**Sclerocarya birrea** (Anacardiaceae) stem-bark extract corrects glycaemia in diabetic rats and acts on β-cells by enhancing glucose-stimulated insulin secretion

Ivonne Gisèle Makom Ndifossap1,2, Francesca Frigerio1, Marina Casimir1, Florence Ngueguim Tsafack2, Etienne Dongo3, Pierre Kamtchouing2, Théophile Dimo2 and Pierre Maechler1

1Department of Cell Physiology and Metabolism, Faculty of Medicine, University of Geneva, 1 rue Michel-Servet, 1211 Geneva 4, Switzerland

Departments of 2Animal Biology and Physiology and 3Organic Chemistry, Faculty of Science, University of Yaoundé 1, PO Box 812, Yaoundé, Cameroon

(Correspondence should be addressed to P Maechler; Email: pierre.maechler@unige.ch)

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**Abstract**

*Sclerocarya birrea* is a plant widely used as traditional medication for the treatment of diabetes in sub-Saharan regions. However, the mechanism of action is unknown and only hypoglycaemic effects of *S. birrea* extract (SBE) in diabetic rats have been reported to date. Here, we tested aqueous extracts of *S. birrea* on insulin-secreting INS-1E cells and isolated rat islets. Following 24 h of treatment at 5 μg/ml, the extract markedly potentiated glucose-stimulated insulin secretion. Neither basal insulin release nor non-nutrient stimulation was affected. The potentiation of the secretory response at stimulatory glucose appeared after 12 h of treatment. No acute effects were observed and, at the effective concentration, SBE was safe regarding cell integrity and differentiation. The mechanism of action of the SBE was related to glucose metabolism as both ATP generation and glucose oxidation were enhanced following the 24-h treatment. In streptozotocin-induced diabetic rats, SBE administration corrected glycaemia and restored plasma insulin levels after 2 weeks of treatment. These data show direct action of *S. birrea* on insulin-secreting cells and favour further delineation for use of the plant in the management of diabetes.


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**Introduction**

Diabetes represents a growing health problem in Africa with rapidly increasing prevalence, in particular in sub-Saharan regions (Gill et al. 2009). Associated with economic constraints, it is crucial to identify effective and low-cost medications. Traditional remedies in Africa rely mostly on medicinal plants. Among them, *Sclerocarya birrea* ((A. Rich.) Hochst.) (Anacardiaceae) is one of the plant species widely used in Africa against various diseases, including diabetes (Dieye et al. 2008). In a cross-sectional survey conducted in Dakar, 43% of patients attending consultancy at the hospital declared using *S. birrea* (Dieye et al. 2008). This shows the wide use of the plant for the treatment of various diseases, although investigations on biological effects are lacking.

*S. birrea* stem-bark extracts have been shown to exert hypoglycaemic effects in animal models. In rats with streptozotocin-induced diabetes, acute administration of *S. birrea* stem-bark extract reduces blood glucose levels (Ojewole 2003, Dimo et al. 2007). Chronically, the efficacy of *S. birrea* stem-bark extract administered for 5 weeks was shown to be similar to metformin treatment with regards to lowering of glycaemia (Gondwe et al. 2008).

Despite these promising in vivo studies, cellular and molecular mechanisms responsible for hypoglycaemic effects of the plant have not been investigated. Blood glucose control depends on the normal regulation of insulin secretion from the pancreatic β-cells and the action of insulin on its target tissues. In the consensus model of glucose-stimulated insulin secretion, glucose phosphorylation initiates its metabolism (Iynedjian 2009), ultimately leading to plasma membrane depolarisation (Ashcroft 2006) and the ensuing cytosolic calcium rise inducing insulin exocytosis (Elansson et al. 2008). Additional signals, generated by mitochondrial metabolism, amplify the calcium signal (Maechler et al. 2006). This process is referred to as metabolism-secretion coupling, since glucose oxidation promotes ATP generation necessary for the triggering of exocytosis signals. Elevation of plasma insulin was reported in stem-bark-treated diabetic rats (Dimo et al. 2007). Accordingly, it was of importance to investigate putative direct effects of *S. birrea* on insulin-secreting cells.

