Saponins from the traditional medicinal plant *Momordica charantia* stimulate insulin secretion *in vitro*

Amy C. Keller[a], **Jun Ma**[b], **Adam Kavalier**[a], **Kan He**[c], **Anne-Marie B. Brillantes**[d], and **Edward J. Kennelly**[*]

[a]Lehman College and The Graduate Center, City University of New York, Bronx, NY 10468

[b]Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, MD 20740

[c]Naturex, Inc., South Hackensack, NJ 07606

[d]Armgo Pharma Inc., New York, NY 10032

Abstract

The antidiabetic activity of *Momordica charantia* (L.), Cucurbitaceae, a widely-used treatment for diabetes in a number of traditional medicine systems, was investigated *in vitro*. Antidiabetic activity has been reported for certain saponins isolated from *M. charantia*. In this study insulin secretion was measured in MIN6 β-cells incubated with an ethanol extract, saponin-rich fraction, and five purified saponins and cucurbitane triterpenoids from *M. charantia*, 3β,7β,25-trihydroxycucurbita-5,23(*E*)-dien-19-al (1), momordicine I (2), momordicine II (3), 3-hydroxycucurbita-5,24-dien-19-al-7,23-di-*O*-β-glucopyranoside (4), and kuguaglycoside G (5). Treatments were compared to incubation with high glucose (27 mM) and the insulin secretagogue, glipizide (50 µM). At 125 µg/ml, an LC-ToF-MS characterized saponin-rich fraction stimulated insulin secretion significantly more than the DMSO vehicle, p=0.02. At concentrations 10 and 25 µg/ml, compounds 3 and 5 also significantly stimulated insulin secretion as compared to the vehicle, p 0.007, and p= 0.002, respectively. This is the first report of a saponin-rich fraction, and isolated compounds from *M. charantia*, stimulating insulin secretion in an *in vitro*, static incubation assay.

Keywords

Momordica charantia; alternative medicine; hypoglycemia; insulin secretion; diabetes; bitter melon

Introduction

Diabetes is currently one of the most prevalent and serious diseases worldwide. According to the World Health Organization, diabetes causes nearly 5% of deaths worldwide, and is

[*]Corresponding author: Edward J. Kennelly, phone: 718-960-1105, fax: 718-960-8236, kennelly@lehman.cuny.edu.

Declarations of Interest

The authors have no conflicts of interest to disclose.

expected to rise by 50% in the next 10 years (World Health Organization, 2010). As of 2007, 7.8% of the United States population suffers from diabetes, and in that year alone, 1.6 million new cases were diagnosed (Centers for Disease Control and Prevention, 2010).

Type 2 diabetes, also known as non-insulin dependent diabetes mellitus (NIDDM), is responsible for 90% to 95% of diagnosed diabetes (Centers for Disease Control and Prevention, 2010). The progression of type 2 diabetes can be characterized both by insulin resistance, and loss of normal β-cell activity, such as hyperplasia of the pancreas and gradual decrease of insulin secretion (Talchai et al., 2009). As impaired β-cell function is central to the progression of type 2 diabetes, novel therapies that regulate insulin secretion and prevent β-cell damage and subsequent impaired function may be integral to the future of treatment (Talchai et al., 2009).

In addition to widely used modern medications, people throughout the world increasingly rely on complementary and alternative medicine, including plant-based traditional medicines, as a form of health care (Egede et al., 2002). In 2007, during the 12 months prior to an interview by the authors, 4 out of 10 adults used alternative and complementary medicine, 17.7% of this being natural products (Barnes et al., 2008). In another study, during an interview of Dominican patients in an emergency room setting, 24% were using alternative medicine for their emergency complaint (Allen et al., 2000). A study done in the United States in 2002 reported that 19% of subjects interviewed had used natural products in the form of herbal remedies, functional foods, and supplements in the prior 12 month period (Barnes et al., 2002). With this prevalence of herbal medicine, it is of paramount importance that these herbs are investigated.

