Anti-diabetic properties of the Canadian lowbush blueberry *Vaccinium angustifolium* Ait.

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Abstract

Incidence of type II diabetes is rapidly increasing worldwide. In order to identify complementary or alternative approaches to existing medications, we studied anti-diabetic properties of *Vaccinium angustifolium* Ait., a natural health product recommended for diabetes treatment in Canada. Ethanol extracts of root, stem, leaf, and fruit were tested at 12.5 μg/ml for anti-diabetic activity in peripheral tissues and pancreatic β cells using a variety of cell-based bioassays. Specifically, we assessed: (1) deoxyglucose uptake in differentiated C2C12 muscle cells and 3T3-L1 adipocytes; (2) glucose-stimulated insulin secretion (GSIS) in β TC-tet pancreatic β cells; (3) β cell proliferation in β TC-tet cells; (4) lipid accumulation in differentiating 3T3-L1 cells; (5) protection against glucose toxicity in PC12 cells. Root, stem, and leaf extracts significantly enhanced glucose transport in C2C12 cells by 15–25% in presence and absence of insulin after 20 h of incubation; no enhancement resulted from a 1 h exposure. In 3T3 cells, only the root and stem extracts enhanced uptake, and this effect was greater after 1 h than after 20 h; uptake was increased by up to 75% in absence of insulin. GSIS was potentiated by a small amount in growth-arrested β TC-tet cells incubated overnight with leaf or stem extract. However, fruit extracts were found to increase ^{3}H-thymidine incorporation in replicating β TC-tet cells by 2.8-fold. Lipid accumulation in differentiating 3T3-L1 cells was accelerated by root, stem, and leaf extracts by as much as 6.5-fold by the end of a 6-day period. Stem, leaf, and fruit extracts reduced apoptosis by 20–33% in PC12 cells exposed to elevated glucose for 96 h. These results demonstrate that *V. angustifolium* contains active principles with insulin-like and glitazone-like properties, while conferring protection against glucose toxicity.

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Enhancement of proliferation in β cells may represent another potential anti-diabetic property. Extracts of the Canadian blueberry thus show promise for use as a complementary anti-diabetic therapy.

Introduction

The incidence of type 2 diabetes mellitus (T2DM) has reached epidemic proportions in western and developing countries, with an estimated 194 million people afflicted (International Diabetes Federation, 2005). Peripheral insulin resistance is a key feature of T2DM and results from a combination of sedentary lifestyle, unhealthy dietary habits, and genetic predisposition (Skyler, 2004). Insulin resistance is also implicated in a number of life-threatening disorders collectively referred to as the metabolic syndrome (Hansen, 1999).

Current medication options for the treatment of diabetes are relatively limited, have non-negligible side-effects, and must often be prescribed in combination (Cheng and Fantus, 2005). The use of natural health products as complementary or alternative approaches to existing medications is growing in popularity. While these have been the object of very few rigorous scientific studies, it is clear that many plants possess hypoglycaemic activity, some having considerable anti-diabetic potential (Marles and Farnsworth, 1995; Yeh et al., 2003). Indeed, the widely prescribed insulin-sensitizer metformin was derived from guanidine, a molecule isolated from *Galega officinalis* L. (French lilac) (Bailey and Day, 2004; Witters, 2001). Moreover, *Trigonella foenum-graecum* L. (Fenugreek) is a plant long-consumed around the world for its anti-diabetic properties. It contains abundant amounts of the unconventional amino acid 4-hydroxy-isoleucine, which, along with its derivatives, is currently being developed as a novel anti-diabetic molecule (Broca et al., 2004, 1999).

Various members of the *Vaccinium* genus, including *Vaccinium myrtillus* L (European blueberry or bilberry) and *Vaccinium macrocarpon* Ait. (American cranberry), are reputed to possess anti-diabetic activity (Blumenthal, 1998; Chambers and Camire, 2003) and have been used extensively as traditional medicines for the treatment of diabetic symptoms (Jellin et al., 2005). A recent survey identified *Vaccinium angustifolium* Ait. (Canadian lowbush blueberry) as one of the anti-diabetic plants most highly recommended by Quebec traditional practitioners, alongside fenugreek (Haddad et al., 2003). However, despite such claims and widespread use, the majority of published studies have focussed on the anti-oxidant properties of this species (Costantino et al., 1992; Kalt et al., 2001; Kay and Holub, 2002; Lyons et al., 2003; Rimando et al., 2004; Sweeney et al., 2002; Wang and Jiao, 2000) and its anti-diabetic potential has not been the object of rigorous scientific investigation. The purpose of the present study was to test *V. angustifolium* for insulin-like activity, insulinotropic activity, glitazone-like activity, and cyto-protective activity in cell-based bioassays.

Materials and methods

Plant material and preparation

Samples of wild *V. angustifolium* Ait. were harvested near la Vérendrye Wildlife Reserve, Quebec, Canada. Voucher specimens are stored at the University of Ottawa. After collection, the plant was washed and separated into four organs: fruits, leaves, roots, and stems. Fruits were frozen at $-80\,^{\circ}C$, leaves were preserved in ethanol (95%), while roots and stems were air-dried and then stored in darkness at room temperature (RT). Preserved leaves were filtered, crushed, and re-extracted three times with fresh ethanol at RT for 12 h. The four ethanolic phases were recovered, pooled, dried at $40\,^{\circ}C$ by rotary evaporator, and lyophilized. Dried roots and stems were ground on a Wiley mill to mesh size 40 and extracted three times in ethanol at RT for 12 h. The ethanolic phase was then dried and lyophilized, as above. Frozen fruits were crushed and extracted as for roots and stems. Lyophilized extracts were stored in the dark at $4\,^{\circ}C$.

