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THE HISTOLOGICAL, IMMUNOHISTOCHEMICAL AND BIOCHEMICAL EFFECTS OF n-HEXANE FRACTION OF Moringa oleifera ON THE PANCREAS OF STREPTOZOTOCIN-INDUCED DIABETIC WISTAR RATS

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ABSTRACT

Background: **Diabetes mellitus (DM) is an aggressive challenge of public health concern in international context. Several studies provide evidences on the antidiabetic benefits of Moringa oleifera, but there are limited data on the effect of the n-hexane fraction of Moringa oleifera (n-HFMO) on the histoarchitecture, biochemistry and immunohistochemistry of the pancreatic beta cells, hence this study.**

Methods: **Forty-eight Wistar rats weighing between (200-220) g were used for this study. They were randomly divided into 6 groups of 8 rats each. Group 1 received 2ml/kg/day of distilled water orally for 42 days. Diabetes was induced in groups 2 to 5, by intraperitoneal administration of streptozotocin (STZ) for 5 days, groups 2 was left untreated while groups 3-6 were treated with n-HFMO 125, 250, 500 mg/kg/day and Insulin 5 IU/kg/day respectively for 42 days. After the experiment, the animals were sacrificed and the blood samples were collected for serum analyses.**

Results: **The administration of n-HFMO at 500 mg/kg and insulin, produced restorative effect and improved serum -amylase levels significantly in group 5 compared with group 2, but insulin treatment did not produce a statistically significant improvement in group 6. There was distortion of pancreatic histoarchitecture in group 2, revealing atrophy and effacement of beta cells, with increased deposition of collagen around the blood vessels and stroma indicating fibrosis in group 2. Immunohistochemical analysis revealed no immunoreactivity in any of its fields, whereas group 2 showed intense immunoreaction, with high IL-2 receptor expression on the destructive T-cells on Fiji image J software for analyses of images.**

Conclusion: **This concluded that n-HFMO demonstrated antihyperglycaemic, anti-inflammatory, antioxidant potentials and was able to improve the pancreatic -cell function.**

Keywords: n-hexane fraction of Moringa oleifera, Streptozotocin, Diabetes, Wistar rats, Pancreas,

INTRODUCTION

Diabetes mellitus (DM) is a fast-growing problem of public health in an international context.[1] Over 425 million people were estimated to be living with the disease in 2017, with forecasts indicating that the number of people living with DM (PLWD) will reach approximately 35.35% by 2045.[1] Type 2 diabetes mellitus (T2DM) represents the most common type of DM that contributes to more than 90 percent of morbidity and mortality due to diabetes across the globe.[2]

The exact pathogenesis of T2DM is still poorly understood, but the development is mainly linked to insulin resistance and pancreatic β-cell dysfunction, the processes that play a central role in glucose homeostasis.[3,4] These pathological processes are driven by mechanisms such as the dedifferentiation of β-cells, mitochondrial dysfunction, oxidative stress and inflammation.[5]

Type 2 Diabetes Mellitus (T2DM) is a polygenic and multifactorial, complicated disease that is affected by a substantial number of risk factors. Pre-obesity and obesity form significant risk factors, whereas the progressive age levels predisposed to a significant extent.[6] Other risk factors like genetic tendency, unhealthy eating habits, environmental and work-related exposures, smoking, drinking, and lack of exercises only increase the risk of diseases.[6] There were reports that the etiology of DM is multifactorial but lifestyle contribution and especially sedentary behavior and unhealthy diets are closely linked with the development of DM.[4]

Glycaemic control and patient outcomes have also been enhanced with the introduction of antidiabetic therapies such as insulin, biguanides, sulphonylureas, α-glucosidase inhibitors, and other classes of drugs. Nevertheless, the application of these agents is regularly limited due to the high cost, low accessibility, especially in developing nations, poor pharmacokinetic activity, and the presence of intolerable adverse effects like hypoglycaemia, dizziness, and lactic acidosis.[7,4] It is these constraints that have led to an increased interest in the establishment of effective ethnomedicinal alternatives which are usually viewed as affordable, have easy access, and are reasonably safe to be used over extended period.

Plants used in medicine have an extensive history as they have served in the treatment of diseases due to their therapeutic effects, low side effects, low cost and great availability. Their pharmacological actions can be explained in large part by bioactive phytochemicals which include carotenoids and have various health-promoting actions.[8] The findings from several studies provide evidence on the antidiabetic benefits of Moringa oleifera, however there are limited data on the effect of the n-hexane fraction of Moringa oleifera (n-HFMO) on the immunohistochemistry, biochemistry and histomorphology of the pancreatic beta cells of Wistar rats with T2DM, hence this study. Moringa oleifera (MO) is used as a food source and for traditional medicine, due to probable antidiabetic, antioxidant, anti-inflammatory, and anti-lipidaemic properties. These are explained by the presence of numerous phytochemicals in the leaves.[9] It has shown promising antidiabetic benefits, particularly in reducing blood glucose, combating oxidative stress and inflammation.

Moringa oleifera is also known as "drumstick". It is a tree belonging to the family Moringaceae, genus Moringa, originally native from the Himalayans but currently cultivated in many tropical and subtropical regions around the world including Nigeria.[10] Various parts of MO, e.g leaves, flowers, fruits, and roots have been used as food and in traditional medicine [11]. For example, leaves are the most commonly used part which contain many nutrients including beta-carotene; vitamins B, C, and E; minerals (calcium, iron, potassium, magnesium, etc.); essential and non-essential amino acids; and carbohydrates; among others.[12] Moringa oleifera has been used in traditional medicine for the treatment of various conditions and, more recently, has been proposed to be of benefit in numerous diseases including cardiovascular, diabetes, cancer, neurological, gastroenterological, and inflammatory [13]. It is reported in animal models, that MO has antihyperglycaemic activity[14] which can improve induced diabetic effects in rats.[15]

Specific objectives of the study are;
To evaluate the effects of n-HFMO on biochemical markers of pancreatic function (insulin, C-peptide, pro-insulin, β-amylase, HbA1c, and RBS) in STZ-induced diabetic Wistar rats. To assess the histomorphological changes in the pancreas of STZ-induced diabetic Wistar rats following n-HFMO treatment and to determine the immunohistochemical expression of IL-2 in the pancreas of STZ-induced diabetic Wistar rats following n-HFMO treatment and; Compare the effects of n-HFMO with insulin treatment in STZ-induced diabetic Wistar rats.

