VERNONIA AMYGDALINA UPREGULATES HEPATIC ENZYMES AND IMPROVES LIVER MICROANATOMY IN EXPERIMENTAL DIABETES MELLITUS

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Summary

This work was designed to study the blood glucose response of streptozotocin-induced hyperglycaemic rats to a chronic regimen of the ethanolic leaf extract of *Vernonia amygdalina*. Besides, hepatic morphology and the activity of glucose 6 phosphate dehydrogenase and lactate dehydrogenase were assessed. Twenty adult Wistar rats of both sexes (8 weeks old; body weight of 151 g on average) were randomly assigned into one of the following groups of four animals each: control, diabetic, diabetic+*Vernonia amygdalina*, diabetic+chlorpropamide, and *Vernonia amygdalina* alone. *Vernonia amygdalina* and chlorpropamide were administered orally at 400 mg/kg bw/d and 14.3 mg/kg bw/d respectively for 42 days. By the end of the 3rd week, normoglycemia was established in hyperglycaemic rats treated with *Vernonia amygdalina*, while hepatic levels of glucose 6 phosphate dehydrogenase and lactate dehydrogenase were significantly elevated in these animals at 42 days ($P<0.05$). Besides, plasma lactate dehydrogenase levels were significantly reduced in all the treatment groups compared to control ($P<0.05$). In addition, hepatic microanatomy was comparable to control in all the treatment groups except untreated diabetic rats. In the latter, hepatic sinusoids were occluded with congestion of the central veins, while hepatocytes appeared swollen. Our findings show that chronic treatment with ethanolic leaf extract of *Vernonia amygdalina* produces normoglycemia in hyperglycaemic rats; and this effect is not associated with liver injury.

Key words: Vernonia amygdalina, glucose 6 phosphate dehydrogenase, lactate dehydrogenase, liver.

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Introduction

Complementary and alternative medicine describes a diverse group of medical and health care systems and products not currently considered to be part of conventional medicine (1). Promising antidiabetic botanicals, from which new antidiabetic drugs may be derived, include *Coccinia indica*, *Momordica charantia*, *Gymnema sylvestre, Azadirachta indica* and *Vernonia amygdalina* (1,2).

In long-standing diabetes mellitus, the morphology and function of the liver are impaired. Liver biopsy findings in type 1 diabetics with hepatomegaly are comparable to hepatic findings in Mauriac's syndrome and include marked glycogen deposition in hepatocytes (3). In type 2 diabetes, impaired insulin action results in non-alcoholic fatty liver disease, including steatosis and steatohepatitis (4).

Furthermore, chronic hyperglycemia of diabetes mellitus is associated with oxidative stress. Excessive glucose delivered to the mitochondria overdrives the electron transport chain, resulting in overproduction of superoxide anion (5). Besides, hyperglycemia can lower the activity of antioxidant enzymes such as SOD and glutathione reductase, perhaps via glycation (6). Glutathione synthesis was also reportedly inhibited under hyperglycemic conditions (7).

In oxidative stress, upregulation of glucose 6-phosphate dehydrogenase (G6PDH) has been reported (8). G6PDH is the first and rate-limiting enzyme in the pentose phosphate pathway. Its activity involves generation of NADPH, and this is required for maintaining glutathione in its reduced state (for the detoxification of free radical and lipid hydroperoxides) (9). Besides, NADPH maintains the catalytic activity of catalase, and thus contributes to the reduction of H_2O_2 (10). Thus, G6PDH level of a tissue may suggest the antioxidant status of that tissue.

In this work, we report the blood glucose responses of hyperglycemic rats to a chronic regimen of the leaf extract of the African bitter leaf (*Vernonia amygdalina* Del). Besides, the morphology of the liver and the activity of hepatic G6PDH and LDH are reported.

