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ORIGINAL ARTICLE

Histomorphological and morphometric studies of the pancreatic islet cells of diabetic rats treated with extracts of *Annona muricata*

D.O. Adeyemi[1], O.A. Komolafe[1], O.S. Adewole[1], E.M. Obuotor[2], A.A. Abiodun[1], T.K. Adenowo[3]

[1]Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife, Nigeria
[2]Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria
[3]Department of Anatomy, Olabisi Onabanjo University, Ikenne, Nigeria

Microanatomical changes in the pancreatic islet cells of streptozotocin induced diabetic Wistar rats were studied after treatment with methanolic extracts of Annona muricata *leaves. Thirty adult Wistar rats were randomly assigned into three groups (control, untreated diabetic group, and* A. muricata-*treated diabetic group) of ten rats each. Diabetes mellitus was experimentally induced in groups B and C by a single intra-peritoneal injection of 80 mg/kg streptozotocin dissolved in 0.1 M citrate buffer. The control rats were intraperitoneally injected with an equivalent volume of citrate buffer. Daily intra peritoneal injections of 100 mg/kg* A. muricata *were administered to group C rats for two weeks. Post sacrifice the pancreases of the rats were excised and fixed in Bouin's fluid. The tissues were processed for paraffin embedding and sections of 5 μm thickness were produced and stained with H & E, Gomori aldehyde fuchsin, and chrome alum haematoxylin-phloxine for demonstration of the β-cells of islets of pancreatic islets. Histomorphological and morphometric examination of the stained pancreatic sections showed a significant increase in the number, diameter, and volume of the β-cells of pancreatic islets of the* A. muricata-*treated group (5.67 ± 0.184 N/1000 μm^2, 5.38 ± 0.093 μm and 85.12 ± 4.24 μm^3, respectively) when compared to that of the untreated diabetic group of rats (2.85 ± 0.361 N/1000 μm^2, 2.85 ± 0.362 μm and 69.56 ± 5.216 μm^3, respectively). The results revealed regeneration of the β-cells of islets of pancreatic islet of rats treated with extract of* A. muricata*. (Folia Morphol 2010; 69, 2: 92–100)*

Key words: *Annona muricata*, diabetes mellitus, streptozotocin, pancreas, histology, histomorphometry

INTRODUCTION

For a long time, it was believed that the endocrine pancreas belonged to a category of tissues that were finally differentiated and irreplaceable in the adult. This was mainly supported by the low replication rate of endocrine cells in adulthood [48]. In the light of many recent data, this point of view has been drastically changed, and nobody disputes today that that endocrine pancreas is a plastic organ and that β-cell mass is dynamic, especially because of its significant capacity for adaptation to changes in insulin demand [5]. This property has been demon-

Address for correspondence: D.O. Adeyemi, Department of Anatomy and Cell Biology, Faculty of Basic Medical Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria, tel: +2348034498833, e-mail: waledavade@yahoo.com

strated in physiological as well pathophysiological conditions such as pregnancy [43] and obesity [28]. Increase in β-cell mass may occur through increased β-cell replication, increased β-cell size, decreased β-cell death, and differentiation of β-cell progenitors (neogenesis) [19].

Diabetes mellitus is one of the most common metabolic disorders with a worldwide prevalence estimated at between 1% and 5% of the world population [25]. It is estimated that in the year 2000, 171 million people had diabetes, and this is expected to double by the year 2030 [6]. Conventionally, insulin-dependent diabetes mellitus is treated with exogenous insulin [17] and non insulin-dependent diabetes mellitus with synthetic oral hypoglycaemic agents like sulphonylureas and biguanides [42]. However, the hormone fails as a curative agent for complications of diabetes [34], and synthetic oral drugs produce adverse health effects [41]. Different medicinal systems are using active plant constituents, which were discovered as natural hypoglycaemic medicine, and came from the virtue of traditional knowledge. *Annona muricata* has been found to contain numerous bioactive compounds useful for the management of various ailments, including diabetes mellitus, in folkloric medicine. The management of *Diabetes mellitus* depends on continuous hypoglycaemic therapy, which may not be consistently adhered to by the patient. This research therefore investigates whether or not extracts of *A. muricata* could provide lasting hypoglycaemic control through regeneration of the destroyed β-cells of the pancreatic islets of experimentally induced diabetic Wistar rats.

