

***In vitro* investigation of potential anti-diabetic activity of the corm extract of *Hypoxis argentea* Harv. Ex Baker**

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The corms of *Hypoxis argentea* are widely used as a traditional remedy for diabetes mellitus in South Africa. In this study, we investigated the effects of non-toxic concentrations (12.5–100 µg mL⁻¹) of the aqueous extract of *H. argentea* (HAA) corms on glucose uptake, pancreatic beta cell proliferation, and adipocyte differentiation. HAA stimulated glucose uptake in HepG2 cells up to 19.6 % and 17.0 % in L6 myotubes. Live-cell imaging microscopy revealed significant increases ($p < 0.001$) in total INS-1 cell numbers exposed to HAA, although no effect was observed on adipogenesis in 3T3-L1 pre-adipocytes. HAA produced weak to moderate inhibition of porcine pancreatic α -amylase, α -glucosidase, porcine pancreatic lipase, dipeptidyl peptidase IV (DPP IV) activities, as well as protein glycation. Our results suggest that the acclaimed anti-diabetic effects of *H. argentea* could be mediated by its promotion of glucose utilization and preservation of pancreatic beta cell populations while preventing fat accumulation in adipocytes.

Keywords: *Hypoxis argentea*, diabetes, HepG2, INS-1, 3T3-L1, L6 myotubes, glucose uptake, adipogenesis

Diabetes mellitus is currently considered the third most significant chronic, non-communicable disease, responsible for most deaths and disabilities in the world (1), after cancer and cardiovascular diseases. With worldwide prevalence predicted to rise to double figures in 2035, due to increasing levels of modernized, sedentary lifestyles, diabetes mellitus has now emerged as a global public health challenge (2, 3). The huge financial burden of managing diabetes as a chronic health problem using conventional anti-diabetic medication, as well as the propensity for development of adverse effects have led to the exploration of alternative anti-diabetic therapy using herbs and other plant-related remedies. However, many of these traditional remedies are yet to be scientifically validated with respect to their anti-diabetic efficacy.

Plants are generally known to be rich in compounds including phenolics, flavonoids, and terpenoids, which are reported to possess hypoglycemic properties (4, 5). In effect, effective management of diabetes mellitus in many parts of the world can benefit immensely

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from the use of plant products, since they offer valuable sources of agents for long-term glycemic control with fewer side effects (6, 7). In Africa, several plants are used traditionally for anti-diabetic management, although many of them are yet to be scientifically validated.

Hypoxis argentea Harv. ex. Baker (*Hypoxidaceae*) is a perennial herb with leaves enclosed in a sheath arising directly from an underground adventitious rootstock (corm), in what appears to be a false stem, while a slender stalk from the middle of the leaves bears an inflorescence of bright yellow, often star-shaped flowers. The corms are traditionally used in the Eastern Cape province of South Africa for the management of diabetes mellitus and other ailments. *H. argentea* corms are dark brown in color externally and white internally. When sliced, the corms exude a greyish resinous juice. The corms are reportedly crushed and soaked in cold water and administered orally to diabetic patients (8).

Previous investigations involving other *Hypoxis* species have identified a nonlignan glycoside called hypoxoside, or chemically [(*E*)-1,5-bis(4'- β -D-glucopyranosyloxy-3'-hydroxyphenyl)pent-4-en-1-ynyl] as the most important and common phytochemical constituent (9, 10). Hypoxoside, as well as its hydrolytic product, rooperol, are believed to contribute to the anti-diabetic activities reported for some *Hypoxis* species (11). However, no reports of any study investigating the anti-diabetic properties of *H. argentea* were found in the literature. Recent investigations relating to anti-diabetic therapy development have explored approaches that involve the preservation and/or expansion of insulin-secreting, pancreatic β cell mass, either by stimulating increased β cell proliferation and/or decreasing β cell apoptosis (12). These efforts are based on the understanding that β cell mass declines considerably in diabetic patients. In the present study, we sought to investigate the anti-diabetic potential of an aqueous extract of the *Hypoxis argentea* corms using different relevant cell-based bioassays in rat insulinoma (INS-1) cells, rat skeletal muscle (L6 myotubes), human hepatocellular carcinoma (HepG2) cells and murine pre-adipocytes (3T3-L1). The effects of the extract on enzymes relevant to intestinal carbohydrate and fat digestion, as well as non-enzymatic protein glycation, were also investigated.

EXPERIMENTAL

Plant material and preparation of plant extract

Whole plant samples, including the underground corms of *H. argentea*, were collected from localities within Alice district and Grahamstown, Eastern Cape, South Africa. Specifically, the plant can be found at the following coordinates: 33° 14.699'S 26° 35.653'E, along Botha's Ridge, Grahamstown, South Africa. Plant materials were authenticated at the Selmar Schonland Herbarium, Rhodes University, South Africa, and the Giffen Herbarium, University of Fort Hare, South Africa, where voucher specimens are kept.

The corms were initially separated from the rest of the plant and washed with clean tap water, after which they were sliced and oven dried at 30 °C. They were then milled into a coarse powder. About 200 g of ground plant material was soaked in distilled water (1 litre) for about 24 hours. Due to the slimy nature of the mixture, it was initially filtered through a thick layer of sterile cotton wool before a repeat filtration using Whatmann No. 1 filter paper. The resulting filtrate was frozen at -40 °C in an acetone chilling machine and later dried in a freeze-dryer. The aqueous extract was chosen for these studies as the corms are reportedly soaked in water for their traditional medicinal use. The extraction solvent

and processes were selected to resemble, as much as possible, the approach of ethno-medicinal use by local healers in the Eastern Cape.

All spectrophotometric measurements were performed using a BioTek® PowerWave XS microplate reader (BioTek Instruments Inc., USA).

High performance-liquid chromatography coupled with time-of-flight mass spectrometry analysis (HPLC-TOF-MS/MS)

A high performance Agilent 1260 Infinity liquid chromatography (HPLC) system combined with an AB SCIEX 5600 Triple TOF hybrid mass spectrometer (MS) (Applied Biosystems Sciex, USA) equipped with an electrospray ionization source in negative mode was used to acquire mass spectra of the *H. argentea* aqueous extract. The LC system consisted of a 4.6 × 50 mm reverse phase column (Poroshell 120, EC-C18, diameter 7 µm, Agilent Technologies, USA). The mobile phase for gradient elution consisted of two solvents: solvent A: water with 0.1 % formic acid and solvent B: acetonitrile with 0.1 % formic acid. The elution gradient used was as follows: 75 % of solvent A for 0–8 min, followed by 25 % of solvent A for 8–15 min, and then 2 min with 75 % solvent A, giving a total run time of 17 minutes. The injection volume was 5.0 µL and the flow rate was 1 mL min⁻¹.

