ETHANOLIC LEAF EXTRACT OF *Vernonia amygdalina* IMPROVES ISLET MORPHOLOGY AND UPREGULATES PANCREATIC G6PDH ACTIVITY IN STREPTOZOTOCIN-INDUCED DIABETIC WISTAR RATS

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Summary

Potential botanical sources of antidiabetic therapies are currently being explored. In this work, we studied the effect of ethanolic leaf extract of *Vernonia amygdalina* on glycemia, pancreatic islet microanatomy, glucose-6-phosphate dehydrogenase and lactate dehydrogenase activity of the pancreas and liver in streptozotocin-induced diabetes mellitus. Hyperglycemia was induced in fasted adult Wistar rats with an intraperitoneal dose of streptozotocin (65 mg/kg bw/d). Leaf extract of *Vernonia amygdalina* and chlorpropamide were administered orally at 400 mg/kg bw/d and 15 mg/kg bw/d, respectively, for 6 weeks. The pancreas was processed for histological studies and pancreatic levels glucose-6-phosphate dehydrogenase and Lactate dehydrogenase were estimated in the plasma and tissue homogenates. Normoglycemia was established in *Vernonia amygdalina*-treated diabetic rats at the end of week 3. Necrosis was observed in the islets of untreated diabetic rats, while a degree of histological improvement was observed in islets of *Vernonia amygdalina*-treated diabetic rats and upregulation of the enzymes activity had occurred in the pancreas of this group compared with control ($P<0.05$). The present study suggests that chronic treatment with ethanolic leaf extract of *Vernonia amygdalina* improves hyperglycemia and ameliorates islet lesions in diabetic rats.

Key words: Pancreatic islets, *Vernonia amygdalina*, G6PDH, LDH.

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Introduction

In diabetes mellitus, progressive loss of β cell function and subsequent death of these cells are key events (1). Type 1 diabetes is a result of autoimmune destruction of β cells, and this event usually commences in childhood in susceptible individuals (2). In contrast, insulin resistance, β-cell dysfunction and ultimate β-cell failure are major findings in type 2 diabetics. In these patients, relative insulin deficiency occurs and fasting hyperglycemia is evident (3).

Furthermore, oxidative stress is a major event in diabetes mellitus, and it is a result of increased generation of reactive oxygen species and/or downregulation of cellular antioxidant activities (4), etc. In oxidative stress, upregulation of glucose 6-phosphate dehydrogenase (G6PDH) has been reported in the liver (5) and embryonic tissues (6). G6PDH is the first and rate-limiting enzyme in the pentose phosphate pathway; and its activity involves generation of NADPH and D-ribose 5-phosphate. The former is required for maintaining glutathione in its reduced state (for the detoxification of free radical and lipid hydroperoxides) (7). Besides, NADPH maintains the catalytic activity of catalase, and thus contributes to the reduction of H_2O_2 (8). Thus, G6PDH level is an indication of the antioxidant status of a tissue.

Alternative therapies in the management of diabetes mellitus include the use of the leaf extract of *Vernonia amygdalina* Del (African bitter leaf) (9). The report of Ebong et al (10) showed that the ethanolic extract of *V. amygdalina* produced normoglycemia in alloxan-induced diabetic rats. In the present work, we studied pancreatic islet morphology and pancreatic G6PDH activity in hyperglycemic rats treated with an ethanolic leaf extract of *V. amygdalina*.

Materials and Methods

Animals

A total of twenty-five adult male Wistar rats were bred at the animal holdings of the Department of Anatomy, University of Ilorin. Animals had an average weight of 167 g and were eight weeks old at day 0 of experiment. They were exposed to 12-hour light, 12-hour dark cycle at 22–24 ^{0}C. All animals were maintained on a pelletized rodent feed (Bendel Feeds, Iwu, Nigeria), and water was given freely.

Collection and Extraction of *Vernonia amygdalina* Leaves

Fresh mature leaves of *V. amygdalina* were collected from Tanke Garden (Ilorin) between December 2008 and January 2009. The botanical identification and authentication of the leaf sample was done at the Herbarium Section, Department of Plant Biology, University of Ilorin, Nigeria (Voucher No.10). The leaves were air-dried at room temperature (24 ^{o}C), and the dried leaves were milled into powder and weighed (873 g). The powder was infused with 5 L of 70% ethanol for 72 hours (11). The solution was filtered using chess material and the extract was evaporated to dryness at 40 ^{0}C in the laboratory oven (Gallenkamp, UK). The dried residue (crude extract; 45g) was stored at 4 ^{0}C.

