Sapium ellipticum (Hochst) Pax ethanol leaf extract modulates glucokinase and glucose-6–phosphatase activities in streptozotocin induced diabetic rats

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ABSTRACT

Objective: To examine the effects of *Sapium ellipticum* (*S. ellipticum*) extract on hepatic activities of glucokinase and glucose-6-phosphatase in streptozotocin (STZ)-induced diabetic Wistar rats.
Methods: STZ-induced diabetic Wistar rats (four groups, *n* = 8) were used in this study. *S. ellipticum* was assessed at two different doses, 400 and 800 mg/kg BW, in comparison with metformin (12 mg/kg BW) as a standard antidiabetic drug. All treatments were done orally (*p.o*), twice daily at 8 h interval for a period of 21 days. Glucose-6-phosphatase and glucokinase activities were respectively determined. Hepatic and muscle glycogen contents were estimated as well.
Results: STZ caused significant decrease in glucose-6-phosphatase activity and concomitant increase in glucokinase activity. *S. ellipticum* especially at 400 mg/kg significantly restored glucokinase activity by 40.31% and glucose-6-phosphatase activity by 37.29%. These effects were though significantly lower than that of metformin which enhanced glucokinase activity by 94.76% and simultaneously inhibited glucose-6-phosphatase activity by 49.15%. *S. ellipticum* also improved hepatic glycogen level by 32.37% and 27.06% at 400 and 800 mg/kg, respectively. HPLC-MS analysis of active *S. ellipticum* fractions in dynamic MRM mode (using the optimized compound-specific parameters) revealed among other active compounds, the presence of amentoflavone, which has been associated with anti-diabetic function.
Conclusions: The ability of *S. ellipticum* extract to concurrently inhibit glucose-6-phosphatase and activate glucokinase in this study suggests that it may be a treatment option for type 2 diabetes patients and the presence of amentoflavone in the plant extract may account for its anti-diabetic potential.

1. Introduction

Fasting hyperglycaemia in all forms of diabetes mellitus occurs primarily as a result of upsurge in hepatic glucose synthesis (HGS) [1,2]. Two specific enzymes, glucokinase (GK) and glucose-6 phosphatase (Glu-6-Pase) play crucial role in hepatic glucose production, utilization and homeostasis[3-7]. These enzymes catalyze very important enzymatic steps in the regulatory pathways of glucose in the liver. Glu-6-Pase catalyzes the final step of glycogenolysis and gluconeogenesis in which Glu-6-Pase is hydrolysed to yield glucose and phosphate[7]. Glu-6-Pase activity is markedly increased in insulin-deficient diabetic rats or during short period of fasting[2].

Conversely, GK activity, which allows the liver to utilize glucose, is decreased during fasting. It catalyzes the rate limiting step of glycolysis by phosphorylating glucose to Glu-6-Pase. GK also functions as a glucose sensor in ensuring appropriate secretion and release of insulin vis-à-vis plasma glucose concentration. The enzyme is activated by high plasma glucose concentration (> 7.5 mmol/L) and becomes deactivated when the glucose level drops to normal level (< 5.5 mmol/L)[8]. These observations indicate that both GK and Glu-6-Pase are important regulators of HGS in diabetic conditions. Compounds that modulate the activity of these enzymes have been reported to enhance their regulatory function in drug-

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induced diabetes in animal model[4,9], and consequently contribute to the management of diabetes mellitus.

Sapium ellepticum (Hochst) Pax (*S. ellepticum*) enjoys huge therapeutic applications in the local treatment of a number of diseases[10,11], including diabetes (ethno-botanical survey). It belongs to the family Euphorbiaceae and is commonly referred to as jumping seed tree. *S. ellipticum* is widely distributed in eastern and tropical Africa. In southwest part of Nigeria, particularly among the Ilorin indigenes, the plant is popularly known as aloko-ạgbọ.

A few scientific investigations have been carried out on it. Adesegun *et al.*[12] in their *in vitro* study credited substantial antioxidant properties to the stem bark extract of the plant. Cytotoxicity screening of selected Nigerian plants used in traditional cancer treatment on HT29 (colon cancer) and MCF-7 (breast cancer) cell lines (HeLa cervix adenocarcinoma cells) indicated that *S. ellipticum* leaf extract expressed the highest cytotoxic activity among other plants with anticancer potential which was comparable to the reference drug, ciplastin[13]. The phythochemical constituents, *in vitro* antioxidant capacities and antiplasmodial activities of *S. ellipticum* stem bark extracts were documented by Nana *et al.*[10]. Edimealem *et al.*[14] in their study demonstrated the presence of lupeol, lupeol acetate and stigmasterol in the stem bark extract of *S. ellipticum*. This present study investigated the effects of the plant leaf extract on glucose metabolizing enzymes such as glucokinase and glucose-6-phosphatse.