Materials and Methods

Animals

A total of twenty-five adult Wistar rats (15 females and 10 males) were bred at the animal holdings of the Department of Anatomy, University of Ilorin. Animals weighed 151 g on average and were eight weeks old at the start of the experiment. They were exposed to 12-hour light, 12-hour dark cycle at 22-24 ^{0}C. All animals were maintained on a pelletized growers feed (Bendel Feed & Flour Mill Ltd., Ewu, Nigeria). Rat pellets and water were 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 (873g). The powder was infused with 5L of 70% ethanol for 72 hours (11). The solution was filtered using chess material and the solvent was removed by distillation at 40^{0}C in a Soxlet extractor (Stuart Scientific, UK). The extract was then 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 Wistar rats were injected with a single i.p. dose of streptozotocin (STZ) (Sigma, St. Louis, MO, USA) at 65 mg/kg b.w (12). Just prior to the induction of diabetes, streptozotocin was dissolved in 0.1M cold citrate buffer, pH 4.5 (13). Control animals were injected intraperitoneally with citrate buffer alone at a single dose of 1.2 ml/kg b.w. All animals were allowed free access to feed and water after STZ injection, and they were left undisturbed for a minimum of 72 hours for hyperglycemia to develop (2,13). Thereafter, fasting blood glucose levels of the animals were measured with One Touch Ultra Mini Glucometer (LifeScan Inc. Milpitas, CA, USA). Animals with blood glucose ≥ 250mg/dl were considered hyperglycemic (14).

Administration of *V. amygdalina* Extract

A total of twenty adult Wistar rats (twelve surviving diabetic rats and eight normal rats) were randomly assigned into one of the following treatment groups of four animals each: control, diabetic, diabetic+*Vernonia amygdalina,* diabetic+chlorpropamide, and *Vernonia amygdalina* alone. *Vernonia amygdalina* was dissolved in distilled water and administered orally at 400 mg/kg bw/d (2), while chlorpropamide (Neimeth Int'l Pharm, Lagos, Nigeria) was given orally at 14.3 mg/kg b.w/d (2). Administration of both drugs was for 42d and was done at 9:00–10:00 hr each day.

Blood Glucose, Feed Intake and Body Weight

A One Touch Ultra Mini Glucometer (LifeScan Inc., Milpitas, CA, USA) was used to estimate the blood glucose of treated and control animals. Blood was obtained from the dorsal vein of the animals. Blood glucose was estimated at day 0 and at 1, 3, 5, and 7 hours after the first dose of the extract. Thereafter, measurement was done twice a week for six weeks. Feed intake was monitored on a daily basis during the experimental period. Body weight of the animals was also recorded twice a week.

Termination of Treatment

All animals were fasted and killed under diethyl ether anaesthesia 24 hours after the last treatment day. Blood was collected into heparinised tubes and centrifuged at 2067 for 20 min in a desktop centrifuge model 90-1 (Jiangsu Zhangji Instruments Co., China) (15). Plasma was stored at -20^0 until analysed. Laparatomy was performed on each animal and part of the liver was rinsed in normal saline and fixed in Bouin's fluid for histopathological studies. Besides, portions of the liver were homogenized in 0.1M phosphate buffer (pH 7.4) in a potter homogeniser (GPE, Bedfordshire, England). The homogenate was centrifuged at 1000 g for 10 min at 4 ^{0}C (16), and the supernatant was used to assay for G6PDH and LDH.

Tissue Processing and Enzyme Assay

The liver was fixed in cold Bouin's fluid and stained with haematoxylin and eosin to demonstrate the hepatocytes. Photomicrographs were taken with a JVC colour video digital camera (JVC, China) mounted on an Olympus light microscope (Olympus UK Ltd, Essex, UK). The levels of LDH and G6PDH liver of treated and control rats (as well as plasma LDH levels) were estimated according to the methods of Weisshaar *et al* (17) and Lohr and Waller (18), 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 student's t test. $P<0.05$ was considered statistically significant. All graphs were drawn using Microsoft Excel 2007 (Microsoft Corporation, USA).

