Effects of extract of leaves of Newbouldia laevis on the activities of some enzymes of hepatic glucose metabolism in diabetic rats

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In diabetes mellitus and insulin resistance, the activity of the enzymes involved in hepatic glucose homeostasis is disturbed. Promoting the physiologic functions of these enzymes and restoring homeostatic control by medicinal plants could be beneficial in the management of diabetes. This study investigates the effects of extract of leaves of Newbouldia laevis on some key enzymes of hepatic glucose metabolism in streptozotocin-induced diabetic rats. Diabetes was induced in rats by intravenous injection of streptozotocin. Diabetic rats were treated orally with N. laevis extract for four weeks. At the end of the treatment, the activities of hepatic glucokinase and glucose 6-phosphatase were evaluated. Effect of N. laevis extract on glucose 6-phosphatase activity was also assessed in vitro using enzyme obtained from rabbit liver. The levels of hepatic glycogen, pancreatic insulin and serum insulin were also determined. Treatment of diabetic rats with N. laevis extract resulted in a significant increase ($P < 0.05$) in the activity of hepatic glucokinase when compared with diabetic control. Extract of N. laevis showed significant inhibitory effect against the activity of glucose 6-phosphatase in both in vivo and in vitro studies. The level of hepatic glycogen in treated rats significantly increased ($P < 0.05$) compared to untreated diabetic rats. Although there was a slight increase in serum and pancreatic insulin levels of treated diabetic rats, the difference was not significant ($P > 0.05$) when compared to diabetic control. Based on the results of this study, we conclude that N. laevis leaf extract stimulates the activity of hepatic glucokinase and inhibits the activity of glucose 6-phosphatase in streptozotocin-induced diabetic rats. It could serve as a good alternative remedy in the management of diabetes mellitus.

Key words: Liver, glucokinase, glucose 6-phosphatase, diabetes mellitus, Newbouldia laevis, glucose.

INTRODUCTION

Liver, in synergy with peripheral tissues, plays an important role in the regulation of blood glucose levels. It is estimated that liver absorbs one third of the postprandial glucose and effectively convert glucose into glycogen for storage through the process of glycogenesis (Moore et al., 2012). Liver maintains stable blood glucose levels in the fasting state by producing glucose through the processes of glycogenolysis and gluconeogenesis.
The key enzymes that play prominent roles in hepatic glucose homeostasis include glucokinase and glycogen synthase which catalyze the process of glycogenesis; and the major enzymes responsible for the regulation of gluconeogenesis are glucose 6-phosphatase, pyruvate carboxylase, phosphoenolpyruvate carboxykinase and fructose 1, 6-bisphosphatase (Eid et al., 2006). Hepatic glucose homeostatic control is also influenced at the hormonal level. The key hormones involved in glucose homeostasis are insulin and glucagon. When there is an increase in blood glucose concentration, insulin release is increased and glucagon release is suppressed. As a result, glucokinase is stimulated, glycogen synthase is activated and glycogen phosphorylase is inhibited leading to enhanced glycogen synthesis. On the other hand, glucose 6-phosphatase catalyzes the dephosphorylation of glucose 6-phosphate to glucose as the terminal step in gluconeogenesis and glycogenolysis (Dentin et al., 2007). In type 2 diabetes and insulin resistance, the control of hepatic glucose metabolism is disturbed and this leads to increased hepatic glucose output and hyperglycemia. Therefore targeting the pathways of these hepatic enzymes could lead to significant reduction of blood glucose level in type 2 diabetic patients (Tahrani et al., 2011; Agius, 2007). One way through which this could be achieved is to investigate the effects of medicinal plants on these pathways.

