Selected plant species from the Cree pharmacopoeia of northern Quebec possess anti-diabetic potential

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Abstract: Type II diabetes is a major health problem worldwide. Some populations, such as aboriginal peoples, are particularly at risk for this disease. In the Cree Nation of Quebec, Canada, prevalence in adults is approaching 20%, and the consequences are compounded by low compliance with modern medicine. In 2003, we conducted an ethnobotanical study of Cree medicinal plants used for the treatment of symptoms of diabetes. This served as the basis for a project designed to identify efficacious complementary treatment options more readily accepted by this population. The present study assesses the in vitro anti-diabetic potential of extracts from the 8 most promising plants to emerge from the ethnobotanical study. Cell-based bioassays were employed to screen for (*i*) potentiation of glucose uptake by skeletal muscle cells (C2C12) and adipocytes (3T3-L1); (*ii*) potentiation of glucose-stimulated insulin secretion (GSIS) and insulin production by pancreatic beta cells (INS 832/13); (*iii*) potentiation of triglyceride accumulation in differentiating 3T3-L1 cells; (*iv*) protection against glucose toxicity and glucose deprivation in pre-sympathetic neurons (PC12-AC). Additionally, anti-oxidant activity was measured biochemically by the diphenylpicrylhydrazyl (DPPH) reduction assay. All plant extracts potentiated basal or insulin-stimulated glucose uptake to some degree in muscle cells or adipocytes. Adipocyte differentiation was accelerated by 4 extracts. Five extracts conferred protection in PC12 cells. Three extracts displayed free radical scavenging activity similar to known anti-oxidants. None of the plant extracts enhanced GSIS or insulin content in INS 832/13 beta cells. It is concluded that the Cree pharmacopoeia contains several plants with significant anti-diabetic potential.

Key words: type II diabetes, medicinal plants, cell-based bioassays, insulin action and secretion, insulinomimetic, insulin-sensitizing, cytoprotection, anti-oxidant potential, natural health products.

Résumé : Le diabète de type II est un problème de santé majeur mondial. Certaines populations, comme les populations autochtones, sont particulièrement à risque de contracter cette maladie. Chez les Cris du Québec, au Canada, la prévalence chez les adultes approche 20 % et les effets sont aggravés par une faible acceptation de la médecine moderne. En 2003, nous avons mené une étude ethnobotanique des plantes médicinales utilisées par les Cris pour le traitement des symptômes du diabète. Cette étude a servi de base à un projet visant à identifier des options de traitement complémentaires efficaces plus facilement acceptés par cette population. La présente étude évalue le potentiel antidiabétique in vitro d'extraits des 8

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plantes les plus prometteuses ayant ressorti de l'étude ethnobotanique. Nous avons utilisé des dosages biologiques sur cellules pour détecter : (*i*) la potentialisation de la captatition de glucose par les cellules musculaires squelettiques (C2C12) et les adipocytes (3T3-L1); (*ii*) la potentialisation de la sécrétion d'insuline stimulée par le glucose et la production d'insuline par les cellules bêta pancréatiques (INS 832/13); (*iii*) la potentialisation de l'accumulation des trigylcerides durant la différenciation des cellules 3T3-L1; (*iv*) la protection contre la toxicité du glucose et la carence de glucose dans les neurones pré-sympathiques (PC12-AC). De plus, nous avons analysé biochimiquement l'activité antioxydante par un dosage de réduction du diphénylpicrylhydrazyl (DPPH). Tous les extraits de plantes ont potentialisé dans une certaine mesure la capture de glucose basale ou stimulée par l'insuline dans les cellules musculaires et les adipocytes. Les 4 extraits ont accéléré la différenciation des adipocytes. Cinq extraits ont induit une protection dans les cellules PC12. Trois extraits ont présenté une activité de piégeage des radicaux libres similaire à celle d'antioxydants connus. Aucun extrait n'a augmenté la sécrétion ou la teneur en insuline dans les cellules bêta INS 832/13. Nous concluons que la pharmacopée crie contient plusieurs plantes ayant un important potentiel antidiabétique.

Mots clés : diabète de type II, plantes médicinales, dosages biologiques sur cellules, action et sécrétion insuliniques, insulinomimétique, sensibilisant à l'insuline, cytoprotection, potentiel antioxydant, produits de santé naturels.

[Traduit par la Rédaction]

Introduction

The incidence of type II diabetes (TIID) has reached epidemic proportions worldwide (WHO 2004). Certain populations, including many aboriginal populations, are especially at risk due in part to a genetic predisposition (Neel 1999) and recent changes in their diet and lifestyle (Berkes and Farkas 1978; Hegele 2001). Amongst the First Nations of Canada, the incidence rate of TIID is nearing 20% in adults, or 3 to 5 times higher than in the rest of the Canadian population (Health Canada 2002). The severity of the consequences of this exceptionally high incidence rate is compounded by low compliance with modern pharmaceuticals (Young et al. 2000).

Insulin resistance and pancreatic β-cell dysfunction, both precursors to the loss of glycaemic control and TIID, have complex etiologies affected by lifestyle and genetic factors (Kahn 2003). In unmanaged or advanced TIID, chronic hyperglycaemia is linked to serious and potentially life-threatening complications such as cardiovascular disease, nephropathy, and neuropathy (Bate and Jerums 2003). Current treatment options for the management of TIID include a number of drugs that increase insulin sensitivity or potentiate insulin secretion (Krentz and Bailey 2005), but which have relatively low efficacy and activity (Goldfine 2001).