Annona muricata is a plant which belongs to the family *Annonaceae*. It is a medicinal plant that has been used as a natural remedy for a variety of illnesses. Several studies by different researchers have demonstrated that the bark as well as the leaves have anti-hypertensive, vasodilator, anti-spasmodic (smooth muscle relaxant), and cardio depressant (slowing of heart rate) properties in animals [18]. Researchers have re-verified *A. muricata* leaf's hypotensive properties in rats [3, 8]. Other properties and actions of *A. muricata* documented by traditional uses include its use as anti-cancerous [38, 50], anti-diabetic [51], anti-bacterial [49], anti-fungal [22], anti-malarial, anti-mutagenic (cellular protector), emetic (induces vomiting), anti-convulsant [36], sedative, insecticidal, and as a uterine stimulant. It is also believed to be a digestive stimulant, antiviral, cardio tonic (tones, balances, and strengthens the heart), febrifuge (cures fever), nerviness (balances/ /calms the nerves), vermifuge (expels worms), pediculicide (kills lice), and as an analgesic. Padma et al. [40] confirmed the anti-viral activity of ethanolic extracts of *A. muricata* against the *Herpes simplex* virus. Extracts of *A. muricata* have been shown to have anti-parasitic [7], anti-rheumatic, astringent [12], anti-leishmanial, and cytotoxic effects [24, 30]. *A. muricata* has also been shown to be effective against multi-drug resistant cancer cell lines [30, 37]. Extracts of *A. muricata* were also shown to be effective against the cancer cell line U973 [24], and haematoma cell lines *in-vitro* [10]. Extracts were also shown to be lethal to the fresh water mollusc, *Biomphalaria glabrata*, which acts as a host to the parasitic worm *Schistosoma mansoni* [12, 31].

Streptozotocin-induced hyperglycaemia in rats is considered a good model for the preliminary screening of agents active against type 2 diabetes [23] and is widely used. Generally, destruction of β-cells starts three days after streptozotocin (STZ) administration and reaches its peak at three to four weeks in rats [2]. Streptozotocin-induced diabetes in laboratory animals has been widely used for research on diabetes and its long-term complications. Control animals in these studies are usually injected with citrate buffer solution. Streptozotocin is a potent DNA methylating agent and acts as a nitric oxide donor in pancreatic islet cells. Although the β-cell cytotoxic action of STZ is not fully understood, it is thought to be mediated by the inhibition of free radical scavenger enzymes, thereby enhancing the production of superoxide. The latter has been implicated in lipid oxidation, DNA damage, and sulfhydryl oxidation.

MATERIAL AND METHODS

Plant material

Leaves of *Annona muricata* (family: Annonaceae) were collected from Mowe, Ogun State, Nigeria in February 2006. The leaves were botanically identified by a taxonomist in the Department of Botany of Obafemi Awolowo University, Ile-Ife, and a voucher specimen was deposited in the University's Botany Department Herbarium.

Preparation of methanolic extract of *Annona muricata* leaves

A. muricata leaves were air dried at room temperature for two weeks. The air-dried leaves were powdered in a warring blender (Christy and Norris

— 47362, England) at the Department of Pharmacognosy of Obafemi Awolowo University, Ile Ife. Then 600 g of the powdered leaf was macerated in 5 litres of 70% methanol for 72 hours at room temperature. The mixture was filtered, and the filtrate was concentrated *in vacuo* at 35°C using a vacuum rotary evaporator (RE 100B, Bibby Sterilin, United Kingdom). The wet residue was freeze-dried using a vacuum freeze drier (FT33 — Armfield, England) and was stored until use. The percentage yield of extraction was calculated as follows:

$$Percentage\ Yield = \frac{weight\ of\ extract}{weight\ of\ the\ powdered} \times 100\%$$

