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Effect of methanol extract of *musa sapientum* leaves on protein glycation and erythrocyte antioxidant status in alloxan-induced diabetic Wistar rats

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Abstract

Background: Increased generation of free radicals from protein glycation has been associated with compromised integrity of erythrocytes in diabetes. *Musa sapientum* has been reported to possess anti-diabetic properties and this study investigated the effect of methanol extract of *Musa sapientum* on protein glycation and erythrocyte integrity.

Methods: Forty-two male Wistar rats (180-200g) were randomly grouped into seven:1 (control), 2 (diabetic untreated), 3 (normal extract-treated (250mg/kg)), 4 (normal metformin-treated (150mg/kg)), 5 (diabetic extract-treated (250mg/kg)), 6 (diabetic metformin-treated (150mg/kg)), 7 (diabetic insulin-treated (1IU/kg)). Diabetes was induced with single intraperitoneal injection of 120mg/kg alloxan. Animals were treated for 14 days and blood (3mls) was collected from retro-orbital plexus to determine serum fructosamine level, erythrocyte superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities. Glycated haemoglobin (HbA1c) level was estimated using a conversion formula. Animals were sacrificed thereafter by cervical dislocation and pancreatic tissues were excised and stained with haematoxylin and eosin for histological study. Statistical significance at $P< 0.05$ was analyzed by one-way ANOVA and Newman-Keuls' post-hoc test.

Results: Diabetic rats treated with extract, metformin and insulin had significant reduction in serum fructosamine level by 62.64%, 74.63% and 56.05 % respectively while HbA1c level reduced by 45.06%, 50.62% and 40.57% respectively. Activities of erythrocyte SOD and GPx were increased in the extract-treated group. Histological studies showed regeneration of islet cells in the diabetic extract-treated rat which was comparable to normal.

Conclusion: The extract inhibited protein glycation, regenerated the islet cells and improved erythrocyte antioxidant status in diabetic rats.

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Keywords: *Diabetes, Protein glycation, Musa sapientum, erythrocyte SOD and GPx*

Résumé

Contexte: La génération augmentée de radicaux libres provenant de la glycation des protéines a été associé avec l'intégrité compromise des érythrocytes dans le diabète. Musa sapientum a été référé à posséder des propriétés antidiabétiques et cette étude a investiguée l'effet de l'extrait de méthanol de Musa sapientum sur la glycation des Protéines et de l'intégrité des érythrocytes.

Méthodes: Quarante-deux rats Wistar (180-200g) ont été regroupés au hasard en groupe de sept: 1 (témoin), 2 (diabétiques non-traité), 3 (traité avec extrait normale (250mg / kg)), 4 (traité avec metformine normale (150 mg / kg)), 5 (traité avec extrait diabétique (250 mg / kg)), 6 (traité avec metformine diabétique (150 mg / kg)), 7 (traité avec insuline diabétique (1 UI / kg)). Le diabète était induit avec une injection intra-péritonéale de 120 mg / kg d'alloxane. Les animaux étaient traités pendants 14 jours et le sang (3 ml) était recueilli provenant du plexus rétro-orbitaire pour déterminer le niveau sérique de fructosamine, dismutase super-oxyde érythrocytaire (SOD) et la glutathion des activités peroxydase (GPx). Le niveau d'hémoglobine glyquée (HbA1c) était estimé en utilisant une formule de conversion. Les animaux étaient sacrifiés après cela par dislocation cervicale et les tissus pancréatiques étaient excisées et colorées avec hématoxyline et éosine pour l'étude histologique. La signification statistique à $p <0,05$ a été analysé par une voie ANOVA et le test post-hoc de Newman-Keuls.

Résultats: Les rats diabétiques traités avec l'extrait, la metformine et l'insuline avaient réduction significative du niveau de fructosamine sérique par 62,64%, 74,63% et 56,05% respectivement, tandis que le niveau HbA1c réduit par 45,06%, 50,62% et 40,57% respectivement. Les activités des SOD érythrocytes et GPx étaient augmentés dans le groupe traité avec l'extrait. Les études histologiques ont montré régénération des îlots de cellules chez les rats Traités avec extrait diabétique ce qui était comparable à la normale.

