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Research article – Histology and cell biology

Morphological study of the effects of aqueous leaf extract of *Xylopia aethiopica* on the pancreas in diabetic rats

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Abstract

To investigate the histological and immunohistochemical effects of aqueous leaf extract of *Xylopia aethiopica* on the pancreas in streptozotocin-induced diabetic rats, 30 adult Wistar rats were divided into three groups (n=10). Group A was the control (administered with equivalent volume of citrate buffer), group B animals were made diabetic by a single intraperitoneal injection of streptozotocin dissolved in citrate buffer (65 mg/kg), group C animals were made diabetic as above and treated with 200mg/kg body weight of aqueous leave extract of *Xylopia aethiopica* for 25 days. Upon animal sacrifice, the pancreas were excised, fixed in 10% formol saline and processed for light microscopy and immunohistochemistry.. The results revealed destruction of the islet cells in the untreated diabetic group as compared with the controls. The extract treated group was characterized by recovery/regenerative processes indicated by improvement in islet morphology. In untreated diabetic rats immunoreactive β-cells were sparse, at variance from the controls. The group treated with aqueous leaf extract of *Xylopia aethiopica* revealed more intense staining for insulin and significant ($p<0.05$) increase in the percentage of immunolabelled surface area when compared with the untreated diabetic group, suggesting the ability of β-cells to secrete insulin in the extract treated rats. We conclude that the aqueous leaf extract of *Xylopia aethiopica* improves recovery process of β-cells in streptozotocin-induced diabetic rats and might become useful in the management of diabetes related complications.

Key words

Xylopia aethiopica, pancreas, immunohistochemistry, histology, diabetes mellitus

Introduction

Diabetic mellitus is a chronic disease characterized by relative or absolute deficiency of insulin, resulting in glucose intolerance. It develops in 4-5 million persons in the United States (approximately 2% of the population) (Wolosin and Edelman, 2000; Piyachaturawat et al., 1991). The classic symptoms of diabetes mellitus result from abnormal glucose metabolism (Wolosin and Edelman, 2000). Deficiency of insulin results in failure of glucose transfer from the plasma into the cells. This situation is also called "starvation in the midst of plenty". The body responds as if it was in the fasting state, with stimulation of glucogenolysis, gluconeogenesis and lipolysis producing ketone bodies (Bastaki et al., 2010). Pancreatic exocrine dysfunction occurs in up to 80% of

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individuals with type 1 diabetes but is rarely significant enough to lead to any clinical problems with digestion (Wolosin and Edelman, 2000). The pancreas has a tremendous reserve, and a modest reduction in pancreatic enzyme secretion rarely leads to difficulty in digesting or absorbing carbohydrate, fat, or protein. In many animal species, streptozotocin (STZ) induces diabetes that resembles human hyperglycemic non ketotic diabetes mellitus (Weir et al., 1981). This effect has been extensively studied and appears to be mediated through a lowering of β cell nicotinamide adenine dinucleotide (NAD^+) and results in histopathologic alteration of pancreatic islet beta cells (Karunanayake et al., 1974). In immune-mediated diabetes, the rate of β-cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults) (Zimmet et al., 1994). Some patients, particularly children and adolescents, may present with ketoacidosis as the first manifestation of the disease. Others have modest fasting hyperglycemia that can rapidly change to severe hyperglycemia and/or ketoacidosis in the presence of infection or other stress. Still others, particularly adults, may retain residual β-cell function sufficient to prevent ketoacidosis for many years; such individuals eventually become dependent on insulin for survival and are at risk for ketoacidosis. At this latter stage of the disease, there is little or no insulin secretion, as manifested by low or undetectable levels of plasma C-peptide. Immune-mediated diabetes commonly occurs in childhood and adolescence, but it can occur at any age, even in the 8th and 9th decades of life (Gavin et al., 2003).

