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Evaluation of the Ameliorative Property of *Vernonia amygdalina* Fractions in Streptozotocin Induced Diabetes

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Authors' contributions

This work was carried out in collaboration between all authors. Authors EAO and ECA designed the study, wrote the protocol and interpreted the data. Authors MEA and AO anchored the field study. Author CUO gathered the initial data and performed preliminary data analysis. While authors EAO and CUO managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

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ABSTRACT

Medicinal plants play a major role in the management of Diabetes mellitus especially in developing countries.
Aims: The present study examines the bioactive constituents of *Vernonia amygdalina* and the effect of extract and fractions of *Vernonia amygdalina* on some biochemical indicators and antioxidant level in Diabetes mellitus management in Wistar rats.

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Study Design: Multi-arm pre-post interventional study.
Place and Duration of Study: Department of Physiology, faculty of basic medical sciences, Delta state university, Abraka. The study lasted for 28 days.
Methodology: The fresh *Vernonia* leaves were air-dried, crushed and soaked in ethanol for 48 hours after which the ethanolic extract was sieved out and allowed to dry. The resultant ethanol-free juice was subjected to liquid-liquid fractionation using solvents of varying polarity. The rats were divided into 7 groups (n=5) as follows, non-diabetic group, diabetic groups (crude, chloroform, ethyl acetate, Benzene and Butanol) received 300 mg/kg *Vernonia amygdalina* extract and the Metformin group received 50 mg/kg metformin.
Results: Administration of Crude ethanolic leaf extracts of *Vernonia amygdalina* at dose of 300 mg/kg/day produce maximum fall (81.45%) in the fasting glucose level in diabetic rats and decreased aspartate aminotransferase (11%), alanine aminotransferase (82), alkaline phosphatase (21%), Bilirubin levels and triglycerides and low density lipoprptein-cholesterol (17%), while increasing the levels of serum proteins (69%) in the diabetic Wistar rats after 28 days of treatment. Administration of the fractions at 300 mg/kg/day produce a decrease in the fasting glucose level in diabetic rats, Chloroform (65.85%), ethyl acetete (69.65%), Benzene (45.59%) and Butanol (37.31%) respectively and decreased aspartate aminotransferase (11-66%), alanine aminotransferase (77-86%), alkaline phosphatase (60.75%), Bilirubin levels in the diabetic Wistar rats after 28 days of treatment.
Conclusion: The finding showed that *Vernonia amygdalina* extract and fractions reduced the hyperglycaemia induced by streptozotocin in Wistar rats. It possesses significant antioxidant properties and ameliorated the toxic effect of Diabetes mellitus as evident by its effect of biochemical indicators. It also contains bioactive compounds with potent antidiabetic properties.

Keywords: Diabetes; streptozotocin; metformin; Vernonia amygdalina; liver enzymes.

1. INTRODUCTION

Diabetes mellitus is grossly reflected by profound changes in protein metabolism and by a negative nitrogen balance and loss of nitrogen from most organs. Increased urea nitrogen production in the disease may be accounted for by enhanced catabolism of both muscle and plasma proteins leading to elevated serum creatinine and urea levels. Management of Diabetes mellitus without any side effects is still a challenge to the medical system. There is an increasing demand by patients to use natural products with antidiabetic activity, because insulin and oral hypoglycaemic drugs have undesirable side effects [1].

The complications of diabetes are linked to oxidative stress induced by hyperglycemia which overcomes the body's natural anti-oxidant system [2]. In the later stages of diabetes, lipid metabolism is affected and seen as hyperlipidema and hypercholesterolemia which are risk factors in artherosclerosis [3]. There is also possibility of liver damage in diabetes due to increased gluconeogenesis and ketogenesis. The hyperglycemia of diabetes has been attributed to pancreatic islet cell damage, resulting in lack of insulin production or when insulin is still being produced, resistance to it at the tissue membrane receptors. Streptozotocin has been shown to be cyto-toxic to the pancreas, selectively destroying pancreatic beta cells in mammals [4] within a period of 72 hrs [5] this was quite evident in the course of this study.

