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Natural products-characterized moringa oleifera leaves methanolic extract and anti-diabetic properties mechanisms of its fractions in streptozotocin-induced diabetic rats

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ABSTRACT

Background: The study characterized natural products and investigated the mechanisms of anti-diabetic properties in *Moringa oleifera* leaves methanolic extract in Streptozotocin-induced diabetic male rats.

Methods: the natural bioactive compounds in different fractions were obtained by column chromatography on 60-120 silica gel mesh and assayed using gas chromatography-mass spectrometric (GC-MS) technique. Also, possible anti-diabetic properties mechanisms of the fractions of extracts investigated using standard methods.

Results: Lethality study indicated safety of the extract at 5000mg/kgbwt. The GC- MS analysis of the methanolic extract revealed seven out of eleven common bioactive constituents with reported anti-diabetic activity. Results of possible anti-diabetic properties mechanisms based on studied glucose homeostasis-related bio-indicators showed significant ($P<0.05$) increase in serum lipoprotein lipase, whole blood GLUT 1, liver homogenate G-6-P-DH, Intestine homogenate Na^+ /K^+-ATPase, Muscle homogenate GLUT 4 and liver homogenate AMP-Kinase activities in rats administered with the drug (glibenclamide) and fractions 3,4,5 compared with the diabetic group. A significant ($P<0.05$) inhibition of serum α-amylase activity in rat administered drug and fractions 3,4,5 was observed when compared with diabetic control and fractions 1 and 2 . Histological outcomes of rats' pancreas corroborated these findings.

Conclusion: Thus, characterized natural bioactive constituents of *M. oleifera* fractions exerted concerted potent anti-diabetic activity in streptozotocin-induced diabetic rats by probable mechanisms involving concerted α-amylase inhibition, AMP-kinase and Lipo-protein lipase activation and glucose transport Proteins (GLUT-1 and GLUT-4) and liver homogenate Glucose-6-phosphate dehydrogenase up-regulation.

1. Introduction

Diabetes mellitus (DM), an endocrine disease, results to complex and multifarious disorders known to alter the metabolism of various bio-molecules, including carbohydrate[1]. Its symptoms are therefore characterized by high level of fasting and post-prandial blood sugar which manifests mainly as hyperglycaemia following abnormal insulin secretion and/or insulin resistance-related alteration in fuel metabolism[1]. Global onset of diabetes mellitus is expected to rise from 171 million to 366 million in 2030 with increasing prevalence rate trend[2]. In 2016, an estimated 1.6 million death was attributed to high blood glucose and diabetes[3]. Type-1-diabetes mellitus (T1DM),

the major type, results from insulin deficiency arising from autoimmune destruction of Beta (β) cells of the pancreas and leads to the associated abnormal metabolism and hyperglycaemia. The other type, type-2- diabetes mellitus (T2DM) arises due to tissue insensitivity to insulin and compensatory secretion of the hormone by islet beta cells of the pancreas[3]. Predisposing risk factors to DM include increased consumption of energy rich meals, sedentary lifestyle and obesity. According to American Diabetes Association[4], an individual is considered diabetic with blood glucose level above 126 mg/dL after an overnight fast and more than 200 mg/dL after an oral glucose load of 75g (oral glucose tolerance test, OGTT).

The streptozotocin (STZ), a broad-spectrum antibiotic, could induce particularly T1DM by its capacity to destroy beta-cells of the islets, which leads to a reduction in insulin release by β-cells leading to high blood glucose referred to as hyperglycemia[5]. On the other hand, glibenclamide, a standard anti-diabetic drug, is involved in stimulating insulin secretion from pancreatic β-cells principally by inhibiting ATP sensitive (K_{ATP}) channels in the plasma membrane and thus reducing blood glucose level[6]. Conventional pharmacologic regimen for DM present with adverse effects warranting need for hence the attendant increasing use of alternative perhaps medicinal plant with anti-diabetic activity and with minor side effects. It is difficult to regenerate the β-cells once they are destroyed, however a number of herbal compounds including *M. oleifera* have been reported to have this effect on chemically-induced diabetic rats[7].

Moringa oleifera Lam., (*M. oleifera*) offers a whole lot of promises in that direction. It is commonly called drumstick tree and belongs to the family *Moringacaea*. It originated from India, where different parts of the plant have served as food and medicine [8]. Medicinal benefits of *M. Oleifera* in many regions of Africa abound, including as self-medication against particularly, diabetes[7,8]. Notwithstanding global acceptance of possible anti-diabetic properties of *M. Oleifera*, limited if any scientific data exist on the anti-diabetic properties mechanisms of *M. olefera* leaf extract in relation to the glucose homeostasis-related bio-indicators including glucose transport proteins (GLUT 1, GLUT 4), Adenosine-monophosphate-Kinase (AMP-K) and Na^+/K^+-ATPase. These formed the basis for this study aimed at investigating the natural products-characterized *M. Oleifera* leaves methanolic extract and possible anti-diabetic properties mechanisms of its fractions in streptozotocin-induced diabetic rats.