For centuries, medicinal plants have been employed in the management of diabetes mellitus and are well recognized as important source of new drugs. Therefore, in the search for novel anti-diabetic drugs, the potentials of medicinal plants should be diligently explored (Newman and Cragg, 2007). Such efforts have yielded positive results in the past. For instance, metformin is an anti-diabetic drug derived from *Galega officinalis*, a plant prescribed in medieval times to relieve the symptoms of diabetes (Andrade-Cetto, 2012). Metformin lowers blood glucose levels without causing overt hypoglycemia or stimulating insulin secretion. It reduces fasting plasma glucose by 25% by suppressing hepatic gluconeogenesis from substrates such as pyruvate, lactate, glycerol and amino acids. It also modulates mitochondrial respiration by increasing intramitochondrial level of calcium ions (Jaya et al., 2010). There are other plants that are used in the treatment of diabetes which may contain principles that have similar or even better therapeutic effects than metformin. Many of these plants, including *Newbouldia laevis*, have not been subjected to proper scientific investigation. *N. laevis* is an angiosperm which belongs to the Bignoniaceae family. Its common names are ‘Fertility tree’ and ‘African border tree’. The extract of the leaves has been reported to lower blood glucose level in diabetic rats (Owolabi et al., 2011). It was also shown to attenuate glycation of hemoglobin and lipid peroxidation in diabetic rats (Kolawole et al., 2013a). In the present study, we investigated the effects of extract of the leaves of *N. laevis* on insulin secretion and the activities of glucokinase and glucose 6-phosphatase in streptozotocin-induced diabetic rats.

**MATERIALS AND METHODS**

**Preparation of plant extract**

Leaves of *N. laevis* were collected from the premises of Government House Annex, Osogbo, Nigeria. The leaves were identified and authenticated by Mr. Odowo of Forest Research Institute of Nigeria (FRIN) and a voucher specimen was deposited in the herbarium of the institute (voucher specimen no: FHI 107753). After the leaves have been thoroughly washed with clean distilled water, they were air-dried under shade in the laboratory for 5 days and then pulverized using an electric grinding machine. The powder sample (400 g) was extracted with 80% ethanol at 70°C by continuous hot percolation using a Soxhlet apparatus. The extraction was carried out for 24 h and the resulting ethanol extract (NdEt) was concentrated at 40°C in a rotary evaporator. The solid sample obtained weighed 40.2 g (yield = 10 %). The crude ethanol extract was kept in an air-tight container and stored in a refrigerator at 4°C until the time of use.

**Experimental animals**

Male Wistar rats weighing 150 to 180 g were obtained from the Animal Holding Unit of the Department of Pharmacology and Therapeutics, Ladoke Akintola University of Technology (LAUTECH), Nigeria. The animals were housed in polypropylene cages in a well-ventilated area in the laboratory complex. The animals were maintained under standard laboratory conditions of temperature (22 ± 2°C), relative humidity (55-65%) and 12 h light/dark cycle. They were allowed to acclimatize for two weeks before the experiment. During the experimental period, animals were fed with a standard balanced commercial pellet diet (Ladokun Feeds Ltd. Ibadan, Nigeria) and potable tap water *ad libitum*.

**Ethical consideration**

All experimental procedures were conducted in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH, 1985) as well as Ethical Guidelines for the Use of Laboratory Animals in LAUTECH, Nigeria.

**Induction of diabetes mellitus**

Experimental diabetes was induced in rats which had fasted for 12 h by a single intravenous injection of a freshly prepared solution of streptozotocin (STZ) (60 mg/kg body weight) dissolved in 0.1 M cold citrate buffer, pH 4.5 (Chen et al., 2005). The rats were allowed to drink 5% glucose solution overnight to overcome drug-induced hypoglycemia. Estimation of fasting blood glucose (FBG) was done 72 h after injection of STZ to confirm induction of diabetes and then on the 7th day to ensure a stable diabetic condition. Fasting blood glucose was estimated by One Touch® glucometer (Lifescan, Inc. 1995 Milpas, California, USA). Blood sample for the FBG determination was obtained from the tail vein of the rats and those with blood glucose value ≥ 200 mg/dl were selected for the study.