Care and management of animals

Thirty healthy adult Wistar rats (*Rattus norvegicus*) weighing between 150 g and 250 g were used for the experiment. They were kept in individual cages under natural light and dark cycles at room temperature. They were maintained on standard rat pellet (Ladokun feeds, Ibadan, Nigeria) and water given *ad libitum.* The animals were randomly assigned into three groups (A, B, and C) of ten rats each. Group A was the control, non-diabetic group of rats, group B was the experimentally induced diabetic group without *A. muricata* treatment, while group C was the experimentally induced diabetic group treated with methanolic extracts of *A. muricata*. There was a pre-experimental period of four weeks during which the body weight and blood glucose levels were monitored in the animals before the commencement of the experiment. The rats received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health [35].

Acute toxicity testing

The median lethal dose (LD$_{50}$) of *A. muricata* leaf extract was determined in mice using the method of Abdel-Barry et al. [1]. The mice fasted for 16 hours and were randomly divided into 6 groups of 10 mice each. Graded doses of PNE (20, 50, 100, 200, 400, and 600 mg/kg) were separately administered intraperitoneally (ip) to the mice in each of the 'test' groups. The extract was dissolved in distilled water, and the average volume injected was 0.3 mL. Each of the mice in the control group was given an equivalent volume of the distilled water used in dissolving the extract. The mice in both the 'test' and 'control' groups were allowed free access to food and water. The mortality in each cage was assessed 24 hours, 48 hours, and 72 hours after administration of the extract. The percentage mortality in each group was calculated and plotted against the log$_{10}$ of the extract dose. A regression line was fitted by the method of least squares, and confidence limits for the lethal dose (LD$_{50}$) values were calculated by the method of Abdel-Barry et al. [1].

Induction of experimental diabetes and *Annona muricata* extract administration

Diabetes mellitus was induced in the rats in groups B and C by a single intraperitoneal injection of STZ (80 mg/kg body weight) dissolved in 0.1 M sodium citrate buffer (pH 6.3) [3], while group A 'control' rats were injected with volumes of the citrate buffer equivalent to the volume of STZ administered intraperitoneally. Diabetes was allowed to develop and stabilize in these STZ-treated rats over a period of 48 hours. The rats were fasted overnight (16 h) but still allowed free access to water before STZ administration. The daily body weight and the weekly blood glucose levels were monitored in the animals for the next four weeks. After four weeks of experimental-induction of diabetes, group C rats were given daily intraperitoneal injections of 100 mg//kg of extracts of *A. muricata* dissolved in distilled water for two weeks, and the animals were monitored for another four weeks.

Determination of body weight and blood glucose level

The body weights of the animals were measured using a top loader weighing balance. Blood samples were obtained from the tail vein of the animals and their fasting blood glucose level was determined in mmol/L using a digital glucometer (Accu-chek® Advantage, Roche Diagnostic, Germany). The animals were fasted for a period of 16 hours before their blood glucose levels were measured.

Surgical procedures

A mid-line incision was made through the anterior abdominal walls of the rats under slight anaesthesia using sodium pentobarbital (6.4 mg/100 g body weight i.m.), and the pancreatic tissues were excised and weighed after the fat and lymph nodes had been removed. The pancreas is located at the junction of the supra-colic and infra-colic compartments of the abdominal cavity as it extends transversely across the posterior abdominal wall between the duodenum on the right and the hilum of the

spleen on the left; the pancreas was isolated from the surrounding organs and weighed. The relative weight (%) was calculated from the body weight at sacrifice and the absolute pancreatic weight as follows:

$$\text{Relative pancreatic weight} = \frac{\text{absolute pancreatic weight}}{\text{body weight at sacrifice}} \times 100\%$$

Histological procedure

The splenic part of the pancreas of each rat was fixed in Bouin's fixative for 24 hours and processed via the paraffin wax embedding method of Drury and Wallington [13]. Paraffin-embedded sections were cut at 5 μm and stained with haematoxylin and eosin (HE), Gomori aldehyde fuchsin, and chrome alum haematoxylin phloxine for light microscopic examination of the pancreatic islets architecture. The sections were examined under a Carl Zeiss research microscope (Axioskope 40, Germany) with a digital camera attached. Digital photomicrographs of the pancreatic sections were taken at various magnifications.