2. Materials and Methods

2.1 Sample Collection and Crude Extract Preparation

Fresh matured leaves of *M. oleifera* were harvested from Isuikwuato Local Government Area of Abia State. The leaves were authenticated by a taxonomist at the Herbarium Unit of Forestry and Environmental Management Department of Michael Okpara University of Agriculture, Umudike, Abia State. Voucher specimen with voucher No VIH001/1011 was deposited at the Herbarium Unit of the department. The leaves were washed with distilled water to remove dust and dirt before being shade dried under natural air flow and surrounding's temperature (25 ^{o}C) for 4 days until a constant weight was attained. The dried leaves were blended into fine powder using electric blender (model QBL-18L40) and stored in air tight plastic containers. A portion of the powdered leaves (4000g) was packed in a glass column to saturation with 10 liters of methanol. The packed column was allowed to stand for 72 hrs. The column was eluted and the filtrate collected. The methanol was evaporated using a rotary evaporator (model YARONG RE-52). The extract yield obtained was 142 grams.

2.2 Isolation of fractions from Methanolic extract of *M. oleifera*

The crude methanolic extract of *M. oleifera* (142g) was fractionated by column chromatography on silica gel (60-120 mesh) as the stationary phase using the wet packing method. Varying solvent combinations of petroleum ether, chloroform and methanol of increasing polarity were used for the elution. The fractions were collected and kept at 4 ^{0}C in the refrigerator for further analysis. Thin layer chromatography (TLC) analysis was performed on the fractions for identifications using solvent system of chloroform: methanol (70:30). Retention factor was calculated based on the formula described by Stahl[9]. The fractions with similar retention factor (R_f) values were pooled and labeled accordingly as Fraction 1 to Fraction 5.

2.3 Animal study

Eighty-five (85) male albino rats of the Wistar strain (140-180 g) obtained from the animal house of the Department of Biochemistry, University of Nigeria, Nsukka, Enugu State, Nigeria, were used. The animals were kept in metal cages with steel base in the animal house of the Department of Biochemistry, Michael Okpara University of Agriculture, Umudike, Nigeria. All animals were housed at 25 °C and had free access to drinking water and their diets (Vital Feed with 8% crude proteins and 2800kcal metabolizable

energy). The animals were acclimatized for two weeks to their environment and diets before commencement of experiment. All chemicals used in the study were of analytical grade. The ethical guidelines provided by the ethical committee of College of Natural Science, Micheal Okpara University of Agriculture Umudike were followed strictly on the treatment and handling of animals. The authors certify that the animals were handled in accordance with the national institute of Health Guide for the care and use of laboratory animals[10]. All chemicals used in the study were of analytical grade.

2.4 Procedure for LD_{50} determination of *M. oleifera*

The acute toxicity of the *M. oleifera* leaf methanol extract was determined in two phases using Lorke's method[11]. A confirmatory test was carried out by administering 5000 mg/kg to two animals and observed for mortality. LD_{50} was calculated using the formula; $LD_{50}= (M_0 + M_1)/2$, where M_0= highest dose that gave no mortality; M_1= lowest dose that gave mortality

2.5 GC-MS analysis

GC–MS analysis on the extract was carried as described by Igwe and Okwu[12]. The mass spectra of the fractions of the plant extracts were compared with the spectra of the known components stored in the National Institute of Standards and Technology (N1ST) library. The name of the compound, molecular formula, molecular weight and structure of the components of the test materials were ascertained.

2.6 Induction of diabetes

Freshly prepared solution of streptozotocin (STZ) (250 mg dissolved in 40 mL of freshly prepared sodium citrate buffer 0.1 M, pH 4.5) was injected intraperitoneally to 85 rats a dosage of 65mg/kg body weight at fasting state. Blood was collected from the tail vein and blood glucose concentration was analyzed using a blood glucose meter (Double G glucometer, USA). Seventy-five (75) rats that were diabetic were used for the study in the two phases. The STZ-treated rats with fasting blood glucose levels > 200 mg/dL after seven days of induction of STZ were considered to be diabetic and were used for the study. The blood glucose levels of all experimental rats were checked every week throughout the duration of the experiment.

2.7 Experimental Design for Animal Treatment

A complete randomized experimental design comprising of eight treatment groups replicated thrice was used for the study. STZ-induced diabetic rats with stable diabetic condition were allotted into 7 subgroups (groups 2 to 8) with five animals *per* group while the non-diabetic rats formed the first group. The diabetic rats were treated with different fractions of *M. oleifera* leaf and Glinbenclamide. The groups were as follows: Group 1: Normal rats received only water (Normal control); Group 2: Diabetic rats received 5 mg/kg bwt of glibenclamide (standard drug control); Group 3: Diabetic rats received 2% of the vehicle, (dimethyl sulfoxide). Group 4: Diabetic rats administered 500 mg/kg bwt of methanol fraction 1 (F1); Group 5: Diabetic rats administered 500 mg/kg bwt of methanol fraction 2 (F2); Group 6; Diabetic rats administered 500 mg/kg bwt of methanol fraction 3 (F3). Group 7: Diabetic induced rats administered 500 mg/kg bwt of methanol fraction 4 (F4); Group 8 Diabetic induced rats administered 500 mg/kg bwt of methanol fraction 5 (F5). The administration of extract fractions and water were given orally for 28 days.