Of traditional medicines used for diabetes treatment, *Momordica charantia*, L. (Cucurbitaceae) is reported to be the most common (Marles and Farnsworth, 1995). The hypoglycemic activity of various preparations of *M. charantia* is well substantiated with many recent mouse models of diet-induced obesity and type 2 diabetes (Miura et al., 2001; Miura et al., 2004; Nerurkar et al., 2008; Shih et al., 2008). Also, recent studies have begun to show that isolated triterpene glycosides known as saponins may be responsible, at least in part, for the antidiabetic activity seen in *M. charantia*. For example, the isolated cucurbitane triterpenoids 5β,19-epoxy-3β,25-dihydroxycucurbita-6,23(*E*)-diene, and 3β, 7β, 25-trihydroxycucurbita-5,23(*E*)-dien-19-al lowered blood sugar in diabetic mice (Harinantenaina et al., 2006), while a saponin-rich fraction isolated from *M. charantia* was also found to lower blood sugar and small intestine disaccharidase activity in rats (Oishi et al., 2007). In another study, momordicosides Q, R, S, and T increased GLUT4 translocation via the AMPK pathway *in vitro*, and momordicoside T improved glucose tolerance in mice fed a high fat diet during a glucose tolerance test (Tan et al., 2008).

Although the insulin secretion activity of *M. charantia* has begun to be explored *in vitro* (Xiang et al., 2007), the exact mechanisms of the plant's activity have yet to be fully elucidated. As this plant is used so widely throughout the world in traditional medicine for diabetes, knowledge of its bioactivity, including potential toxicity, and possible mechanisms of action are critical for informed public health decisions. It is also important to assess the potential efficacy of pure compounds, both acting together in a concentrated fraction of plant extract, and independently.

We hypothesized that the hypoglycemic activity of *M. charantia* is due, at least in part, to the stimulation of insulin secretion in pancreatic β-cells by cucurbitane triterpenoids. To test this hypothesis, we investigated the insulin secretion activity of *M. charantia* ethanol extract and an LC-ToF-MS characterized saponin-rich fraction; additionally, the five cucurbitane triterpenoids and saponin compounds 3*β*,7*β*,25-trihydroxycucurbita-5,23(*E*)-dien-19-al (1),

momordicine I (2), momordicine II (3), 3-hydroxycucurbita-5,24-dien-19-al-7,23-di-*O*-*β*-glucopyranoside (4), and kuguaglycoside G (5) were also tested to ascertain the potential mechanism of the glucose-lowering activity of *M. charantia* in MIN6 β-cells, a mouse insulinoma β-cell line (Fig. 1). Of the clonal cell lines appropriate for testing potential insulin secretion, the glucose-stimulated insulin secretion previously observed with MIN6 cells was close to that of normal islets (Ishihara et al., 1993). Because the function of MIN6 cells closely resembles normal physiologic β-cell function, we chose this cell line to test our hypothesis in order to more precisely extrapolate a true pancreatic islet insulin secretion response. Extracts and compounds were tested in a static incubation assay, and insulin secretion and cell viability were measured.

Materials and methods

Plant material

Dried fruits, including seeds (1.94 kg), of *M. charantia* were identified by Naturex, Inc., and extracted in 75% ethanol, resulting in 578 g total solid extracted material. Spare extracted material was deposited in Naturex's herbarium (South Hackensack, New Jersey). This resulting 75% ethanol-extracted material was tested in the cells and referred to as the ethanol extract, of which aliquots (24 g) were partitioned sequentially with methylene chloride and water-saturated butanol, repeatedly. The average yield of the dried butanol extract was 5.67% w/w of starting material. All dried butanol extracts were combined, and subsequently used for experiments.

Compound isolation

Compounds 1-5 were isolated from *M. charantia* as described previously (Ma et al., 2010).

β-Cell assay

MIN6 β-cells were grown at 37 °C with 5% CO_2 in DMEM media (Invitrogen, California) with 15% fetal bovine serum (Invitrogen, California), 1% penicillin/streptomycin (Invitrogen, California), 1:200 gentamicin (Invitrogen, California), and 1% basel medium eagle (Invitrogen, California). Cells were trypsinized off the plate, split, and replated into 6-well plates and grown until confluent. The ethanol extract, saponin-rich fraction, and isolated compounds were re-suspended in 100% DMSO to make a stock solution of 125 mg/ml. The assay was performed similarly to a previously described method (Persaud et al., 1999). Cells were incubated with Kreb's Ringer buffer (KRB) for 60 min on the plate. Cells were then rinsed with fresh KRB twice, and an aliquot of the second wash was saved as a baseline insulin measurement. Cells were then incubated for 60 min with KRB (negative control), 50 µM glipizide with 27 mM glucose in KRB (positive control), or 0.1% DMSO in KRB (vehicle control). The ethanol extract and saponin-rich fraction were tested at 25, 75, and 125 µg/ml concentrations, while compounds 1-5 were tested at 5, 10, and 25 µg/ml. Treatment solutions were collected at 60 min, and insulin concentrations were determined by ELISA (ALPCO, New Hampshire).

