric ions in alkaline medium with protein peptide bonds in the sample resulting in the formation of a coloured complex. The concentration of albumin was measured according to the bromocresol-green (BCG) method as described by Dumas et al.[41] using Randox Diagnostic kit. This principle is based on the quantitative binding of plasma albumin to the indicator 3, 3, 5, 5-tetrabromocresol sulphonephthalein (bromocresol-green) to form a stable albumin–BCG complex which absorbs maximally at 578 nm while the estimation of total bilirubin concentration in the plasma was carried out as reported by Schlebusch et al.[42] using Randox kits.

Hyperlipidaemic biomarkers

Total cholesterol concentration was assayed by the method described by Kelly[43] based on the formation of quinoneimine from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. The level of high-density lipoprotein (HDL) cholesterol was measured by the method of Lopes-Virella et al.[44] based on the addition of phosphotungstic acid in the presence of magnesium ions while triglyceride concentration was determined by the method of Fossati and Prencipe[45] after enzymatic hydrolysis with lipases with formation of quinoneimine from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under catalytic influence of peroxidase.

Immunohistochemical evaluation of the pancreas

The pancreas was removed after sacrifice and rinsed in ice-cold normal saline. After fixing in formaldehyde, the processed tissue samples were sectioned at 3 microns on rotary microtome and placed on hot plate at 70°C for 1 h. Sections were washed in two changes in xylene, three changes in alcohol and distilled water before heated in citric acid; pH 6.0 for 25 min in a process called antigen retrieval. Peroxidase blocking was done on the sections by covering sections with 3% H_2O_2 for 15 min. Subsequently, the sections were washed with PBS and protein blocking using avidin was carried out for 15 min before endogenous biotin in tissue was blocked with biotin for another 15 min. After washing with PBS, sections were incubated with mouse anti-insulin antibody (primary antibody) for 1 h and biotinylated goat anti-mouse immunoglobulin antibody (secondary antibody) for 15 min. They were thereafter, labelled with horseradish peroxidase at 37°C for 30 min. The localization of the antigen was indicated by a brown colour obtained with 3-amino-9-ethyl-carbazole (AEC) as chromogenic substrate for peroxidase activity. Slides were counterstained with haematoxylin for microscopic observation after dehydrated in alcohol, cleared in xylene and mounted in DPX. The specificity of the immunohistochemical staining was checked by omission of mouse anti-insulin antibody. All these gave negative results. Control pancreas sections with (+) signals were used as a positive control.

Insulin immunoreactivity image analysis

For immunoreactivity evaluation, the signal intensity from the tissue sections was measured. More than 5 islets in each rats group were randomly selected and transferred to a pathology image analysing system (VNT, Beijing, China). Grey density from the captured images of the staining signals of the islets selected was calculated as a staining intensity per unit area (mm^2). Calibration of the insulin immunoreactivity was from 0 to 10.

GC-qTOF/MS analysis

Sample preparation

The sample from the hexane fraction of *K. africana* fruit was prepared for gas chromatography time-of-flight mass spectrometer (GC-TOF-MS) by a simple modified method of Kaneria et al.[46] Briefly, 1 g of the hexane fraction of *K. africana* fruit extract was steeped in 2 ml each of pyridine and N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), respectively. The sample was vortexed and oven-incubated at 40°C for 30 min, then cooled at 25°C. The resulting mixture was filtered with 0.25-µm polyvinylidene difluoride (PVDF) membrane syringe-like filter and extracted using sodium sulphate cartridge, then concentrated to 1 mL with TurboVap at 55°C with nitrogen before subjected to GC-qTOF-MS analysis.

Instrumentation and analytical method

A gas chromatographic machine was used in screening the hexane fraction of *Kigelia africana* fruit extract at Department of Chemistry, University of South Africa, South Africa. This GC machine was hyper-aerated to a time-

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of-flight mass spectrophotometer (5975C) with triple-axis detector equipped with an auto injector (10-µl syringe). The injector, transfer line and ion source temperature were maintained at 250, 350 and 250°C, respectively. The TOF software version 4.32 (LECO Corp., St. Joseph, MI, USA) was used in data acquisition, processing and instrumental control. The GC separation was achieved using a RXI-5SilMS (30 m × 0.25 mm ID × 0.25 µm df) column with cross-band similar to 5% diphenyl/ 95% dimethylpolysiloxane (serial # 1280721; Restek, Bellefonte, PA, USA). The carrier gas was helium operated at 1.4 ml/min, and ion source was operated at 70 eV. The modulation period was set to 2 s with a 0.6-s hot pulse and 0.4-s lag time between cooling stages. The total elution time was 20 min. The metabolite was identified by comparing the mass spectra obtained with those of the standard mass spectra obtained from the National Institute of Standards and Technology (NIST) library 14.

