

IMMUNOHISTOCHEMICAL AND BIOCHEMICAL EFFECTS OF *ANNONA MURICATA* LINN. (ANNONACEAE) LEAF AQUEOUS EXTRACT ON PANCREATIC β -CELLS OF STREPTOZOTOCIN-TREATED DIABETIC RATS

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Running Title

Protective effects of *Annona muricata* on the morphology of pancreatic β -cells

Summary

Numerous bioactive compounds have been found in *Annona muricata* plants for management, control, and/or treatment of plethora of human ailments, including diabetes mellitus. In view of this, the present study was undertaken to evaluate the possible protective effects of leaf aqueous extract of *Annona muricata* Linn. on morphology of pancreatic β -cells and oxidative stress induced by streptozotocin (STZ)-diabetic rats. Diabetes mellitus was induced in Groups B and C rats by a single intraperitoneal injection of STZ, (75 mg/kg body weight). Group A rats received an equivalent volume of citrate buffer (pH 6.3). Group C rats were further treated with *A. muricata* leaf aqueous extract (AME, 100 mg/kg/day, p.o.) as from day 5 post STZ injections, and stopped on the 30th day of the study period. Post-euthanization, pancreatic tissue were excised and processed for immunohistochemical staining and biochemical assays for antioxidant enzymes and cholesterol such as reactive oxygen species (ROS), glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), thiobarbituric acid reactive substances (TBARS), triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL) and low density lipoprotein (LDL). In diabetic state, Image analysis of pancreatic β -cells demonstrated weak immunoreactive stain with respect to control rats. The morphology of *A. muricata*-treated rats' pancreases showed strong immunoreactive stain. STZ treatment significantly decreased ($p < 0.05$) GSH-Px, SOD, GSH, HDL and pancreatic/serum insulin levels. However, STZ treatment equally increased blood glucose concentrations, ROS, MDA, TC, TG, LDL and altered islets microanatomy. The *A. muricata*-treated rats showed a significant decrease ($p < 0.05$) in elevated blood glucose, ROS, MDA, TC, TG and LDL. Furthermore, *A. muricata*-treatment significantly increased ($p < 0.05$) antioxidant enzymes' activities, as well as pancreatic/serum insulin contents. In conclusion, the findings of the present study indicate that *A. muricata* treatment has protective beneficial effects on pancreatic tissues subjected to STZ-induced oxidative stress possibly through decreasing lipid peroxidation and indirectly enhancing production of endogenous antioxidants.

Key Words: *Annona muricata* Leaf Aqueous Extract; Immunohistochemical; Morphological changes; Oxidative stress and Antioxidants.

Introduction

Diabetes mellitus is basically a disease of glucose metabolism resulting from dysfunction of pancreatic β -cells and insulin resistance, but at later stages of the disease, lipid metabolism is also affected. Streptozotocin (STZ), an antibiotic produced by *Streptomyces achromogenes*, possesses pancreatic β -cell cytotoxic effect [1]. Streptozotocin has been widely used for inducing diabetes mellitus in a variety of animals. STZ causes degeneration and necrosis of pancreatic β -cells [2; 3]. Although the mechanism of the β -cell cytotoxic action of STZ is not fully understood, experimental evidence has demonstrated that some of its deleterious effects are attributable to induction of metabolic processes, which lead onto an increase in the generation of reactive oxygen species (ROS) [4]. Apart from production of ROS, STZ also inhibits free radical scavenger-enzymes [5]. The superoxide radical has been implicated in lipid peroxidation, DNA damage, and sulfhydryl oxidation [6; 7].

There are several reasons why use of medicinal plants should be studied: herbal remedies may have recognizable therapeutic effects [8]; they may also have toxic side-effect [9]. All parts of the *A. muricata* tree are used in natural medicine in the tropics including the bark, leaves, root and fruit-seeds. Generally the fruit and fruit juice is taken for worms and parasites, to cool fevers, to increase mother's milk after childbirth (lactagogue), and as an astringent for diarrhea and dysentery. The crushed seeds are used as a vermifuge and antihelmintic against internal and external parasites and worms. The bark, leaves and roots are considered sedative, antispasmodic, antidiabetic, antihypertensive, smooth muscle relaxant and nervine and a tea is made for various disorders for those purposes [10]. Many bioactive compounds (ellagic acid, tannins, flavonoids, polyphenolic compounds) and phytochemicals have been found in *A. muricata* as scientists have been studying its properties since the 1940's. Its many uses in natural medicine have been validated by this scientific research [11]. The earliest studies were between 1941 and 1962. Several studies by different researchers demonstrated that the leaf, bark, roots, stem and seed extracts are antibacterial *in vitro* against numerous pathogens [12; 13; 14] and that the bark has antifungal properties [15]. Much of the recent research on *A. muricata* has been on a novel set of phytochemicals (*Annonaceous acetogenins*) that are found in the leaves, seeds and stem which are cytotoxic against various cancer cells [16; 17; 18].

A. muricata (Linn.) (family, Annonaceae) commonly called "Soursop" is a small, upright evergreen tree growing 5 to 6 meters in height. Young branchlets are rusty-hairy, the malodorous leaves, normally evergreen, are alternate, smooth, glossy, dark green on the upper surface, lighter beneath; oblong, elliptic or narrowobovate, pointed at both ends, 6 - 20 cm long and 2 - 6 cm wide. The flowers are borne singly, may emerge anywhere on the trunk, branches or twigs. They are short stalked, 4 - 5 cm long, plump, and triangular-conical, the 3 fleshy, slightly spreading, outer petals yellow-green, the 3 close-set inner petals pale-yellow [19; 20]. The fruit is more or less oval or heart-shaped, some times irregular, lopsided or curved, due to improper carper development or insect injury. The size ranges from 10-30 cm long and up to 15 cm in width and the weight may be up to 4.5-6.8 kg.