Histomorphometric analysis

All histomorphometric studies were carried out on an Olympus research microscope (Olympus WF10X, Japan) with a linear scale-ocular micrometer and an area-measuring ocular grid inserted into the eyepiece. The ocular micrometer and the ocular grid were calibrated with a 1 mm stage micrometer (Graticles Tonbridge, Kent, England). Twenty-four histological stained sections (eight from each group) were used for morphometric analysis; 202 islet profiles were examined at different magnifications in the non-serial pancreatic sections to estimate (a) the number of islets in each section of the pancreas, (b) the area of the pancreatic tissue section, (c) the area of the pancreatic islet, (d) the diameter of the islets, (e) the number of β-cells of the pancreatic islets, and (f) the diameter of β-cells of the pancreatic islets. The islet area was measured in each pancreatic section at 400$\times$ magnification using the area-calibrated ocular grid. The number of islets and the area of pancreatic tissue were quantified in these sections at 40$\times$ magnification using the ocular grid. The number of islets was expressed as *N/10 mm²* of the pancreatic parenchyma. The β-cell profiles were determined by direct counting method at 1000$\times$ magnification using the ocular grid, and the point counting method of Weibel [52] was used to estimate the numerical density of the β-cells per 1000 μm²

of the islet profile (N/1000 μm²). With an ocular micrometer and a graticule of a calibrated linear scale, the major axis (a) and minor axis (b) at right angles to the major axis of the islets were measured. The profile diameter of the islets (D_i) was calculated from the equation $D_i = \sqrt{ab}$ [54].

Similar steps were followed to measure the diameter (D_n) of the β-cells. A total of 150 β-cells were measured at a magnification of 1000$\times$ (50 from each group). The mean corrected islet diameter (D_i) and the mean corrected nuclear diameter (D_n) were used to calculate the mean islet volume and mean nuclear volume [54].

$$V = \frac{4\pi}{3} \times (D/2)^3$$

Statistical analysis

The data were analysed using descriptive and inferential statistics. All values are presented as mean $\pm$ standard error of mean (SEM) for ten rats in each of the three group of rats. The significance of difference in the means of all parameters reported for the three groups of animals was determined using paired sample student t-test, and a p-value of < 0.05 (two tailed) was considered as significant.

RESULTS

Physical observation

Prior to STZ administration, there was no significant difference in the average weights of all the rats in the three groups. By the end of the first week after diabetes mellitus was experimentally induced, the weights of diabetic rats in groups B and C were significantly reduced despite the increase in food and fluid intake in these animals. This weight loss continued for four weeks after STZ administration (Fig. 1). The animals manifested alopecia and poly-urea, shown by marked wetness of the ventral body surface of the animals. However, the weight of the animals in group C gradually increased with treatment with extracts of *A. muricata* over the period of five weeks (Fig. 1). In addition, there was an improvement in the physical outlook of the *A. muricata* treated animals over time. At the end of the experiment, there was a significant increase in the body weights of *A. muricata* treated diabetic rats when compared to the untreated diabetic rats (Fig. 2); while no significant difference existed between the weights of the treated rats and the control.

