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Effects of *Sclerocarya birrea* Stem-Bark Extracts on Glucose Uptake, Insulin Synthesis and Expression of Selected Genes Involved in the Synthesis and Secretion of Insulin in Rat Insulinoma Pancreatic Beta Cells

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The present study reported the effects of *Sclerocarya birrea* stem-bark (SBSB) extracts on glucose uptake, insulin synthesis and the expression of glucose transporter 2 (GLUT2), glucokinase, pancreatic duodenal homeobox-1 (PDX-1), musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) and pre-proinsulin genes in rat insulinoma (RIN)-m5F pancreatic beta cells. The amount of glucose taken-up by RIN-m5F cells was measured using a glucose oxidase-based assay kit. Intracellular and secreted insulin were measured using an enzyme linked immunoassay kit. Pre-proinsulin gene expression was determined using the conventional polymerase chain reaction (PCR) technique, while the expressions of GLUT2, glucokinase, PDX-1 and MafA genes were evaluated using quantitative real-time PCR technique. Of the four SBSB extracts investigated in the study, only the SBSB hexane extract positively affected all the study variables in RIN-m5F cells compared with the DMSO control. Thus, the SBSB hexane extract contains phytochemicals capable of enhancing insulin synthesis partly through up-regulation of the expression of GLUT2, glucokinase, PDX-1, MafA and pre-proinsulin genes.

Keywords: *Sclerocarya birrea*, Glucose uptake, Insulin synthesis, Gene expression, RIN-m5F pancreatic beta cells.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disease, which, if not treated properly, may result in both microvascular and macrovascular complications that severely effect the quality of life of affected patients and raise the cost of their diabetic care [1,2]. The disease is characterized by sustained hyperglycaemia due to the abnormalities in either insulin secretion, insulin action or both [3,4]. Its prevalence is increasing steadily, and it is estimated that by 2025, 300 million people worldwide will be affected by this disease [2,5]. Current medications for diabetes mellitus are either expensive, have undesirable side effects or are not readily accessible [6]. In light of these treatment problems, research is conducted all over the world with the aim of discovering novel, safe and cost-effective antidiabetic agents from medicinal plant species with alleged antidiabetic activity [7-9].

Sclerocarya birrea (A. Rich) Hochst, subspecies *caffra* (Sond) Kokwaro, family Anacardiaceae, is widely distributed in Southern Africa. The stem-bark, leaves and roots of *S. birrea* are traditionally used to treat, manage and control a variety of human ailments, including diabetes mellitus [10,11]. Oral administration of moderate to high doses (100 to 800 mg/kg body weight) of water *S. birrea* stem-bark (SBSB) extract to streptozotocin-induced diabetic rats significantly lowered their blood glucose level [12,13]. It has thus been speculated, based on plasma insulin measurements, that the observed blood glucose lowering effect of the SBSB extract could be related to the stimulation of insulin secretion from the pancreatic beta cells [12,14]. It was reported that water SBSB extract had the capacity to correct hyperglycaemia in diabetic rats by enhancing insulin secretion from pancreatic beta cells [15]. In addition, a flavonoid compound, (-)-epicatechin-3-galloyl ester, isolated from the stem-bark of *S. birrea,* had insulin secretory properties [16]. In all of the research studies cited above, only polar SBSB extracts were investigated for their insulin secretion stimulatory activities. There is therefore a need to investigate also, the effects of non-polar SBSB extracts on glucose stimulated insulin

secretion (GSIS) by pancreatic beta-cells. Furthermore, the mechanism(s) through which SBSB extracts enhance insulin secretion from pancreatic beta cells is currently unknown.

Antidiabetic agents that stimulate insulin secretion from pancreatic beta cells may do so by either enhancing one or more processes involved in the synthesis and secretion of insulin, such as glucose uptake, transcription of pre-proinsulin, translation and post-translational processing of proinsulin in pancreatic beta cells [3,17] or up-regulating the expression of genes whose protein products are involved in the synthesis and secretion of insulin, such as the genes for GLUT2, glucokinase enzyme and transcription factors (pancreatic duodenal homebox-1 [PDX-1] and MafA) involved in the expression of pre-proinsulin gene [17-19]. In this study, the effects of crude polar (water and ethanol) and non-polar (hexane and ethyl acetate) SBSB extracts on glucose uptake, expression of GLUT2 and glucokinase enzyme genes, expression of selected insulin genes associated transcription factors (PDX-1 and MafA), expression of pre-proinsulin and the total amount of insulin synthesized by RIN-m5F pancreatic beta cells were investigated.

EXPERIMENTAL

Plant material collection, identification and extraction: Fresh *Sclerocarya birrea* stem-bark (SBSB) was collected in April 2014 at Sefako Makgatho Health Sciences University (25º37′06.2″S, 28º01′26.7″E) Gauteng Province, South Africa. The taxonomic identity of the plant was confirmed by Mr. J.J. Meyer at the South African National Biodiversity Institute (SANBI, Pretoria). The voucher specimen of the plant species and material were deposited in the herbarium of SANBI with the unique identification number PRE0996412-0.

The collected plant material was thoroughly rinsed with running tap water, dried in the shade at room temperature, ground into fine powder using a laboratory mill (Polymix DX-MFC 90D, Kinematica, Italy) and stored in closed containers at room temperature till extraction. Dried fine powder (50 g) was then extracted sequentially with 150 mL of hexane, ethyl acetate, ethanol and water according to the method described by Jeyaseelan *et al*. [20] with some modifications. The resultant hexane, ethyl acetate and ethanol fractions were concentrated under *vacuo* using a Büchi rota vaporator (Lasec, SA) at 37 ºC whereas the water extract was freeze-dried using the Vacutec freeze-dryer (Lasec, SA). Twenty mg/mL stock solutions were then prepared by dissolving each crude extracts in 100% DMSO.