2. Materials and methods

2.1. Collection of S. ellipticum leaves

Fresh *S. ellipcitum* leaves were harvested in the month of December, 2012 from a forest in a suburb of Ibadan, southwest of Nigeria. The harvested leaves were taxonomically authenticated by a botanist (Mr. TK Odewo) at the Lagos University Herbarium (LUH), Nigeria, and a specimen was deposited and assigned a voucher number of LUH 5423.

2.2. Preparation of S. ellipticum leaf extracts

The plant material was free of extraneous materials, air dried at room temperature and milled into a fine powder using a Waring blender. A total of 300 g powdered sample was macerated in 2.5 L extracting solvent (ethanol). The mixture was allowed to stand for 72 h and stirred intermittently with a glass rod to facilitate extraction. Sieving of the mixture was achieved with a muslin cloth (maximum pore size 2 mm). The resulting filtrate on sieving was further filtered through Whatman filter paper No. 42 and subsequently reduced in volume with a rotary evaporator at 40 °C. Final elimination of solvent and drying was done using a regulated water bath at 40 °C.

2.3. Induction of diabetes mellitus with streptozotocin in experimental rats

Single intraperitoneal (*i.p.*) dose (55 mg/kg BW) of freshly prepared streptozotocin (STZ) was administered to a batch of normoglycaemic rats starved for 16 h.

2.4. Experimental design and management of animals

Eight normal animals constituted a control group (Group 1). Thirty-two STZ-treated Wistar rats were randomly assigned to four groups (Groups 2, 3, 4 and 5) containing eight animals each. Animals in Group 1 were administered olive oil (0.5 mL) and served as normal control. Group 2 animals were left untreated and served as diabetic control. Groups 3 and 4 were respectively treated with 400 and 800 mg/kg BW of *S. ellipticum* and the last group was treated with metformin (12 mg/kg BW), a reference anti-diabetic drug. All treatments were done orally (*p.o.*), twice daily at 8 h interval for a period of 21 days. All procedures for maintenance and sacrifice (care and use) of animals were carried out according to the criteria outlined by the National Academy of Science published by the National Institute of Health[15] and approved by the Ethical Committee of the College of Bioscience, Federal University of Agriculture, Abeokuta.

2.5. Preparation of tissue homogenate and post-mitochondrial fraction (PMF)

At the end of 21 days of treatments, the rats were fasted over night and sacrificed by cervical dislocation. The liver of each rat was harvested, rinsed with ice-cold 1.15% KCl solution, blotted and immediately suspended in an appropriate homogenization buffer depending on the assay.

2.5.1. Estimation of hepatic glucokinase activity in rats
Glucokinase activity was measured as described by Zhang *et al.*[16] with slight modification. Briefly, 100 mg of liver tissue was homogenized in 1 mL of ice-cold homogenizing buffer containing 100 mmol/L KCl, 25 mmol/L HEPES (N'-2-Hydroxyethylpiperazine-N'-2 ethanesulfonic acid), 7.5 mmol/L $MgCl_2$, 4 mmol/L dithiothreitol (pH 7.4) using Potter Elvehjem type homogenizer. The resultant homogenate was then lysed overnight at 4 °C.

2.5.2. Estimation of hepatic Glu-6-Pase activity in rats
Glu-6-Pase activity was assayed according to the method of Baginsky *et al.*[17] by estimation of the inorganic phosphate liberated from Glu-6-Pase.

One gram of frozen liver tissue was homogenized in ice-cold sucrose solution using Potter Elvehjem type homogenizer. The homogenate was centrifuged sequentially at 13 254 r/min for 30 min, then at 129 238 r/min 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.

2.5.3. Estimation of hepatic glycogen content in rats
Glycogen contents in hepatocytes were determined by the reported method[18]. A total of 100 mg of the tissue sample was digested in 1 mL of 30% KOH. The digested tissue was dissolved in 1.25 mL of

95% ethanol, and centrifuged at 3 000 r/min for 15 min to obtain a precipitate which was purified by re-dissolving in 1.25 mL of 95% ethanol and re-centrifuging a couple of times, and then analyzed for its glycogen content.