Oxidative stress is the excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS)

[21; 22]. ROS include free radicals such as superoxide (O_2^-), hydroxyl (OH^\cdot), peroxy (RO_2^\cdot), hydroperoxyl (HRO_2^\cdot) as well as nonradical species such as hydrogen peroxide (H_2O_2), and hydrochlorous acid ($HOCl$) [23]. RNS include free radicals like nitric oxide (NO) and nitrogen dioxide (NO_2^\cdot), as well as nonradicals such as peroxyxynitrite ($ONOO^-$), nitrous oxide (HNO_2) and alkyl peroxyxynitrates ($RONOO$) [24]. Of these reactive molecules, O_2^- , NO and $ONOO^-$ are the most widely studied species and play important roles in the diabetic cardiovascular complications. Oxidative stress in diabetes, sources from (i) nonenzymatic source resulting from biochemical oxidation of glucose, glucose reacts with protein in nonenzymatic manner leading to the development of advanced glycation end product (AGE) [25]. (ii) enzymatic sources which includes nitric oxide synthetase (NOS), NAD(P)H oxidase and xanthine oxidase [26; 27]. (iii) mitochondria respiratory chain, during oxidative phosphorylation process, electrons are transferred from electron carriers NADH and $FADH_2$ to oxygen, thus generating ATP in the process [28].

Annona muricata has a long history of use in herbal medicine in the tropical areas in South and North America including the Amazon and Western Nigeria. All parts of the tree are claimed to be used in natural medicine, however, no scientific studies have been done to establish hypolipidemic and antioxidative effects of the AME. Therefore, in view of this, the present study was designed to evaluate the hypoglycemic, hypolipidemic and antioxidative. In addition, possible morphological changes of pancreatic β -cells' effects of *A. muricata* leaf aqueous extract in experimental models of diabetes mellitus in Wistar rats.

Materials and Methods

Animal care and monitoring

Healthy, male and female, Wistar rats (*Rattus norvegicus*) weighing 250–300 g (averaging 12 weeks old) and normal Mice (*Mus domesticus*) were used in this study. They were housed under standard laboratory conditions of light, temperature ($21\pm 2^\circ C$) and relative humidity ($55\pm 5\%$). The animals were given standard rat pellets and tap water *ad libitum*. The rats were randomly divided into three experimental groups: A (control), B (STZ-treated), C (STZ + *A. muricata*-treated). The control group animals consisted of ten rats, while groups B and C consisted of forty rats. The mice were used for acute toxicity testing of the crude plant extract, while the rats were used for hypoglycemic and morphological evaluations of the plant's extract. Maintenance and treatment of animals were in accordance with the principles of the "Guide for care and use of laboratory animals in research and teaching" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH) publication 86-23 revised in 1985.

Plant material

Fresh leaves of *Annona muricata* (Linn.) (family, Annonaceae) (locally known as "Soursop in English, and "Abo" in Yoruba language of Western Nigeria) were collected

in Ibadan, Nigeria between April and May 2006. The leaves were identified, by the Taxonomist/Curator of the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria, as those of *A. muricata* Linn. (family, Annonaceae). Voucher specimen of the plant has been deposited in the Herbarium of the University's Botany Department.

Preparation of *Annona muricata* leaf aqueous extract

A. muricata fresh leaves were air-dried at room temperature. One kilogram of the air-dried leaves of the plant was milled into fine powder in a Waring blender, department of Pharmacognosy, Obafemi Awolowo University, Ile-Ife, Nigeria. The powdered leaf was macerated in distilled water and extracted twice, on each occasion with 2.5 litre of distilled water at room temperature for 48 h. The combined aqueous extract solubles were concentrated to dryness under reduced pressure at $60^{\circ}\text{C}\pm 1^{\circ}\text{C}$ in a rotary evaporator. The resulting aqueous extract was freeze-dried, finally giving 36.23 g (i.e., 3.62% yields) of a light green, powdery crude aqueous leaf extract of *A. muricata* (AME). Aliquot portions of the crude plant extract residue were weighed and dissolved in distilled water for use on each day of our experiments.

Acute toxicity testing

The median lethal dose (LD_{50}) of *A. muricata* leaf aqueous extract (AME) was determined in the rat using a modified method of [29]. Mice fasted for 16 h were randomly divided into groups of eight mice each. Graded doses of AME (50, 100, 200, 400, 800, 1600 and 3200 mg/kg) were separately administered intraperitoneally (i.p.) to the mice in each of the test groups. Each of the mice in the control group was treated only with distilled water (3 ml/kg, i.p.). The mice in both the test and control groups were then allowed free access to food and water, and observed over a period of 48 h for signs of acute toxicity. The number of deaths (caused by the extract) within this period of time was noted and recorded. Log dose-response plots were constructed for the plant's extract, from which the LD_{50} of the leaf aqueous extract was determined.

Evaluations of hypoglycemic activity of *A. muricata* leaf aqueous extract (AME)

The test compound (i.e., *A. muricata* leaf aqueous extract (AME, 50-400 mg/kg) were administered orally to the groups of fasted normal (control) and fasted diabetic 'test' rats. 1, 2, 3, 4 and 8 h following oral administrations of the test compound to the animals, blood glucose concentrations (G_t) were again determined and recorded. In each case and for each dose, the rats were restrained in a cage, and blood samples (0.02 ml) were collected from the tail vein of each rat for blood glucose analysis. Percentage glycemic variation was calculated as a function of time (t) by applying the formula:

$$\% \text{ glycemic change} = G_o - G_t \times 100/G_o$$

[where G_o and G_t represent glycemic values before (i.e., 0-time or 0-h glycemic values), and glycemic values at 1, 2, 3, 4 and 8 hours after, oral administrations of 'test' compound respectively].

Induction of experimental diabetes

Diabetes mellitus was induced (in groups B and C 'test' rats) by single intraperitoneal (i.p.) injection of STZ (75 mg/kg), freshly dissolved in 0.1mol/l citrate buffer [30]. Control rats were injected with only citrate buffer solution (pH 6.3) intraperitoneally. The 'test' animals in groups B and C became diabetic within 48 hours after STZ administration. Diabetic state was confirmed by measuring basal blood glucose concentration 48 hours after STZ injection. Diabetes was allowed to develop and stabilize in these STZ-treated rats over a period of 3-5 days. All animals in groups A, B and C were kept and maintained under laboratory conditions of temperature, humidity, 12-h day – 12-h night cycle; and were allowed free access to food (standard pellet diet) and water *ad libitum*. Before the commencement of our experiments, both the control normal (normoglycemic) and STZ-treated, diabetic (hyperglycemic) test rats were fasted for 16-h, but still allowed free access to water throughout. At the end of the 16-h fasting period – taken as 0 time (i.e., 0 h) – blood glucose levels (initial glycemia, G_0) of the fasted normal and STZ-treated, diabetic rats were determined and recorded. Fasted STZ-treated rats with blood glucose concentration ≥ 18 mmol/L were considered to be diabetic, and used in this study. The test compound [i.e., *Annona muricata* leaf aqueous extract (AME, 100 mg/kg/day p.o.)] was administered orally (by orogastric intubation) to the group C fasted diabetic rats. The administration of plant's aqueous extract was commenced as from the 5th day post STZ injections and stopped on the 30th day of study period.