Conclusion: L'extrait a inhibé la glycation des protéines, régénéré les îlots de cellules et a amélioré le statut d'érythrocytes antioxydant Chez les rats diabétiques.

Mots-clés: *Diabète, glycation des Protéines, Musa sapientum, SOD érythrocytaire et GPx*

Introduction

Diabetes is a metabolic disorder that modifies a number of physiologically important mammalian proteins such as serum proteins [1], haemoglobin [2], membrane proteins [3] and protein in the eyes [4]. Diabetes-induced glycation of proteins subsequently causes an increase in the generation of free radicals affecting body cells, including red blood cells (RBCs) [5]. It is believed that the underlying causes of complications associated with diabetes are hyperglycemia and consequent protein glycation which culminates in increased production of free radicals [6, 7]. Oyedeji *et al.* [5] reported that associated modifications of RBC membrane proteins as a result of hyperglycemia consequently reduce erythrocyte survival, antioxidant activities and increase lipid peroxidation. Endogenous enzymatic and non-enzymatic antioxidants such as glutathione, catalase, glutathione peroxidase, superoxide dismutase, vitamin A, C and E, melatonin, coenzyme Q, glutathione reductase and others help mop up generated free radicals thus protecting the cells from damage [8, 9, 10]. Exogenous agents and plants have well been reported to possess anti-diabetic and antioxidant properties that help up-regulate the activities of endogenous antioxidants. Experimental animals treated with *Garcinia kola* root and *Musa sapientum* leaf extracts respectively showed significant reduction in lipid peroxides and significant increase in the activities of antioxidant enzymes in diabetes [11, 12].

Musa sapientum is an herbaceous plant of Musaceae family, commonly known as 'banana'. The anti-diabetic potential [13, 14] and the anti-ulcerative properties [15] of various parts of the plant have also been demonstrated. The extract of *Musa sapientum* leaves has been reported to contain saponins, antioxidant properties [12] but there is dearth of information on its possible effects on protein glycation and erythrocyte antioxidant status. This study therefore seeks to investigate the possible effect of methanol extract of *Musa sapientum* leaves on protein glycation and erythrocyte antioxidant status in diabetes.

Materials and methods

Collection and preparation of plant extract

Fresh leaves of *Musa sapientum* were collected and air-dried at room temperature. Dried *Musa sapientum* leaves were grounded to powder using an electronic mill (Binatone) and subjected to extraction processes using n-hexane (for 72 hours), then ethyl acetate and finally methanol for 72 hours with constant daily stirring. The methanol filtrate was collected using Whatman no. 1 filter paper and evaporated to dryness under reduced pressure using rotary evaporator. The dried extract was stored in dry sterilized containers at 4°C until use. The aqueous solution was prepared by dissolving 11.25g of extract in 100mls of water and labeled as MEMSL (Methanol Extract of *Musa sapientum* Leaves). The dose of MEMSL used was 250mg/kg body weight.

Phytochemical screening

The methanol extract of the leaves of *M. sapientum* was screened for the presence of phenolic compounds, flavonoids, tannins, terpenoids according to the method of Trease and Evans [16]; Sofowora, [17] and Khandelwal [18].

Estimation of total phenols

The amount of total phenols in the plant leaves was estimated using the method of Singleton *et al.* [19] which is based on the reaction of phenols with phosphomolybdic acid in Folin-Ciocalteau reagent to produce a blue-coloured complex in alkaline medium measured spectrophotometrically at 765nm.

Animals

Forty-two healthy male Wistar rats weighing (180-200g) and having blood glucose (70-90mg/dL) were maintained on standard rat pellet (Vita feeds, Nigeria), and water *ad libitum* at a photo-periodicity of 12 hours light and 12 hours night in the animal house of the Faculty of Basic Medical Sciences. The animals were acclimatized to laboratory conditions for two weeks before the start of the experiment. All experiments were performed in accordance with the public health policy on Humane Care and Use of Laboratory Animals of National Institute of Health [20].