Cell cultures: RIN-m5F (ATCC® CRL-11065™) insulinoma cells were donated by Dr. O.O. Olaokun (University of Pretoria), whereas, H-4-II-E (ATCC® CRL1548™) liver cells were donated by Prof. L.J. Shai (Tshwane University of Technology). H-4-II-E liver cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) antibiotic cocktail solution (10000 units/mL penicillin and 0.1 g/L streptomycin) in a humidified atmosphere of 5% at 37 ºC. RIN-m5F cells were cultured in RPMI-1640 supplemented with 10% FBS containing 10 mM HEPES, 1 mM sodium pyruvate and antibiotics. Cell culture media were changed every 3-4 days and cells were seeded for subsequent experiments once they have reached 80-90% confluence.

MTT viability assay: The effect of SBSB extracts on the viability of H-4-II-E was determined using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay described by Mosmann [21], with some modification. Briefly, cultured H-4-II-E cells were seeded in 96-well plates at a density of 1×10^5 cells/well and incubated for 24 h at 37 ºC in a humidified atmosphere of 5% CO_2. Cells were then incubated with 100 µL/well of different concentrations of SBSB extracts (3.125-200 µg/mL) for 72 h. DMSO (1% v/v) and hydrogen peroxide 1% v/v) served as vehicle and positive control, respectively. Thereafter, the medium was removed and replaced with 200 µL of 5 mg/mL MTT solution, followed by incubation at 37 ºC for 1 h. When the formazan crystals were formed, 100% pre-warmed DMSO was added to all the wells, followed by incubation of the plates in the dark for 4 h, after which, the absorbance at 570 nm was measured using a microplate spectrophotometer (Multiskan Accent Thermo Labsystems, Finland). Percentage cell survival rate was calculated from triplicate experiments using the formula:

$$\text{Survival rate (\%)} = \frac{A_{\text{treated cells}} - A_{\text{blank}}}{A_{\text{untreated cells}} - A_{\text{blank}}} \times 100$$

The LC_{50} (plant extract concentration corresponding to 50% survival rate) was calculated from a plot of percentage survival rate *versus* concentration of the plant extracts.

Glucose uptake assay: Glucose uptake by RIN-m5F pancreatic beta cells was determined using a modified method described by Roffey *et al*. [22] with some modifications. In brief, RIN-m5F pancreatic beta cells cultured as described under cell culture section, seeded at a density of 2×10^5 cells/well in a 96-well microtiter plates and incubated for 72 h at 37 ºC in a humidified atmosphere of 5% CO_2. Following incubation, cells were washed once with serum-free RPMI-1640 and incubated in 100 µL/well of the same medium for 2 h at 37 ºC. Thereafter, cells were further washed twice in 1X glucose-free Krebs-Ringer-bicarbonate HEPES (KRBH) buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 10.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 20 mM HEPES, pH 7.4) supplemented with 0.1% bovine serum albumin (BSA) and incubated in 100 µL/well of the same medium for 30 min at 37 ºC. Following this incubation, the medium was removed and replaced with 100 µL/well of KRBH buffer supplemented with 0.1% BSA, 15 mmol/L glucose and SBSB extracts at increasing concentrations (3.12, 6.25, 12.5, 25, 50 and 100 µg/mL). All concentrations used were less than the LC_{50} for SBSB extracts (Table-1).

TABLE-1
CYTOTOXIC EFFECTS (EXPRESSED AS LC_{50}) OF FOUR *S. birrea* STEM-BARK EXTRACTS DETERMINED USING THE MTT ASSAY ON H-4-II-E CELLS

Extract	Yield (%)	LC_{50} (µg/mL)
Hexane	0.28	121.6 ± 1.82
Ethyl acetate	3.50	324.1 ± 8.65
Ethanol	4.74	128.1 ± 3.64
Water	2.62	202.8 ± 4.29

LC_{50} = Extract concentration with 50% inhibition growth of cells

The cell mixtures were subsequently incubated in a 5% CO_2 at 37 ºC for 60 min, prior to determination of glucose uptake by cells. Thereafter, the amount of glucose left in the supernatant medium was determined using a glucose oxidase-based assay kit (KAT Medical Laboratory, South Africa), following the manufacturer's protocol. The amount of glucose uptake effected by the different SBSB extracts was then calculated as the difference between the initial supplemented amount of glucose (15 mmol/L) and the amount of glucose left in the culture media after the incubation. Results were expressed as mean ± standard error of the mean of three independent triplicate experiments.

RNA extraction, cDNA synthesis and conventional PCR analysis: The effect of SBSB extracts on the expression of the pre-proinsulin gene was determined using conventional (end-point) PCR according to the method described by Vanderford *et al*. [23], with some modification. Briefly, RIN-m5F pancreatic beta cells (3×10^5 cells/well in a RPMI-1640 media supplemented with 10% (v/v) FBS, 10 mM HEPES, 1 mM sodium pyruvate and 100 µM β-mercaptoethanol) were cultured (3 mL) in a 6-well plate and incubated for 48 h at 37 ºC in a 5% CO_2 incubator. The cells were further incubated under the same conditions and subsequently treated with different concentrations of SBSB extracts (25, 50 and 100 µg/mL in a complete RPMI-1640 medium containing 15 mmol/L glucose) for 16 h. Untreated RIN-m5F pancreatic beta cells served as a negative control whereas DMSO only, treated cells served as solvent control.