HPLC analyses

Root, stem, leaf, and fruit extracts were characterized by HPLC–MS analysis. Fruit extracts have also been characterized by others (Kalt et al., 1999; Kalt et al., 2001; Lyons et al., 2003; Rimando et al., 2004; Schmidt et al., 2004). All analyses were performed using a Hewlet-Packard Chemstation series 1100 chromatograph (Agilent, Palo Alto, CA, USA), with a photo-diode array detector and an APCI/MS. A Waters YMC ODS-AM narrow bore column (100 mm × 2 mm i.d.; 3 μm particle size) was used in a 50 °C oven at a flow rate of 0.3 ml/min. Elution conditions with a mobile phase system of methanol (solvent A) and trifluoroacetic acid (0.05%) in water (pH 3.4; solvent B) were optimized for MS detection as follows: initial conditions 8:92 (A:B),

held for 5 min, then changing to 13:87 in 2 min, then to 30:70 in 14 min, 60:40 in 3 min, 100:0 in 2 min, then isocratic elution with 100:0 for 2 min, finally returning to the initial conditions in 2 min, which is held for 5 min to re-equilibrate the column. The total analysis time was 35 min. The sample injection volume was 1 µl, and the elution profiles were also monitored on-line at 325 and 280 nm with the DAD. MS detection was performed in positive ionization mode, the optimized conditions were: APCI conducted at 300 °C with the vaporizer at 400 °C; nebulizer pressure: 40 psi; nitrogen (drying gas) flow rate: 6.0 l/min; fragmentation voltage: 20 V; capillary voltage: 3000 V; corona current: 3.0 µA. The MS data were collected in scan mode for ions from 100 to 800 mass units.

Cell culture

Cell biology reagents were purchased from Invitrogen Life Technologies (Burlington, ON) unless otherwise noted. C2C12 murine skeletal myoblasts and 3T3-L1 murine pre-adipocytes were obtained from American Type Cell Collection (ATCC; Chicago, IL). β TC-tet murine pancreatic β cells were kindly provided by Dr. Shimon Efrat (Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel) (Fleischer et al., 1998; Milo-Landesman and Efrat, 2002). In this line derived from transgenic mice expressing the SV40T antigen (Tag) under control of the tetracycline gene regulatory system, growth arrest can be induced by shutting off Tag expression in the presence of tetracycline. PC12-AC cells are a clonal derivative of the PC12 pheochromocytoma cell line (ATCC) and can be differentiated into a sympathetic neuronal phenotype with exposure to nerve growth factor.

All cells were cultured at 37 °C in a humidified 5% CO_2, 95% air atmosphere. C2C12 myoblasts were cultured in high glucose (2.5 g/l) Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 10% horse serum (HS), and penicillin–streptomycin, until 80% confluence. Myoblasts were then differentiated into myotubes over 6 days in DMEM containing 2% HS. By the end of this period, confluence was attained and all cells were multinucleated. 3T3-L1 cells were proliferated in high-glucose DMEM containing 10% FBS and antibiotics. Upon 80% confluence, differentiation was initiated by adding 250 µM 3-isobutylmethylxanthine (IBMX), 1 µM dexamethasone (DMX), and 670 nM insulin to this medium for 2 days. Differentiation was then continued in DMEM containing 10% FBS and 670 nM insulin for approximately 10 days. At this point, confluence was attained and over 90% of cells contained lipid droplets visible under phase-contrast microscopy. For adipogenesis assays, proliferating cells were allowed to attain confluence before initiating differentiation as above. Cells were then differentiated for a total of 5–6 days. β TC-tet cells were grown in high-glucose DMEM containing 15% HS, 2.5% FBS, and antibiotics until 80% confluent. PC12-AC cells were cultured in RPMI 1640 containing 11 mM glucose, 10% HS, 5% newborn calf serum, and antibiotics until 80% confluent.

V. angustifolium extracts were solubilized in dimethyl sulphoxide (DMSO) for application to cell cultures; final DMSO concentration was fixed at 0.1% for all assays. All extracts were applied at a dose of 12.5 µg/ml except in the PC12-AC survival assay where concentrations ranging from 0.5 to 25 µg/ml were tested. The selection of the 12.5 µg/ml dose was based on pilot cytotoxicity experiments indicating that root and stem extracts are slightly toxic at 25 µg/ml; at this concentration viability was reduced by approximately 10%, as assessed by Trypan blue dye exclusion (results not shown). Overnight incubation at 12.5 µg/ml was confirmed to have no effect on the morphology or viability of the various cell types employed herein.

Glucose transport assay

To screen for insulin-like activity and for potentiation of insulin action, basal and insulin-stimulated glucose uptake were measured in differentiated C2C12 skeletal myotubes and in differentiated 3T3-L1 adipocytes incubated with extracts of *V. angustifolium*. Both of these cell types exhibit insulin-regulated glucose uptake and possess Glut-1 and Glut-4 glucose transporters (Klip and Paquet, 1990; Sweeney et al., 2002). Confluent and differentiated cells, grown in 12-well plates were incubated with either vehicle (DMSO) alone, extract in vehicle, or positive control in vehicle for either 1 or 18–21 h prior to assay. For 1 h treatments, differentiation medium was replaced with serum-free medium 1 h before the start of incubation period and the incubation was performed in serum-free medium. For 18–21 h treatments, the last 3 h of incubation were performed in serum-free medium. Following the incubation period, cells were rinsed twice with a Krebs-phosphate buffer (20 mM HEPES, 4.05 mM Na_2HPO_4, 0.95 mM NaH_2PO_4, 136 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM glucose, 0.5% BSA, pH 7.4) at 37 °C. Cells were then treated with 0, 1, or 100 nM insulin in this buffer for 30 min at 37 °C, in the presence or absence of extracts. Cells were then washed twice with glucose-free Krebs-phosphate buffer at 37 °C and treated with 0.5 µCi/ml 2-deoxy-D-[1-^{3}H]-glucose (TRK-383, Amersham Biosciences, UK) in this buffer for exactly 10 min at 37 °C without extracts. Following this, cells were placed on ice and immediately washed three times with ice-cold Krebs-phosphate buffer, lysed with 0.1 M NaOH for 30 min, and scraped. The lysate was added

to 1 ml of liquid scintillation cocktail (Ready-Gel 586601, Beckman Coulter) and incorporated radioactivity was measured in a scintillation counter. Metformin (400 μM) applied for 18–21 h was used as a positive control in C2C12 experiments (Klip and Paquet, 1992; Kumar and Dey, 2002). For 3T3-L1 experiments, fenugreek seed ethanolic extract, a known hypoglycaemic agent (Vats et al., 2002), was used as a positive control at its maximum non-toxic dose of 75 μg/ml (cytotoxicity data not shown).