Mechanism of action n-HFMO
The n-hexane fraction of Moringa Oleifera contains flavonoids, quercetin, kaempferol, phenolic acids, chlorogenic acid and caffeoylquinic acid.[16] These seem to confer antihyperglycaemic properties, acting as competitive inhibitors of the sodium-glucose linked transporter type 1 (SGLT1) in the mucosa of small intestine thus reducing the intestinal absorption of glucose.[17]

MATERIALS AND METHODS

Study Setting: Forty-five adult male Wistar rats weighing between 180–220g were procured from Animal house of the College of Health Sciences, Obafemi Awolowo University, Ile-Ife, Osun State. The rats were housed in plastic cages under standard laboratory conditions, with natural light/dark cycle. Animals were acclimatized for two weeks prior to the commencement of the experiment and provided with standard rat chow and free access to water.

Study design: It is an experimental study, that comprises 48 adults male Wistar rats weighing between (200 - 220) g were used for this study. These rats were randomly divided into 6 groups (1 - 6) of 8 rats each. All rats were fed high fat rat diet over a period of 4 weeks and given water ad libitum. Animals were kept and maintained under standard laboratory conditions. Group A (normal control) received 2ml/kg/day of distilled water orally for 42 days. Diabetes was induced in groups B, C, D and E by intraperitoneal administration of streptozotocin dissolved in 0.1M sodium citrate buffer (pH 4.5) for 5 days. Twenty-four-hours post STZ, the test groups C, D and E were treated with nHFMO 125 mg/kg/day, 250 mg/kg/day, 500 mg/kg/day, respectively for 42 days. Group F was the test group that was treated with Insulin 5 IU/kg/day subcutaneously for 42 days after induction of diabetes.

Sample size calculation: Turkey's Test, ANOVA, α-level was set at < 0.05

Sampling procedure: Blood samples were collected by cardiac puncture into plain bottle for serum analysis. Rats were sampled in the caudal tail vein to measure the RBG level in the blood. All the measurements were done with a glucometer. Before sampling, a tail was rubbed by gently massaging a peripheral blood flow, disinfected using an alcohol swab before being punctured with a lancet. The first droplet was discarded and the second droplet put on a glucose test strip to be examined. All readings were recorded properly and rats with blood glucose levels above 200 mg/dL (11.1 mmol/L) were considered diabetic.

Ethical considerations: Experimental procedures were conducted in accordance with institutional ethical guidelines for animal research and approved by Health Research Ethics Committee (HREC), Institute of Public Health (IPH) Obafemi Awolowo University (OAU) Ile-Ife. The animals were given humane care according to the guidelines of HREC, IPH, OAU.

Study procedure: The excised pancreas tissues were harvested and fixed in 10% neutral buffered formalin. The tissues were processed for paraffin embedding and processed via paraffin wax embedding method of Dury and Wallington, (1980). The tissues were dehydrated at room temperature through ascending grades of ethanol of 50%, 70%, 80%, 90%, and absolute ethanol twice. The dehydrated tissues were cleared at room temperature in two changes of xylene each lasting one hour. The tissues were further infiltrated in two changes of molten paraffin wax at 600.C for one hour each, then the tissues were embedded in paraffin wax using multi block plastic embedding mould. The paraffin blocked tissues were trimmed, soften on an ice and mounted for sectioning on a rotary microtome. Sections of 5ìm thick were produced from the tissue blocks using a rotary microtome (Laboid LBM-RM2, London, England). The sections were collected and transferred onto a floating bath at 400.C to allow spreading of the folded ribbons of sections. The sections were picked and mounted on a new clean glass slides and were dried at 400.C on a slide drier to enhance adherence of the section to the slides and stained. Haematoxylin & Eosin was employed to assess general histoarchitecture of the pancreas, Mason trichome and VVG to assess for collagen deposition and aldehyde fuschin to assess beta cells. Immunohistochemical stain was done to show the presence of antigen- antibody complex and inflammatory markers with IL-2 in pancreatic injury.

Extraction of n-hexane fraction of Moringa Oleifera

Preparation of sample

Fresh Leaves of MO were washed in distilled water, thereafter the air-dried leaves were pulverized. The powdered form was then soaked in 70% ethanol for 72 hours at room temperature.

Procedure for solvent extraction

10 g of the ethanol extract was mixed in n-hexane, in a ratio o f 1:3 (w/w) for extract to solvent. The mixture was intermittently agitated with magnetic stirrer and shaken. For better efficiency, a mixture of solvents n-hexane:acetone:ethanol (5:4:1) were used to break down the matrix. The extraction was carried out at 40°C for 60 minutes. The mixture was centrifuged and then filtered with Whatman's filter paper no 1 to separate the n-hexane leaf extract of Moringa oleifera solid residue from the solvent phase.

Fractionation and purification

The extract was transferred to a separating funnel and distilled water was added in enhancing the phase separation to allow the lipophilic n-hexane to separate from the aqueous layer. The n-hexane layer was then washed in distilled water to remove impurities. The extract was dried using anhydrous sodium sulfate to remove remaining traces of water.

