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Marrubiin, a constituent of *Leonotis leonurus*, alleviates diabetic symptoms

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ABSTRACT

Aims: Marrubiin and an organic extract of *Leonotis leonurus* were tested *in vitro* and *in vivo* for their antidiabetic and anti-inflammatory activities.
Materials and methods: INS-1 cells were cultured under normo- and hyperglycemic conditions conditions. An *in vivo* animal model confirmed the biological activities of marrubiin and the organic extract observed in the studies *in vitro*.
Results: The stimulatory index of INS-1 cells cultured under hyperglycemic conditions was significantly increased in cells exposed to the organic extract and marrubiin, relative to the hyperglycaemic conditions. Insulin and glucose transporter-2 gene expressions were significantly increased by the organic extract and marrubiin. Similarly, the extract and marrubiin resulted in an increase in respiratory rate and mitochondrial membrane potential under hyperglycaemic conditions. Marrubiin increased insulin secretion, HDL-cholesterol, while it normalized total cholesterol, LDL-cholesterol, atherogenic index, IL-1β and IL-6 levels in an obese rat model.
Conclusion: The results provide evidence that marrubiin, a constituent of *Leonotis leonurus*, alleviates diabetic symptoms.

Introduction

Pancreatic β-cells secrete insulin which is the primary hormone that maintains physiological glucose homeostasis. Hyperglycemia and/or free fatty acids (FFAs) have been shown to trigger insulin deficiency in type 2 diabetes (T2D). In T2D a deficiency of insulin is caused by the loss of β-cells function and mass, as they fail to compensate against insulin resistance (IR) (Choi et al. 2007). Obesity is closely associated with IR which is a pre-diabetic state that often results in T2D. Exposure to FFAs for a long time could hinder glucose-stimulated insulin secretion (GSIS), insulin gene expression and cause apoptosis of cultured β-cells or isolated islets (Choi et al. 2007). GSIS relies heavily on mitochondrial metabolism since glucose oxidation is tightly associated with ATP production (Affourtit and Brand 2008). An increase in cytosolic [Ca^{2+}] occurs when blood glucose levels increase, as a result mitochondrial [Ca^{2+}] increases, leading to the activation of the respiratory chain through the stimulation of Ca^{2+}-sensitive NADH-generating dehydrogenases. The resulting NADH and $FADH_2$ are fed into the electron transport chain, thereby increasing ATP production through an increase of the mitochondrial membrane potential (Affourtit and Brand 2008).

Leonotis leonurus (Lamiaceae) R.Br a plant indigenous to South Africa contains the diterpenoid labdane lactones premarrubiin and marrubiin (M) (Fig. 1). Previous studies have shown that the leaf extract of this plant possessed hypoglycaemic effects in a streptozotocin (STZ)-induced diabetic rat model (Ojewole 2005; Scott et al. 2004). *L. leonurus* has been reported to be traditionally used to treat hypertension (Van Wyk et al. 2000). Ojewole (2003) evaluated the aqueous extract for its cardiovascular and hypotensive effects in rats and observed that the arterial blood pressures and heart rates of normal, anaesthetized spontaneously hypertensive rats were significantly reduced (Ojewole 2003). This study was undertaken to investigate the mechanism of the hypoglycaemic activity of *L. leonurus* extracts and to determine if M is one of the compounds responsible for the biological effects observed.

Methods and materials

All the reagents used were of an analytical grade.

Abbreviations: AI, atherogenic index; ASP, aspirin; AUC, area under the curve; C, control; CVD, cardiovascular diseases; DM, diabetes mellitus; FFAs, free fatty acids; Glut, glucose transporter; GSIS, glucose-stimulated insulin secretion; IL, interleukin; IPGTT, intraperitoneal glucose tolerance test; HGC, hyperglycaemic control; IPITT, intraperitoneal insulin tolerance test; IR, insulin resistance; LC, lean control; M, marrubiin; MET, metformin; MMP, mitochondrial membrane potential; NMMU, Nelson Mandela Metropolitan University; NC, negative control; NGC, normoglycaemic control; OC, obese control; OL, organic extract; PFP, pentafluorophenyl; RIA, radioimmunoassay; STZ, streptozotocin; SU, sulfonylurea; T2D, type 2 diabetes; TZDs, thiazolidinediones.

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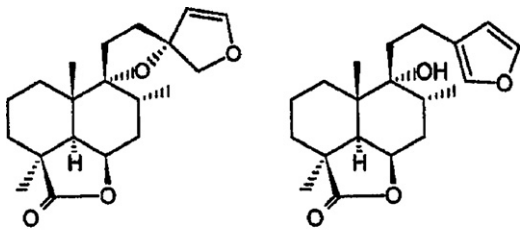

Fig. 1. The chemical structures of premarrubiin and marrubiin (Van Wyk et al. 2000).

Ethical clearance

Ethical clearance for this study was approved by the Nelson Mandela Metropolitan University (NMMU) Animal Ethics Committee (Ref: A09-SCI-BCM-001).

Plant material

L. leonurus was collected at NMMU, South campus and examined by Mr C. Weatherall-Thomas (the curator of the Herbarium of the Department of Botany, Nelson Mandela Metropolitan University) with reference number 19302.