2.6. Column chromatography fractionation of S. ellipticum crude extract

S. ellipticum crude extract (12 g) was subjected to column chromatography to separate the extract into its component fractions using a column with size of 3.5 cm × 50 cm. Silica gel (60 G) was used as the stationary phase while varying solvent (hexane, ethyl acetate and methanol) combinations of increasing polarity were used as mobile phase. Fractions of similar TLC mobility and band formation pattern (RF value) were pooled together and those with two or more bands were subjected to another round of column and thin layer chromatographic procedures.

2.7. HPLC–MS analysis of Bioactive S. ellipticum fractions

The reported method[19] was employed in the HPLC–MS analysis of *S. ellipticum* fractions. Agilent Technologies 1200 series HPLC coupled with Agilent 6410B series electro spray ionization triple-quad MS/MS was used to perform the analyses. The samples were in each case first subjected to separation using a Zorbax Eclipse XDB-C18 (Agilent) rapid resolution column (50 mm × 4.6 mm i.d., 1.8 m particle size) held at 50 °C. Then 0.05% aqueous formic acid (A) and methanol (B) constituted the binary mobile phase. Samples were dissolved in mobile phase (ratio 1:1) to obtain a final concentration of 2 mg/mL. Working standards were prepared by serial dilutions (1:1) of standard mixture with solvents A and B. The injection volume was 5 L, and elution was 1 mL/min with gradient program as follows: 0 min 30% B, 6 min 70% B, 9 min 100% B, 12 min 100% B, re-equilibration time 3 min. Eluted components were detected by MS, using the ion source parameters as follows: nebulization gas (N_2) pressure was 40 psi, drying gas (N_2) flow was 9 L/min and temperature 350 °C, capillary voltage 4 kV, negative polarity. Data were acquired in dynamic MRM mode, using the optimized compound-specific parameters (retention time, pre-cursor ion, product ion, fragmentor voltage, collision voltage). Agilent Mass Hunter Workstation software-Qualitative Analysis (version B.03.01) was used to determine the peak areas for samples.

2.8. Statistical analysis

Data analysis was performed using statistical software, Prism graphpad, version 6.4. The statistical significance of difference between groups was analyzed using One-way ANOVA followed by independent-sample *t* test. The level of significance was set at $P < 0.05$. The results are presented as mean ± SD or mean ± SEM.

3. Results

3.1. Hepatic glucokinase and Glu-6-Pase activities

Hepatic activities of Glu-6-Pase and glucokinase were respectively elevated and decreased by 54.85% and 118.51% in the diabetic

control rats relative to the normal control animals. *S. ellipticum* extract especially at 400 mg/kg significantly ($P < 0.05$) curtail these alterations by increasing glucokinase activity by 40.31% and inhibiting Glu-6-Pase activity by 37.29% compared to the diabetic control animals. However, the effects were significantly ($P < 0.05$) lower than that of metformin which enhanced glucokinase activity by 94.76% (Figure 1) and simultaneously inhibited Glu-6-Pase activity by 49.15% (Figure 2).
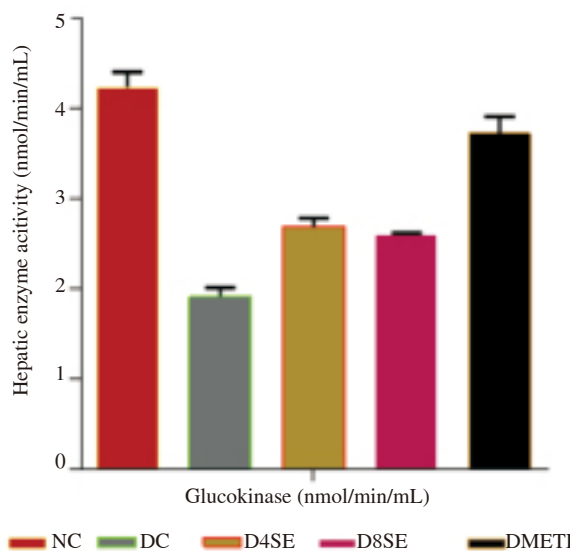

Figure 1. Effects of *S. ellipticum* on glucokinase activity in streptozotocin-induced diabetic rats.
NC: Normal control; DC: Diabetic control; D4SE: 400 mg/kg of *S. ellipticum*; D8SE: 800 mg/kg of *S. ellipticum*; DMETF: Metformin.

