Journal of Anatomical Sciences

Email:anatomicaljournal@gmail.com

J Anat Sci 13 (1)

The Therapeutic Role of the Aqueous Extract of *Anonna muricata* Leaf (AMLE) on the Persistent Hyperglycemia in Alloxan- Treated Wistar Rats.

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ABSTRACT

Persistent hyperglycemia is a key feature of Diabetes (DM). Eventhough there are numerous orthodox treatment options in circulation, alternative treatment options that are efficacious and safe are continuously sought. The aim of this study was to ascertain the effect of *Anonna Muricata* leaf extract (AMLE) in treating persistent hyperglycemia in Wistar rats. Thirty six rats (150–200g) were randomly divided into six groups (A, B, C, D, E and F). Group A (negative control) was treated with saline-only, group B (positive control), group C, D, E and F were all treated with single dose of Alloxan (125mg/kg body weight intraperitoneally). After confirming the persistent hyperglycemia in all the Alloxan treated groups using glucometer, animals from group C, D and E were then treated with 50, 100 and 200 mg/kg body weight of AMLE. While those from group F were treated with metformin at 200mg/kg. Blood sugar was also measured at the end of the experiment, on the 21st day. The result suggested that the AMLE had a lowering effect on hyperglycemia to the same extent with the metformin. The histopathological examination revealed a distortion of the islet cell mass as well as the exocrine tissue in the Alloxan-only treated groups. A dose-dependent, progressively increasing similarity in the section from the animals treated with AMLE was observed when compared to the sections from the control. Thus, the AMLE has a therapeutic role in lowering of glucose level and reversal of pathologic effect of Alloxan in the pancreatic tissue of Wistar.

Keywords: Annona muricata extract, Aqueous; Diabetes, Alloxan, metformin.

INTRODUCTION

The pancreas is a soft, pale, and finely lobulated grey gland that lie transversely across the posterior abdominal wall[1]. Exocrine and endocrine pancreas produce pancreatic juice containing enzymes such as trypsin and chymotrypsin as well as hormones such as insulin and glucagon that play vital role in the metabolism of carbohydrates, proteins, and fats[2]. Beta (β) cell mass of the pancrease are a very dynamic set of cells with significant capacity for adaptation to changes in insulin demand[3]. These changes have been reportedly associated with β-cell replication, increased β-cell size, decreased β-cell death, and differentiation of β-cell progenitors (neogenesis)[4].

Alloxan is a drug with diabetogenic activity when administered parenterally, (i.e., intravenously, intra-peritoneally or subcutaneously) in rodents. It is selectively toxic to insulin-producing pancreatic beta cells following its uptake via the GLUT2 glucose transporter[5,6]. There is an Alloxan-sulphur group that allows selective inhibition of glucose-induced insulin secretion through inhibition of glucokinase and also selectively kills the pancreatic insulin-producing beta-cell[7]. Diabetes mellitus (DM) is a chronic disorder that is characterised by hyperglycemea mostly due to abnormalities in glucose metabolism including the inhibition of glucose-induced insulin release[8]. It is a very common endocrine disease accompanied with various metabolic disorders including disturbances of carbohydrate, fat, protein metabolism and complications like atherosclerosis, retinopathy, neuropathy and nephropathy[8,9,10]. It is poised to affect the developing countries of the world much more than their developed counterparts[11].

For various reasons in recent years, the popularity of alternative medicine has increased. Surveys conducted in Australia and United States indicate that almost 48.5% and 34% of the respondents had used at least one form of unconventional therapy, including herbal medicine[12]. Globally, there is a trend for the evaluation of effective plants constituents for management of many health conditions for which modern drugs are either absent, expensive or have poor safety[13]. This has led to the increasing demand for readily available and relatively cheap herbal products with anti-diabetic activity[14].

Annona muricata L. is a species of the Annonaceae family known for its edible fruit and therapeutic potential for diseases including diabetes and cancer[15,16].