Insulin secretion assay

The β TC-tet cell line was used to screen extracts of *V. angustifolium* for the potentiation of glucose-stimulated insulin secretion (GSIS). This pancreatic β-cell line releases insulin in response to physiological concentrations of glucose in a dose-dependent manner. Changes in cell secretory properties (basal secretion, GSIS, and a shift in glucose sensitivity) can be detected by measuring insulin secretion released into the medium in response to incremental concentrations of glucose. β TC-tet cells were seeded in 12-well plates at a density of 2.5×10^5 cells/well. Upon reaching 60–80% confluence, growth medium was supplemented with 1 μg/ml tetracycline for 48 h. Growth-arrested cells were then incubated 18 h with or without 12.5 μg/ml of plant extracts in growth medium. Cells were rinsed with and pre-incubated for 1 h at 37 °C in a Krebs–Ringer buffer (10 mM HEPES, 25 mM NaHCO$_3$, 2 mM NaH$_2$PO$_4$, 118 mM NaCl, 5 mM KCl, 2.5 mM CaCl$_2$, 1 mM MgSO$_4$, 0.1% fatty-acid-free BSA, pH 7.4) containing no glucose, in presence or absence of extracts. Insulin secretion was then assessed over a 2 h period in the presence or absence of extracts in buffer containing 0.5 mM IBMX and either 2 mM glucose (basal secretion), 6, 10 or 16 mM glucose (GSIS), or 2 mM glucose plus 50 mM K$^+$ (non-fuel secretagogue) (buffer adjusted to 50 mM KCl and 73 mM NaCl). The insulin released into the medium at the end of the secretion period was determined by radioimmunoassay (RIA), as described below. Three replicates were performed for each experimental condition. Samples of incubation media were centrifuged 3 min at 4 °C at 3000 g to remove any cells, and supernatants were stored at −20 °C until assayed for insulin. Cellular insulin content was measured in cells exposed to 2 mM glucose (basal secretion). Intracellular insulin was extracted overnight at 4 °C in 0.2 mM HCl−75% ethanol. These samples were briefly sonicated and centrifuged at 30,000 g for 5 min before measurement of insulin in the supernatant by RIA. All insulin concentrations were expressed per well of growth-arrested cells. RIA was performed on samples diluted between 100 and 1000 times using a rat insulin RIA kit (# RI-13 K, Linco

Research, St-Charles, MO) according to the manufacturer's instructions.

β cell proliferation assay

To test for a proliferative effect of *V. angustifolium* extracts on β cells, extracts were applied to replicating (non-growth arrested) β TC-tet cells and incorporation of ³H-thymidine was evaluated. Cells were seeded in 24-well plates at a density of 1.0×10^5 cells/well and incubated in growth medium for 24 h. Incubation was continued for another 48 h in growth medium while one group was treated with tetracycline (1 μg/ml) in order to arrest growth. Replicating cells were then incubated 24 h in the presence or absence of extracts. A measure of 1 μCi/ml of methyl ³H-thymidine (#2404105, MP Biomedicals, Irvine, CA) was added to medium over the last 6 h of treatment. Cells were then rinsed three times in PBS and lysed with 0.1 M NaOH for 30 min and scraped. The lysate was added to 1 ml of liquid scintillation cocktail and incorporated radioactivity was measured in a scintillation counter. Four replicates were performed for each experimental condition. Average counts from tetracycline-treated (growth-arrested) wells were considered as non-specific incorporation and were subtracted from all other measures.

Adipocyte differentiation assay

As an indirect screen for glitazone (thiazolidinedione)-like activity in *V. angustifolium* extracts, 3T3-L1 pre-adipocytes differentiating in the presence of extracts were assessed for accelerated differentiation over non-treated cells by measuring the accumulation of triglycerides at the end of the treatment period, as is often done to determine PPAR-γ agonist activity (Harmon and Harp, 2001; Ljung et al., 2002; Norisada et al., 2004; Tontonoz and Spiegelman, 1995). Cells were grown in 24-well plates. One day after attaining confluence, proliferation medium was replaced with differentiation medium containing IBMX, DXM, and insulin, as described above, with either vehicle (DMSO) alone, extract in vehicle, or positive control in vehicle. This medium was changed after 24 h. After 48 h, medium was replaced with differentiation medium containing only insulin, as above, with or without plant extracts or controls. This medium was changed every 24 h. Rosiglitazone (10 μM; Alexis Biochemicals, Hornby, ON), a PPAR-γ agonist of the thiazolidinedione family, was used as a positive control, while vehicle in proliferation medium was used as a negative control. Experiments were terminated after the first visual detection of intracellular lipid droplets by phase-contrast microscopy in vehicle-treated cells, typically by day 5 or 6 of the

incubation period. At this time, micrographs were taken of live cells with a 40 × objective. Intracellular lipids in live cells were then stained with AdipoRed fluorescent reagent (Cambrex Bio Science, Walkersville, MD), a Nile red derivative, as per manufacturer's protocol. Briefly, cells were washed in PBS (8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.68 mM KCl, pH 7.4), then 1 ml of PBS was added to each well followed by 30 μl of reagent. After a 15 min incubation at ambient temperature, fluorescence was measured with a plate reader (Wallac Victor 2, Perkin-Elmer, St-Laurent, QC) with a 485 nm excitation filter and a 572 nm emission filter. Four replicates were performed for each condition. The mean value obtained from the negative control condition was considered as background and subtracted from all other readings.

