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Extracts from *Leonurus sibiricus* L. increase insulin secretion and proliferation of rat INS-1E insulinoma cells

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ABSTRACT

Ethnopharmacological relevance: Traditional Mongolian medicine (TMM) uses preparations from herbs as one form of medication for the treatment of a diversity of diseases including diabetes mellitus (DM). We evaluated the effect of extracts from the plant *Leonurus sibiricus* L. (LS), used in TMM to treat typical symptoms of type 2 DM, on insulin secretion, electrophysiological properties, intracellular calcium concentration and cell proliferation of INS-1E insulinoma cells under standard cell culture conditions (SCC; 11.1 mM glucose).

Materials and methods: Insulin secretion was measured by ELISA, electrical properties were assessed by whole cell patch clamping, intracellular calcium concentration (Ca_i) by Fluo-4 time lapse imaging, insulin receptor expression was verified by RT-PCR and cell proliferation assessed by CellTiter-Glo® cell viability assay.

Results: Insulin released from INS-1E cells into the culture medium over 24 h was significantly increased in presence of 500 mg/L aqueous LS extract (LS OWE) as well as methanolic LS extract (LS MeOH/H2O) but not in the presence of the butanol-soluble extract (LS MeOH/BuOH). Acute application of LS OWE resulted in a depolarization of the cell membrane potential paralleled by an initial increase and subsequent decline and silencing of action potential frequency, by KATP channel inhibition, persisting depolarization and an increase in Ca_i. The electrophysiological effects were comparable to those of 100 µM tolbutamide, which, however failed to elevate insulin secretion under SCC. Furthermore all LS extracts stimulated INS-1E cell proliferation.

Conclusions: The finding that extracts from *Leonurus sibiricus* L. enhance insulin secretion and/or foster cell proliferation may provide possible explanations for the underlying therapeutic principles in the empirical use of LS-containing formulations in DM and DM-related disorders as applied in TMM.

Abbreviations: AP, action potential; Ca_i, intracellular calcium concentration; DM, Diabetes mellitus; T2DM, type 2 diabetes mellitus; DMrD, DM-related disorders; GLUT-4, glucose transporter isoform 4; IK_{ATP}, ATP-sensitive K^+ conductance/currents; IR, insulin receptor; LS, *Leonurus sibiricus* L.; LSMeOH/BuOH, *Leonurus sibiricus* purified methanolic extract; LS MeOH/H_2O, *Leonurus sibiricus* water-soluble part of the methanolic extract; LS OWE, *Leonurus sibiricus* original water extract; SCC, standard cell culture condition; T2DM, Type 2 Diabetes mellitus; TMM, Traditional Mongolian Medicine; TOL, tolbutamide; V_{mem}, cell membrane potential; WHO, World Health Organization.

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1. Introduction

Driven by socio-economic needs empiricism-based traditional healing methods have gained worldwide attention with respect to evidence based scientific evaluation of their medical efficiency, unraveling the mode(s) of action(s) as well as identification of active constituents. However, national pharmacopoeias or other regulations regarding formulae, analytical composition, physical constants, main chemical properties and mode(s) of preparation of traditional herbal medicines do not exist in the majority of countries practicing traditional medicines (WHO, 2005). Traditional Mongolian medicine (TMM), using herbs as one form of medication, exists for more than 2500 years. 200–250 medicinal plants are commonly used and more than 800 plants have been registered in Mongolia as medicinal plants. TMM has developed from traditional Tibetan medicine by replacing shamanistic practices with medical theories, techniques and medications (Ligaa, 1996).

In the year 2010 worldwide approximately 285 million people were affected by Diabetes mellitus (DM) with type 2 (T2DM) making up about 90% of the cases (Melmed et al., 2011). The incidence of T2DM is increasing rapidly (Wild et al., 2004), and the disease is a major socio-economic cost factor for all health care systems (WHO, 2013). Since antiquity, DM or DM-related disorders (DMrD) have been treated with herbal medicines, such as plants and their extracts. In Mongolia, different medicinal plants are used in the treatment of DM or DMrD, such as the prescription BAIMAI-SAN, which consists of 19 different ingredients (Liu et al., 2011). However, to date, only a small number of scientific studies have addressed the application of medicinal plants, traditionally used in TMM, to treat DM and the underlying mechanisms of action still have to be identified.

T2DM is characterized by insulin resistance of peripheral tissues and defective insulin secretion which results from intrinsic β-cell dysfunction and reduced β-cell mass (Bonner-Weir and O'Brien, 2008; de Koning et al., 2008). Studies demonstrated a 63% reduction in islet mass of T2DM patients as compared to normo-glycemic control subjects and a loss of β-cell mass even at early stages of the disease due to increased rates of apoptosis (Butler et al., 2003). Both, the peripheral tissues as sites of insulin action and the site of hormone production itself, the β-cell, are possible targets for medicinal plant constituents used in the treatment of T2DM.

Two genera, *Dryopteris* and *Filix*, used in Mongolia as medicinal plants, have been reported to ameliorate diabetic symptoms. Diabetic rats treated with extracts of these plants showed improved plasma glucose responses as assessed by oral glucose tolerance test and decreased glucose clearance rates during low-dose euglycemic clamp studies were markedly improved by a single treatment with these extracts. However, insulin levels were not changed in animals treated with the plant extracts. The authors concluded that the effect on plasma glucose levels was due to an improvement in insulin resistance triggered by an activation of the insulin-signaling pathway in skeletal muscle and therefore an increased amount of the glucose transporter GLUT-4 present in the plasma membrane (Khookhor et al., 2007). Effects on GLUT-4 translocation to the plasma membrane have also been described for other herbal constituents, such as the stilbenoid resveratrol (Penumathsa et al., 2008). On the β-cell level, marrubiin, an organic extract from *Leontis leonurus* R.Br, has been shown to increase insulin secretion in INS-1 cells both at 11.1 mM and 33.3 mM glucose, and to alleviate diabetic symptoms in a rat obesity animal model (Mnonopi et al., 2012). Furthermore, nuciferine, the main alkaloid from leaves of *Nelumbo nucifera*, a plant used in traditional Vietnamese medicine that exerts anti-diabetic effects, stimulates insulin secretion in isolated islets from CD1 mouse pancreas and INS-1E cells (Nguyen et al., 2012).