The n-hexane fraction was concentrated using a rotary evaporator at 40°C under low pressure to obtain a concentrated extract in a hood to a small volume, for higher purity fraction, ethanol was added to the concentrated extract as an anti-solvent at low temperatures (0°- 4°C) to precipitate the extract. The resulting extract was collected by vacuum filtration, dried and kept at low temperature.

$$\%\text{Yield} = \frac{\text{Weight of n-hexane – free extract X 100}}{\text{Weight of fresh M.O(g)}}\%(g)^{18}$$

Streptozotocin preparation

STZ induces diabetes mellitus by selectively destroying pancreatic β-cells through DNA alkylation, leading to insulin deficiency and persistent hyperglycemia. STZ was prepared following the methods recommended by Ghasemi and Jeddi (2023). STZ solution was prepared and maintained in 0.1M ice-cold acidic (pH 4.5) citrate buffer, and was used immediately after its preparation. To prevent degradation due to its photosensitivity, STZ was prepared in tube wrapped with aluminum foil.

Induction of diabetes
Diabetes was induced in overnight-fasted Wistar rats through a five-day series of low dose intraperitoneal injection of STZ (45 mg/kg) dissolved in citrate buffer. To prevent acute hypoglycemia caused by STZ-induced β-cell dysfunction, the animals were provided with 5% dextrose water ad libitum for 24 hours post STZ administration, Ghasemi and Jeddi (2023).

Animal care
Ethical clearance was obtained from health research ethics committee (HREC), institute of public health (IPH) Obafemi Awolowo University (OAU) Ile-Ife. The animals were given humane care according to the guidelines of HREC, IPH, OAU.

Experimental design
Preceding the experimental interventions, animals were fasted of food (16 hours) but provided water ad libitum. The end of the fasting period was the time zero (0 h) and this was when venous blood glucose concentrations were measured with glucose assay kit using Glucose Oxidase – Peroxidase (GOD-POD) method and values gotten were recorded.

Forty-eight (48) adults male Wistar rats weighing between (200 - 220) g were used for this study. These rats were randomly divided into 6 groups (1 - 6) of 8 rats each. All rats were fed high fat rat diet over a period of 4 weeks. Animals were kept and maintained under standard laboratory conditions. Group 1 (normal control) received 2ml/kg/day of distilled water orally for 42 days. Diabetes was induced in groups 2, 3, 4, 5 and 6 by intraperitoneal administration of streptozotocin dissolved in 0.1M sodium citrate buffer (pH 4.5) for 5 days. Twenty-four-hours post STZ, the test groups 3, 4, 5 and 6 were treated with oral n-HFMO 125, 250, 500 mg/kg/day, and Insulin 5 IU/kg/day subcutaneously respectively for 42 days. At the end of the experiment, the rats were given 2 weeks of recovery period, then sacrificed under ether anesthesia. The blood samples were collected by cardiac puncture into plain bottle for serum analysis.

Random blood glucose estimation
The rats were sampled in the caudal tail vein to measure the RBG level in the blood. All the measurements were done with a glucometer. Before sampling, a tail was rubbed by gently massaging a peripheral blood flow, disinfected using an alcohol swab before being punctured with a lancet. The first droplet was discarded and the second droplet put on a glucose test strip to be examined. All readings were recorded properly and rats with blood glucose levels above 200 mg/dL (11.1 mmol/L) were considered diabetic.

Histological, immunohistochemical and biochemical studies
The excised pancreas tissues were harvested and fixed in 10% neutral buffered formalin. The tissues were processed for paraffin embedding and processed via paraffin wax embedding method of Dury and Wallington, (1980). The tissues were dehydrated at room temperature through ascending grades of ethanol of 50%, 70%, 80%, 90%, and absolute ethanol twice. The dehydrated tissues were cleared at room temperature in two changes of xylene each lasting one hour. The tissues were further infiltrated in two changes of molten paraffin wax at 600.C for one hour each, then the tissues were embedded in paraffin wax using multi block plastic embedding mould. The paraffin blocked tissues were trimmed, soften on an ice and mounted for sectioning on a rotary microtome. Sections of 5ìm thick were produced from the tissue blocks using a rotary microtome (Laboid LBM-RM2, London, England). The sections were collected and transferred onto a floating bath at 400.C to allow spreading of the folded ribbons of sections. The sections were picked and mounted on a new clean glass slides and were dried at 400.C on a slide drier to enhance adherence of the section to the slides and stained. Haematoxylin & Eosin was employed to assess general histoarchitecture of the pancreas, Mason trichome and VVG to assess for collagen deposition and aldehyde fuschin to assess beta cells. Immunohistochemical stain was done to show the presence of antigen- antibody complex and inflammatory markers with IL-2 in pancreatic injury.

Photomicrography
Tissues slides were scanned with Motic Scanner with SVS format and the scanned slides were viewed and analyzed.

Data Collection were done for Pro Insulin, Insulin, C pepetide, β amylase, Hemoglobin A1c and Random blood glucose, which is as shown below;

Statistical analysis
Data were analyzed by using GraphPad prism version 8.4.3.686, one way analysis of variance (ANOVA), followed by Turkey's test for multiple comparison. The results were expressed as Means ± S.E.M $p<0.05$ was considered statistically significant.