In vitro glucose uptake in 3T3 L1 adipocytes

3T3 L1 cells derived from mouse 3T3 cells were fully differentiated after cell culture in glucose-free Dulbecco's modified Eagles medium (DMEM) as confluent monolayers into adipocytes and were exposed to the hexane fraction of *Kigelia africana* fruit extracts at lower concentrations of 0.05, 0.5, 5 and 50 µg/ml as described by Ayeleso et al.[47] Glibenclamide (1 µM) was added as positive control as well as metformin (1 µM) as a reference drug control. The plates were mixed together, sonicated and incubated for 3 h. After the incubation period, glucose uptake in 3T3 L1 adipocytes was determined using pulse labelling with [3H]-2-deoxyglucose (3H-2-DOG) in glucose-free DMEM containing the extract and fractions for 15 min. Liquid scintillation counting (Parkard-Tricarb, USA) was employed for the evaluation of the intracellular 3H-2-DOG. Glibenclamide and metformin were expressed as % relative to the media control, while the activities of the fraction were expressed as femtomole (fmol).

Statistical analysis

All data were analysed using GraphPad Prism 6 (GraphPad, San Diego, CA, USA) and presented as mean ± SEM. Analysis of variance (ANOVA) was used to compare between different groups. Probability levels ≤0.05 and ≤0.01 were accepted as statistically significant.

Results

Body weight gains of rats

As shown in Table 1, the effect of the hexane fraction of *K. africana* fruit had caused a per cent increase in body weight. An increase was mostly noticed in Group D with 42.28 ± 0.03% increase. The remarkable decrease in per cent body weight seen in diabetic group (Group B) was not significant when compared with normal control group (Group A) but had significant differences when compared with the rest of the groups as shown in Table 1.

Changes in fasting blood glucose levels

The fasting blood glucose (FBG) level was within the normal range between 90 and 100 mg/dl initially. After the STZ induction, there was a surge in the blood glucose level with respect to time in the different groups with an average mean of 195.27 ± 2.33 mg/dl. A significant reduction was seen in groups C, D, E and F treated with the hexane fraction as well as glibenclamide when compared with normal group (Group A). After 28 days of treatment, the mean fasting blood glucose levels were found to have reduced in a dose-dependent manner with significant differences at $P \leq 0.05$ between groups B, C, D, and F. The pattern of alteration in Table 1 showed that *K. africana* fruit can effectively reduce the level of sugar *in vivo*.

Effect on antioxidant enzymes and biochemical activities in rats

Table 1 depicted the levels of these antioxidant enzymes in the liver. SSignificant differences were found between normal control group (Group A) and diabetic group (Group B) as well as between diabetic groups fed with *K. africana* fruit extracts. This demonstrates the remarkable antioxidant capacities of the fruit. Diabetic group (Group B) showed elevated increase in catalase activity and decrease in superoxide dismutase activity with significant differences shown in Table 1. In addition, the levels of total protein, bilirubin and albumin concentrations were measured. As seen in Table 1 and Data S1, there was a decreased value of albumin alongside elevated value of bilirubin whereas total protein concentration of diabetic rats was not statistically significant when compared with normal control groups. Diabetic rats with various interventions are found to have decreased level of bilirubin and increase in albumin concentration when compared with control groups. There was elevated level of total protein concentration but was not statistically significant when compared with normal control groups without diabetes.

Effect on lipid profile of the rats

The lipid profile analysis showed decreased values of high-density lipoprotein cholesterol concentration. As shown in Table 2, there were an elevated values of triglyceride concentration in the blood and liver of

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Table 1 Effect of hexane fraction of *Kigelia africana* fruit on body weight, fasting blood glucose (FBG), antioxidant enzymes (Catalase and Superoxide dismutase) and biochemical parameters of experimental rats

	Group A (Control group)	Group B (Diabetic group)	Group C (Diabetic group + 100 mg/kg B. W. HF)	Group D (Diabetic group + 200 mg/kg B. W. HF)	Group E (Diabetic group + 400 mg/kg B. W. HF)	Group F (Diabetic group + 10 mg/kg Glib)
	Body weight (g)					
Initial body weight (g)	124.11 ± 1.28	125.47 ± 1.74	128.93 ± 0.77	123.24 ± 1.69	125.17 ± 1.66	127.61 ± 1.04
Final body weight (g)	164.79 ± 0.92	160.49 ± 1.06*	167.21 ± 0.87*	175.35 ± 1.72*	164.53 ± 0.54*	175.81 ± 0.83*
	Fasting blood glucose (mg/dL)					
Initial FBG (mg/dl)	93.34 ± 1.07	98.45 ± 1.01	90.30 ± 0.75	93.34 ± 1.23	96.43 ± 1.01	92.23 ± 1.43
Final FBG (mg/dl)	95.50 ± 1.48	350.30 ± 4.82*	246. 20 ± 2.88*	223.40 ± 1.02*	145.60 ± 8.52*	110.60 ± 2.89
	Antioxidant enzymes (catalase and superoxide dismutase) in the liver					
CAT activity (μmol/min/ml)	1.028 ± 0.19	1.544 ± 0.003*	0.35 ± 0.003*	0.19 ± 0.003*	0.83 ± 0.007	0.95 ± 0.005
SOD activity (μmol/min/ml)	0.713 ± 0.026	0.168 ± 0.015*	0.219 ± 0.029*	0.255 ± 0.009*	0.363 ± 0.019*	0.481 ± 0.026
	Biochemical parameters in the plasma					
Total protein (g/dl)	23.03 ± 0.37	29.96 ± 0.34*	23.96 ± 0.22	23.01 ± 0.15	23.28 ± 0.90	23.40 ± 0.58
Albumin (g/dl)	4.15 ± 0.16	3.12 ± 0.17	3.63 ± 0.06	3.21 ± 0.09	3.61 ± 0.17	4.44 ± 0.03
Total bilirubin (μmol/l)	90.82 ± 0.72	224.29 ± 3.90*	154.81 ± 2.30*	132.31 ± 4.34*	108.53 ± 5.70	95.18 ± 7.90