**Treatment of experimental animals**

Rats were divided into a group of non-diabetic and three groups of STZ - diabetic rats. Each of the four groups consisted of six rats. The
grouping was as follows: Group I = non-diabetic control; Group II = diabetic control; Group III = diabetic rats treated with NLet (500 mg/kg); Group IV = diabetic rats treated with metformin (200 mg/kg) or glibenclamide (5 mg/kg). The drugs were administered orally everyday for 28 days using a sterile syringe fitted with a sterile cannula. Rats in groups I and II were treated orally with distilled water for the four weeks. On Day 29, the rats were euthanized under chloroform vapor. The jugular vein was exposed and cut with a sterile scalpel blade, and the rats were bled into specimen bottles. Blood samples were transferred to sterilized centrifuge tubes and allowed to clot at room temperature. The blood samples were centrifuged for 10 min at 1500 rpm. The serum obtained was used for serum insulin assay.

Tissue preparation

After 28 day treatment with the plant extract (NLet) and the reference drugs, the rats were sacrificed by cervical dislocation. Segments of the liver and pancreas tissues were excised separately from rats in all the experimental groups. The tissues were washed with phosphate buffered saline (pH 7.4) containing 0.16 mg/ml of heparin to remove any red blood cells (erythrocytes) and clots (Prasad et al., 1992). Liver and pancreas homogenates were prepared and stored at -20°C for the assays. Liver homogenate was used for glucokinase, in vivo glucose 6-phosphatase and hepatic glycogen assays while pancreas homogenate was used for the pancreatic insulin assay.

Glucokinase assay

Glucokinase activity was measured as previously described (Zhang et al., 2009; Davidson and Arion, 1987). About 100 mg liver tissue was homogenized in 1 ml ice-cold homogenization buffer containing 100 mM KCl, 25 mM HEPES, 7.5 mM MgCl2, 4 mM dithiothreitol (pH 7.4), and then lysed overnight at 4°C. Supernatant of the tissue extract was obtained after centrifugation at 3000 rpm for 10 min at 4°C, and then supplemented with 1 mM NAD, 4 mM ATP, and 100 or 0.5 mM glucose at pH 7.4. The enzymatic reaction was started by the addition of 0.2 unit of glucose 6-phosphate dehydrogenase and incubated for 10 min at 30°C. NADH generated by glucokinase was measured by a spectrophotometer at 340 nm. The enzymatic activity of glucokinase was calculated as the difference between activities calculated at 100 and 0.5 mM glucose. Hexokinase activities were calculated as μM/mg protein. Protein concentration in liver extract was measured by Lowry method (Lowry et al., 1951).

In vivo glucose 6-phosphatase assay

Glucose 6-phosphatase (G6Pase) activity was assayed according to the method of Baginsky et al. (1974) by estimation of inorganic phosphate (P) liberated from glucose 6-phosphate (G6P). The ultracentrifugation method of Pari and Satheesh (2006) was used to prepare liver microsomal fractions. For this assay, 1 g of frozen liver tissue was homogenized in ice-cold sucrose solution with a Polytron homogenizer. The homogenate was centrifuged sequentially at 11,000 g for 30 min, then at 105,000 g for 1 h using an ultracentrifuge (Beckman Inc., CA, USA). The solid pellet was re-suspended in ice cold sucrose/EDTA solution and used as the source of the enzyme. Tubes were divided into samples, blanks and standard. To each were added 0.1 mL of sucrose/EDTA buffer (0.25 mM, pH 7.0), 0.1 mL of G6P (100 mM), and cadoxylate buffer solution. This was followed by the addition of 0.1 mL of sample to the sample tube, 0.1 mL of sucrose/EDTA solution to the blank and 0.1 mL of different concentration of K2HPO4 (0.5, 1, 1.5 and 2 mM) to the standard tube. All tubes were incubated at 37°C for 15 min and the enzyme activity was then terminated by adding 2 mL TCA/ascorbate (10%/2%). The tubes were centrifuged at 3000 g for 10 min. To 1.0 mL of this clean supernatant were added 0.5 mM ammonium molybdate (1%) and 1 mL of Na-arsenite/Na-citrate (2%/2%). The tubes were then allowed to stand for 15 min at room temperature and absorbance was read at 840 nm. The amount of inorganic phosphate liberated by the enzyme was calculated by comparing with absorbance values of the standard. G6Pase activity was expressed as units/mg of protein. One unit of glucose-6-phosphatase activity is defined as the amount of P, liberated/min at 37°C under the assay conditions.