Cell viability

Immediately after incubation with treatment solutions, cells were trypsinized and resuspended in PBS buffer (Invitrogen, California). Cells (10 µl) were added to an equal amount of Trypan Blue (Invitrogen, California) and counted on a hemocytometer. Cells excluding Trypan Blue were assumed viable. Both live and dead cells were counted and are presented as an average of four counts for each treatment.

DNA extraction

For a total cell count, DNA was extracted by incubating cells overnight with 1% SDS/10 mM EDTA/10 mM Tris lysis buffer and 1% proteinase K. Phenol-chloroform 1:1 was added, and cells were centrifuged. Sodium acetate-isopropanol 1:3 was added to the supernatant, and material was incubated at -80 °C for 60 min. Precipitated DNA was then washed twice with 70% ethanol, and air-dried. DNA was reconstituted with 10:1 tris-EDTA buffer (pH 8.0), and the concentration was read with a spectrophotometer at 260 nm. The conversion factor of 5.8 µg of DNA = 1.0×10^6 cells was used.

LC-ToF-MS analysis

Samples were analyzed by LC-MS using a Waters LCT Premiere XE Time of Flight (ToF) mass spectrometer (Waters MS Technologies, Manchester, UK). Ionization was achieved using a multi-mode EScI source in electrospray (ESI) mode at the following conditions: +ESI capillary 3000 V, -ESI capillary 2800 V, both + and – ESI: cone: 20 V, aperture 1: 0 V, ion guide 1: 0 V, multichannel plate (MCP): 2600 V. Nitrogen was used for both cone and desolvation gases, with a cone gas flow of 20 l h-1, and desolvation gas flow of 600 l h-1 at 400 °C. The source temperature was 120 °C. Leucine-enkephaline was used as a reference mass and infused by a secondary reference probe. The reference mass was scanned once every five scans for each positive and negative data collection. Both positive and negative ESI data were collected using a scan time of 0.2 s, with an interscan time of 0.01 s, and a polarity switch time of 0.3 s. MS data were collected in centroid mode using MassLynx V4.1 Scn 727.

LC separation was conducted using a Waters Alliance 2695 HPLC coupled to a Waters 2998 PDA. Separation was achieved on a 150×2.0 mm 2.6 µm Kinetex C-18 column (Phenomenex, California) held at a constant temperature of 45 °C, using the following solvent gradient system: (A) 0.1% formic acid in water, (B) 0.1% formic acid in acetonitrile at a flow of 0.2 ml/min: 0-15 min B:10-70%, 15-20 min B:70-100%, 20-42 min B:100%.

Statistics

The JMP software, version 8.0, was used to determine statistical significance between measured values. Significant differences were determined using least square means contrast within a one or two-way ANOVA.

Results

We tested a saponin-rich fraction of the total ethanol extract from *M. charantia* to investigate the mechanism of action behind the plant's hypoglycemic activity. This fraction stimulated insulin secretion above that of the DMSO vehicle at the 125 µg/ml concentration (Fig. 2, p=0.02). The activity of the saponin-rich fraction at 125 µg/ml was 4.9-fold greater than that of the DMSO vehicle, while the positive control's activity was 20.2-fold greater than the DMSO vehicle (Fig. 2).

To ascertain that the potentially active compounds present in the saponin-rich fraction of *M. charantia* were saponins, the fraction was characterized using LC-ToF-MS. These compounds are characterized by their dammarane-type triterpene skeleton, found in cucurbitanes (Hostettmann and Marston, 1995). The cucurbitane saponins found in *M. charantia* are glycosylated at carbons 7 and 23, and sometimes contain an aldehyde group attached at carbon 9 (Fig. 1) (Miro, 1995). Using the extracted ion chromatogram which corresponds to a common saponin basal skeleton with m/z 437.3290, we were able to characterize most of the ionizable components in the extract as saponins (Fig. 3).

