Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2017.05.009

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

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ARTICLE INFO

Article history:
Received 1 Aug 2016
Received in revised form 8 Sep, 2nd revised form 12 Oct 2016
Accepted 6 Dec 2016
Available online 31 May 2017

Keywords:
Sapium ellipticum
Glucose homeostasis
Diabetes
Glucokinase
Glucose-6-phosphatase

ABSTRACT

Objective: To examine the effects of *Sapium ellipticum* (SE) leaf extract on the hepatic activities of glucokinase and glucose-6-phosphatase in streptozotocin-induced diabetic Wistar rats.

Methods: STZ-induced diabetic Wistar rats (four groups, $n = 8$) were used in this study. SE was assessed at two different doses, 400 and 800 mg/kg BW, in comparison with metformin (METF) (12 mg/kg BW) as a reference antidiabetic drug. All treatments were done orally (p.o), twice daily at 8 h interval for a period of 21 days. Glucokinase and glucose-6-phosphatase activities were respectively determined using standard protocols. 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. SE extract especially at 400 mg dosage significantly reversed the alterations by increasing glucokinase activity by 40.31% and inhibiting glucose-6-phosphatase activity by 37.29% compared to diabetic control animals. However, the effects were significantly lower than that of METF which enhanced glucokinase activity by 94.76% and simultaneously inhibited glucose-6-phosphatase activity by 49.15%. The extract also improved hepatic glycogen level by 32.37 and 27.06% at 400 and 800 mg dosage respectively. HPLC-MS analysis of some SE 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 antidiabetic function.

Conclusions: The ability of SE 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 which enables the liver to produce glucose catalyzes the final step of glycogenolysis and gluconeogenesis in which glucose-6-Phosphate is hydrolysed to yield glucose and phosphate [7]. 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 activity of glucose-6-Phosphatase becomes a necessary counter-regulatory response which is often triggered by glucagon and other insulin antagonistic hormones. Hence, Glu-6-Pase activity is markedly increased in insulin-deficient diabetic rats or during short period of fasting [2].

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Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

On the other hand, 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 glucose -6-phosphate. 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 mM) and becomes deactivated when the glucose level drops to normal (<5.5 mM) [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-induced diabetes in animal model [4,9], and consequently contribute to the management of diabetes mellitus.

Sapium ellepticum (*S. ellipticum*) (Hochst) pax enjoy huge therapeutic application in the local treatment of a number of disease conditions [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-ẹgbọ*.

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 and colleagues [14] in their study demonstrated the presence of Lupeol, lupeol acetate and stigmasterol in the stem bark extract of *S. ellipticum*. This present study sought to investigate 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 Sapium ellipticum leaves

Fresh *S. ellipticum* (SE) leaves were harvested in the month of December, 2012 from a forest in a suburb of Ibadan, south-west of Nigeria. The harvested leaves were taxonomically authenticated by a botanist (Mr. T.K. Odewo) at the Lagos University Herbarium (LUH), Nigeria, where a specimen was deposited and assigned a voucher number, LUH 5423.

2.2. Preparation of Sapium ellipticum leaf extracts

The plant material was freed of extraneous materials; air dried at room temperature and milled to a fine powder, using a Waring blender. 300 g of the powdered sample was macerated in 2.5 L of the 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 normoglycemic 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. Group 1 animals 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 of *S. ellepticum* 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]. This was 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 homogenizing 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 homogenization buffer containing 100 mM KCl, 25 mM HEPES (N′-2-Hydroxyethylpiperazine-N′-2 ethanesulphonic acid), 7.5 mM $MgCl_2$, 4 mM 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 glucose 6-phosphatase activity in rats

Glucose 6-phosphatase (G6Pase) activity was assayed according to the method of Baginsky *et al.* [17] by estimation of the inorganic phosphate (Pi) liberated from glucose 6-phosphate (G6P).

One gram of frozen liver tissue was homogenized in ice-cold sucrose solution using Potter Elvehjem type 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.

2.5.3. Estimation of hepatic glycogen content in rats

Glycogen contents in hepatocytes were determined by the method of Seifter with slight modifications as reported in Methods in Enzymology Vol. 111 [18].