Herbs such as *Xylopia aethiopica* (*X. aethiopica*) posses hypoglycemic and antihyperlipidemic effects in adult Wistar rats (Blanche et al., 2013). Recently, Gometi et al., (2014) reported a significant decrease in the liver function test following treatment of diabetic rats with extract of *X. aethiopica*. Virtually every part of *X. aethiopica* has medicinal value. It will be interesting to note that ailments such as candidiasis, fever, cough, dyspepsia and skin infections can be treated using *X. aethiopica* (Fleischer et al., 2008). Several reports have demonstrated the antimicrobial potential of *X. aethiopica* (Boakye-Yiadom et al., 1977; Thomas, 1989; Tatsadjieu et al., 2003; Asekun and Adeniyi, 2004; Okigbo et al., 2005). Despite reports on the antihyperglycemic effect of *X. aethiopica* (Ogbonnia et al., 2008; Baldé et al., 2006), the morphological transformation within the pancreas leading to the hypoglycemic state is yet to be thoroughly investigated. The adoption of complementary medicine in modern era has increased tremendously. This is because some of the synthetic drugs being used today eventually have some adverse effects on the patients. In view of the up surge in the use of herbal therapy, it becomes therefore very important to under study its effects on pancreatic morphology.

The aim of this study was to investigate the effect of aqueous leaf extract of *X. aethiopica* on the pancreas in STZ-induced diabetic rats using histological and immunohistochemical studies. This will further elucidate on the mode of action of this hypoglycemic herbal therapy.

Materials and Methods

Animal care

Thirty male albino rats of the Wistar strain were maintained under standard laboratory conditions of light, humidity and temperature. The animals were acclima-

tized for two weeks before the commencement of the research work. Animals were fed with standard rat diet and given water liberally. All the animal experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Public Health, Obafemi Awolowo University (Ethics No: IPHOAU/12/61).

Induction of experimental diabetes

Streptozotocin (65 mg/kg body weight) dissolved in 0.1 mol/L sodium citrate buffer (pH 6.3) was injected intraperitoneally to animals in groups B and C while animals in group A received an equivalent volume of 0.1 mol/L sodium citrate buffer (pH 6.3). Before the commencement of the experiments, all the animals were fasted for 16 h, but still allowed free access to water. At the end of the 16 h fasting period – taken as 0 h – blood glucose levels (initial glycemia) were determined and recorded. Three days after hyperglycemic status had been confirmed, the leaf extract of *X. aethiopica* in aqueous solution was administered orally through gavages at a concentration of 200 mg/kg body weight/rat/day.

Determination of blood glucose level

Blood glucose level was done using a digital glucometer (Accu-chek® Advantage, Roche Diagnostic, Germany) consisting of a digital meter and the test strips. Blood sample was obtained from the tail vein to determine the hyperglycemic state on 3rd day. This was again determined on the 14th and 28th day.

Experimental design

The animals were divided into three groups as follows, with ten animals in each group:

Group A: Control rats were administered intraperitoneally with an equivalent volume of 0.1mol/L sodium citrate buffer.

Group B: Rats made diabetic through intraperitoneal STZ (65 mg/kg body weight) dissolved in 0.1 mol/L sodium citrate buffer (pH 6.3).

Group C: Rats made diabetic with STZ, as above, and then treated orally with aqueous leaf extract of *X. aethiopica* (200 mg/kg body weight/day/rat) in aqueous solution for 25 days after hyperglycemia was confirmed, which happened on the third day from streptozotovcin injection which was taken as day 0 of the extract administration. The leaves of *X. aethiopica* were procured from a local market in Ile-Ife, Nigeria.

Animal sacrifice

All the animals were treated humanely and sacrificed under chloroform anesthesia twenty four hours after the last administration. Blood samples were collected by cardiac puncture. The animals were opened up and the pancreas was excised and fixed for light microscopy.

Biochemical assays

Serum was obtained after centrifuging the blood samples for 5 min at 5000 rpm in a Benchtop Refrigerated centrifuge (Centurion Scientific centrifuge, R8000 series, UK). The serum obtained was used for the determination of triglyceride (Footsati and Prencipe, 1982), total cholesterol (Allain et al., 1974) and high-density lipoprotein-cholesterol (Allain et al., 1974) using respective diagnostic commercial kits from Randox, Northern Ireland. Very low-density lipoprotein cholesterol and low-density lipoprotein cholesterol were calculated adopting Friedewald's equation (Friedewald et al., 1972).