Its methanolic extract had been reported to have anti-diabetogenic effect in earlier studies, however, the long standing African tradition of making aqueous herbal extract for management of medical condition can be harnessed for its acceptability, availability and cheap nature in the management of diabetes rather than relying on the ethanolic processing. This study is aimed at evaluating the persistent-hyperglycemia lowering effect (anti-diabetogenic features) of the aqueous extract of Annona muricata L in relation to standard anti-diabetes drugs (Metformin) in Alloxan-diabetes models of adult Wistar rats. The outcome of the current study may strengthen the role of the advocates for the extract (AMLE), and may provide the much needed scientific evidence for its therapeutic effectiveness in both reversing the persistent-hyperglycemia of Alloxan-treated Wistar rats with its associated pancreatic β- cells dysfunction[17].

MATERIALS AND METHODS

Ethical approval: Ethical approval was sort and obtained from the ethical committee of ESUCOM to carry out the research.

Study Protocols: The entire study protocol was presented to the departmental board of anatomy department of the Enugu State College of Medicine, Parklane and it was reviewed and accepted.

Experimental Animals and Study Groupings: Twenty-four (24) Wistar rats (*Rattus norvegicus*), weighing 150–200g obtained from the department of Pharmacology and Toxicology, University of Nigeria, Nsukka were used for this study. They were housed in standard cages in the animal house of the department of Anatomy, Enugu State College of Medicine, Parklane under standard conditions (including the cycle for light and darkness). The animals were given free access to food (standard rat pellets) and drinking water. The rats were randomly divided into seven experimental groups

Table 1: Summary of Animal Grouping

GROUPS	RATS PER CAGE	INDUCING AGENT/ EXTRACT	DOSAGE
Group A (negative control)	4	Normal saline	0.9%
Group B (positive control)	4	Alloxan	125mg/kg body weight
Group C	4	Alloxan (125 mg/kg/body weight) +AMLE extract (low dose)	50mg/kg
Group D (negative control)	4	Alloxan (125 mg/kg/body weight) + AMLE extract 2 (medium dose)	100mg/kg
Group E (protective group)	4	Alloxan (125 mg/kg/body weight) +AMLE extract 3 (high dose)	200mg/kg
Group F	4	Alloxan + metformin (standard drug)	200mg/kg

Preparation of the Aqueous Leaf Extract of *Annona muricata* Plant: Fresh leaves of *Annona muricata* (Linn.) (Family: Annonaceae) were collected in Awgu local Government, Enugu state Nigeria between January - July 2018. The leaves were identified by the student researchers and the memebers of the supervisory-academic staff in the department of Anatomy-Enugu State College of Medicine, Parklane. The seeds were also authenticated and a voucher number of BUKHAN 0341 was obtained from herbarium unit in the Department of plant biology, Bayero University, Kano

The leaves were air-dried at room temperature and one kilogram (1 kg) of the air-dried leaves was milled into fine powder in a blender. The powdered leaf was macerated in distilled water and extracted twice with 2.5 1itre of distilled water at room temperature for 48hrs (with occasional shaking) on each occasion. The aqueous extracts was concentrated and eventually dried at 60±1°C in a rotary evaporator. The resulting aqueous extract was freeze-dried with a final yield of 36.23 g (3.62% yields) of a light green, powdery crude aqueous leaf extract of *A. muricata* (AMLE). This crude aqueous extract was stored in aliquot and refrigerated for use in this study as the need arises.

Experimental Procedure: The Diabetes mellitus model was developed as in an earlier study[18]. It involved a single intraperitoneal injection of 125 mg/kg/body weight of Alloxan that was freshly dissolved in 0.9% saline. The diabetic state was confirmed by measuring basal blood glucose concentration (≥ 200 mg/dl) after an 8 hour food fasting period (water was freely available during this 8 hours fasting) 72 hours after the Alloxan injection. The blood sample was obtained from the tail vein of the animals and their fasting blood glucose determined in mg/dl using a digital glucometer (Accu-check®). The administration of AMLE as well as the comparator standard drugs (metformin and glibenclamide) to the different animals in the experimental groups was commenced from the 7th study day for an additional 21 days.