According to the Report of Market Research on Mongolian Traditional Medicinal Drugs, 2007, *Leonurus sibiricus* L. (LS) is one of the most common traditional medicinal raw materials originating from domestic markets (HSUM and MoH, 2007). It is used to treat menstrual irregularities, amenorrhea, malaria and hypertension (Taehakkyo and Yŏn'guso, 1998), and is known for several beneficial properties, which have been investigated in a number of scientific studies. LS was shown to exert anti-inflammatory activity by decreasing levels of inflammatory cytokines secreted from human mast cells (Shin et al., 2009). Moreover, LS can induce expression of nitric oxide and tumor necrosis factor-α in mouse peritoneal macrophages (An et al., 2008) and stimulate the murine uterus in vitro (Shi et al., 1995). Furthermore, it was shown to exert antibacterial activity (Ahmed et al., 2006), to have analgesic as well as anti-inflammatory activity in rats (Islam et al., 2005), and to possess potent antioxidant capacity (Lee et al., 2010). In Mongolian literature, LS is mentioned as a treatment for typical symptoms of T2DM such as thirst ((Khaidav and Choijamts, 1965) and personal communication with Prof. Narantuya Samdan, WHO). It is also used to treat DM in the traditional medicine of Bangladesh (Mohammed Rahmatullah et al., 2010). As atherosclerosis is a well-known diabetic complication, it is of interest that treatment with a LS herb extract has been shown to affect the atherogenic process. Thus, C57BL/6 mice on an atherogenic diet supplemented with a LS herb extract displayed decreased plasma cholesterol levels, reduced plasma triglyceride levels, and an increased HDL-cholesterol concentration compared to animals receiving the atherogenic diet alone (Lee et al., 2010). LS also leads to a reduction of intracellular reactive oxygen species, which play an important role in the etiology of atherosclerosis (Singh and Jialal, 2006). Furthermore, it decreases the expression of adhesion molecules such as the lectin-like ox-LDL receptor (LOX-1) (Lee et al., 2010). LOX-1 may mediate the incorporation of ox-LDL into endothelial cells, a process supposed to promote endothelial dysfunction, the first stage of atherosclerosis (Vita et al., 1990). Additionally, LOX-1 expression was shown to be elevated in proatherogenic settings such as T2DM. Through its different effects on atherosclerosis development, LS may therefore retard the progression of T2DM and its resulting morbidities.

These observations, as well as the ancient tradition of using LS to treat the symptoms of DM and DMrD in TMM, led us to investigate a potential effect of LS on the production site of insulin, the pancreatic β-cell. Three extracts were monitored: an aqueous extract (LS OWE), corresponding to the traditional way of administration, a purified methanolic extract (LS MeOH/BuOH), and the water-soluble part of the methanolic extract (MeOH/H_2O).

2. Materials and methods

2.1. Plant material

The aerial parts of *Leonurus sibiricus* were harvested at Tsetserleg in the Mongolian province Archangai (latitude 47° 28′ 8.22″; longitude 101° 26′ 32.14″) in summer 2009 (collection number 01/09/mon). The species was identified by E. Ganbold, Mongolia. A reference specimen is kept at the herbarium of the School of Pharmacy, Health Sciences University of Mongolia, Ulaanbaatar, Mongolia (herbarium number 10080805).

2.2. Preparation of plant extracts

For this study, three different extracts were prepared. As LS is an ingredient of several traditional prescriptions, which are usually taken as crude powder together with a glass of water, we prepared an aqueous crude extract (original water extract, LS

OWE), which should reflect this common administration in traditional Mongolian medicine. In parallel, an extract was prepared using methanol as polar organic solvent. The use of methanol provides enrichment of less polar compounds and a softer handling during the purification process compared to the acidic aqueous solutions (shorter evaporation times, evaporation at lower temperatures). In order to obtain purified fractions of high polarity, the dissolved methanolic extract was partitioned between solvents of different polarity, yielding the two extracts used for our analysis, the butanol-soluble extract (LS MeOH/BuOH), and the water-soluble extract (LS MeOH/H_2O).

LS OWE: 5 g powdered dried plant material (drug) were suspended in 125 mL water, and, after adjustment to pH 2 with trifluoroacetic acid, the suspension was shaken gently at 40 °C for 2 h to simulate gastric conditions. Trifluoroacetic acid was used because it could be removed by vacuum evaporation after extraction. The extracts were obtained by subsequent freeze-drying. The yield (2.1 g) is given as drug to extract ratio (DER 2.4:1).

LS MeOH/BuOH and LS MeOH/H_2O: 80 g of the powdered plant material were extracted by accelerated solvent extraction (ASE 200 Accelerated Solvent Extractor, Dionex). Each of the eight extraction chambers were filled with 10 g powder and treated with methanol. Each chamber underwent three extraction cycles consisting of 5 min heat up time, 2 min static time, 10% flush volume, and 60 s nitrogen purge. All resulting solutions were combined and dried under reduced pressure at 40 °C. The DER of the resulting raw methanolic extract amounted to 6:1. It was re-dissolved in methanol and purified by liquid–liquid extraction using three solvents of different polarity consecutively. The extraction with light petroleum was performed to deplete chlorophyll. The remaining methanolic layer was partitioned against dichloromethane followed by water-saturated butanol. The whole procedure resulted in four extracts after evaporation of the solvents under reduced pressure: the light petroleum extract (DER 23:1), and the dichloromethane extract (DER 38:1) were not further investigated; the butanol extract (LS MeOH/BuOH; DER 48:1), and the aqueous layer which remained after the partition with water saturated butanol (LS MeOH/H_2O; 17:1) were used for the study. For experiments extracts were dissolved in double distilled water (LS OWE and LS MeOH/H_2O) or methanol (LS MeOH/BuOH) to receive stock solutions at concentrations of 50 mg/ml (LS OWE and LS MeOH/H_2O) or 500 mg/ml (LS MeOH/BuOH). Respective MeOH-solvent controls were used for all experimental approaches in this study.

2.3. Chemicals and reagents

All salts and chemicals used for the preparation of experimental solutions and cell culture media were p.a. grade.

2.4. Cell culture

INS-1E cells were grown under standard cell culture conditions (SCC) in RPMI 1640 medium containing 11.1 mM D-glucose, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B at 37 °C, 5% CO_2 and 95% air. Subcultures were established twice a week by trypsin/EDTA treatment. Cells between passages 90 and 110 were used (Merglen et al., 2004).

2.5. Insulin ELISA

For ELISA experiments, cells were seeded in poly-D-lysine coated 96-well plates (Sarstedt) at a density of 8,000 cells/well and grown under standard culture conditions for 48 h prior to the start of the experiments. To avoid FCS-induced cross-reaction of serum insulin, experiments were performed in serum-free RPMI 1640 medium. Cells were incubated for 24 h in serum-free medium containing 500 mg/L LS OWE, LS MeOH/H_2O, or MeOH/BuOH. Untreated and solvent-treated cells served as controls. The insulin released into the medium during the 24-hour (LS) or 2-hour (tolbutamide) incubation period was measured using a rat insulin ELISA kit (Mercodia) according to the manufacturer's instructions. Subsequently, cell numbers were assessed by using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) (see cell proliferation assays in this section). Data (ng insulin/mL medium) were converted to ng insulin/cells in the respective well and further to ng insulin/100,000 cells.