Histological procedures

Pancreatic tissues were excised from sacrificed animals, weighed, and fixed in aqueous Bouin's solution for 48 h and were sequentially embedded in paraffin wax blocks according to the standard procedure, sectioned at 5 μ thickness. They were further deparaffined with xylol, and histologic observations were performed after staining for functional pancreatic tissues by Aldehyde fuchsin trichrome method described by Stevens [31]. The slides were examined using light microscope interfaced with Olympus DR10 digital camera system.

Biochemical assays:

Blood Glucose and serum insulin estimations

Blood samples were obtained by repeated needle puncture of the same tail tip vein. Samples were obtained 1 day before STZ-treatment, and on various days after induction of diabetes mellitus. Diabetes was allowed to develop and stabilize in these STZ-treated rats over a period of 3-5 days. Blood glucose concentrations were determined by means of Bayer Elite[®] Glucometer, and compatible blood glucose test strips [32]. The mean fasting blood glucose levels for normal, nondiabetic rats were found to vary between 4.01 ± 0.04 and 4.20 ± 0.13 mmol/L. Fasted STZ-treated rats with blood glucose concentrations ≥ 18 mmol/L were considered to be diabetic, and used in this study. Serum insulin concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Crystal Chem, Chicago, III).

Preparation of pancreas homogenates

The rats were sacrificed under light halothane anesthesia and pancreata were excised, rinsed in ice-cold physiological saline, and homogenized with Potter Elvehjem homogenizer. 20% homogenates were prepared in 6.7 mM phosphate buffer solution, pH 7.4 and centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatant was used for antioxidant enzyme assays. For the determination of lipid peroxidation, pancreatic tissues were homogenized in 1.15% KCl solution to obtain a 10% (w/v) homogenate. Protein content of pancreas homogenates was determined by using bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Company, Rockford, IL), Smith and Rennie's method, [33].

Reactive oxygen species (ROS)

Amount of ROS activity in pancreata was measured using 2',7'-dichlorofluorescein diacetate (DCF-DA) which gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The assay was performed as described by Socci *et al.* [34]. Briefly, pancreas was homogenized (10 mg) in 1 ml of ice-cold 40 mM Tris-HCl buffer (pH 7.4) and further diluted to 0.25% with the same buffer and placed on ice. The sample was divided into two equal fractions. In one fraction 40 µl of 1.25 mM DCF-DA in methanol was added for ROS estimation. Another fraction in which 40 µl of methanol was added, served as a control for tissue auto fluorescence. All samples were incubated for 15 min in a 37°C water bath. Fluorescence was determined at 488 nm excitation and 525 nm emission using a fluorescence plate reader (Tecan Spectra Fluor Plus, Germany). Result are expressed as nmol min⁻¹ mg⁻¹ protein

Reduced GSH and oxidized glutathione GSSG activities

Pancreatic GSH and GSSG contents were measured as described by Hissin and Hilf [35]. For measuring GSH contents, 4 ml of pancreatic homogenate was precipitated by adding 1 ml of 25% metaphosphoric acid and centrifuged at 10,000 x g (Ultracentrifuge, Hitachi, Japan) for 30 min. Supernatant was diluted 20 times with same buffer and 100 µl of orthophthaldehyde (OPT) was added. In addition, for GSSG assay, 0.5 ml supernatant was incubated at room temperature with 200 µl of 0.04 mol/l N-ethylmaleimide solution for 30 min and to this mixture 4.3 ml of 0.1 mol/l NaOH was added. A 100 µl sample of this mixture was taken for the measurement of GSSH using the exact procedure described above for GSH assay except that 0.1 mol/l NaOH was used as the diluent instead of phosphate buffer. Samples were incubated at room temperature for 15 min and fluorescence was measured using spectrofluorometer (Tecan Spectra Fluor Plus Germany) at 350 nm (E_x)/420 nm (E_m).

Superoxide Dismutase Activity (SOD)

Pancreatic SOD activity was assayed by the method of Kakkar *et al.* [36]. Reaction mixture contained 1.2 ml of sodium pyrophosphate buffer (0.052 mM, pH 7.0), 0.1 ml of phenazine methosulphate (PMS) (186 µM), 0.3 ml of nitro blue tetrazolium (NBT) (300

μM). 0.2 ml of the supernatant obtained after centrifugation (1500 x g, 10 min followed by 10,000 x g, 15 min) of 5% pancreatic homogenate was added to reaction mixture. Enzyme reaction was initiated by adding 0.2 ml of NADH (780 μM) and stopped precisely after 1 min by adding 1 ml of glacial acetic acid. Amount of chromogen formed was measured by recording color intensity at 560 nm. Results are expressed as units/mg protein.

Glutathione Peroxidase Activity (GSH-Px)

Glutathione peroxidase (GSH-Px) activity was measured by NADPH oxidation, using a coupled reaction system consisting of glutathione, glutathione reductase, and cumene hydroperoxide [37]. 100 μL of enzyme sample was incubated for five minutes with 1.55 ml stock solution (prepared in 50 mM Tris buffer, pH 7.6 with 0.1 mM EDTA) containing 0.25 mM GSH, 0.12 mM NADPH and 1 unit glutathione reductase. The reaction was initiated by adding 50 μL of cumene hydroperoxide (1 mg/ml), and the rate of disappearance of NADPH with time was determined by monitoring absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme that transforms 1 μmol of NADPH to NADP per minute. Results are expressed as units/mg protein.