Induction / determination of diabetes

Diabetes was induced by a single intra-peritoneal (*ip*) injection of alloxan monohydrate (Sigma Aldrich, Germany) (120mg/kg body weight) [21] after 18h fasting. Blood glucose levels from tail vein were monitored at 12h interval for three days after alloxan injection. Diabetes was confirmed 72 hours after alloxan injection using AccuChek Active glucometer (Roche Diagonistics, Germany). Animals with constant fasting blood glucose (FBG) values above 250mg/dL [22] were recruited for the study.

Experimental procedure

The animals were divided into seven groups of six rats each.

Group 1: Normal untreated (NU) rats with free access to water and food
Group 2: Diabetic untreated (DU) rats with free access to water and food
Group 3: Normal rats treated orally with MEMSL (250 mg/kg) daily for 14 days (NMEMSL)
Group 4: Normal rats treated orally with metformin (150mg/kg/day) daily for 14days (NMETF)
Group 5: Diabetic rats treated orally with MEMSL (250 mg/kg) daily for 14days (DMEMSL)
Group 6: Diabetic rats treated orally with metformin (150mg/kg/day) daily for 14days (DMETF)
Group 7: Diabetic rats treated with 1IU/kg (*i.m.*) insulin for 14 days (DINSL)
The blood glucose and body weight of the animals were monitored throughout the study.

Biochemical assay
After 14 days of treatment, 3mls of blood was collected through retro-orbital plexus under mild ether anaesthesia into plain and heparinized serum bottles (1.5ml each). The blood samples in the plain bottles were allowed to clot for one hour and centrifuged at 3500 rpm for 10minutes. Serum was carefully aspirated into clean sterile plain bottles and kept frozen at (-20°C) for biochemical assay while heparinized whole blood was used immediately for antioxidant enzyme analysis.

Estimation of serum fructosamine
Serum was allowed to de-freeze at room temperature and assayed for fructosamine level using quantitative colorimeric fructosamine kit (Fortress Diagonistics, UK).

Estimation of glycated haemoglobin (HbA1c)
Estimation of glycated haemoglobin (HbA1c) was done using the prediction formula of Cohen *et al.* [23] for calculating the HbA_{1c} level from available data of fructosamine level.

$\%\ HbA_{1c} = 0.017 \times \text{Fructosamine}\ (\mu M) + 1.61$

Estimation of antioxidant enzymes
Heparinized blood sample was centrifuged at 3500 rpm for 10minutes to separate red blood cells (RBCs). A volume of 0.5ml of RBCs from centrifuged heparinized blood sample was washed four times with 5ml of normal saline before being subjected to biochemical assay. Determination of the erythrocyte SOD and GPx activity in the RBCs was done using test kits (Fortress Diagonistics, UK). Assay for SOD was performed according to the principle of Wooliams, *et al.* [24]. This method involved generation of superoxide radicals by xanthine and xanthine oxidase to react with 2-(4-iodophenyl)-3-(4-nitophenol)-5-phenyltetrazolium chloride, while GPx assay was done based on the principles of Pippenger, *et al.* [25].

Histopathological Studies
The animals were sacrificed by cervical dislocation and an incision was made in abdomino-pelvic region to excise the pancreas fixed in 10% formalin. The fixed pancreatic tissues were evaluated for histopathology using Hematoxylin and Eosin staining procedures of Culling [26].

Statistical analysis
Results were expressed as Mean ± SEM and analyzed using one-way analysis of variance (ANOVA) and the Newman-Keuls post-hoc test for test of significance at ($P<0.05$) (Graphpad Prism 5.01, USA)

Results
Phytochemical screening of MEMSL showed presence of phenolic compounds, flavonoids and tannins while terpenoids were found to be absent (Table 1).