The animals were sacrificed by cervical dislocation and the pancreas of each of the animals was immediately dissected out, fixed in 10% phosphate buffered formalin for a minimum of 48 hours and prepared for

sectioning. The sections were cut as 5µm thick sections using a rotary microtome and the cut sections were prepared for Hematoxylin and Eosin stainingafter mountanting with DPX.

The data for blood glucose levels obtained in this study were expressed as means (± SD) and analyzed using one-way analysis of variance (ANOVA) in the statistical package for social sciences SPSS version 20.0 and the values of $p<0.05$ were taken to be statistical significance.

RESULTS

Weight of study animals: The animals used in this study were weighted intermittently according to their groups (group A-negative control; group B - Alloxan-only treatment; group C- E alloxan treatment followed by AMLE treatment at 50, 100 and 200 mg/kg body weight and group F- alloxan treatment followed by 200 mg/kg body weight of metformin). The weighing was done at baseline, 7th day and at the end of the experiment (day 21)) with the results presented in Figure 1 and 2.

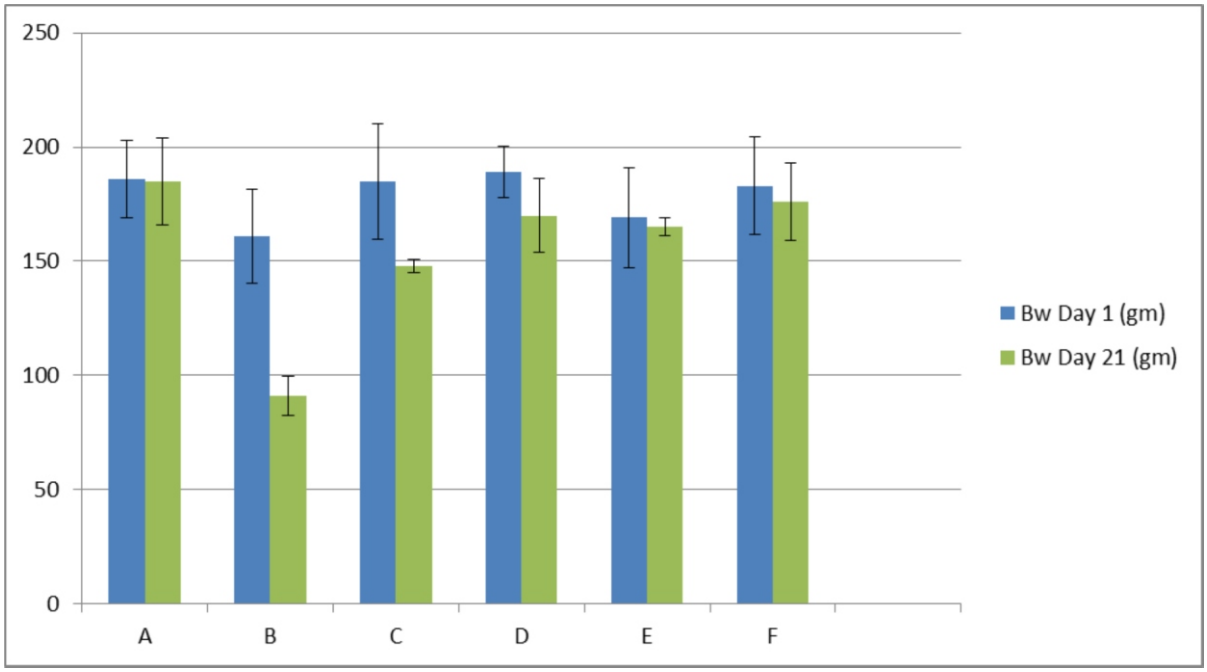


Figure 1: A summary of the weight of Wistar rats at baseline and at the end of the hyperglycemia and AMLE therapeutic effect experiment (Paired T test comparison for group A, P= 0.97; group B, P= 0.003; group C: P= 0.104; group D: P=0.121; group E: P=0.638; group F: P=0.504)