Figure 2. Effects of *S. ellipticum* on Glu-6-Pase activity in streptozotocin-induced diabetic rats.
NC: Normal control; DC: Diabetic control; D4SE: 400 mg/kg of *S. ellipticum*; D8SE: 800 mg/kg of *S. ellipticum*; DMETF: Metformin.
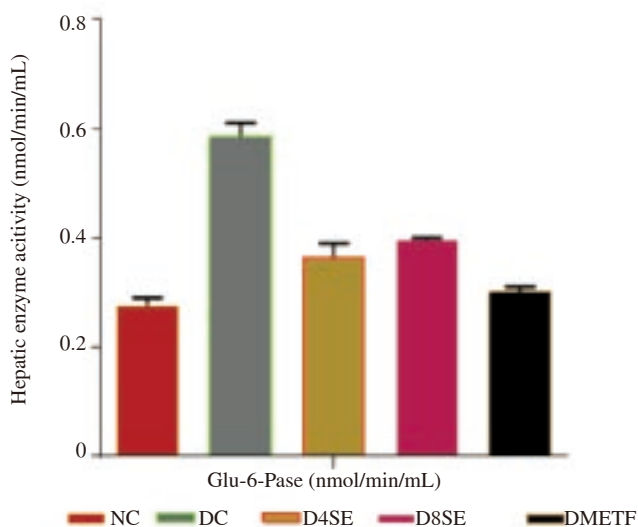
3.2. Hepatic glycogen level

The extract at the administered doses improved hepatic glycogen contents in rats, with 400 mg/kg producing a significantly ($P < 0.05$) greater improvement (32.37%) compared to 800 mg/kg (27.06%). The enhancing effects of both doses were significantly lower than
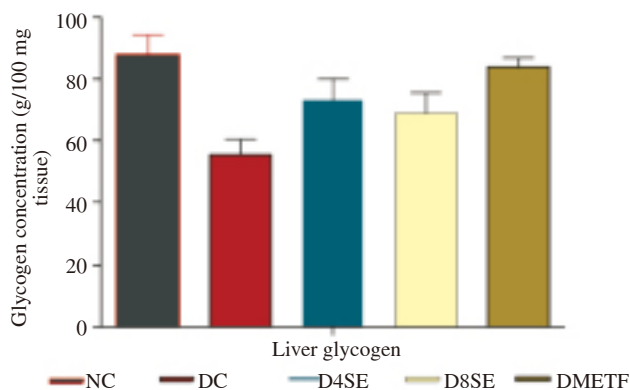
that of metformin in both tissues (Figure 3).

Figure 3. Effects of *S. ellipticum* and METF on liver glycogen concentration in STZ-Treated rats.
NC: Normal control; DC: Diabetic control; D4SE: 400 mg/kg of *S. ellipticum*; D8SE: 800 mg/kg of *S. ellipticum*; DMETF: Metformin.

3.3. HPLC-MS analysis

HPLC-MS analysis of *S. ellipcitum* fractions in dynamic MRM mode (using the optimized compound-specific parameters) revealed the presence of amentoflavone with a precursor m/z value of 536, product m/z value of 373, V collision value of 33, V fragmentor value of 220 and retention time of 5 min 30 s.

4. Discussion

The large increase in glucose-6-phosphatase activity and concomitant decrease in glucokinase activity in the liver of diabetic rats noted in this study affirms the diabetogenic ability of streptozotocin and accounts for its common use in evaluating the anti-diabetic potential of medicinal plants in animal model.

Glucose-6-Phosphatase is one of the glucose metabolizing enzymes in the liver which catalyzes the final step of glycogenolysis and gluconeogenesis in which glucose-6-phosphate is hydrolysed to yield glucose and phosphate[20,21]. The enzyme is like a double edged sword with the unusual capability to balance the concentrations of free glucose and stored glucose as glycogen. When body cells are energy-starved due to unavailability of glucose, the increased activity of glucose-6-phosphatase becomes a necessary counter-regulatory response which is often triggered by glucagon and other insulin antagonistic hormones. This explains the usual increase in the enzyme activity in STZ-induced cellular glucose shortage.