Data were presented as Mean ± SEM (n = 5). Group B was compared with Group A, Groups C – F were compared with Group A. CAT, catalase; FBG, fasting blood glucose; Glib., glibenclamide; HF, hexane fraction; SOD, superoxide dismutase. *$P < 0.05$ and **$P < 0.01$.

diabetic rats. In diabetic groups fed with different dosages of the hexane fraction of *K. africana* fruits, the concentrations of triglyceride, high-density lipoprotein cholesterol and total cholesterol were comparable with normal control groups (Group A). The results showed that 'good' cholesterol levels were lower in diabetic rats and the hexane fraction had the capacity to elevate the levels of these cholesterol molecules as well as normalize triglyceride and total cholesterol concentrations.

Evaluation of the immunohistochemistry of the pancreas

Figure 1 depicted the immunohistochemical analysis of the pancreas in diabetic rats. In normal controls (Group A), the islets of Langerhans showed normal structure with a large central core that was formed by insulin-secreting β-cells. Across the groups, immunoreactivity occurred in the endocrine pancreas evident by diaminobenzidine (DAB) brown areas at the region of the islet of Langerhans. It is important to note that the colouration is more intense at the central portion of the core than at the periphery. However, insulin-producing β-cells of Group B (diabetic groups) showed almost complete negative reaction to insulin evident by optimal haematoxylin-counterstained areas including in the islet. Exocrine pancreas was negative to insulin antibody across the groups evident by haematoxylin-counterstained areas (non-brown areas). Groups C–F otherwise showed immunoreactivity by the restoration of the immunoreactivity β-cells when compared with diabetic group (Group B).

Morphometric analysis

The statistical determination of average *grey value* showed significant drop ($P = 0.0075$) in negative control rats (16.26 ± 1.46) when compared with normal control (89.56 ± 6.37). This is seen in Figure 1a. Intensities of insulin were significantly high in Groups C, D, E and F when compared with Group B ($P < 0.0001$). Comparison made within extract-treated groups showed no significant difference across the groups, although average grey values decreased insignificantly down the groups with the maximum in Group C (93.44 ± 3.82), followed by D (90.79 ± 5.42), then E (85.95 ± 3.05). Comparison made between positive control rats and extract-treated rats indicated no significant difference at all doses ($P = 0.3585$) although the intensity was insignificantly higher in this

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Table 2 Effect of hexane fraction of *Kigelia africana* fruit on lipid profile in the plasma and liver

Groups	Cholesterol concentration in the liver (mmol/l)	Cholesterol concentration in the plasma (mmol/l)	Triglyceride concentration in the liver (mmol/l)	Triglyceride concentration in the plasma (mmol/l)	HDL concentration in the liver (mmol/l)	HDL concentration in the plasma (mmol/l)
A (Control Group)	5.28 ± 0.10	5.56 ± 0.31	10.02 ± 0.06	11.92 ± 0.07	5.17 ± 0.23	6.12 ± 0.04
B (Diabetic Group)	4.49 ± 0.12	5.11 ± 0.39	11.66 ± 0.24	11.99 ± 0.15	4.02 ± 0.05	5.29 ± 0.17
C (Diabetic Group + 100 mg/kg B. W. HF)	4.95 ± 0.44	6.55 ± 0.19	8.76 ± 0.19	11.56 ± 0.20	4.64 ± 0.02	6.08 ± 0.15
D (Diabetic Group + 200 mg/kg B. W. HF)	4.24 ± 0.34*	7.64 ± 0.13*	9.59 ± 0.07	12.25 ± 0.12	4.72 ± 0.07	6.12 ± 0.06
E (Diabetic Group + 400 mg/kg B. W. HF)	4.71 ± 0.51	6.85 ± 0.21	8.55 ± 0.30	10.71 ± 0.39	4.42 ± 0.05	6.31 ± 0.19
F (Diabetic Group + 10 mg/kg Glib)	5.32 ± 0.99	7.94 ± 0.19	9.41 ± 0.33	11.44 ± 0.34	4.78 ± 0.09	6.29 ± 0.16

Data were presented as Mean ± SEM (n = 5). Group B was compared with Group A, Groups C–F were compared with Group A. HF, hexane fraction; Glib., glibenclamide; HDL, high-density lipoprotein. *P < 0.05.

group than in any of the extract-treated groups (99.90 ± 7.499).

Selected area covered by chromogen dropped significantly (P = 0.03) in negative control rats (0.49 ± 0.009) when compared with normal control (11.39 ± 1.92) as seen in Figure 1b. The extract significantly increased endocrine cell distribution at all doses (P = 0.0001) when compared with negative control, at 6.59 ± 0.70, 6.13 ± 0.29, 6.10 ± 0.82 for groups C, D, E respectively. Groups C and E also had significantly reduced endocrine cell distributions when compared with normal control, while group D is not significantly different from normal control (0.09). No significant difference exists in extract-treated groups. Group C and E showed significantly decreased distribution (P = 0.0003) when compared with group F (13.94 ± 0.21).