Regarding the in vivo hypoglycaemic effect of *S. birrea* stem-bark, chronic treatments have been reported only with organic extracts (Dimo et al. 2007, Gondwe et al. 2008), whereas aqueous extracts have been shown to be effective in acute conditions (Ojewole 2003). In the present study, we tested chronic effects of aqueous extracts of *S. birrea* stem-bark...
in vivo in diabetic rats and in vitro on insulin-secreting cells. Rats with nicotinamide–streptozotocin-induced diabetes were treated over a 14-day period, revealing efficiency of the treatment. For in vitro studies, we used both the well-differentiated insulinoma INS-1E cell line as well as the isolated rat islets. Aqueous extracts of *S. birrea* stem-bark were found to promote glucose metabolism and insulin secretion, demonstrating for the first time direct effects of *S. birrea* on insulin-secreting cells.

**Materials and Methods**

**Preparation of plant extract**

Fresh stem-bark of *S. birrea* (A. Rich.) Hochst. (Anacardiaceae) was collected in Garoua (North Province, Cameroon) and certified at the Department of Plant Biology and Physiology, University of Yaoundé I. A voucher specimen documenting the collection was deposited at the National Herbarium (Yaoundé) under the reference 7770 HNC.

One kilogram of air-dried stem-bark of *S. birrea* was minced, powdered and macerated in 3 l distilled water for 48 h at room temperature. The water extracts were filtered through Whatman no. 3 filter paper and were concentrated under reduced pressure at 35°C, yielding 160 g of a dark-brown (16%) *S. birrea* extract (SBE).

For each series of experiments, the extract was weighed and dissolved in distilled water to obtain a 30 mg/ml stock solution.

**Animals and diabetes induction**

For *in vivo* studies, we used male Wistar rats weighing 200–250 g obtained from in-house breeding (Faculty of Science, University of Yaoundé I). The rats were kept and maintained in laboratory animal units under standard conditions of temperature and humidity with 12 h light:12 h darkness cycle and free access to chow diet and water. Authorisation for the use of animals was obtained from the Institutional Animal Ethics Committee (N° FW-IRB00001954).

Diabetes was induced by an i.p. injection of 110 mg/kg nicotinamide 15 min before an i.v. (penile vein) injection of 65 mg/kg streptozotocin (Sigma Chemicals) in 0.9% sodium chloride solution. Non-diabetic control rats were injected with vehicle only.

Three days after diabetes induction, fasting blood glucose levels were determined using a glucometer (Boehringer Mannheim). Diabetes was allowed to develop and to stabilise over a period of 2 weeks before starting the treatments.

**Animal treatments and glucose tolerance test**

SBE at a dose of 150 or 300 mg/kg body weight, glibenclamide (Glib) at the dose of 10 mg/kg (positive control) and distilled water at 10 ml/kg (control group) were administered orally by gastric intubation, and each group consisted of six rats. Doses of SBE were selected based on previous animal studies (Ojewole 2003, Dimo et al. 2007) and use by traditional practitioners.

Chronic treatment was conducted by daily administration of the test compounds for 14 consecutive days with measurements of body weight and fasting glycaemia at the indicated times. At the end of the treatment period, rats were fasted overnight, killed, and plasma collected for analysis of insulin levels using a commercial kit (Mercodia, Uppsala, Sweden).

Acute effects were evaluated in fasted animals during a glucose tolerance test. Immediately after oral glucose (5 g/kg) administration, test compounds were administered as described above, and glycaemia was recorded over a 5-h period.

**Cell culture and treatments**

INS-1E cells (Merglen et al. 2004) were cultured in complete RPMI-1640 medium supplemented with 5% heat-inactivated FCS, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. Rat islets were isolated from adult male Wistar rats by collagenase perfusion (Carobbio et al. 2004) and cultured in complete RPMI-1640 medium.

INS-1E cells were seeded in 24-well plates and cultured in complete RPMI-1640 medium. After 3 days, the cells were incubated with the indicated concentrations of SBE (stock solution: 10 mg/ml dissolved in bidistilled water) for time periods as described.