Medicinal plants are widely used in management of diseases all over the world [6,7]. One medicinal plant that has continued to receive a lot of attention due to the numerous curative potentials that it has demonstrated is *Vernonia amygdalina* commonly referred to as bitter leaf which is purported to possess antioxidant activity from radical scavenging [8]. Ingestion of crude *Vernonia amygdalina* and raw chewing by healthy human subjects were found to control post-prandial blood glucose without inducing severe hypoglycaemia [9].

The present study was performed to assess the antidiabetic properties of the leave extract and fractions of *Vernonia amygdalina* on biochemical enzymes in streptozotocin-induced diabetic Wistar rats.

2. MATERIALS AND METHODS

2.1 Chemicals

Streptozotocin (STZ) (Batch 1378), Address: Sigma. Aldrich Co.3050 Spruce Street St. Louis Mo 63103 U.S.A 314-771-5765, Acetic Acid

(Batch – 70419322), H_2SO_4 SL 704l419322, E. Merk, Darmstadt 2.5l, Weigh: 1.05lg, Ethyl Acetate (1502 batch 1356o517) Gato Perez, 33-P.1. Masden Gsa 08181 sentmenat Spam. Shelf Life 5/2017, Hydrochloric acid Gunsgdong Guanghua Chemical Factory Shantou Guondghuo China, Mgcl (Production: Moo6112), Batch No: 5h160911, N-Butanol, Guangdong Guanghua Sci-Tech Co., Ltd. Shatou, Guangdong, China, 515000, Benzene (Batch No. 704 L419322). Gato Perez, 33-P.1. Masden Gsa 08181sentmenat Spam. Shelf Life 5/2017, Liver Enzymes [ALT, AST, ALP] ALT= Batch-295830, ALP= Batch-213577. Randox Laboratory Ltd, 55 Diamond Road Crumlin Country Antrim United Kingdom. Expiring Date 2016 -02, LDH (Batch - 43930). Chloroform Plot No. D-22 Tarapur Midc, Boisar. Dist Thane 401 506. Metformin; Merck Sante s.a.s, 2 rue de pressoir vert 45400 Semoy, France.

2.2 Methods

2.2.1 Source of *Vernonia amygdalina*

Fresh leaves of *Vernonia amygdalina* were collected at the staff quarters, located at Site III, Delta State University Abraka, Delta State, Nigeria and was authenticated by Mr. Adeniji A. Kehinde of the Forestry Research Institute of Nigeria, Ibadan, with herbarium number; FHI 110336. The plants were transported to Emma-Maria Scientific and Research Laboratory Abraka for extraction.

2.2.2 Extraction procedure

The fresh *Vernonia* leaves were air-dried, crushed and soaked in ethanol for 48 hours after which the ethanolic extract was sieved out and allowed to dry. The resultant ethanol-free juice was subjected to liquid-liquid fractionation using solvents of varying polarity from non-polar to highly polar according to [10].

2.2.3 Ethical approval

Permission was obtained from the Bioethics Committee for the Use of Animals for Research of the Faculty of Basic Medical Science, Delta State University Abraka, Nigeria.

2.2.4 Handling of animals

Thirty (30) adult male *Wistar* rats, weighing between 100-250 g were used in this research. The animals were bred and purchased from the Emma-Maria Laboratory Animal unit, Abraka, Delta State and transported in plastic basket to the College of Health Sciences Laboratory Animal Facility, Delta State University, Abraka.

They were housed in an environment of normal ambient temperature and the lighting period was about 12 hours daily. The relative humidity was between 40 and 60%, they were kept in stainless steel cages, supplied with clean drinking water and fed *ad libitum* with standard commercial pelleted feed (Vital feed, UAC, Lagos).