Five pure compounds isolated from *M. charantia* fruit were tested in the β-cell insulin secretion assay. Although in a previous study, glycoside compound momordicoside U was found to be moderately active in the β-cell assay at both 10 and 25 μg/ml concentrations, (Ma et al., 2010) its aglycone, compound 1 (Fig. 1), did not stimulate insulin secretion.

Compound 3 (Fig. 4) significantly stimulated insulin secretion at 0.1 μg/1×10^6 cells for both concentrations 10 and 25 μg/ml, 7.3 and 7.1 times more than the vehicle (Fig. 3A, p=0.006, 0.007, respectively), while the positive control's activity was 14.3 times above the vehicle (Fig. 3A). However, its aglycone, compound 2, was not active.

Compound 5 (Fig. 4) stimulated insulin secretion at 0.1 μg/1×10^6 cells at both 10 and 25 μg/ml concentrations, 8.1 and 7.8 times the vehicle (Fig. 3B, p=0.002); the positive control stimulated insulin secretion 13.7 times the vehicle (Fig. 3B). The aldehyde of compound 5, compound 4 (Fig. 1), showed no activity.

To ensure that the insulin secretion was due to treatment-induced insulin secretion and not cell death, cells were stained with Trypan Blue and counted for viability. The viability of cells exposed to compound 2 at 25 μg/ml was significantly less as compared to the vehicle (Table 1C, p=0.002). As there was no significantly lower cell viability in any of the other treatment groups for either the ethanol extract or saponin-rich fraction, or pure compounds tested when compared to the DMSO control (Table 1), insulin secretion due to cell death was ruled out.

Discussion

Saponins are well known bioactive phytochemicals, and have been investigated for a multitude of activities, including antimicrobial, cytotoxic, anti-inflammatory, and immunostimulatory (Hostettmann and Marston, 1995; Francis et al., 2002). Although saponins are known to have hypoglycemic activity (Harinantenaina et al., 2006; Oishi et al., 2007; Tan et al., 2008), the cellular and molecular mechanisms of action are only beginning to be explored, and may be varied.

The saponin-rich fraction from *M. charantia*, along with compounds 3 and 5, stimulated insulin secretion in MIN6 pancreatic β-cells in a concentration-dependent manner. The compounds were compared to a strong positive control; thus, it is possible to compare the strength of activity relative to both positive and negative controls. As the saponin-rich fraction and compounds 3 and 5 stimulated insulin secretion significantly above the vehicle, but less than a highly active positive control, we conclude that the saponin-rich fraction and isolated compounds' activity is robust. The saponin-rich fraction was also found to contain saponins as detected by LC-ToF-MS, suggesting that these compounds may be responsible for part or all of the hypoglycemic activity of *M. charantia*. These results are consistent with previous reports of hypoglycemic activity of saponins (Francis et al., 2002; Harinantenaina et al., 2006; Oishi et al., 2007; Tan et al., 2008).

The ranges in bioactivity of compounds 1-5 may be due to their differences in structure. The previously tested compound, momordicoside U (Ma et al., 2010), along with the active compound 3, are monodesmoside cucurbitanes as opposed to their aglycones, inactive compounds 2 and 4, respectively (Fig. 1). Although compound 4 is a bidesmoside and is inactive, it also contains an aldehyde group whereas the bidesmoside compound 5 does not, and is the only compound tested to contain both of these moieties (Fig. 1). Cytotoxicity was observed only when testing the 25 μg/ml concentration of compound 2, and may help explain its lack of bioactivity in this insulin secretion assay, in addition to the structural differences mentioned above. Some saponins are known to be cytoxic at certain

concentrations, and this is consistent with our findings for compound 2 (Oleszek et al., 1999; Francis et al., 2002).

These detailed structural differences, especially glycosylation, most likely account for the bioactivity and lack thereof observed. It has been previously reported that both the type of sugar and other moieties along with the specific triterpene skeleton factor greatly in a saponin's bioactivity (Gauthier et al., 2009). For example, when investigating lupane-type saponins for cytotoxic activity, it was reported that the different sugar moieties reportedly explained the variation in activity of both monodesmosides and bidesmosides (Gauthier et al., 2009). The authors also mention that the basal skeleton of saponins can impact the mechanism of action of bioactivity, in addition to general activity (Gauthier et al., 2009). In other studies, saponin functional groups, such as sugars, also influenced the compounds' effects on cell membranes (Francis et al., 2002). Two saponins differing in only one glucose group, and neither having an acyl group, showed significantly different insulin absorption activity (Francis et al., 2002). In another study, the presence or absence of sugar moities affected several saponins' anticancer activity (Wang et al., 2007).