Cell viability was assessed by trypan blue exclusion (Altman et al. 1993). Briefly, INS-1E cells that were seeded in 24-well plates for 3 days were cultured in the presence of SBE (0, 10, 20 and 40 μg/ml) for another 24-h period. Then, the cells were dispersed with trypsin–EDTA before exposure to the membrane impermeant dye, trypan blue (0-4% w/v). The presence of the dye within cells was visualised by light microscopy, and the numbers of stained cells versus unstained cells were determined. Moreover, apoptosis index was estimated using ethidium bromide staining assay (Ribble et al. 2005). In brief, following the 24-h period of exposure to increasing concentrations of SBE, INS-1E cells cultured in 24-well plates were stained with 40 μg/ml ethidium bromide, and fluorescence excited at 485 nm was recorded at 510 nm on a fluorimeter (Fluostar Optima, BMG Labtechnologies, Offenburg, Germany).

**Real-time quantitative RT-PCR**

Total RNA was extracted from treated and non-treated INS-1E cells using the RNeasy Mini Kit (Qiagen), and 2 μg were converted into cDNA as previously described (Rubi et al. 2005). The real-time PCR measurement of cDNAs was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland) to measure duplex DNA formation and normalised to the expression of both transcription factor IIb (TFIIb) and α-tubulin as control housekeeping genes. The primers used in the real-time RT-PCR are listed in Table 1.
Insulin secretion assay

The secretory responses were tested in INS-1E cells and rat islets. Briefly, after washing with glucose-free Krebs–Ringer bicarbonate Hepes buffer (KRKH, containing 135 NaCl, 3.6 KCl, 10 Hepes (pH 7.4), 5 NaHCO$_3$, 0.5 NaH$_2$PO$_4$, 0.5 MgCl$_2$ and 1.5 CaCl$_2$ in millimolar) with 0.1% BSA as insulin carrier, the cells were stimulated with the indicated glucose concentrations for 30 min. Non-nutrient-stimulated insulin release was induced by the calcium-raising agent KCl (30 mM) at basal 2.5 mM glucose. The secretory response to mitochondrial substrates was tested at basal 2.5 mM glucose using 2 mM pyruvate, 10 mM α-ketoglutarate and 5 mM methyl succinate (Maechler et al. 1998a, 2006). Regarding the isolated pancreatic islets, these were washed, hand-picked and distributed into 3 ml tubes in KRKH for static incubation over a 30-min period (Rubi et al. 2004). Incubations were stopped by putting plates (for INS-1E cells) or tubes (for islets) on ice, supernatants were collected for insulin secretion, and cellular insulin contents were determined from acid–ethanol extracts. Insulin secretion was measured by RIA using rat insulin as standard (Linco Research, St Charles, MO, USA).

Cellular ATP and glucose oxidation

Cellular ATP levels were monitored in a thermostated plate reader (Fluostar Optima) in INS-1E cells expressing the ATP-sensitive bioluminescent probe luciferase in the presence of 100 μM luciferin as described (Maechler et al. 1998b, Merglen et al. 2004). Changes in luminescence were monitored at basal 2.5 mM glucose before stimulation during 20 min with 15 mM glucose followed by the addition of the mitochondrial poison azide (2 mM). For glucose oxidation, INS-1E cells were incubated for 1 h with KRKH containing 15 mM glucose traced with 0.1 μCi [U-14C]glucose (de Andrade et al. 2006). Formation of $^{14}$CO$_2$ was counted in a liquid scintillation counter (LKB Wallac 1217 Rackbeta counter, Turku, Finland). Glucose oxidation was normalised to cellular protein contents.

Statistical analysis

Unless otherwise indicated, data are the means±S.E.M. of at least three independent experiments. Differences between groups were assessed by the Student’s t-test for single comparison and by one-way analysis followed by Bonferroni t-test for multiple comparisons. A P value lower than 0.05 was considered as significant.

Results

Treatment of diabetic rats with SBE

At the end of the 2-week treatment period, i.e. 4 weeks after diabetes induction, diabetic control rats exhibited body weights 10% lower compared with non-diabetic animals (P<0.02) see Fig. 1A. Glib treatment maintained body weights comparable to non-diabetic rats, although SBE did not.

Fasting plasma glucose levels were dramatically increased 2 weeks after diabetes induction by streptozotocin, reaching values of 252±13 mg/dl in diabetic controls (Fig. 1B). After 7 days of daily treatment, both Glib and SBE, 300 mg/kg, markedly reduced glycaemia to 121±8 and 119±9 mg/dl respectively. SBE at the dose of 150 mg/kg was also efficient in correcting plasma glucose when compared with diabetic controls (152±14 vs 252±12 mg/dl, P<0.001). After 14 days of treatment, both Glib and SBE, 300 mg/kg, groups exhibited glycaemia similar to non-diabetic rats. SBE at 150 mg/kg reduced plasma glucose levels to 133±9 mg/dl, i.e. ~46% compared with diabetic controls (P<0.001).