2.2.5 Induction and treatment

For the diabetic study, hyperglycaemia was induced using Streptozotocin dissolved in sodium citrate buffer. Prior to induction, their fasting blood glucose of the animals were checked after an overnight fast. Streptozotocin was prepared by dissolving 2 g of Sodium Citrate in 100 ml of water to yield 0.1 mole of citrate buffer; 0.6 g of Streptozotocin was dissolved in 10 ml of citrate buffer to yield 60 mg of Streptozotocin. 1 ml of the resultant solution was injected into the animals through the lateral tail vein and their fasting blood glucose was assessed using ACCUCHEK active blood glucometer, 72 hours after induction. A 50% increase in pre-induction fasting Blood glucose level was considered to be diabetic. Diabetes was not induced in animals for the normoglycaemic study.

2.2.6 Treatment group

The diabetic study which comprised of 30 animals divided into seven (7) groups (n=5). Group 1 (negative control) induced diabetes but untreated, Group 2 was induced diabetes and treated with 50 mg/kg of Metformin, Group 3 was induced diabetes with 60 mg of streptozotocin and treated with 300 mg/kg of Crude *Vernonia amygdalina*, Group 4 was induced diabetes and treated with 300 mg/kg of Benzene fraction, Group 5 was induced diabetes and treated with 300 mg/kg of Chloroform fraction, Group 6 was induced diabetes and treated with 300 mg/kg of Ethyl acetate fraction, Group 7 was induced diabetes and treated with 300 mg/kg of Butanol fraction. Body weight and fasting blood glucose level were measured using electronic weighing balance and the one touch glucometer weekly measurement [11].

2.2.7 Sacrificing of animals and sample collection

The animals were sacrificed using cervical dislocation after an overnight fast in order to

determine their final fasting blood glucose level prior to sacrificing. The animal was pinned on the board and a laparotomy was carried out to expose the internal organs; blood was culled by cardiac puncture using 5ml syringes into a blood sample container. The pancreas organ were harvested, weighed and sectioned. Part of the organs was preserved in formo-saline in preparation for histopathology.

2.2.8 Biochemical analysis

Biochemical analysis was carried out on the samples collected to determine the enzyme activity level of glucose metabolic pathways, Liver function, antioxidant activities, lipid peroxidation, renal function, and lipid profile as shown below;

2.2.9 Procedures of analysis

AST, ALT and ALP were assay by the method of King and Armstrong [12], Levels of bilirubin was measured using colorimetric. The protein content in the serum and liver were estimated by the method of Lowry et al. [13]. All spectrophotometric measurements were carried out in a UV-visible spectrophotometer (N752).

2.3 Histopathological Examination

The process of preparation of tissue for histological examinations is separated into the following stages: Firstly, the tissue was processed by impregnating the specimen into embedding medium to provide a support and suitable consistency for the microtomy sectioning using different graded solutions of alcohol from 70% to 100% to dehydrate, the tissue. Thereafter the tissue was processed using the paraffin wax method with an automatic tissue processor by the following schedule. The sample was embedded in paraffin wax at 70ºC and cut with a rotary microtone 4 µ. The staining technique employed in this study was the haematoxylin and eosin staining techniques. Stained tissue images were captured using digital microscopic eyepiece 'Scoptek' Dcm 500, 5.0 mega pixels connected to USB 2.0 computer.

2.4 Statistical Analysis

All the data are expressed as mean ± standard error of mean SEM. Statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD). The SPSS software (version 20) was used in the statistical analysis using multiple comparison tests, and a p-value of less than or equal to 0.05 ($p \leq 0.05$) was considered significant.

3. RESULTS AND DISCUSSION

3.1 Effect of *Vernonia amygdalina* on Biochemical Parameters in Experimental Rats

Results from statistical analysis are presented in the figure below with the following designations;

All values designated (a) showed significant increase when compared to diabetic control.

All values designated (b) showed significant decrease when compared with diabetic control.

All values designated (*) showed significant increase when compared with control.

All values designated (#) showed significant decrease when compared with control.

3.1.1 Effect of *Vernonia amygdalina* crude and fraction on the fasting glucose level of experimental rats

The level of blood glucose in normal and diabetic rats at initial and final of administration was showed in Fig. 1. There was a significant elevation of blood glucose level in diabetic group as compared to normal control rats ($p \leq 0.05$). The administration of metformin (50 mg/kg) and crude extract and fractions of *Vernonia amygdalina* (300 mg/kg) reduced the blood glucose in diabetic rats as compared to the diabetic control rats. The 21st day treatment with metformin and butanol and chloroform fractions and crude of *Vernonia amygdalina* resulted in significant hypoglycemic effect in diabetic group.