Plant extraction and marrubiin quantification

The extraction procedure used is a modified version described by Mnonopi et al. (2011), Rivett (1964) and Knöss et al. (1997) where they isolated and quantified M from *L. leonurus* and *Marrubium vulgare* L. (Lamiaceae), respectively. M isolated from the *L. leonurus* leaf organic (OL) extract was quantified as described by Mnonopi et al. (2011). For some experiments completed OL1 = 2.5 μg/ml, OL2 = 5 μg/ml, OL3 = 10 μg/ml, M1 = 125 ng/ml, M2 = 250 ng/ml, and M3 = 500 ng/ml.

Cell culture and GSIS

INS-1 rat insulinoma cells were maintained at 37 °C, in a humidified atmosphere supplemented with 10% CO_2 in RPMI 1640 media containing glutamax, 5% FBS, 10 mM Hepes, 50 mM 2-mercaptoethanol and 1 mM sodium pyruvate. The INS-1 cells were exposed to OL (2.5–10 μg/ml) and M (125–500 ng/ml) in 11.1 mM glucose RPMI (normoglycaemic conditions) and 33.3 mM glucose (hyperglycaemic conditions) RPMI for 48 h. GSIS was conducted thereafter, and chronic, basal, stimulated, and insulin content samples were collected (Maedler et al. 2001). Insulin content was determined using the insulin radioimmunoassay (RIA) kit (Linco Research), which was evaluated using a liquid scintillation analyser (TRI-CARB 2300TR).

Cell respiration

INS-1 cells were treated with the OL3 extract and M3 in RPMI for 48 h under both normo- and hyperglycaemic conditions, respectively. After the treatment, a cell count was determined using Trypan Blue and a haemocytometer. Cell respiration rates were determined using the polarographic method. RPMI (1 ml) was used to set the oxygen limit (to 100%), while aliquots of treated cells (1 ml, 10^6 cells/ml) were placed in fresh media within the 37 °C incubation chamber. The oxygen consumption of these cells was measured over 500 s, and captured and analyzed using LabChart 6. Oxygen consumption rates were calculated from the initial steady traces of oxygen uptake and expressed per 10^6 viable cells. Oligomycin (100 μM) was used as a negative control (NC).

Mitochondrial membrane potential

The mitochondrial membrane potential (MMP) in INS-1 cells was determined by using the MMP detection kit (BD Biosciences). Cells were exposed to experimental conditions as indicated in section 2.5. Camptothecin (100 μM) was used as the NC. After treatment, cells were trypsinized, and resuspended in culture media (1 ml). Cells (20 000 cells/ml) from each experimental condition were dyed using JC-1 dye, and quantified using the flow cytometry (Beckman Coultier, Cytomics FC 500).

qPCR

INS-1 cells were exposed to the OL extract (10 μg/ml) or M (500 ng/ml). RNA was isolated for qPCR using an RNeasy Mini kit (Qiagen). Total RNA was quantified spectrophotometrically and cDNA synthesized using a QuantiTect Reverse Transcription kit. qPCR was performed using the iQ SYBR Green Supermix (BIO-RAD) and the BIO-RAD iQ detection system. GeNorm was used to determine the stability of reference genes tubulin, GAPDH and cyclophilin A (M value all less than 1.5), and generate normalization factors across all experimental conditions, relative to the target genes, glucose transporter (Glut)-2 and insulin.

Obesity animal model

Two-week-old male Wistar rats, weighing an average 69.34 ± 5.9 g were obtained from the University of Cape Town Animal Unit and housed in pairs/triplicates in standard animal cages. They were exposed to a 12L:12D photoperiod cycle. Animals were randomly divided into 7 groups, containing 6 rats each: lean control (LC) group, the obese control (OC) group, the obese metformin (MET) group, the obese sulfonylurea (SU) group, the obese aspirin (ASP) group, the obese OL treatment group and the obese marrubiin (M) treatment group (Ashour et al. 2009). The lean rats were fed rat chow and the obese rats were fed on a cafeteria diet as described by Mnonopi et al. (2011). After a treatment period of 2 weeks, intraperitoneal glucose tolerance test (IPGTT), intraperitoneal insulin tolerance test (IPITT), fasting insulin-, triglyceride-, cholesterol levels and pro-inflammatory markers (IL-1β and IL-6) were evaluated.

Intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT)

After the treatment period, rats were fasted for 15 h. Rats were injected intraperitoneally with either glucose (2 g/kg body weight) or insulin (1 unit/kg body weight). After administration of either glucose or insulin, blood glucose levels were determined from the tail vein at 0, 30, 60 and 120 min. Blood glucose was determined using a OneTouch Select AW 06505401A (Lifescan glucometer). Area under curve (AUC) of glucose (AUC_g) and insulin (AUC_i) were calculated using the following formula: $AUC = (\text{concentration}_0 + \text{concentration}_1)/2 \times \text{time}_{1-0}$ (Chiou 1978).

Plasma insulin, triglycerides and cholesterol levels

Fasting plasma insulin concentrations were determined using a insulin RIA kit (Linco Research). Arterial blood was collected using a 1 ml syringe that contained 100 μl of 0.105 M sodium citrate, and centrifuged at 300 × *g* for 15 min to obtain plasma. Post-experimental plasma triglyceride levels were determined with the GPO-PAP kit (Roche Diagnostics) and plasma cholesterol using the CHOD-PAP kit (Roche Diagnostics).