The weight of the study animals were essentially similar at base line (day 1) and this pattern was maintained throughout the study for the animals in the control group (group A). For the animals in group C-F that were receiving the AMLE treatment, the weight appears to be unperturbed at the end of the study (day 21) (all paired t test had $P > 0.05$). Those animals in group B had a statistically significant decrease in weight ($P = 0.003$; df= 3; $T = 8.650$) at the end of the study compared to the baseline weight (Figure 1). It is pertinent to note that the by the 7th day of the study, the weight records for the animals in group B-F were similar and smaller compared to heavier weights of the animals in the control group (A) and these differences in weight were statistically significant (ANOVA; $F(df=5, 17) = 7.90$, $P = 0.001$). Figure 2

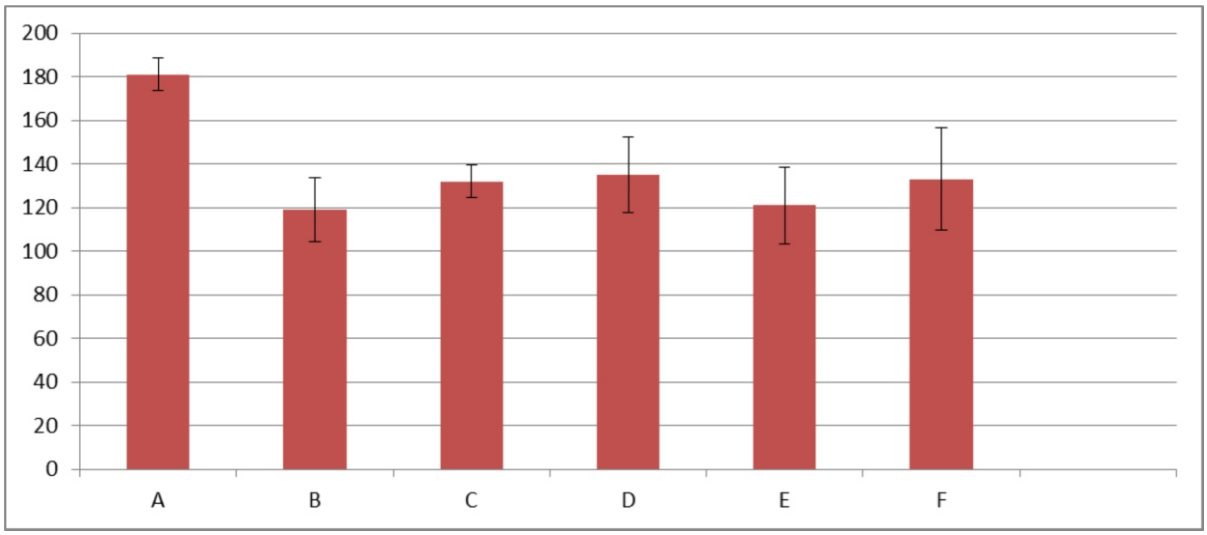


Figure 2: A summary of the weight of Wistar rats at seventh day after treatment with alloxan (B-F) or water (A) with one way analysis of variance (ANOVA; F (df=5, 17) = 7.90, P =0.001) and LSD posthoc test suggesting a significant difference in weight for all groups (B-F) when compared to group A

Alloxan-Hyperglycemia (Diabetic) models: A single preliminary evaluation of the glucose levels among all the groups of animals that received alloxan just before treatment with extract and/or standard anti-diabetic medication (metformin) (group C, D, E, F) was observd to be similar to the high glucose levels obtained for the alloxan-only treatment group (group B) Figure 3