After treatment, the rats were sacrificed humanely and blood was drawn from their heart using 10 mL syringes into ethylenediamine-tetra-acetic acetic acid (EDTA) tubes for the determination of GLUT 1 while the rest of the blood samples were collected in plain tubes which were kept at room temperature (25°C) and allowed to clot. Serum was separated within one hour of blood clot by centrifugation at 3000rpm for about 20 min after which they were harvested using Pasteur pipette and the sera were used for the assay of **lipoprotein lipase.** The intestine was minced and homogenized with phosphate buffer and centrifuged at 5000 rpm for 15 mins at 4°C and the homogenate used for the analysis of intestine homogenate Na^+/K^+-ATPase activity and **intestinal amylase.** A section of the liver was homogenized with phosphate buffer and the homogenate used for analysis of liver homogenate Glucose-6-phosphate dehydrogenase activity and AMP-Kinase activity. The muscle was trimmed off and homogenized with TES buffer for the analysis of GLUT 4 protein

2.8 Determination of Glucose transport Proteins (GLUT) and AMP-kinase activity

The GLUT 1 and GLUT 4 Enzyme Linked Immunosorbent Assay (ELISA) kit were used for the assay of whole blood GLUT 1 and muscle homogenate GLUT 4 using the methods described by Li and McNeil[13]. The AMP-Kinase Elisa kit was used for the assay of liver homogenate AMP-Kinase activity following manufacturer's instruction. The addition of stop solution changes the intensity of the color

solution which was measured at 450nm and 440nm respectively using a micro plate reader. The GLUT and AMP-kinase activities respectively were determined by comparing the optical density of each sample to the standard curve.

2.9 Extraction of liver tissue for analysis of glucose 6-phosphate dehydrogenase

The liver was washed in ice-cold physiological saline and stored in the freezer until analyzed. Ten percent homogenate (w/v) of the liver was prepared in 0.1 M phosphate buffer (pH 7.4) at 4°C using a homogenizer and centrifuged at 1000 rpm for 10 min to remove the nuclei and the cell debris. The supernatant was further centrifuged at 5000 rpm for 60 min at 4°C and the supernatants were analyzed for Glucose-6- phosphate dehydrogenase activity using the methods described by Gancedo and Gancedo[14]. The enzyme activity is expressed as micromole of NADP oxidized/min/mg protein.

2.10 Estimation of Intestinal amylase, Lipoprotein lipase and Na^+/K^+-ATPase activity

Caraway- Somogyi iodine/potassium iodide (IKI) method reported by Cheesebough[15] was used for amylase assay**.** The disappearance of the blue color that starch gives with iodine solution is the measure of the extent to which the starch has been hydrolysed by the α-amylase. Lipoprotein lipase determination was achieved using the glycerol method described by Korn[16], which is divided into 2 stages; the incubation stage and the glycerol determination stage. At the end of the reaction, the optical density was measured at 570nm.The activity of Na^+/K^+-ATPase was measured using the method of Suhail and Rizvi[17].

2.11 Histopathological studies

The method described by Palipoch and Punsawad[18], was used for tissue preparation. The fixed slides were viewed under light microscope and photomicrographs were captured (400x). Photomicrographs were taken with a computer having a Microscopic Analysis Software (Scopelmage-9.O) connected to an Olympus digital light microscope (Olympus UK Ltd. Essex, UK).

2.12 Statistical analysis

Statistical analysis of the data was carried out with SPSS version 22.0 using one way analysis of variance (ANOVA). The results were reported as mean ± SD. The Duncan Multiple Range test (complemented with Student's T test) post- hoc test was used for comparison of the means of the various doses and fractions. Significant difference was accepted at 95 % confidence level of probability ($p<0.05$).

3. Results

3.1 LD_{50} determination of *M. oleifera*

Result of lethal dose studies (LD_{50}) of methanol extract of *M. oleifera* was shown in Table 1. No mortality and no sign of toxicity were recorded at highest dose of 5000 mg/kg bwt.

Table 1 Showing the LD_{50} of the methanol extract of *M. oleifera*

Group	Concentration (mg/kgbwt)	Mortality/Signs of toxicity
Phase 1	-	-
1	200	Nil
2	500	Nil
3	1000	Nil
Phase 2	-	-
1	2000	Nil
2	3000	Nil
3	5000	Nil; Decreased appetite
Confirmatory test	5000	No death/ no sign of toxicity

N=2 animals per group

3.2 GC-MS Analysis of methanol extracts of *M. oleifera*

The GC-MS analysis of fraction 3, 4 and 5 of *M.oleifera (*Table 2*)* revealed the presence of seven compounds reported to possess anti-diabetic effects

Table 2: GC-MS analysis of Methanol extract of *M. oleifera*

Compound name	Mol. Form	% content	RT (min)	Mol. Wt
Hexadecanoic acid	$C_{17}H_{34}O_2$	11..82	24.892	270
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	6.45	25.358	256
9,12-octadecadienoic acid	$C_{19}H_{34}O_2$	9.62	27.125	294
9-octadecenoic acid ethyl ester	$C_{19}H_{36}O_2$	6.30	27.242	296
Octadecanoic acid	$C_{19}H_{38}O_2$	3.50	27.608	298
Palmitic acid	$C_{16}H_{32}O_2$	17.10	29.01	256
Dodecane	$C_{12}H_{25}Cl$	1.37	42.38	204
Cis-9-Hexadecenal	$C_{16}H_{30}O_2$	1.53	31.01	238
Eicosanoic acid	$C_{20}H_{40}O_2$	6.21	28.02	312
Tetradecen-1-ol	$C_{14}H_{28}O$	3.09	23.18	212
Stearic acid	$C_{20}H_{40}O_2$	2.59	21.17	226

Mol. Form= molecular formula; wt= weight; RT= retention time.