Chemical profiling analysis using gas chromatography-mass spectrometer

Peak identification was done by evaluating, and then, comparing retention times (t_R), mass spectra of each identified compounds with the reference standards, the National Institute of Standards and Technology (NIST) library database as well as literatures. The GC chromatogram of the hexane fraction is shown in Figure 2. As shown, 376 metabolites were detected with selected compounds of interest shown in Table 3 and Data S2, and most prominent compounds have been analysed using their mass spectra. This is achieved by matching with similarity index, and those with peak area of about 90% were selected to be fully present on the various peaks. The selected peak number, retention time, peak area, *m/z* values, molecular formula, suggested names of the identified metabolites and reference database identification number (ID) are displayed. Polyphenols are present in abundance in the fraction, and this suggested its antioxidant as well as antidiabetic properties. Of importance is the identification of 2,4-di-tert-butylphenol, a bacterial and marine metabolite, an antioxidant and a polyphenol. The volatile organic compound, 2,4-di-tert-butylphenol (2,4-DTBP), revealed the structure $C_{14}H_{22}O$ (Figure 3) and could be the reason for the potent antioxidant activity of the hexane fraction of *K. africana* fruit extract.

Glucose uptake in 3T3 L1 cells

Kigelia africana hexane fraction was investigated further for glucose uptake in 3T3 L1 mouse adipocyte cell lines using 2-deoxyglucose uptake measurement kit. Deoxyglucose uptake assay is an indirect approach to *Mef2a* gene transcriptional expression or Glut-4 concentration and translocation. The aim is to have a concise information on whether the different concentrations of the hexane fraction can express *Mef2a* gene before gene expression study. *Mef2a* gene is a transcriptional regulator of *Glut-4* gene. Surprisingly, the hexane fraction expressed up-regulation glucose uptake. The effects were seen relative to controls which are metformin and glimepiride. As presented in

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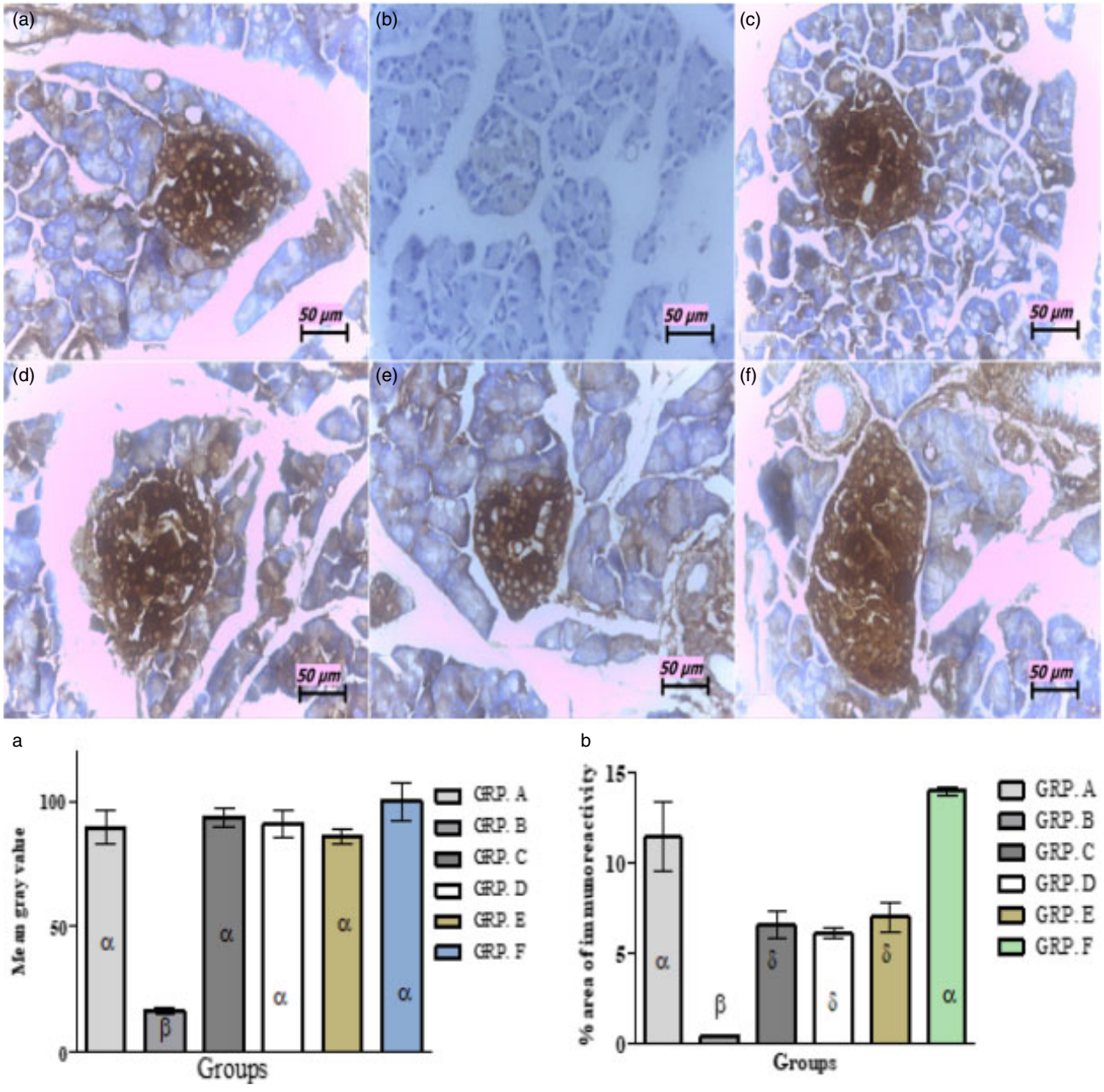