2.6. Cell membrane potential and intracellular Ca^{2+} recordings

INS-1E cells were seeded on poly-D-lysine-coated glass coverslips and used for patch clamp or Ca^{2+} imaging after 24–48 h. The coverslips were transferred to a recording chamber and mounted on a Nikon TE2000-U inverted microscope. Experiments were performed at room temperature. The extracellular solution contained (in mM): 135 NaCl, 5.6 KCl, 2.5 $CaCl_2$, 1.5 $MgCl_2$, 10 HEPES-free acid (FA), 5 glucose, pH 7.4 (adjusted with NaOH), 290 mOsm/kg. Osmolalities were measured with a vapor pressure osmometer (Wescor). Solution exchange was performed with a gravity-flow perfusion system at a flow rate of 1–2 mL/minute. LS OWE (500 mg/L) and/or tolbutamide (50 or 100 μM) were added to the extracellular solution as indicated.

Patch clamp recordings were performed in the conventional whole-cell patch clamp configuration. Patch electrode resistances were 3–5 MΩ. An EPC-10 amplifier and PatchMaster/FitMaster software (HEKA) were used for data acquisition and analysis. Membrane potential recordings were performed in the zero-current clamp mode. The pipette solution contained (in mM) 130 KCl, 4 $MgCl_2$, 2 $CaCl_2$, 20 HEPES-FA, 10 EGTA. The pH was titrated to 7.2 with KOH (296 mOsm/kg). For voltage clamp recordings of the K_{ATP} conductance an intracellular solution containing (in mM) 4 KCl, 2 $MgCl_2$, 10 HEPES-FA, 10 EGTA, 4 Mg-ATP, 43 mannitol, with a pH 7.3 (KOH) and 296 mOsm/kg was used. K_{ATP} currents were measured during 500-ms pulses to −80 and −60 mV at 10-s intervals from a holding potential of −70 mV as previously described (Hambrock et al., 2007; Jakab et al., 2008). In this range of potentials the membrane conductance is predominately determined by K_{ATP} currents (Drews et al., 1998).

For monitoring intracellular Ca^{2+}, cells were loaded with Fluo-4/AM for 30 min at 37 °C followed by 30 min at room temperature. The light source equipped with a mercury short arc lamp and an integrated shutter (LEJ Leistungselektronik Jena GmbH) was coupled to the microscope (Nikon TE2000-U) via a liquid light guide. The light was passed through a filter cube comprising a 340–380 nm excitation filter, a 440 nm dichroic mirror and a 435–485 nm emission filter. 16-bit gray-scale images were captured for 200 ms at 10-second intervals with a cooled CCD camera (SensiCam, pco). Camera and shutter were controlled by TILLvisION software, which was also used for analysis. For each experiment, all cells in the field of vision were analyzed (approximately 20 cells). Data are presented as fluorescence intensities obtained from background-subtracted images.

2.7. RT-PCR of rat insulin receptor

Total RNA was extracted from INS-1E cells using RNAbee reagent (ams biotechnology) based on phenol/chloroform extraction and isopropanol precipitation according to the manufacturer's recommendations. 1 μg of isolated RNA was used for the reverse transcriptase (RT) reaction with oligo-dT18 primers performed

with the first strand cDNA synthesis kit (Fermentas). mRNA levels of rat insulin receptor were determined by PCR (35 cycles, annealing temperature 61 °C) using specific primers (GenBank accession # NM_017071.2; forward: 5′-CATGAATTCCAGCAACTT-GATGTGC-3′, reverse: 5′-CAGGTTGTTGCCCCCTCGGATGT-3′), yielding a 172-bp fragment. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

2.8. Cell proliferation assays

For the assessment of the effect of LS extracts on cell proliferation, INS-1E cells were seeded in poly-D-lysine-coated standard 96-well plates at a density of 8000 cells/well. Cells were grown for 48 h before incubation with LS extracts for further 24 and 48 h, respectively. Cell numbers were assessed by using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) following the manufacturer's instructions at three different time points, i.e. at time point zero (the time point of the addition of the LS-extracts to the cells), as well as 24 and 48 h after addition of LS-extracts. The cell numbers at the time point zero were set to 100% and the cell numbers assessed at the other two time points were normalized to these values.

2.9. LC-MS analyses

HPLC-ESI-MS^n analyses were performed on an UltiMate 3000 RSLC-series system (Dionex, Germering, Germany) coupled to a 3D quadrupole ion trap mass spectrometer equipped with an orthogonal ESI source (HCT, Bruker Daltonics, Bremen, Germany). HPLC separation was carried out on a LiChrospher 100 RP18e column, 4.6 × 250 mm, 5 μm (Agilent, CA, USA) at 25 °C using water (pH 2.8 with formic acid) and methanol as mobile phase A and B, respectively. The flow rate was 1.0 mL/min and the following gradient program was used for the LS OWE sample: 10% B (0 min), 50% B (80 min), and 95% B (81 min). The gradient program for the LS MeOH/BuOH sample was as follows: 10% B (0 min), 30% B (20 min), 30% B (42 min), 50% B (82 min), and 95% B (83 min). UV data were recorded from 190 to 400 nm. The eluent flow was split roughly 1:8 before the ESI ion source, which was operated slightly different for the LS OWE sample (capillary voltage: ±3.7 kV, nebulizer: 32 psi (N_2), dry gas flow: 9 L/min (N_2), and dry temperature: 350 °C) and for the LS MeOH/BuOH sample (capillary voltage: +3.7/−4.0 kV, nebulizer: 22 psi (N_2), dry gas flow: 8 L/min (N_2), and dry temperature: 320 °C). The mass spectrometer was operated in an automated data-dependent acquisition (DDA) mode to obtain MS^2 and MS^3 spectra (collision gas: He, isolation window: 4 Th, fragmentation amplitude: 1.0 V). The aglycone part of the flavonoids was identified by spectrum matching to an in-house library containing the MS^n spectra of 57 reference flavonoids using ESI compass 1.3 for HCT (Bruker Daltonics).

2.10. HPLC-UV-DAD analyses

HPLC-UV-DAD analyses were performed on a Prominence LC-20AD coupled to a Prominence SPD-M20 Diode Array Detector (Shimadzu Corporation, Kyoto, Japan) and to a low temperature-evaporative light scattering detector (ELSD-LT, Shimadzu Corporation, Kyoto, Japan). The chromatographic system was identical to the one employed for the MS analyses. For the sample solution of the LS OWE 25 mg were dissolved in 100 μL aqueous methanol, 10 μL thereof were injected. For the sample solution of the LS MeOH/BuOH 1.0 mg of the MeOH/BuOH extract were dissolved in 100 μL methanol, 5 μL thereof were injected. The temperature of the ELSD was set to 40 °C at an air pressure of 360 kPa.

2.11. Statistics and data presentation

Data are expressed as mean ± SEM. Statistical analysis was carried out using unpaired or paired Student's *t*-test or the general linear model with one fixed and one random effect, as applicable. *P* values < 0.05 between groups were considered as statistically significant. Data were analyzed and plotted using GraphPad Prism version 5.0d for PC (GraphPad Software).