Table 1: Phytochemical screening of methanolic extract of *Musa sapientum* leaves (MEMSL)

Phytochemicals	Test	Presence
1. Phenolic compounds	a. Ferric chloride test	+
	b. Lead acetate test	+
2. Flavonoids	a. Sodium Hydroxide test	+
	b. Conc. H_2SO_4 test	+
3. Tannins	Extract + Distilled H_2O + 10% $FeCl_3$	+
4. Terpenoids	Extract + ethanol + acetic anhydride + H_2SO_4	-

Total phenolic content of MEMSL was estimated according to the method of Singleton *et al.* [19] to be 2.48 ± 0.00 mgGAE. *mgGAE – milligramme Gallic Acid Equivalent. Result expressed per unit gramme of extract.* Value expressed in mean ± standard deviation

Table 2: Effects of methanolic extract of *Musa sapientum* leaves, metformin and insulin on blood glucose

Experimental Groups	Blood Glucose (mg/dL) 72 hours after alloxan injection	Values after 14days of treatment	% Reduction
1 (Normal Untreated)	90.4 ± 3.23	73.6 ± 1.03	18.58*
2 (Diabetic untreated)	397.4 ± 24.91	474.2 ± 13.93	-19.33
3 (Normal MEMSL-treated)	87.6 ± 0.24	75.2 ± 1.66	14.16*
4 (Normal Metformin-treated)	94.4 ± 0.98	82.6 ± 3.75	12.50*
5 (Diabetic MEMSL-treated)	360.8 ± 30.33	194 ± 44.67	46.23*
6 (Diabetic Metformin-treated)	375.0 ± 19.00	192.6 ± 42.03	48.64*
7 (Diabetic Insulin-treated)	287.2 ± 10.82	135.8 ± 13.17	52.72*

*Values are mean ± SEM; n=6. *Significantly different from Group 2 (Diabetic untreated) (P < 0.05)*

Administration of MEMSL, metformin and insulin on diabetic groups significantly ($p < 0.05$) reduced blood glucose level by 46.23%, 48.64% and 52.72% respectively compared to diabetic untreated group (table 2)

Diabetic animals treated with MEMSL, metformin and insulin showed significant reduction ($P < 0.05$) of 62.64%, 74.73% and 56.05% respectively in serum fructosamine level compared to diabtic untreat (figure 1)".

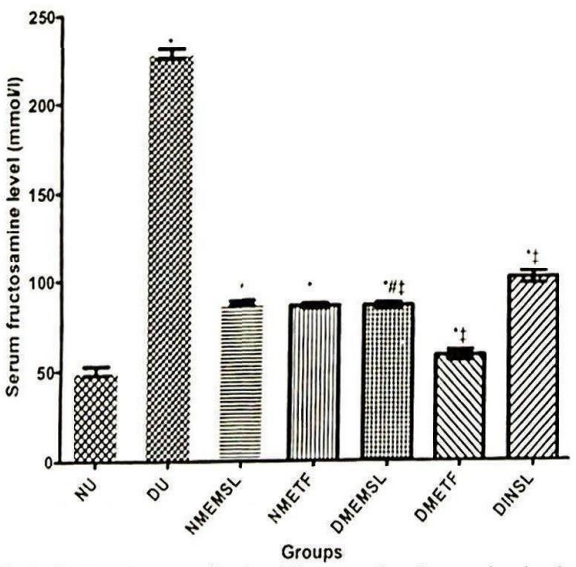

Fig.1: Serum fructosamine level in control and treated animals. *Values are mean ± SEM, n=6, *compared with NU (P < 0.05), †compared with DU (P < 0.05), ‡compared with DMETF and DINSL (P < 0.05).* NU: Normal Untreated. DU: Diabetic Untreated. NMEMSL: Normal MEMSL-treated. NMETF: Normal Metformin-treated DMEMSL: Diabetic MEMSL-treated. DMETF: Diabetic Metformin-treated. DINSL: Diabetic Insulin-treated