Induction of Diabetes Mellitus

To induce hyperglycemia, seventeen fasted adult male Wistar rats were injected with a single intraperitoneal dose of streptozotocin (Sigma, St. Louis, USA) at 65 mg/kg b.w in 0.1 M

citrate buffer, pH 4.5 (12,13). Control animals were injected intraperitoneally with citrate buffer alone (1 ml/kg b.w). All animals were allowed free access to feed and water after streptozotocin (STZ) injection, and they were left undisturbed for a minimum of 72 hours for hyperglycemia to develop (10,13). Thereafter, fasting blood glucose levels of the animals were measured with One Touch Ultra Mini Glucometer (LifeScan Inc., CA, USA). Animals with blood glucose ≥ 250 mg/dl were considered hyperglycemic (14).

Administration of *Vernonia amygdalina* Leaf Extract

A total of twenty adult Wistar rats (twelve surviving diabetic rats and eight normoglycemic rats) were randomly assigned to one of the following treatment groups of four animals each: control, diabetic, diabetic + *Vernonia amygdalina,* diabetic + chlorpropamide, and *Vernonia amygdalina* alone. *Vernonia amygdalina* leaf extract was dissolved in distilled water and administered at 400 mg/kg bw/d (10), chlorpropamide (Neimeth Int'l Pharm, Lagos, Nigeria) was also dissolved in distilled water and administered at 15 mg/kg b.w/d (10). Both drugs were given orally for 42d at 9:00–10:00 hour each day.

Blood Glucose, Feed Intake and Body Weight

A One Touch Ultra Mini Glucometer (LifeScan, CA, USA) was used to estimate the blood glucose of treated and control animals. Blood was obtained from the dorsal vein of the animals, and glucose readings were taken at 8.00–9.00 hours (three times a week, for six weeks). At day 0, blood glucose was taken at 0, 1, 3, 5 and 7 hours post-dose. Feed intake was also monitored on a daily basis while body weight of the animals was recorded twice a week.

Termination of Treatment

All animals were fasted and sacrificed under diethyl ether anaesthesia 24 hours after the last treatment day. Blood was collected into heparinised tubes and centrifuged at 860 x g for 20 minutes in a desktop centrifuge model 90-1 (Jiangsu Zhangji Instruments Co., China) (15). Laparatomy was performed on each animal and the splenic parts of the pancreas were fixed in pre-cooled Bouin's fluid for histological studies. Portions of the pancreas and liver were also homogenized in 0.1 M phosphate buffer (pH 7.4) in a potter homogeniser (GPE, Bedfordshire, England). The homogenate was centrifuged at 10, 000 x g for 10 minutes at 4 ^{0}C (16) and the supernatant was analysed for G6PDH in the pancreas and LDH in the pancreas and liver.

Tissue Processing and Enzyme Assay

Splenic parts of the pancreas were fixed in cold Bouin's fluid and stained in aldehyde-fuchsin to demonstrate the β and α cells of the pancreatic islets (17). Photomicrographs were taken with a JVC digital camera (JVC, China) mounted on an Olympus light microscope (Olympus, Essex, UK). The levels of LDH and G6PDH in the pancreas and liver of treated and control rats (as well as plasma LDH levels) were estimated according to the methods of Weisshaar (18), and Lohr and Waller (19), respectively, using LDH and G6PDH kits from Randox Laboratories (UK). Absorbance was read at 320 nm in a spectrophotometer (Camspec M105 Spectrophotometer, UK).

Statistical Analysis

The data were analyzed for statistical significance using ANOVA. $P<0.05$ was considered statistically significant. Graphs were drawn using Microsoft Excel 2007 (Microsoft Corporation, USA).