Changes in the blood glucose level

Prior to STZ administration, the fasting blood glucose levels did not differ significantly between
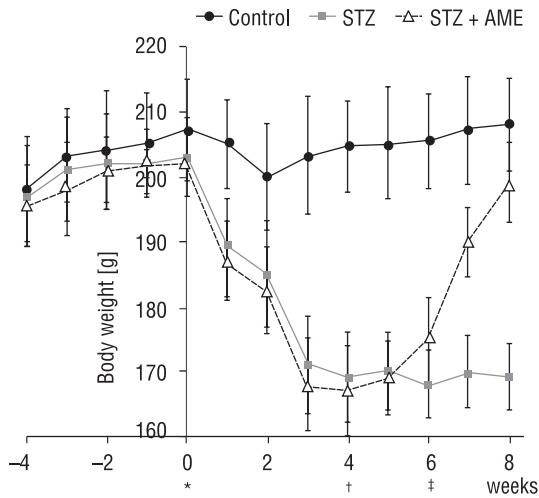
Figure 1. Weekly changes in the body weight of the rats after streptozotocin (STZ) and *A. muricata* extract (AME) administration; *STZ — administration; †AME — administration begins; ‡AME — administration ends.
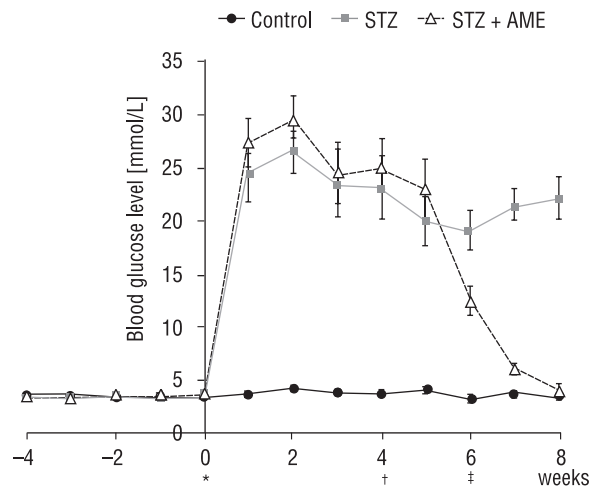

Figure 3. Weekly changes in the blood glucose level of rats after streptozotocin (STZ) and *A. muricata* extract (AME) administration; *STZ — administration; †AME — administration begins; ‡AME — administration ends.
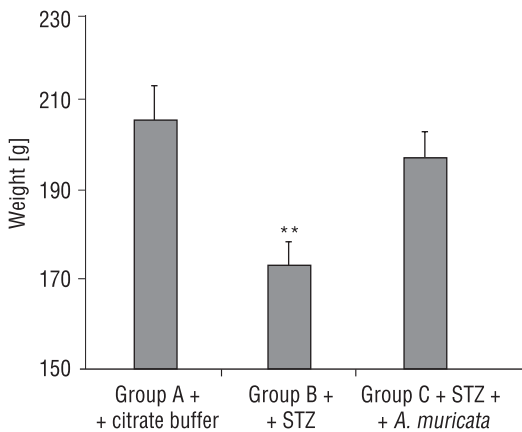

Figure 2. Weight of control, untreated diabetic, and *A. muricata* extract (AME)-treated diabetic rats; **statistical significant difference exists when compared with controls (p < 0.05).

Figure 4. Blood glucose level of control, untreated diabetic, and *A. muricata* extract (AME)-treated diabetic rats; **statistical significant difference exists when compared with controls (p < 0.05).
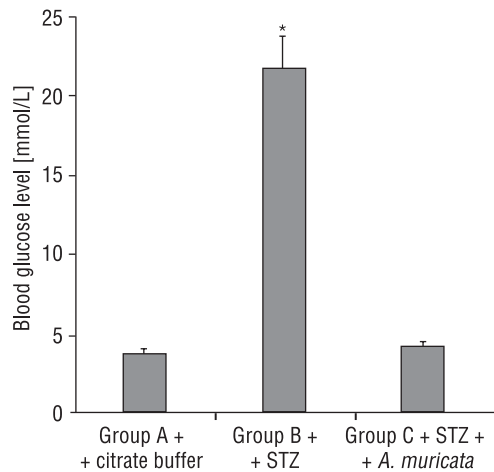
the three groups of experimental animals. Twenty-four hours after administration of STZ, the blood glucose level was significantly higher in animals from groups B and C. The blood glucose levels of animals in group C gradually decreased with treatment with extracts of *A. muricata* over the period of five weeks (Fig. 3). Control rats treated with citrate buffer were euglycaemic throughout the period of the experiment. At the end of the experiment there was a significant reduction in the blood glucose levels of groups C rats compared to those of group B rats. In addition, there was no significant difference between the blood glucose level of rats in groups A and C (Fig. 4)