As compounds 1-5 all shared the same basal skeleton, we observed that the sugar moities, and possibly the presence of an aldehyde group, may have been responsible for their bioactivity or lack thereof, in agreement with the studies described above.

Also, the pattern of insulin secretion in response to the cells' incubation with the saponin-rich fraction is different than the response seen with compounds 3 and 5 (Fig. 2). The response from the saponin-rich fraction is concentration-dependent from 25 to 125 μg/ml, whereas the response to compounds 3 and 5 levels off between 10 and 25 μg/ml (Figs. 2 and 4). As certain bioactive saponins have previously been reported as being agonists, this phenomena may suggest that compounds 3 and 5 are binding to specific receptors, resulting in the observed bioactivity (Yang et al., 2008).

Momordica charantia is one of the most popular medicinal plants used worldwide for diabetes treatment (Marles and Farnsworth, 1995), and is also a widely used food (Basch et al., 2003). With the prevalence of this plant used both for diabetes treatment and food, it is crucial that health practitioners and researchers know and investigate the possible mechanisms of action of its hypoglycemic activity. The more that is known about the efficacy and mechanism of action of *M. charantia*, the better its use in health care settings throughout the world. All of this points to the potential to provide more informed care, and to avoid any potential ill effects such as herb-drug interactions, among other risks associated with herbal medicine.

Although studies suggest the consistent, but limited bioavailability of saponins (Xu et al., 2003; Podolak et al., 2010), we are currently investigating the saponin-rich fraction in an animal model of type 2 diabetes. In summary, our data support our hypothesis that *M. charantia* may work to lower blood glucose via promoting insulin secretion, and we theorize that this mechanism may contribute substantially to the plant's overall hypoglycemic effect.