Thiobarbituric Acid Reactive Substances (TBARS)

The product of the reaction between malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) were measured by a modified method of Ohkawa *et al.*, [38]. For each sample to be assayed, four tubes were set up containing 100, 150, 200 and 250 μL of tissue homogenate, 100 μL of 8.1% SDS, 750 μL of 20% acetic acid, and 750 μL of 0.8% aqueous solution of TBA. The volume was made up to 4 ml with distilled water, mixed thoroughly and heated at 95°C for 60 minutes. After cooling, 4 ml of n-butanol was added to each tube, the contents mixed thoroughly, and then centrifuged at 3000 rpm for 10 minutes. The absorption of the clear upper (n-butanol) layer was measured using a Shimadzu UV-1601 (Japan) spectrophotometer at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex $1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ and was expressed in μmol TBARS/g tissue protein.

Determination of Serum Cholesterol, Lipoproteins and Triglyceride

Blood samples were collected from tail vein of rats after 10-12 h of overnight fasting, transferred to sterilized centrifuge tubes and allowed for clotting at room temperature. The blood samples were centrifuged for 10 min at 4,000 x g to obtain serum. The serum were stored in freezer at 0° for later analysis of total cholesterol (TC) and triglyceride (TG) high and low-density lipoproteins (HDL and LDL)-cholesterol. Aliquots of serum were taken for determination of total cholesterol by enzymatic calorimetric method of Allain *et al.* [39], and triglycerides by enzymatic glycerol phosphate oxidase/ peroxidase method of Cheng *et al.*, [40]; Fossati and Prencipe., [41] Auto analyzer (Express plus, Ciba corning USA) and Elitech kit were used. Serum high density lipoprotein (HDL)-cholesterol was performed by precipitation of chylomicrons, very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) with sodium phosphotungstic

acid and magnesium chloride [42]. Centrifugation left only the HDL in the supernatant; their cholesterol content was determined by method of Virella *et al.*, [43]. Estimation of low density lipoprotein (LDL)-cholesterol was done by using empirical formula of Friedewald *et al.* [44] for samples with TG levels < 400 mg/dL. $[\text{LDL-cho}] = [\text{Total chol}] - [\text{HDL-cho}] - ([\text{TG}]/2.2)$ where all concentrations are given in mmol/L

Immunohistochemical procedures

Pancreatic tissues were excised and weighed after the fat and lymphnodes had been removed. The splenic parts of the pancreas of each rat were fixed in 4% paraformaldehyde fixative, dehydrated and were embedded in paraffin-wax. Each pancreatic block was serially sectioned (5 μ) thickness throughout its length to avoid any bias due to changes in islet distribution or cell composition, and thereafter mounted onto poly-L-lysine slides. For each pancreas, 10 sections were randomly chosen at a fixed interval through the block (every 30th section), a procedure that has been shown to ensure that selected sections are representative of the whole pancreas [45]. Sections were immunostained for insulin, using a peroxidase indirect labeling technique. The sections were incubated for 1 h with guinea-pig anti-insulin serum (final dilution 1:1,000, Ref. 64-104-1; Aurora, OH), washed in PSB 6 min. Thereafter, sections were incubated for 45 min with peroxidase-conjugated rabbit anti-guinea pig IgG (final dilution 1:20; Dako, Carpinteria, CA), washed in PSB 6 min. The activity of the antibody-peroxidase complex was revealed with 3,3'-diaminobenzidine-tetrahydrochloride, using a peroxidase substrate kit (DAB; Biosys-Vector, Compiègne, France), nuclei stained with hematoxylin. Sections were mounted in Dako faramount, evaluated by a bright field microscope and were photographed (Nikon Optiphot 2, Tokyo). A standard concentration of hematoxylin was added as a counterstain.

Pancreatic insulin content determination

The splenic regions of the pancreatic tissues from euthanized rats were weighed, homogenized, and protein was extracted for 4 h at 4°C in acid-ethanol solution, on various experimental days, (75% ethanol, 23.5% distilled water, 1.5% concentrated HCl). After overnight incubation at 4°C, the suspensions were centrifuged, and the supernatants were collected and assayed for insulin contents, using a competitive ELISA kit [46]. Plates were coated with rabbit anti-guinea-pig Ig secondary Ab (Organon Teknika, Durhan, NC), followed by incubation with a guinea-pig anti-human insulin Ab (Cortex Biochem, San Leandro, CA). Following two washing steps, various extract dilutions or insulin standards (Linco Research, St. Louis, MO) were mixed with constant concentration of HRP-conjugated rat insulin (Organon Teknika) for 4 h at room temperature, or at 4°C overnight, before competitive capturing was allowed for 3 h. After washing five times, Sigma FAST OPD tablets (Sigma, St. Louis, MO) were used as substrate. Results were analyzed using ceres 900 C ELISA-reader with KC3 software (Bio-Tek Instruments, Winooski, VT). Pancreatic extract total protein was measured by a protein assay (Bio-Rad, Richmond, VA) according to the manufacturer's recommendations using BSA standards (Bio-Rad).

Statistical Analysis

The data obtained were expressed as means (\pm SEM), and analyzed using repeated measures of variance. The differences between the means were analyzed statistically with one-way analysis of variance (ANOVA; 95% confidence interval). Values of $p < 0.05$ were taken to imply statistical significance.

Results**Effects of diabetes on body weight and serum insulin**

Forty-eight hours after STZ administration, all animals that had been treated with STZ displayed glucosuria, hyperglycemia, hypoinsulinemia and a moderate loss of body weight. Average blood glucose concentrations and serum insulin levels of the STZ-treated experimental animals are shown in Table 1. The baseline weight of the rats at the beginning of the study was similar in all groups. At the end of the study period (2 months), diabetic animals in group B presented with weight loss. The initial and final body weights were not significantly ($p > 0.05$) different in control and AME-treated groups.