Hundred milligram of the tissue sample was digested in 1 mL of 30% KOH. The digested tissue was dissolved and in 1.25 mL

of 95% ethanol, centrifuged at 3000 rpm 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 SE crude extract

SE crude extract (12 g) was subjected to column chromatography to separate the extract into its component fractions using a column size of 3.5 × 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 into a pre-weighed beaker 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 SE fractions

The method of Orčić *et al.*, [19] was employed in the HPLC–MS analysis of SE fractions. Agilent Technologies 1200 series HPLC coupled with Agilent 6410B series electrospray 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. 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 (N2) pressure was 40 psi, drying gas (N2) 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 (ver.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 the one-way analysis of variance (ANOVA) followed by independent-sample *t* test. The level of significance was set at $p < 0.05$. The results are presented as mean ± SEM.

3. Results

3.1. Hepatic glucokinase and glucose-6-phosphatase 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 compared to the normal control animals. SE extract especially at 400 mg dosage significantly ($p < 0.05$) reversed these alterations by increasing glucokinase activity by 40.31% and inhibiting glucose-6-phosphatase activity by 37.29% compared to the diabetic control animals. However, the effects were significantly ($p < 0.05$) lower than that of METF which enhanced glucokinase activity by 94.76% (Figure 1) and simultaneously inhibited glucose-6-phosphatase activity by 49.15% (Figure 2).

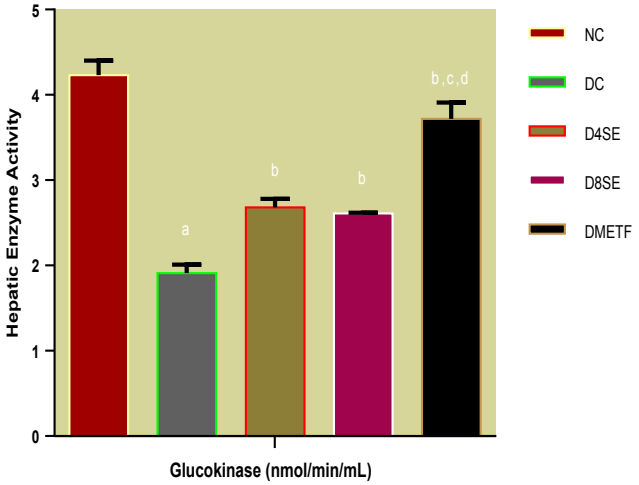

Figure 1. Effects of SE on Glucokinase activity in streptozotocin-induced diabetic rats.
Values are expressed as mean ± SEM of 8 rats. NC= Normal control, DC = Diabetic control, D4SE = Diabetic animals treated with SE (400 mg/kg BW), D8SE = Diabetic animals treated with SE (800 mg/kg BW), DMETF = Diabetic animals treated with metformin (12 mg/kg BW). *P* value < 0.05 = significant, a = significant when compared to CN, b = significant when compared to DC, c = significant when compared D4SE d = significant when compared to D8SE, f = significant when compared to DMETF.

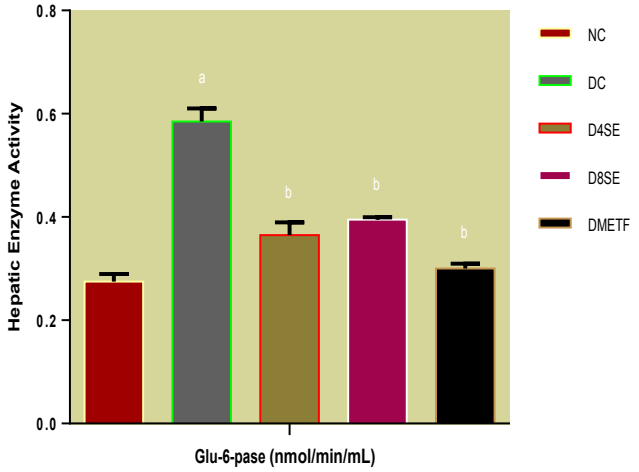

Figure 2. Effects of SE on Glu-6-Pase activity in streptozotocin-induced diabetic rats.
Values are expressed as mean ± SEM of 8 rats. NC= Normal control, DC = Diabetic control, D4SE = Diabetic animals treated with SE (400 mg/kg BW), D8SE = Diabetic animals treated with SE (800 mg/kg BW), DMETF = Diabetic animals treated with metformin (12 mg/kg BW). *P* value < 0.05 = significant, a = significant when compared to CN, b = significant when compared to DC, c = significant when compared D4SE d = significant when compared to D8SE, f = significant when compared to DMETF.