Acknowledgments

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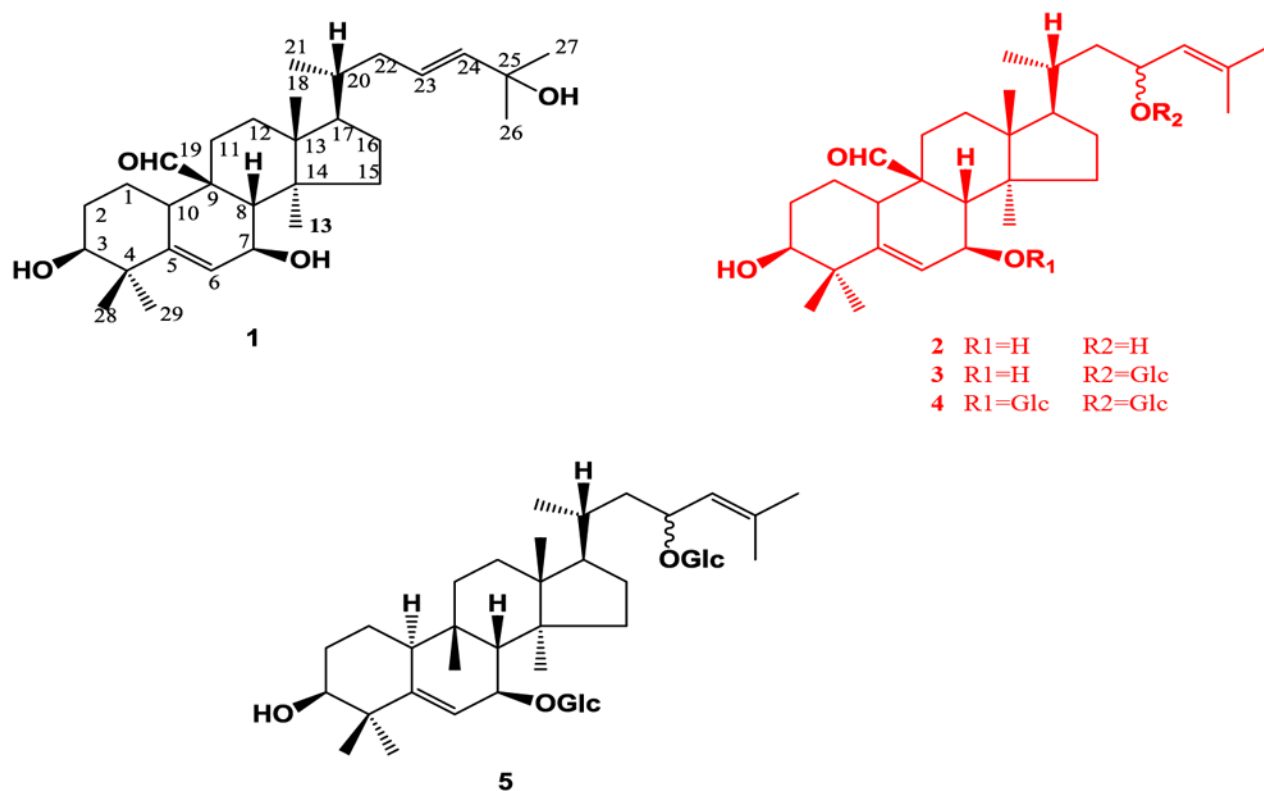
Fig. 1.
Compounds 3β,7β,25-trihydroxycucurbita-5,23(E)-dien-19-al (1), momordicine I (2), momordicine II (3), 3-hydroxycucurbita-5,24-dien-19-al-7,23-di-O-β-glucopyranoside (4), kuguaglycoside G (5).
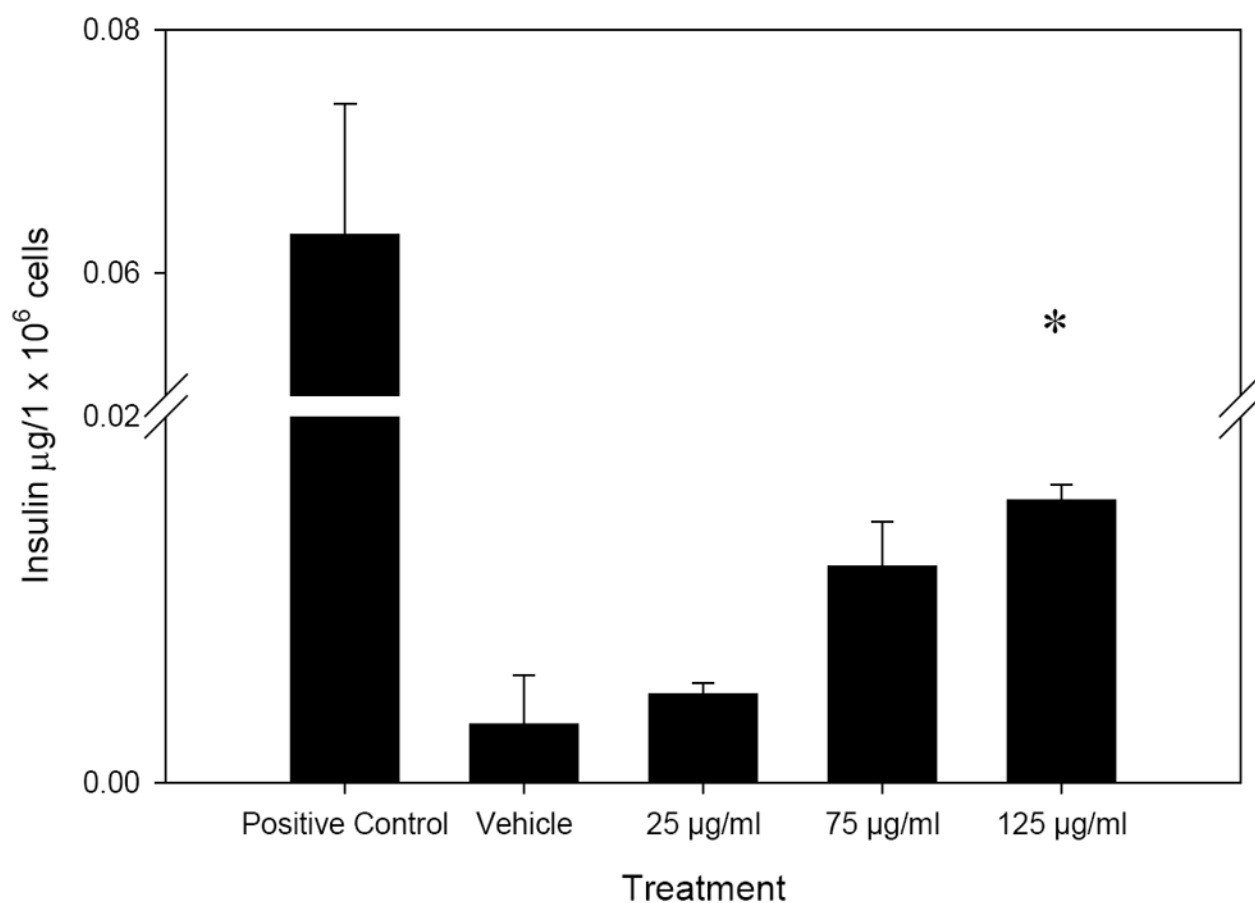
Fig. 2.
Insulin secretion activity of the saponin-rich fraction, after 60 min incubation