Many plants used worldwide as part of traditional folk medicine are known to have anti-hyperglycaemic and (or) other anti-diabetic properties (Marles and Farnsworth 1995), and important anti-diabetic compounds have been developed from some of these. For example, guanidine was isolated from *Galega officinalis*, a plant used medicinally in Europe for centuries (Bailey and Turner 1996), and was subsequently developed into metformin, the ubiquitously prescribed insulin-sensitizer. Nonetheless, the majority of plants traditionally used throughout the world to treat symptoms of TIID remain to be explored pharmacologically for anti-diabetic activity.

Like many of the Canadian First Nations, the Cree of Eeyou Istchee (CEI), inhabiting northern Quebec, have experienced a tremendous rise in the incidence of TIID (Kuzmina and Dannenbaum 2004; Légaré 2004). In an effort to provide complementary or alternative anti-diabetic treatment options that would be more culturally acceptable to this population and other aboriginal populations of Canada, we have undertaken a project that consists of (*i*) identifying natural products employed in Cree traditional medicine to treat symptoms of diabetes, (*ii*) screening these products for anti-diabetic activity in vitro and in vivo, and (*iii*) introducing standardized extracts of efficacious anti-diabetic natural products into diabetes intervention programs within this population. We have recently conducted an ethnobotanical survey of natural products used by CEI elders and healers of Mistissini, Quebec, to treat various symptoms of TIID (Leduc et al. 2005). The purpose of the present study was to evaluate the anti-diabetic potential of the most highly ranked plant species identified in that survey using a series of cell-based and biochemical assays.

Materials and methods

Plant materials

Plant species evaluated are listed in Table 1. Plants were harvested in Mistissini, Quebec, Canada (Leduc et al. 2005), as per the instructions of the elders and healers of this community. Plants were identified by a plant taxonomist (Dr. A. Cuerrier) and voucher specimens were deposited in the Marie-Victorin herbarium of the Montreal Botanical Garden, Montreal, Que., Canada. The plants were air dried and transported to the University of Ottawa for extraction. Plants were washed and separated by organ parts. For each plant, the appropriate part to be evaluated (Table 1), as determined by traditional use, was ground using a Wiley Mill with a 2 mm filter (Arthur H. Thomas Co., Swedesboro, N.J.). Ground plant material was extracted twice for 24 h in 10 mL of 80% ethanol per gram dry material on a mechanical shaker and then filtered using Whatman paper. The first and second extracts were combined and dried using a rotary evaporator followed by lyophilization. The seed of *Trigonella foenum-graecum* L. (Fenugreek), for use as a positive control, was purchased from Lone Wolf Herb (Phippen, Sask.) and extracted similarly. All lyophilized extracts were preserved at 4 °C and protected from light.

Table 1. List of plant species investigated and their respective concentrations tested in each cell line or assay.

Species	Species abbreviation	Family	Plant part	C2C12/ 3T3-L1 (μg/mL)	INS 832/13 (μg/mL)	Glucose toxicity (μg/mL)	Glucose deprivation (μg/mL)
Abies balsamea (L.) Mill.	*A. balsamea*	Pinaceae	Inner bark	50	25	10	10
Alnus incana subsp. rugosa (Du Roi) R.T. Clausen	*A. incana*	Betulaceae	Inner bark	50	10	10	10
Larix laricina K. Koch	*L. laricina*	Pinaceae	Inner bark	25	25	10	10
Picea mariana BSP	*P. mariana*	Pinaceae	Cones	10	10	1	10
Pinus banksiana Lamb.	*P. banksiana*	Pinaceae	Cones	15	1.5	10	10
Rhododendron groenlandicum (Oeder) Kron & Judd	*R. groenlandicum*	Ericaceae	Leaves	75	75	10	10
Sarracenia purpurea L.	*S. purpurea*	Sarraceniaceae	Whole plant	100	200	30	30
Sorbus decora C.K. Schneid.	*S. decora*	Rosaceae	Inner bark	15	7.5	1	1

Assessment of total phenolics

Preliminary phytochemical analyses were conducted on the 8 plant extracts. Total phenolic content was determined using the Folin–Ciocalteau method (Singleton and Rossi 1965) with modifications made to reduce volumes. Briefly, individual extracts were added to freshly diluted Folin–Ciocalteau reagent (BDH, Toronto, Ont.) and allowed to equilibrate for 5 min before adding a 7.5% anhydrous $NaHCO_3$ solution. After 2 h of incubation at room temperature (RT), the absorbance of the mixture was measured at 725 nm. Eighty percent ethanol was used as a blank and quercetin as a standard. Results are expressed as quercetin equivalents (μg) per mg of extract. All tests were conducted in triplicate.

High-performance liquid chromatography (HPLC) analyses

All analyses were performed using a Hewlett-Packard Chemstation series 1100 chromatograph (Agilent, Palo Alto, Calif.) with a photodiode array detector and an atmospheric pressure chemical ionization quadrupole mass spectrometer (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 changed to 13:87 in 2 min, to 30:70 in 14 min, to 60:40 in 3 min, then to 100:0 in 2 min, then to isocratic elution with 100:0 for 2 min, finally returning to the initial conditions in 2 min, which was 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 diode-array detector (DAD). Spectral scans from 190 nm to 600 nm were made throughout the elution of each peak detected.