3.1.2 Effect of *Vernonia amygdalina* crude and fraction on the liver function of experimental animals

Fig. 2 showed the level of serum AST, ALT and ALP in normal and experimental rats. There was a significant elevation ($p \leq 0.05$) of serum AST, ALT and ALP activity in diabetic rats as compared to control, crude extract and fractions of *Vernonia amygdalina* rats. Also the administration of Metformin (50 mg/kg) significantly decreased ($p \leq 0.05$) the serum AST, ALT and ALP activity in diabetic rats as compared to diabetic rats.

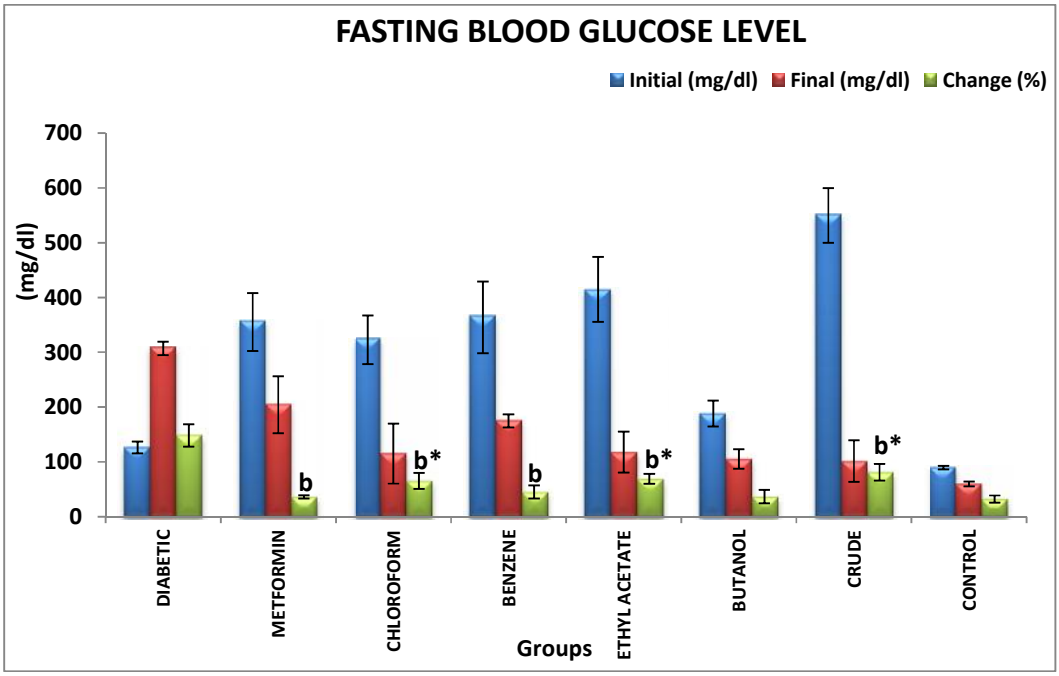

Fig. 1. Effects of *Vernonia amygdalina* fractionates on the fasting blood glucose level of treated STZ induced diabetic and non-diabetic experimental rats expressed as Mean±S.E.M