3.3 Effect of fractions of *M. oleifera* leaves extracts on Fasting Blood Glucose

Effects of *M. oleifera* on FBG in table 3 shows that animals in groups administered fractions 3, 4, and 5 were able to reverse their blood glucose to normal at the end of the treatment while fraction 1 and 2 showed no such effect. The animals treated with fraction 1 and 2 were still diabetic at the end of the 28 days treatment.

Table 3: FBG levels (mg/dl) of diabetic rats administered fractions of *M. oleifera* methanolic extract.

Treatment	Pre- induction	After-induction	WK 1	WK 2	WK 3	WK 4
Normal control	85.00± 7.91	NI	84.00± 3.39	82.00± 7.21	83.00± 1.58	80.20 ±2.28^{a}
Drug control	84.00± 8.3	436.40±7.03	254.20± 4.24	143.20± 4.66	89.20± 6.64	84.20 ±4.21^{a}
Diabetic control	87.00 ±10.36	425.20±9.76	414.60±13.04	303.20± 4.77	308.80±4.44	239.0± 1.73^{b}
Fraction1	76.60± 6.84	423.00±10.0	289.40± 6.21	231.20 ±4.08	205.00±1.82	201.0± 2.37^{bc}
Fraction2	75.40±12.42	399.40±13.6	280.8 ±5.79	215.20± 3.47	186.40±3.73	198.0± 2.29^{c}
Fraction3	76.80±13.66	347.60±7.64	251.60± 5.86	164.60 ±6.33	107.60±2.28	105.6 ±1.49^{d}
Fraction4	74.80 ±9.95	322.40±5.84	259.80 ±3.27	213.20± 2.37	124.80±3.03	98.20± 9.33^{e}
Fraction5	76.80 ±3.70	428.60±10.6	308.80± 8.05	235.60 ±6.40	126.00±4.84	112.20± 9.83^{d}

$^{a-e}$Means with different superscripts along each column are significantly different at P< 0.05. NI=No induction

3.4 Effect of administration of fractions of M. oleifera extracts on serum lipoprotein lipase and Amylase

Result in Table 4 shows the level lipoprotein lipase, intestine amylase, G6P-D and Na^+/K^+-ATPase in rats administered fractions of *M. oleifera*. The result showed a significant (P<0.05) increase in lipase, G6P-D and Na^+/K^+-ATPase activity in groups administered fraction 3, 4, 5 when compared with diabetic control group. The result of the intestinal amylase showed a significant decrease (P<0.05) in the treated groups compared to diabetic control group. Treatment with fractions 1 and 2 showed no significant difference.

Table 4: Effect of administrations of fractions of *M. oleifera* leaf methanolic extract on serum lipoprotein lipase and intestinal amylase in STZ- induced diabetic wistar rats

Treatment	G6P-D (µ/mg)	L. lipase (IU/L)	Intestinal amylase(IU/L)	Na^+/K^+-ATPase Specific activity(IU/L)
Normal control	$3.78^b \pm 0.27$	$3.31^b \pm 0.12$	$31.21^c \pm 1.04$	$0.66^a \pm 0.06$
Drug control	$3.60^b \pm 0.17$	$3.21^b \pm 0.42$	$32.97^c \pm 0.75$	$0.62^{ab} \pm 0.17$
Diabetic control	$2.02^c \pm 0.03$	$2.15^c \pm 0.13$	$61.04^a \pm 0.86$	$0.62^{ab} \pm 0.17$
Fraction1	$2.23^c \pm 0.14$	$2.19^c \pm 0.23$	$57.58^a \pm 2.18$	$0.52^c \pm 0.14$
Fraction2	$2.23^c \pm 0.12$	$2.11^c \pm 0.13$	$59.20^a \pm 3.68$	$0.51^c \pm 0.01$
Fraction3	$3.63^b \pm 0.23$	$3.09^b \pm 0.14$	$37.38^c \pm 0.89$	$0.59^b \pm 0.01$
Fraction4	$3.66^b \pm 0.17$	$3.06^b \pm 0.24$	$37.58^c \pm 1.52$	$0.61^{ab} \pm 0.05$
Fraction5	$3.79^b \pm 0.56$	$3.33^b \pm 0.17$	$41.40^b \pm 5.56$	$0.59^b \pm 0.01$

[a-c]Means with different superscripts along each column are significantly different at $P < 0.05$

3.5 Effects of Fractions of *M. oleifera* leaf extract on GLUT 4, GLUT 1 and AMP Kinase

Result of effect of fractions of *M. oleifera* leaf extract on GLUT 4, GLUT 1 and AMP Kinase expressions in STZ- induced diabetic male wistar rats were shown in Table 5. There was significant increase ($P<0.05$) in the levels of GLUT 4, GLUT 1 and AMP-Kinase in groups administered standard drug (glibenclamide) and fractions 3,4, and 5 of *M. oleifera* leaf extract when compared with the diabetic control group. Groups administered fraction 1 and 2 showed no ($P>0.05$) significant difference in the levels of GLUT 1, 4 and AMP Kinase expressions relative to the diabetic control.