with MIN6 β-cells. The (*) denotes significantly different activity as compared to DMSO vehicle, n=3. Error bars represent standard error.
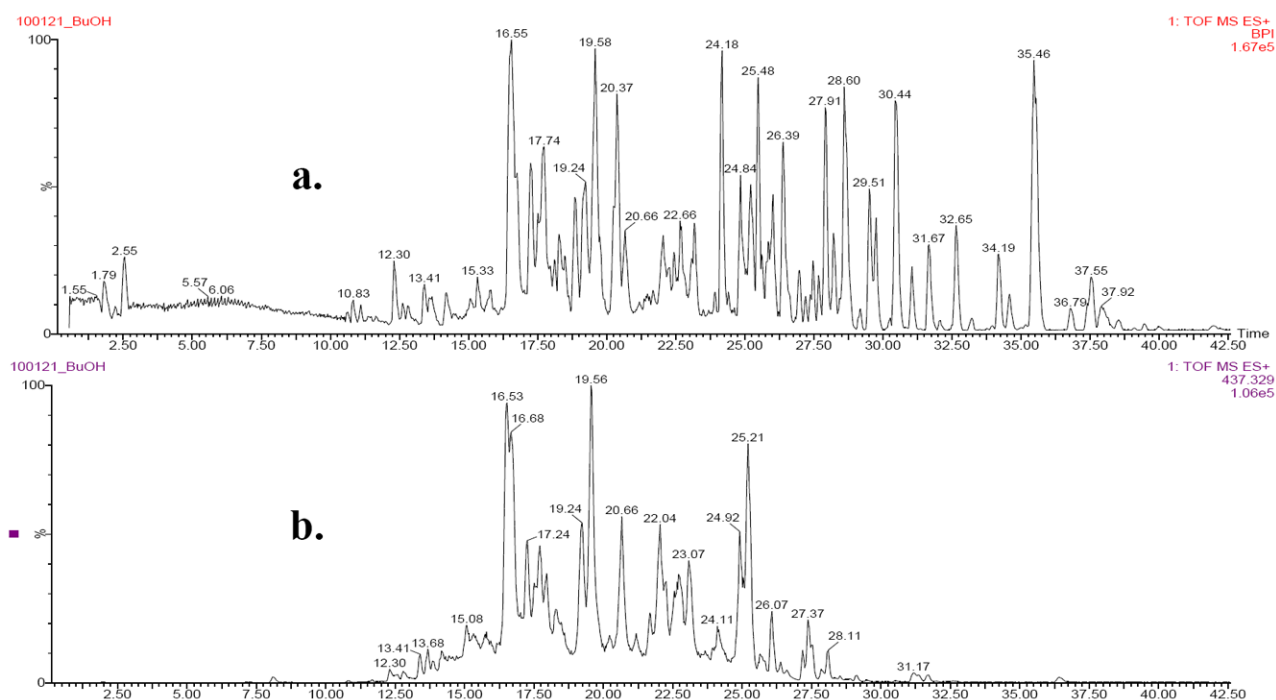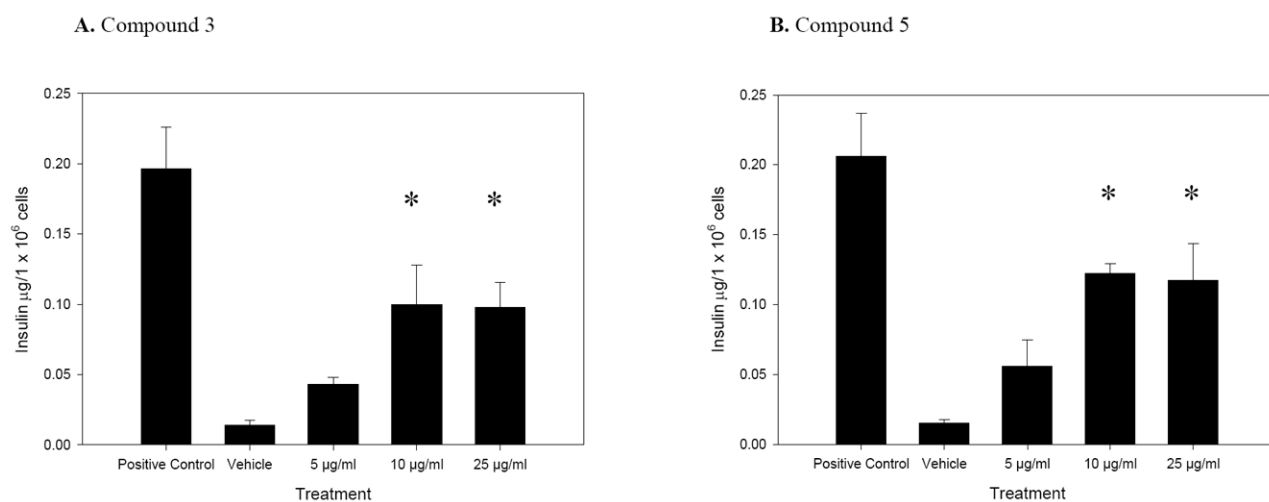
Fig. 3.
Base peak intensity chromatogram (a), derived from a total ion chromatogram, of saponin-rich fraction of *M. charantia*. Extracted ion chromatogram (b) for a common cucurbitane skeleton, $m/z = 437.3290$.