At the end of the 2-week treatment period, plasma insulin concentrations in diabetic controls were reduced to 8.5±1.3 compared with 12.7±0.9 mU/ml in non-diabetic animals (P<0.001), see Fig. 2A. Both Glib and SBE, 300 mg/kg, treatments restored insulin levels towards normal values (P<0.001 versus diabetic control group). These data show efficient hypoglycaemic effects of chronic SBE treatment correlating with increased circulating insulin.

Acute effects of SBE were tested in vivo during an oral glucose tolerance test (Fig. 2B). Animals received simultaneously glucose load and treatment, both by gavage. Glib rapidly lowered plasma glucose close to non-diabetic levels (163±7 mg/dl at 1 h), an effect maintained over the 5-h recording period. SBE at 300 mg/kg also reduced glycaemia towards non-diabetic levels, although the effects were slightly delayed compared with Glib. At 150 mg/kg, SBE treatment significantly reduced plasma glucose levels at all time points, resulting in glycaemia of 168±12 mg/dl at 5 h (~32% versus diabetic controls, P<0.001).

Table 1: Primers used for gene expression analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers sequence</th>
</tr>
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<tbody>
<tr>
<td>Glucokinase</td>
<td>5'-GTGATGGCGTCGTGATCAAG-3'</td>
</tr>
<tr>
<td>Insulin</td>
<td>3'-GATTCCAGTCCGGTGTA-5'</td>
</tr>
<tr>
<td>Glut2</td>
<td>5'-TCGCCCACTCTTGATGCA-3'</td>
</tr>
<tr>
<td>Pdx1</td>
<td>3'-AGAACAGAGCAAGCAACCTT-3'</td>
</tr>
<tr>
<td>Tfam</td>
<td>5'-GCCGAGGAAGAAATCTGCTGAAA-3'</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>3'-ACAGATAGGCGAGCTCAATG-3'</td>
</tr>
<tr>
<td>Carboxylase</td>
<td>5'-GCCAATTCGATGCCATCTCACCTGTTGC-3'</td>
</tr>
<tr>
<td>Cox1</td>
<td>3'-AGCTGCTCTGACTCTGATT-3'</td>
</tr>
<tr>
<td>TFBb</td>
<td>5'-TGTTCTGCACTGTGACCTT-3'</td>
</tr>
<tr>
<td>Tubulin</td>
<td>3'-GAACCAAGGTTGATGTCGGA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGCGGCAACGATGTCGGA-3'</td>
</tr>
<tr>
<td></td>
<td>3'-GTGCGGATGGGGATACATG-5'</td>
</tr>
</tbody>
</table>

For glucose oxidation, INS-1E cells were incubated for 1 h with KRKH containing 15 mM glucose traced with 0.1 μCi [U-14C]glucose (de Andrade et al. 2006). Formation of $^{14}$CO$_2$ was counted in a liquid scintillation counter (LKB Wallac 1217 Rackbeta counter, Turku, Finland). Glucose oxidation was normalised to cellular protein contents.
SBE treatment and INS-1E insulin-secreting cell preservation

In a previous study, SBE did not exhibit toxic effects in Chang liver cells exposed for 48 h to 12.5 mg/ml extract (van de Venter et al. 2008). In order to determine the safety threshold in insulin-secreting cells, we increased SBE concentrations up to 50 mg/ml over a 24-h incubation period. Trypan blue assay was performed on INS-1E cells following the 24-h culture period in the presence of increasing concentrations of SBE (Fig. 3A). This revealed that concentrations up to 10 mg/ml SBE did not induce toxic effects. Using ethidium bromide staining assay, we measured the apoptosis index showing significant cell death at 50 mg/ml SBE (Fig. 3B). According to these dose–responses, the concentration 5 mg/ml below the safety threshold was selected for the rest of the study.