Table 5: The effect of fractions of *M. oleifera* leaf methanolic extract on muscle homogenate GLUT 4, blood GLUT 1 and liver homogenate AMP-Kinase expressions

Groups	muscle GLUT 4(ng/ml)	Blood GLUT 1 (ng/ml)	liver (nmol/L) AMP-Kinase
Normal control	$28.71^c \pm 1.19$	$14.89^c \pm 0.32$	$0.91^b \pm 0.04$
Drug control	$30.50^b \pm 2.88$	$18.84^{ab} \pm 0.77$	$0.86^c \pm 0.05$
Disease control	$22.68^e \pm 1.48$	$12.50^d \pm 1.25$	$0.71^d \pm 0.02$
F1	$22.99^e \pm 0.27$	$12.50^d \pm 0.56$	$0.73^d \pm 0.43$
F2	$22.76^a \pm 0.55$	$12.50^d \pm 0.87$	$0.73^d \pm 0.03$
F3	$34.19^a \pm 0.88$	$18.57^{ab} \pm 0.76$	$0.97^{ab} \pm 0.16$
F4	$33.57^b \pm 1.25$	$18.09^b \pm 1.05$	$1.01^{ab} \pm 0.09$
F5	$34.43^a \pm 1.19$	$18.07^b \pm 0.61$	$1.04^{ab} \pm 0.18$

[a-c]Means with different superscripts along each column are significantly different at $P < 0.05$

3.6 Histologic changes in the pancreas of STZ-induced diabetic rats treated with fractions of *M. oleifera* leaf

The result of the histologic studies showed that out of the five fractions studied only fractions 3, 4 and 5 exhibited the ability to regenerate the partially destroyed pancreatic acini and reduced vacuolation of pancreatic acini and Langerhans islets cells resulting in absence of necrosis. Fraction 1 and 2 did not show any significant activity, because the histoarchitecture of the pancreas were not restored when compared with the normal and disease control.

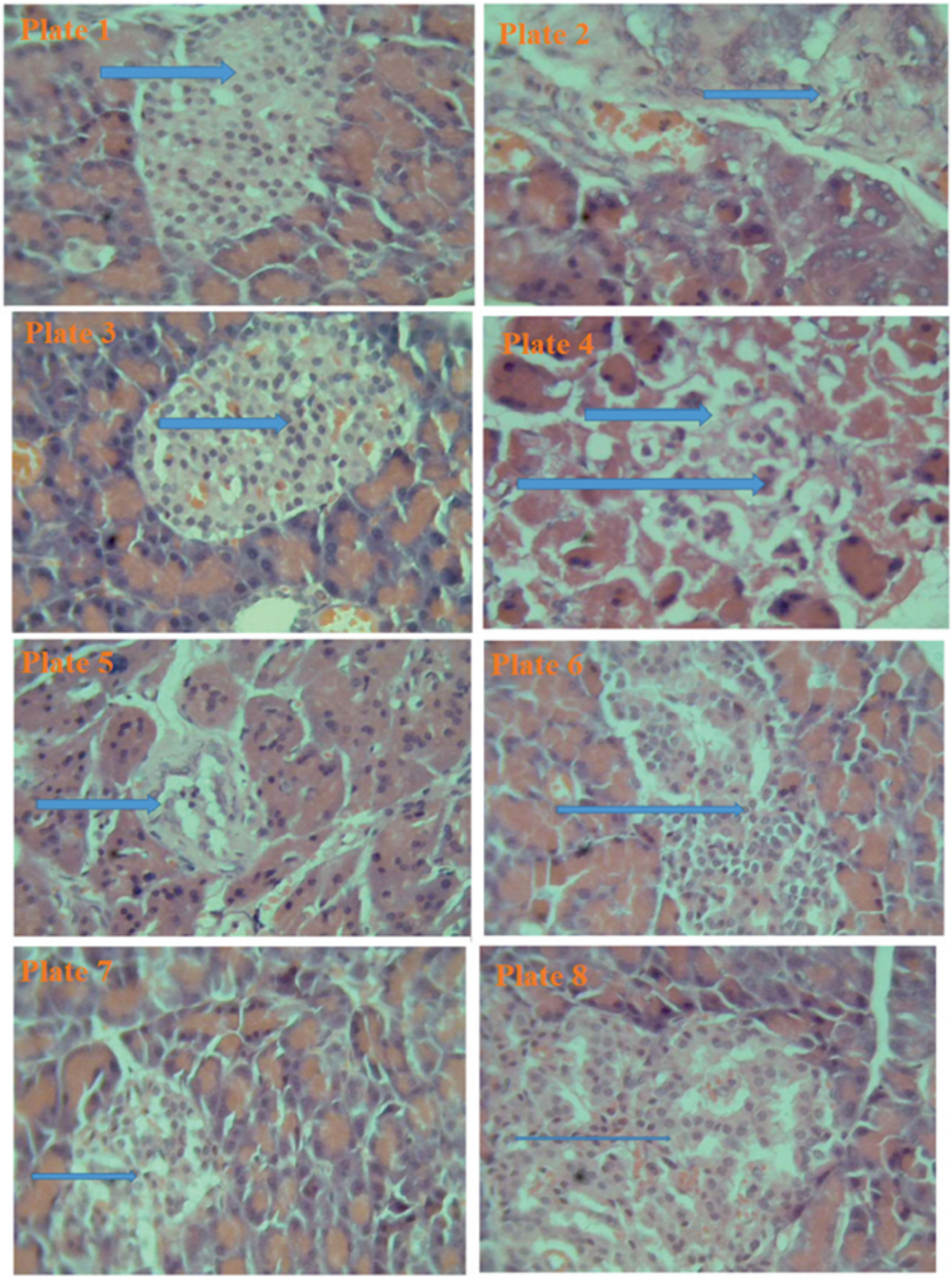
Plate 1
Plate 2
Plate 3
Plate 4
Plate 5
Plate 6
Plate 7
Plate 8