Glycated haemoglobin (HbA1c) level in diabetic-MEMSL treated, diabetic metformin treated and

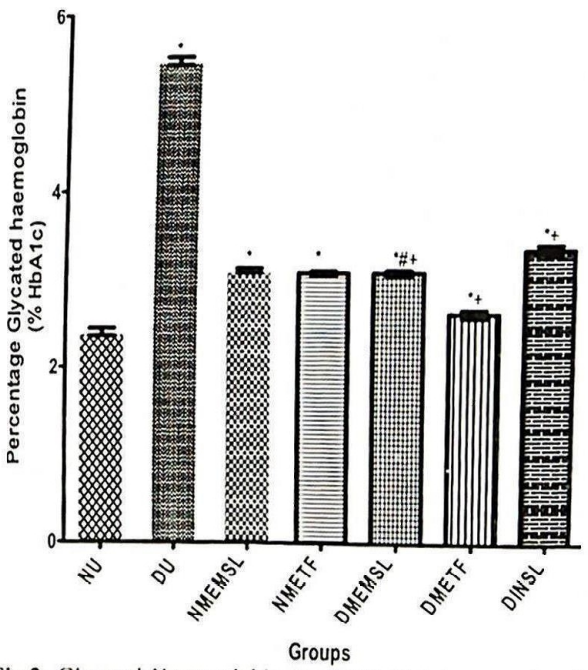

Fig.2: Glycated Haemoglobin level (HbA1c) in control and treated animals

*Values are mean ± SEM, n=6, *compared with NU (P < 0.05), †compared with DU (P < 0.05), ‡compared with DMETF and DINSL (P < 0.05).* NU: Normal Untreated. DU: Diabetic Untreated. NMEMSL: Normal MEMSL-treated. NMETF: Normal Metformin-treated. DMEMSL: Diabetic MEMSL-treated. DMETF: Diabetic Metformin-treated. DINSL: Diabetic Insulin-treated

diabetic insulin treated was significantly reduced ($P < 0.05$) compared to diabetic untreated (Fig.2).

The superoxide dismutase (SOD) activities in RBCs of diabetic groups treated with MEMSL, metformin and insulin increased significantly ($P < 0.05$) when compared with diabetic untreated (Figure 3). Also, the glutathione peroxidase (GPx) activities in the RBCs of all diabetic treated groups increased significantly compared to diabetic untreated (figure 4).

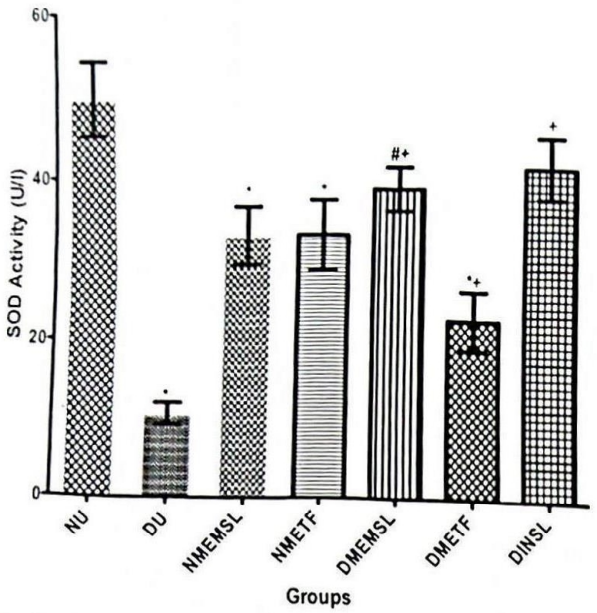

Fig.3: Activity of superoxide dismutase (SOD) in the erythrocytes of normal and treated animals
*Values are mean ± SEM, n=6, *compared with NU (P < 0.05),‡compared with DU (P < 0.05), #compared with DMETF (P < 0.05).* NU: Normal Untreated. DU: Diabetic Untreated. NMEMSL: Normal MEMSL-treated. NMETF: Normal Metformin-treated. DMEMSL: Diabetic MEMSL-treated. DMETF: Diabetic Metformin-treated. DINSL: Diabetic Insulin-treated