Protection of PC12-AC cells from glucose toxicity

To test for cytoprotective activities against glucose toxicity, viability assays were performed on PC12-AC cells subjected to chronically elevated glucose in the presence of extracts or vehicle (0.1% DMSO). Cells were seeded in 96-well plates at a density of 6.25×10^3 cells/ well and cultured for 24 h at 37 °C. Complete medium was then replaced with serum-free medium adjusted to 150 mM glucose (approx. 14-fold greater than normal concentration for this cell line) and supplemented with 0.025% BSA, with extracts or vehicle (DMSO) for 96 h. While these conditions feature a supra-physiological concentration of glucose (the concentrations observed in severe unmanaged diabetes can attain levels 7-fold greater than normal), they are necessary to induce apoptosis in approximately 40% of the cells over the 4-day experimental duration and to provide adequate experimental resolution. Under this paradigm, toxicity is due to glucose per se and not osmotic stress since the substitution of L-glucose by D-glucose abolishes toxicity (Koshimura et al., 2002). Viable cell counts were determined by a modified WST-1 viability assay (Cell Proliferation Reagent; Roche, Laval, QC). Ten microlitres of WST-1 tetrazolium salt reagent was added to each well, as per the manufacturer's instructions, and plates allowed to incubate for 75 min before colorimetric analysis of formazan content was made by measurement of absorbance at $\lambda = 420/620$. Number of live cells per well was calculated from absorbance based on experimentally prepared standard curves of known number of PC12-AC cells. High glucose vehicle controls were included on every plate and pooled for statistical analysis. Eight replicates were performed for each experimental condition. Data were expressed as a percentage of live cell number measured in normal glucose conditions.

Statistical analysis

Results were analysed by one-way analysis of variance with Fisher, Bonferonni or Dunnett post hoc test. Comparisons of insulin secretion dose–response curves were performed using AllFit software (André DeLéan, Université de Montréal (DeLean et al., 1978)). Statistical significance was set at $p \leqslant 0.05$. All data are reported as mean ± SEM.

Results

Phytochemical characterization of extracts

HPLC–MS methods were developed to characterize the root, stem, leaf, and fruit crude ethanolic extracts of *V. angustifolium*. Extracts of the four organ parts differed significantly in composition and content of major phytochemical classes (Table 1). Root extract contained significant amounts of vanillic acid, was virtually devoid of flavanols and their glycosides, and exhibited moderate amounts of epicathecins and proanthocyanidins. Stem extract was richest in catechin, epicathecin and proanthocyanidins. Its content in several flavonols and their glycosides was high, and chlorinergic acid was the most abundant phenolic acid present. Leaf extract was very abundant in chlorogenic acid, several flavanols and glycosides, as well as catechin and epicathecin. Finally, fruit extracts contained chlorogenic acid and small amounts of flavanols and glycosides. Fruit extract was the only organ part to contain anthocyanins. Fruit extracts have been reported by others to be rich in total phenolics and anthocyanins (Kalt et al., 1999, 2001; Lyons et al., 2003; Rimando et al., 2004; Schmidt et al., 2004).

Enhancement of deoxyglucose uptake in differentiated C2C12 muscle cells and 3T3-L1 adipocytes

Extracts of *V. angustifolium* were tested for insulin-like or insulin-sensitizing properties by assessing insulin-dependent and -independent glucose uptake in two insulin-responsive and Glut4-containing cell lines: differentiated C2C12 myotubes and differentiated 3T3-L1 adipocytes (Berti and Gammeltoft, 1999; Calderhead et al., 1990; Galante et al., 1995; Rosen et al., 1978; Sarabia et al., 1990). In C2C12 cells, 100 nM of insulin induces approximately a 40% increase in uptake of labelled deoxyglucose. Metformin can be used as a positive control, and overnight incubation with 400 μM metformin induces an approximate 40% increase over basal or insulin-stimulated uptake. In 3T3-L1 cells,

Table 1. Phytochemical characterization of root, stem, leaf, and fruit crude ethanolic extracts of *V. angustifolium*

Compound	Content (μg compound/mg extract) $\pm$ SEM			
	Root	Stem	Leaf	Fruit
Caffeic acid	Not detected	0.39 ± 0.04	1.22 ± 0.06	Not detected
Chlorogenic acid	0.64 ± 0.12	1.04 ± 0.08	104.0 ± 1.9	3.08 ± 0.02
Vanillic acid	0.66 ± 0.23	Trace	Not detected	Not detected
Q-3-galactoside	Not detected	4.95 ± 0.13	6.54 ± 0.15	0.62 ± 0.03
Q-3-glucoside	Not detected	1.45 ± 0.09	11.65 ± 0.27	0.82 ± 0.03
Q-3-arabinoside	Not detected	7.47 ± 0.24	9.15 ± 0.21	Trace
Q-3-rhamnoside	Not detected	0.11 ± 0.014	4.87 ± 0.16	Trace
Quercetin	Not detected	0.13 ± 0.01	3.07 ± 0.12	0.07 ± 0.0003
Catechin	1.02 ± 0.17	13.83 ± 0.52	20.55 ± 1.75	Trace
Epicatechin	14.09 ± 0.85	32.21 ± 1.13	24.15 ± 1.18	Trace
Proanthrocyanidin B1	0.73 ± 0.15	8.99 ± 0.27	Not detected	Not detected
Proanthrocyanidin B2	4.28 ± 0.27	10.84 ± 0.36	Not detected	Not detected
Anthocyanins	Not detected	Not detected	Not detected	Abundant

100 nM of insulin induces approximately a 400% increase in transport.

Overnight incubation of C2C12 cells with 12.5 μg/ml of root, stem, or leaf extracts prior to the deoxyglucose transport assay resulted in significant ($p < 0.05$) enhancement of uptake by 15–25% in the presence or absence of insulin, as compared to vehicle (Fig. 1A). Incubation with fruit extract only enhanced uptake in the presence of insulin. Reducing extract incubation time to 1 h abolished all enhancement of uptake in C2C12 cells (Fig. 1B).