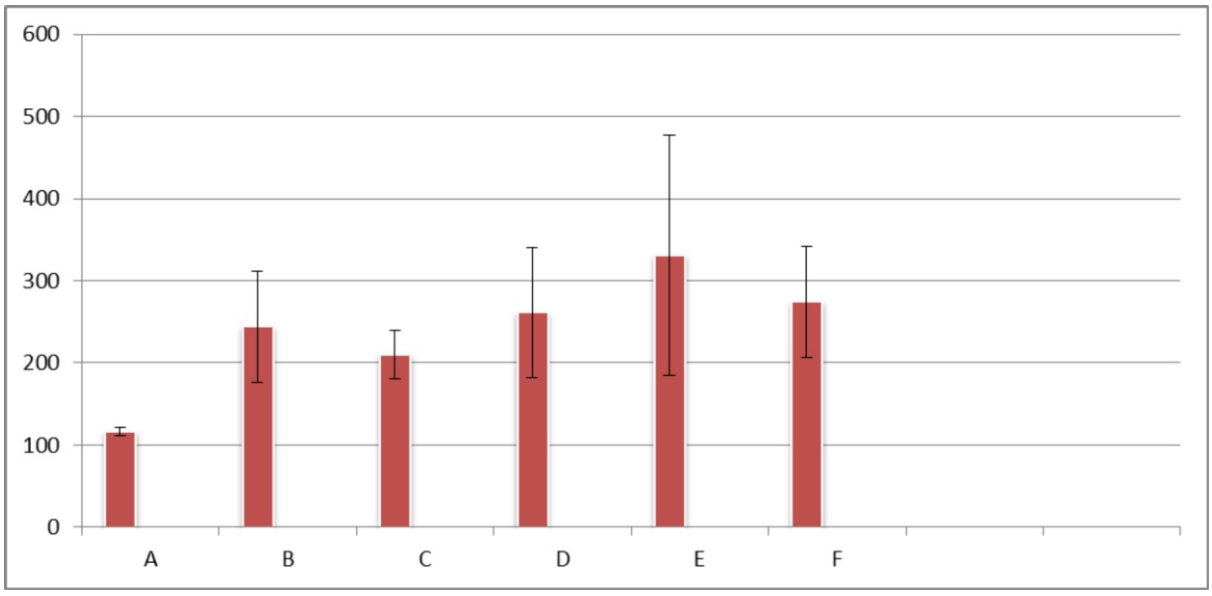


Figure 3: Single glucose levels measurements on the seventh day after Alloxan treatment- Diabetes model (in group B-F) or distilled water treatment-control model (group A). The one way analysis of variance (ANOVA) evaluation was statistically significant (F (5, 18) = 3.34; P = 0.026) and the LSD post hoc analysis revealed that the statistically significant difference was between animals in the control group (A) with all the other study groups (B,D,E, F) except those in group C

Thus, the hyperglycemia was observed for the entire groups (B-F) that received alloxan at the start of the study, while the animals that received only placebo (distilled water) had a normal glucose level. To furtther emphasize the resuls for alloxan related diabetic model obtained in this study, the result for the test of glucose levels at baseline as well as at the end of the 21 days of experiment were singled out for closer assessment. Expectedly, the glucose levels were below the diabetic-hyperglycemia levels in the entire study animals excepts those in the alloxan-only treatment group (group B) at the end of the 21 days study duration. Figure 4**.**