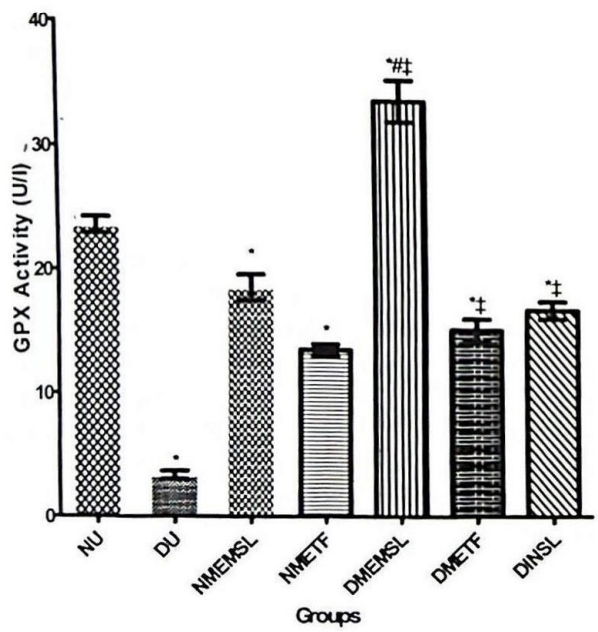

Fig. 4: Activity of glutathione peroxidase (GPx) in the erythrocytes of normal and treated animals
*Values are mean ± SEM, n=6, *compared with NU (P <0.05),‡compared with DU (P < 0.05), #compared with DMETF and DINSL (P < 0.05).* NU: Normal Untreated. DU: Diabetic Untreated. NMEMSL: Normal MEMSL-treated. NMETF: Normal Metformin-treated. DMEMSL: Diabetic MEMSL-treated. DMETF: Diabetic Metformin-treated. DINSL: Diabetic Insulin-treated

Histological observation of the pancreatic tissues of diabetic group treated with MEMSL showed restoration of the cyto-architecture and granulation of the islet cells (Plate 5) and this is comaprable to normal (Plate 1).

Histological observation

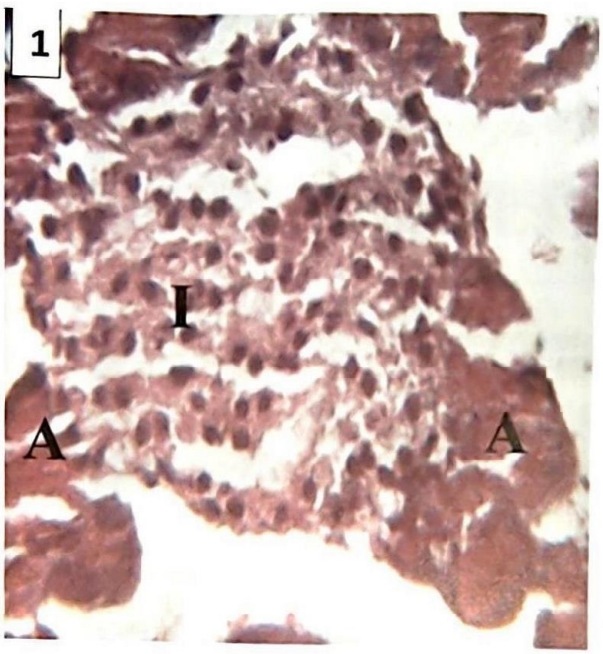

Plate 1 (Normal untreated)
Prominent and well circumscribed islet cells (1) and well defined exocrine acinar cells (A)