3. Results

3.1. Effects of Leonurus sibiricus extracts on insulin release under standard cell culture conditions (SCC)

To determine the effect of extracts from LS on the amount of insulin released from rat INS-1E cells, insulin levels were determined by ELISA. Insulin released into the culture medium under SCC, i.e. in the presence of 11.1 mM glucose, over 24 hours was significantly increased in presence of 500 mg/L LS OWE as well as LS MeOH/H_2O from 19.5 ± 7.0 ng to 30.3 ± 7.7 ng and 33.1 ± 7.5 ng per 100,000 cells (n=5), respectively (Fig. 1). No effect was observed at concentrations of 100 mg/L. In contrast, in a separate series of experiments, exposure to 100 μM tolbutamide (a concentration twice as high as necessary to elicit a maximum electrophysiological response in our experiments; see Fig. 2), which is known to enhance insulin secretion in INS-1E cells kept at 2.5 or 3.3 mM glucose, respectively (Merglen et al., 2004; Taguchi et al., 2008), did not significantly enhance insulin secretion from 15.8 ± 7.0 to 19.0 ± 9.5 ng insulin/100,000 cells under SCC conditions within 2 h (n=6; p=0.27).

3.2. Effects of Leonurus sibiricus extracts on INS-1E single cell electrical activity

To investigate if the increased insulin secretion induced by LS was due to stimulation of electrical activity, we performed whole-cell patch clamp recordings of the cell membrane potential (V_{mem}). As shown in fig. 2B, LS OWE (500 mg/L) acutely and significantly depolarized INS-1E cells from −61.5 ± 1.5 mV to −31.3 ± 1.4 mV within ~1.5 min (n=4). In one out of five experiments LS OWE was without effect. Fig. 2A shows a recording of a spontaneously

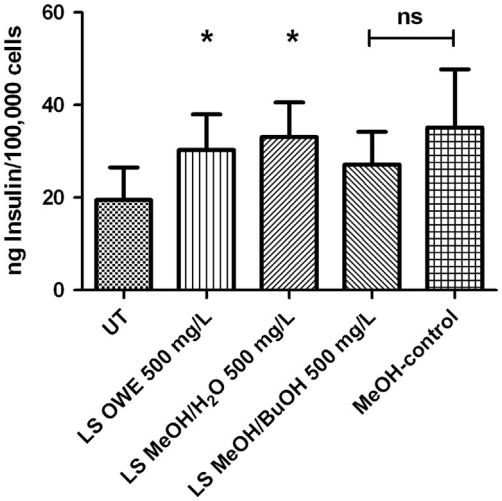


Fig. 1. Levels of insulin released from INS-1E insulinoma cells during 24 h of culture with LS extracts (500 mg/L) measured by ELISA. A statistically significant difference is evident in cells treated with 500 mg/L LS OWE as well as with LS MeOH/H_2O ($n=5$), compared to basal secretion in untreated cells, whereas the MeOH/BuOH extract is without effect, compared to its MeOH-solvent control (*$p < 0.05$; ns, not significant; unpaired *t*-test).

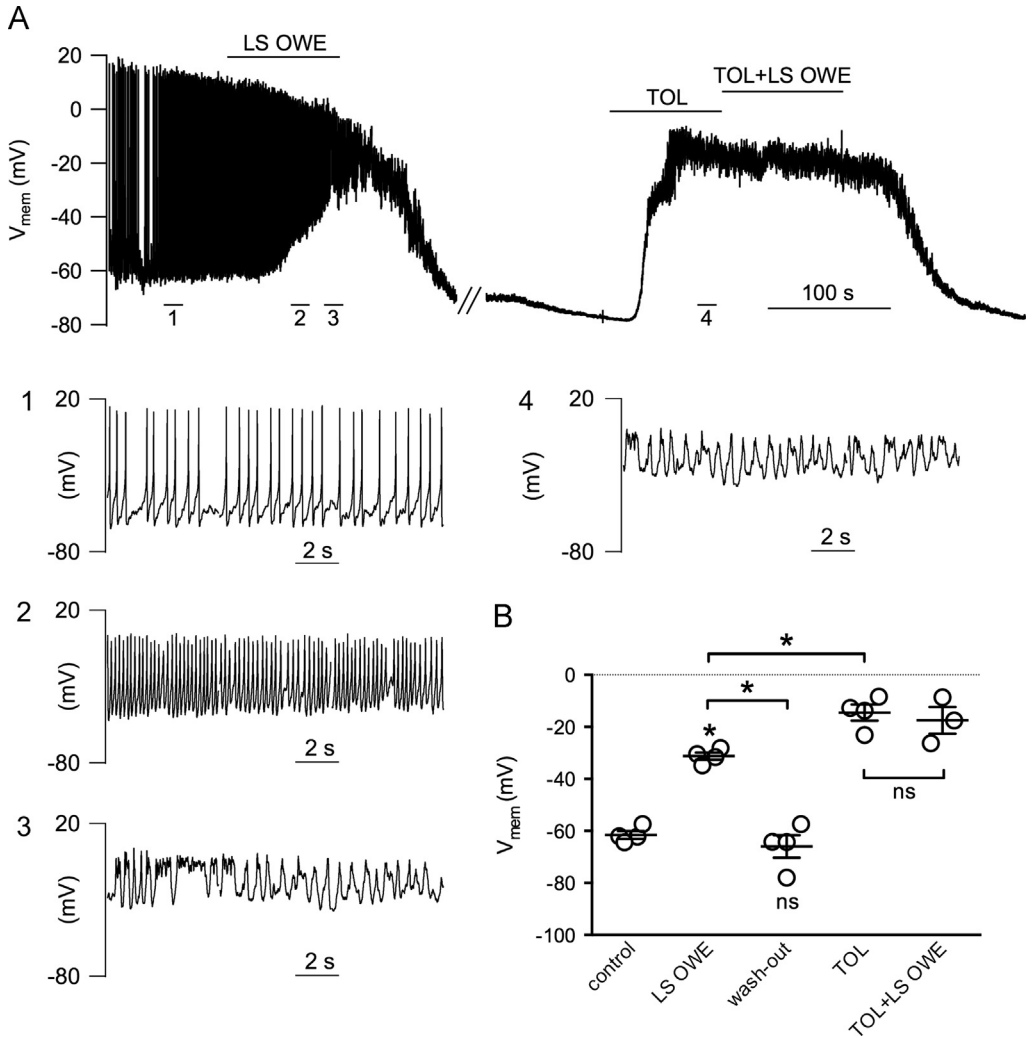


Fig. 2. LS OWE reversibly depolarizes INS-1E cells. (A) Whole-cell zero-current clamp recording in absence and presence of LS OWE (500 mg/L), during wash-out of the extract followed by the application of tolbutamide (TOL, 100 μM), LS OWE on top of TOL and final wash-out of both substances. 1–4 show 15-second segments of the upper recording on an expanded time scale before (1), during application of LS OWE (2, 3) and tolbutamide (4), as indicated in (A). (B) Summary of patch clamp data ($n=3$, mean ± SEM; 1way ANOVA with Bonferroni's multiple comparison post-test; $^*p<0.05$; ns, not significant).