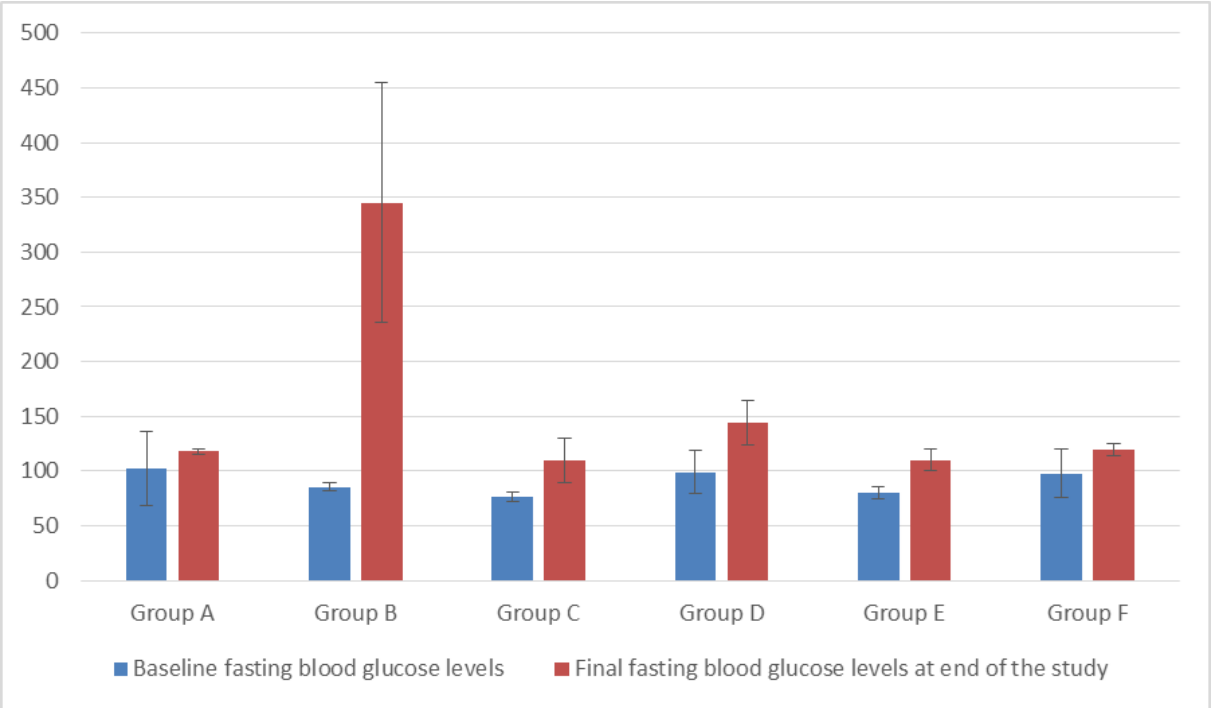
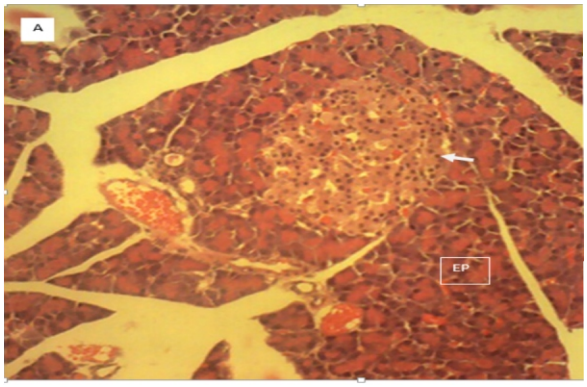
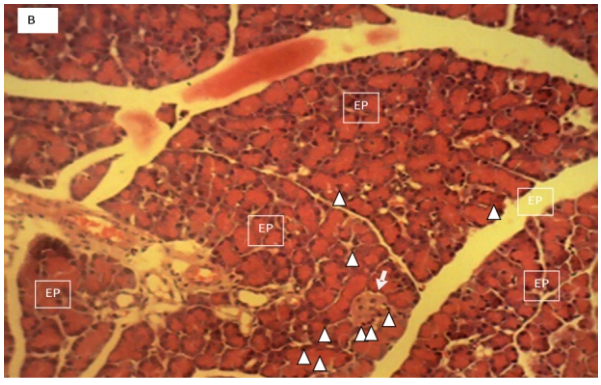


Figure 4: Comparison of the change in fasting glucose levels at onset and end of the study for the effect of distilled water only (A), alloxan-treatment only(B) and /or with extract of AMLE/metformin (C-F) among Wistar rats. (Paired T test comparison for group A, P= 0.439; group B, P= 0.019; group C: P= 0.025; group D: P=0.072; group E: P=0.004; group F: P=0.182) Thus, it is only the animals in the alloxan-only treated group that had a glucose measurement value that is in keeping with hyperglycemia. The other animals in groups that were treated with alloxan as well as either an extract of AMLE or standard anti-diabetic medication (metformin) had a glucose measurement similar to the values measured at baseline and characterized as non-diabetic value.

Histopathological Result: The photomicrographs from the prepared slides of the pancreatic tissue obtainable from the study animals in the different experimental groups were all captured using the Motic™ compound light microscope. These photomicrograph were represented by the photomicrgraph presented in Plate 1 (A-F).