Figure 1: Showing photomicrographs of pancreas of experimental animals following treatment with *M. oleifera* fractions. Plate 1: represents pancreas of normal control rat showing normal islets of Langerhans interspersed among the acini as compact spherical mass (Arrow) without necrosis. Cells of the pancreas were all present in their normal proportions (Magnification: 400x). Plate 2: represents pancreas section of animal induced with diabetes but was not treated (diabetic control). Beta cells were partially destroyed resulting to necrosis and vacuolations of pancreatic acini and shrunken Langerhans islets cells (arrow) (Magnification 400x) Plate 3: showed the pancreas section from experimental animals administered 5mg/kgbwt of glibenclimide (drug control) showing restoration of pancreatic histology. Beta cells of the pancreas regenerated (arrow), there was no necrosis and vacuolations of pancreatic acini and Langerhans islets cells. Plate 4: showed the pancreas section from experimental animal administered Fraction 1 (F1) of *M. oleifera* leaf extract. Beta cells had necrosis and vacuolations of pancreatic acini and Langerhans islets cells. Result showed minor effects of fraction 1 on the Pancreas. Plate 5: pancreas section from experimental animal administered Fraction 2 (F2) of *M. oleifera* leaf extract showing shrunken islets of Langerhans, displaying degenerative and necrotic changes. Result showed that fraction 2 was unable to restore or regenerate the β- cells of the pancreas. Plate 6: represents pancreas section from experimental animal administered fraction 3 (F3) of *M. oleifera* leaf extract. Treated diabetic rat showed improvement of histoarchitecture, reduction in vacuolization concomitantly with an increase in size of islets. There was no necrosis. Plate 7: is the pancreas section from experimental animal administered Fraction 4 (F4) of *M. oleifera* leaf extract. Treated diabetic rat showed near normal morphology, size and cellular density of islets (arrow). It revealed that the islet beta cells of the pancreas has been regenerated showing no necrosis and vacuolations of pancreatic acini (Magnification 400x). Plate 8: pancreas section from experimental animal administered Fraction 5 of *M. oleifera* leaf extract Treated diabetic rat showed near normal morphology, size and cellular density of islets. The islet beta cells of the pancreas have been regenerated showing no necrosis and vacuolations of pancreatic acini and Langerhans islets cell

4. Discussion

Increase in vegetable utilization is very important because they contribute in alleviation of nutritional deficiencies and also in management of diseases. This is made possible because of bioactive components they possess which have been shown to exert pharmacological and biological activities in biological system[19]. The GC-MS assay has proven to be a reliable tool for the identification of these natural products in medicinal plants. The GC-MS analysis of the methanol extract of *M. oleifera* revealed the presence of eleven bioactive compounds reported to possess anti-diabetic properties. Most of the phytochemicals identified have many reported pharmacological activities relevant to the study.

Previous studies have shown that di-n-octyl phthalate with percentage composition of 30.53% can exert antimicrobial, anti-inflammatory and antioxidant properties[20]. Palmitic acid with percentage composition of 17.10% possesses antioxidant, hypocholestrolemic and alpha-amylase inhibitory activities[20,21]. Hexadecanoic acid (11.82%) possess antioxidants and anti-inflammatory, hypocholesterolemic, 5-α-reductase inhibitory effect and in all anti-diabetic activities[21]. Octadecenoic acid (7.76%) posseses antidiabetic, insulin secretion, alpha glucosidase inhibitory effect, hypocholestrelemic and antioxidant activities, octadecenoic acid ethyl ester (6.30%) can decrease blood cholesterol, inhibit alpha glucosidase, show anti-fungi and antioxidant activities. For octadecanoic acid (3.15%), it has been reported to possess hypocholesterolemic activity, exhibit antimicrobial, anti-arthritis and alpha glucosidase inhibitory[21]. Acute toxicity studies carried out revealed that *M. oleifera* leaf was safe at the dose administered.