Results

Body Weight and Feed Intake

Figure 1 shows gain in body weight of each group of animals relative to the initial weight at week 0. By the 6th week of treatment, average body weights of animals in the untreated diabetic, *V. amygdalina*-treated diabetic, and chlorpropamide-treated diabetic rats were significantly lower than control ($P<0.05$). The least gain in body weight was recorded in the untreated diabetic rats (Fig. 1). The quantities of feed taken by each group of animals are expressed as feed intake/animal/d (Fig. 2). By the 6th week, feed intake in non-diabetic rats treated with *V. amygdalina* and diabetic rats treated with chlorpropamide was significantly less than control ($P<0.05$). Feed intake in untreated diabetic rats and *V. amygdalina*-treated dietetic rats was not significantly different from control ($P>0.05$).

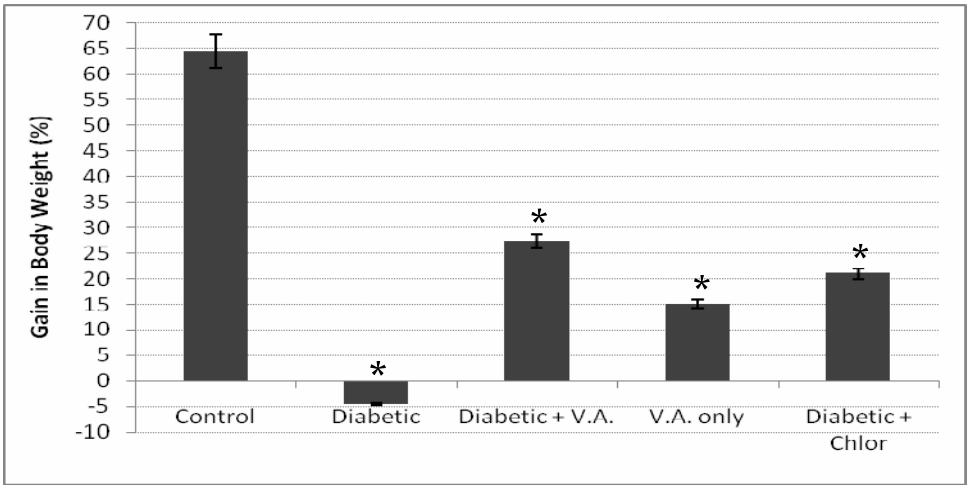

Figure 1. Change in body weights of animals. Values are Mean±SEM. *Significantly different from control, $P<0.05$. (V.A. = *Vernonia amygdalina*; Chlor =chlorpropamide).

Blood Glucose

In the first day of treatment, blood glucose was assessed at 0, 1, 3, 5 and 7 hours post-dose. At one hour post-dose, blood glucose in diabetic rats treated with *V. amygdalina* dropped by 25%. At 7 hrs post-dose, no significant difference in blood glucose occurred between the diabetic and treated groups ($P>0.05$ vs. diabetic group). Figure 3 shows blood glucose levels of the treatment groups on a weekly basis. By the 6th week, blood glucose in diabetic rats treated with *V. amygdalina* was not different from control ($P>0.05$). In contrast, blood glucose was significantly high in the untreated diabetic and chlorpropamide-treated diabetic rats ($P<0.05$), but significantly low in non-diabetic rats that received *V. amygdalina* alone ($P<0.05$).

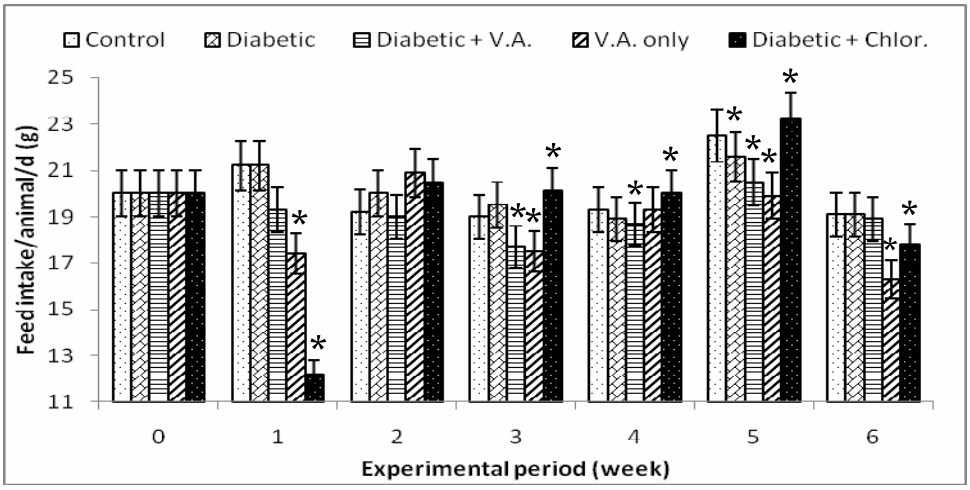

Figure 2. Feed intake of treated and control rats. Values are Mean±SEM. *Significantly different from control, P<0.05. (V. A. = *Vernonia amygdalina*; Chlor.=chlorpropamide).