Histology

The tissue was excised and fixed in 10% formol-saline and processed for light microscopic study. The processing was done using automatic tissue processor (ASP 200S, Leica, Wetzlar, Germany). This included dehydration of the samples through graded ethanol, clearing in isopropanol and infiltration with paraffin wax at 56°C. The samples were immediately embedded in paraffin wax using Leica EG1150 H. A rotary microtome (Leica RM 2135) was used to obtain serial sections 5 μm thick which were stained with hematoxylin and eosin.

Immunohistochemistry

Sections of the pancreas were immunostained for insulin. Slides were de-waxed, rehydrated, rinsed in 1% PBS (10 mmol/L Na_2HPO_4, 1.8 mmol/L KH_2PO_4, 137 mmol/L NaCl and 2.7 mmol/L KCl; pH 7.4), and immersed in antigen retrieval solution (1:50 Low pH Dako-Envision; Dako, Glostrup, Denmark) and subjected to heat-induce antigen retrieval for 10 min in a microwave (LG, Englewood Cliffs, New Jersey; 1200W at medium-High level). The slides were rinsed in 1% PBS + Tween 20 and 1% PBS for 3 min. each. The sections were treated to block endogenous peroxidase by 3% H_2O_2 for 5 min, rinsed in 1% PBS + Tween 20, incubated with Sniper (Biocare Medical, Concord, California; it is used for blocking nonspecific background staining commonly found with immunohistochemistry) for 30 min and rinsed again in 1% PBS + Tween 20 and 1% PBS for 5 min each rinse. Sections were later incubated for 2 h at room temperature with polyclonal guinea pig anti-insulin primary antibody (Ready-to-use, Dako Autostainer/Autostainer Plus). After rinsing in 1% PBS + Tween 20 and 1% PBS, 3 drops of Horseradish peroxidase (Dako REAL EnVision-HRP, Rabbit/Mouse) were added to the slides, and then washed in 1% PBS + Tween 20 and 1% PBS for 5 min each. Three drops of substrate chromogen (Dako DAB Chromogen,) solution was added to the slides for 3 min. Thereafter, the slides were washed with distilled water and counter stained with Mayer's hematoxylin, dehydrated, cleared in xylene and mounted with DPX (Belami fine chemicals, Maharashtra, India). The antibody labeled the cytoplasm of insulin producing beta-cells in the pancreas deep brown. The sections were examined and photographed using a Zeiss (Oberkochen, Germany) Axioscope A1 microscope with digital camera attached.

Quantitative analysis of immuno-labeled surface area

Five areas of the pancreas per specimen were randomly selected and photographed at magnification x400. The intensity of labeling with anti-insulin antibody was evaluated with Zeiss AxioVision Image analysis software package version 4.8.3. The size of the measurement frame was expressed as μm^2. This measurement corresponded to a mean area frame of 23485.01μm^2. Intensity of immunoreactivity was expressed as percent labeled surface area / frame surface area.

Statistical analysis

Statistical analysis was carried out using statistical package SPSS version 17.0 (SPSS, Cary, NC, USA) with Duncan's Multiple Range Test option. One-way analysis of variance was used to evaluate statistical significance of the differences between animal groups. A value of $p \leq 0.05$ was considered significant. Data are reported as mean ± standard deviation (SD).

Results

Body weight

There was a decline in the average body weight of the diabetic rats after 14 days of induction. On the contrary, the extract-treated group showed a gradual increase in weight from the period of commencement of the treatment until the 28th day (Table 1).

Blood glucose level

The average blood glucose level increased significantly 48 h after the administration of STZ. This increment was sustained in the untreated diabetic animals till the 28th day. However, the blood glucose level was significantly ($p<0.05$) lowered in the extract-treated group when compared with the untreated diabetic group from the period of commencement of the treatment until the 28th day (Table 2).