Blood glucose and serum insulin concentrations

Mean blood glucose concentrations and serum insulin levels of the STZ-treated experimental animals are shown in Table 2. In our control set of experiments, pretreatment of the rats with citrate buffer alone did not significantly modify ($p > 0.05$) the serum insulin and blood glucose concentrations. As shown in Table 2. Fig. 1, induction of diabetes resulted in a significant increase in blood glucose level of rats, there was a gradual rise in the blood glucose concentrations as from day 1 following injection of STZ, and the values were significantly higher ($p < 0.05$) than those of control animals (Table 2). Furthermore, high levels of blood glucose concentrations of the STZ-treated rats were persistently observed throughout the study period (21.9 ± 0.4 mmol/l). The blood glucose concentrations in the AME-treated group, (6.2 mmol/l) significantly reduced in value ($p < 0.05$). Also AME treatment caused a significant increase ($p < 0.05$) in the lowered serum insulin levels in STZ-diabetic rats.

Table 1. Various parameters recorded in control, STZ-treated and STZ + AME-treated diabetic rats just before and after treatment.

Parameters		Control	STZ-treated	STZ + AME-treated
Body weight (g)	Initial	246±12	248±14 ^a	244±10 ^a
	Final	251±09	221±16 ^b	254±09 ^a
Pancreatic weight (g)	Initial	0.29±0.2	0.27±0.5	0.27±0.3
	Final	0.27±0.1	0.22±0.4	0.26±0.8
Blood glucose (mmol/l)	Initial	4.1±0.4	18.2±0.3 ^c	8.6±0.2 ^a
	Final	4.2±0.5	21.9±0.4 ^c	5.2±0.6 ^a
Serum insulin (μU/ml)	Initial	12.8±1.5	8.5±1.4 ^d	10.3±1.2 ^a
	Final	12.2±1.1	4.9±1.2 ^d	11.2±1.4 ^a

Values are expressed as means (±SEM) of 10 rats. ^a Insignificant difference ($p>0.05$) between treated groups and control. ^{b,c,d} Significant difference ($p<0.05$) between treatment and control groups.

Table 2. Changes in blood glucose concentrations and serum/pancreatic insulin contents in control, STZ-treated and STZ + AME-treated diabetic rats.

Blood glucose concentration (mmol/L)							
	Control	Treated					
Days	0	10	20	30	40	50	60
STZ-treated	4.2±0.6	18.8±0.2 ^b	20.2±0.4 ^b	21.4±0.3 ^a	21.2±0.1 ^b	22.1±0.3 ^b	21.9±0.2 ^b
STZ + AME-treated	4.3±0.2	8.6±0.4 ^b	7.6±0.2 ^a	6.5±0.3 ^a	5.9±0.4 ^a	5.3±0.8 ^a	5.2±0.6 ^a
Serum insulin concentration (μU/ml)							
STZ-treated	12.8±1.5	8.5±1.4	6.3±1.4 ^c	5.9±1.3 ^c	5.7±2.3 ^c	5.5±1.2 ^c	4.9±1.2 ^c
STZ + AME-treated	12.2±1.2	10.3±1.2 ^a	10.6±1.1 ^a	10.9±1.7 ^a	11.5±1.2 ^a	11.0±0.9 ^a	11.2±1.4 ^a
Pancreatic insulin content (μU/mg)							
STZ-treated	15.1±2.4	9.6±2.1	8.9±2.1 ^d	7.7±2.3 ^d	6.9±2.0 ^d	6.5±2.3 ^d	6.6±2.1 ^d
STZ + AME-treated	15.3±2.1	11.6±2.5 ^a	12.1±2.3 ^a	12.9±2.2 ^a	13.3±2.3 ^a	13.2±2.0 ^a	14.1±2.2 ^a

Values are expressed as means (±SEM) of 10 rats. ^a Insignificant difference ($p>0.05$) between treated groups and control. ^{b,c,d} Significant difference ($p<0.05$) between treatment and control groups.

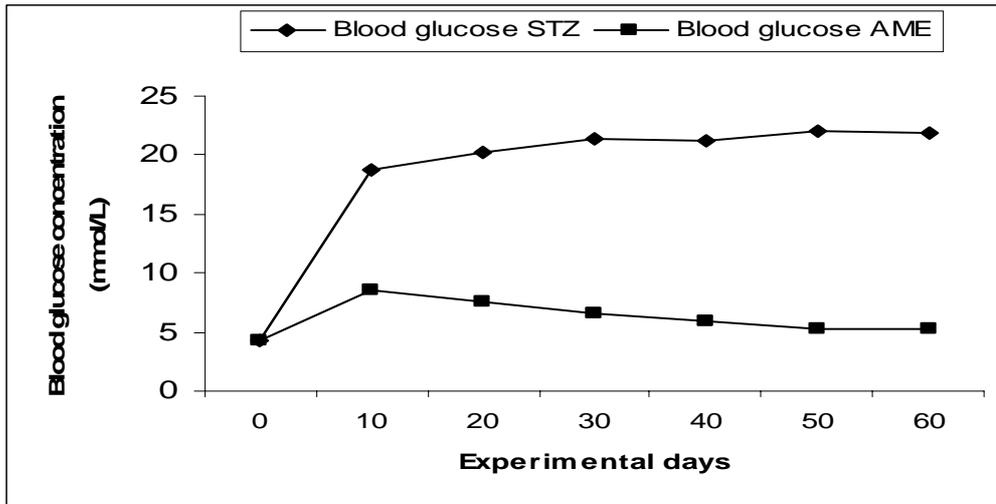


Fig. 1, Blood glucose concentrations in STZ-treated and STZ + AME-treated diabetic rats. The figure illustrates ameliorative effects of AME by restoring blood glucose to normal level.