Changes in the pancreatic weights

The mean weights of the pancreases were significantly higher in the control and *A. muricata*-treated rats than in the untreated diabetic rats. In addition, the relative ratio of the pancreas to the body weight of the rats at sacrifice expressed as a percentage was significantly higher in the control and *A. muricata*-treated groups of rats when compared to the untreated diabetic rats (Fig. 5). However, there was no significant difference between the pancreatic weights of the control rats and that of the rats treated with extracts of *A. muricata* (Fig. 5).
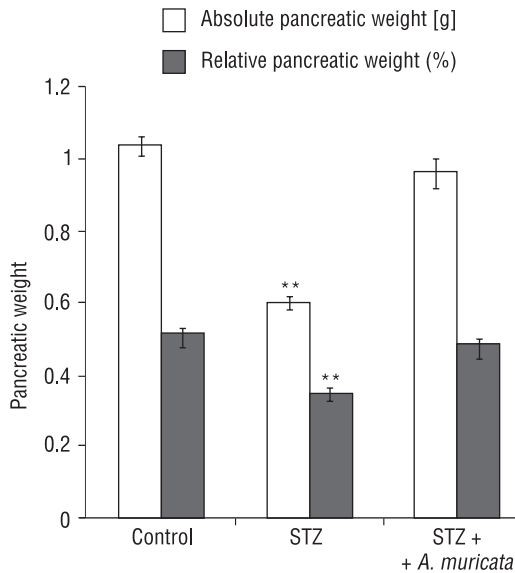
Figure 5. Pancreatic weights of control, untreated diabetic, and *A. muricata* extract (AME)-treated diabetic rats; **statistical significant difference exists when compared with controls (p < 0.05).

Morphological observations

The histological appearance of the pancreatic islet cells of the control was normal. Microscopic examination of the pancreatic sections of the untreated diabetic group revealed a breakdown of micro-anatomical features including degenerative and necrotic changes, and shrunken in the pancreatic islet of Langerhans, β-cell degranulation, pycnotic β-cell nuclei, decreased islet cellular density, and severe vacuolation (Fig. 6) in the islet, as well as a severe reduction in the number of cells in the islets; though the pancreatic acinar epithelium, and ductal and connective tissues appeared normal. The morphology of the pancreas of *A. muricata*-treated diabetic rats revealed remarkable improvement in the islet of Langerhans. There was an increase in the islet cellular density, with an increase in granulation, but vacuolation was reduced or absent in many islets (Fig. 6).