Results

Body Weight and Liver Weight

At the end of the treatment period, change in body weight of the animals was estimated. As shown in fig. 1, untreated diabetic rats had negative body weight gain. In contrast, appreciable gain in weight occurred in normal and diabetic rats treated with the extract of *Vernonia amygdalina* (*V. amygdalina*). Table 1 shows the weight of the liver relative to the body weight of animals at 42d. In untreated diabetic rats, the liver constituted 5.3% of body weight ($P<0.05$ vs. control), as opposed to 3.52% in control rats and 3.8% in diabetic rats treated with *V. amygdalina.*

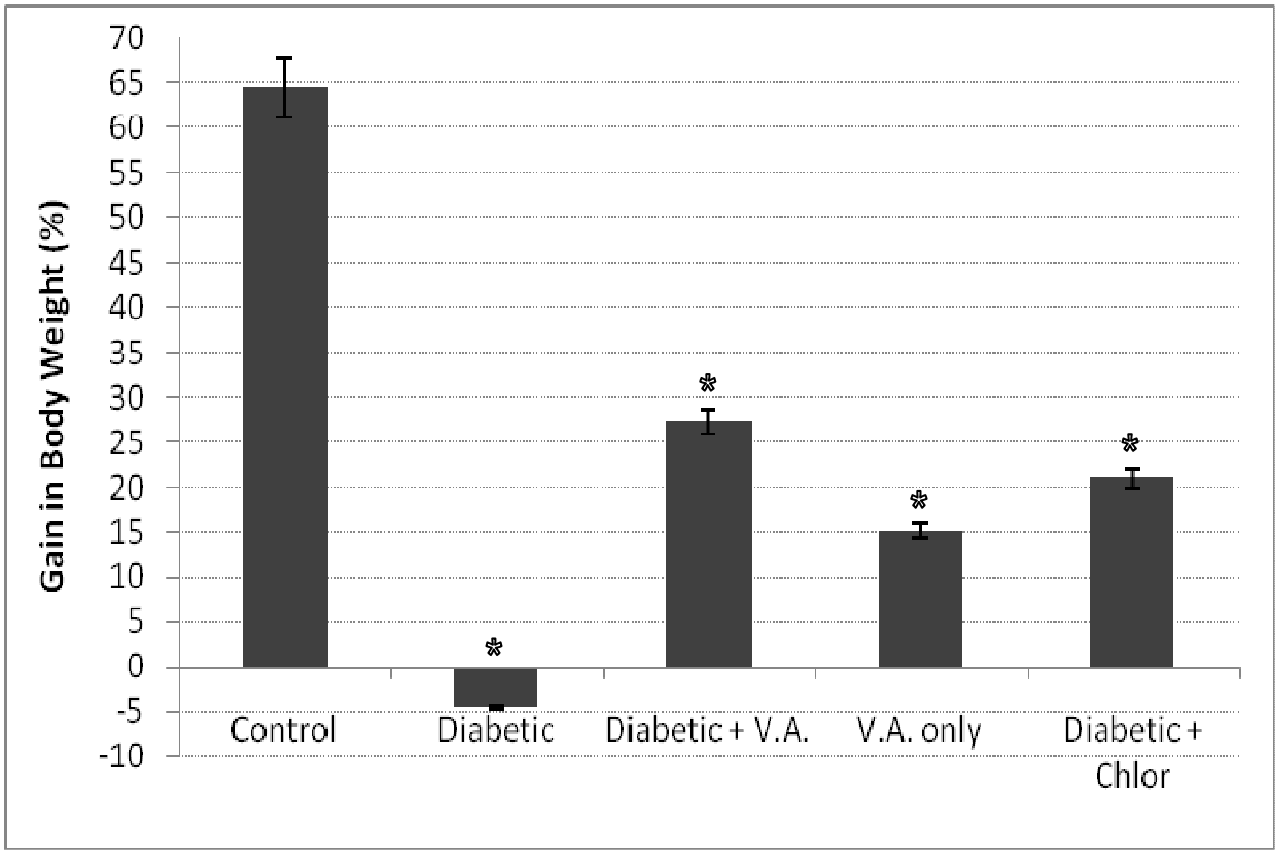

Figure 1. Change in body weights of animals. Values are Mean±SEM of four rats. *significantly different from control, $P<0.05$. (V.A. = *Vernonia amygdalina*; Chlor.=chlorpropamide)

Table 1 Relative weight of the liver (%)

Control	3.52±0.01
Diabetic	5.31±0.05*a*
Diabetic+V.A.	3.79±0.01*a*
Diabetic+Chlor.	4.85±0.02*a*
V.A. only	2.63±0.02*a*

a= P < 0.05 compared with control

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 2 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 (P<0.05) in untreated diabetic and chlorpropamide-treated diabetic rats, but significantly low (P<0.05 vs. control) in non-diabetic rats that received *V. amygdalina* alone.