A. Compound 3

B. Compound 5

Fig. 4.
A and B. Insulin secretion activity of compounds 3 and 5, after 60 min incubation with MIN6 β-cells. Positive control is 27 mM glucose together with 50 μM glipizide, and vehicle is DMSO. The (*) denotes significantly different activity as compared to vehicle, n=3. Error bars represent standard error.

Table 1

A-F. β-Cell viability was measured by staining cells with Trypan Blue immediately after static incubation, and is an average of counting four quadrants on a hemocytometer. Viability was assessed as percentage (%) live cells and is ± standard error, n=3. Error bars represent standard error.

A:	
Treatment Live Cells (%)	
Ethanol extract 25 µg/ml	77 ± 5
Ethanol extract 75 µg/ml	76 ± 4
Ethanol extract 125 µg/ml	73 ± 5
Saponin-rich fraction 25 µg/ml	94 ± 5
Saponin-rich fraction 75 µg/ml	93 ± 2
Saponin-rich fraction 125 µg/ml	93 ± 3
B:	
Treatment Live Cells (%)	
Compound 1 (0.005 mg/ml)	97 ± 1
Compound 1 (0.010 mg/ml)	97 ± 1
Compound 1 (0.025 mg/ml)	93 ± 3
C:	
Treatment Live Cells (%)	
Compound 2 (0.005 mg/ml)	96 ± 1
Compound 2 (0.010 mg/ml)	95 ± 1
Compound 2 (0.025 mg/ml)	89 ± 3*
D:	
Treatment Live Cells (%)	
Compound 3 (0.005 mg/ml)	98 ± 0
Compound 3 (0.010 mg/ml)	98 ± 0
Compound 3 (0.025 mg/ml)	99 ± 0
E:	
Treatment Live Cells (%)	
Compound 4 (0.005 mg/ml)	98 ± 0
Compound 4 (0.010 mg/ml)	99 ± 0
Compound 4 (0.025 mg/ml)	98 ± 0
F:	
Treatment Live Cells (%)	
Compound 5 (0.005 mg/ml)	97 ± 0
Compound 5 (0.010 mg/ml)	95 ± 1