In vitro glucose 6-phosphatase assay

The effect of NLet on glucose 6-phosphatase activity was performed using glucose 6-phosphatase from rabbit liver (Sigma, USA). The method of Baginsky et al. (1974) was adapted. Fifty microlitres (50 μL) of each concentration of the plant extract, sodium orthovanadate or metformin were transferred into separate test tubes followed by 100 μL of enzyme solution (glucose 6-phosphatase in 0.25 M sodium acetate buffer, pH 6.7). The mixture was then pre-incubated in a water bath at 37°C for 20 min. Thereafter 100 μL of the substrate solution (glucose 6-phosphate in 0.25 M sodium acetate buffer pH 6.7) was added into each of the test tube to start the reaction. The mixture was again incubated at 37°C for 15 min When the reaction was complete, 500 μL of Molybdate reagent (2 g of (NH4)2Mo7O24H2O in 2.45 M H2SO4 w/v) and 500 μL of reducing agent (1.0% Elon (p-methyl aminophenol sulphate) in 3.0% sodium bisulphites (NaHSO3) w/v) were added. The intensity of the blue solution was measured with a spectrophotometer at 660 nm. Glucose 6-phosphatase inhibitory activity was expressed as percentage inhibition. The graph of percentage inhibition was plotted against extract concentration.

Hepatic glycogen assay

Hepatic glycogen content was measured according to the method of Postle and Bloxham (1980). Frozen tissue (100 mg) was placed in chilled citrate buffer (0.1 M, pH 4.5) and extracted with 1.5 mL of 6% (w/v) perchloric acid (HClO4). This was then centrifuged at 2000 g for 15 min and the supernatant (0.5 mL) was neutralized with 10% (w/v) KOH. The glycogen in the supernatant was hydrolyzed by α-amylglucosidase (50 U/mL; Sigma, USA) in sodium acetate buffer (50 mM/L; pH 4.8) overnight at room temperature. Glucose released from glycogen was estimated by glucose assay kit (Sigma, USA). The glycogen content of the liver samples was calculated as the difference between the glucose level with and without amylglucosidase incubation. Glycogen content was expressed as mg/g wet tissue.

Insulin assay

Serum insulin level was measured by an enzyme-linked immunosorbent assay (ELISA) procedure using rat insulin ELISA kit (Mercodia, USA). Briefly, the solid phase two-site enzyme immunoassay is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microtitration well. After washing three times, unbound enzyme labeled antibody was removed. The bound conjugated insulin was detected by reacting with 3, 3’, 5, 5’-tetramethylbenzidine. The reaction was stopped by adding acid to give a colorimetric end-point and optical density was measured with a microplate autoreader at a wavelength of 450 nm.
The serum insulin was expressed as μg/L. The same procedure was followed for pancreatic insulin assay using supernatant of pancreas homogenate as sample. The results were expressed as ng/g wet weight of tissue.

**Phytochemical analysis**

Tests were carried out on the extract using standard procedures to identify its phytoconstituents as described by Trease and Evans (2002), Harborne (1984), and Sofowora (1993). The extract was tested for alkaloids, flavonoids, tannins, terpenoids, saponin, phenolic compounds and cardiac glycosides.

**Statistical analysis**

Data obtained from the experiments are expressed as mean ± standard error of mean (SEM). For statistical analysis, data were subjected to one-way analysis of variance (ANOVA) followed by Student’s t-test. A level of \( P < 0.05 \) was taken as significant. GraphPad Prism version 5.0 for windows was used for these statistical analyses (GraphPad software, San Diego California USA).