active cell before the application of LS OWE; tracing 1 depicts the electrical activity recorded under control conditions. Along with depolarizing the cell, LS OWE caused an initial increase and a subsequent decline of action potential (AP) frequency with persisting depolarization, whereby the AP amplitude gradually decreased (tracings 2 and 3). The LS OWE-induced depolarization was fully reversible (Fig. 2B); V_{mem} after wash-out of LS OWE was -66.0 ± 4.3 mV (n=4). Spontaneous electrical activity, however, did not recur. Subsequent application of the sulfonylurea tolbutamide (100 μM) rapidly depolarized V_{mem} to -14.5 ± 3.1 mV (n=4) with low amplitude APs similar to those observed during LS OWE application (Fig. 2A, tracing 4). Tolbutamide was significantly more effective in depolarizing V_{mem} than LS OWE. LS OWE (500 mg/L) applied on top of tolbutamide did not add to the effect of the sulfonylurea (-17.5 ± 5.1 mV; n=3; Fig. 2B).

The apparent resemblance of the LS OWE effect to the effect of tolbutamide led us to test if the herb extract might likewise act on the ATP-sensitive K^+ conductance (IK_{ATP}). After establishing the whole-cell patch clamp configuration, V_{mem} was clamped to -70 mV and voltage pulses to -80 and -60 mV with durations of 500-ms were applied every 15 s. In this range of potentials, the whole-cell conductance of β-cells is mainly determined by IK_{ATP} (Drews et al., 1998). Clamping the intracellular ATP concentration by dialyzing the cytoplasm with the pipette solution yielded stable control currents within 2–5 min (Fig. 3). Similar to tolbutamide (50 μM), LS OWE (500 mg/L) brought a significant and reversible inhibition of the K_{ATP} current, reaching a plateau after 1–2 min. An inhibitory effect of LS was observed in 3 out of 5 experiments. Exemplary time courses and current tracings are shown in Figs. 3A and B for LS and Figs. 3C and D for tolbutamide. LS OWE caused an inhibition to $62.0 \pm 8.5\%$ and $57.7 \pm 7.4\%$ of control currents at -80 and -60 mV, respectively (Fig. 3E). Tolbutamide (50 μM) reduced IK_{ATP} to $34.7 \pm 7.5\%$ (at -80 mV) and $31.7 \pm 4.3\%$ (at -60 mV) of control values. The inhibition by tolbutamide measured at -60 mV was significantly stronger compared to LS OWE.

3.3. *Effect of Leonurus sibiricus extracts on intracellular Ca^{2+} (Ca_i)*

To assess whether the altered electrical behavior of INS-1E cells under LS OWE is paralleled by a rise in Ca_i, cells were loaded with the Ca^{2+} sensitive fluorescent dye Fluo-4/AM and acutely superfused with extracellular solution containing LS OWE (500 mg/L), tolbutamide (100 μM) and LS OWE plus tolbutamide. On average, LS OWE and tolbutamide led to a reversible rise of Ca_i up to $30 \pm 10\%$ and $68 \pm 14\%$ ($n=3$) compared to control conditions, respectively (Fig. 4B), with a high variability of single cell responses (Fig. 4A). Peak responses were followed by a decline of the signal in the persisting presence of drugs. In two out of

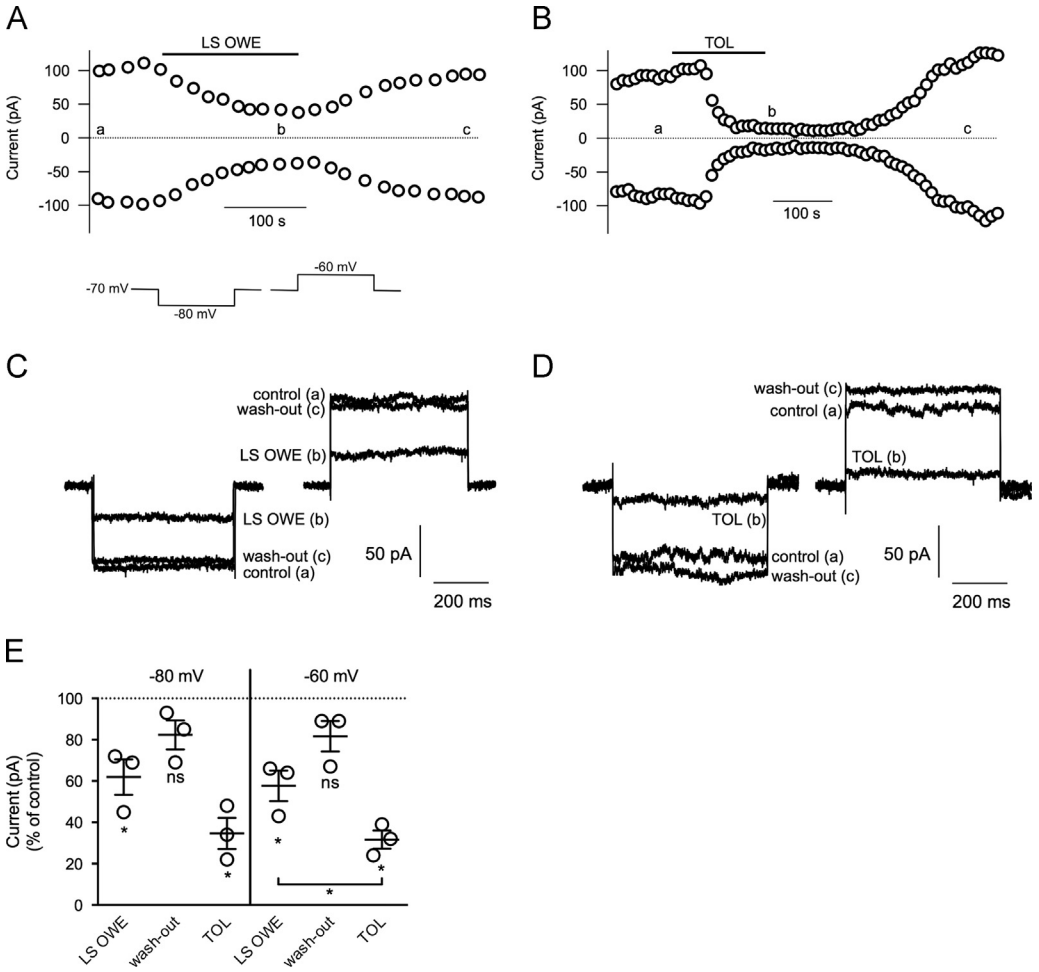


Fig. 3. Inhibition of the ATP-sensitive K^+ current (IK_{ATP}) in INS-1E cells by LS OWE (whole-cell patch clamp recordings). Voltage pulses to −80 and −60 mV with durations of 500-ms were applied every 15-seconds from a holding potential of −70 mV (insert). (A+C) IK_{ATP} recorded in the absence and presence of LS OWE (500 mg/L) or tolbutamide (TOL, 50 μM). Each data point represents the average current during a 500-ms voltage pulse. Tracings were zero-offset subtracted so that currents recorded at −80 and −60 mV appear as negative and positive deflections, respectively. (B+D) Corresponding original current tracings at the time-points a (control), b (presence of LS OWE or TOL) and c (wash-out) as indicated in A+C. (E) Scatter blot summarizing the results of 3 individual experiments. Data are shown as % of control currents. $^*p < 0.05$ (unpaired *t*-tests).