Overnight incubation of 3T3-L1 cells with root or stem extract significantly enhanced transport at 1 nM insulin, but not at 100 nM or in the absence of insulin (Fig. 1C). Reducing incubation time with root or stem extract to 1 h resulted in significantly enhanced transport at 0, 1, and 100 nM insulin (Fig. 1D). Overnight incubation with leaf extract inhibited uptake at all insulin concentrations, while fruit extract had no effect (Fig. 1C). At 1 h, leaf and fruit extracts did not affect uptake (Fig. 1D).

Potentiation of GSIS in β TC-tet pancreatic β cells

In order to test whether extracts of *V. angustifolium* possess insulinotropic activity, tetracycline-treated (growth-arrested) insulin-secreting β TC-tet cells were incubated overnight with or without 12.5 μg/ml of each extract. Insulin secretion was assessed over a 2 h incubation in conditions of basal secretion, glucose-stimulated secretion, or potassium-stimulated secretion, in the continued presence or absence of extract. In this model, insulin secretion increases 2.5-fold above basal levels at 6 mM glucose, 5-fold at 10 mM, and 7-fold at 16 mM in cells incubated with vehicle only (DMSO). The use of growth-arrested cells allows to distinguish

proliferative effects (see below) from insulinotropic effects.

Treatment with leaf extract resulted in a small but statistically significant increase in GSIS (Fig. 2A). While differences in insulin secretion between extract-treated and vehicle-treated cells at each of the four tested glucose concentrations were not significant as analysed by ANOVA, a global comparison of the dose–response curves (modelled as four-parameter sigmoidal curves) using the AllFit method (DeLean et al., 1978) revealed that the curves are significantly different ($F = 11.5$; $p < 0.001$). Furthermore, this global difference can be explained in large part by a combination of differences in ED50 and maximal response of the curves ($F = 5.19$; $p = 0.019$); any changes in minimal response did not contribute significantly to the global difference. Therefore, treatment with leaf extract does not significantly affect basal secretion, but slightly increases GSIS magnitude and sensitivity. The dose–response curves for the stem extract treatment and the vehicle treatment were also significantly different ($F = 3.11$; $p < 0.05$) when analysed globally (Fig. 2B).

Secretion stimulated by potassium-induced depolarization was not altered, indicating normal cellular function (results not shown). Likewise, intracellular insulin content was not significantly affected by the treatment (results not shown).