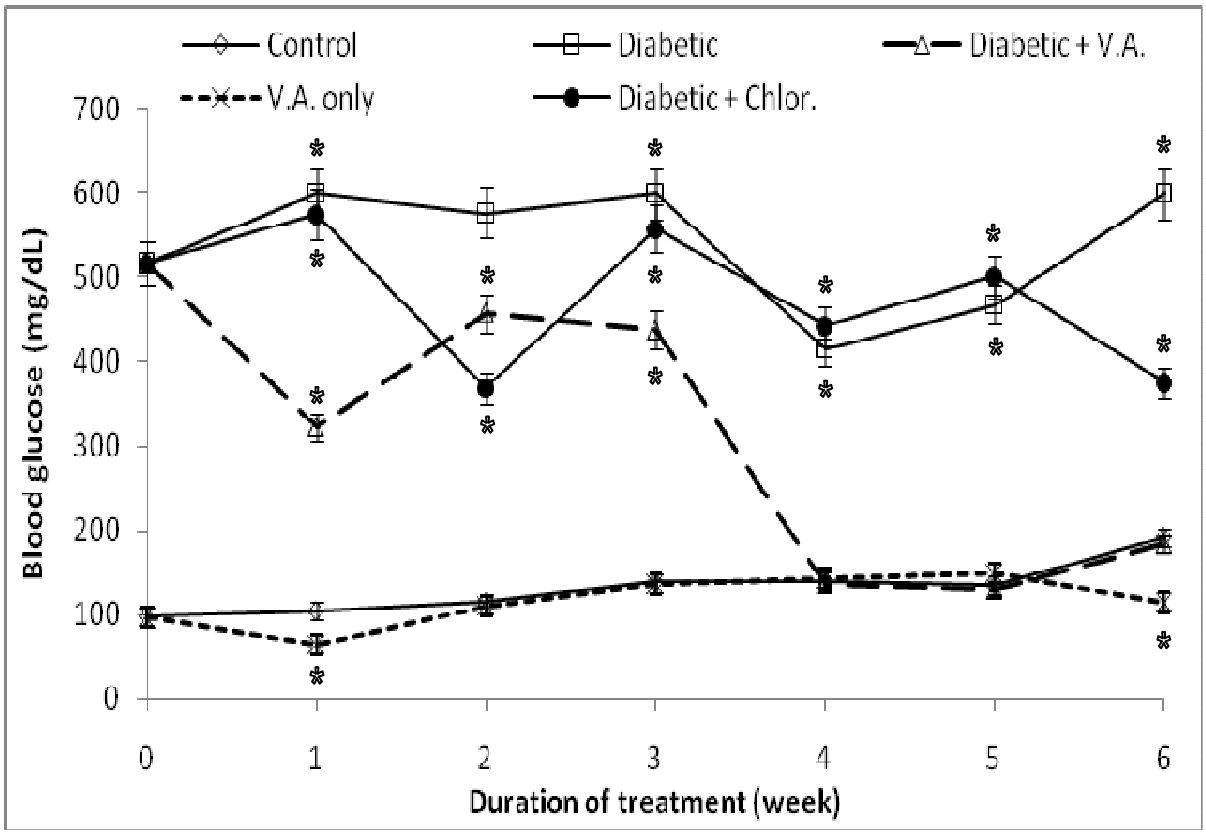

Figure 2. Blood glucose levels on a weekly basis. Values are Mean±SEM of four rats. *Significantly different from control, P<0.05. (V.A. = *Vernonia amygdalina*; Chlor. =chlorpropamide).