MS detection was performed in both positive and negative ionization modes. For the positive mode, the optimized conditions were as follows: APCI conducted at 300 °C with the vaporizer at 400 °C; nebulizer pressure, 40 psig; nitrogen (drying gas) flow rate, 6.0 L/min; fragmentation voltage, 20 V; capillary voltage, 3000 V; corona current, 3.0 μA. For the negative mode, the optimized conditions were as follows: APCI conducted at 350 °C with the vaporizer at 400 °C; nebulizer pressure, 60 psig; nitrogen flow rate, 6.0 L/min; fragmentation voltage, 160 V; capillary voltage, 3000 V; corona current, 15.0 μA. The MS data were collected in scan mode for ions from 100 to 800 mass units.

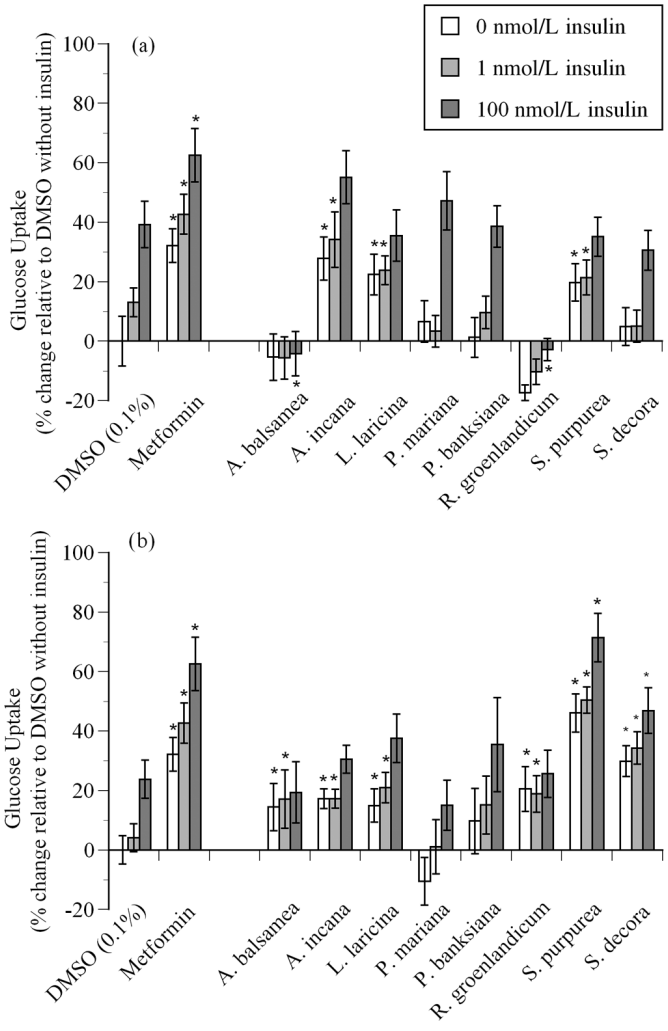
Metabolomics-based analyses

Over 200 purified phenolic compounds were injected into the HPLC system and the ultraviolet (UV) absorbance spectra were scanned and saved into a searchable library. Peaks from the Cree plant extracts were screened against this library and similarities in the absorbance spectra greater than 95%, as determined by Chemstation software (Agilent), were considered a match. Matches were further corroborated by a visual inspection of the spectral match, a retention time match, and a match of either a major ion or a major ion fragment in the unknown peak's mass spectrum corresponding to the mass spectrum of the library entry molecule or its fragments. Co-chromatography of the matching standard compounds and the plant extract was then performed to confirm any matches in the extract.

Cell culture

Cell culture media were purchased from Invitrogen Life Technologies (Burlington, Ont.) unless otherwise noted. Other reagents were purchased from Sigma–Aldrich (Oakville, Ont.) unless otherwise noted. All cells were cultured at 37 °C in a humidified 5% CO_2 : 95% air atmosphere. C2C12 murine skeletal myoblasts and 3T3-L1 murine preadipocytes were obtained from the American Type Cell Collection (ATCC; Chicago, Ill.). C2C12 myoblasts were cultured in high glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), and penicillin–streptomycin antibiotics, until 80% confluent. Myoblasts were then differentiated into myotubes over 6 d in DMEM containing 2% HS, resulting in 100% multinucleated cells by the end of this period. 3T3-L1 cells were cultured in DMEM containing 10% FBS and antibiotics. Upon 80% confluence, differentiation was initiated by adding 250 μmol/L 3-isobutylmethylxanthine (IBMX), 1 μmol/L dexamethasone (DMX), and 670 nmol/L insulin to this medium for 2 d. Differentiation was then continued in DMEM containing 10% FBS and 670 nmol/L insulin for approx. 10 d until more than 90% of the cells contained lipid droplets visible under

Fig. 1. Insulin-dependent and -independent ^{3}H-deoxyglucose uptake in differentiated C2C12 muscle cells treated for (*a*) 1 h and (*b*) 18–21 h with 0.1% DMSO, 400 μmol/L metformin, or plant extracts. Results are expressed as mean % of vehicle treated without insulin ± SE (n = 4–7) and metformin (n = 9). *$p \leq 0.05$ compared with the respective DMSO condition.