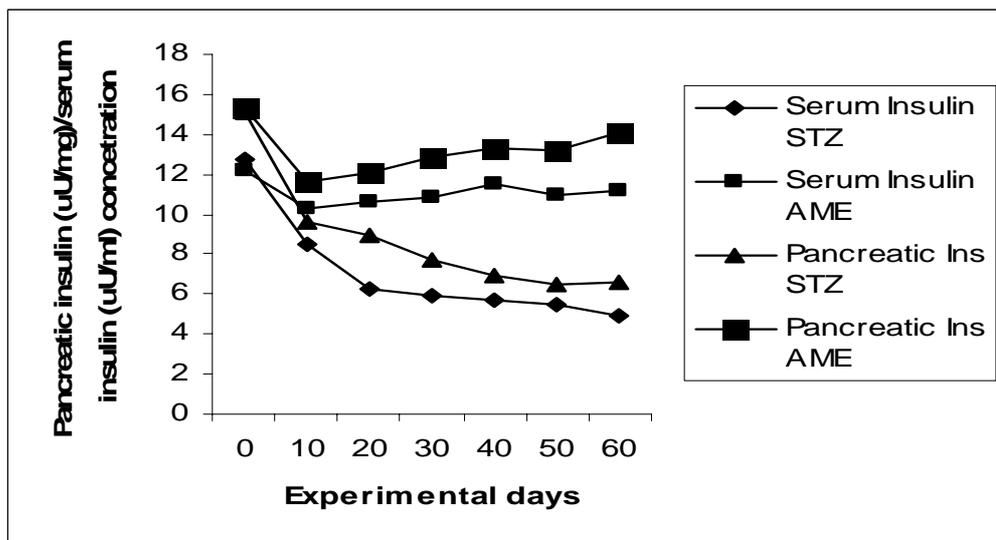


Fig. 2, Serum insulin and pancreatic insulin concentrations in STZ-treated and STZ + AME-treated diabetic rats. The figure illustrates protective effects of AME on the pancreatic tissues with subsequent significant increase in insulin content of endocrine pancreas and maintenance of normal serum insulin level.

Biochemical findings

Table 3 shows the effects of *A. muricata* on biochemical variables suggestive of oxidative stress in STZ-treated animals. There was clear evidence that STZ-induced pancreatic injury was associated with free radical injury and oxidative stress. Oxidative stress was characterized by increased lipid peroxidation and/or altered non-enzymatic and enzymatic antioxidant systems. Effects of STZ and STZ + AME treatments on pancreatic tissue’s ROS, GSH, SOD, GSH-Px and TBARS are presented in Table 3. The pancreatic antioxidant activity of GSH, SOD, GSH-Px and pancreatic insulin significantly decreased

($p < 0.05$), while pancreatic TBARS and ROS significantly increased in the STZ-treated, diabetic group of rats. The control group rats maintained optimal value activity of the antioxidants studied. *Annona muricata* treatment significantly ($p < 0.05$) decreased the elevated ROS and TBARS, but also significantly increased ($p < 0.05$) the reduced antioxidant enzyme activities. Furthermore, AME proved significantly better in restoring the altered activity of antioxidant enzymes like ROS, GSH, SOD, GSH-Px and TBARS, and insulin towards their normal values in the pancreas.

Serum total cholesterol, triglycerides, HDL and LDL cholesterol and (T-chol/HDL-chol) in control, STZ-treated and STZ + AME-treated rats are shown in Table 4. Serum total cholesterol, triglycerides, LDL cholesterol and (T-chol/HDL-chol) were significantly elevated ($p < 0.05$) in untreated diabetic rats as compared to control normal rats. Similarly, HDL cholesterol significantly reduced ($p < 0.05$) in untreated diabetic rats. All lipids parameters tested were improved towards normal values after the treatment with AME.

Table 3. Pancreatic tissue ROS (nmol min⁻¹mg⁻¹ protein), GSH (U/g protein) SOD (U/mg protein), GSH-Px (U/mg protein), TBARS (nmol/mg protein) and Insulin (μ U/mg protein) of all groups, A (control), B (STZ-treated) and C (STZ- +AME-treated) rats.

Parameters	Control	STZ-treated	STZ + AME-treated
Pancreatic ROS	0.13±0.1	0.29±0.4 ^b	0.14±0.5
Pancreatic GSH	7.22±1.3	3.82±1.2 ^b	6.6±1.2 ^a
Pancreatic SOD	24.9±1.4	13.7±1.3 ^b	25.7±1.1 ^a
Pancreatic GSH-Px	0.49±0.4	0.28±0.3 ^b	0.51±0.2 ^a
Pancreatic TBARS	79±15	148±17 ^b	85±14 ^a

Values are expressed as means (\pm SEM) of 10 rats for all groups. ^a Insignificant difference ($p > 0.05$) when compared with control groups. ^b Significant difference ($p < 0.05$) in the same row between treatment and control groups.

Table 4. Serum lipid profile in control, STZ-treated and STZ + AME-treated diabetic rats.

Experimental days	STZ-treated rats				STZ + AME-treated rats			
	HDL	LDL (mmol/l)	TC	TRIG (mmol/l)	HDL	LDL (mmol/l)	TC	TRIG (mmol/l)
0	0.88±1.4	1.02±0.3	2.5±1.9	1.3±0.2	0.84±0.2	1.01±0.2	2.4±1.8	1.2±0.3
10	0.72±1.3 ^a	1.11±0.2 ^a	2.7±1.8 ^a	1.9±0.3 ^a	0.89±0.4 ^a	1.00±0.4 ^a	2.3±1.9 ^a	0.9±0.2 ^a
20	0.64±1.4 ^a	1.70±0.5 ^c	3.3±1.5 ^d	2.1±0.5 ^a	0.92±0.3 ^a	0.92±0.4 ^a	2.3±1.5 ^a	0.9±0.4 ^a
30	0.59±1.3 ^b	1.91±0.4 ^c	3.6±1.7 ^d	2.3±0.4 ^c	1.05±0.4 ^b	0.68±0.1 ^c	2.1±1.3 ^a	0.8±0.3 ^c
40	0.58±1.1 ^b	2.01±0.2 ^c	3.7±1.6 ^d	2.5±0.3 ^e	1.13±0.1 ^b	0.65±0.4 ^c	2.1±1.6 ^a	0.7±0.1 ^c
50	0.52±1.2 ^b	1.96±0.3 ^c	3.7±1.8 ^d	2.9±0.5 ^e	1.24±0.5 ^b	0.44±0.2 ^c	2.0±1.5 ^a	0.7±0.4 ^c
60	0.51±1.3 ^b	2.12±0.4 ^c	3.9±1.9 ^d	2.9±0.2 ^e	1.22±0.2 ^b	0.42±0.3 ^c	1.9±1.7 ^a	0.6±0.2 ^c

Values are expressed as means (\pm SEM) of 10 rats for all groups. ^a Insignificant difference ($p > 0.05$) when compared with control groups. ^{b,c,d,e} Significant difference ($p < 0.05$) in the same column between AME treatment and control groups. Values for control group being the 0 day of experiment and T-chol/HDL-chol in STZ-treated rats was 5.57±1.5 when compared with control rats 2.14±1.7.