Table 1 – Effects of *Xylopia aethiopica* on the body weight (g) of control rats (group A), rats made diabetic by streptozotocin (group B) and diabetic rats treated with *X. aethiopica* extract (group C). Mean ± standard deviation; N = 10 in each group.

	0 day	14 days	28 days
GROUP A	165.80±16.31^{c}	238.60±21.50^{b}	241.00±20.73^{b}
GROUP B	144.28± 7.60^{b}	156.57±39.11^{a}	143.14±54.97^{a}
GROUP C	100.14±22.71^{a}	182.85±21.51^{a}	187.57±23.79^{a}

In each column, values indicated with different letters (a, b, c) differ significantly from each other ($p < 0.05$) while values indicated with the same letter do not differ significantly from each other.

Table 2 – Effects of *Xylopia aethiopica* on the blood glucose level (mg/dl) of control rats (group A), of rats made diabetic by streptozotocin (group B) and diabetic rats treated with *X. aethiopica* extract (group C). Mean ± standard deviation; N = 10 in each group.

	0 day	3 days	14 days	28 days
GROUP A	111.40±7.23^{b}	111.00± 5.38^{a}	102.60± 19.80^{a}	112.60± 5.02^{a}
GROUP B	86.71±9.12^{a}	283.28±29.70^{b}	353.71±102.13^{b}	361.85±110.97^{b}
GROUP C	113.57±4.23^{b}	316.28±37.78^{b}	120.57± 3.99^{a}	107.16± 22.68^{a}

In each column, values indicated with different letters (a, b) differ significantly from each other ($p < 0.05$) while values indicated with the same letter do not differ significantly from each other.

Table 3 – Effects of *Xylopia aethiopica* on the blood lipid levels (mmol/L) of control rats (group A), of rats made diabetic by streptozotocin (group B) and diabetic rats treated with *X. aethiopica* extract (group C). Mean ± standard deviation; N = 10 in each group.

	TC mmol/L	TG mmol/L	HDL-C mmol/L	LDL-C mmol/L	VLDL-C mmol/L
GROUP A	0.62±0.26^{a}	0.55±0.29^{a}	1.48±0.22^{a}	0.37±0.28^{a}	0.24±0.13^{a}
GROUP B	1.70±0.63^{b}	0.80±0.44^{a}	1.32±0.49^{a}	1.33±0.57^{b}	0.36±0.20^{a}
GROUP C	1.57±0.46^{b}	0.61±0.55^{a}	1.16±0.75^{a}	1.30±0.33^{b}	0.27±0.25^{a}
	P<0.06	P<0.605	P<0.632	P<0.004	P<0.601

TC = total cholesterol; TG = triglycerides; HDL-C = high density lipoprotein cholesterol; LDL-C = low density lipoprotein cholesterol; VLDL-C = very low density lipoprotein cholesterol. In each column, values indicated with different letters (a, b) differ significantly from each other ($p < 0.05$) while values indicated with the same letter do not differ significantly from each other.

Lipid profile

The result showed that total cholesterol, triglyceride, low density lipoprotein cholesterol and very low density lipoprotein cholesterol were increased when the untreated diabetic group was compared with the extract treated animals. The high density lipoprotein cholesterol was not significantly different in all the groups as shown in Table 3.

Histopathology

The results revealed multiple vascular congestion and destruction of islets cells in the untreated diabetic group as compared with the control group. The microanatomy of the islet in the extract-treated group was characterized by recovery/regenerative processes as evident by the reduction in vascular congestion and improvement in islet morphology (Fig. 1).