Next, expression patterns of genes playing a key role in pancreatic β-cells were analysed following a 24-h culture period with 5 mg/ml SBE (Fig. 3C). In particular, we measured expression of metabolic genes (glucokinase (Gck) and Glut2 (Slc2a2)), of mitochondrial function (pyruvate carboxylase (Pcx) and COX1 (Ptgs1)), of insulin and of TFs relevant for β-cell function (Pdx1 and Tfam). Expression of the tested genes was not affected by SBE treatment, showing preserved differentiation of INS-1E cells upon SBE exposure.

Effect of SBE on insulin secretion

Acute effects of SBE on insulin secretion were tested in INS-1E cells cultured in 24-well plates (Fig. 4A). Sub-confluent INS-1E cells were exposed to 5 μg/ml SBE for 1 h and then assayed for insulin secretion over a 30-min incubation period. In control cells, 15 mM glucose stimulated insulin release 3.0-fold over basal release at 2.5 mM glucose, in accordance with previous reports (Merglen et al. 2004). Acute SBE treatment did not modify basal release or glucose-stimulated insulin secretion.

Next, a time course of SBE exposure (5 μg/ml) was conducted over a 12-h culture period terminated by glucose-induced insulin secretion assays (Fig. 4B). Insulin release stimulated by 15 mM glucose was potentiated in SBE-treated cells by 166% (P<0.05) after 12 h compared with non-treated stimulated controls. Insulin release at 2.5 mM glucose was not modified by SBE treatment.

Figure 1 Effects of chronic SBE treatment on body weight and glycaemia in diabetic rats: diabetes was induced by streptozotocin injection (at time pre-diabetic), followed by a period of 2 weeks to stabilise the diabetic state before initiation of treatments (day 1). Daily oral administration of water (non-diabetic and diabetic control), of SBE (150 and 300 mg/kg) and of glibenclamide (10 mg/kg, Glib) was continued for 14 days. Body weights (A) and fasting glycaemia (B) were recorded. *P<0.05 versus diabetic control, n=6.

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Figure 2 Effects of SBE treatment on insulin levels and glucose tolerance test in diabetic rats: diabetes was induced by streptozotocin injection followed by a period of 2 weeks to stabilise the diabetic state. (A) After 14 days of indicated daily treatments, fasted rats were killed, and plasma insulin levels were determined. (B) Fasted untreated rats with stabilised diabetes received one single acute treatment immediately after oral glucose load and glycaemia were monitored over 5 h. *P<0.05 versus diabetic control, n=6.
Insulin secretion evoked by nutrient and non-nutrient secretagogues was then compared between control and 5 mg/ml SBE-treated cells following a 24-h culture period (Fig. 5A). Stimulatory glucose (15 mM) induced a 3.3-fold response in controls and a 6.4-fold response in SBE-treated cells, corresponding to a 146% potentiation at high glucose (P < 0.05). Non-nutrient-induced insulin release was evoked by depolarising concentrations of KCl (30 mM) in order to raise cytosolic calcium levels. Such metabolism-independent secretory response was not modified by SBE exposure (Fig. 5A). Insulin secretion was also tested by stimulating INS-1E cells with mitochondrial substrates (Fig. 5B). In control cells, both 2 mM pyruvate and 5 mM methyl succinate significantly raised insulin secretion compared with basal release (4.5-fold, P < 0.001; 1.8-fold, P < 0.01 respectively). On the contrary, α-ketoisocaproate did not stimulate insulin release, tested at both 5 mM (not shown) and 10 mM (Fig. 5B). SBE-treated INS-1E cells exhibited similar responses to pyruvate and methyl succinate compared with control cells. However, 10 mM α-ketoisocaproate evoked a 1.9-fold secretory response (P < 0.005), corresponding to a 50% increase compared with corresponding non-treated control (P < 0.05). Insulin contents were not changed in INS-1E cells after 24 h of culture in the presence of 5 µg/ml SBE compared with controls (1828 ± 309 vs 1942 ± 268 ng/well respectively, n = 12).

Primary β-cells were used to validate further the effects of SBE on insulin-secreting cells. Isolated pancreatic rat islets were cultured in the presence of SBE (5 µg/ml) for 24 h prior to measurements of glucose-stimulated insulin secretion (Fig. 5C). Basal insulin release at 2.8 mM glucose was not...
affected by SBE exposure. Stimulatory glucose concentration (16.7 mM) induced an 8.8-fold secretory response in control islets. SBE-treated islets exhibited a 12.2-fold glucose response, potentiating glucose-stimulated insulin secretion by 30% (P < 0.02) compared with controls.