In the present study, compared to diabetic control rats, hepatic glucose-6-phosphatase activity was significantly decreased in both *S. ellipcitum* and metformin treated diabetic animals, with the latter exhibiting greater reduction in glucose-6-phosphatase activity. This observation is consistent with the claim of Tahrani *et al.*[22] and Jung *et al.*[23] who posited that metformin is an established inhibitor of glucose-6-phosphatase. The ability of *S. ellipcitum* extract to reverse the STZ-induced upsurge in glucose-6-phosphatase is suggestive of a pro-insulin effect for the extract. The extract may have promoted insulin secretion from the beta cells of the pancreas, consequently enhancing glucose mobilization into the cells and slowing down the process of glycogenolysis and gluconeogenesis in which glucose-6-phosphatase is a key player. This postulation is in line with a previous report by Massillon *et al.*[24] who noted that control of blood glucose level in diabetes with insulin usually results in decreased gucose-6-phosphatase gene expression and activity. It also explains the relative improvement in hepatic glycogen level observed in *S. ellipcitum* and metformin treated diabetic rats compared to diabetic control animals in which the activity of the enzyme was significantly elevated. Anti-diabetic effects of *S. ellipcitum* extract have been noted in another work in our laboratory, and deactivation of glucose-6-phosphatase activity as observed in this study is probably one of the mechanisms by which *S. ellipcitum* extract elicits its antidiabetic potential. Meanwhile, like *S. ellipcitum*, a number of other plants have been reported to exhibit hypoglycemic activity primarily through inhibition of glucose-6-phosphatase. Few examples are *Cecropia obtusifolia*[25], *Malmea depressa*[26] and *Tinospora cordifolia*[9]. When compared to *S. ellipcitum*, the significantly greater effect of metformin on glucose-6-phosphatase activity may be responsible for the higher glycogen contents observed in the liver of diabetic rats treated with the reference drug.

Glucokinase is another glucose metabolism enzyme; it is found mainly in the liver and pancreas. Pancreatic glucokinase plays a very important role (as a glucose sensor) in ensuring appropriate secretion and release of insulin vis-à-vis plasma glucose concentration. The enzyme like its liver counterpart is activated by high plasma glucose concentration (> 7.5 mmol/L) and becomes deactivated when the glucose level drops to normal (< 5.5 mmol/L)[8]. According to Alberti *et al.*[27], within normal physiological glucose concentrations hepatic glucokinase is usually in its inactive form, bound to regulatory protein. However, secondary to upsurge in plasma glucose concentration, the enzyme is activated and released from its bound protein molecule into the cytoplasm where it facilitates the breakdown of glucose through glycolysis as one of the means of maintaining glycemic balance.

In this study, hepatic glucokinase activity was observed to decrease significantly in diabetic control animals compared to normal control animals, suggesting a compromise in enzymatic regulatory mechanism. The suppressed enzyme activity is likely a result of STZ induced conformational alteration in the enzyme molecule. Zhang *et al.*[16] had previously associated the decreased activity of glucokinse observed in similar studies with STZ induced free radical damage. This observation is very important because of the role of the enzyme in hepatic glucose homeostasis. It catalyzes the rate limiting step of glycolysis by phosphorylating glucose to glucose-6-phosphate. Down regulation of glucokinase activity will therefore contribute to the onset and progression of diabetes mellitus. This assertion is further validated by the report of Zhang *et al.*[16] which associated gene mutation in glucokinase with a form of diabetes known as maturity onset diabetes of the young. Compounds that elicit stimulatory effect on the activity of glucokinase have been reported to enhance the regulatory function of the enzyme in drug-induced diabetes in animal model[28,29].

In this study, treatments of diabetic rats with *S. ellipcitum* extract significantly improved hepatic glucokinase activity in rats. The

extract may possess the chemical or structural requirements capable of directly stimulating glucokinase or correcting the STZ-induced conformational alteration likely responsible for the decrease in the enzyme activity.

Interestingly, Patil *et al.*[29] had previously identified amentoflavone as the antidiabetic principle in *Biophytum sensitivum*, a plant well known for its antidiabetic function. Hence, the presence of the flavonoid in *S. ellipcitum* is likely responsible for its significant effects on glucose metabolizing enzymes such as glucokinase and glucose-6-phosphatase, as noted in the present study.

The ability of *S. ellipcitum* to respectively activate and inhibit key enzymes (glucokinase and glucose-6-phosphatase) of glycolytic and glycogenolytic pathways in this study suggests that it may be a treatment option for type 2 diabetes patients whose fasting hyperglycaemia is primarily a result of increased hepatic glucose output, and the presence of amentoflavone in the plant extract as previously reported[30] may account for its anti-diabetic potential.

Acknowledgment

Conflict of interest statement

We declare that we have no conflict of interest.

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