Hepatic and Plasma Enzyme Activity

At 42d, upregulation of G6PDH and LDH activity had occurred in the liver of untreated diabetic and treated animals (Fig. 3). In contrast, plasma levels of LDH decreased significantly ($P<0.05$) in all the treatment groups compared to control ($P<0.05$).

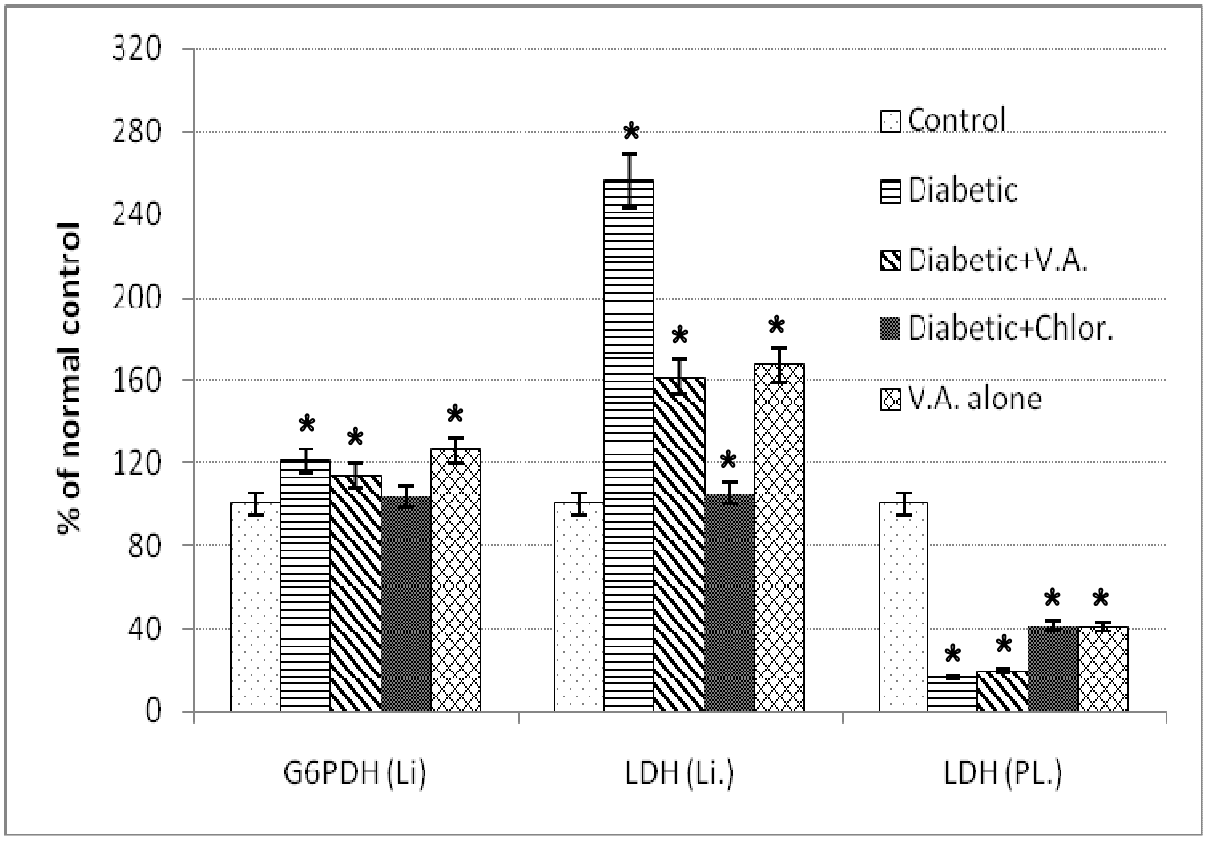

Figure 3. Hepatic levels of G6PDH and LDH, and plasma levels of LDH expressed as percentage of non-diabetic control. Values are Mean±SEM of four animals. *Significantly different from control, $P<0.05$. [G6PDH (Li) = hepatic G6PDH; LDH (Li) = hepatic LDH; LDH (PL) = Plasma LDH; Chlor. = Chlorpropamide; V.A. = Vernonia amygdalina].