Total RNA was extracted from plant extract treated and untreated RIN-m5F pancreatic beta cells using GeneJet RNA purification kit (Thermo Fisher Scientific). The extracted RNA was reverse-transcribed using the cDNA RevertAid M-MulVRT kit (Thermo Fisher Scientific). The PCR-mediated amplification of the pre-proinsulin gene was performed using KAPATaq ReadyMix DNA polymerase kit (Lasec) using MyCycler (Bio-Rad). The following pre-proinsulin primers used in this study were: forward; 5′-TGCCCAGGCTTTTGTCAAAC-3′; reverse; 5′-CTCCAGTGCCAAGGTCTGAA-3′. The PCR conditions were 95 ºC for 4 min (initial denaturation), followed by 30 cycles of 95 ºC for 30 s, 59 ºC for 30 s and 72 ºC for 2 min. This was followed by final extension phase at 72 ºC for 4 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was co-amplified as internal PCR control using primers: forward; 5′-ACTTTGGCATTGTGGAAGG-3′, reverse; 5′- ACACATTGGGGGTAGGAACA-3′. PCR products were then separated on a 1.25% agarose gel and detected using ChemiDoc Image system (Bio-Rad). Quantification of mRNA levels was performed with ImageLab software version 5.2.1 Program (Bio-Rad).

Quantitative real-time polymerase chain reaction analysis: The effects of SBSB extracts on the expressions of GLUT2, glucokinase, PDX-1 and MafA genes were determined using real-time quantitative polymerase chain reaction (qPCR). Briefly, RIN-m5F pancreatic beta cells were cultured as described above and treated with SBSB extracts at two different concentrations (50 and 100 µg/mL). RNA extraction and cDNA synthesis were also carried out as described above. qPCR was performed with the use of 2X SensiFAST SYBR Hi-ROX PCR Mix (Bioline Celtic Diagnostics), according to the manufacturer's protocol and amplification on StepOnePlus™ Real-Time PCR detection System (AB Applied Biosystems, Bio-Rad). Primer pairs used in this assay are listed in Table-2. The cycling conditions were as follows: 40 cycles each of 2 min at 95 ºC for initial denaturation, 30 s for final denaturation at 95 ºC, annealing for 30 s at 60 ºC and extension for 10 s at 72 ºC, followed by termination in a final cooling step for 30 s at 72 ºC. Relative expression levels of GLUT2, Glucokinase, PDX-1 and MafA genes were evaluated using the CT method (ΔΔCt), (where Ct is the average threshold cycle number from three independent experiments) and normalized to the relative expression of the GAPDH, a housekeeping gene.

TABLE-2
PRIMERS USED FOR GENE EXPRESSION ANALYSIS IN RIN-m5F CELLS

Genes amplified	Primer sequence
MafA	5'-AGGCCTTCCGGGGTCAGAG-3'
	3'-TCGCTCTTCACGGTCGAGGT-5'
PDX-1	5'-AACCCGAGGAAAACAAGAGG-3'
	3'-GTTCAACTCACTGCCAGCTC-5'
Rat GLUT2	5'-TGGGTTCCTTCCAGTTCG-3'
	3'-GTATGCTGTGGTCTGCGGA-5'
Rat glucokinase	5'-TGACAGAGCCAGGATGGAG-3'
	3'-CCGTCACCTCGCACTTCT-5'
GAPDH	5'-ACCACACAGTCCATGCCATCAC-3'
	5'-TCCACCACCCTGTTGCTGA-3'

Total amounts of insulin synthesized by RIN-m5F pancreatic beta cells: The total amount of insulin synthesized by RIN-m5F pancreatic beta cells upon stimulation by different SBSB extracts was calculated from the sum of GSIS amount and intracellular insulin amount. RIN-m5F pancreatic beta cells cultured as described under cell culture section were seeded in a 24-well plate at a density of 2.5×10^5 cells/well) in RPMI-1640 for 48 h at 37 ºC in a humidified atmosphere of 5% CO_2. Cells were washed twice with PBS and were subsequently equilibrated for 20 min in 1X Krebs-Ringer bicarbonate HEPES (KRBH) buffer containing 0.1% BSA and 1.1 mmol/L glucose and then incubated at 37°C in 5% CO_2. Cells were further washed twice with 1X glucose-free KRB-HEPES buffer and then incubated in the presence of SBSB extracts concentrations (25, 50 and 100 µg/mL) containing 15 mmol/L glucose for 30 min at 37 ºC in 5% CO_2 incubator. The plates were then divided in to two parts with one-half for GSIS secretion assay and other half for intracellular insulin content assay.

GSIS amount was measured according to the procedure described by Zhang *et al*. [24], using rat insulin enzyme-linked immunosorbent assay (ELISA) (Sigma-Aldrich). The intracellular insulin content was determined by adding 500 µL acid-ethanol (75% v/v ethanol, 1.5% v/v conc. HCl) to the incubated cells. Aliquots of the medium of RIPA lysed cells were then used to measure the intracellular amount of insulin using rat ELISA kit (Sigma-Aldrich). Both the GSIS amount and the intracellular insulin amount were normalized by dividing them by respective amounts of protein measured using Lowry protein assay kit (Thermo-Fisher Scientific).

Data analysis: Data were expressed as mean ± standard error of the mean (SEM) and displayed graphically using histo-

grams. Student's *t*-test assuming equal variance determined at a $p < 0.05$ and $p < 0.001$ levels of statistical significance was used to detect significant differences between the results of SBSB treated RIN-m5F cells and those of untreated RIN-m5F pancreatic cells or DMSO only treated cells. Data analysis was performed using Microsoft 16 Excel software.