low power magnification. For adipogenesis assays, differentiation was initiated one day after reaching confluence and cells were differentiated for a total of 6 d. INS832/13 cells (Hohmeier et al. 2000), kindly provided by C.B. Newgard (Duke University), are a clonal derivative of the INS rat insulinoma cell line (Asfari et al. 1992) that exhibit superior glucose-stimulated insulin secretion (GSIS). INS832/13 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with 11 mmol/L glucose and supplemented with 10% FBS, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 μmol/L beta-mercaptoethanol, and antibiotics, until 80% confluence. PC12-AC cells are a clonal derivative of the PC12 pheochromocytoma cell line (ATCC; Chicago, Ill.) and can be differentiated into a sympathetic neuronal phenotype with exposure to nerve growth factor. PC12-AC cells were cultured in RPMI 1640 medium containing 11 mmol/L glucose, 10% HS, 5% newborn calf serum, and antibiotics until 80% confluent.

Determination of plant extract concentrations for bioassays

Plant extracts were solubilized in dimethyl sulfoxide (DMSO) for application to cell cultures; final DMSO concentration was 0.1%, unless otherwise stated. Aliquots of plant extracts were stored at –20 °C. Plant extracts were used at a maximal non-toxic concentration (Table 1). For C2C12 and 3T3-L1 cells, this was determined by the absence of morphological changes and by Trypan Blue dye exclusion after overnight incubation with extracts (results not shown). For INS832/13, this was determined by the absence of caspase-3/7 activity measured using the Caspase-Glo 3/7 luminescent-based assay (Promega, Madison, Wis.) after 18 h incubation with various concentrations of plant extracts in complete RPMI with 3 mmol/L glucose (results not shown). Alternatively, the PC12 cytoprotection assays were conducted using an extract concentration range from 1 to 100 μg/mL, and the dose conferring maximum cell viability was selected. The diphenylpicrylhydrazyl (DPPH) assays were performed using an extract concentration range from 1 to 75 ppm (described below).

Glucose-uptake 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 plant extracts. Both of these cell types exhibit insulin-regulated glucose uptake and possess GLUT-1 and GLUT-4 glucose transporters (Berti and Gammeltoft 1999; Calderhead et al. 1990). Extracts were tested at maximal non-cytotoxic concentration (Table 1), as described above. Confluent and differentiated cells, grown in 12 well plates, were incubated with vehicle (DMSO) alone, extract in vehicle, or positive control in vehicle for either 1 h (short-term incubation) or 18–21 h (long-term incubation) prior to assay. To control for inter-plate variability, replicates were performed on separate plates, and each plate also contained vehicle. Replicates were also from at least 2 separate experiments. For short-term experiments, differentiation medium was replaced with serum-free medium 1 h before the start of the incubation period, and the incubation was performed in serum-free medium. For long-term experiments, the last 3 h of incubation were performed in serum-free medium. Following the incubation period, cells were rinsed twice with Krebs-phosphate buffer (20 mmol/L HEPES, 4.05 mmol/L Na_2HPO_4, 0.95 mmol/L NaH_2PO_4, pH 7.4, 136 mmol/L NaCl, 4.7 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 5 mmol/L glucose, 0.5% BSA) at 37 °C. Cells were then treated with 0, 1, or 100 nmol/L 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. Cells were then treated with 0.5 μCi/mL 2-deoxy-D-[1-^{3}H]-glucose (TRK-383, Amersham Biosciences, Baie d'Urfé, Que.) in this buffer for exactly 10 min at 37 °C without extracts. Cells were then placed on ice and immediately washed 3 times with ice-cold Krebs-phosphate buffer. Cells were then lysed with 0.1 mol/L NaOH for 30 min and scraped. The lysate was added to 1 mL of liquid scintillation cocktail (Ready-Gel 586601; Beckman Coulter Inc., Fullerton, Calif.) and incor-

Table 2. Total phenolics and identified marker compounds of each plant extract.

Plant	Yield (%)[a]	Total phenolics (μg/mg)[b]	Identified phenolic marker compounds
A. balsamea	15.3	97.6	p-Coumaric acid, gallocatechin
A. incana	26.1	305.9	Catechins
L. laricina	23.8	208.0	Taxifolin, hydroxystilbenes
P. mariana	21.0	163.7	p-Coumaric acid, hydroxystilbenes
P. banksiana	9.0	318.0	Taxifolin, catechin, procyanidins
R. groenlandicum	31.0	188.5	Chlorogenic acid, catechins, procyanidins, quercetin glycosides
S. purpurea	25.2	85.4	Taxifolin, flavonol glycosides (quercetin, kaempferol, myricitin)
S. decora	8.9	59.6	Quercetin and quercetin glycosides

[a]Yield is expressed as (mass of recovered extract / mass dry plant material) × 100%.
[b]Total phenolics expressed as quercetin equivalents (μg) / mg extract.

porated radioactivity was measured in a scintillation counter. Metformin (400 μmol/L) applied for 18 to 21 h was used as a positive control in C2C12 experiments (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.