Hepatic Histology

Figure 4 (A-D) shows microscopic anatomy of the livers in control and treated groups at 42d. In these groups, hepatic microanatomy was comparable to control, except in untreated diabetic rats, where hepatic sinusoids had become occluded, central veins were congested and hepatocytes appeared swollen (Fig. 4B).

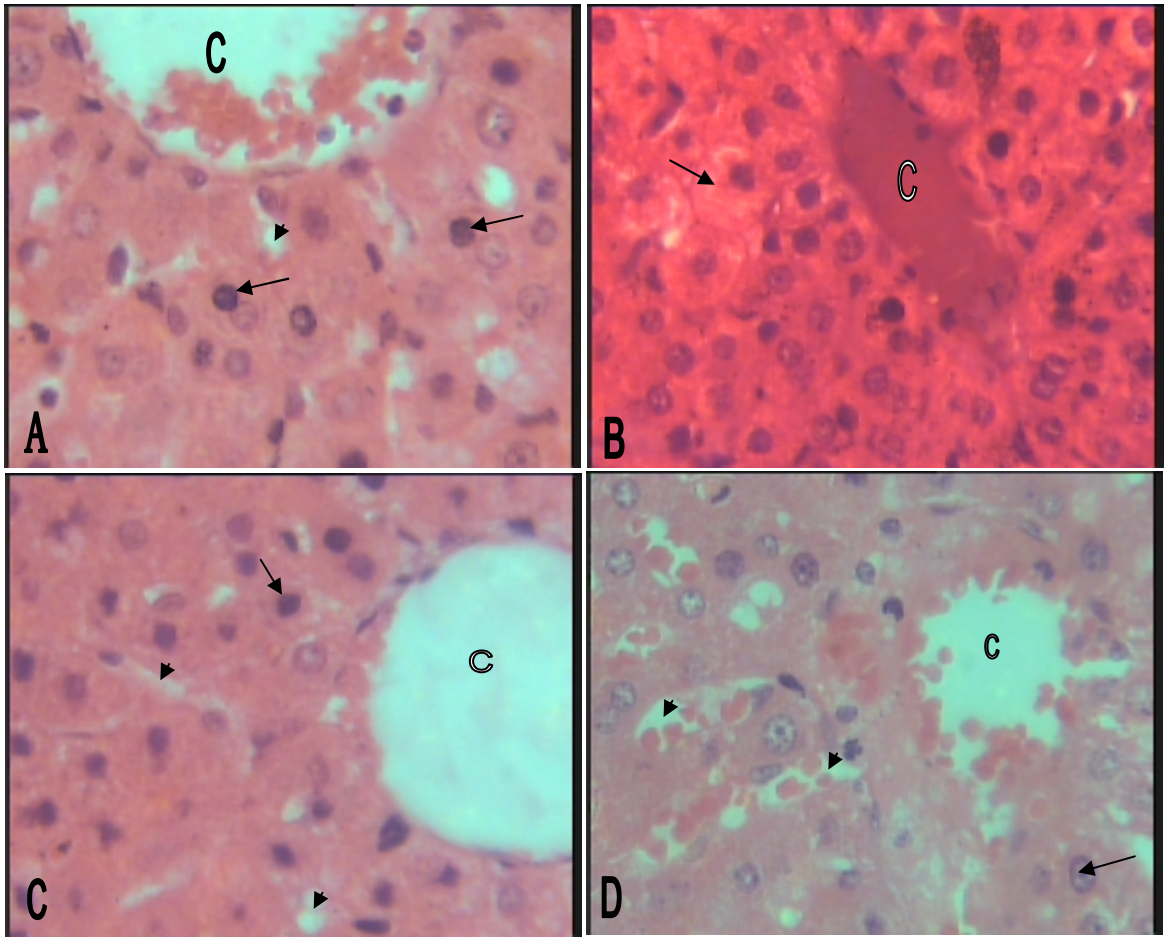

Figure 4. Liver of rats at 42d of treatment**. A,** Control group. The morphology of the liver is normal as indicated by intact central vein (c), sinusoids (arrowhead), and hepatocytes (arrows). **B,** *Diabetic group*. Sinusoids have become largely occluded, perhaps due to swollen hepatocytes (arrows); central vein (c) is also congested. **C**, *Diabetic+V. amygdalina.* The liver has normal morphology (c, central vein; arrow, hepatocyte; arrowheads, sinusoids). **D**, *Diabetic+chlorpropamide*. Hepatic morphology is comparable to control. Arrows indicate hepatocytes; arrowheads indicate sinusoids. H & E stain; x400.

Discussion

In diabetic Wistar rats, oral administration of *V. amygdalina* did not produce normoglycemia in the first day of treatment. Furthermore, hyperglycemia persisted in these animals till the end of week 3. By the end of the 3rd week however, normoglycemia was established (Fig. 2) and this was maintained till euthanasia at 42d. This finding shows that the leaf extract of *V. amygdalina* possesses hypoglycemic activity in rats when administered chronically at 400 mg/kg bw/d. This is consistent with the reports of Ebong *et al* (2), Nwanjo (12), and Akah and Okafor (19). Ebong *et al* (2) 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. These findings thus corroborate the present report.

At the moment, the mechanism of hypoglycemic activity of *V. amygdalina* is a subject of future research. However, certain flavonoids in *V. amygdalina* may confer hypoglycemic property on the leaf extract of this plant (20). According to the report of Igile *et al* (21), the leaves of *V. amygdalina* contains 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* (22). In addition, *V. amygdalina* leaves had been reported to contain bioactive sesquiterpene lactones such as vernolide and vernodalol (23). 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* (24) showed that flavonoid such as quercetin improves hyperglycemia and islet morphology in STZ-induced diabetic rats. Besides, Adewole and Caxton-Martins (25) 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 may play significant roles in the establishment of normoglycemia in diabetic rats. Similar inference had been drawn by Ojewole (26).