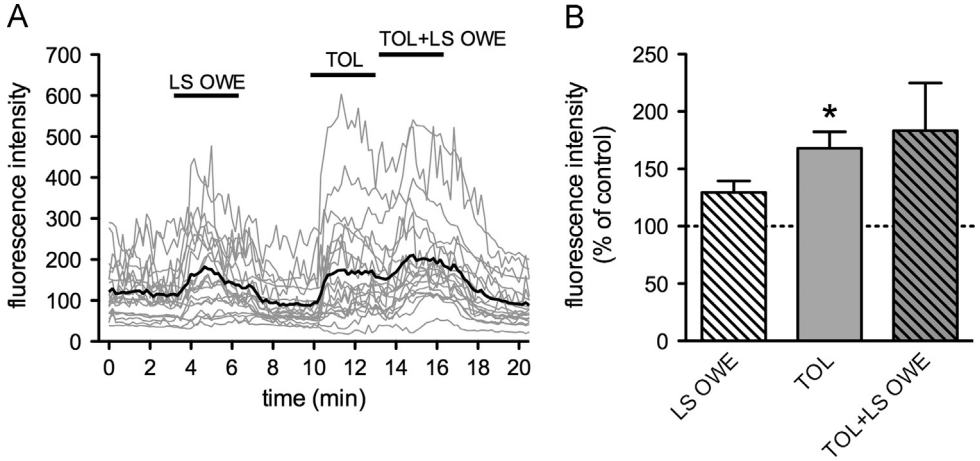


Fig. 4. LS OWE induces an acute and reversible rise in intracellular Ca^{2+} (Ca_i). Cells were loaded with Fluo-4/AM and the fluorescence intensity was measured in the absence or presence of drugs as indicated (500 mg/L LS OWE; 100 μM tolbutamide, TOL). Images were acquired at 10-s intervals. (A) Responses of single cells (gray tracings) and mean response of all 20 cells in the field of view (black tracing) of an individual experiment. (B) Bar graph showing the average of the mean responses of 3 individual experiments (LS OWE and TOL, $n=3$; paired *t*-test; TOL+LS OWE, $n=2$; $^*p < 0.05$). Data are given as % change in fluorescence intensity compared to control conditions.

three experiments we applied LS OWE on top of tolbutamide, as shown in Fig. 4A, and observed that LS OWE could elicit a further Ca^{2+} peak. However, similar to the observation concerning V_{mem} recordings, there was no additive effect to the overall response of tolbutamide alone.

3.4. Insulin-receptor expression

To verify the expression of the insulin-receptor (IR) in INS-1E cells, which is the prerequisite for an auto/paracrine effect of the hormone, we performed RT-PCR. As shown in Fig. 5 the expected amplicon (172-bp fragment) can be detected using primers specific for the rat IR α-subunit.

3.5. Influence of Leonurus sibiricus extracts on cell proliferation

Insulin acts as a growth factor in β-cells. Given the expression of insulin-receptors in INS-1E cells, an altered proliferative behavior of INS-1E cells, caused by the increased amounts of insulin secreted from the cells due to LS treatment, is plausible. We therefore assessed the effect of the three LS extracts, OWE, MeOH/

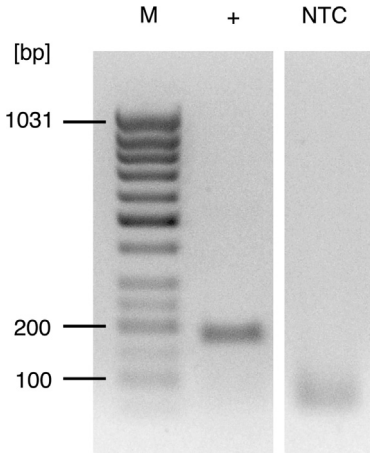


Fig. 5. Expression of the insulin-receptor (IR) in INS-1E cells. The sample containing cDNA template (+) yields a specific amplification product for the IR α-subunit (172 bp). NTC, non-template control; M, 50-bp DNA size marker. (1.5% agarose gel, ethidium bromide staining).

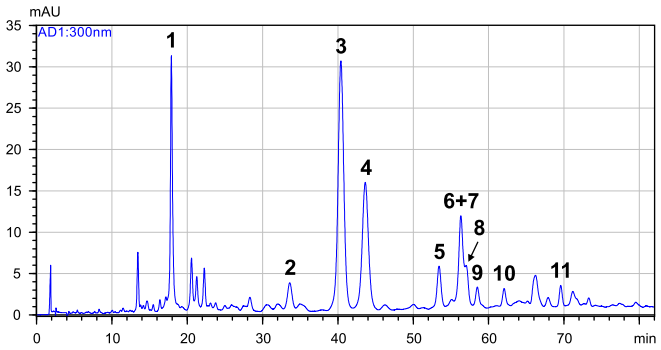


Fig. 7. HPLC chromatogram of the LS MeOH/BuOH extract showing the DAD response at 300 ± 2 nm. The extract was also analyzed by LC-MS and the proposed structures of corresponding compounds 1–11 can be found in Table 1.

H_2O and MeOH/BuOH (500 mg/L each) on cell proliferation 24 and 48 h after treatment. The data are shown in Fig. 6. All extracts significantly increased the number of cells compared to untreated cells or the respective methanol control (0.1%). After 24 h, LS OWE increased the number of cells to 173 ± 10%, LS MeOH/H_2O to 173 ± 9% compared to 149 ± 4% of untreated cells, while LS MeOH/BuOH had no significant effect (176 ± 11%) compared to the 0.1% methanol-control (152.5 ± 2.6%). After 48 h, LS OWE caused an increase in cell number to 215.4 ± 5.2%, LS MeOH/H_2O to 218.9 ± 7.1% compared to untreated cells (189.1 ± 1.9%). LS MeOH/BuOH also enhanced cell proliferation compared to the control (223.8 ± 7.5% vs. 194.1 ± 2.3%).