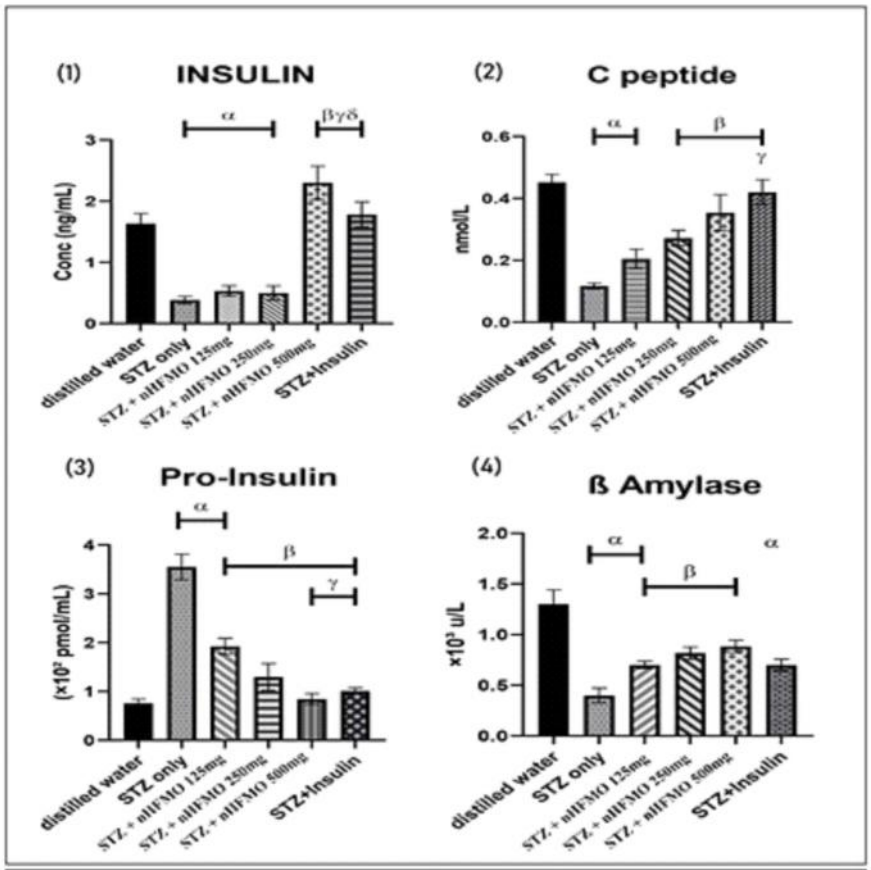
All quantitative results obtained from this study were expressed as mean ± standard error of the mean (SEM), to provide a measure of central tendency (mean) along with the precision of the estimate (SEM). The number of animals per group (n) was clearly indicated in the methodology as n=8, individual animals were used to calculate group means, while

Insulin (ng/mL)

S/N	Val 1	Val 2	Val 3	Val 4	Val 5	Val 6
1	1.1	2.3	1.6	0.8	0.9	3.1
2	0.3	0.2	0.5	0.6	0.3	0.4
3	0.3	0.5	0.7	0.6	0.8	0.3
4	0.7	0.3	0.1	0.5	0.9	0.5
5	2.9	1.3	1.9	2.0	2.7	3.0
6	2.2	1.8	1.7	2.5	1.3	1.2
C-Peptide (nmol/L)						
S/N	Val 1	Val 2	Val 3	Val 4	Val 5	Val 6
1	0.3	0.4	0.5	0.28	0.39	0.84
2	0.12	0.18	0.13	0.09	0.07	0.11
3	0.21	0.17	0.08	0.25	0.3	0.22
4	0.35	0.2	0.21	0.4	0.24	0.23
5	0.45	0.5	0.38	0.23	0.81	0.21
6	0.31	0.48	0.29	0.54	0.44	0.46
Pro-Insulin ($\times 10^2$ pmol/mL)						
S/N	Val 1	Val 2	Val 3	Val 4	Val 5	Val 6
1	0.7	0.93	1.1	0.54	0.58	0.69
2	2.5	3.56	4.2	3.9	4.0	3.12
3	1.36	1.66	2.51	1.86	2.17	2.0
4	1.96	0.89	0.99	1.33	0.43	2.17
5	0.93	0.76	0.88	1.31	0.55	0.63
6	1.1	1.24	0.99	0.81	0.93	1.0
β Amylase ($\times 10^3$ u/L)						
S/N	Val 1	Val 2	Val 3	Val 4	Val 5	Val 6
1	0.8	1.1	1.4	1.8	1.2	1.5
2	0.4	0.6	0.5	0.3	0.1	0.5
3	0.6	0.8	0.7	0.8	0.6	0.7
4	1.0	0.8	0.6	0.7	0.9	0.9
5	0.8	0.9	1.1	1.0	0.7	0.8
6	0.5	0.7	0.8	0.7	0.9	0.6
Hb A1c (%)						
S/N	Val 1	Val 2	Val 3	Val 4	Val 5	Val 6
1	1.5	1.9	4.3	4.8	5.3	5.9
2	7.4	7.3	8.6	8.9	9.5	10.0
3	6.9	7.9	6.1	6.5	8.9	8.8
4	6.4	7.3	6.8	5.1	6.7	7.1
5	4.9	5.1	6.3	5.4	6.1	5.3
6	5.8	6.6	5.0	6.6	6.3	5.2
RBS (mmol/L)						
S/N	Val 1	Val 2	Val 3	Val 4	Val 5	Val 6
1	2.8	3.7	4.5	5.1	4.3	3.3
2	10.9	17.8	8.8	15.1	18.2	20.2
3	11.3	10.2	7.6	8.3	12.1	11.9
4	4.8	7.9	9.1	9.8	10.8	9.7
5	5.6	8.5	7.4	3.3	4.8	9.1
6	7.1	7.8	8.0	6.6	4.9	5.9

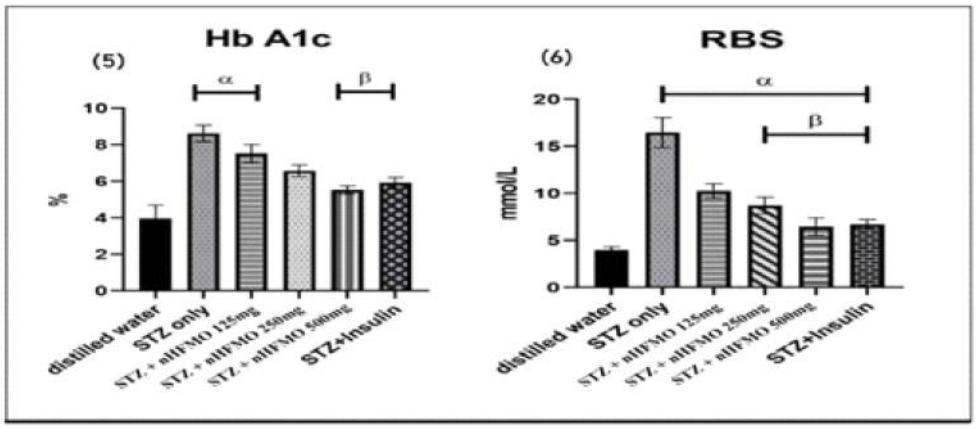
repeated measurements within the same animal was averaged before inclusion in group-level analysis. Statistical comparisons were performed between experimental and control groups using appropriate parametric tests with assumptions of normality and homogeneity of variance. A p-value < 0.05 was considered statistically significant. Statistical analyses were conducted using validated software GraphPad Prism, to ensure reproducibility and transparency. Results were presented in graphs with error bars representing SEM