In the present study, *V. amygdalina* did not produce normoglycemia in diabetic rats until the end of week 3 of treatment (Fig. 2); and this suggests that viable β cells may be required for the hypoglycemic effect of *V. amygdalina.* This observation is further substantiated by our finding in non-diabetic rats treated with *V. amygdalina*. In these animals, pancreatic islets were comparable to control (fig. not shown), and blood glucose levels were significantly lower than control at week 1 (Fig. 2). This finding 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 owing to significant reduction in β-cell mass, 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 3, hepatic LDH activity in diabetic and non-diabetic rats treated with *V. amygdalina* had increased significantly at 42d ($P<0.05$ vs. control). This finding suggest that chronic exposure to *V. amygdalina* upregulates the glycolytic enzyme LDH, thereby promoting glucose disposal via increased glycolysis in hepatocytes. However, Upregulation of hepatic LDH in untreated diabetic rats was associated with fasting hyperglycemia. This suggests that pyruvate, generated from enhanced glycolysis in these animals, was reduced to lactate, and that the latter served as a substrate for gluconeogenesis, thereby contributing to glucose output from the liver. In diabetes mellitus, one factor that contributes to chronic hyperglycemia is increased hepatic glucose output via gluconeogenesis (28). However, in diabetic rats treated with *V. Amygdalina*, although hepatic LDH was also significantly upregulated at 42d, the animals were normoglycemic. Upregulation of hepatic LDH in these animals, in the absence of hyperglycemia, suggests that lactate was not serving as substrate for gluconeogenesis in the liver. One possibility is that LDH facilitated the conversion of lactate to pyruvate; and the latter was oxidised in the tricarboxylic acid cycle. Such probable effect of *V. amygdalina* will thus result in significant fall in glycemia, as reported in this study.