3.6. Phytochemical characterization of the of Leonurus sibiricus plant extracts

The main phenolic constituents in the LS MeOH/BuOH extract were identified as chlorogenic acid, flavonoid glycosides and most prominently glycosylated phenyl ethanoids such as verbascoside and lavandulifolioside by LC-MS (see structural formulae, Fig. 7, and Table 1). These typical phenyl ethanoids were not detected in the LS OWE. The latter was characterized by a high content of stachydrine, which was identified by LC-MS and confirmed by TLC analysis comparing the R_f values with a reference compound after detection with Dragendorff reagent. After depletion of stachydrine,

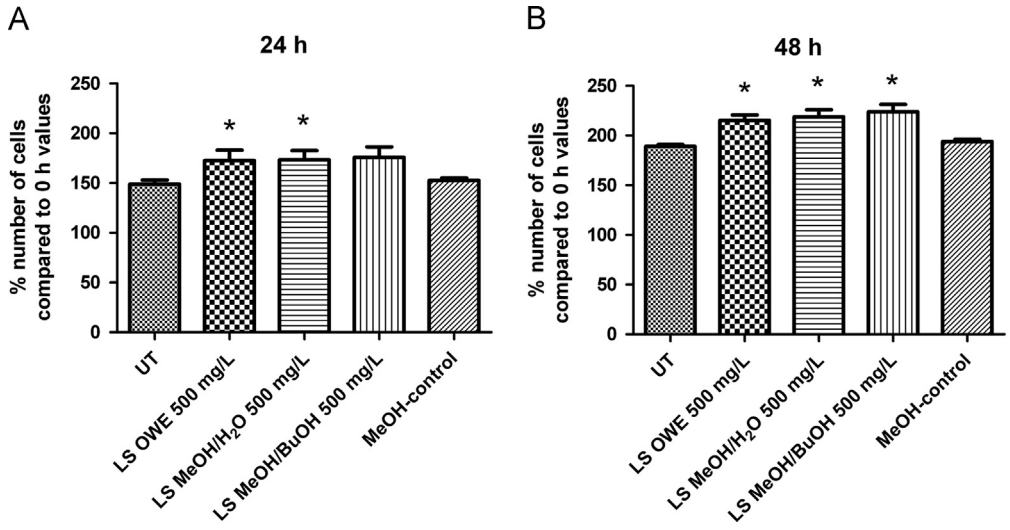


Fig. 6. Increased proliferation rates of INS-1E cells treated with LS OWE, MeOH/H_2O and MeOH/BuOH (500 mg/L) after 24 (A) and 48 (B) hours. * Statistically significant difference compared to control conditions ($p < 0.05$, Mann-Whitney test or unpaired t-test with Welch's correction).

Table 1
Proposed structure and MS data of the secondary plant metabolites identified in the LS MeOH/BuOH extract.

#	Proposed structure[a]	[M-H]$^-$	Main fragment ions (> 10% Rel. Int.)	References
1	Chlorogenic acid	353.1	MS^2 [353.1]: 190.8	Ritter et al. (2010)
2	Quercetin-*O*-hexosyl hexoside[b]	625.2	MS^2 [625.2]: 445.1, 300.9, 299.9, 270.9, 254.9 MS^3 [299.9]: 270.9, 254.8, 178.7, 150.7	–
3	Lavandulifolioside[c]	755.3	MS^2 [755.3]: 623.2, 593.3, 461.1 MS^3 [593.3]: 461.2 MS^3 [461.1]: 315.0, 314.1, 178.8, 160.8, 144.8, 135.9, 134.9, 133.9	Krasteva et al. (2011) and Ritter et al. (2010)
4	Verbascoside[c]	623.2	MS^2 [623.2]: 461.2 MS^3 [461.2]: 315.0, 160.8, 134.9	Li et al. (2012) and Ritter et al. (2010)
5	Lavandulifolioside-isomer	755.3	MS^2 [755.3]: 623.2, 593.3, 461.2 MS^3 [593.3]: 461.2 MS^3 [461.2]: 315.0, 297.0, 160.8, 135.9, 134.8	Cai et al. (2005)
6	Leonoside A	769.3	MS^2 [769.3]: 637.3, 607.2, 593.3, 461.2 MS^3 [637.3]: 491.1, 461.2 MS^3 [593.3]: 461.2	Calis et al. (1992)
7	Quercetin-*O*-deoxyhexosyl hexoside[b]	609.2	MS^2 [609.2]: 300.9, 300.0, 270.9 MS^3 [300.9]: 272.9, 270.9, 254.8, 178.8, 150.7	Fernando Rolim de Almeida et al. (2008) and Hayashi et al. (2001)
8	Verbascoside-isomer	623.2	MS^2 [623.2]: 461.1 MS^3 [461.1]: 315.0, 160.8, 134.9	–
9	Leucoceptoside A	637.3	MS^2 [637.3]: 461.1 MS^3 [461.2]: 315.0, 297.0, 160.8, 142.8, 134.9	Tasdemir et al. (1999)
10	Leonoside A-isomer	769.3	MS^2 [769.3]: 637.3, 593.3, 461.1 MS^3 [637.3]: 491.1, 461.2 MS^3 [593.3]: 461.1	–
11	Leonoside B	783.3	MS^2 [783.3]: 651.3, 607.3 MS^3 [607.3]: 475.2	Calis et al. (1992)

[a] The identification of the compounds is based either on direct comparison of the experimental MS data with literature data or on congruence of the MS- and UV-data with the structure of known secondary metabolites from *Leonurus sibiricus* or other *Leonurus* species.
[b] The aglycone part of the flavonoids as identified by spectrum matching to an in-house library containing the MS^n spectra of 57 reference flavonoids using ESI compass 1.3 for HCT (Bruker Daltonics, Bremen, Germany).
[c] These constituents were confirmed by comparison of the retention times and UV-spectra with reference compounds.

quercetin glycosides and derivatives of phenolic carbonic acids were found as minor phenolic components of the LS OWE (data not shown).

As stachydrine was found to be present in LS OWE in high amounts, we tested whether stachydrine alone was the active compound accounting for the augmented insulin secretion from LS OWE-treated INS-1E cells. However, the sole application of stachydrine had no effect on insulin secretion in this cell line (3 individual experiments, data not shown).