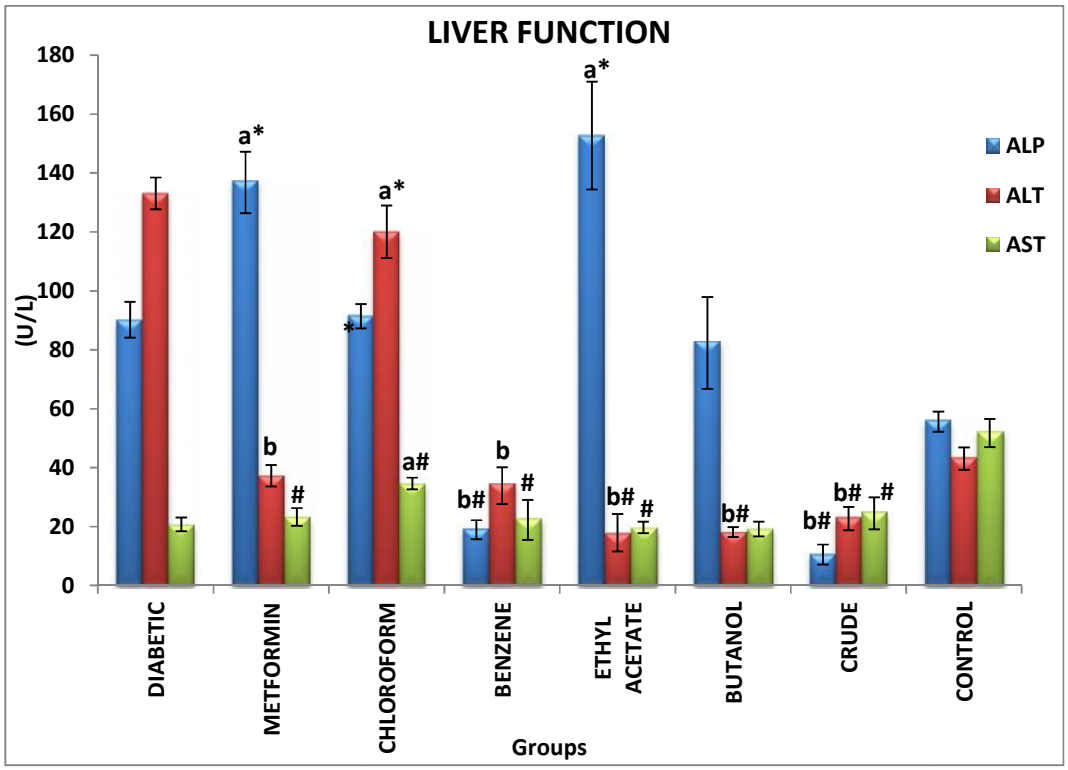

Fig. 2. Effects of *Vernonia amygdalina* on the level of hepatic biomarker enzymes of treated STZ-induced diabetic experimental rats expressed as Mean±S.E.M

3.1.3 Effects of *Vernonia amygdalina* on the level of bilirubin in treated STZ-induced diabetic experimental rats

Fig. 3 showed the level of serum bilirubin in normal and experimental rats. There was a significant decrease ($p \leq 0.05$) in serum direct and indirect bilirubin activity in diabetic rats as compared to control. The administration of crude extract and butanol fractions of *Vernonia amygdalina* significantly increased ($p \leq 0.05$) the serum direct and indirect bilirubin enzymes level in diabetic treated rats as compared to diabetic rats.

3.1.4 Effects of *Vernonia amygdalina* on the level of total protein in the serum of treated STZ-induced diabetic experimental rats

Fig. 4 showed the level of serum total protein in normal and experimental rats. There was a significant increase ($p \leq 0.05$) in serum total protein in diabetic rats as compared to control. The administration of crude extract and butanol fractions of *Vernonia amygdalina* significantly decreased ($p \leq 0.05$) the serum direct and indirect bilirubin enzymes level in diabetic treated rats as compared to diabetic rats.

3.1.5 Effect of *Vernonia amygdalina* crude and fraction on the histological morphology of the pancreas of experimental animals

The cyto-restorative property of *V. amygdalina* was determined by assessing its effect on the microscopic anatomy of the pancreas of diabetic rats as shown in the Plates 1-8.

3.2 Discussion

In this study, the blood glucose reducing potency of *Vernonia amygdalina* crude extract and fractions was observed, with crude showing 81.5% reduction in fasting blood glucose level in diabetic rats; fractions showed 69.7%, and 65.9% reduction, higher than that of metformin which showed 36.5% reduction. An earlier study by Nwanjo [14] purported the hypoglycaemic property of *Vernonia amygdalina*. The result of this study shows that administration of *Vernonia amygdalina* crude extract and fractions caused a significant decrease in fasting blood glucose level as has been observed by authors like Osinubi [15] and Ekam et al. [10].