RESULTS AND DISCUSSION

There is a growing interest among researchers to discover new, safe and effective natural products from medicinal plants, especially those with known and scientifically proven anti-diabetic properties [9,15,25]. The present study investigated the effects of *Sclerocarya birrea* stem-bark (SBSB) extracts on glucose uptake, expression of GLUT2 and glucokinase enzyme genes, expression of selected insulin gene associated transcription factors (PDX-1 and MafA), expression of pre-proinsulin gene and on the total amount of insulin synthesized by in RIN-m5F pancreatic beta cells.

Effects of SBSB extracts on glucose uptake: Effects of SBSB extracts at different concentrations on glucose uptake by RIN-m5F pancreatic beta cells were determined and the result are shown in Fig. 1.

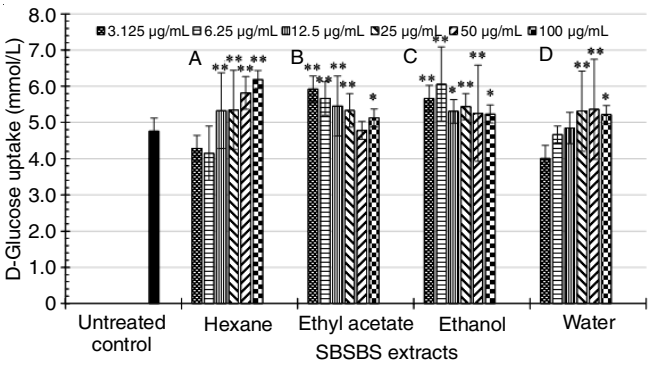

Fig. 1. Effect of SBSB extracts on glucose uptake in RIN-m5F pancreatic beta cells cultured in the presence of 15 mmol/L glucose for 60 min. Data are mean glucose uptake ± SEM of three independent experiments, each performed in triplicate, (n = 9). Standard error of the average relative expression is indicated by the error bars in the figure * indicates statistical significance at $p < 0.05$ and ** indicates statistical significance at $p < 0.001$ when compared with untreated RIN-m5F pancreatic beta control cells

As shown in Fig. 1a, the SBSB hexane extract at concentrations from 12.5 µg/mL to 100 µg/mL significantly enhanced glucose uptake by treated RIN-m5F pancreatic beta cells compared with untreated RIN-m5F pancreatic control cells in a dose-dependent manner ($p < 0.001$). With the exception of 50 µg/mL concentration, all SBSB ethyl acetate extract at concentrations tested, significantly enhanced glucose uptake by RIN-m5F pancreatic beta cells compared with untreated RIN-m5F pancreatic beta control cells. However, enhanced glucose uptake decreased with increasing concentration of the extract (Fig. 1b). All the concentrations of SBSB ethanol extracts significantly enhanced glucose uptake by RIN-m5F pancreatic beta cells. However, the enhancement of glucose uptake was not dose-dependent as in the case of SBSB hexane extract (Fig. 1c). *Sclerocarya birrea* stem-bark (SBSB) water extract at concentrations ranging from 3.12 µg/mL to 50 µg/mL enhanced glucose uptake in a dose-dependent manner. However, significant differences compared with untreated control were only observed only for the 25 µg/mL and 50 µg/mL ($p < 0.001$) as well as the 100 µg/mL ($p < 0.001$) extract concentrations. The highest enhancement of glucose uptake by RIN-m5F pancreatic beta cells by all SBSB extracts of different concentrations was observed with the SBSB hexane extract at a concentration of 100 µg/mL (23.1%), followed respectively by the SBSB ethanol extract at a concentration of 6.25 µg/mL (21.4%), SBSB ethyl acetate extract at a concentration of 3.13 µg/mL (19.6%) and the SBSB hexane extract at a concentration of 50 µg/mL (18.2%).

The uptake or entry of glucose into pancreatic beta cells is a necessary step in both the regulation of insulin synthesis and secretion [3,26]. However, the entry of glucose in pancreatic beta cells does not depend on the action of insulin, instead, it is regulated in rodents by the GLUT2 and the enzyme, glucokinase [18,27]. Whereas most studies have investigated the effect of plant extracts on glucose uptake by insulin dependent cells like muscle and adipose tissue [14,28], very little or no study have investigated the effect of antidiabetic medicinal plants on glucose uptake by non-insulin dependent pancreatic beta cells. The results of the current study indicate that SBSB hexane and water extracts at concentrations ranging from 12.5 µg/mL to 100 µg/mL, significantly enhanced glucose uptake by RIN-m5F pancreatic beta cells in a concentration dependent manner when compared with untreated RIN-m5F control cells. On the other hand, *S. birrea* ethyl acetate and ethanol extracts appeared to have initially enhanced glucose uptake by RIN-m5F at a concentration 3.12 µg/mL and 6.25 µg/mL respectively, but later suppressed it as the concentration of the extracts increased from 12.5 µg/mL to 100 µg/mL. This observation suggests that highly non-polar and highly polar constituents of SBSB extracts may enhance glucose uptake by RIN-m5F cells, whereas the moderately polar and non-polar constituents might suppress glucose uptake by these cells.

Effects of *S. birrea* stem-bark on insulin synthesis and secretion: Effects of antidiabetic agents on the total amount of insulin synthesized by pancreatic beta cells can be assessed by measuring and calculating the sum of the intracellular insulin content left after GSIS and the GSIS amount of insulin after the stimulation of pancreatic beta cells with such an antidiabetic agent [29]. Thus, in the current study, the total amount of insulin synthesized by RIN-m5F pancreatic cells after 30 min incubation and stimulation of these cells with SBSB extracts was assessed.