TOF-MS parameters were as follows: the declustering potential (DP) was 60 V, while collision energy (CE) was 35 V. Product ion parameters were as follows: ion spray voltage floating (ISVF): 4500; ion source gas 1 (GS 1): 31.026 kPa, ion source gas 2 (GS 2): 31.026 kPa and temperature (TEM): 450 °C. TOF masses were acquired using the Analyst Software for masses ranging from 100–1000 Da, while spectra were recorded in the ESI negative mode between *m/z* 50 and 1000.

Data were analyzed by ACD/Spectrus Processor 2017.2 software (Advanced Chemistry Development, Inc. Ontario, Canada). Putative identification of the major metabolites (peaks) in the extract was carried out using the RIKEN plant-specific MS/MS spectral database (ReSpect) (RIKEN, Japan).

Routine maintenance of cell cultures

HepG2 cells (human liver cancer cell line) were purchased from Highveld Biological, (Johannesburg, South Africa). INS-1 cells were a gift from Prof. Guy Ritter (University of Bristol, UK). L6 cells were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). HepG2 and INS-1 cell lines were maintained routinely in fresh culture medium consisting of EMEM and RPMI-1640, containing 25 mmol L⁻¹ HEPES, 2 mmol L⁻¹ glutamine supplemented with 10 % fetal bovine serum (FBS). L6 myotubes and 3T3-L1 pre-adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, Highveld Biological, Johannesburg, South Africa), with 10 % fetal bovine serum. Culture media were changed every 2–3 days. All cell cultures were incubated at 37 °C in a humidified atmosphere with 5 % CO₂.

INS-1 cell proliferation assay

The proliferative effect of *H. argentea* aqueous extract was studied in two assays on INS-1 cells, the crystal violet assay and live cell fluorescence imaging.

Crystal violet assay. – This assay was carried out according to the method of Feoktistova *et al.* (13), with slight modifications. INS-1 cells were seeded at a density of 8,000 cells per

well in 96-well micro-titer plates with 100 μL of complete medium. The extract was re-suspended in DMSO (0.25 %, *V/V*) and added to the wells already containing complete medium to reach concentrations of 12.5, 25 and 50 $\mu\text{g mL}^{-1}$. The plates were incubated for about 48 hours at 37 °C in a humidified incubator and 5 % CO_2 . GABA (100 $\mu\text{mol L}^{-1}$) was used as the positive control, while DMSO (0.25 %, *V/V*) served as the vehicle (untreated) control. Briefly, for the crystal violet assay, the treatments together with spent culture medium were removed by aspiration and were replaced with 100 μL of formaldehyde in PBS (10 %, *V/V*) to fix the cells. After about 1 hour, the fix solution was removed and 100 μL crystal violet solution, 0.1 %, *m/V* (0.1 g of crystal violet dissolved in 100 mL distilled water and filtered through Whatman No. 1 filter paper) was added to each well, followed by incubation of the plates at room temperature for about 30 min. Excess dye was removed by washing the plates with distilled water while the plates were dried by tapping on a paper towel. The dye taken up by the cells was extracted with 10 % acetic acid (100 μL per well). The absorbance of the wells was read at 595 nm. Cell viability was expressed as a percentage.

Live-cell fluorescence imaging of INS-1 cells

INS-1 cells were treated in culture in the same way as for the Crystal violet assay. Stock solution of nucleic acid stain, Hoechst 33342[®] [2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1*H*-benzimidazole trihydrochloride trihydrate, Thermo Fisher Scientific Inc., USA] of 10 mg mL^{-1} was prepared in distilled water.

The stock solution was further diluted 1:2,000 in PBS. All medium was removed from the cultured cells and replaced with 100 μL of Hoechst solution. The plates were incubated for about 20 min at room temperature away from light using an aluminum foil paper. Propidium iodide (PI) (50 $\mu\text{g mL}^{-1}$) was then added shortly before acquiring the images. Image acquisition was performed with an ImageXpress Micro XLS[®] microscope (Molecular Devices[®], USA) using blue and red filters with a 40X objective. Nine fields were acquired per well and cells were scored by defined dimensions and analyzed by the MetaXpress[®] software.

Glucose uptake assay in HepG2 cells

Glucose utilization assays were performed with simple modifications of the methods used by Deutschlander *et al.* (14). HepG2 cells were initially dislodged by brief exposure to 0.25 % trypsin in phosphate-buffered saline, then seeded in new growth medium in 96-well culture plates at a density of 6,000 cells per well in 200 μL of growth medium per well. The cells were allowed to adhere and grow in a humidified incubator (5 % CO_2 at 37 °C) for three days. Two cell-free rows were included to serve as blanks for the glucose utilization assay. On day 3, without changing the medium, 10 μL -aliquots of the plant extract (prepared at final concentrations of 25 and 100 $\mu\text{g mL}^{-1}$) and the positive controls, metformin (1 $\mu\text{mol L}^{-1}$) and berberine (50 $\mu\text{mol L}^{-1}$) were added to each well (six replicates for respective wells). The cells were incubated for further 48 hours. At the end of incubation, all spent medium was removed and then replaced with 25 μL of incubation buffer (RPMI 1640 diluted with PBS containing 0.1 % BSA) containing 8 mmol L^{-1} glucose. The plates were then incubated for a further 3 h at 37 °C. Ten μL of the incubation medium from each well was transferred to a new 96-well plate, and the glucose concentration in the medium was determined by adding 200 μL of glucose oxidase reagent

(SERA-PAK Plus, Bayer, Germany) and developed for 15 min at 37 °C. Absorbance was measured at 510 nm. Thereafter, glucose utilization was calculated as the difference between the cell-free and cell-containing wells. Glucose utilization was expressed as percentage of the untreated controls.

Glucose uptake assay in L6 myotubes

L6 myoblasts were seeded into flat-bottom 96-well plates at a density of 3,000 cells per well, maintained in DMEM supplemented with 10 % fetal bovine serum and incubated in a 5 % CO₂ humidified atmosphere at 37 °C. They were allowed to adhere and grow to about 90 % confluence, after which the culture medium was replaced with DMEM containing 2 % horse serum. The culture was then left for an additional five days to allow differentiation into myotubes. Two cell-free rows were included to serve as blanks for the glucose utilization assay. Forty-eight hours prior to the glucose utilization assay, the culture medium was replaced and an aliquot (10 µL) of each plant extract (prepared at final concentrations of 12.5, 25 and 50 µg mL⁻¹) was added using five replicates for each treatment. The cells were incubated in the presence of the extracts or insulin (positive control, 1 µmol L⁻¹) for a further 48 h. The spent medium was removed and replaced with 25 µL incubation buffer containing 8 mmol L⁻¹ glucose (RPMI 1640 medium diluted with PBS containing 0.1 % BSA) and this was incubated for 3 h at 3 °C. An aliquot of 10 µL of the medium from each well was transferred into a new 96-well plate and the glucose concentration was determined as described for HepG2 cells.