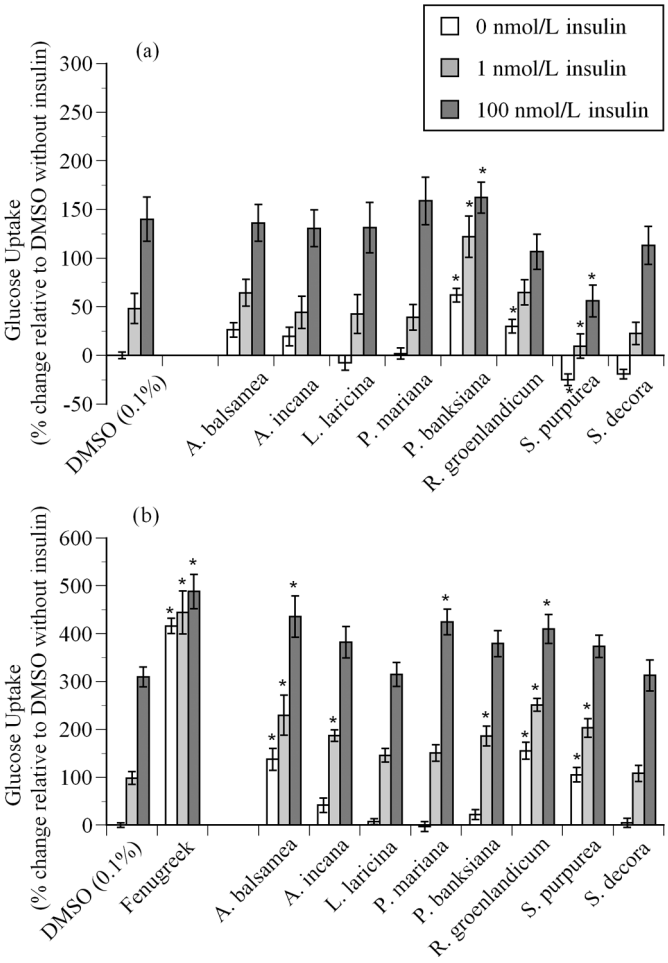
Insulin-secretion assay

Since several phytochemicals are known secretagogues (Hii and Howell 1985), the INS832/13 rat insulinoma cell line was used to screen the plant extracts for potentiation of GSIS and to investigate their effects on insulin content. The INS832/13 pancreatic β-cell line releases insulin in response to physiological concentrations of glucose. Changes in cell-secretory properties (basal secretion, GSIS, as well as shifts in glucose sensitivity) can be detected by measuring insulin secretion released into the medium in response to incremental concentrations of glucose. Extracts were tested at maximal non-cytotoxic concentrations (Table 1). Cells were seeded at a density of 1.5×10^5 cells per well in 12 well plates and incubated at 37 °C for 24 h in complete RPMI with 11 mmol/L glucose. Cells were then incubated for 18 h in a medium containing either vehicle (DMSO) or extract as well as glucose that was adjusted to 3 mmol/L to confer glucose sensitivity to the cells. The final concentration of DMSO was 0.1% for all extracts except for *S. purpurea*. Following the 18 h pre-treatment, cells were rinsed with Krebs-Ringer buffer (10 mmol/L HEPES, 0.5 mmol/L NaH_2PO_4 (pH 7.4), 135 mmol/L NaCl, 3.6 mmol/L KCl, 2 mmol/L $NaHCO_3$, 1.5 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, and 0.07% fatty-acid-free BSA) containing 2 mmol/L glucose and incubated in this buffer for 1 h at 37 °C in the presence or absence of extracts. Then, insulin secretion was assessed over a 1 h period in the presence or absence of extracts in Krebs-Ringer containing either 2 mmol/L glucose (basal secretion), 4 or 11 mmol/L glucose (GSIS), or 2 mmol/L glucose and 30 mmol/L KCl (non-fuel secretagogue). The insulin released into the medium at the end of the secretion period was determined by radioimmunoassay (RIA), as described below. Four replicates from 2 separate experiments were performed for each experimental condition. Performance of the secretion assay is routinely tested with known secretagogues (incretins, phorbol 12-myristate 13-acetate (PMA)). Samples of incubation media were centrifuged at 3000*g* for 3 min at 4 °C to remove any cells, and supernatants were stored at –20 °C until assayed for insulin. Insulin measurements were normalized to the total protein content of each well, as assessed by the BCA protein assay (Pierce, Rockford, Ill.). Cellular insulin content was measured in cells exposed to 2 mmol/L glucose (basal secretion). Intracellular insulin was extracted in a 0.2 mmol/L HCl – 75% ethanol mixture, and samples were kept overnight at 4 °C. These samples were briefly sonicated and centrifuged at 30 000*g* for 5 min before measurement of insulin in the supernatant by RIA. RIA was performed as previously described (Roduit et al. 2004). Briefly, samples were placed on ice and appropriate dilutions were prepared for analysis of basal and stimulated insulin secretion and of cellular insulin content in a phosphate buffer (50 mmol/L Na_2HPO_4 (pH 7.5), 25 mmol/L EDTA, 1% BSA RIA-grade, 0.01% thimerosal). Diluted samples were incubated in 12 mm × 75 mm polypropylene RIA tubes with rat ^{125}I-insulin (Linco Research, St-Charles, Mo.) and a primary antibody against rat insulin (Linco, No. 1013). The tubes were incubated in the dark at 4 °C overnight before the secondary antibody (Linco, No. 2020) was added and then incubated for 2 h at 4 °C. Tubes were then centrifuged at 5350*g* for 15 min and the radioactivity in the pellet was measured. Human insulin was used as a standard.