Diabetes as a metabolic disease interferes with the normal metabolic processes in tissues and organs, resulting in alterations in normal secretory activities. ALT, AST, and ALP activities were over expressed in liver of streptozotocin induced diabetic rats compared to the normoglycaemic control group. The administration of *Vernonia amygdalina* significantly decreased these enzyme levels. Similar hepatoprotective effect by *Vernonia amygdalina* was reported by Leelaprakash *et al*., [16] in CCl_4 hepatotoxcity. The reversal of elevated serum intracellular enzyme levels by *Vernonia amygdalina* extract and fractions in diabetic rats may be attributed to the ability of the plant to cause stabilizing of the hepatic cell membrane preventing enzymes leakages. In addition, it was reported that the reversal of increased levels of transaminases to nearly normal predicts the restoration of hepatocytes and regeneration of hepatic parenchyma [17]. This observation is consistent with earlier report on hepatoprotective potentials of leaf extracts of *Vernonia amygdalina* in mice [18].

The ethyl acetate and butanol fractions of the plant significantly decreased the level of Serum bilirubin in treated diabetic rats, this effect was however not observed with the other fractions. Serum protein play an important role in cellular maintenance, growth and functioning of the body, serving as the basic structural molecule of all tissues in the body, its level are very important markers of health status of the body. Ravi et al. [19] reported that the characteristics loss of body weight associated with diabetes is due to excessive break down of tissue protein and an increased muscle wasting in diabetes. Similarly, hepatic complications in diabetes is associated with an alteration in the levels of serum total protein and albumin levels and this serves as a marker for hepatic damage [20,21]. The level of serum protein in untreated diabetic rats where seen to be significantly lower than that of healthy rats of the control group which could be attributed to the diabetogenic activity of streptozotocin and the resultant hepatic complications. Administration of *Vernonia amygdalina* fractions decreased the level of serum proteins in this study, implying a reversal of body protein catabolism due to the effect of diabetes. Increase in protein level in liver by extract of *Vernonia amygdalina* leaves and its fractions were reported in diabetic animals by Akah et al. [22] same was also observed in this study. This may have resulted from effective regulation of protein metabolism due to the plant administration. In diabetes, there is increased protein catabolism with inflow of amino acids to

liver, which feed gluconeogenesis and accelerated ureagenesis, resulting in hypoproteinemia and hypoalbuminemia which may have been the case in the present study. Histopathological studies carried out in this work showed that the architectural distortion and cell destruction that resulted from the diabetogenic effect of streptozotocin induction could not be ameliorated by *Vernonia amygdalina.*