Figure 1 Representative light micrographs of formalin-fixed, paraffin-embedded sections of pancreas stained with insulin antibody and visualized using diaminobenzidine (DAB). Observe positive insulin immunoreactivities in a, c, d, e, f (brown areas) and near negative immunoreactivity in b (showing mostly the haematoxylin-counterstained area). [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 4, the % glucose uptake at 50 µg/ml was 214 fmol/mg and was significantly different when compared with controls (metformin and glibenclamide).

Discussion and Conclusion

Discussion

Hyperglycaemia is the most common predominant symptom of *diabetes mellitus*, and it is very dangerous for diabetic patients. Elevated blood glucose levels are specifically thought to lead to cell death from oxidative stress through β-cell massive destruction. Immunohistochemical evaluations are therefore indispensable diagnostic tools of anatomical histopathology that gives information on β- and α-cell mass, endocrine cell distribution within the pancreas, islet β-cell composition and islet morphology in diabetic rats.[48] In this study, we observed immunohistochemically that streptozotocin significantly reduced

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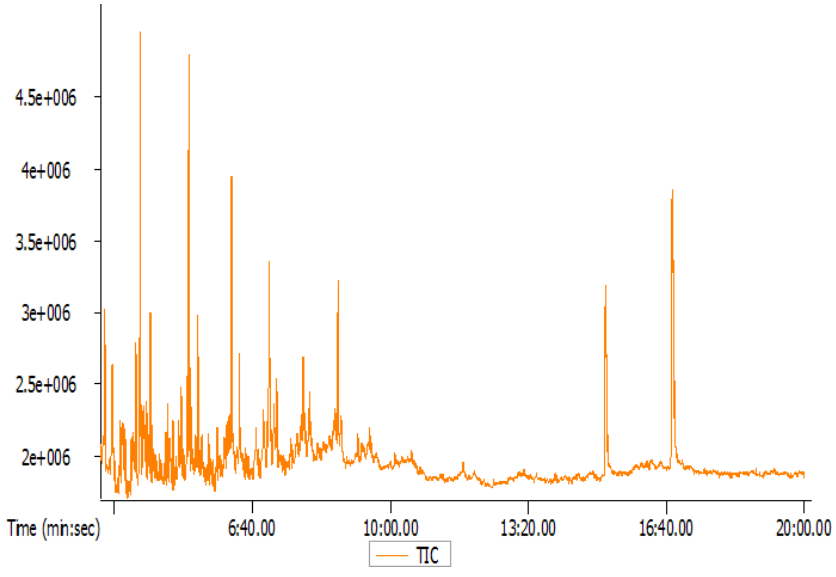

Figure 2 Gas chromatographic chromatogram of hexane fraction of *Kigelia africana* fruit extract. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 3 Selected metabolites profiled in hexane fraction of *Kigelia africana* fruit extract using gas chromatography-TOF-mass spectrometer