Adipocyte differentiation assay

Thiazolidinediones, commonly known as glitazones, are a class of anti-diabetic drugs that act by binding to peroxisome proliferator-activated receptor gamma (PPARγ). In differentiating 3T3-L1 adipocytes, treatment with PPAR agonists confers accelerated acquisition of insulin-responsiveness and earlier formation of intracellular lipid droplets. To screen for glitazone-like activity, 3T3-L1 pre-adipocytes differentiating in the presence of plant extracts were assessed for accelerated differentiation as measured by enhanced accumulation of triglycerides (Norisada et al. 2004; Tontonoz et al. 1995). One day post-confluence cells grown in 24 well plates were switched from the proliferation medium to the differentiation medium (containing IBMX, DXM, and insulin, as described above) with vehicle (DMSO) alone, extract in vehicle, or positive control in vehicle. This medium was changed after 24 h. After 48 h, the medium

Fig. 2. Insulin-dependent and -independent ^{3}H-deoxyglucose uptake in differentiated 3T3-L1 adipocytes treated for (*a*) 1 h and (*b*) 18–21 h with 0.1% DMSO, 75 μg/mL fenugreek seed extract, or plant extracts. Results are expressed as mean % of vehicle treated without insulin ± SE (n = 6–8). *$p \leq 0.05$ compared with the respective DMSO condition.



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 μmol/L; Alexis Biochemicals, Hornby, Ont.) was used as a positive control, whereas 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 around day 5 or 6 of the incubation period. At this time, micrographs were taken of live cells. Intracellular lipids 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 phosphate buffered saline (PBS; 8.1 mmol/L Na_2HPO_4, 1.47 mmol/L KH_2PO_4 (pH 7.4), 137 mmol/L NaCl, and 2.68 mmol/L KCl), then 2 mL of PBS were added to each well followed by 2 × 30 μL of reagent. After 15 min incubation at room temperature, fluorescence was measured with a plate reader (Wallac Victor 2, Perkin-Elmer, St-Laurent, Que.) 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 the background and subtracted from all other readings.

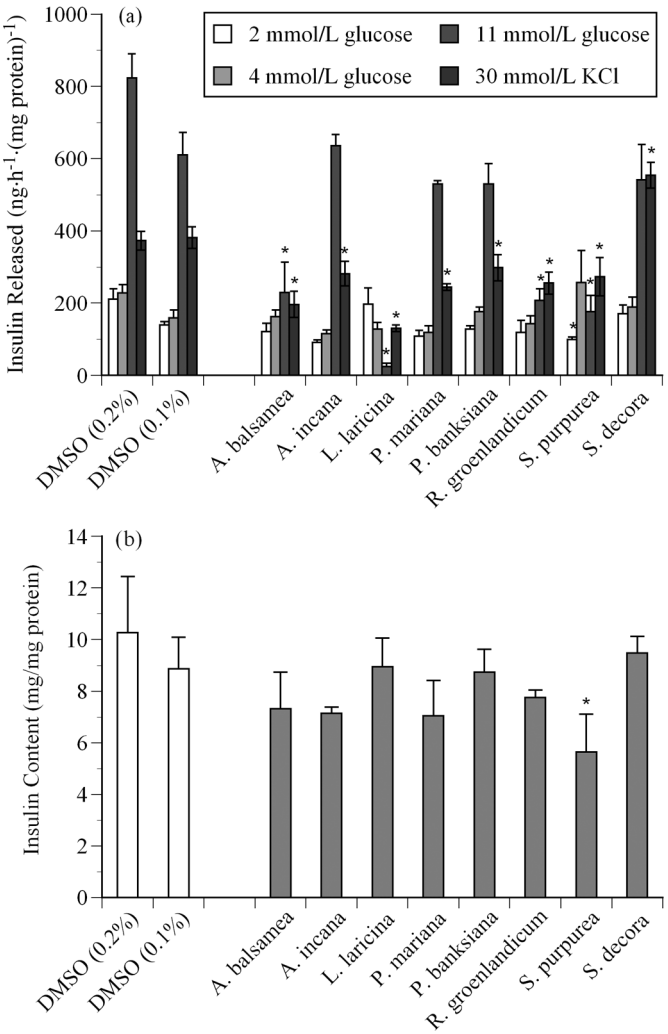
Protection of PC12-AC cells from glucose toxicity and glucose deprivation

To test for cytoprotective activities against glucose toxicity or glucose deprivation, viability assays were performed on PC12-AC cells subjected to 96 h of elevated or reduced glucose conditions 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 1.1 mmol/L glucose (low glucose) or serum-free medium adjusted to 150 mmol/L glucose (high glucose) supplemented with 0.025% BSA and 0.1% DMSO, with extracts or vehicle (DMSO). Under this paradigm, toxicity resulting from high glucose conditions is not the result of osmotic stress but is due to glucose per se, since the substitution of D-glucose for L-glucose abolishes toxicity (Koshimura et al. 2002). Viable cell counts were determined by a modified WST-1 viability assay (Cell Proliferation Reagent; Roche, Laval, Que.). Ten microlitres of WST-1 tetrazolium salt reagent was added to each well, as per the manufacturer's instructions, and plates were allowed to incubate for 75 min before colorimetric analysis of formazan content was made by measuring the absorbance at λ = 420/620. The number of live cells per well was calculated from absorbance based on experimentally prepared standard curves of known numbers of PC12 cells. Low glucose and high glucose vehicle controls were included on every plate and pooled for statistical analysis. Each condition was conducted with a minimum of 8 replicates from 2 separate experiments. Data were expressed as a percentage of live cell number measured in either high or low glucose control conditions.