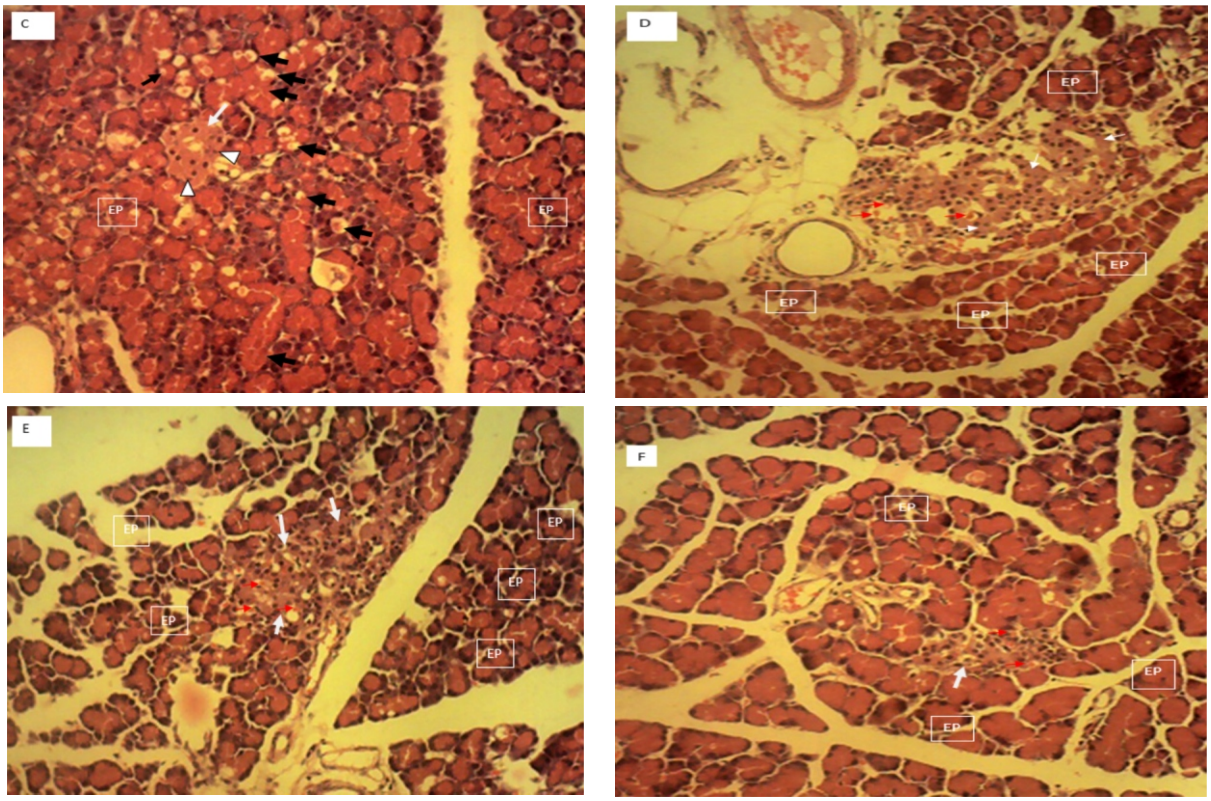


Plate 1: Representative photomicrograph sections from the pancreas of animals in the (A) control group (0.9% saline for 14 days) with preserved pancreatic islets represented as white arrow and exocrine pancreas (labelled as EP); (B) Diabetic model (DM) (single 125mg/kg body weight of Alloxan intraperitoneally) with depletion of pancreatic islets (arrow), congestion (C) and exocrine pancrease (EP) cells as well as pyknosis (white arrow head); (C) DM treated with 50mg/kg of AMLE extract for fourteen days features as in (B) with vacoulation (black arrow); (D) DM treated with 100mg/kg of AMLE extract for fourteen days showing features as in (A) with prominent features of vasculature (very small red arrow); (E) DM treated with 200mg/kg of AMLE extract for fourteen days showing features as in (A) with prominent vasculature (very small red arrow); (F) DM treated with 200mg/kg of metformin for fourteen days showing features as in (A) with prominent vasculature (very small red arrow) (H&E x400).The histoarchitectural features observed across the different tissue sections included evidence of normal pancreatic tissues as in group A, D,E and F) as well as features of distorted pancreatic tissue architecture (group B).