Increase in proliferation of β TC-tet pancreatic β cells

In order to test whether extracts of *V. angustifolium* can stimulate β cell proliferation, geometrically replicating insulin-secreting β TC-tet cells were incubated overnight with or without 12.5 μg/ml of
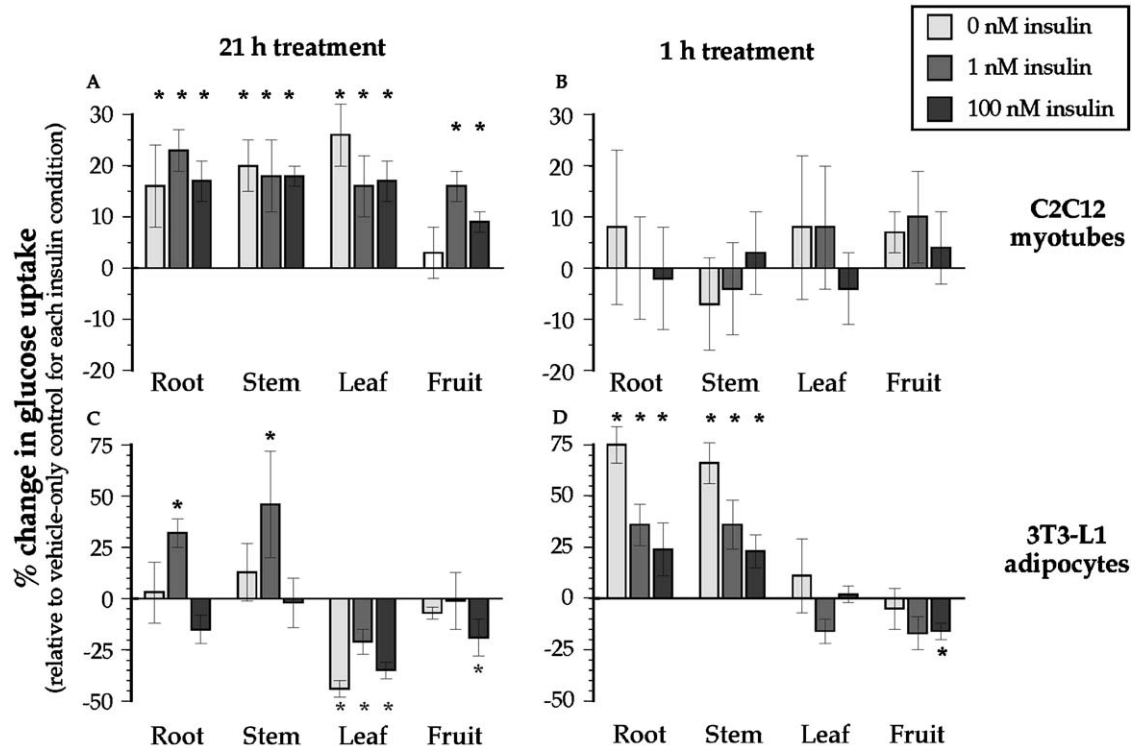
Fig. 1. Insulin-like and insulin-sensitizing effects of *V. angustifolium* extracts were assessed in differentiated and confluent C2C12 skeletal muscle cells and 3T3-L1 adipocytes. These cell lines express insulin-responsive GLUT-4 glucose transporters. Cells were incubated with 12.5 μg/ml of root, stem, leaf, or fruit extracts or with vehicle only (0.1% DMSO) for 18–21 h or for 1 h and were then subjected to a ^{3}H-deoxyglucose uptake assay in the presence of 0, 1, or 100 nM insulin. For each concentration of insulin, uptake is expressed as mean counts of incorporated radioactivity per well normalized by the mean counts measured in cells treated with vehicle only plus that same concentration of insulin. Means$\pm$SEM are presented for 3–6 replicates. * indicates a significant difference from vehicle only ($p \leqslant 0.05$).
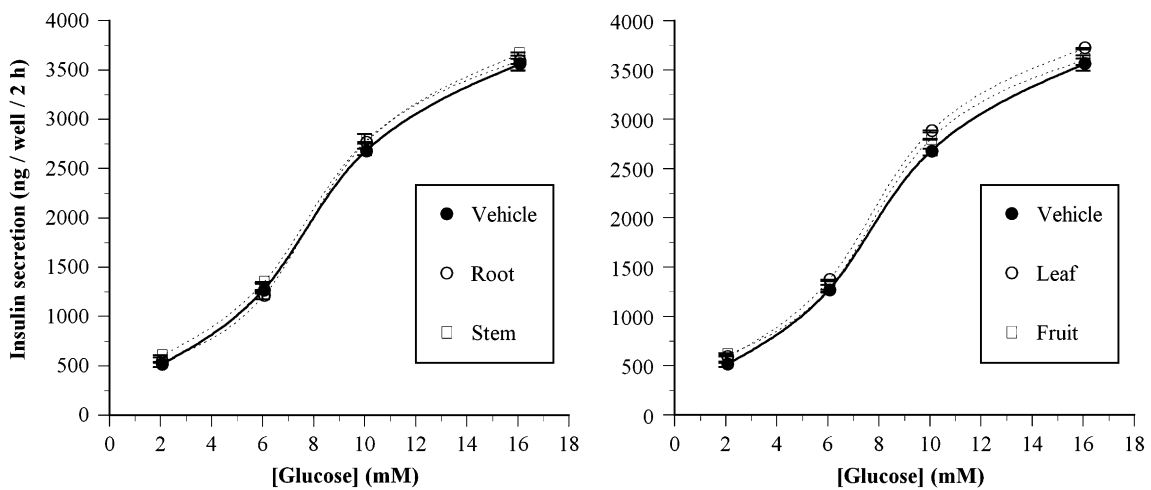
Fig. 2. Effects of *V. angustifolium* extracts on glucose-induced insulin secretion were assessed in growth-arrested β TC-tet pancreatic β cells. Cells were incubated with 12.5 μg/ml of root, stem, leaf, or fruit extracts or with vehicle only (0.1% DMSO) for 18 h. Insulin secretion over a 2 h period in response to 2, 6, 10, or 16 mM glucose was measured by RIA. Leaf extract and stem extract dose–response curves were statistically different from the vehicle dose-response curve. No differences were observed in the secretory response to 50 mM potassium or in intracellular insulin content (not illustrated). Means$\pm$SEM are presented for three replicates. Curves were globally analysed using the AllFit method (DeLean et al., 1978).
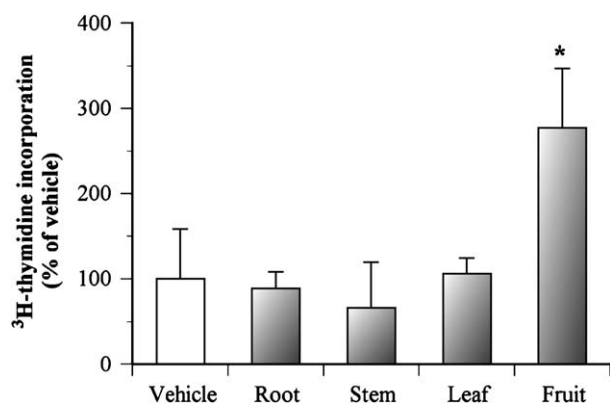
Fig. 3. Proliferative effects of *V. angustifolium* extracts on pancreatic β cells were assessed in replicating β TC-tet cells. Cells were treated with 12.5 μg/ml of root, stem, leaf, or fruit extracts or with vehicle only (0.1% DMSO) for 24 h. A measure of 1 μCi/ml of ^{3}H-thymidine was added for the final 6 h of treatment and incorporated radioactivity was quantified. Non-specific incorporation (incorporation into growth-arrested cells) was subtracted and results are expressed relative to vehicle treatment. Means ± SEM are presented for four replicates. * indicates a significant difference from vehicle only ($p \leqslant 0.05$).

each extract and ^{3}H-thymidine was added to the medium during the final 6 h of the treatment. Treatment with fruit extract was found to increase radioactivity incorporation by 2.8-fold while other extracts had no effect (Fig. 3).

Increase in lipid accumulation in differentiating 3T3-L1 cells

A hallmark of glitazone (thiazolidinedione)-like activity is enhanced adipogenesis mediated through PPARγ receptor activation. The presence of glitazone (thiazolidinedione)-like activity in *V. angustifolium* extracts was indirectly assessed by testing for increased lipid accumulation in differentiating 3T3-L1 pre-adipocytes treated with extracts over a 6-day period. Intracellular triglyceride content was measured using the AdipoRed fluorescent reagent at the end of the treatment period. At this time point, lipid droplets were observable by phase-contrast microscopy techniques in a small percentage of cells exposed to vehicle only while nearly all cells exposed to 10 μM rosiglitazone contained visible lipid droplets. Rosiglitazone treatment resulted in a 6.8-fold enhancement of lipid accumulation, as compared to vehicle only in differentiation medium. Treatment with *V. angustifolium* extracts resulted in 4-fold increases for root and stem extracts and a 6.5-fold increase for leaf extract (Fig. 4B). Treatment with fruit extract did not result in a significant increase.