4. Discussion

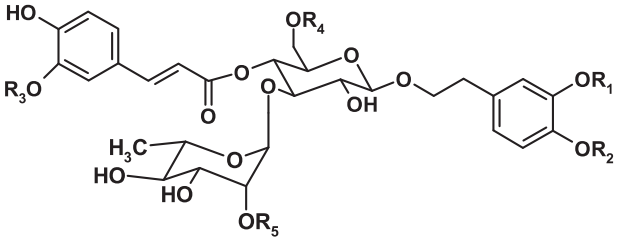
In the present study we determined whether extracts from *Leonurus sibiricus* L., a plant used in the treatment of DM and/or DMrD in TMM, affect pancreatic β-cell function. We have chosen rat INS-1E cells as a T2DM cell culture surrogate, as these cells are maintained at 11.1 mM glucose as standard cell culture condition (SCC), thus mimicking a hyperglycemic metabolic situation. If pre-incubated at glucose concentrations of 2.5 mM, these cells exhibit a dose-dependent glucose-induced insulin release with a plateau phase at 15–20 mM and a 50% effective concentration at 10.4 mM (Merglen et al., 2004), which is close to the SCC glucose concentration of 11.1 mM as used in this study. Therefore the experimental conditions chosen still allow for the detection of at least a shallow elevation of insulin release, while simultaneously simulating T2DM conditions. While under these conditions 100 μM tolbutamide had no stimulatory effect on insulin release within 2 h of treatment, exposure of the cells to aqueous extracts from LS for 24 h resulted in an increased secretion. This effect was observed for OWE and MeOH/H_2O extracts, whereas the MeOH/BuOH extract showed no effect (Fig. 1). This suggests that the active compound(s) in LS responsible for the stimulation of insulin secretion is/are very polar and highly soluble in water. According to the conventional stimulus-secretion-coupling model insulin secretion is driven by elevated concentrations of plasma glucose and other physiological secretagogues like amino acids, triglycerols and free fatty acids. Glucose uptake and metabolism results in an increased ATP/ADP ratio thereby closing of ATP-sensitive K^+ (K_{ATP}; KIR 6.2/ SUR1) channels, depolarization of the cell membrane potential (V_{mem}) and stimulation of insulin exocytosis by Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Ashcroft et al., 1989; Henquin, 2000; Satin et al., 1995). In adherence to that model, we tested the effect of LS OWE on the electrical behavior of INS-1E cells. Acute application of LS OWE resulted in closure of K_{ATP} channels, and depolarization of V_{mem}, which was paralleled by an initial increase and subsequent decline of action potential (AP) frequency with persisting V_{mem} depolarization, gradually decreasing amplitudes and silencing of the APs. Washout of LS OWE caused a repolarization of V_{mem} without spontaneous electrical activity. Subsequent application of tolbutamide elicited a depolarization with low amplitude APs similar to those observed during application of LS OWE. This behavior was not further modified upon additional application of LS OWE (Fig. 2). LS OWE- and tolbutamide-induced depolarizations of V_m were paralleled by an increase in Ca_i (Fig. 4), most likely reflecting the opening of voltage-activated Ca^{2+} channels. In further experiments we could show that, similar to tolbutamide, LS OWE inhibits K_{ATP} channels (Fig. 3). In a previous study on the effect of the phytostilbene resveratrol on the electrophysiological and secretory behavior of INS-1E cells, we found that the compound inhibits K_{ATP} channels (IK_{ATP}), L- and T-type voltage-dependent Ca^{2+} channels and swelling-activated Cl^- currents (ICl_{islet}, $ICl_{glucose}$, ICl_{swell}) (Jakab et al., 2008). Even though both, hyperpolarizing IK_{ATP} as well as depolarizing Ca^{2+}- and Cl^- currents, were inhibited by resveratrol, the net effect was an inhibition of electrical activity (APs) and insulin release. Since LS OWE represents a mixture of compounds, it is likely that it exerts multiple effects on different ion conductances – probably with different temporal and dose-response profiles. The net effect of LS OWE is a depolarization and stimulated insulin release, for which the block of K_{ATP} channels provides a plausible explanation. However, we cannot exclude that the effect of LS on V_{mem} and Ca_i are due to additional effects on other ion conductances (e.g., inhibition of further K^+ currents, or activation of depolarizing ion conductance(s) like Ca^{2+}- or Cl^- channels, or a combination thereof). It has previously been shown

that aqueous extracts from *Leonurus cardiaca*, another species of the genus *Leonurus* used to treat tachyarrhythmia and other cardiac disorders, act as a mixed inhibitor of ICa.L- and IKr-channels as well as a modulator of If- channels (Ritter et al., 2010). The natural polyphenolic flavenoid quercetin has recently been found to stimulate insulin secretion in INS-1 cells and rat isolated pancreatic islets by direct activation of L-type calcium channels (Bardy et al., 2013). Since quercetin-*O*-hexosyl hexoside and quercetin-*O*-deoxyhexosyl hexoside are consitutents of the LS extract (Table 1 and Fig. 7), it may be assumed that the observed effects of LS on insulin secretion/proliferation may at least in part arise from a comparable effect. In mouse pancreatic islet cells the mitochondrial uncoupling protein 2 (UCP2) could be identified as a target of genipin, a compound found in *Gardenia jasminoides Ellis* fruits, which is used in traditional Chinese medicine to treat T2DM symptoms; the substance was shown to inhibit UCP2, to increase mitochondrial membrane potential and ATP levels, to inhibit K_{ATP} channels and to stimulate insulin secretion (Zhang et al., 2006).

Besides its metabolic action on peripheral tissues, insulin also acts in an auto/paracrine manner as e.g. shown in MIN6 cells (Muller et al., 2006), which is important for cell proliferation and survival and hence the maintenance of the β-cell mass of pancreatic islets. Recent studies have further concluded that insulin is a positive regulator of its own production (Leibiger et al., 1998; Xu et al., 2000) and β-cell-specific insulin receptor (IR) knockout mice manifest reduced islet size, progressive glucose intolerance, as well as reduced insulin content (Kulkarni et al., 1999). In the current study, we show that LS extracts stimulate INS-1E cell proliferation. Since we also demonstrate the presence of the IR in INS-1E cells, the LS-boosted proliferation rates may be explained by such an auto/paracrine action of the hormone. Moreover, it cannot be excluded that LS constituents have a direct effect on cell proliferation. With respect to that it is noteworthy to mention that not only LS OWE and LS MeOH, but also LS MeBuOH was able to stimulate INS-1E cell proliferation (Fig. 6) despite its inability to increase insulin secretion (Fig. 1). Clearly the cellular signaling pathways mediating the proliferative effects of LS extracts need to be identified in further studies.

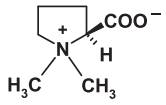
In conclusion we show that extracts from *Leonurus sibiricus* increase insulin secretion of rat INS-1E insulinoma cells that can be explained at least in part by a transiently increased electrical activity and elevation of the intracellular calcium concentration. Moreover, treatment with LS extracts stimulates INS 1-E cell proliferation. These findings may provide explanations for the empirical use of LS formulations in the treatment of diabetes mellitus and its related disorders in traditional medicine.

Structural formulae



Verbascoside = Acteoside (R_1–R_5 = H)
Lavandulifolioside (R_1, R_2, R_3, R_4 = H; R_5 = arabinose)
Leucosceptoside A (R_1, R_2, R_4, R_5 = H; R_3 = Me)
Leonoside A (R_1, R_2, R_4 = H; R_3 = Me; R_5 = arabinose)
Leonoside B (R_1, R_3 = Me; R_2, R_4 = H; R_5 = arabinose)

Stachydrine



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