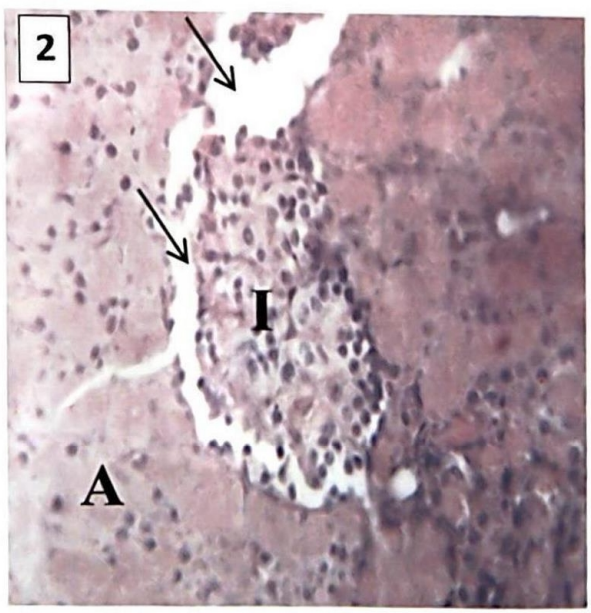

Plate 2 (Diabetic untreated)
The secretory acinar cells are present (A) necrotic islet cells (1) that appeared degenerated with lesion around the islet cells indicated with arrows

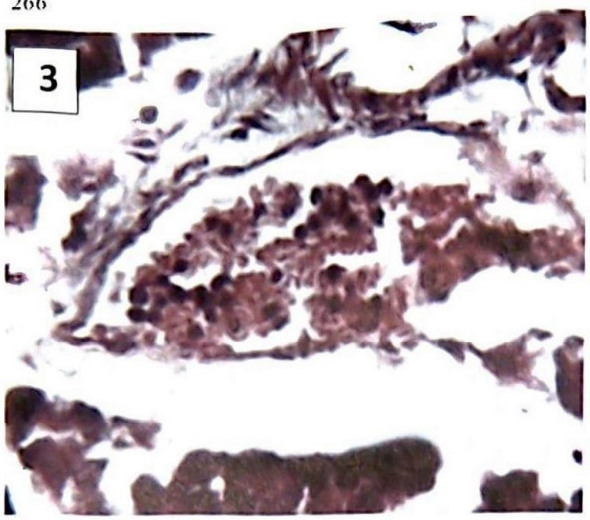

Plate 3 (Diabetic MEMSL-treated)
Shows normal architecture of the acinar cells (A) and islet cells (I) that are clearly defined and prominent with marked vascular congestion indicated with arrows

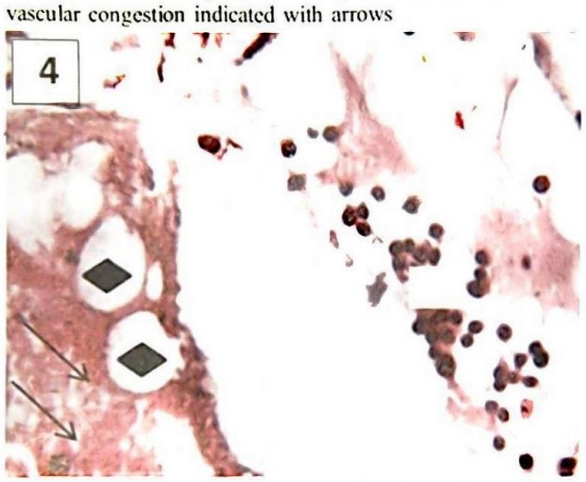

Plate 4 (Diabetic Metformin-treated: Presence of severe infiltration of connective tissue by inflammatory cells (indicated with box), necrotic acinar cells indicated with arrows and few normal islet cells (I)