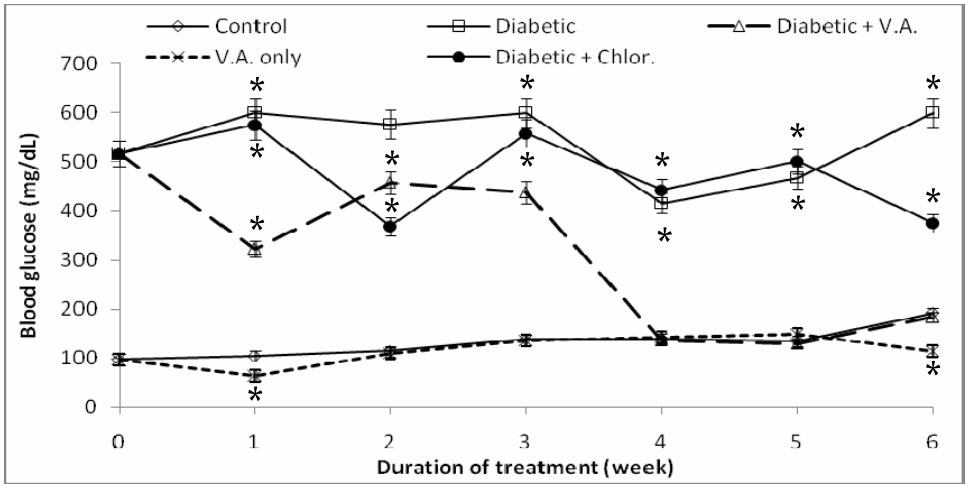

Figure 3. Blood glucose levels of control and treated rats. Values are Mean±SEM of four rats. *Significantly different from control, P<0.05. (V.A. =*Vernonia amygdalina*; Chlor. =chlorpropamide).

Glucose 6 Phosphate Dehydrogenase Activity of the Pancreas

Figure 4 shows changes in G6PDH activity of the pancreas in the different treatment groups relative to control. At 42d, significant increases in the levels of this enzyme had occurred in the pancreas of *V. amygdalina*-treated diabetic and non-diabetic rats (P<0.05).

Lactate Dehydrogenase (LDH) Activity of the Pancreas and Liver

Figures 4 show changes in LDH activity in the pancreas and liver of treated and control rats. By the 6th week of treatment, significant increases in the activity of this enzyme had occurred in all the treatment groups (P<0.05).