DM. There was a significant reduction in insulin concentration of groups treated with STZ only, n-HFMO 125 mg/kg and 250 mg/kg when compared with the normal control group ($p<0.05$), and this effect



Data were expressed as mean ± SEM. 'α' is significantly different from distilled water group, 'β' is significantly different from STZ only group, 'γ' is significantly different from STZ+nHFMO 60mg group and 'δ' is significantly different from group 4.

Figure 1: showed the effect of n-HFMO on insulin in adult male Wistar rats with STZ-induced



Data were expressed as mean ± SEM. 'α' is significantly different from distilled water group, 'β' is significantly different from STZ only group, 'γ' is significantly different from STZ+nHFMO 60mg group and 'δ' is significantly different from group 4.

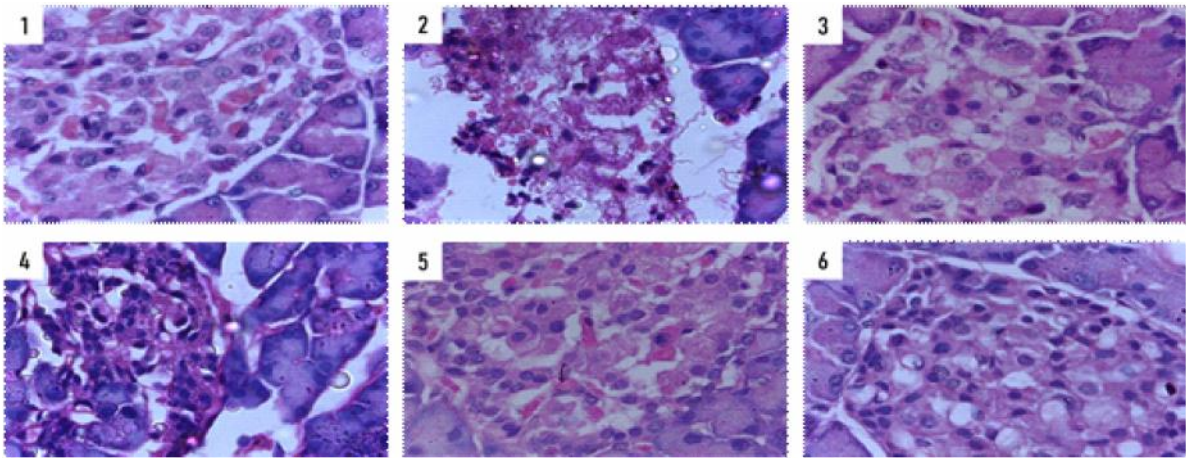
Figure 2; showed the effect of n-HFMO on C-peptide in adult male Wistar rats with STZ-induced DM

was reversed in groups treated with n-HFMO 500 mg/kg and insulin 5 IU/kg. The restoration of insulin concentration was seen in groups treated with n-HFMO 250 mg/kg and 500 mg/kg, and this was found significantly different from groups treated with STZ only, n-HFMO 125 mg/kg and 250 mg/kg ($p<0.05$).

Figure 2 showed the effect of n-HFMO on C-peptide in adult male Wistar rats with STZ-induced DM. C-

Histological ev aluation

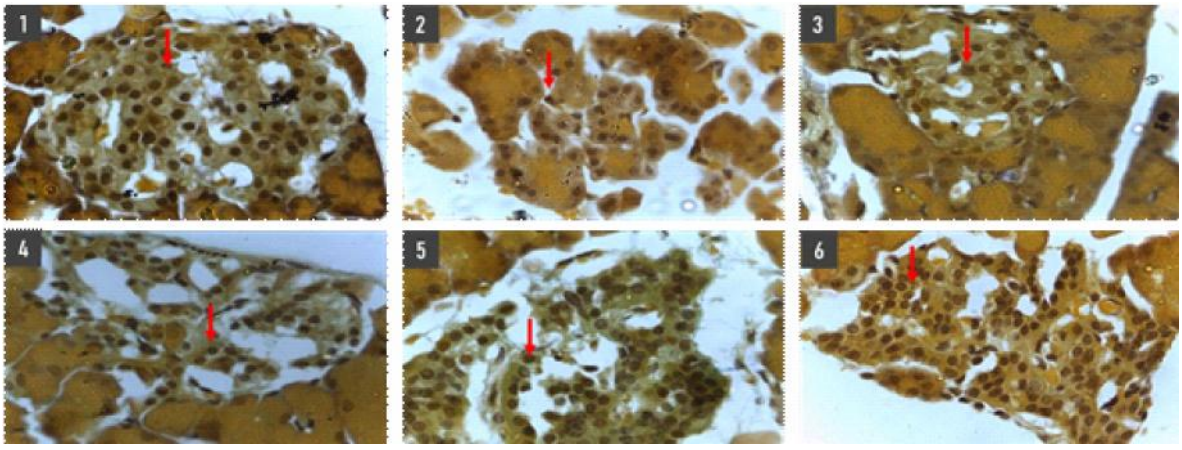
Plate 1



Sample photomicrograph of the pancreas showing the effect of n-HFMO on STZ induced DM

(1)showed the histoarchitecture of the pancreas appearing relatively normal. A distortion of the Islet of Langerhans was found in group (2) indicating degeneration of beta cells. However, a restoration of tissue integrity was seen in groups, (3), (4), (5) and (6). H & E X1000.