Morphometric analysis

The results of the morphometric analysis revealed a significant reduction in the numerical density of islets (number of islet/pancreas), islet area, islet diameter, numerical density of β-cells (number of β-cells per islet), volume of islets, and volume of β-cells in the untreated diabetic group of rats when compared with the control group. However, these morphometric parameters were significantly increased in the *A. Muricata*-treated rats when compared with those of the untreated diabetic rats (Table 1).
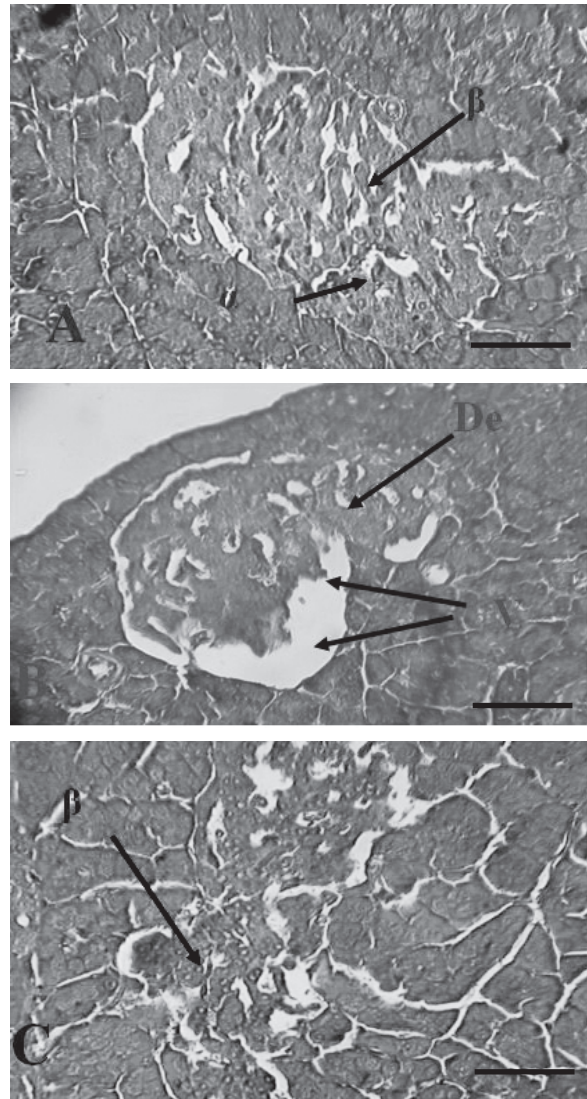

Figure 6. Photomicrograph of pancreatic islet of normal (control), untreated diabetic, and *A. muricata* treated diabetic rats stained with H & E (A, B, and C respectively). Note the clusters of centrally placed beta-cells and peripherally placed alpha-cells in **A.** Degranulation (De) and vacuolation (V) of the pancreatic islets of diabetic rats in **B.** Also note the increase in the beta-cell density with greatly reduced vacuolation of islet in **C.** Bar = 4.55 μm.

DISCUSSION

Diabetes mellitus is a major global health problem, which is becoming an epidemic. The latest World Health Organization publication (global burden of disease) estimates diabetes in adults to be around 173 million [53], around two thirds of these living in developing countries [32]. It is a metabolic disease characterized by high-blood glucose levels resulting from defects in insulin secretion, insulin action, or both [39]. The prevalence of diabetes mellitus is increasing and it is expected to increase by 5.4% by 2025 [33]. An increase in sedentary life-

Table 1. Histomorphometric parameters of the pancreatic islet of control, untreated diabetic, and *A. muricata*-treated diabetic rats

	Group A (Control)	Group B (STZ)	Group C (STZ + *A. muricata*)
Number of islets/pancreas [N/10 mm²]	18.75 ± 0.733[a]	4.79 ± 0.443[b]	8.53 ± 0.565[c]
Area of islets [mm²]	0.0108 ± 0.0010[a]	0.0052 ± 0.0006[b]	0.0069 ± 0.0007[a]
Diameter of islets [μm]	130.79 ± 12.825[a]	63.75 ± 8.410[b]	97.69 ± 10.702[a]
Number of β-cells/islet [N/1000 μm²]	10.64 ± 0.733[a]	2.85 ± 0.362[b]	5.67 ± 0.184[c]
Diameter of β-cells [μm]	5.49 ± 0.110[a]	4.92 ± 0.137[b]	5.38 ± 0.093[c]
Volume of islets [μm³]	$1.49 \times 10^6 \pm 3.953 \times 10^5$ [a]	$2.10 \times 10^5 \pm 9.493 \times 10^4$ [b]	$6.53 \times 10^5 \pm 1.989 \times 10^5$ [a]
Volume of β-cells [μm³]	115.29 ± 6.550[a]	69.56 ± 5.216[b]	85.12 ± 4.24[c]

a, b, and c between row signify that means with different letters differ significantly at p < 0.05 two tailed t-test while means with the same letter do not differ significantly at p < 0.05

style, consumption of energy-rich diet, and obesity are some of the factors causing the rise in the number of diabetics. However, Asia and Africa are identified as regions with the greatest potential, where diabetics could rise two- or threefold above the present level [4]. In traditional African societies, phytotherapy is highly valued and widely utilized. The majority of the population use plant materials as their source of primary healthcare [15].