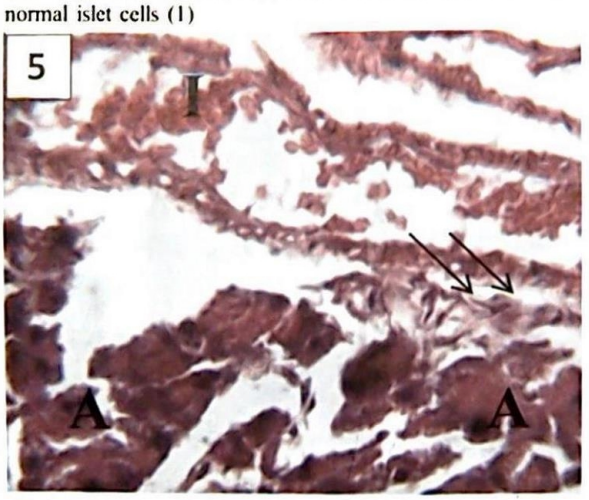

Plate 5 (Diabetic insulin-treated):
Normal acinar cells (A) and islet cells (I) with moderate vascular congestion and haemorrhage indicated with arrows

Plates 1-5: Photomicrographs (x 400) of pancreas of normal and treated groups.\
Staining: Haematoxylin and Eosin

Discussion

Regulation of blood glucose level within physiological limit is important for survival of cells while increased blood glucose level (hyperglycaemia) can initiate different physiological and biochemical disturbance that disrupt cellular activities [2, 27]. It has been reported that hyperglycaemia accelerates non-enzymatic glycation of proteins as well as production of free radicals [7, 27]. Sacks [28] and American Association of Clinical Chemistry [29] reported that serum fructosamine and HbA1c levels are good indicators of protein glycation. The anti-diabetic activity of MEMSL and the increase in body weight observed in the extract treated group in this study agrees with earlier report of Adewoye and Ige [12], Eleazu and Okafor [30] and Kim *et al.* [31].

However, the stimulatory effect of insulin on body weight as observed in this study agrees with the report of Pinheiro *et al.* [32]. Metformin did not stimulate any weight gain in the diabetic group studied and this is supported by the report of Colay [33]. According to Sacks [28], effective reduction of blood glucose by 60mg/dL in diabetics will result in 75µmol change in fructosamine and 2% change in HbA1c. The reduction in protein glycation observed in this study may well be the effect of the phytochemicals present in the plant extract. Flavonoids, phenols, saponins and tannins present in the plant extract have been reported to possess anti-diabetic and anti-glycation activities [34-36].

Studies have also reported that metformin reduces protein glycation through deactivation of methylglyoxal to D-lactate and/or direct inhibition of glycation process [37, 38] while insulin acts by reducing blood glucose level and enhancing endocytotic uptake of glycated products [39, 40]. It is also possible that the mechanism of reducing protein glycation by the extract is similar to that utilized by metformin and insulin. Diabetes induces generation of free radicals and could overwhelm the activities of antioxidant enzymes that protect against complications [7, 8, 10].

In this study the SOD and GPx activities were stimulated in the erythrocytes of diabetic extract treated group. This could possibly be that the phytochemicals scavenged the generated free radicals thereby enhancing the activity of the antioxidant enzymes [41, 42]. According to Memisogullari *et al.* [43] and Balajee and Dahan [44], metformin up-regulates the activities of antioxidant enzymes in erythrocytes of diabetic patients and rats. Similar result was observed in the diabetic metformin treated and diabetic insulin

treated groups which showed up-regulation in the activities of SOD and GPx in the erythrocytes.

Evidence from animal model of diabetes indicated low glucose utilization and clearance due to necrosis of the islet cells [45] which was reported to affect the integrity of the islet cells and its functions in glucose homeostasis. Similar report was made in the histology of the pancreatic tissue where the integrity of the islet cells was distorted. However the pancreas of diabetic group treated with MEMSL showed regeneration of islets and this was supported by Coskun *et al.* [46] and Adeyemi *et al.* [9] who reported that phytochemical contents of plant extract cause proliferation and regeneration of islet cells in diabetic rats.

Conclusion

The extract inhibited protein glycation, regenerated the islet cells and improved erythrocyte antioxidant status in diabetic rats. The present study revealed that methanol extract of *Musa sapientum* leaves possesses the potentials that could be of great benefit in the management of diabetes and its complications.

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