Plate 2



Sample photomicrograph of the pancreas showing the effect of n-HFMO on STZ-induced DM

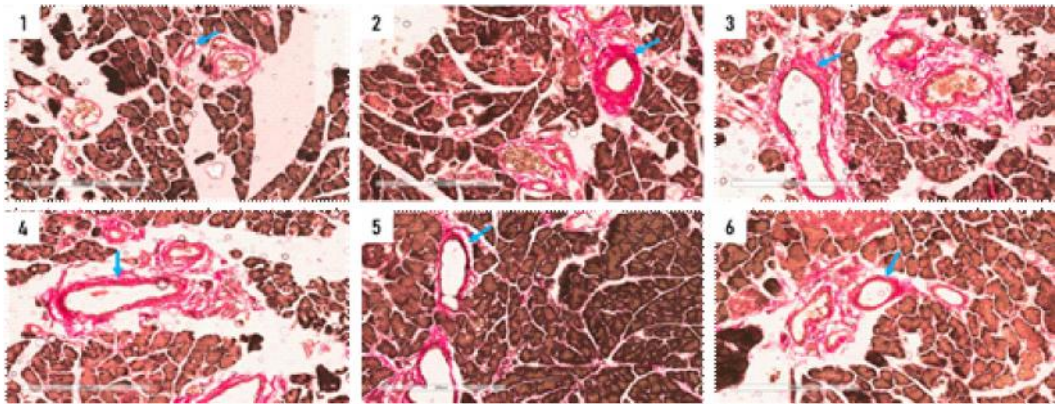
(1)Showed a relatively normal islet of Langerhans with it beta cells (red arrow) highlighted, which are stained dark-brown. Whereas, the dark-brown stained cells were found reduced in group (2) and (3) indicating degeneration of beta cells. Groups (4), (5) and (6) showed that the interventions provided protected cells from the toxicity of induced DM. Aldehyde fuschin X1000.

peptide was reduced in the experimental groups induced with DM. A significant improvement was seen in groups treated with n-HFMO 500 mg/kg and insulin when compared with group 2 ($p<0.05$). Statistics showed that the efficacy of the lowest of n-HFMO was limited when compared with the normal control group ($p>0.05$).

Figure 3 showed the effect of n-HFMO on Pro-insulin level of adult male Wistar rats with STZ-induced DM. Relative to the normal control group, there was a significant rise in Pro-insulin levels of the STZ only and n-HFMO treated groups ($p<0.05$), demonstrating the low efficacy of the lowest dose of n-HFMO in comparison with the other doses. Nevertheless, n-HFMO and insulin reduced proinsulin levels significantly compared to the group treated with STZ only ($p<0.05$). n-HFMO 500 mg/kg and insulin showed the highest efficacy.

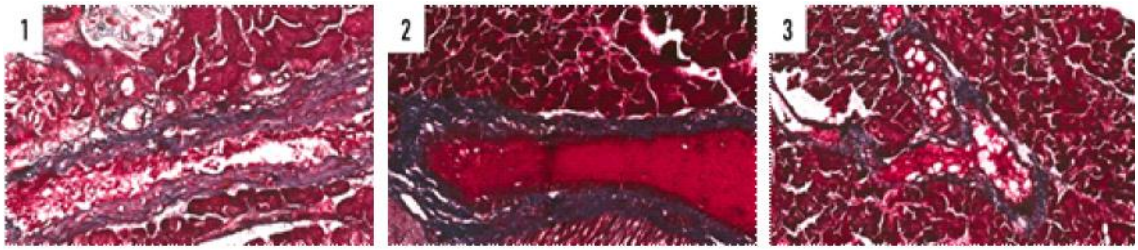
Figure 4 showed the effect of n-HFMO on beta amylase protein of adult male Wistar rats with STZ-induced DM. There was a significant reduction of beta amylase in STZ only treated groups when compared to the normal control group ($p<0.05$). Nevertheless, the administration of n-HFMO increased protein levels significantly, when compared with the STZ only treated group ($p<0.05$). Insulin

Plate 3



Sample photomicrograph of the pancreas showing the effect of n-HFMO on STZ-induced DM
(1) showed thin collagen content around the blood vessel (white arrow) which appears normal. Increased collagen deposition was found around the blood vessel of (2), (3), (4), (5) and (6) indicating fibrosis. (3) showed signs of tissue integrity restoration, however a better recovery was seen in (4), (5) & (6). VVG X200