**RESULTS**

**Hepatic enzymes**

The activity of glucokinase was significantly reduced in STZ-diabetic rats compared to nondiabetic control. Treatment of diabetic rats with NLet and metformin resulted in a significant increase \( (P < 0.05) \) in the activity of hepatic glucokinase when compared with diabetic control as shown in Figure 1. *In vivo*, G6Pase activity of STZ-diabetic rats was significantly increased \( (P < 0.05) \) compared to that of non-diabetic control. Treatment of diabetic rats with NLet and metformin for 28 days caused significant decrease \( (P < 0.05) \) in the activity of G6Pase compared to diabetic control (Figure 2). *In vitro* study also demonstrated that NLet has inhibitory effect against the activity of glucose 6-phosphatase in rabbit liver (Figure 3). The inhibitory activity of NLet \( (IC_{50} = 752.8 \, \mu g/ml) \) was about half that of sodium orthovanadate \( (IC_{50} = 355.4 \, \mu g/ml) \). Metformin failed to inhibit rabbit glucose 6-phosphatase *in vitro*.

**Hepatic glycogen content**

Hepatic glycogen level was significantly decreased \( (P < 0.05) \) in diabetic rats compared to non-diabetic control. After treating the animals with NLet \( (500 \, mg/kg) \) and metformin \( (200 \, mg/kg) \) for 28 days, the level of hepatic glycogen in both NLet-treated and metformin-treated groups significantly increased \( (P < 0.05) \) compared to diabetic control (Figure 4). However there was a significant difference \( (P < 0.05) \) between NLet-treated and metformin-treated groups.

**Serum and pancreatic insulin levels**

Serum and pancreatic insulin levels were significantly decreased \( (P < 0.05) \) in diabetic control rats compared to the non-diabetic group. Although there was a slight
**Figure 2.** *In vivo* effect of *N. laevis* extract on rat liver Glucose 6-phosphatase activity. Values are mean ± SEM (n = 6). *P* < 0.05 compared with diabetic control rats; †*P* < 0.05 compared with non-diabetic control. NDC = Non-diabetic control; DC = Diabetic control; TD = Treated diabetic rats.

**Figure 3.** *In vitro* effect of *N. laevis* extract on rabbit liver glucose 6-phosphatase activity. Values represent mean ± SEM of three replicates.
increase in both serum and pancreatic insulin levels of NLet–treated diabetic rats, the difference was not significant (\( P > 0.05 \)) when compared to diabetic control as shown in Figures 5 and 6, respectively.

**Phytochemical screening**

The results of the analysis show that the crude ethanolic extract of *N. laevis* leaves contain tannins, saponin,
flavonoids, terpenoids and phenolic compounds. Cardiac glycosides and alkaloids were not detected. The results of the analysis are presented below (Table 1).

**DISCUSSION**

Glucokinase is one the enzymes that play key role in hepatic glucose homeostasis. It catalyzes the rate limiting step of glycolysis by phosphorylating glucose leading to the synthesis of glucose -6-phosphate (Postic et al., 2004). In pancreatic β-cells, glucokinase acts as a glucose sensor, ensuring that insulin release is appropriate to the plasma glucose concentration. In the liver, glucokinase is essential for this tissue to carry out its unique metabolic functions (Agius, 2008). Under basal glucose concentrations (< 5.5 mM), hepatic glucokinase is bound to the glucokinase regulatory protein (GKRP) in
the nucleus (Zimmet et al., 2005). However, when subjected to high glucose concentration (10-30 mM), glucokinase is released from the GKRP into the cytoplasm where it exists in the unbound active form (Alberti et al., 2005). Impairment of glucokinase activity plays a major role in precipitating glucose intolerance and the development of diabetes mellitus. Glucokinase gene mutation has been associated with a form of diabetes known as maturity onset diabetes of the young (Zhang et al., 2009).

In the present study, the activity of glucokinase significantly decreased in STZ-diabetic rats. In the NLet-treated diabetic rats, there was an increase in the activity of this enzyme which was significant ($P < 0.05$) when compared with the diabetic control. This is probably a reflection of the slight increase in plasma insulin concentration following treatment with NLet. Glucokinase activity is impaired in pancreas and liver following the damage caused by free radicals that are generated by streptozotocin in diabetic rats (Zhang et al., 2009; Srinivasan and Ramaraoc, 2007). Therefore the observed effects of the extract could also be due to direct activation of glucokinase. Activators of glucokinase have been reported to enhance the activity of the enzyme in drug-induced diabetic animals (Priyadarsini et al., 2012).