Furthermore, figure 3 shows upregulation of G6PDH in the liver of diabetic and non-diabetic rats treated with the leaf extract of *V. amygdalina*. Glucose 6 phosphate dehydrogenase is the

first and rate-limiting enzyme in the hexose monophosphate shunt (HMS) pathway in which glucose is the substrate (29). Thus, hepatic upregulation of this enzyme, as observed in this study (Fig. 3) would result in increased disposal of glucose via the HMS pathway, and this could partly account for the normoglycemia reported in *V. amygdalina*-treated diabetic rats from the end of week 3 of treatment. Future study may also consider the assessment of LDH ad G6PDH activity in skeletal muscles cells of diabetic rats treated with *V. amygdalina* leaf extract.

In untreated diabetic rats, significant loss in body weight had occurred at the end of six weeks of chronic hyperglycemia (Fig. 1). Loss of body weight is a feature of diabetes mellitus in human, and it is owing to depletion of body adiposity as a result of marked reduction in plasma levels of insulin (30). In contrast, body weight loss was not as marked in *V. amygdalina*-treated rats compared to untreated diabetic animals. In *V. amygdalina*-treated rats, up to 27% increase in body weight was recorded, in contrast to –4% gain 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 ingested food are taken up and metabolized by body cells, and excess caloric intake is stored as increased adipose tissue, thereby leading to increased adiposity and body weight gain (30).

However, although body weight gain in diabetic and non-diabetic rats treated with *V. amygdalina* was higher than what obtained in untreated diabetic animals, it was less than the control value (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. Such anorexic effect of *V. amygdalina* may be due to increased production of the hormone leptin by adipocytes. The present data and observation suggest 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 (31). The report of Bjorbaek *et al* (32) 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).

Microscopic study of haematoxylin and eosin-stained sections of the liver in untreated diabetic rats shows extensive occlusion of the sinusoids (Fig. 4B). This suggests swelling of hepatocytes; and may explain the significant increase ($P<0.05$) in liver weight in these rats at 42d (Table 1). In diabetes mellitus, swelling of hepatocytes could arise from accumulation of glycogen in these cells – a condition referred to as hepatic glycogenosis of diabetes mellitus (or glycogenic hepatopathy). Glycogenic hepatopathy, recently reported as under-recognised in diabetic patients with hepatomegaly, is a common complication in type 1 diabetics whose blood glucose is poorly-controlled with insulin (3,33). Up to 80% of type 1 diabetes patients could be affected (34). In such patients, marked accumulation of glycogen occurs in hepatocytes when insulin treatment leads to increased uptake of glucose by hepatocytes, followed by rapid conversion of glucose to glycogen, and subsequently, trapping of the latter (35). Apart from diabetes, hepatic glycogenosis could also occur in Mauriac's syndrome (36), glycogen storage disease (37), and following short-term high dose steroid therapy (38). In this work, the condition may arise from upregulation of glycogen synthase. This enzyme had been shown to be upregulated in alloxan- and streptozotocin-induced diabetes (39,40). In the latter model, total glycogen synthase was reported to be significantly higher than control 40d after induction of diabetes. Besides, the work of Ferrannini *et al* (41) showed that longstanding insulin deficiency might actually enhance glycogen synthase activity. Future

studies may thus consider diastase-controlled staining of liver sections in period acid-Schiff (to test the presence of glycogen in hepatocytes) as well as assay for the activity of glycogen synthase in the liver.

In contrast to findings in diabetic rats, morphological (and biochemical) studies suggest that chronic administration of *V. amygdalina* to rats is not associated with liver injury. As shown in fig. 4, hepatic microanatomy of *V. amygdalina*-treated animals was comparable to control. Besides, plasma levels of LDH (Fig. 3) suggest that oral administration of *V. amygdalina* to diabetic rats does not produce cytotoxicity.

In conclusion, data from the present study show that chronic treatment with ethanolic leaf extract of *V. amygdalina* (i) produces normoglycemia in hyperglycaemic rats, (ii) possesses no adverse effects on hepatic morphology and (iii) is not cytotoxic. Thus, alternative and complimentary approach to the management of diabetes mellitus in human may thus include the use of the leaf extract of *V. amygdalina* as an antidiabetic therapy.

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