3.2. Hepatic glycogen level

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

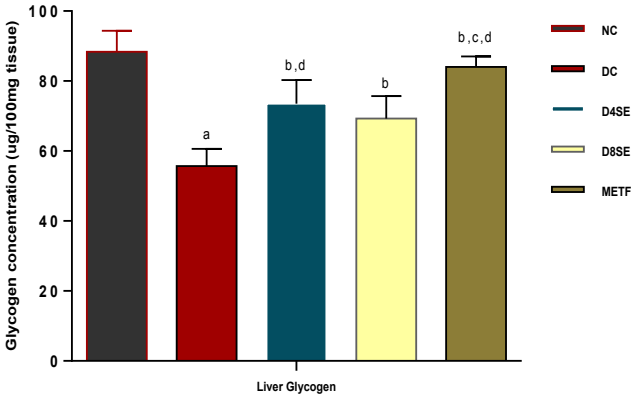

Figure 3. Effects of SE and METF on liver Glycogen concentration in STZ-Treated rats.
Values are expressed as mean ± SEM of 8 rats. NC = Normal control, DC = Diabetic control, D4SE = Diabetic animals treated with SE (400 mg/kg BW), D8SE = Diabetic animals treated with SE (800 mg/kg BW), DMETF = Diabetic animals treated with metformin (12 mg/kg BW). *P* value < 0.05 = significant, a = significant when compared to CN, b = significant when compared to DC, c = significant when compared D4SE d = significant when compared to D8SE, f = significant when compared to DMETF.

3.3. HPLC-MS analysis

HPLC-MS analysis of SE fractions in dynamic MRM mode (using the optimized compound-specific parameters) revealed the presence of amentoflavone (Table 1).

Table 1
Detection of Amentoflavone in SE fraction through HPLC-MS analysis.

SE fraction	Precursor *m/z*	Product *m/z*	V Collision (V)	V Fragmentor (V)	Compound	Time (min: sec)
F2	536	373	33	220	Amentoflavone	5.30

F2 = *Sapium ellipticum* extract fraction 2.

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 SE and METF treated diabetic animals, with the latter producing 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 SE 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.* [5], 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 SE and METF treated diabetic rats compared to diabetic control animals in which the activity of the enzyme was significantly elevated. Anti-diabetic effects of SE extract has 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 SE extract elicits its antidiabetic potential. Meanwhile, like SE, a number of other plants have been reported to exhibit hypoglycemic activity primarily through inhibition of glucose-6-phosphatase. Few examples are *Cecropia obtusifolia* [24], *Malmea depressa* [25] and *Tinospora cordifolia* [9]. When compared to SE, the significantly greater effect of METF 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 play 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 mM) and becomes deactivated when the glucose level drops to normal (<5.5 mM) [8]. According to Alberti *et al.* [26] 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 release from its bound protein molecule into the cytoplasm where it facilitates the breakdown of glucose through glycolysis as one of the means of maintaining glycaemic balance [27].

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 as 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 to 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 contributes 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 [27,28].

In the current study, treatments of diabetic rats with SE 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.* [28] 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 SE 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 SE to respectively activate and inhibit key enzymes (glucokinase and glucose-6-phosphatase) of glycolytic and glycogeneolytic pathways in this study suggests that it may be a treatment option for type 2 diabetes patients whose fasting hyperglycaemia is primarily as result of increased hepatic glucose output, and the presence of amentoflavone in the plant extract may account for its anti-diabetic potential.

Conflict of interest statement

We declare that we have no conflict of interest.

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