Glucose 6-phosphatase (G6Pase) catalyzes the final step in both glycogenolytic and gluconeogenic pathways, cleaving phosphate from glucose 6-phosphate to liberate free glucose into the circulation (Smith et al., 2005). It is thus uniquely situated to regulate both circulating concentrations of glucose and the storage of excess glucose as glycogen. In diabetic condition, glucose 6-phosphatase activity is elevated and the symptoms associated with hyperglycemia are exacerbated. In this study, in vivo activity of G6Pase in the rat liver was significantly reduced ($P < 0.05$) in both NLet- and metformin-treated groups when compared to diabetic control group. The hypoglycemic activity of NLet reported by Owolabi et al. (2011) could be due to the suppression of G6Pase activity as reported for other hypoglycemic agents such as vanadate compounds (Mosseri et al., 2000). Normalization of serum blood glucose concentration in diabetes with insulin usually results in decreased G6pase gene expression and activity (Massillon et al., 1996). However, the same report also indicated that correction of hyperglycemia in diabetic rats leads to normalization of hepatic gene expression of G6Pase regardless of the circulating insulin concentration. This indicates that in vivo gene expression of G6Pase in the diabetic liver is regulated by glucose independent of insulin. Therefore the reduction in G6Pase activity in rat liver observed in this study after treatment with NLet may be attributed to the hypoglycemic effect due to insulin or other mechanisms independent of insulin. In vitro study also confirmed the inhibitory activity of NLet against G6Pase. The inhibitory activity of NLet was about half that of sodium orthovanadate. This result is promising especially when one takes into consideration the toxicity of sodium orthovanadate. It was earlier reported that NLet has low toxicity profile with LD$_{50}$ > 5g/kg body weight in mice (Kolawole et al., 2013b). Metformin is a well known inhibitor of G6Pase in vivo (Jung et al., 2006). However the results of this study showed that metformin failed to inhibit the activity of hepatic G6Pase in vitro. Enzyme inhibitors are known to act via different mechanisms (Lieberman and Marks, 2009). Some inhibitors induce conformational changes in the structure of an enzyme; such inhibitors could be expected to inhibit enzymes in vitro. Other enzyme inhibitors influence the activity of enzymes by suppressing the synthesis of enzymes at the genetic level, and as such will show no inhibitory effect in vitro. The result of this study suggests that metformin belongs to the latter group of inhibitors.

The results of this study indicate that hepatic glycogen level was significantly decreased in diabetic rats compared to non-diabetic control. After the 28-day treatment with NLet and metformin, the level of hepatic glycogen in both NLet-treated and metformin-treated groups significantly increased compared to diabetic control. A significant difference was also observed between NLet-treated and metformin-treated groups. This may be a reflection of insulin level in each experimental group. *Nebouldia laevis* extract appears to have very little insulinotropic effect and the stimulation of glycogen synthase activity in the NLet-treated group was not as pronounced as in the group treated with metformin. Metformin on the other hand appears to have stimulated the activities of glycogen synthase and glucokinase better than NLet. The slight increase in serum and pancreatic insulin levels observed in diabetic rats following treatment with NLet could be due to the protection of the functional β-cells from further deterioration so that they remain active and produce insulin. Studies have shown that the presence of phytochemicals such as flavonoids, phenols, saponins, tannins and terpenoids in plants are responsible for their anti-diabet ic properties (Narendrer et al., 2011; Momoh et al., 2011). Phytochemical analysis of NLet. revealed the presence of tannins, flavonoids, saponins, terpenoids and phenolic compounds, while cardiac glycosides and alkaloids were not detected. The active ingredients responsible for antidiabetic activities of NLet probably reside in one or more of these phytochemicals.

**Conclusion**

Our data indicate that extract of the leaves of *N. laevis* promotes good hepatic glucose homeostasis in streptozotocin-induced diabetic rats by enhancing the activity of hepatic glucokinase and inhibiting the activity of glucose 6-phosphatase. It could serve as a good alternative medicine in the management of diabetes mellitus.
Structural elucidation of the active components of the plant could also provide a lead molecule for the development of novel antidiabetic drug.

Conflict of Interests

The author(s) have not declared any conflict of interests

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