Evaluation of anti-oxidant activity using the DPPH assay

The anti-oxidant activity of the plant extracts was evaluated by quantifying the reduction of the stable free-radical DPPH (Cotelle et al. 1996; McCune and Johns 2002). DPPH was purchased from TCI America (Portland, Ore.). Ascorbic acid was employed as the reference anti-oxidant, and quercetin, catechin, and epicatechin were employed as additional standards. Sample concentrations were measured in ppm and calculated as follows: ppm = (g sample / L ethanol × (1/0.789 g/mL)) × 1000. A standard curve for ascorbic acid was obtained using 18 concentrations between 0–100 ppm. Each plant extract was tested at 0, 5, 10, 25, 50, and 75 ppm, while standards were tested at 0, 5, 10, 25, 50, 75, and 100 ppm. Five hundred microlitres of sample was combined with 3 mL of 100 μmol/L DPPH in 100% ethanol. Test tubes were vortexed and the reaction was allowed to proceed for 10 min. The samples were transferred to cuvettes and absorbance was measured at 517 nm. Ethanol was used as the blank. For each sample, the concentration required to achieve the IC_{50} of ascorbic acid was extrapolated from the experimental data points. All treatments were carried out in 3 to 6 replicates.

Fig. 3. (*a*) Insulin secretion and (*b*) total insulin content in INS832/13 cells exposed to plant extracts. Cells were exposed to extracts or vehicle (DMSO) for 18 h in complete Roswell Park Memorial Institute (RPMI) 1640 medium with 3 mmol/L glucose. Secretion assays were performed in Krebs-Ringer buffer containing 2, 4, or 11 mmol/L glucose or 2 mmol/L glucose and 30 mmol/L KCl in the presence and absence of extracts. The final DMSO concentration was 0.1% with one exception; *Sarracenia purpurea* was tested at 0.2% DMSO to allow for its maximal nontoxic dose. Nevertheless, 0.2% DMSO controls are presented and were identical to 0.1% DMSO. Results are expressed as the mean ± SE (n = 3). *$p \leq 0.05$ compared with respective DMSO controls.



Statistical analysis

Data were analyzed by one-way analysis of variance with the appropriate post-hoc test using StatView software (SAS Institute Inc., Cary, N.C.). Statistical significance was set at $p \leq 0.05$. Results are reported as means ± SE.

Results

Total phenolic content and phytochemical markers

Total phenolic content of the various plant extracts, as assessed by the Folin–Ciocalteau method, varied substantially between plant species. Two extracts, *Pinus banksiana* and *Alnus incana*, contained over 30% phenolics by weight while *Abies balsamea*, *S. purpurea* and *Sorbus decora* all contained less than 10% phenolics by weight (Table 2). To further characterize the extracts phytochemically, a metabolomics-based approach was employed to identify some marker compounds (Table 2). A comprehensive phytochemical analysis of CEI medicinal plants is in preparation.

Glucose transport in insulin-responsive cell lines

All plant extracts were tested for insulinomimetic or insulin-sensitizing properties by assessing insulin-independent and -dependent glucose uptake in 2 insulin-responsive and GLUT-4-containing cell lines: differentiated C2C12 myotubes and differentiated 3T3-L1 adipocytes (Berti and Gammeltoft 1999; Calderhead et al. 1990). Cells were exposed to maximal non-toxic doses of extracts for 1 h (Figs. 1*a* and 2*a*) or 18–21 h (Figs. 1*b* and 2*b*) immediately prior to measurement of glucose uptake. All of the extracts tested significantly increased either basal or insulin-stimulated uptake in at least one of the cell lines and after at least one extract exposure duration (Figs. 1 and 2; Table 3). Basal uptake was increased by up to 47% in muscle cells and by up to 155% in adipocytes as compared with vehicle alone, whereas insulin-stimulated uptake was increased by up to 46% in muscle cells and up to 153% in adipocytes as compared with vehicle plus 1 nmol/L insulin. With the exception of *Picea mariana*, all extracts affected basal glucose uptake thereby exhibiting potential insulinomimetic activity. In most of these cases, insulin-stimulated uptake was not increased as much as basal uptake and exhibited a tendency towards saturation at the highest insulin concentration tested, much like the effect of metformin on muscle cells. Both muscle-specific (*Larix laricina*) and adipocyte-specific effects (*P. banksiana*) were observed. Effects were greater after 18–21 h of exposure to the extracts than after 1 h of exposure, with the exception of *A. incana* in C2C12, *L. laricina*, in C2C12 and *P. banksiana* in 3T3-L1. Also, after 18–21 h of treatment of C2C12 myotubes with *S. purpurea* and *S. decora* the response was as high as or greater than that of cells treated with 400 μmol/L metformin (Fig. 1*b*).