Effects of SBSB extracts on intracellular insulin content, GSIS and the total amount of insulin synthesized by RIN-m5F pancreatic beta cells (intracellular insulin amount plus GSIS) are shown in Fig. 2. The SBSB ethyl acetate (50 µg/mL) and water (50 µg/mL) extracts significantly suppressed insulin synthesis (total amount synthesized) (Fig. 2c) and GSIS (Fig. 2b) in treated RIN-m5F pancreatic beta cells when compared with untreated RIN-m5F pancreatic control cells ($p < 0.001$). However, all other SBSB extracts at concentrations investigated in the study, significantly enhanced both insulin synthesis and GSIS in treated RIN-m5F pancreatic beta cells when compared

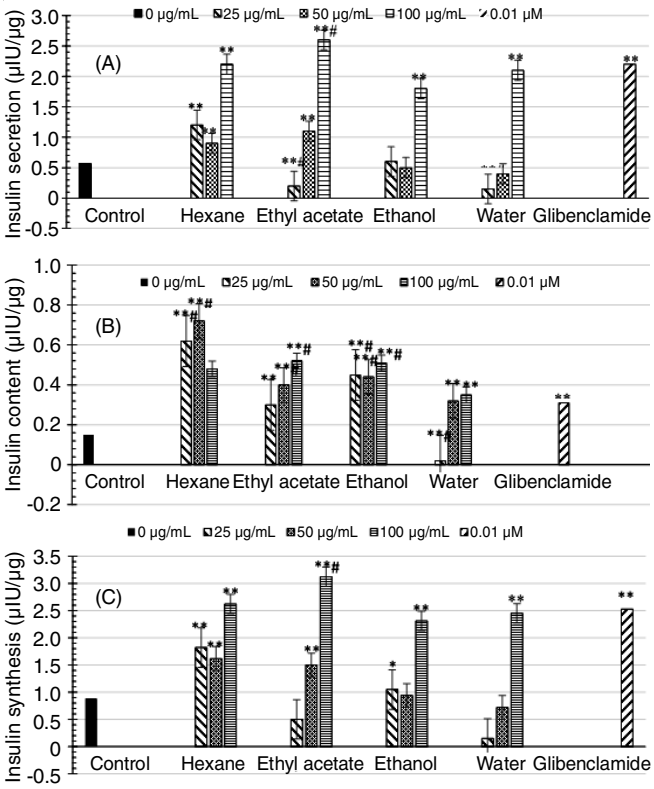

Fig. 2 Effects of SBSB extracts on (A) intracellular insulin concentration, (B) glucose stimulated insulin secretion and (C) total amount of insulin synthesized by RIN-m5F pancreatic beta cells. Data are mean insulin concentration ± SEM of three independent experiments, each performed in triplicate, (n = 9). Standard error of the insulin concentration is indicated by the error bars in the figure * indicates statistical significance at $p < 0.05$ and ** indicates statistical significance at $p < 0.001$ when compared with untreated RIN-m5F pancreatic beta control cells. # indicates statistical significance at $p < 0.001$ when compared with 0.01 µM glibenclimide

with untreated RIN-m5F pancreatic beta control cells. The SBSB hexane extracts (50 and 100 µg/mL), the SBSB ethyl acetate (100 µg/mL) and SBSB significantly enhanced GSIS compared with 0.01 µM glibenclamide, whereas only the SBSB ethyl acetate extract (100 µg/mL) significantly enhanced total insulin synthesis compared with 0.01 µM glibenclamide.

All the SBSB extracts under investigation significantly enhanced GSIS at 100 µg/mL compared with untreated control cells. In this regard, the hexane, ethyl acetate, ethanol and water extracts enhanced the production of 2.4, 2.6, 1.8 and 2.1 µIU/µg protein insulin, respectively. However, the amount of insulin secreted by RIN-m5F cells upon stimulation by *S. birrea* stem-bark extracts were significantly lower than that produced by the standard antidiabetic agent, glibenclamide at 0.01 µM. The amount of intracellular insulin left after GSIS were very low compared to the amount of insulin secreted during GSIS, and it ranged from 0.38 µIU/µg protein for the water extract and 0.56 µIU/µg protein for ethyl acetate extract. The amount of intracellular insulin left after GSIS were more or less similar to that which was left after the glibenclamide stimulated insulin secretion.

Effects of SBSB extracts on the expression of GLUT2 and glucokinase gene: As mentioned above, glucose entry in to pancreatic beta cells does not depend on the action of insulin, instead it is regulated in rodents by the GLUT2 and the enzyme, glucokinase [18,27]. Thus, as one of the possible mechanisms of action for the observed enhancement of glucose uptake by SBSB extracts, the effect of extracts of SBSB extracts on the expression of both GLUT2 and glucokinase enzyme in RIN-m5F pancreatic beta cells were also investigated in this study.

The effects of SBSB extracts on the expression of GLUT2 gene in RIN-m5F pancreatic beta cells are shown in Fig. 3a. The SBSB hexane extract (50 µg/mL), SBSB ethyl acetate extract (100 µg/mL) and the SBSB ethanol extract (100 µg/mL) significantly up-regulated the expression of the GLUT2 gene 2.7, 5.5 and 1.3-fold, respectively, when compared with same gene expression in untreated RIN-m5F pancreatic beta cells ($p < 0.05$). On the other hand, SBSB hexane extract (100 µg/mL), SBSB ethyl acetate extract (50 µg/mL), SBSB ethanol extract (50 µg/mL), SBSB water extract (50 µg/mL) and SBSB water extract (100 µg/mL) significantly down-regulated the expression of GLUT2 0.5, 0.3, 0.3 and 0.7-fold, respectively, compared with the expression of same gene in untreated cells ($p < 0.05$).