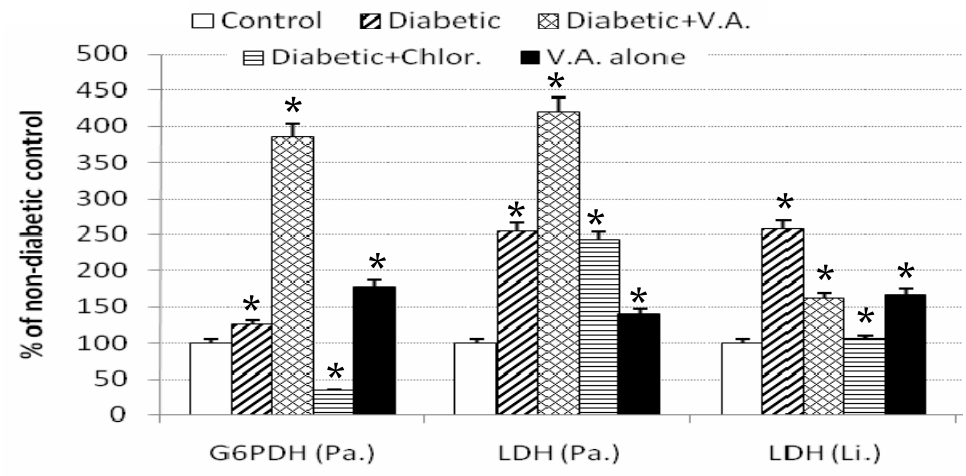

Figure 4. Enzyme levels in the pancreas and liver of treated and control rats. Values are Mean±SEM. *Significantly different from control, P<0.05; [V.A. = *Vernonia amygdalina*; Chlor. =chlorpropamide; G6PDH (Pa.) = pancreatic G6PDH; LDH (Pa.) = pancreatic LDH; LDH (Li.) = hepatic LDH; LDH (Pl.) = Plasma LDH].

Microscopic Anatomy of Islets of Langerhans of Treated and Control Rats

Figure 5 (A□D) shows the histology of the pancreatic islets. Islets of control rats showed viable β cells as demonstrated by aldehyde fuchsin stain (Fig. 5 A). In contrast, islets of diabetic rat showed necrotic changes, with paucity of islet β cells (Fig. 5 B). However, treatment of diabetic rats with *V. amygdalina* resulted in improvement of islet histology as shown in figure 5 C. In these rats, viable β cells were observable in the islet. Microanatomy of pancreatic islets of *V. amygdalina*-treated non-diabetic rats was similar to control.

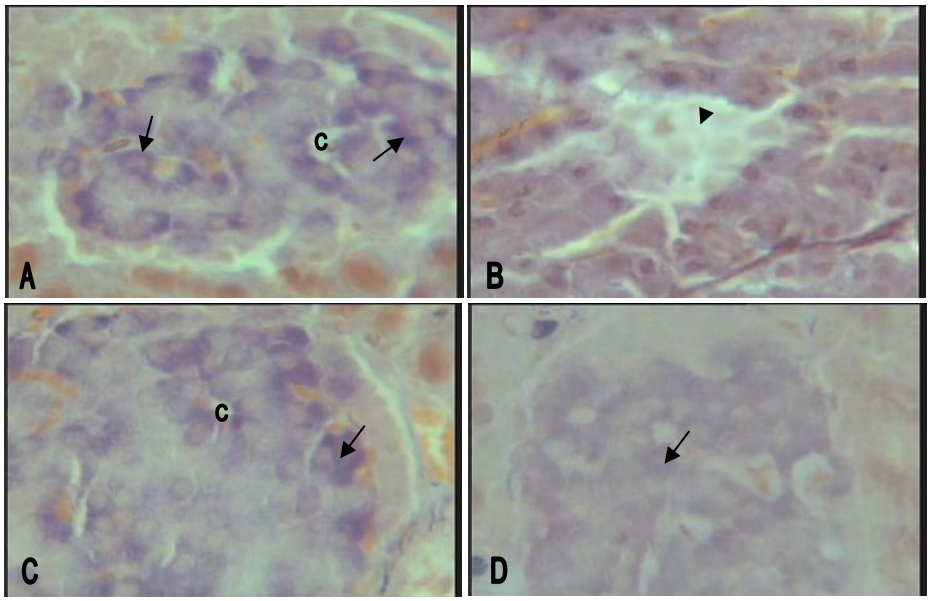

Figure 5 (A-D). Histology of pancreatic islet at 42d of treatment. ***A*,** Control group. The islet is normal; **β** cells are stained purple-violet (arrows), while α cells appear orange; *c* indicates capillary. ***B*,** Diabetic group. Necrosis of islet cells is observable (arrowhead). ***C*,** Diabetic+*V. amygdalina.* Viable β cells are observable in the islet (arrow); capillaries (*c*) can also be seen. ***D*,**

Diabetic+chlorpropamide. Islet cellularity is less in this group compared with control. Aldehyde-fuchsin stain; x400.

Discussion

In this study, hypoglycemic activity of the ethanolic leaf extract of *V. amygdalina* was investigated in STZ-induced hyperglycaemic Wistar rats. Besides, the histology of the endocrine pancreas and the activity of LDH and G6PDH were studied in the pancreas and liver.