Immunohistochemistry

The immunohistochemical staining of the pancreas of the diabetic group showed that immunoreactive β-cells were sparse compared with the control group. The group

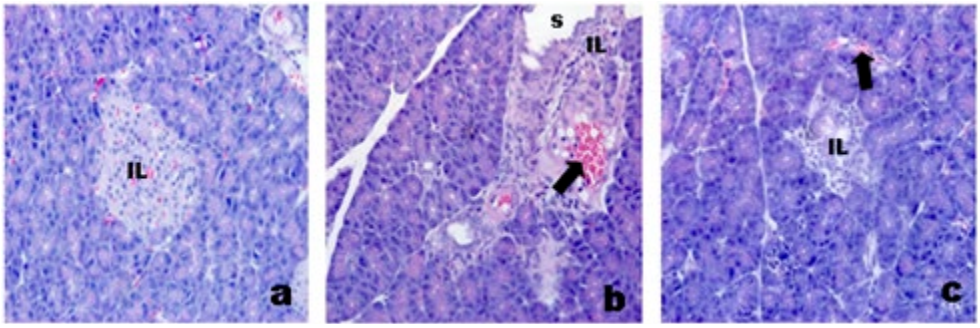

Figure 1 – Photomicrograph of the pancreas in: **(a)** a control rat, note the islet (IL); **(b)** a rat induced to become diabetic with streptozotocin, note the disruption of islets (IL), multiple congested blood vessels (arrow) and lumen of an exocrine duct (S); **(c)** a diabetic rat treated with aqueous leaf extract of *X. aethiopica,* note the better islet morphology (IL) and reduced congestion of blood vessels (arrow) as compared with panel (b). Hematoxylin and eosin, X400.

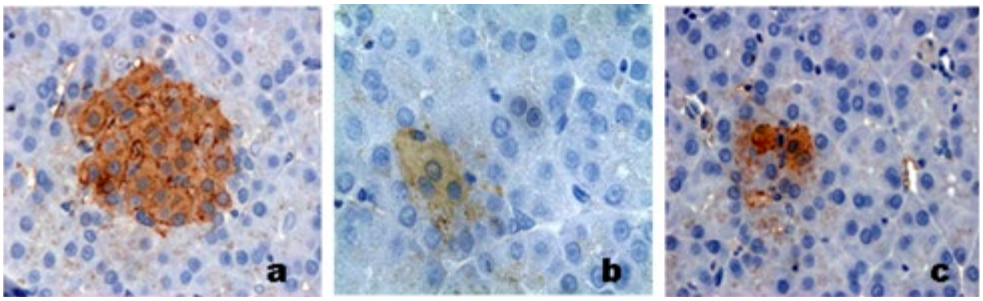

Figure 2 – Photomicrograph of the pancreas showing insulin immunoreactivity of β cells (dark brown) in: **(a)** a control rat, note the intense immunoreactivity; **(b)** a rat induced to become diabetic with streptozotocin, note the reduced immunoreactivity; **(c)** a diabetic rat treated with aqueous leaf extract of *X. aethiopica*, note the improvement in immunoreactivity as compared with panel (b). Indirect immunohistochemistry for insulin, X400.

Table 4 – Morphometric analysis of insulin immuno-labeling of control rats (group A), of rats made diabetic by streptozotocin (group B) and diabetic rats treated with *X. aethiopica* extract (group C). Mean ± standard deviation; N = 10 in each group.

	GROUP A	GROUP B	GROUP C
Field surface area (μm^2)	23485.01	23485.01	23485.01
Immuno labeled surface area (%)	5.31 ± 5.35^b	0.45 ± 1.01^a	4.72 ± 2.08^b

In each row, values indicated with different letters (a, b) differ significantly from each other ($p < 0.05$) while values indicated with the same letters do not differ significantly from each other.

treated with aqueous leaf extract of *X. aethiopica* revealed better staining pattern for insulin than the diabetic group (Fig 2). The results of image analysis are shown in Table 4. The mean percentages (± SD) of the relative surface area stained for insulin

were 5.31±5.35, 0.45±1.01, 4.72±2.08 for control, diabetic and extract-treated diabetic groups respectively (N = 10 for each group). There was a significant effect of *X. aethiopica* on the expression of insulin when compared with the diabetic group.