MTT cytotoxicity assay

For each of the glucose uptake assays in HepG2 and L6 cells, toxicity assays were conducted on the cells, after determination of glucose concentration, to determine the proportion of cells that actually participated in glucose uptake. To achieve this goal, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg mL⁻¹ in RPMI-1640) was added to the appropriate wells of each plate, which were then incubated at 37 °C for 3 h. MTT is a yellow tetrazolium dye that is reduced by live cells to a purple formazan, the absorbance of which is directly proportional to cell density. At the end of incubation, the medium was aspirated and 200 µL of DMSO was added to solubilize the formazan crystals formed in the cells. The absorbance of the wells was read at 540 nm.

3T3-L1 pre-adipocyte differentiation assay

The ability of the plant extract to induce differentiation and triglyceride accumulation was studied in 3T3-L1 pre-adipocytes according to the method of Ramirez-Zacarias *et al.* (15) with slight modifications. The cells were seeded into 48-well culture plates at a density of 6,000 cells per well and allowed to grow to 100 % confluence. Differentiation was induced in two-day post-confluent cells by treatment with plant extracts (prepared at 12.5, 25 and 50 µg mL⁻¹) or the positive control (rosiglitazone, 1 µmol L⁻¹) for 48 h. The cells were then cultured for an additional 8 days in normal culture medium (DMEM supplemented with 10 % FBS) and the medium was replaced every 2–3 days.

On the tenth day, the spent culture medium was removed and the cells were washed gently with PBS. The cells were fixed by adding 500 µL of 10 % formaldehyde in

PBS per well. Fixing was allowed for 1 hour at room temperature. The fix solution was then aspirated and the cells were stained by adding 200 μL of pre-warmed Oil Red O (prepared initially as a stock solution of 0.5 % *m/V* in isopropanol). A working solution was prepared by adding 4 mL of distilled water to 6 mL of the stock solution. Staining was allowed for 15 min at 37 °C. The plates were then washed extensively with water to remove excess dye, followed by gentle drying in an oven at 37 °C. Triglyceride accumulation was quantified by extracting the Oil red O stain with isopropanol (250 μL per well), after which 200 μL was transferred to a new 96-well plate) and the absorbance was measured at 520 nm. Excess isopropanol was removed, the plates were washed with water and dried at 37 °C and were then stained with Crystal Violet as previously described to normalize cell density.

α -Amylase inhibition

The plant extract was prepared at concentrations of 50, 100 and 200 $\mu\text{g mL}^{-1}$ in phosphate buffer (pH 6.8). Five μL of enzyme solution (10 mg porcine pancreatic amylase solubilized in 100 mL phosphate buffer) was pipetted into the appropriate well of a 96-well plate. Thereafter, 15 μL of test samples (plant extract), positive control (acarbose, 100 $\mu\text{mol L}^{-1}$) or phosphate buffer were added to the enzyme in respective wells. Control wells without enzyme and without the substrate (starch) were also included. The plate was pre-incubated for 15 minutes at 37 °C to allow interaction of the enzyme with different compounds. The reaction was started by the addition of 20 μL starch solution to the wells, and the plate was again incubated for 30 min at 37 °C. The reaction was terminated by adding 10 μL of 1 mol L^{-1} HCl to each well, followed by 75 μL of an iodine reagent. Absorbance was measured at 580 nm.

α -Glucosidase inhibition

The ability of the plant extract to inhibit α -glucosidase activity was measured using the chromogenic method (16) with slight modifications. It measures the rate of release of *p*-nitrophenol (PNP) from *p*-nitrophenyl- α -D-glucopyranoside (PNP-GLUC), which serves as substrate for the enzyme. The released PNP gives a yellow color, the absorbance of which can be measured spectrophotometrically. The reaction mixture within wells of a 96-well micro-titer plate consisted of 5 μL of plant extract (prepared at concentrations of 50, 100 and 200 $\mu\text{g mL}^{-1}$) or the positive control, epigallocatechin gallate (EGCG, 10 $\mu\text{g mL}^{-1}$) and 20 μL of α -glucosidase solution (α -glucosidase type 1 from Baker's yeast was prepared at a concentration of 0.58 $\mu\text{g mL}^{-1}$). This was pre-incubated at 37 °C for 5 min and the initial background absorbance was read at 405 nm. Ten μL of PNP-GLUC was then added and the reaction mixture was incubated again for 20 min at 37 °C. The reaction was terminated by the addition of 25 μL of sodium carbonate solution (100 mmol L^{-1}). The absorbance was measured again at 405 nm. Controls without enzyme and without the substrate (PNP-GLUC) were also included in the assay.

Protein glycation inhibition

The formation of fluorescent advanced glycation end-products when the protein, gelatin, was glycated with glyceraldehyde, was measured in this assay. Gelatin solution (50

μL) was first pipetted into the wells of a 96-well plate. Then, 40 μL of the positive control, aminoguanidine (20 mmol L^{-1}) or the plant extracts (at 50 and 100 $\mu\text{g mL}^{-1}$), was added to the respective wells. Thereafter, 10 μL of 500 mmol L^{-1} glyceraldehyde solution was added to each well. The plates were sealed and incubated at 37 °C for 24 hours. The fluorescent intensity was measured at an excitation wavelength of 370 nm and emission wavelength of 440 nm using a Biotek® Synergy MX fluorimeter (BioTek Instruments Inc., USA). The experiment was conducted in quadruplicates and fluorescence quench controls were also included for each sample.

Lipase inhibition assay

In this assay, the conversion of the substrate, *p*-nitrophenol phosphate (pNPP), was used to study lipase inhibition. Briefly, 10 μL of the extract (prepared at concentrations of 50, 100 and 200 $\mu\text{g mL}^{-1}$), positive control (Orlistat, 100 $\mu\text{mol L}^{-1}$) and DMSO (vehicle) was pipetted into the respective wells of a 96-well plate. Freshly prepared porcine pancreatic lipase was added at four times the volume of the test samples, positive and negative controls (40 μL). The plates were initially incubated at 37 °C for 15 min. Thereafter, 170 μL of the substrate solution was added to the wells. The substrate solution was a mixture of two solutions including solution A containing 1 mg mL^{-1} pNPP in 10 % isopropanol, while solution B consisted of 20 mg gum arabic, 40 mg sodium deoxycholate and 100 μL of Triton X-100, all dissolved in 18 mL of Tris-HCl buffer (pH 8.0). The plate was then incubated at 37 °C for 25 min and the absorbance was read at 405 nm.

Dipeptidyl peptidase IV (DPP IV) inhibition

The method described by Al-Masri *et al.* (17), with slight modifications, was used in determining DPP IV inhibition. The chromogenic substrate Gly-Pro-*p*-nitroanilide (GPPN) was cleaved by DPP IV to release *p*-nitroanilide (pNA), a yellow colored product which can be measured colorimetrically. Briefly, 35 μL of extracts (prepared at 50 and 100 $\mu\text{g mL}^{-1}$) and the positive control (Diprotin A, 50 $\mu\text{g mL}^{-1}$) were initially mixed with 15 μL of the DPP IV enzyme solution in the respective wells of a 96-well plate. The assay included a no-sample blank in which the samples were replaced with buffer and a no-enzyme blank in which the enzyme solution was also replaced by the buffer. The plate was pre-incubated at 37 °C for 5 minutes to allow interactions of extracts and the enzyme, after which a background absorbance was measured. Thereafter, 50 μL of the substrate solution (Gly-Pro-pNA) was added to all wells and the plate was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 25 μL of 25 % acetic acid. Absorbance was measured at 410 nm.