The effects of SBSB extracts on the expression of glucokinase enzyme gene in RIN-m5F pancreatic beta cells are shown in Fig. 3b. The SBSB hexane extract (50 µg/mL), SBSB ethyl acetate (100 µg/mL), SBSB ethanol extract (100 µg/mL) and SBSB water extract (100 µg/mL) significantly up-regulated the expression of glucokinase gene 3.2, 9.9, 7.4 and 7.6-fold, respectively, compared with the expression of same gene in untreated cells ($p < 0.001$) (Fig. 3). On the other hand, SBSB hexane extract (100 µg/mL), SBSB ethyl acetate extract (50 µg/mL), SBSB ethanol extract (50 µg/mL) and SBSB water extract (50 µg/mL) significantly down-regulated the expression of the glucokinase gene 0.01, 0.6, 0.6. and 0.4-fold, respectively in treated RIN-m5F pancreatic beta cells compared with the expression of same gene in untreated cells.

Present observation is that SBSB hexane extract at a concentration of 50 µg/mL significantly up-regulated the expression of both GLUT2 and the glucokinase enzyme compared to the untreated control cells. However, this up-regulation surprisingly, could not be observed at a higher concentration of 100 µg/mL. On the other hand, stem-bark ethyl acetate extract of *S. birrea* at 100 µg/mL but not at 50 µg/mL significantly up-regulated the expression of both GLUT2 and the glucokinase enzyme in RIN-m5F cells in comparison with untreated control cells. Compared with untreated control cells, both the ethanol and water extract of stem-bark of *S. birrea* at 100 µg/mL, significantly up-regulated the expression of enzyme, glucokinase, but not that of GLUT2. Taken together, these observations suggested that *S. birrea* stem-bark hexane, ethyl acetate, ethanol and water extracts may enhance glucose uptake by RIN-m5F pancreatic beta cells, in part, though the up-regulation of either the GLUT2 or glucokinase enzyme or even both. As to why the SBSB hexane extract up-regulated the expression of both GLUT2 and the glucokinase enzyme at 50 µg/mL but not at 100 µg/mL is not clear but may be related to the toxic effect of the extract at a higher concentration on the cultured cells [30].

Effects of SBSB extracts on PDX-1 and MafA genes: Plant extracts that enhance or promote the secretion of insulin