Histopathological findings

The histology of pancreatic islet cells was normal in control group (Fig. 3A). Histologically, in diabetic rats with no treatment, the most consistent findings in the sections of pancreatic tissues were breakdown of micro-anatomical features such as extensive β -cell degranulation, decreased cellular density, and an indistinct boarder between the endocrine and exocrine regions. Also there was a diffused degenerative and necrotic changes, and shrunken in the islet of Langerhans (Fig. 3B). The nucleus of necrotic cells showed either pyknosis or marginal hyperchromasia, and the cytoplasm were filled by hydropic changes (Fig. 3B). The α -cells, exocrine pancreatic acinar epithelium, ductal and connective tissues appeared normal (Fig. 3A and 3C). Even though pancreatic islets of diabetic rats were of low cellularity, there was no evidence of inflammatory cell infiltration (Fig. 3B). In diabetic rats treated with *A. muricata*, there was a remarkable improvement in the islet of Langerhans with distinct cellularity changes, majority of the cells showed viable islet of Langerhans, with increase in granulation (Fig. 3C).

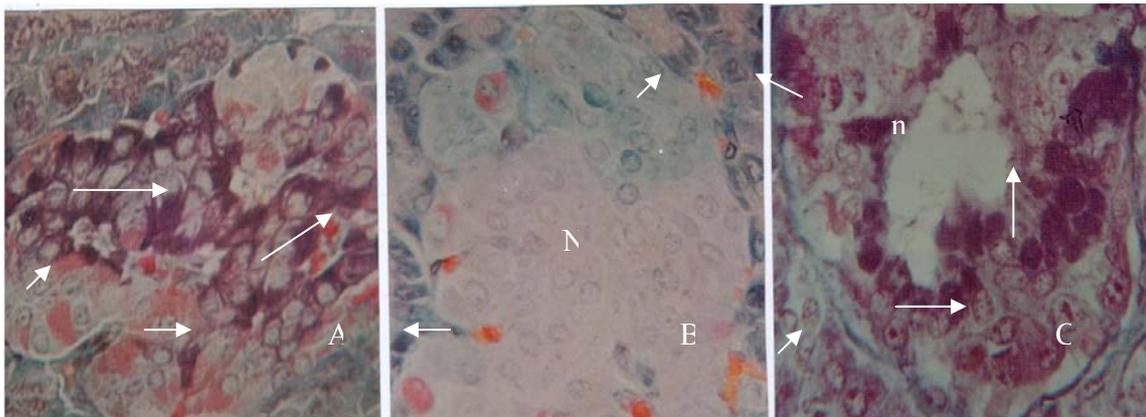


Figure 3. Aldehyde fuchsin trichrome staining of the pancreatic tissues. Arrows show β -cells with purple colour, while arrowheads show α -cells in orange colour. (A) Control group: showing normal cells in the islet of Langerhans and showing distinct granules filling the entire islet of Langerhans that are strongly stained purple, α -cells are seen mostly at the periphery. (B) Diabetic untreated group: shrunken islet of Langerhans displaying degenerative and necrotic changes (N), nuclear shrinkage and pyknosis were evident with cytoplasmic vesiculation in the centre of the islet of Langerhans, and decreased cellular density. (C) STZ+AME-treated: still retained residual necrotic area (n) and enhanced amount of β -cells with moderate staining granules, and distinct endocrine and exocrine boarder, α -cell still present within the islet of Langerhans X 160.

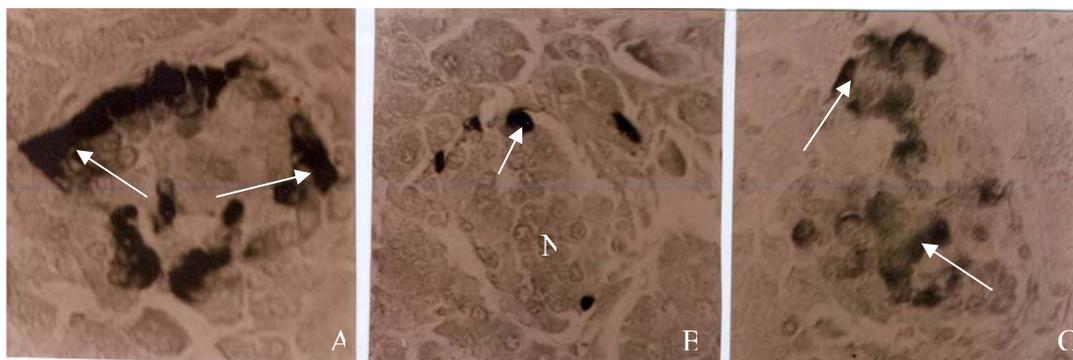


Figure 4. Pancreatic sections immunostained for insulin using a peroxidase indirect labeling technique, represented by dark granules. (A) Control group: showing normal β -cells in the islet of Langerhans that are strong staining with the anti-insulin antibody. Immunoperoxidase, haematoxylin counterstain. (B) Diabetic untreated group: showing shrunken degranulated necrotic (N) islet with weak immunoreactive β -cells in the islet of Langerhans. Immunoperoxidase, haematoxylin counterstain. (C) STZ-AME treated: still retained considerable amount of β -cells with remarkable anti-insulin antibody staining granules. Immunoperoxidase haematoxylin counterstain X 160.