Data analysis

Six or eight replicates were performed for the cell-based assays, while four replicates were used for the enzymatic assays. Values for each treatment were compared with the untreated controls and, where required, the respective positive controls.

Statistical analysis was carried out using Graph Pad Prism software Version 5.01 and the test of significance was done using Student's *t*-test (two-tailed). Differences between means were considered statistically significant at *p* values between 0.01 and 0.05.

RESULTS AND DISCUSSION

INS-1 proliferation

In this study, the *in vitro* assays of the effects of the aqueous extract of *H. argentea* (HAA) on mouse insulinoma (INS-1) cell numbers indicated that the extract showed some potential to stimulate proliferation of pancreatic beta cells. With Hoechst 33342[®] staining, HAA, at all the different concentrations of 12.5, 25 and 50 $\mu\text{g mL}^{-1}$ produced a significant increase ($p < 0.01$) in the number of live INS-1 cells, up to 112.6, 115.5 and 113.1 %, resp., relative to the untreated control (100 %) (Fig. 1). This effect was similar to that obtained with the positive control, GABA, which produced cell viability up to 116.2 %. At the same time, propidium iodide staining revealed marginal decreases ($p < 0.05$), compared to the untreated controls, in the percentage of dead cells when INS-1 cells were exposed to HAA, producing cell deaths of only 0.47, 0.41 and 0.36 % at 12.5, 25 and 50 $\mu\text{g mL}^{-1}$, resp., while

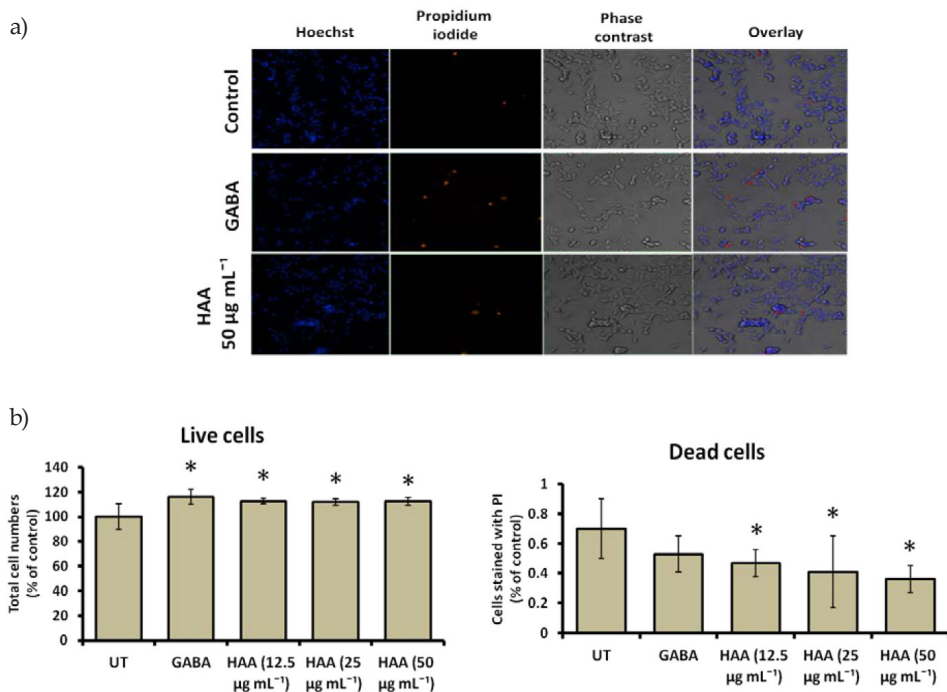


Fig. 1. Proliferative effects of the *Hypoxis argentea* aqueous extract in pancreatic β cells using ImageXpress micro XLS analysis: a) Hoechst (blue) and propidium iodide (red) staining of INS-1 cells following 48 h exposure to the aqueous extract of *Hypoxis argentea* (50 $\mu\text{g mL}^{-1}$) and the positive control (GABA, 100 $\mu\text{mol L}^{-1}$). Blue staining indicates live cells, red staining indicates dead cells, b) percentage of live and dead INS-1 cells achieved by MultiWave scoring. Values are expressed as percentage of control (mean \pm SD, $n = 8$). *Significant increase at $p < 0.01$ for live cells or $p < 0.05$ for dead cells, compared to the control.

GABA – gamma-amino butyric acid, HAA – *Hypoxis argentea* aqueous extract, UT – untreated control

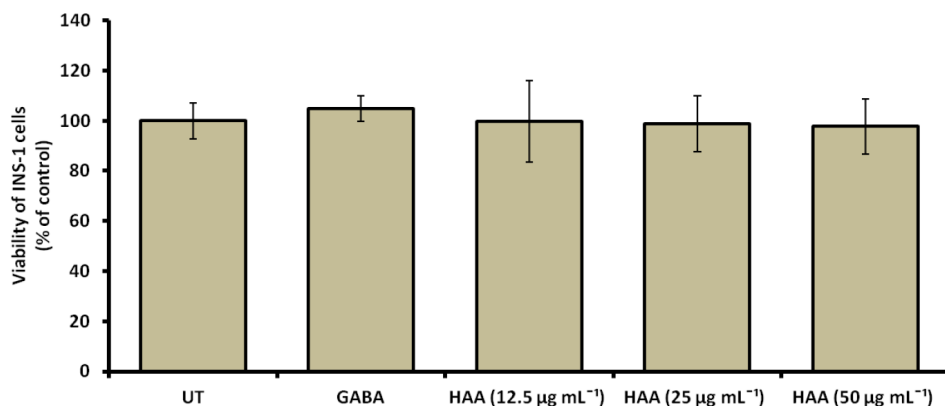


Fig. 2. Crystal violet assay of aqueous extracts of *Hypoxis argentea* on INS-1 cells (expressed as % of the control \pm SD, $n = 8$).

GABA – gamma-amino butyric acid, HAA – *Hypoxis argentea* aqueous extract, UT – untreated control

control wells had 0.70 % cell death. Crystal Violet staining of INS-1 cells in a separate assay merely revealed a tendency for HAA to maintain cell populations at near control values, producing 99.7, 98.8 and 97.8 % cell viability at the respective concentrations of 12.5, 25 and 50 $\mu\text{g mL}^{-1}$ (Fig. 2). There were no significant differences between cell viability in cells exposed to the different extract concentrations compared to that of cells exposed to GABA. The results obtained from both assays have, however, shown clearly that HAA was not toxic to INS-1 cells at the concentrations tested.