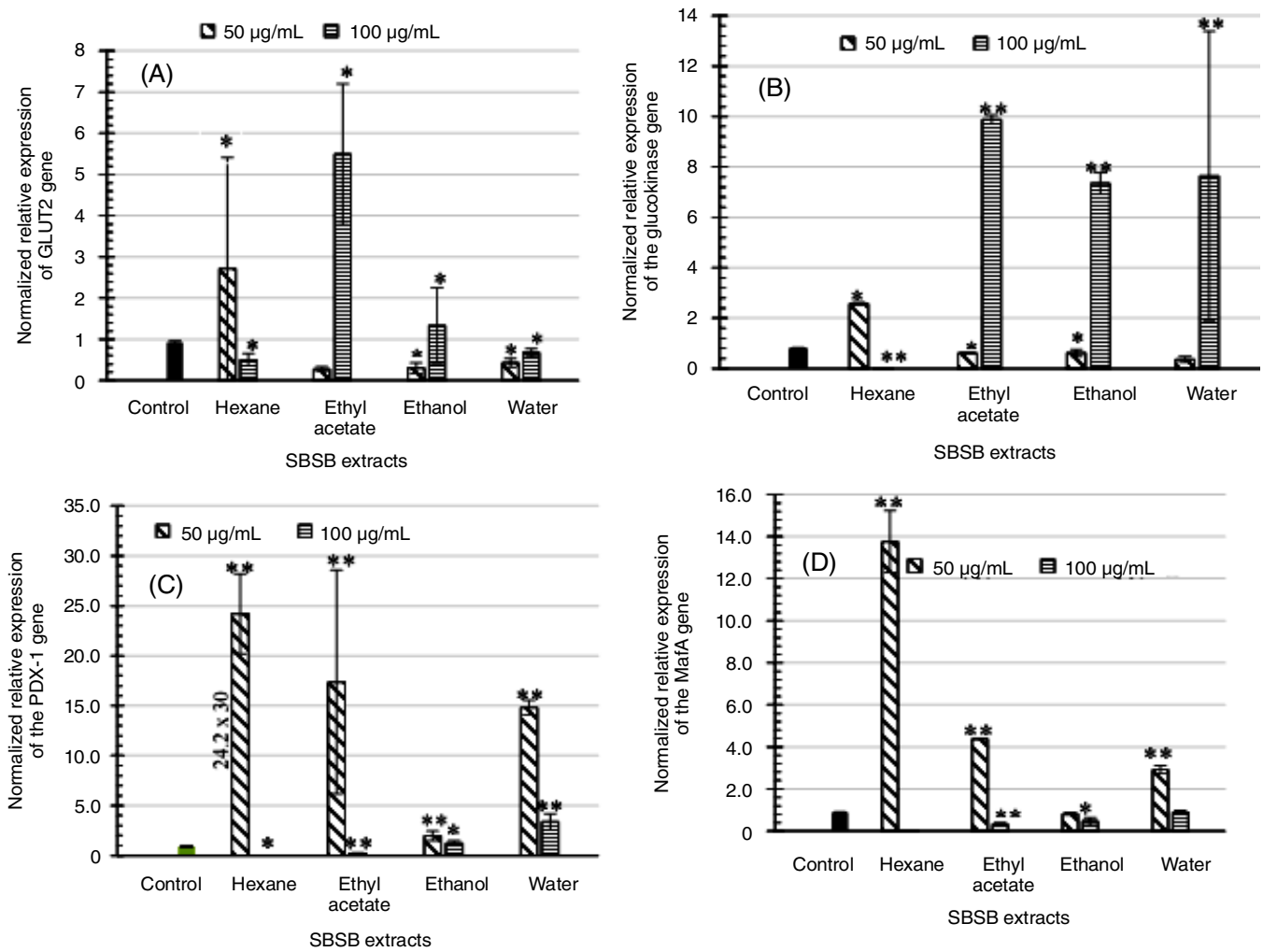

Fig. 3. Effects of SBSB extracts on the relative expression of (A) GLUT2, (B) glucokinase (C) PDX-1 and (D) MafA mRNAs in RIN-m5F pancreatic beta cells. Treatments with SBSB extracts at concentrations of 50 µg/mL and 100 µg/mL were performed in triplicate. Data are expressed as the relative fold change in mRNA expression normalized to the GAPDH house keeping reference gene. Standard error of the average relative mRNA expression is indicated by the error bars in the figure. * indicates statistical significance at $p < 0.05$ and ** indicates statistical significance at $p < 0.001$ when compared with untreated RIN-m5F pancreatic beta control cells

by pancreatic beta cells may do so though stimulation of insulin synthesis [17,31], in particular, the up-regulation of the genes of transcription factors such as PDX-1 and MafA that are involved in the transcription of insulin gene. In light of this, the effect of *S. birrea* stem-bark extracts on the expression of insulin gene transcription factors, PDX-1 and MafA as well as on the expression of insulin gene itself were also investigated.

As shown in Fig. 3c, SBSB hexane extract (50 µg/mL), SBSB ethyl acetate extract (50 µg/mL), SBSB ethanol extract (50 µg/mL), SBSB ethanol extract (100 µg/mL), SBSB water extract (50 µg/mL) and the SBSB water extract (100 µg/mL) significantly up-regulated the expression of the PDX-1 gene 726, 17.4, 2.0, 1.2, 14.8 and 3.4-fold, respectively, in treated RIN-m5F pancreatic beta cells when compared with untreated cells ($p < 0.05$). On the other hand, the SBSB hexane extract (100 µg/mL) and the SBSB ethyl acetate extract (100 µg/mL) significantly down-regulated the expression of the PDX-1 gene 0.02 and 0.2-fold, respectively, in treated RIN-m5F pancreatic beta cells compared with the expression of the same gene in untreated control cells. The effects of SBSB extracts on the expression of the MafA gene in RIN-m5F pancreatic beta cells are shown in Fig. 3d. In the current study, hexane, ethyl acetate and water SBSB extracts, but not the SBSB ethanol extract, also up-regulated the expression of MafA gene 13.8, 4.4 and 2.9-folds, respectively compared with untreated control cells at a lower concentration of 50 µg/mL but not at a higher concentration of 100 µg/mL. The findings of this aspect of the present study revealed that compared with untreated control cells, PDX-1 gene was significantly up-regulated by all the SBSB extracts under investigation at a lower concentration of 50 µg/mL but not at a higher concentration of 100 µg/mL with hexane, ethyl acetate, ethanol and water extracts, respectively.

Effect of SBSB extracts on pre-proinsulin gene: The effects of SBSB extracts at concentrations of 25, 50 and 100 µg/mL were investigated using the conventional end-point PCR technique. The expression profile of pre-proinsulin in the SBSB extract treated RIN-m5F pancreatic beta cells compared to the DMSO control is shown in Fig. 4.

The SBSB hexane extract (100 µg/mL), SBSB ethyl acetate extract (100 µg/mL) and SBSB ethanol extract (50 and 100 µg/mL) significantly up-regulated the expression of pre-proinsulin mRNA compared to the expression of pre-proinsulin gene in the DMSO control ($p < 0.05$). The highest fold increase (7.7) in pre-proinsulin mRNA was produced by the SBSB ethanol extract while the lowest fold increase (2.0) in pre-proinsulin mRNA was observed with the SBSB ethanol extract. On the other hand, the SBSB water extract at concentrations of 50 and 100 µg/mL significantly down-regulated the expression of pre-proinsulin mRNA compared to the expression of pre-proinsulin gene in DMSO only treated control cells.

With exception of water extract, other SBSB extracts investigated in this study, up-regulated the expression of insulin gene with hexane, ethyl acetate and ethanol extracts showing respectively 5.1, 1.5 and 7.7-folds up-regulation of insulin gene expression. Taken together, these findings simply or suggest that SBSB extracts, in particular, their non-polar phytochemical components have the capacity to enhance the expression or transcription of insulin gene, as well as that of its two transcription factors, PDX-1 and MafA. The up-regulation of transcription factors correlates with an increase in insulin secretion and insulin expression, especially because PDX-1 is a key regulator of insulin expression and release by the pancreatic beta cells, as well as beta cell maturation [32]. For instance, mature beta cells with PDX-1 deficiency are associated with glucose intolerance, suggesting a critical role of PDX-1 in maintaining beta cell function. Similarly, MafA is a transcription factor that specifically binds to a conserved insulin enhancer element, RIPE3b/C1-A2 and activates insulin gene expression [33]. The results suggest that ethyl acetate and hexane extracts may induce their hypoglycaemic effects by enhancing insulin expression and release *via* PDX-1 and MafA genes, as well as activation of glycolysis by activating glucokinase in pancreatic beta cells.