**Effect of SBE on ATP generation and glucose metabolism**

Insulin secretion results pointed to metabolic effects following chronic SBE treatment. Accordingly, glucose-induced ATP generation and glucose oxidation were measured in INS-1E cells following a 24-h exposure to 5 μg/ml SBE.

Cellular ATP levels were monitored in INS-1E cells expressing luciferase (Fig. 6A). Control cells kept at 2.5 mM glucose showed stable basal ATP levels. Raising glucose to 15 mM induced sustained elevation of cellular ATP, which was abruptly disrupted after addition of the mitochondrial poison azide. In SBE-treated cells, glucose-stimulated ATP generation was markedly increased compared with controls, before similar azide-induced drop.

Finally, glucose metabolism was assessed by measurements of 14C–glucose oxidation to 14CO2, reflecting the coupling of glycolytic rate to mitochondrial activity (Casimir et al. 2009). In SBE-treated INS-1E cells, glucose oxidation was increased by 49% (P < 0.05) compared with control cells. These data show enhanced glucose metabolism following chronic treatment with SBE.
Discussion

SBE is widely used as traditional remedy against diabetes in Africa (Dieye et al. 2008). However, very little is known about the mechanisms of action conferring its hypoglycaemic effects. Here, we showed that glycaemia of diabetic rats was efficiently corrected by SBE treatment over a 2-week period. SBE at a dose of 300 mg/kg exhibited an efficiency that was similar to that of Glib, and SBE at a dose of 150 mg/kg significantly lowered blood glucose after 1 week of treatment. Plasma insulin levels were increased by SBE treatment in vivo, indicating an action on pancreatic β-cells. Accordingly, we then investigated putative direct effects of SBE on β-cells.

Noteworthy, 150 mg/kg SBE efficiently reduced glycaemia although plasma insulin levels were similar to diabetic controls. This suggests that tissues other than pancreatic β-cells also contributed to the lowering of blood glucose. Indeed, SBE treatment was shown previously to increase hepatic glycogen storage to similar extent than metformin (Gondwe et al. 2008). Such liver-specific effects could possibly explain the blood glucose lowering effect observed in rats as soon as 1 h after the first administration of SBE. Among other molecules, SBE contains epicatechin-3-galloyl ester (Galvez Peralta et al. 1992) that is also present in green tea and is shown to improve glucose tolerance in diabetic mice and human subjects (Tsuneki et al. 2004). In the present study, acute treatment of insulin-secreting cells with SBE did not modify the secretory response of insulin-producing cells. Therefore, pancreatic β-cells are not likely to contribute to very early hypoglycaemic effects of SBE.

When tested in vitro on insulin-secreting cells, SBE exhibited strong potentiation of the secretory response at stimulatory glucose concentrations following a 24-h exposure period. This effect was observed both in insulinoma INS-1E cells and in primary pancreatic islets. Importantly, SBE did not stimulate insulin release at basal glucose, a property that prevented undesired hypoglycaemia in vivo. Toxicity assays and gene expression analysis showed the absence of cytotoxicity at effective concentrations as well as preserved differentiation of the insulin-secreting cells.

At the cellular level, the mechanism of action of SBE was associated with enhanced glucose metabolism. Indeed, SBE treatment increased both glucose oxidation and ATP generation upon glucose stimulation. Because these effects pointed to mitochondria as putative target of SBE action, expression of associated genes as well as secretory responses to mitochondrial substrates was tested. Corresponding results did support mitochondrial specificity; with the exception of α-ketoisocaproate-induced insulin release in SBE-treated INS-1E cells. Therefore, data suggest that SBE promoted overall metabolic pathway of glucose metabolism leading to ATP formation.

In conclusion, this study provides the first evidence for direct action of SBE on pancreatic β-cells, enhancing glucose-stimulated insulin secretion. This correlated with hypoglycaemic effects in diabetic rats associated with restored levels of plasma insulin. Further investigations should identify the active molecules present in SBE. Clinical data with large cohorts of patients are also missing and should challenge S. birrea as an effective tool for the management of diabetes. These studies should be conducted to identify low-cost medications accessible for the increasing populations of diabetic patients in sub-Saharan regions (Gill et al. 2009).

Declaration of interest

The authors declare that there is no conflict of interest that could have prejudiced the impartiality of the research reported.

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