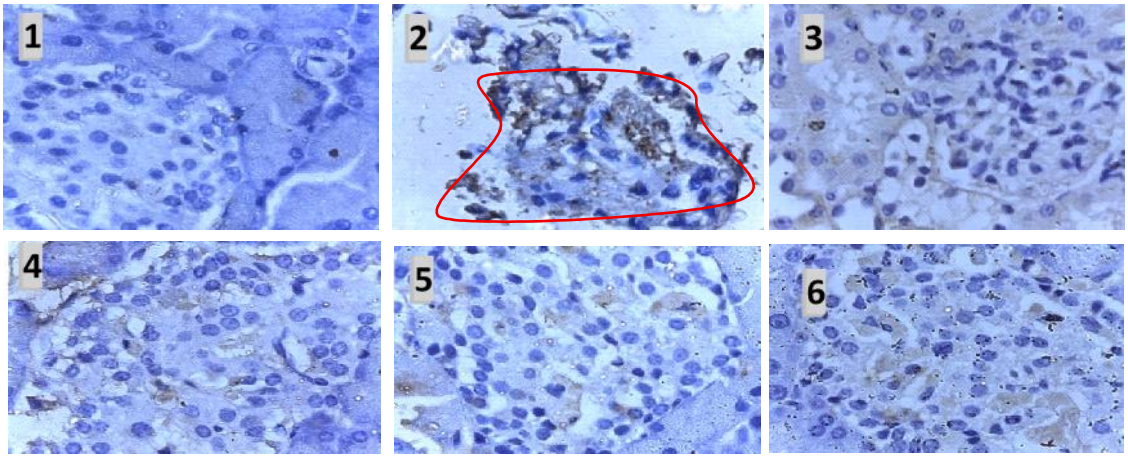
Plate 4



Sample photomicrograph of the pancreas showing the effect of n-HFMO on STZ-induced DM
(1) showed the normal collagen (blue-green) content around the blood vessel. Whereas, collagen was found abundant in (2) compared to (1) characterized by an intense blue-green stain around the blood vessel indicating fibrosis. (3), (4), (5) & (6) showed signs of tissue integrity restoration MT X200

Immunohistochemical evaluation

Plate 5



Representative photomicrograph of the pancreas showing the effect of n-HFMO on STZ-induced DM
(1) showed the normal group with no immune reactivity of IL-2 (blue). Whereas, immunoreactivity was profound in (2) (areas circled in red) characterized by areas of intense brown discoloration majorly in the central portion of the tissue. (3) & (6) showed signs of mild immune reactions characterized by some areas of light brown colors, 4 and 5 showed near normal reactivities with tissue integrity restoration. IL-2 X200

increased beta amylase level but this impact was insignificant when compared with the STZ only treated groups ($p>0.05$).

Figure 5 showed the effect of n-HFMO on hemoglobin A1c of adult male Wistar rats with STZ-induced DM. Relative to the distilled water group there was a significant rise of Hb A1c in the STZ only treated group ($p<0.05$). A reduction of Hb A1c was seen in groups treated with the lowest, medium and highest dose of n-HFMO and also insulin 5 IU/kg. But statistics showed that in comparison with the STZ only group a significant reduction of Hb A1c was caused by the n-HFMO 500 mg/kg and insulin 5IU/kg ($p<0.05$).

Figure 6 showed the effect of n-HFMO on the RBG level of adult male Wistar rats with STZ-induced DM. There was a significant rise in RBS level in all experimental groups compared with the normal control group ($p<0.05$). The interventions provided reduced RBS level, meanwhile a significant effect was seen only in groups treated with n-HFMO 250 mg/kg, 500 mg/kg and insulin 5IU/kg ($p<0.05$).

DISCUSSION

Chronic hyperglycemia has been associated with some biochemical alterations and pathological changes such as necrosis and fibrosis of the pancreas.[18] The biochemical findings of this study demonstrated that DM induced by STZ produced classical features of β-cell dysfunction, including marked reductions in insulin, C-peptide, and β-amylase levels, accompanied by compensatory elevations in pro-insulin concentration, and sustained hyperglycemia. These findings confirm the successful induction of pancreatic injury, consistent with the widely documented β-cell cytotoxic effects of STZ.[19,20,23]

The administration of n-HFMO (particularly at 500 mg/kg) and insulin, produced a significant restorative effect on pancreatic insulin concentration. Figure 1 showed that STZ-induced DM caused a profound reduction in insulin concentration, reflecting a either a severe β-cell dysfunction or death. The potency of low dose and medium doses of n-HFMO (125 and 250 mg/kg) were insufficient to significantly reverse this decline, suggesting a dose-dependent therapeutic efficacy. However, the 500 mg/kg dose restored insulin levels to near-normal values, comparable to the insulin-treated group. This indicated that adequate concentrations of the n-HFMO may enhance β-cell recovery or stimulate residual functional β-cells, likely through antioxidant and membrane-stabilizing effects known to preserve β-cell viability.

Similarly, Figure 2 revealed that C-peptide levels were significantly improved by n-HFMO at 250 mg/kg, 500 mg/kg and by insulin 5 IU/kg, whereas the effect of n-HFMO 125 mg/kg was limited. Since C-peptide is a reliable marker of endogenous insulin production, the improvement seen with the mid and higher n-HFMO dose reinforces the hypothesis that lycopene enhances β-cell functional recovery rather than merely replacing insulin activity. The ability of n-HFMO to elevate C-peptide suggests improved pro-insulin processing and restored secretory dynamics within surviving islets.

The elevated pro-insulin levels in the STZ only group indicate impaired pro-insulin–to–insulin conversion (a hallmark of β-cell stress and dysfunction) and its persistence in group treated with n-HFMO 125 mg/kg demonstrated low efficacy of n-HFMO lowest dose. The significant reduction in proinsulin levels observed in the n-HFMO (500 mg/kg maximum benefit) and insulin-treated groups suggests normalization of prohormone processing pathways. This likely reflects the antioxidant and anti-inflammatory potential of n-HFMO at reducing damage to β-cells. Hence, preserving their ability to synthesize insulin and its precursor, pro-insulin.[21] The dose-dependent response again highlights the necessity of adequate n-HFMO concentrations to achieve therapeutic benefit.

The findings of this study provide insight on the effect of DM on exocrine part of the pancreas based on changes in β-amylase. Chronic hyperglycemia resulted in a significant reduction in this exocrine marker, consistent with reports that chronic hyperglycemia and oxidative stress impair both endocrine and exocrine pancreatic compartments.[22]

However, the treatment with n-HFMO improved â-amylase levels significantly compared to the untreated diabetic group, whereas insulin treatment did not produce a statistically significant improvement. This finding suggested that n-HFMO may exert broader protective effects on the pancreas than insulin alone, possibly by attenuating oxidative damage across both the exocrine and endocrine parts of the pancreas.