From the results described above, the potential of *H. argentea* to stimulate proliferation of INS-1 cells was well demonstrated by image cytometry. The stimulation of β cell proliferation by herbal extracts was proposed to occur by means of direct interactions of the constituents of these extracts with the cells (18). Natural products such as genistein were shown to induce β cell proliferation and insulin secretion by activation of protein kinase A and the extracellular signal-regulated kinase (19). Other flavonoids such as quercetin and catechin are believed to maintain the integrity of β cells *via* their effects in diminishing oxidative stress in β cells (20, 21).

Stimulation of glucose uptake

H. argentea demonstrated promising anti-hyperglycemic potential in HepG2 and L6 cell lines, effects that were largely concentration-dependent. It is believed that plant extracts that promote glucose utilization in skeletal muscle, as obtained in this study, act by mechanisms that mimic insulin-mediated signaling, possibly involving increased mobilization of GLUT 4 molecules to the plasma membrane (22).

The results obtained for the *in vitro* assay of glucose uptake in HepG2 cells in the presence of HAA are represented in Fig. 3a. The results indicate that HAA showed some potential in lowering blood glucose levels, producing a concentration-dependent increase in glucose uptake at 25 $\mu\text{g mL}^{-1}$ (103.9 %) and 100 $\mu\text{g mL}^{-1}$ (119.6 %) relative to the untreated control (100 %). The observed increase in glucose uptake by HAA was statistically signifi-

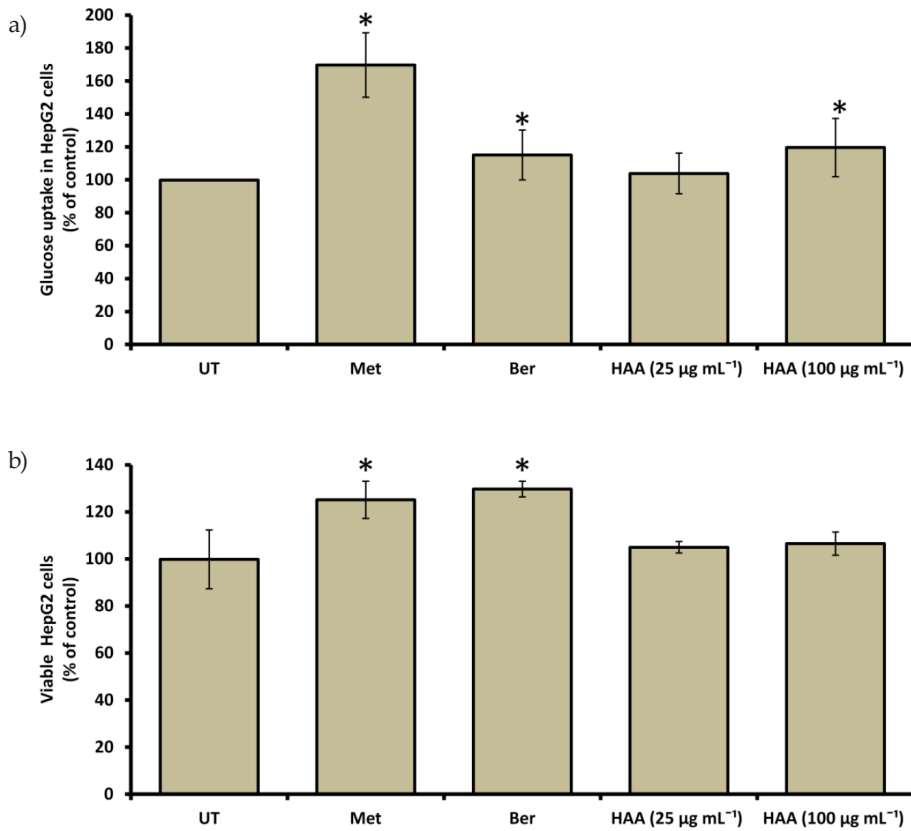


Fig. 3. a) Glucose uptake in HepG2 cells (expressed as % of the control \pm SD, $n = 6$) when exposed to the aqueous extract of *Hypoxis argentea* (HAA) corms, b) toxicity (expressed as % of the control \pm SD, $n = 6$) of the plant extract to HepG2 cells used in the glucose uptake assay. * Significant difference ($p < 0.05$) compared to the untreated control.

Ber – berberine, HAA – *Hypoxis argentea* aqueous extract, Met – metformin, UT – untreated control

cant ($p < 0.05$) at the higher concentration (100 $\mu\text{g mL}^{-1}$) compared to the untreated control. Metformin (1 $\mu\text{mol L}^{-1}$) and berberine (50 $\mu\text{mol L}^{-1}$), used as positive controls, produced increases in glucose uptake up to 169.7 and 115.1 %, resp. HAA produced a significantly lower ($p < 0.01$) amount of glucose uptake compared to metformin. However, the extract exhibited a similar degree of glucose uptake compared to berberine. The observed increases in glucose uptake obtained with the different compounds may be relevant, since the toxicity assay conducted on the cells revealed that neither HAA nor positive controls were toxic to HepG2 cells (Fig. 3b).

Fig. 4a depicts glucose uptake when L6 myotubes were exposed to HAA at different concentrations. The extract again showed a potential to stimulate glucose uptake in L6 myotubes, producing 111.3, 116.3 and 117.0 % glucose uptake, resp., at 12.5, 25 and 50 $\mu\text{g mL}^{-1}$. This effect was statistically significant ($p < 0.05$) at all extract concentrations compared to the