DISCUSSION

The weight changes observed in the current study were mainly a decline in weight following treatment with alloxan and the weight decline was reversed following treatment with AMLE extract or standard anti-diabetic treatment for 2 weeks.

The histo-pathological results in the current study suggest that alloxan treatment played a key role in promoting cellular damage among both the islets cell and the exocrine cell populations which was reversed in a dose-dependent manner with AMLE or metformin treatment. The cellular damage included vascular congestion, features of pyknosis and vacoulation as well as reduction in the size and population of islets cells. The assessment of the pancreatic function of glucose regulation via determination of fasting sugar level corroborated the alloxan associated cellular damage seen on the slides from animals treated with alloxan only. The glucose levels remained persistently high (in the diabetic range) throughout the study in the alloxan-only treated animals. The decline in fasting sugar levels among animals that received AMLE extract or metformin after alloxan treatment suggested that the extract as well as metformin had anti-diabetic effects.

The persistently high levels of fasting blood glucose levels identified in the current study following alloxan injection is in keeping with the high levels of fasting blood glucose levels commonly encountered in diabetics which is associated with absolute or relative deficiency in insulin secretion or insulin action[9,10]. Clinical features of hyperglycemia were associated with the loss of free water and electrolytes in the urine which is commonly described as polydipsia. The polydipsia related depletion of free water, glycogen and triglycerides was characterized with weight loss. Another basis for weight loss was from the associated reduction in muscle mass from diversion of amino acid

into formation of glucose and ketone bodies[19]. Thus, reversal of the polydipsia with its associated depletion of free water, glycogen as well as triglycerides will reverse the weight loss associated with diabetic-hyperglycemia. This literature report was in keeping with the result from the current study, which was characterized with weight loss in the Alloxan-only treatment group, while the introduction of the AMLE extract or metformin arrested the trend of weight loss and ultimately reversed it towards pre- study levels.

Some earlier studies had corroborated the current histo-morphological findings associated with Alloxan exposure in Wistar rats and these studies also reported that the size and number of pancreatic islets were decreased[20,21,22,23]. The studies also reported features of necrosis and atrophy of β-cells of islets of Langerhans and exocrine tissue in alloxan exposure model of diabetes[20,23]. There was no dose dependent damage to the pancreatic tissues following addition of AMLE extract to alloxan treated animals, rather, there was a reversal and normalization of both the hyperglycemia and pancreatic tissue damage associated with alloxan treatment. The normalization of the fasting glucose level was regardless of the dose of AMLE used while the normalization of the tissue section was dose dependent. It had earlier been reported that AMLE is safe and has a very large LD50 and thus this may account for its safety in the current study[24].

The mechanisms through which several extracts including AMLE exert their anti-diabetic effects are not completely known. However, for obtaining similar hypoglycemic results with the standard anti-diabetic therapies such as gliclazide and metformin, it is putatively correct to suggest that some of the mechanisms of action for the extracts may be similar to those of the standard treatments. Stimulating insulin secretion by blocking $K^{\pm}$channels in the pancreatic β cells and increasing tissue uptake of glucose are some of the pancreatic and extrapancreatic approaches for sugar lowering activity of standard therapies[25,26,27]. Additional activities that have been attributed to extracts included reduction in the absorption of glucose from the intestine and inhibition of the absorption of glucose by the kidney tubules[28]. Some other studies revealed that treatment with plant extract was associated with a compensatory regeneration of damaged islets cells with associated increased size and activities[25,29]. In the current study, the precise mechanism via which AMLE extract mediates it anti diabetic activity were not explored, however, the mechanisms may be in the realms of mechanisms already highlighted in the literature or completely novel one. However, the current finding of hypoglycemic effect observed with the 3 doses of AMLE studied with a dose-dependent difference in the normalization of the histo-pathological features of pancreatic tissue is in keeping with the literature reports for extracts promoting the regeneration of damaged islet cells. Thus, the AMLE extract putatively promote a dose dependent regeneration of the islet cell mass.

CONCLUSION

In conclusion, the aqueous extract of *Anonna muricata* leaf has serum glucose lowering effect as well as regeneration of islets cells in a dose dependent manner among Wistar rats following Alloxan-induced persistent hyperglycemia.

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