Peak no.	t_R (min/sec)	NIST similarities	Peak area (%)	*m/z* value	Molecular formula	Name of compound	Reference ID (#CAS)
1	03:31	92.9%	819360	128.17	$C_{10}H_8$	Azulene	275-51-4
2	03:03	94.4%	36025	134.22	$C_{10}H_{14}$	Benzene, 1-ethyl-2, 3-dimethyl-	933-98-2
3	03:04	93.1%	6740.1	134.22	$C_{10}H_{14}$	Benzene, 2-ethyl-1, 3-dimethyl-	2870-04-4
4	03:05	92.2%	52250	134.22	$C_{10}H_{14}$	Benzene, 1-ethyl-2, 4-dimethyl-	874-41-9
5	04:09	92%	159083	176.3	$C_{11}H_{10}$	1H-indene, 1-ethylidene-	2471-83-2
6	04:46	92.5%	55788	204.35	$C_{15}H_{24}$	Trans-'a-Bergamotene	13474-59-4
7	06:55	95.7%	182066	194.23	$C_{11}H_{14}O_3$	Undecanoic acid, Isopropyl ester,10-hydroxy-11-morpholin-4-yl-	4118-51-8
8	05:13	88.2%	164072	206.32	$C_{14}H_{22}O$	2,4-Di-tert-butylphenol	96-76-4
9	08:02	90.7%	23270	268.5	$C_{19}H_40$	Nonadecane	629-92-5
10	03:12	90%	43375	170.33	$C_{12}H_{26}$	2,6-Dimethyldecane	13150-81-7
11	08:31	92.8%	48374	296.6	$C_{21}H_{44}$	Heneicosane	629-94-7
12	06:29	90.9%	113772	224.42	$C_{16}H_{32}$	Cetene	629-73-2
13	03:15	94.2%	80778	170.34	$C_{12}H_20$	Undecane, 2-methyl-	7045-71-8
14	03:16	93.2%	3324485	188.34	$C_9H_{20}O_2SI$	Silane, Cyclohexyldimethoxymethyl-	17865-32-6
15	05:52	91.3%	59332	296.23	$C_{12}H_{25}L$	1-iodo-2-methylundecane	73105-67-6
16	05:13	88.2%	164072	206.32	$C_{14}H_{22}O$	2,4-Di-tert-butylphenol	96-76-4
17	05:18	87.5%	23143	200.36	$C_{13}H_{28}$	n-Tridecan-1-ol	112-70-9
18	03:19	92.9%	567399	258.35	$C_{14}H_{26}O_4$	Oxalic acid, Isobutyl octyl ester	959275-41-3
19	04:21	89.3%	71882	158.28	$C_{10}H_{22}O$	1-Heptanol, 2-propyl-	10042-59-8
20	04:15	93.1%	107421	142.2	C11H10	Naphthalene, 1-methyl-	90-12-0
21	04:04	91.7%	30322	186.33	C18H38	Heptadecane, 2-methyl-	1560-89-0
22	03:59	92.9%	295475	226.44	C16H34	Hexadecane	544-76-3
23	03:23	90.9%	134062	148.24	$C_{11}H_{16}$	Benzene, 1-methyl-4-(1, methylpropyl)-	1595-16-0
24	03:25	92.2%	122121	140.27	$C_{10}H_{20}$	1-Decene	872.05-9
25	03:27	92.9%	1139009	184.36	$C_{13}H_{28}$	Tridecane	629-50-5
26	03:30	68.2%	40248	159.23	$C_8H_{17}NO_2$	Pregabalin	5486-97-1
27	03:30	94.8%	1008021	212.41	$C_{15}H_{32}$	Pentadecane	629-62-9

#CAS, chemical abstracts service database; *m/z* value (mass-to-charge ratio), *m/z* represents mass divided by charge number and the horizontal axis in a mass spectrum and expressed in units of *m/z*. since *z* is always one with MS, the *m/z* value is often considered to be Mass (experimental); NIST, National Institute of Standards and Technology; t_R, retention time (min).

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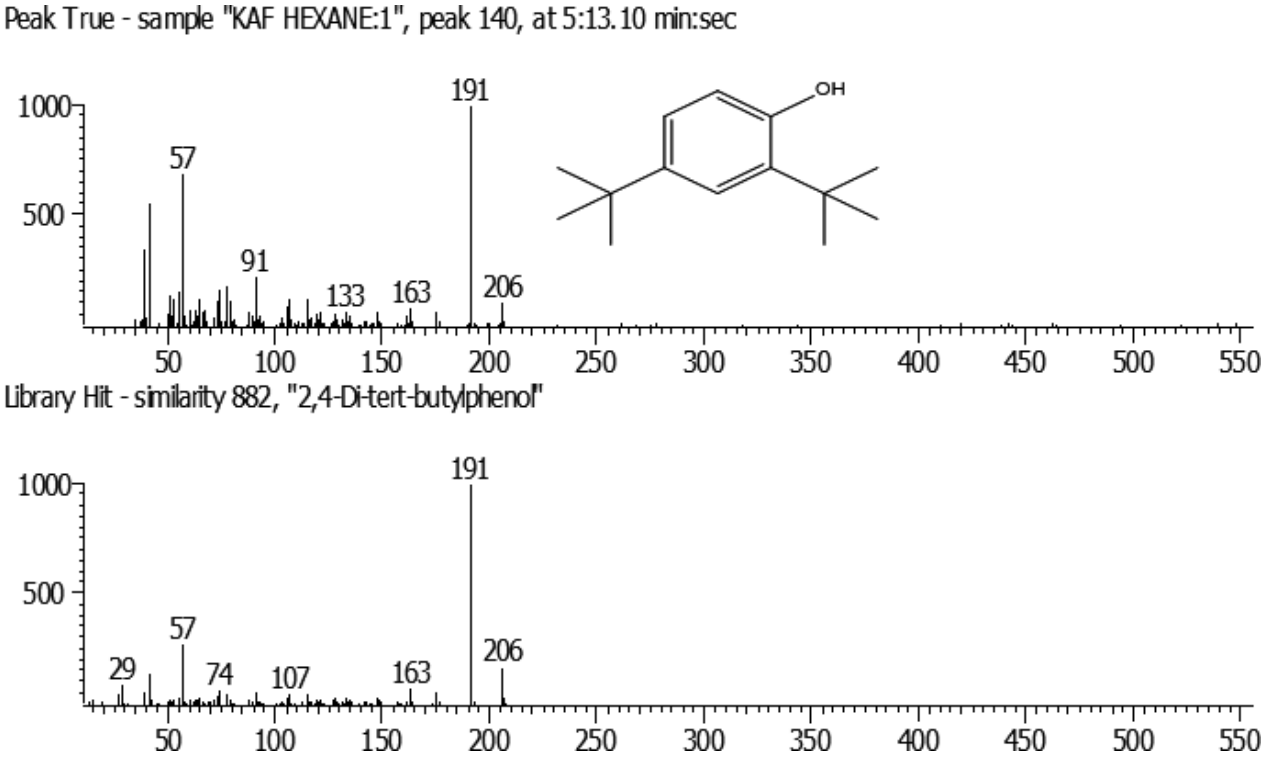

Figure 3 Mass spectral analysis of 2,4-di-Tert-butylphenol (2,4-DTBP).