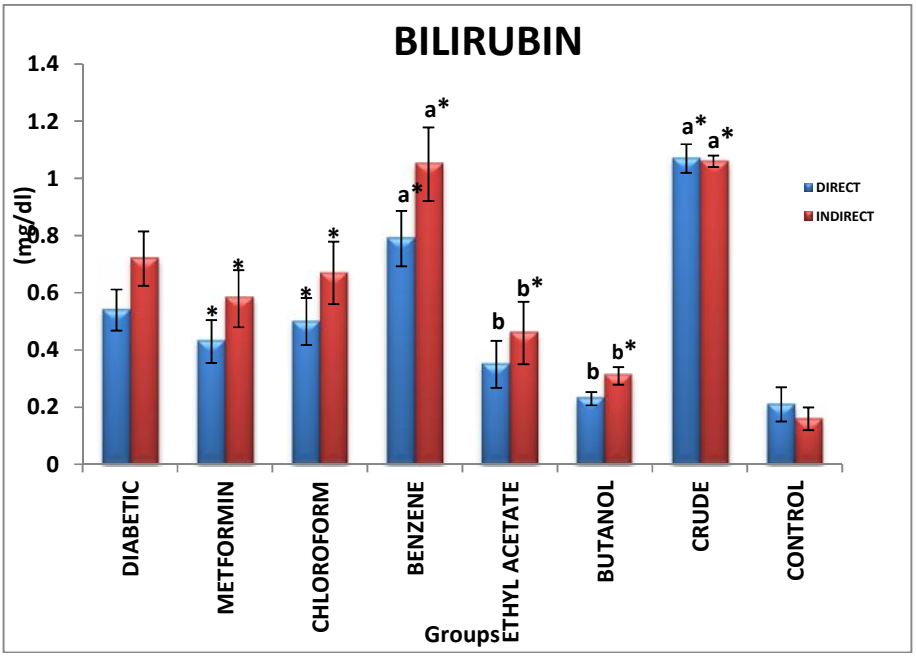

Fig. 3. Effects of *Vernonia amygdalina* on the level of bilirubin in treated STZ-induced diabetic and non-diabetic experimental rats expressed as Mean±S.E.M

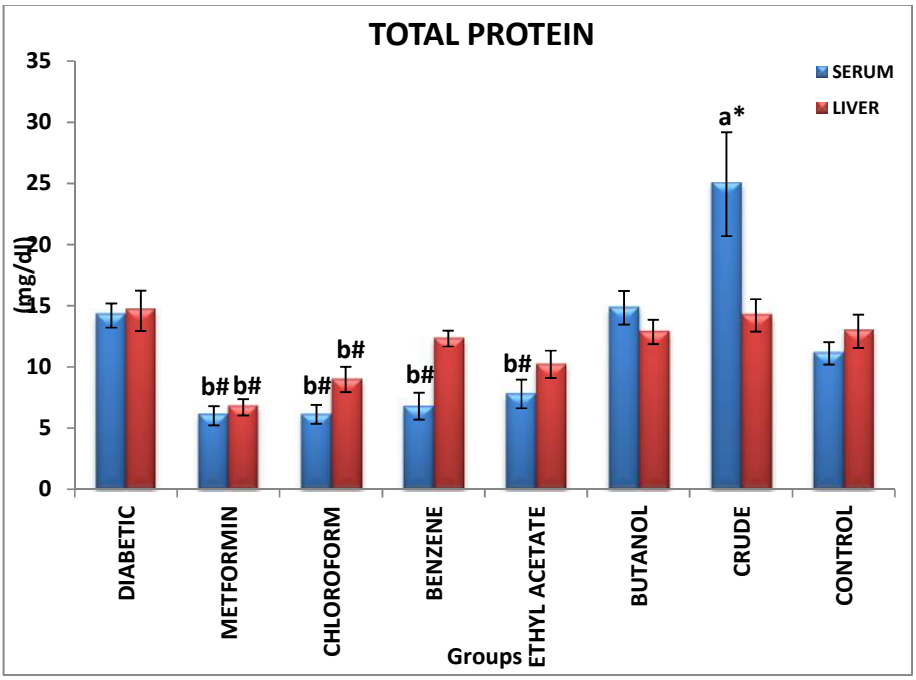

Fig. 4. Effects of *Vernonia amygdalina* on the level of total protein in the serum and liver of treated STZ-induced diabetic and non-diabetic experimental rats expressed as Mean±S.E.M