Significant elevation in the level of fasting blood glucose was observed in STZ-induced diabetic rats as compared to normal rats which might be due to the destruction of beta cells of the pancreas. Oral administration of crude fractions of *M. oleifera* at 500 mg/kg bwt showed significant ($P<0.05$) reduction in blood glucose level when compared with the diabetic control rats. The study showed the ability of *Moringa* leaf to reduce blood glucose level which was also confirmed by the histological findings of the pancreas. Thus, it is reasonable to attribute the pharmacological and biological activities of the extract of *M. oleifera* to the presence of these identified bioactive compound which has been reported to stimulate insulin secretion and increases hepatic glucokinase in an insulin-like manner as earlier reported by Ampa *et al*[7]. Thus, it is plausible that modulation of insulin secretion and or insulin action could be involved in the anti-diabetic effect of *M. oleifera* leaf.

To examine the anti-diabetic properties and probable mechanisms of *M. oleifera* leaf extract fractions, Glut 4, AMP-kinase and GLUT 1 activity were measured in muscle homogenate, liver homogenate and whole blood respectively. The major cellular mechanism that reduces blood glucose when carbohydrate is ingested is insulin stimulated glucose transport into skeletal muscle[22]. The principal glucose transporter protein that mediates this uptake is GLUT 4 which is proposed to be important in maintaining glucose homeostasis. Thus, high level of activity of GLUT 4 enhances insulin sensitivity and increase glucose clearance which gives an added protection against insulin resistance. GLUT 1 proteins are important glucose transport proteins in erythrocytes and are not under the influence of insulin. In type 2 diabetic patients, it has been reported that increase in glycation decreases GLUT 1 glucose uptake[22]. In this study, the GLUT 1 and 4 activity are enhanced significantly ($P<0.05$) in treated groups when compared with diabetic control. Therefore, it may not be unusual to say that the hypoglycemic effect of *M. oleifera* is partly by enhancing the insulin-dependent glucose uptake in site of glucose disposal through the stimulation of glucose carriers which are the major route of glucose transport in cell membrane. In line with this result, Hsu *et al.,*[23] had earlier reported elevated level of GLUT 4 in skeletal muscle of diabetic rat after treatment with Puerarin.

There was a significant ($P<0.05$) increase in AMP-kinase activity in groups treated with fraction 3,4 and 5 when compared with the diabetic control group. AMPK has been observed to cause GLUT 4 deployment to the plasma membrane leading to insulin dependent glucose uptake. AMPK is considered as a therapeutic target for the treatment of diabetes and dyslipidemia and activation of AMPK results in increased lipid and glucose catabolism[24]. The resultant increase in AMP-Kinase activity suggests that the anti-diabetic mechanism in part may be through non-insulin dependent pathway and also through insulin stimulated transport protein (GLUT 4). The result of this study is in agreement with Yueh-Hsiung *et al.*[25] that also reported increase in AMP-Kinase activity in STZ-induced diabetic mice after treatment with triterpenoid compound.

Glucose-6-phosphate dehydrogenase is a key enzyme which catalyzes the first and rate limiting step of the hexose monophosphate (HMP) shunt and it has been observed to decrease in diabetic rats [26]. Administration of *M. oleifera* fractions showed a significant ($P<0.05$) dose-dependent increase in G6P-dehydrogenase level when compared with the diabetic control group. It is possible that the crude fraction of *M. oleifera* leaf increases the influx of glucose into pentose monophosphate shunt in a bid to reduce high blood glucose due to increase secretion of insulin by the regenerating cells of the pancreas. Thus, this will lead to an increased production of the NADPH, with concomitant decrease in oxidative stress. The result of this study is in agreement with the report of Gopinathan and Naveenraj[1] that also report increased level of G-6-PDH in treated diabetic rats.

Lipoprotein lipase (LPL) is the rate-limiting enzyme in triacylglyceride-rich lipoprotein metabolism. In poorly controlled diabetes, deficiency of lipoprotein lipase plays a role in the pathophysiology of hyperlipidemia. The result of this study showed significant ($P<0.05$) dose-dependent increase in the experimental groups. The increased activity of LPL levels after treatment with *M. oleifera* shows its protection against atherosclerosis. This result is in agreement with the report of Ranganathan *et al*.[27] who also reported high levels of LPL activity in diabetic rats treated with plants. Amylases are a group of hydrolases that split complex carbohydrates and are present in a number of organs and tissues. The inhibition of alpha-amylase in this study shows that *M. oleifera* delays carbohydrate digestion leading to reduction in glucose absorption rate which consequently reduce the post prandial plasma glucose rise. Several indigenous medicinal plants have a high potential in inhibiting pancreatic amylase activity and because of this, new pharmacological agents are developed based on inhibition of these enzyme. The result of the present study identifies with the reports of Najafian [28] that also reported decrease in amylase activity in diabetic rats after treatment with plant extracts.