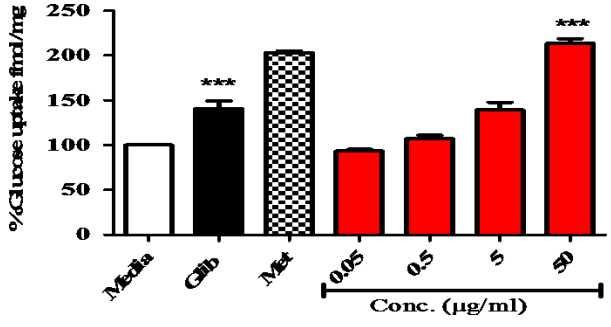

Figure 4 Glucose uptake in 3T3 L1 adipocytes using pulse labelling with [3H]-2-Deoxy-D-Glucose in glucose-free DMEM containing different concentrations of hexane fraction and control standard drugs (metformin and glibenclamide) for 15 min. [Colour figure can be viewed at wileyon linelibrary.com]

endocrine cell distribution and islet β-cell composition in negative control rats evident by low insulin immunoreactivity in this group. This is in consonance with several reports where STZ was implicated in the induction of these deranged features.[49–51] This result corroborates the previous report on general histomorphology where reduction in endocrine cell population including β-cells and karyopyknosis was noticed. This effect could be as result of the oxidative stress inflicted on β-cells by STZ. In a study conducted by Prado et al.,[52] it was reported that at the early stage of diabetes, pancreatic β-cells become stressed which they respond to by triggering a cell death pathway that results in the loss of β-cell function, and ultimately the loss of β-cell mass. It has been reported previously that the pathways that brings about toxic effect on the pancreas have reactive oxygen species formation in common which when produced in excess and over time cause chronic oxidative stress, which in turn causes defective insulin gene expression and insulin secretion as well as increased apoptosis.[53–56]

Spinas[57] and Liu et al.,[58] reported that insulin is synthesized as pre-proinsulin in the ribosomes of the rough endoplasmic reticulum of β-cells and are sensitive to damage by free radicals because of their low levels of free radical scavenging enzymes thus promoting cell death. Low intensity and area covered by chromogen as observed in Group B (diabetic group) could be said to occur as a result of β-cell death that resulted from oxidative damage induced by STZ with the consequent non-synthesis of insulin in the ribosomes of the rough endoplasmic reticulum. However, *Kigelia africana* fruit hexane fraction significantly increased endocrine cell distribution (chromogen intensity) and area covered. This points to its ability to improve and increase β-cell mass, endocrine cell distribution within the pancreas, islet beta cell composition and islet morphology in diabetic rats possibly by its ability to inhibit oxidation, a chemical reaction that produces free radicals, leading to chain

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reactions that can damage the cells of organisms. Hexane fraction of *Kigelia africana* fruit extract has been reported to possess this pharmacological importance by the recent work of Fagbohun et al.[34]

Kigelia africana fruit extracts are able to protect β-cells possibly due to the abundant presence of antioxidants which probably counteracted the effects of free radicals such as reactive oxygen and nitrogen species and this could be justified by our metabolomic study of the chemical compounds present in the fruit by modern quadrupole time-of-flight mass spectrometer coupled with a Gas Chromatographic Machine. From advanced GC-TOF-MS results of the study, the identification of the antioxidant, 2,4-di-tert-butylphenol, is the hallmark of the ability of *Kigelia africana* to mop up free radicals generated from the development of diabetes. The abundance of phytochemicals in the fruit will help any metabolic disease recovery, and the hexane fraction demonstrated a lot of it in its arsenal. A compound of importance with peak area (%) of 819,360, m/z value of 128.17 and compared with NIST 14 library having a reference ID of 275-51-4 was identified as azulene. At retention time of 5.13 min, a metabolite with NIST similarities of 92.5% having a peak area (%) of 55788 and m/z value of 204.35 was identified as Trans-'a-Bergamotene. As shown in Table 3, it is a pheromone that is used as antibiotics against insects. It is noteworthy to state the discovery of 'Pregabalin' for the first time in the fruit of *K. africana*. It is used in the treatment of epilepsy and fibromyalgia.[59] Other uses of pregabalin include neuropathic pain, restless leg syndrome and generalized anxiety.[60]

Superoxide dismutase and catalase are majorly the two antioxidant enzymes that scavenge the ability of free radicals in experimental and clinical studies. The investigation of the activities of these enzymes shed more light on the capacity of *K. africana* fruits. According to Aebi (1984) whose method was used for the determination of catalase activity of this study, a decrease in the activities of these antioxidants can lead to availability of excess superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) which in turn leads to abnormal production of hydroxyl radical (OH^-). From the results of this study, diabetic rats showed a significantly decreased activity of superoxide dismutase and this could possibly be due to a negative shift in the antioxidant status. Superoxide dismutase activities have been reduced from Groups C to F. Superoxide dismutase works in parallel with catalase in eliciting its pharmacological activity. It normally catalyses the dismutation of superoxide anion leading to the formation of hydrogen peroxide (H_2O_2) which is subsequently deactivated into to H_2O by catalase.[61] The change in the activities of these antioxidant enzymes means that the fruit could also provide a scavenging and modulatory effect by increasing the activities of catalase and superoxide dismutase.