Protection against glucose toxicity in PC12-AC cells

The ability of extracts of *V. angustifolium* to protect against cell death induced by chronically elevated glucose was tested in PC12-AC cells. In this model, cells are incubated in serum-free medium containing supra-physiological glucose for 96 h, effectively inducing apoptosis in approximately 40% of cells by the end of the treatment period. PC12-AC cells were incubated under these conditions in the presence or absence of 0.5, 2.5, 10, and 25 μg/ml of extracts in 0.1% DMSO and number of viable cells was quantified by WST-1 tetrazolium salt assay. Stem, leaf and fruit extracts were found to increase the number of viable cells by 20–33%, as compared to vehicle in high glucose (Fig. 5). However, root extract had no effect. While leaf extract was found to only confer protection at 10 μg/ml, stem and fruit extracts exhibited a dose–response effect. Finally, while protection induced by fruit extracts was highest at 25 μg/ml, root and stem extracts were found to be slightly cytotoxic at this concentration.

Discussion

V. angustifolium is reputed to possess anti-diabetic properties (Haddad et al., 2003), and in Canada, extracts of this plant are available as natural health products designed for use as a complementary treatment for diabetes. While similar claims have been partially validated in other species of the *Vaccinium* genus (Blumenthal, 1998; Chambers and Camire, 2003), the anti-diabetic potential of *V. angustifolium* has not been subjected to rigorous scientific investigation and only the plant's anti-oxidant properties have been well documented (Costantino et al., 1992; Kalt et al., 2001; Kay and Holub, 2002; Lyons et al., 2003; Rimando et al., 2004; Sweeney et al., 2002; Wang and Jiao, 2000). Using multiple cell-based bioassays, the present study has confirmed that *V. angustifolium* possesses insulin-like and glitazone-like activities as well as cytoprotective activities. Furthermore, *V. angustifolium* exhibits potentially anti-diabetic effects in pancreatic β cells.

An important finding of this work is that extracts of *V. angustifolium* possess considerable insulin-like properties, as evidenced by enhancement of insulin-dependent and -independent glucose uptake in cell-based assays. Further work is required to elucidate the mechanism by which this effect is mediated. However, the tissue-specific nature of the effect, whereby muscle cells are more greatly affected than adipocytes, combined with the observation that there is no saturation at the highest insulin concentration tested, suggest an insulin-independent mechanism such as an involvement of the AMP-activated protein kinase (AMPK) pathway
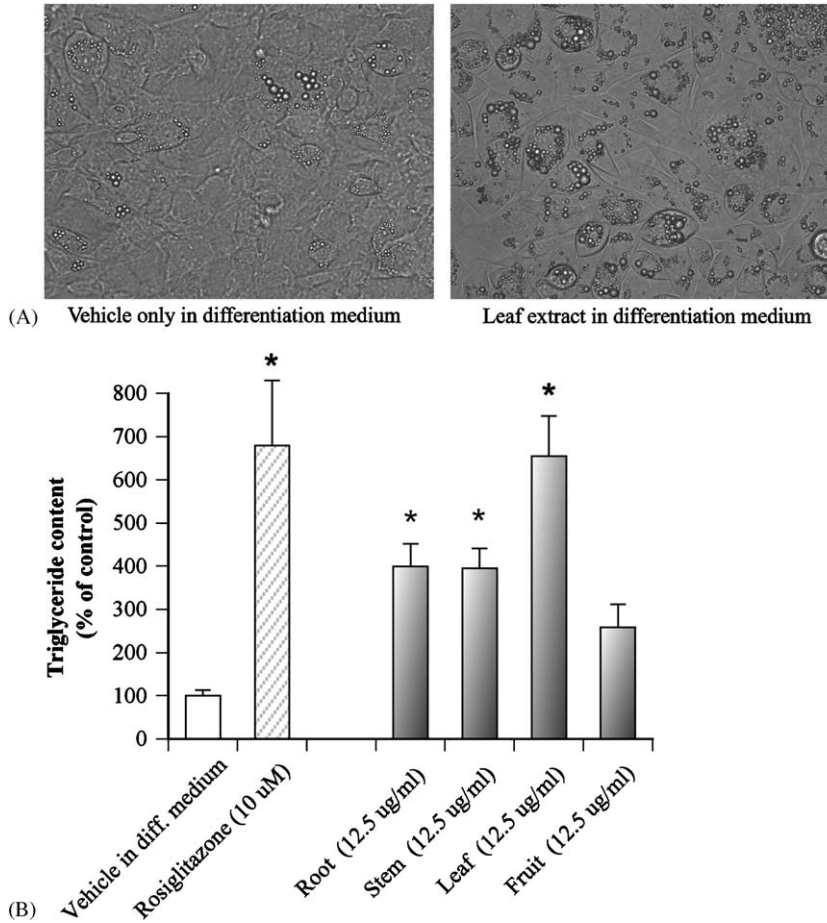
(A)   Vehicle only in differentiation medium   Leaf extract in differentiation medium

(B)