Limitations of the study: A major limitation of the present study is that more valuable information could have been obtained if these investigations were accompanied by investigations of the effect of the plant extracts on the protein/enzyme activity of glucokinase, PDX-1 and MafA. Despite this limitation, a number of strengths related to the current study can be pointed out. Firstly, plant extracts of different polarity obtained through the sequential extraction of each plant material, secondly, the simultaneous effect of SBSB extracts on several parameters involved in the insulin synthesis and secretion pathways and

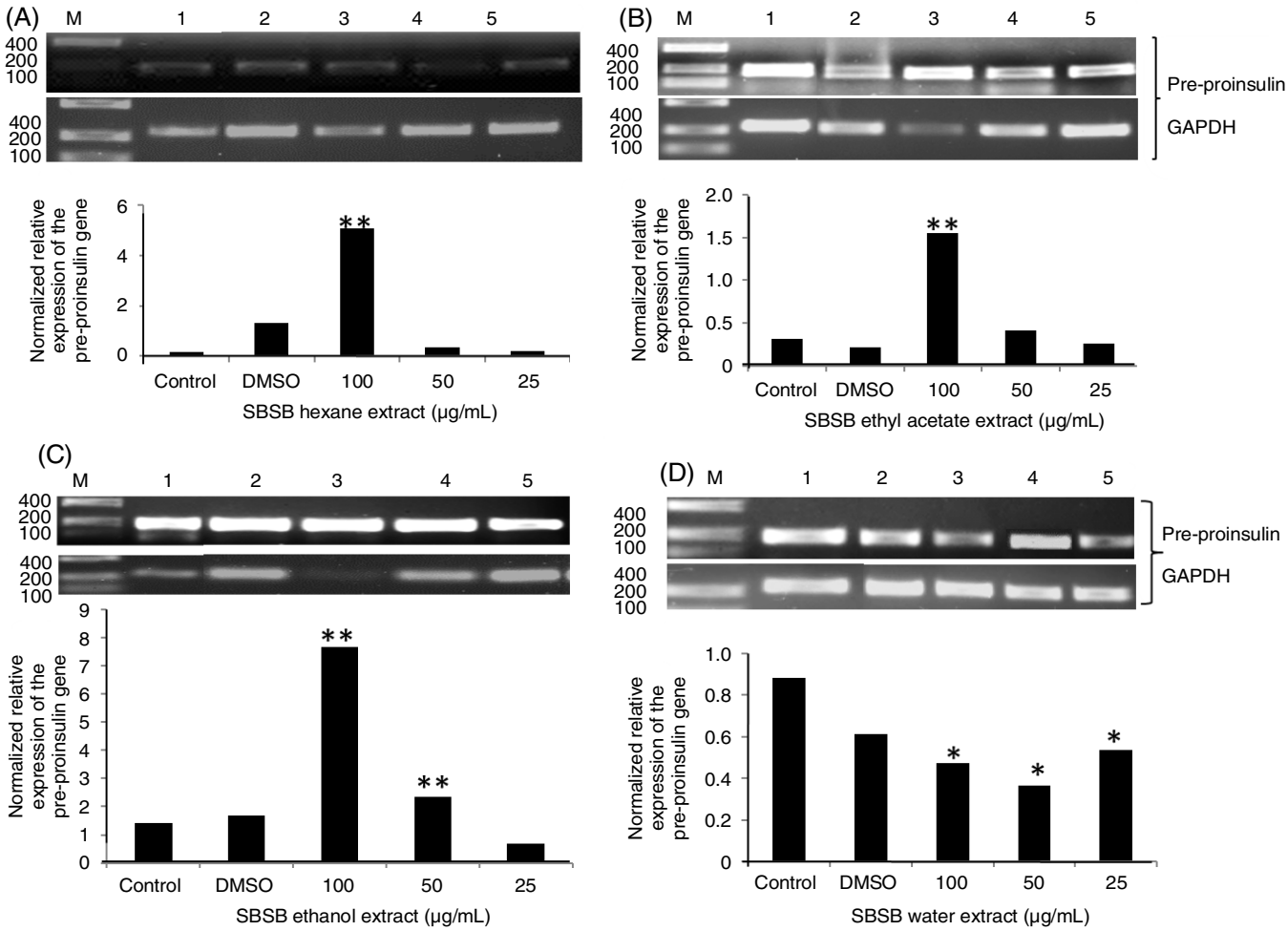

Fig. 4. Effects of SBSB extracts on the relative expression of pre-proinsulin mRNA. (A) Effect of SBSB hexane extract, (B) effect of SBSB ethyl acetate extract, (C) effect of SBSB ethanol extract and (D) effect of SBSB water extract. Data are expressed as the relative fold change in mRNA expression normalized to the GAPDH house keeping reference gene * indicates statistical significance at $p < 0.05$ and ** indicates statistical significance at $p < 0.001$ when compared with the DMSO control

thirdly, the effect of each extract at two or more different concentrations in order to reveal the presence or lack of a dose dependent effect were investigated.

Conclusion

The results of the study suggest that *Sclerocarya birrea* stem-bark (SBSB) hexane and water extracts significantly enhances glucose uptake by RIN-m5F pancreatic beta cells in a dose-dependent manner. The results also, suggest that with the exception of SBSB ethyl acetate (50 µg/mL) and water (50 µg/mL) extracts, all other SBSB extracts at concentrations investigated in the study significantly enhanced both insulin synthesis and GSIS in RIN-m5F pancreatic beta cells compared with control. Whereas, SBSB ethyl acetate, ethanol and water extracts at concentrations investigated in the study, up-regulated the expression of some of the genes and suppressed the expression of some of the genes investigated in the study, SBSB hexane extract at a concentration of 50 µg/mL appears to up-regulate the expression of all the genes under investigation. Overall, it can be concluded that SBSB hexane extract enhances glucose uptake and insulin synthesis in RIN-m5F cells partly through up-regulation of the expression of GLUT2, glucokinase, PDX-1, MafA and pre-proinsulin genes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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