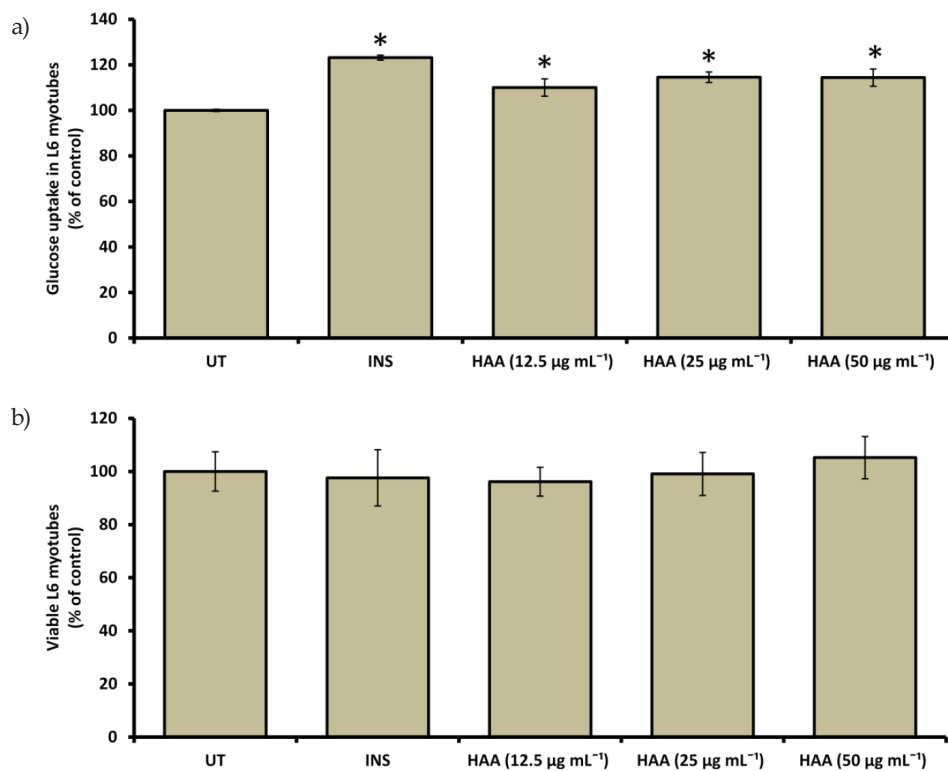


Fig. 4. a) Glucose uptake in L6 myotubes (expressed as % of the control \pm SD, $n = 6$) when exposed to the aqueous extract of *Hypoxis argentea* corms, b) toxicity (expressed as % of the control \pm SD, $n = 6$) of the plant extract to L6 myotubes used in the glucose uptake assay. * Significant difference ($p < 0.05$) compared to the untreated control.

HAA – *Hypoxis argentea* aqueous extract, Ins – Insulin, UT – untreated control

untreated control. The positive control, insulin ($1 \mu\text{mol L}^{-1}$), however, produced slightly better stimulation, up to 123.3 % glucose uptake, compared to the untreated control. When compared with the extract, however, this difference was not statistically significant. Again, HAA showed no potential for toxicity to L6 myotubes at the concentrations tested (Fig. 4b), suggesting that the observed stimulation of glucose uptake by HAA might be a true reflection of its hypoglycemic potential.

Stimulation of glucose uptake obtained in this study with *H. argentea* is supported by previous studies on *H. hemerocallidea*, a prominent member of the *Hypoxis* genus (23). The plant was found to exert a hypoglycemic effect in rats, probably due to its content of phytoosterols and/or sterolins. The present study, however, is the first attempt to investigate the mechanisms of anti-diabetic actions of *H. argentea* in *in vitro* assays. Our observations of increased glucose uptake in HepG2 cells and L6 myotubes could be considered relevant, since the extract did not exhibit any potential toxicity to these cells at the concentrations tested, which were considered to be physiologically relevant from previous studies (24).

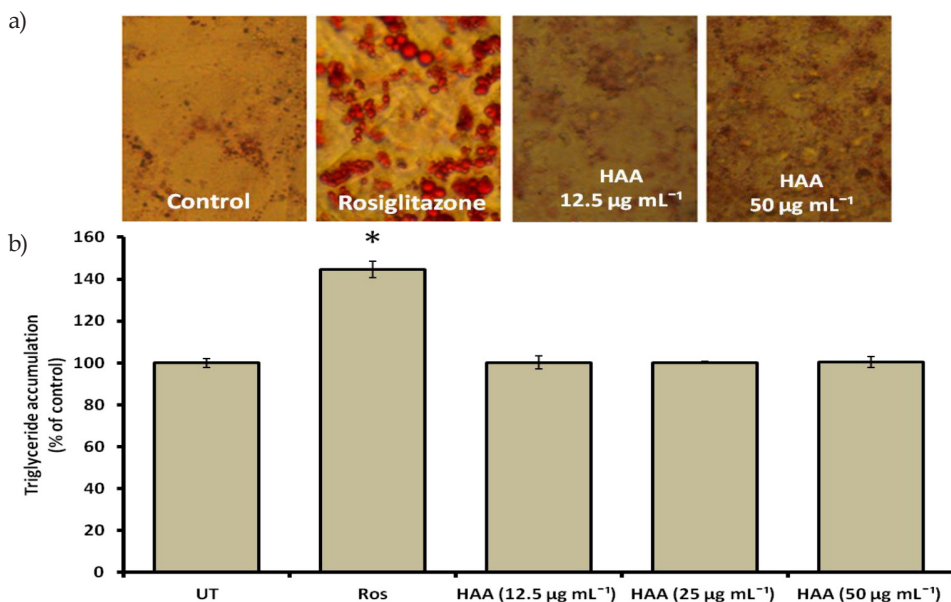


Fig. 5. Effect of the aqueous extract of the *Hypoxis argentea* corms on 3T3-L1 pre-adipocyte differentiation: a) plates show the appearance of Oil Red O stained cells after 10 days of treatment with the plant extract (12.5 and 50 µg mL⁻¹) or rosiglitazone (1 µmol L⁻¹), b) quantitation of the effect of the *Hypoxis argentea* aqueous extract on 3T3-L1 pre-adipocyte triglyceride accumulation. Values are expressed as mean ± SD of four replicates corrected for cell density. * Significant difference ($p < 0.05$) compared to the untreated control.

HAA – *Hypoxis argentea* aqueous extract, Ros – rosiglitazone, UT – untreated control

Triglyceride accumulation

Elevated levels of triglycerides in peripheral tissues can produce lipotoxicity and also contribute to obesity-related type 2 diabetes mellitus. Fig. 5 shows the effect of HAA on triglyceride accumulation in 3T3-L1 pre-adipocytes, as revealed by Oil Red O staining. HAA did not produce any significant alteration in adipocyte differentiation at three concentrations, 12.5, 25 and 50 µg mL⁻¹, when compared to the untreated control. However, Oil Red O staining in pre-adipocytes treated with the positive control, rosiglitazone, showed intense reddish staining, indicating the presence of large amounts of cytoplasmic lipid droplets in the differentiated adipocytes. Spectrophotometric quantification of the amount of stain taken up by the cells indicated slight changes in triglyceride accumulation with values of 100.1, 100.2 and 100.4 % at 12.5, 25 and 50 µg mL⁻¹ of HAA, resp. Rosiglitazone, on the other hand, produced a significant increase ($p < 0.05$) in triglyceride accumulation of up to 144.6 %. Rosiglitazone thus produced a significant increase ($p < 0.05$) in triglyceride accumulation compared either to the extract or the untreated controls.

Drugs such as rosiglitazone are known to induce adipogenesis in pre-adipocytes by increasing the transcription of adipocyte-specific genes required for the formation of tri-

glyceride droplets (25). During differentiation, the level of triglycerides increases in adipocytes *via* mechanisms involving up-regulation of the CCAAT/enhancer-binding proteins (C/EBP α , β and γ) and the peroxisome proliferator-activated receptor gamma (PPAR γ) (26). The results of this study show that *H. argentea* exerts no pro-adipogenic effects at the concentrations tested, since its addition to 3T3-L1 pre-adipocytes did not alter significantly the triglyceride accumulation in these cells.

Certain anti-diabetic drugs are able to mobilize triglycerides from ectopic tissues (such as liver and muscle) into the adipose tissue. This process is beneficial in such ectopic tissues as it prevents the development of insulin resistance, although there is the risk of excessive triglyceride accumulation in adipose tissue, which may result in adipose hypertrophy and might, in turn, pose a risk for the development of type 2 diabetes (27). Thus, recent studies have argued that the ideal anti-diabetic agent is one that neither blocks nor induces adipogenesis and/or triglyceride accumulation (28).