Fig. 4. Lipid accumulation in differentiating 3T3-L1 adipocytes treated for 6 days with 12.5 μg/ml of root, stem, leaf, or fruit extracts or with vehicle only (0.1% DMSO in differentiation medium). Rosiglitazone (10 μM) was used as a positive control. (A) Phase-contrast micrograph of vehicle-treated and leaf-treated cells showing lipid droplets at 400 × . (B) Quantitation of intracellular lipid content in live cells using AdipoRed fluorescent dye. Background (fluorescence in non-differentiated cells treated with proliferation medium only) was subtracted and results expressed relative to the vehicle-treated group. Means±SEM are presented for four replicates. * indicates a significant difference from vehicle only ($p \leqslant 0.05$).
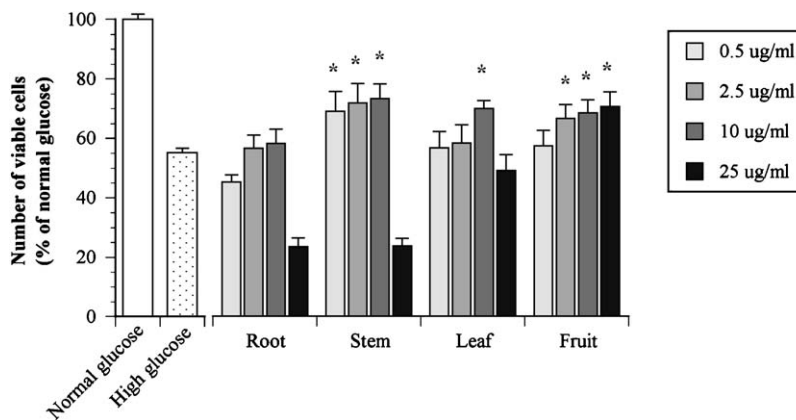
Fig. 5. Cytoprotection against glucose toxicity was measured in PC12-AC cells treated with root, stem, leaf, or fruit extracts or with vehicle only (0.1% DMSO) for 96 h in serum-free medium containing 150 mM glucose. These conditions result in a 40% decrease in number of viable cells in control cells. Number of live cells was quantified by WST-1 viability assay. Results are expressed relative to the normal-glucose vehicle-treated group. Means±SEM are presented for eight replicates. * indicates a significant ($p \leqslant 0.05$) increase over the high-glucose vehicle-treated group.

(Musi and Goodyear, 2003). However, the absence of glucose uptake enhancement in muscle following a 1 h incubation with extracts suggests that regulation may occur at the transcriptional or translational level. Alternatively, cellular uptake of the active compound may require facilitative transport and be subject to the kinetics of such a transport mechanism (Strobel et al., 2005).

In addition, *V. angustifolium* extracts possess important glitazone-like activity, as assessed using a screening assay based on 3T3-L1 adipogenesis. While it remains to be confirmed that such glitazone-like activity is mediated through a mechanism involving PPARγ activation, the results from this adipogenesis assay suggest PPARγ agonism and potential insulin-sensitization as a result of the upregulation of genes coding for key proteins of glucose transport and metabolism. This will require confirmation using a more direct measurement of PPAR activation, such as a reporter gene assay. While it is difficult to correlate results from the adipogenesis assay with results from the glucose-uptake assays due to different time scales, it is possible that PPAR stimulation could account in part for increases in glucose transport observed following 18 h treatment with extracts.

V. angustifolium extracts were found to exert positive effects in pancreatic β cells. The β TC-tet cellular system used here is well suited to distinguish effects on insulin secretion from effects on proliferation. In growth-arrested pancreatic β cells, leaf and stem extracts exerted a subtle anti-diabetic effect on GSIS consisting of both an increase in maximal secretion as well as a leftward shift in the glucose-insulin secretion dose–response curve, without a significant effect on basal secretion. These are highly desirable properties in an anti-diabetic medication, as they minimize the potential for inducing hypoglycaemia, an important concern with sulphonylureas and related insulin secretagogues (Bailey, 1999). While the magnitude of this effect is small, it may prove to be more physiologically relevant when the responsible active compounds are purified. Perhaps more important than the effects of leaf and stem extracts on GSIS is the considerable effect of the fruit extract on proliferation of replicating β cells; in light of the existing evidence of β cell mass plasticity in the adult pancreas (Bouwens and Rooman, 2005), such an effect could represent a valuable anti-diabetic activity potentially capable of retarding β cell decompensation in advanced T2DM.

Complications secondary to chronic hyperglycaemia are numerous and include damage to vascular endothelial cells, kidney podocytes, and neural tissue (Ristow, 2004; Rojas and Morales, 2004; Stas et al., 2004; Wendt et al., 2003). In order to test for anti-diabetic cytoprotection, we chose to use the PC12 cell model and exposed cells to chronically elevated glucose for 4 days. Cell death from hyperglycaemia is thought to be due to oxidative

stress, protein glycation, and other insidious mechanisms. In order to accelerate this process so as to achieve a significant amount of cell death in the 96 h experimental duration, it was necessary to use a supra-physiological concentration of glucose. Nevertheless, *V. angustifolium* extracts exerted a protective effect in PC12 cells under these conditions. While the mechanisms behind these protective effects are unclear, they may occur through a decrease in lipid peroxidation, protein oxidation and/or protein glycation (Kikuchi et al., 2003; Mchugh and Mchugh, 2004), consistent with known anti-oxidant properties of *V. angustifolium* (Costantino et al., 1992; Kalt et al., 2001; Kay and Holub, 2002; Lyons et al., 2003; Rimando et al., 2004; Sweeney et al., 2002; Wang and Jiao, 2000) and of flavonoids in general (Wang, 2000). *V. angustifolium* has previously been reported to confer neuroprotection in an animal model of ischaemic stroke (Sweeney et al., 2002).

The findings of anti-diabetic properties in cell-based bioassays support the ethnobotanical use of *V. angustifolium* in the context of human T2DM. Nonetheless, these promising in vitro results must be ascertained in vivo using animal models of insulin resistance and diabetes. The active molecules and mechanisms of action responsible for *V. angustifolium*'s insulin-like, glitazone-like, cytoprotective, and pancreatic effects remain to be identified. However, the observation that different effects are attributable to extracts from different parts of *V. angustifolium* suggests that *V. angustifolium* contains several active molecules with potential anti-diabetic activity. Since several of these activities are complementary, it may be suitable in the context of diabetes therapy to use the whole plant or a rational combination of various plant parts. Indeed, our results show that significant differences exist in the phytochemical composition of the various parts of the plant. Although it is too early to speculate about active compounds, these differences will guide the future search for the active principles responsible for the various anti-diabetic activities uncovered herein. Our results thus pave the way toward the discovery of novel molecules in various organs of the plant that may contribute to the standardization of preparations of *V. angustifolium* organs or organ mixtures and to the development of novel medications for the treatment of T2DM.

Acknowledgements

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