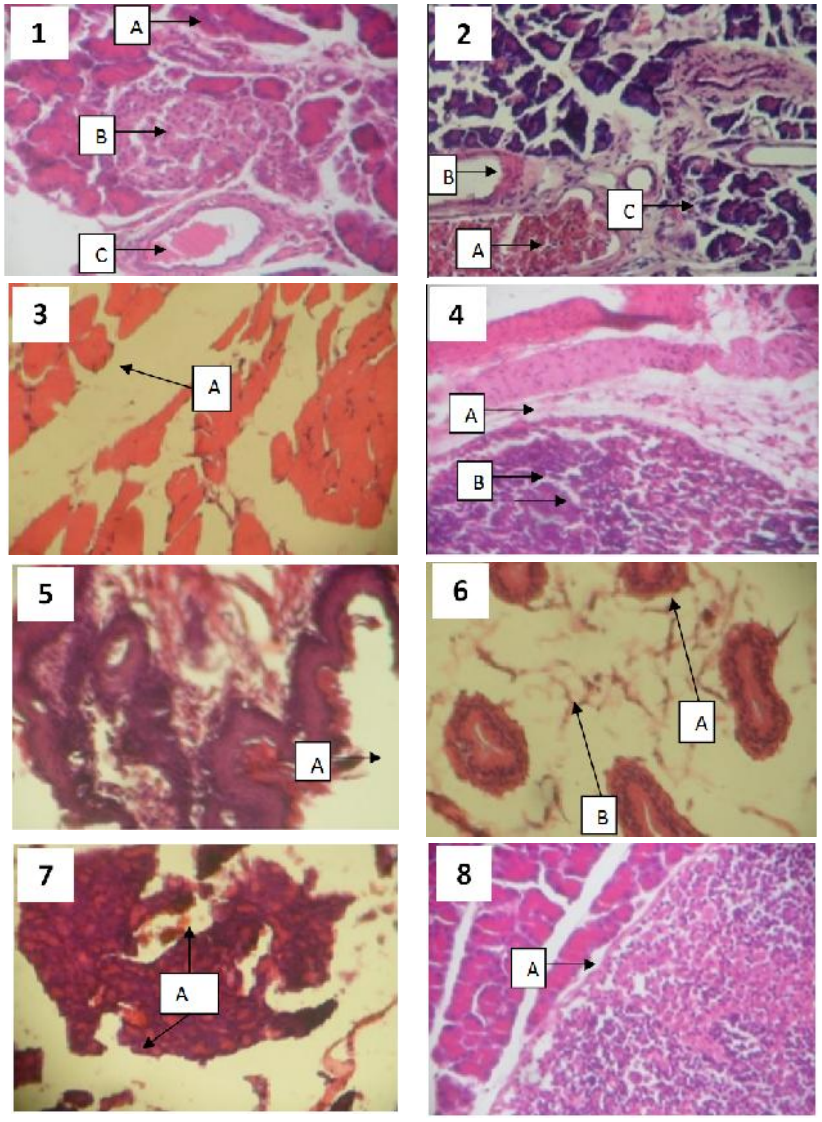

Plate 1. Control Rat pancreas composed of exocrine glands A, islets of Langerhans B and interlobular duct C (H&E x 100);
Plate 2. Negative control: Diabetes induced with STZ, rat pancreas showing moderate interlobular vascular congestion A, dilatation and hypertrophy B as well as mild interlobular infiltrates of chronic inflammatory cells C (H&E x 100);
Plate 3. Diabetes induced with STZ and treated with Metformin rat pancreas showing ghost of stromal fibres A (H& E x 100);
Plate 4. Diabetes induced with STZ and treated with Benzene fraction of *Vernonia amygdalina* rat pancreas showing stratified squamous epithelium keratinized A (H&E x 100);
Plate 5. Diabetes induced with STZ and treated with Chloroform fraction of *Vernonia amygdalina* rat pancreas showing discrete exocrine glands A embedded in fat B (H&E x 100);
Plate 6. Diabetes induced with STZ and treated with ethyl acetate fraction of *Vernonia amygdalina* rat pancreas showing moderate congestion A (H&E x 100)
Plate 7. Diabetes induced with STZ and treatment with butanol fraction of *Vernonia amygdalina* rat pancreas showing some resurgence of pancreatic cells and lymphoid aggregates (H&E x 100)
Plate 8. Diabetes induced with STZ and treatment with crude extract of *Vernonia amygdalina* rat pancreas showing some markedly activated lymphoid aggregates A (H&E x 100)

4. CONCLUSION

This study concludes by stating that the plant *Vernonia amygdalina* indeed possesses hypoglycemic properties as has been purported by some authors, and its protein sparing ability makes it well adapted for the management of disease conditions that results in protein catabolism such as diabetes mellitus. It also demonstrated a significant ability to restore liver enzymatic function following an alteration as a result of diabetic mellitus.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee of Delta State University, Abraka. All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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