Inhibition of carbohydrate and lipid-digesting enzymes and protein glycation

Mechanisms of anti-diabetic action by some compounds may involve the inhibition of carbohydrate-digesting enzymes, including α -amylase and α -glucosidase, as well as processes that lead to the development of diabetic complications (*e.g.*, protein glycation). Table I summarizes the results of the inhibitory activities of HAA against α -amylase, α -glucosidase, lipase, dipeptidyl peptidase IV (DPP IV) and glycation of gelatin. Alpha-amylase and alpha-glucosidase are the key carbohydrate-digesting small intestinal enzymes, which have been recognized as therapeutic targets for modulating post-prandial hyperglycemia. The percentage inhibitory activity of HAA against α -glucosidase at 50, 100 and 200 $\mu\text{g mL}^{-1}$ was 16.3, 19.0 and 31.5 %, resp., compared to the untreated control. These values may be physiologically relevant as higher concentrations of the extract will be present in the gut. EGCG, a known inhibitor of α -glucosidase activity, produced as much as 57.5 % inhibition. However, the extract did not exhibit any appreciable inhibition of α -amylase activity at any of the three concentrations, while acarbose, used as positive control, produced as much as 94.7 % inhibition of α -amylase activity.

Protein glycation, a non-enzymatic glycosylation reaction between reducing sugars and free amino groups of proteins, is one of the consequences of increased levels of glucose in the blood of diabetic patients. HAA produced very weak inhibition (5.5 %) of gelatin glycation at the highest concentration tested (100 $\mu\text{g mL}^{-1}$). This was in contrast to amino-guanidine, which exhibited as much as 65.7 % inhibition. The enzyme lipase plays a key role in fat digestion by hydrolyzing dietary triglycerides to monoglycerides and free fatty acids. Free fatty acids are known to contribute to the development of insulin resistance in type 2 diabetes (29). In this study, HAA showed weak inhibitory activities against porcine pancreatic lipase (Table I). At 50, 100 and 200 $\mu\text{g mL}^{-1}$, the extract produced only 3.2, 5.4 and 5.4 % inhibition of lipase activity, resp., all of which were much lower compared to 58.8 % obtained with Orlistat, which was used as a positive control. DPP IV normally degrades incretin hormones such as GLP-1, which are known to enhance β -cell function. Inhibition of DPP IV leads to increased half-life and circulating levels of incretins. As indicated in Table I, DPP IV inhibition was virtually non-existent for the plant extract at the concentrations tested compared to Diprotin A, the positive control, which exhibited 75.8 % inhibition of DPP IV activity.

Table I. Inhibitory effects of *Hypoxis argentea* on the activities of the key intestinal carbohydrate and fat digesting enzymes and protein glycation

	Inhibition (%)
<i>α</i> -Amylase	
Acarbose	94.7 ± 0.0
HAA (50 µg mL ⁻¹)	NI
HAA (100 µg mL ⁻¹)	NI
HAA (200 µg mL ⁻¹)	NI
<i>α</i> -Glucosidase	
EGCG	57.5 ± 4.8
HAA (50 µg mL ⁻¹)	16.30 ± 4.8*
HAA (100 µg mL ⁻¹)	19.00 ± 6.5*
HAA (200 µg mL ⁻¹)	31.5 ± 4.0*
Lipase	
Orlistat	58.8 ± 0.3
HAA (50 µg mL ⁻¹)	3.2 ± 2.4*
HAA (100 µg mL ⁻¹)	5.4 ± 1.1*
HAA (200 µg mL ⁻¹)	5.4 ± 0.7*
DPP IV	
Diprotin A	75.8 ± 0.3
HAA (50 µg mL ⁻¹)	NI
HAA (100 µg mL ⁻¹)	NI
Protein glycation	
Aminoguanidine	71.0 ± 5.0
HAA (50 µg mL ⁻¹)	13.0 ± 1.0*
HAA (100 µg mL ⁻¹)	12.0 ± 2.0*

EGCG – epigallocatechin gallate, HAA – *Hypoxis argentea* aqueous extract, NI – no inhibition
Values are expressed as mean ± SD, *n* = 4.

* Significantly lower (*p* < 0.05) compared to the positive control.

Compounds in *H. argentea* extract and their possible impact

Compound identification of the *Hypoxis argentea* aqueous extract was carried out by HPLC-TOF-MS/MS. The negative ionization mode was preferred, since it has been reported to be more sensitive for the detection of polyphenols and also has lower limits of detection compared to the positive mode of analysis (30).

Eight major peaks were identified or tentatively identified according to their retention times and fragment ions in the specific plant natural product database, as shown in Fig. 6

Table II. Mass data of major compounds detected in the aqueous extract of the *Hypoxis argentea* corms

n	Compound	Formula	t_R (min)	Ion mode	Calcu- lated m/z	Experimen- tal m/z	Product ion m/z
1	(+)-Epicatechin	C ₁₅ H ₁₄ O ₆	2.523	[M-H] ⁻	290.079	288.936	158.978, 174.956, 187.097, 272.960, 276.936, 288.936, 304.915, 402.918, 418.894
2	L-β-homotryptophan HCl	C ₁₂ H ₁₄ N ₂ O ₂	2.723	[M-H] ⁻	218.25	216.909	111.948, 216.909, 260.873, 276.846, 358.839, 396.797, 418.779
3	Glycyl-L-proline	C ₇ H ₁₂ N ₂ O ₃	3.053	[M-H] ⁻	172.18	171.030	165.041, 171.030, 189.041, 195.050, 223.045, 267.071, 327.092, 385.135
4	Glycyl-L-proline	C ₇ H ₁₂ N ₂ O ₃	3.798	[M-H] ⁻	172.18	171.030	109.029, 171.030, 172.033, 187.097, 191.020, 339.031, 365.049, 513.145
5	D-(-)-quinic acid	C ₇ H ₁₂ O ₆	4.265	[M-H] ⁻	192.17	191.020	111.009, 129.019, 187.097, 191.020, 192.023, 405.028, 406.029, 421.002
6	Dihydrocapsaicin	C ₁₈ H ₂₉ NO ₃	6.428	[M-H] ⁻	307.43	169.015	169.015, 170.017, 187.097, 324.073, 325.076, 341.109, 387.115, 477.173
7	Esculin sesquihydrate	C ₁₅ H ₁₆ O ₉	13.888	[M-H] ⁻	340.079	325.183	265.149, 305.214, 311.167, 325.183, 339.201, 357.206, 409.236, 794.951
8	7-Hydroxy-4-methyl coumarin	C ₁₀ H ₈ O ₃	14.750	[M-H] ⁻	176.17	174.955	174.955, 341.108, 357.208, 397.275, 459.346, 535.384, 614.499, 729.403

and Table II. With regard to bioactivity against diabetes mellitus, compounds at peaks 1, 5 and 8 were deemed relevant. Peak 1 was characterized as epicatechin, displaying the parent ion with m/z 288.936 at 2.523 minutes. Epicatechin is a natural flavonoid found at high concentrations in green tea and known to be important in the management of diabetes (31). Peak 5 was tentatively characterized as quinic acid, with MS/MS spectra giving m/z 191.020 at 4.265 minutes. Quinic acid has been reported to act synergistically with quercetin to alleviate degenerative changes in the liver, kidney and pancreatic tissues in streptozotocin-induced diabetic rats (32). Peak 8 was tentatively identified as a coumarin derivative, 7-hydroxy-4-methyl coumarin, which displayed the deprotonated ion [M-H]⁻ at m/z 174.955. The anti-diabetic activity of coumarin derivatives such as cloricromene has been reported (33).