In modern medicine, the beneficial effects of standard medications on glycaemic levels are well documented; the preventive activity of these medications against the progressive nature of diabetes and its complications produce reasonable result but they are not always effective. Insulin therapy affords glycaemic control in type 1 diabetes, yet its shortcomings, such as ineffectiveness on oral administration, short shelf life, the requirement of constant refrigeration, fatal hypoglycaemia in the event of excess dosage, the reluctance of patients to take insulin injections, and above all the resistance due to prolonged administration, limit its usage [26]. Similarly, treatment of type 2 diabetes patients with sulfonylureas and biguanides is always associated with side effects [20]. Hence the search for a drug with low cost, greater potential, and no adverse side effects is underway in several laboratories around the world.

For various reasons, in recent years, the popularity of alternative medicine has increased. Surveys conducted in Australia and the United States indicate that almost 48.5% and 34% of the respondents, respectively, had used at least one form of unconventional therapy, including herbal medicine [14]. The WHO Expert Committee on Diabetes has also recommended further evaluation of the folkloric

methods of managing this disease because of the high mortality and morbidity arising from its attendant complications and problems associated with the use of conventional antidiabetic agents [9]. This has led to the increasing demand for herbal products with anti-diabetic activity and fewer side effects [27].

Streptozotocin-induced hyperglycaemia in rats is considered a good model for the preliminary screening of agents active against type 2 diabetes [23] and is widely used. Generally, destruction of β-cells starts three days after STZ administration and reaches its peak at three to four weeks in rats [2]. Streptozotocin-induced diabetes in laboratory animals has been widely used for research on diabetes and its long-term complications. Control animals in these studies are usually injected with citrate buffer solution. However, STZ is known to possess pharmacological effects other than its diabetogenic properties [44], and extra pancreatic actions of STZ cannot be excluded. The presence of GLUT2 in liver and kidney might explain the long-term complications seen with hepatic and renal tumours in rats treated with STZ [11, 46]. Because of the extra pancreatic effects of STZ, it can be difficult to distinguish effects secondary to diabetes from those secondary to STZ *per se*. Streptozotocin is a potent DNA methylating agent and acts as a nitric oxide donor in pancreatic islet cells. Although, the β-cell cytotoxic action of STZ is not fully understood, it is thought to be mediated by the inhibition of free radical scavenger enzyme thereby, enhancing the production of superoxide. The latter has been implicated in lipid oxidation, DNA damage, and sulfhydryl oxidation.

Beta-cells are particularly sensitive to damage by nitric oxide and free radicals because of their low

levels of free radical scavenging enzymes [47]. In this present study, almost all the insulin-producing β-cells were degranulated, degenerated, or necrosed in the streptozotocin-treated rats, leading to a decrease in insulin secretion and an increase in blood glucose concentration. However, treatment with extracts of *A. muricata* showed a significant antihyperglycaemic activity in STZ-induced diabetic rats at the end of the experiment. It has been suggested that bioactive compounds from plant sources having antihyperglycaemic activities might act by several mechanisms such as stimulating insulin secretion, increasing repair, or proliferation of β-cells and enhancing the effects of insulin and adrenalin [16, 45]. The results of this present study indicate that the decrease in the blood glucose concentration of diabetic rats by *A.muricata* treatment is due to the regeneration/proliferation in the pancreatic β-cells. The regeneration of the β-cells of the STZ-destructed islets is probably due to the fact that pancreas contains stable (quiescent) cells which have the capacity of regeneration. Therefore, the surviving cells can proliferate to replace the lost cells [21, 29].

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