Immunohistochemical findings

Immunohistochemical staining of the pancreatic tissues in control group showed the presence of strong islet insulin immunoreactivity at a level of 0.61 islet/mm^2 of total pancreatic tissue. This was limited to cytoplasmic staining of individual β -cells (Fig. 4A). The majority of islets from AME-treated pancreases stained positive for insulin suggesting that the architecture of AME-treated rats was normal (Fig. 4C). In contrast, islet cells from diabetic rats were architecturally distorted, containing significantly fewer insulin-positive cells (Fig. 4B). Quantitative image analysis was used to assess the proportion of insulin positive cells per islet in control, STZ-treated and STZ + AME-treated pancreatic sections. The percentage of islets stained for insulin were $72.21 \pm 39\%$, $36.25 \pm 23\%$ and $64.16 \pm 17\%$ ($p < 0.05$) respectively (Fig. 4A, 4B and 4C). Together, these findings further support that AME protected islets from destruction. Of the most interesting is the stability of pancreatic insulin content of the AME-treated rats ($14.1 \pm 2.2 \mu\text{U/mg}$), in contrast with the STZ-treated rats, pancreatic insulin contents were ~ 65 -fold higher (Table 2). This stability in pancreatic insulin values was also observed to reflect in the immunohistochemical staining (Fig. 4C). Immunohistochemical staining intensity was an evidence of protective effects/or regenerative processes leading to increase in β -cell mass, regaining its normal immunostaining for insulin and functional status up to the day of normoglycemia.

Discussion

Diabetes mellitus is probably the fastest growing metabolic disease in the world and as knowledge of the multifactorial/heterogenous nature of the disease increases so does the need for more challenging and appropriate therapies. Traditional plant remedies have been used for centuries in the treatment of diabetes [47], but only a few have been

scientifically evaluated. Therefore, we investigated the effect of aqueous extracts from *Annona muricata* (Linn.) (family, Annonaceae) on lipids profile, in serum, and antioxidant biochemical variables in pancreatic tissue of experimental diabetic rats. Chronic hyperglycemia during diabetes leads to oxidative stress [48], and protein glycation produces free radicals [49]. Along with hyperglycemia and abnormalities in serum lipids [50; 51] diabetes is usually associated with microvascular and macrovascular complications which are the major causes of morbidity and death in diabetic suspects [52]. It can be managed by exercise, diet and pharmaceutical drugs, which are either too expensive or have undesirable side effects or contraindications [53]. The research for more effective and safer hypoglycemic agents therefore has continued to be an area of research of interest [54; 55]. The World Health Organization has recommended and encourages the use of alternative therapy especially in countries where access to the conventional treatment of diabetes is not adequate [56].

The possible sources of oxidative stress in the pathogenesis of diabetes and diabetic complications have been extensively studied for years based on in animal models and in human being. Diabetes mellitus in man and in experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense system and thus promotes *de novo* free radicals generation [57]. Numerous studies have found increased peroxides or ROS and oxidative stress (or both) in different animal models of diabetes [58]. Oxidative stress has recently been shown to be responsible, at least in part, for the β -cell dysfunction caused by glucose toxicity. Under hyperglycemia, production of various reducing sugars such as glucose-6-phosphate and fructose increases through glycolysis and the polyol pathway. During this process, ROS are produced and cause tissue damage [59]. *In vitro* and *in vivo* studies have suggested the implication of oxidative stress in the progression of β -cell dysfunction in type 2 diabetes [60].

An observation in this study correlates with the previous research findings, in that the blood glucose levels significantly increased in STZ untreated diabetic rats [61]. In the present study, the continuous treatment of STZ-diabetic rats with AME for a period of 4 weeks caused a significant decrease in blood glucose levels of treated diabetic rats. The possible mechanism by which aqueous extract of *A. muricata* brings about its hypoglycemic action may be, by potentiating the insulin effect, either by increasing the pancreatic secretion of insulin from the cells of islet of Langerhans or its release from bound insulin [62]. Furthermore we noticed elevated serum lipids in STZ-diabetic rats, diabetes is marked by characteristic alterations in lipoprotein levels, including an elevation of triglycerides and very low density lipoprotein (VLDL) and a decreased HDL concentration [63]. This observation is important because of its association with increased risk of developing cardiovascular diseases. Diabetes-induced hyperlipidemia is attributable to excess mobilization of fat from the adipose due to underutilization of glucose [64]. AME treatment decrease serum total cholesterol, triglyceride and LDL level up to 2.6, 2.4, 2.0-folds respectively and increase HDL level up to 2.2 fold. This preventive /protective effect of AME may be due to inhibition of lipid peroxidation. Our result is consistent with the result of Wolf [65] and El-missiry, [66] who indicated and increase in lipid peroxides and a decrease in antioxidant enzymes in DM.

In the present study, we evaluate possible protective effects of AME against β -cell damage. The current immunohistochemical and histological examination shows that

pancreatic β -cells are destroyed by STZ displaying extensive necrosis, degranulation and hydropic changes. Also, almost all insulin-positive β -cells were degenerated in the STZ-treated rats leading to poor insulin immunoreactive β -cells. AME treatment partially prevents degeneration of β -cells and increased the area of insulin immunoreactive β -cells significantly. Also, we examined the plausible protective and preventive effect of AME in diabetes by decreasing oxidative stress and preservation of β -cell integrity. The result of present study showed an increase in the level of MDA, ROS and a decrease in GSH, SOD and GSH-Px content of the pancreatic tissue of STZ-treated rats which are consistent with those results of Kaneto *et al.*, [60]. AME treatment decreased the elevated MDA, ROS and also increased the reduced antioxidant enzyme activities.

The data obtained in the present study do not allow definite conclusion to be drawn on the mechanisms of action of AME in the experimental animal paradigms used. However, a number of investigators have shown that tannins and other polyphenolic compounds (e.g., coumarins), flavonoids, triterpenoids, saponins, and a host of other plant secondary metabolites possess hypoglycemic, hypotensive, anti-inflammatory, and other pharmacological properties in various experimental animal models [67; 68; 69]. *A. muricata* is known to contain ellagic acid, tannins, flavonoids, polyphenolic compounds, β -sistosterol, and catechins [70; 71; 72; 73]; therefore it is not unreasonable to speculate that some of these chemical compounds especially the coumarins and flavonoids are probably responsible for recovery in the altered biochemical variables and pancreatic tissues.

Based on our findings, we conclude that STZ administration is associated with oxidative stress in pancreatic tissues. Nevertheless, *A. muricata* exhibited antioxidant activity, and is able to diminish and/or prevent, pancreatic oxidative damage produced by STZ. An increased consumption of antioxidants in the diet of individuals is strongly recommended, so that when an individual is subjected to greater oxidative stress, he/she would have better antioxidant defense characteristics, thus counteracting the effects of any pro-oxidant. However, further studies are needed before antioxidants can be used safely as food additives and supplements.

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