For the evaluation of the biochemical parameters of experimental rats, total protein, albumin and bilirubin concentrations were measured. Normally, an assault to the body of diabetic patients causes a reduction in the concentrations of total protein and albumin alongside increase in total bilirubin concentration.[62] The results of this study agreed with the works of Iwasaki et al.,[63] González-Pacheco et al.,[64] Zhang et al.[65] and Zhu et al.[66] The high concentration of bilirubin in diabetic group of experimental rats as found in our study shows that a damage to the liver leads to the release of bilirubin into the bloodstream causing the levels to increase with reduction in the level of albumin and total protein. The impact of *K. africana* will be to normalize the levels of these biochemical biomarkers. There is a correlation between *diabetes mellitus* and lipid concentrations as seen in cardiovascular diseases.[67] One of the major characteristics of *diabetes mellitus* that pertains to its microvascular complications is diabetic dyslipidaemia.[68,69] This is a condition characterized by increased triglyceride concentration, lowered high-density lipoprotein levels and lowered total cholesterol concentration. As found in this study, the diabetic rats are found to have dyslipidaemia. They are prone to cardiovascular diseases as a result of diabetes.[70]

In this study, the effects of *Kigelia africana* fruit extracts were investigated on glucose uptake using 3T3 L1 cell line owing to its potential to differentiate from fibroblasts to adipocytes which makes it widely and extensively used to study adipogenesis and the biochemistry of adipocytes. To our surprise, hexane fraction exhibited an up-regulation glucose uptake relative to standard reference drugs (metformin and glibenclamide). This could be justified by the activation of key transcription factors such as PPARɣ and protein kinase via the inhibition of glycerophosphate dehydrogenase which is also in consonant with what was observed by Ayeleso et al.[47] The metabolites responsible may mimic or perform better than metformin widely recognized as the treatment baseline proposed by Rena et al.[19] From the results of the chemical profiling of the hexane fraction of the fruit of this study, some important metabolites were detected, identified and confirmed. These compounds are azulene, trans-'a-bergamotene, and 2,4-di-tert-butylphenol which were identified in previous studies as bacterial metabolites, antioxidants and pharmaceuticals [71-73]. Furthermore, to evaluate whether the hexane fraction which is the most potent fraction of this study enhances glucose uptake, we used an enzymatic method to measure 2-deoxyglucose (2DG) uptake in differentiated 3T3 L1 cells.

Our results showed significant ($P < 0.001$) stimulatory effect of insulin-dependent glucose uptake in a dose-dependent manner through a mechanism involving the lysosomes via AMPK inhibition. In drug discovery and developments, findings from many studies proposed that the ligand

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occupancy time on the receptor would improve the potency of PPARγ modulators by creating an extended period of protection against kinase activity, that is longer residence times of a drug occupying a target result in improved duration of action times for that drug. The chemical compounds such as 2,4-di-tert-butylphenol detected in this study could be the lead drug target for *diabetes mellitus*. Gene expression studies using *Mef2a*, *Nrf* and *Glut-4* genes are hereby required on the hexane fraction to be able to ascertain the mechanism of action of glucose uptake in the various cells and this study is on the way.

Conclusion

In conclusion, the investigation of this study showed that *K. africana* fruit possesses abundant pharmacological properties of importance ranging from antidiabetic, antihyperglycaemic, maintenance and stability of biochemical integrity and regeneration or protection of pancreatic β-cells of the islet of Langerhans alongside its ameliorative effect in streptozotocin-induced diabetic rats as well as upregulation glucose uptake mechanism in adipocytes. Therefore, *K. africana* fruit is suggested as a modulatory agent against *Diabetes mellitus*.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Ethical approval

Ethics approval of this research project was obtained from the Institute of Public Health, Obafemi Awolowo University, Ile-Ife, Nigeria, with approval number IPH/OAU/12/1263. The experimental protocol was according to the guidelines of Laboratory Animal Care adopted from NIH Publication No. 85-23 principles (NIH Publication Revised, 1985).

Authors contribution

(1). Dr. Oladapo F. Fagbohun (Corresponding Author) involved in research concept and design, collection or assembly of data (present in all the analyses), data analysis and interpretation (all analyses) and writing of article. (2). Dr. Babatunde Olawoye involved in collection or assembly of data (Antioxidant And Lipid Profile Assays) and data analysis. (3). Dr. Adedeji N. Ademakinwa involved in collection or assembly of data (Enzymatic Assays), experimental design and data analysis. (4). Mr. Kehinde A. Jolayemi involved in collection or assembly of data (immunohistochemistry), experimental design, and data analysis. (5). Professor Titus T. A. M. Msagati involved in collection or assembly of data (instrumentation and analytical as well as sample preparation for gc-ms and final approval of the article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1 Data for GC-TOF-MS chromatographic analysis and calculations for antioxidant analysis

Data S2 Gas Chromatography Coupled with TOF Mass Spectrometry Data for the hexane fraction of Kigelia africana fruit

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