HbA1c (Figure 5), a long-term marker (2-3 month glycemic status) of glycemic control, was markedly elevated in the STZ group. All doses of n-HFMO reduced HbA1c, but only the 500 mg/kg dose and insulin produced statistically significant reductions. This again highlights the maximum beneficial efficacy of the highest n-HFMO dose. The reduction in HbA1c indicates improved glucose handling over time, likely

resulting from enhanced insulin secretion and improved pancreatic function. Another glycemic marker RBG was elevated in the diabetic group, especially in the group left untreated. However, the treatment with n-HFMO reduced RBG, but the effect reached statistical significance only at 250 mg/kg, 500 mg/kg, and with insulin 5 IU/kg**.** This suggested that both moderate and high doses of n-HFMO are capable of improving acute glycemic control, with the highest dose exerting the most consistent effects across all parameters. This finding is consistent with the findings of Leh *et al.*, 2021, who reported that T2DM patients with a higher flavonoid intake showed a greater peripheral antioxidant capacity and better glycaemic control'.

Collectively, these results demonstrated a clear dose-dependent protective effect of the n-HFMO, with the 500 mg/kg dose consistently producing the greatest improvements across all measured parameters. The observed biochemical restoration aligns with previous literature describing MO as a potent scavenger of reactive oxygen species capable of stabilizing cellular membranes, reducing lipid peroxidation, and modulating inflammatory pathways. These mechanisms are particularly relevant to STZ-induced â-cell impairment, which is largely driven by oxidative stress. Histological techniques were employed to assess, the damage caused by DM on the pancreas and the recovery made by the provided interventions. H & E, and Aldehyde fuchsin were used to assess the general histoarchitecture of the pancreas and to stain for beta cells respectively. Whereas, MT and VVG were employed to demonstrate the effect of DM on collagen.

Photomicrograph showed that for the untreated diabetic group, there was a distortion of pancreatic histoarchitecture especially with the islet of Langerhans revealing atrophy and effacement of beta cells, with increased deposition of collagen around the blood vessels and stroma indicating fibrosis. The histological alterations on pancreas caused by chronic hyperglycemia induced by STZ is consistent with studies by Nurdiana *et al.*, 2017; Oyouni *et al.*, 2022. The atrophy of the islet revealed by the Aldehyde fuchsin stain in plate 2 justifies the declines in insulin and C peptide levels observed in this study. Rats treated with n-HFMO 125 mg/kg showed disrupted islet morphology in plate 1. This incomplete restoration suggested that the lowest dose provided insufficient protection against STZ induced DM, correlating with the minimal biochemical improvements. In contrast, n-HFMO at 250 mg/kg, 500 mg/kg and insulin 5 IU/kg treatment resulted in enlarged, better-defined islets indicating preservation of tissue integrity correlating with the improved levels of insulin and C-peptides in these groups.

Pancreatic fibrosis is a known consequence of long-term hyperglycemia. In response to elevated RBG level, Hb A1c, reduced insulin production with increase in inflammatory and oxidative biomarkers, fibrosis pathway is initiated.[19,20] Specifically, for Hb A1c, a significant rise in this protein has been linked with higher serum pancreatic fibrotic factors in patients, justifying the fibrotic changes seen in the diabetic group (plate 3 and 4). Excessive collagen was deposited around pancreatic blood vessels and its interstitial space. In the diabetic groups (2-6), increased collagen was deposited, with the STZ only group demonstrating the most prominent fibrosis. A suppression of chronic hyperglycemia induced fibrosis pathways was seen in groups treated with n-HFMO and insulin. Amongst the intervention groups, n-HFMO 125 mg/kg showed elevated collagen content reflecting insufficient suppression of this pathway. The therapeutic benefit of n-HFMO can be linked with its hypoglycemic, antioxidant effect and fibroblast deactivation.

Immunohistochemistry analysis revealed, the normal control free of any immune reactivity in any of its fields, whereas the STZ only group showed an intense immuno reaction, with high IL-2 receptor expression on the destructive T-cells, majorly in the central portion of the slide, groups 3 and 6 showed signs of mild immune reactions characterized by some areas of light brown colors, suggesting an increase in IL-2 positive, activated lymphocytes infiltrating the pancreatic islets denoting, inflammatory changes (Insulitis) groups 4 and 5 showed near normal reactivities with tissue integrity restoration. This is in keeping with the work of Asif *et al.*, 2025 who stated that IL-2, responsive islet cytotoxic NK cells in diabetic state may contribute to inflammatory response in the pancreas of rats induced with diabetes which cascades to B cell destruction and eventual diabetes in the laboratory animals. The ability of n-HFMO to ameliorate histological damage and also improve both endocrine and exocrine pancreatic markers distinguishes it from insulin, which only compensates for endocrine insufficiency. This implies that n-HFMO targets the underlying pathology rather than merely correcting hyperglycemia, as such, n-HFMO may serve as a valuable adjunct in diabetes management due to its cytoprotective properties and broad effects across multiple pancreatic functions.

Limitations

This study was conducted in an animal model (Wistar rats), and findings may not directly translate to humans.

Moreover, the study used male rats only, and sex differences in response to n-HFMO have not been explored, also the duration of treatment was 42 days, which was relatively short, the long-term effects of n-HFMO are yet unknown. Furthermore, no toxicity studies were conducted to assess the safety profile of n-HFMO. Future studies should include female animals, longer treatment durations, larger sample sizes, and detailed mechanistic investigations to further this study.

CONCLUSION

It can be concluded that, nHFMO demonstrated antihyperglycaemic, anti-inflammatory, antioxidant potentials and was able to improve the pancreatic â-cell function and secretion, with long term diabetic control and eventually restore histoarchitectural derangements, in a dose dependent style, which phytochemical potentials transcend endocrine protection to include exocrine aspects of the pancreas making it a potentially valuable adjuvant antidiabetic herbal medication.

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Contributions of authors:

A.B.E. Initiating the research, writing of the literature, methodology and discussion

A.O.S. Conceptualizing the work and provision of funds and guidance

I.M.S. The laboratory bench work and data analyses

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