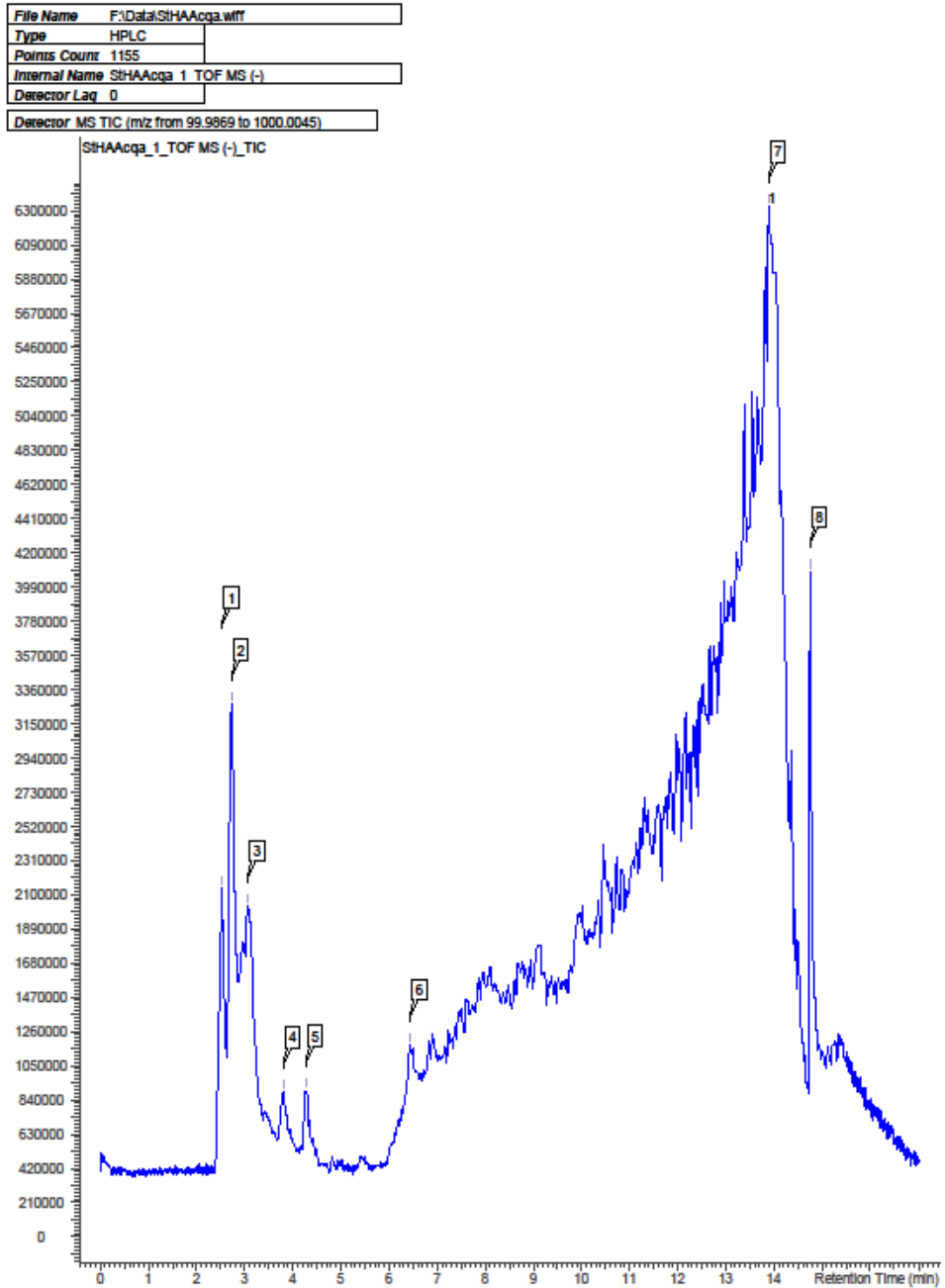


Fig. 6. Total ion chromatogram of the *Hypoxis argentea* aqueous extract analyzed by high performance liquid chromatography coupled with triple time-of-flight mass spectrometry.

CONCLUSIONS

Tentative identification of compounds on the basis of accurate mass spectrometric measurements in the *Hypoxis argentea entea* extract by LC-TOF-MS has revealed the presence of phytochemicals with reported anti-diabetic properties and suggests that the plant warrants further exploration as a potential source of antidiabetic agents. Future detailed analyses utilizing retention time and/or mass spectral data of reference standards are, however, required for the confirmation of their actual identities.

Overall, our results support the traditional medicinal use of *H. argentea* in diabetes mellitus, with its probable mechanisms of action including stimulation of glucose uptake *via* insulin-dependent pathways in skeletal muscle and/or insulin-independent pathways in hepatocytes, as well as the probable stimulation of pancreatic β cell proliferation. We recommend further studies in *in vivo* environments to confirm the promising anti-diabetic potential observed in the *in vitro* cell-based bio-assays.

Acronyms, abbreviations, symbols. – AMPK – adenosine monophosphate protein kinase, BSA – bovine serum albumin, C/EBP – CCAAT/enhancer-binding proteins, DMEM – Dulbecco's modified Eagle's medium, DMSO – dimethyl sulfoxide, DPP IV – dipeptidyl peptidase IV, EGCG – epigallocatechin gallate, EMEM – Eagle's modified essential medium, FBS – fetal bovine serum, GABA – gamma amino butyric acid, GLP-1 – glucagon-like peptide 1, GLUT – glucose transporter, GPPN – Gly-Propara-nitroanilide, HAA – *Hypoxis argentea* aqueous extract, HepG2 – human hepatocellular carcinoma cell line, HPLC-TOF-MS/MS – high performance-liquid chromatography coupled with time-of-flight mass spectrometry, INS-1 – insulinoma cell line, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS – phosphate-buffered saline, PI3-K – phosphatidyl inositol 3-kinase, PKB – protein kinase B, PNP-GLUC – *p*-nitrophenyl- α -D-glucopyranoside, pNPP – *p*-nitrophenol phosphate, PPAR γ – peroxisome proliferator-activated receptor gamma, RPMI – Roswell Park Memorial Institute.

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