Discussion

Diabetes occurs when there is insufficient production of insulin or when the available insulin is not effectively utilized (Awasthi et al., 2014). Estimate suggests that about 346 million people worldwide have diabetes. About 1.5 million people die on a yearly basis as a result of hyperglycemia (WHO, 2015). Low- and middle-income countries are majorly affected by this death. WHO projects that diabetes death will double between 2005 and 2030.

The present study shows a gradual decline in the average body weight of the untreated diabetic group. The reason may be connected with the release of free radicals or glucose intolerance which occurs in ill health conditions like diabetes. Several authors have associated diabetes to the release of free radicals (Keaney and Loscalzo 1999; Haffner 2000; Bonnefont-Rousselot et al., 2000). *X. aethiopica* has been known for its highly rich antioxidant content (Blanche et al., 2013) which may have neutralized the free radical so generated thus leading to the observed increment in the average body weight in the extract treated diabetic animals.

The result also showed that there was a gradual drop in the blood glucose level from the day the extract treatment started (3rd day) till the end of the study (28th day) unlike the consistent increment noticed in the untreated diabetic animals. This observation may be due to the hypoglycemic properties of *X. aethiopica* (Ogbonnia et al., 2008, 2010). Plants with hypoglycemic properties have the tendency to reduce blood glucose level.

Several plasma lipids were increased in the diabetic group when compared with the extract-treated animals, except high density lipoprotein cholesterol which was not significantly different among groups. Triglycerides have been reported to increase in the diabetic state (Suryawanshi et al., 2006). There seem to be a correlation between the level of insulin and bioavailability of triglyceride. For instance, Shih et al., 1997 proposed the increase in triglyceride to be due to insulin insufficiency. Insulin insufficiency leads to derangement in glucose utilization, hyperglycemia and release of fatty acids from adipose tissue for energy production. The excess of these fatty acids which are eventually accumulated in the liver are then converted to triglyceride. The leaf extract of *X. aethiopica* was able to normalize the triglyceride level as compared with the control group. The total cholesterol increment in the diabetic animals in this study may be due to inhibition in cholesterol catabolism as proposed by Suryawanshi et al., (2006). Treatment of diabetic animals with *X. aethiopica* improved total cholesterol levels. Receptors of low density lipoprotein cholesterol decrease with decrease in insulin level (Suryawanshi et al., 2006). In the extract-treated group there was a reduction in low density lipoprotein cholesterol when compared with the diabetic group. The reason for this may be due to the antioxidant and antihyperlipidemic properties of *X. aethiopica*. This may have improved the insulin levels thus increasing the low density lipoprotin cholesterol receptors leading to decrement in the serum levels of the corresponding particles, as shown by previous studies (Suryawanshi et al., 2006; Satheesh and Pari,

2008; Al-Jamal and Alqadi, 2011). The non significant difference noticed in high density lipoprotein cholesterol was not new as Cohen et al., (1979) also reported the same in a similar study. It is therefore presumed that the antihyperlipidemic ability of *X. aethiopica* may be due to its hypolipidemic activity as shown by Nwozo et al.,(2011) and Nwaichi and Igbinobaro (2012). This however might serve as a means of protection against the development of atherosclerosis and other cardiovascular diseases.

There was a disruption of islet morphology, vascular congestion as well as decline in immunoreactive β-cells in the diabetic group, which was significant upon image analysis. These findings indicate dysfunction of the insulin secreting β-cells. The *X. aethiopica* treated group revealed better morphology and significant increase in insulin expression when compared with the diabetic group. This indicates ability of the aqueous extract of *X. aethiopica* to stimulate islets of Langerhans to secrete reasonable level of immunoreactive insulin. This was accomplished by gradual reduction in vascular congestion and regeneration of the immunoreactive β-cells within the islets. Similar study on β-cells had also shown that plant extracts are capable of stimulating regeneration of β-cells (Lukiati *et al.,* 2012).

We therefore conclude that aqueous leaf extract of *X. aethiopica* improves the morphology of islets and stimulates the recovery of β-cells in STZ-induced diabetic rats and, by extension, might be of use in the management of diabetes and related complications.

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