The Na^+/K^+-ATPase is an enzyme that actively transport Na^+ across the brush borders of the enterocyte. In addition to this function, it also co-transports glucose using the energy generated by the Na^+ electrochemical gradient created by this enzyme. The Na^+ gradient also provides a driving force for the sodium dependent glucose transporters (SGLT 1) which are present at high concentration in the intestine with high affinity for glucose[29]. The result of the study showed a significant ($P<0.05$) decrease in Na^+/K^+-ATPase activity in diabetic rats when compared with the normal control groups and other treatment groups. This showed that induction of diabetes with STZ causes impairment of the enzyme in the diabetic control rats which is an indication of impaired glucose translocation within the small intestine and its environment. The impairment could be due to alteration in the enzyme activity or subunit expression. The enzyme inactivation could also be due to

the oxidation of lipids and proteins which are also experienced during diabetes[23]. Na^+/K^+-ATPase is rich in thiol groups and oxidation of thiol groups is known to inhibit enzyme activity. Grindley *et al* [29] also reported significant ($P<0.05$) reduction in Na^+/K^+-ATPase activity in the lower segment of intestine of diabetic rats which agrees with our study.

From the result of the histopathological studies, only fractions 3, 4 and 5 exhibited the ability to regenerate the partially destroyed pancreatic acini and reduced vacuolation of pancreatic acini and Langerhans islets cells resulting in absence of necrosis. Fraction 1 and 2 did not show any significant activity. It is possible that fraction 1 and 2 do not contain the phytochemical agents responsible for regeneration of pancreatic acini cell whereas Fraction 3, 4 and 5 contained bioactive components that may have modulatory effects on biochemical parameters in STZ-induced diabetes male wistar rats.

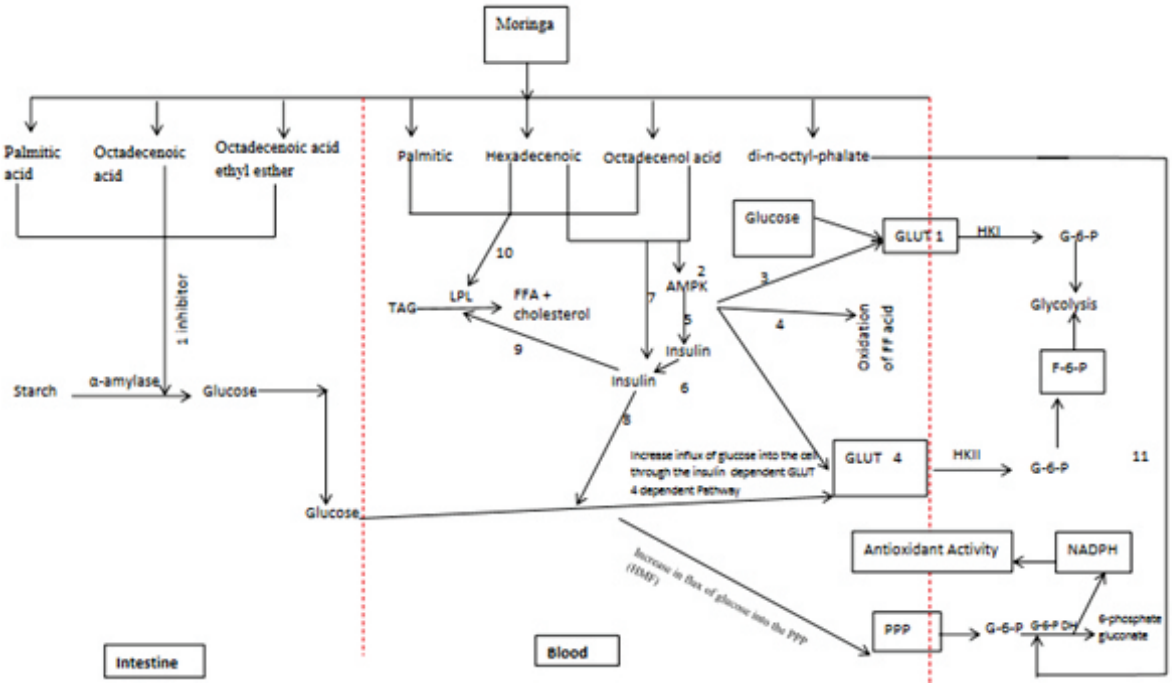

Figure 2: Proposed mechanism of action of fractions of *Moringa oleifera* extract

Summary of mechanism: 1 - inhibition of α- amylase enzyme activity, 2 - activation of AMPKinase by octadecenoic acid, 3 - activation of AMPKinase leads to increase influx of glucose, 4 - activation of AMPKinase in the liver, enhances fatty acid oxidation and decreases production of glucose, 5 - Increase production of insulin, 6 – activation of AMPKinase leads to increase in glucose uptake through an insulin dependent GLUT 4 mechanism, 7 – promotes increase production of insulin by the β-cells, 8–activation of AMP-Kinase leads to increase translocation of GLUT 4, 9 – increase production of insulin leads to activation of lipoprotein lipase activity, 10–Bioactive compounds activates lipoprotein lipase activity, 11–activation of glucose-6-phosphate dehydrogenase. HK I—Hexokinase 1, HK II --Hexokinase 11

5. Conclusion

Thus, characterized natural bioactive constituents of *M. oleifera* fractions exerted concerted potent anti-diabetic activity in streptozotocin-induced diabetic rats by probable mechanisms involving concerted α-amylase inhibition, AMP-kinase and Lipo-protein lipase activation and serum glucose transport Proteins (GLUT-1 and GLUT-4) and liver homogenate Glucose-6-phosphate dehydrogenase up-regulation.

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