In diabetic Wistar rats, oral administration of *V. amygdalina* did not produce normoglycemia in the first day of treatment. Hyperglycemia persisted in these animals till the end of week 3 (Fig. 3). By the end of the 3rd week however, normoglycemia was established and this metabolic state was maintained till euthanasia at 42d. This finding shows that the leaf extract of *V. amygdalina* possesses hypoglycemic activity in rats when administered as a chronic regimen (at 400 mg/kg bw/d). Our finding is consistent with the reports of (10,12,20). Ebong *et al* (10) reported significant improvement in glycemia in diabetic rats treated with 400 mg/kg bw/d for 24d. Besides, Nwanjo (12) reported that the aqueous leaf extract of *V. amygdalina* produced normoglycemia in STZ-induced diabetic rats at week 4 of treatment. Our finding thus corroborates their reports.

The mechanism of hypoglycemic activity of *V. amygdalina* is a subject of further investigation. However, certain flavonoids in *V. amygdalina* may confer hypoglycemic property on the leaf extract of this plant (21). According to the report of Igile *et al* (22) the leaves of *V. amygdalina* contain bioflavonoids such as luteolin, luteolin 7-O-β-glucoside and luteolin 7-O-β-glucuronoside. Besides, several stigmastine type saponins such as vernoniosides A1, A2, B1, B2, D3, A4 and C have been isolated from the leaves of *V. amygdalina* (23). In addition, *V. amygdalina* leaves had been reported to contain bioactive sesquiterpene lactones such as vernolide and vernodalol (24). Thus, it is probable that the hypoglycemic activity of *V. amygdalina*, as reported in the present study, may be a function of its rich flavonoid content. The study of Adewole *et al* (25) showed that flavonoid such as quercetin improves hyperglycemia and islet morphology in STZ-induced diabetic rats. Besides, Adewole and Caxton-Martins (26) reported the beneficial effect of aqueous leaf extract of *Annona muricata* Linn on blood glucose levels of STZ-induced diabetic rats. They concluded that plant bioflavonoids and coumarins might play significant roles in the establishment of normoglycemia in diabetic rats.

In the present study, *V. amygdalina* (at 400 mg/kg bw/d) did not produce normoglycemia in diabetic rats until the end of week 3 of treatment (Fig. 3); and this suggests that viable β cells may be required for the hypoglycemic effect of this botanical. The finding in non-diabetic rats treated with V. amygdalina further substantiates this observation. In these animals, pancreatic islets were intact (Fig. 5), and blood glucose levels were significantly lower than control as early as week 1 (Fig. 3). This suggests that in intact healthy rats, *V. amygdalina* leaf extract is hypoglycemic and can thus lower blood glucose to values below control.

In contrast, hyperglycemia persisted throughout the study period in diabetic rats treated with chlorpropamide. This could be due to significant loss of β cells, as a result of STZ toxicity. Chlorpropamide is an insulin secretagogue (sulphonylurea) and its hypoglycemic activity depends on viable β cells (27). Thus, islet necrosis from STZ toxicity abolished the hypoglycemic activity of chlorpropamide in this study.

As shown in figure 4, hepatic LDH activity in *V. Amygdalina*-treated diabetic and non-diabetic rats had increased significantly at week 6 ($P<0.05$ vs. control). This suggests that chronic exposure to *V. amygdalina* upregulates the glycolytic enzyme LDH, thereby promoting glucose disposal via increased glycolysis in hepatocytes. Such activity of *V. amygdalina* would thus result in a fall in blood glucose, as reported in this study.

Moreover, figure 4 also shows significant increases in the activity of LDH and G6PDH in the pancreas of diabetic and non-diabetic rats treated with *V. amygdalina*. This suggests that *V. amygdalina* may enhance glucose clearance in extra-hepatic tissues. Future work may therefore include an assessment of the activity of these enzymes in skeletal muscles cells, etc, of diabetic rats treated with *V. amygdalina* leaf extract.

In diabetic rats, significant loss in body weight had occurred at the end of six weeks of chronic hyperglycemia (Fig. 1). Weight loss is also a feature of diabetes mellitus in man, and it is owing to depletion of body adiposity as a result of marked reduction in plasma levels of insulin (28). In contrast, weight loss was not as marked in *V. amygdalina*-treated rats as it was in untreated diabetic animals. In *V. amygdalina*-treated rats, up to 27% increase in body weight was recorded, as opposed to 4% loss in weight obtained in untreated diabetic rats (Fig. 1). This relatively higher weight gain in *V. amygdalina*-treated rats (compared to untreated diabetic rats) may be due to increased levels of plasma insulin. Insulin, produced by islets of Langerhans, has a well established role in the regulation of energy metabolism in insulin-sensitive tissues such as skeletal muscle and fat. In the presence of insulin, substrates derived from ingestion of food metabolized by the body cells, and excess caloric intake is stored as increased adipose tissue, thereby leading to increased adiposity and body weight gain (28).

However, although increases in body weight in *V. Amygdalina*-treated diabetic and non-diabetic rats were higher than what obtained in untreated diabetic animals, it was less than body weight gain in the control (Fig. 1). This may be due partly to reduction in feed intake in *V. Amygdalina*-treated animals, starting from the end of the first week of treatment (Fig. 2). Such potential anorexic effect of *V. amygdalina* may be due to increased production of leptin by adipocytes. This observation suggests that chronic administration of *V. amygdalina* may result in upregulation of leptin. Such mechanism had been reported for Exendin-4, a hypoglycemic agent from the saliva of the Gila monster lizard (29). The report of Bjorbaek *et al,* 1998 showed that intravenous administration of leptin to normal fasted rats inhibited food intake, while impaired leptin signaling and/or production results in hyperphagia and obesity (30).

Figure 5 shows histopathology of the pancreas at 42d of treatment. Islets of Langerhans of untreated diabetic rats show necrosis of islet cells. In these animals, necrosis of islet β cells results from exposure to STZ (13). Uptake of STZ into β cells is mediated by GLUT-2 transporters (31). In these cells, STZ mediates its cytotoxicity via the DNA-alkylating activity of its *N*-methyl-*N*-nitrosourea moiety, especially at position O^6 of guanine (32). The transfer of the methyl group from STZ to DNA results in DNA fragmentation and β-cell death (33). Additional mechanisms involved in β-cell toxicity of STZ include over-activation of the poly (ADP-ribose) polymerase (in an attempt to repair damaged DNA), with the resultant depletion of cellular NAD^+ and ATP (34); as well as glycosylation of proteins (35). However, in diabetic rats treated with *V. Amygdalina* leaf extract, increased cellularity of the islets was observed in aldehyde fuchsin-stained sections of the pancreas at 42d (Fig. 5). Besides, upregulation of pancreatic G6PDH had also occurred at this time. The latter suggests that *V. amygdalina* may enhance DNA synthesis, and thus, cell proliferation in pancreatic islets. Increased activity of G6PDH results in the synthesis of ribose-5-phosphate (R-5-P) (36); and

the latter is converted to 5-phosphoribosyl-1-pyrophosphate that acts as the donor of the ribose phosphate unit in nucleotide biosynthesis (36). Thus, G6PDH plays an important role in DNA synthesis and cell proliferation (36). Certain phytochemicals in *V. amygdalina* may thus mediate upregulation of G6PDH (as observed in this study), with the resultant increased DNA synthesis and β-cell proliferation. The report of Kuo and Tang (36) showed that proliferation of NIH 3T3 cells was associated with overexpression of G6PDH.

Furthermore, upregulation of G6PDH had been shown to be associated with enhanced redox status of cells. In addition to the synthesis of the precursor of DNA (R-5-P), G6PDH also generates NADPH. The latter is critical for maintaining glutathione (GSH) in its reduced form; and glutathione is essential for the detoxification of reactive free radicals and lipid hydroperoxides (7). NADPH also maintains the catalytic activity of catalase, and thus, the reduction of H_2O_2 to water and molecular oxygen (7). These beneficial activities of G6PDH may thus promote structural integrity of the islets by ameliorating oxidative stress.

In conclusion, data from the present study show that chronic treatment with ethanolic leaf extract of *V. amygdalina* improves hyperglycemia and ameliorates islet lesions in hyperglycaemic rats. Thus, alternative and complimentary approach to the management of diabetes mellitus in man may thus include the use of the leaves of *V. amygdalina* as an antidiabetic therapy.

Acknowledgement

The authors express their gratitude to Mr Clement Jaiyeoba of the Faculty of Agriculture, University of Ilorin, for his assistance with extraction of *Vernonia amygdalina* leaves.

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