Effect of extracts on GSIS and total insulin content in INS832/13 cells

Basal and GSIS as well as total insulin content were measured in INS832/13 cells after treatment with extracts or vehicle. None of the 8 extracts tested potentiated basal or GSIS (Fig. 3*a*; Table 3) or increased intracellular insulin content (Fig. 3*b*). These results suggest that extracts do not exhibit an anti-diabetic effect at the level of the β cell.

Lipid accumulation in diffentiating 3T3-L1 adipocytes

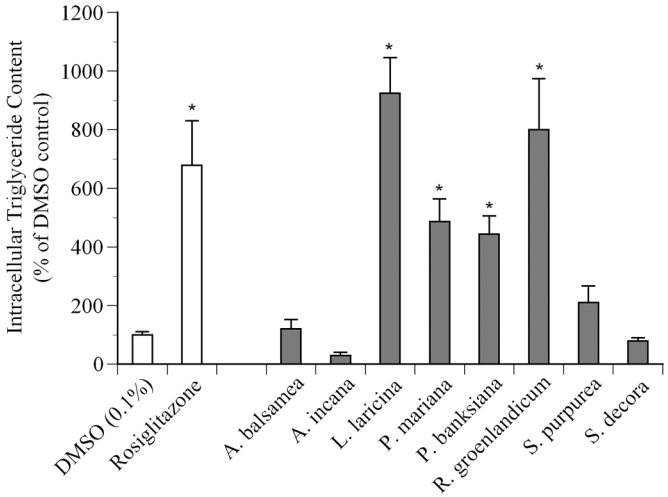
The presence of glitazone-like activity was assessed by testing for increased lipid accumulation in differentiating 3T3-L1 pre-adipocytes exposed to plant extracts. Intracellular triglyceride content was measured by the AdipoRed fluorescent reagent in cells differentiated for 6 d. At this time point, lipid droplets were observed in a small percent of cells exposed to vehicle only while nearly all cells exposed to 10 μmol/L rosiglitazone contained visible lipid droplets.

Table 3. Summary of the anti-diabetic properties of 8 medicinal plants.

Plant species	Insulinotropic effects	Glucose uptake: Muscle cells: No insulin	Glucose uptake: Muscle cells: 1 h	Glucose uptake: Muscle cells: 18–21 h	Glucose uptake: Adipocytes: No insulin	Glucose uptake: Adipocytes: 1 h	Glucose uptake: Adipocytes: 18–21 h	Adipogenesis	PC12 cytoprotection: High glucose	PC12 cytoprotection: Low glucose	Anti-oxidant ranking
A. balsamea		+		+	+		+				5
A. incana		+	+	+			+			+	3
L. laricina		+	+	+				+			6
P. mariana							+	+	+		2
P. banksiana					+	+	+	+		+	1
R. groenlandicum		+		+	+	+	+	+			4
S. purpurea		+	+	+	+		+		+	+	7
S. decora		+		+					+		8

Note: +, positive result.

Fig. 4. Intracellular triglyceride content, measured by AdipoRed fluorescence, in live 3T3-L1 adipocytes incubated for 6 d in the presence of DMSO, plant extracts, or 10 μmol/L rosiglitazone (positive control). Results are relative to DMSO and expressed as the mean ± SE (n = 4). *$p \leq 0.05$ with respect to the DMSO control.



Four of the extracts, *L. laricina*, *P. mariana*, *P. banksiana*, and *R. groenlandicum*, significantly increased triglyceride content between 4–8 fold, as compared with vehicle only (Fig. 4; Table 3). This compared favourably with the positive control rosiglitazone.

Protection of PC12 cells against glucose toxicity or partial glucose starvation

The number of viable PC12 cells was measured after 96 h exposure to extracts and elevated (150 mmol/L) or reduced (1.1 mmol/L) glucose (Fig. 5; Table 3). Consistent with previous studies, cell death was observed in 67% of control cells in the low glucose and 40% of control cells in the high glucose after a 96 h exposure. *Picea mariana*, *S. purpurea*, and *S. decora* were found to significantly protect cells from glucose toxicity. *Alnus incana*, *P. banksiana*, and *S. purpurea* protected cells from glucose deprivation. Only 1 extract, *S. purpurea*, protected cells under both cytotoxic conditions. *Picea mariana*, *S. purpurea*, and *S. decora* extracts conferred complete protection under high glucose conditions, but none of the plants were able to fully protect cells under low glucose conditions.

DPPH assay of free radical scavenging activity

The DPPH reduction assay, with ascorbic acid as the reference anti-oxidant, was employed to quantify the free radical scavenging activity of the extracts. Three other known polyphenolic anti-oxidants, quercetin, catechin, and epicatechin, were included as standards. All extracts had significantly less scavenging activity than ascorbic acid. However, *P. banksiana*, *P. mariana*, and *A. incana* extracts showed elevated free radical scavenging activity as their effect approached that of the polyphenolic anti-oxidant standards (Fig. 6*a*; Table 3). In addition, there was a significant correlation between the total phenolics content and the free radical scavenging activity (Fig. 6*b*).

Fig. 5. Viability of PC12 cells treated with extracts in media containing (*a*) 150 mmol/L glucose and (*b*) 1.1 mmol/L glucose for 96 h. Viability was assessed by the WST-1 assay. Results are expressed relative to high- and low-glucose media, respectively, containing vehicle (DMSO). Results are expressed as the mean ± SE (n = 8–23). *$p \leq 0.05$ compared with viable cell number in the high- or low-glucose condition.

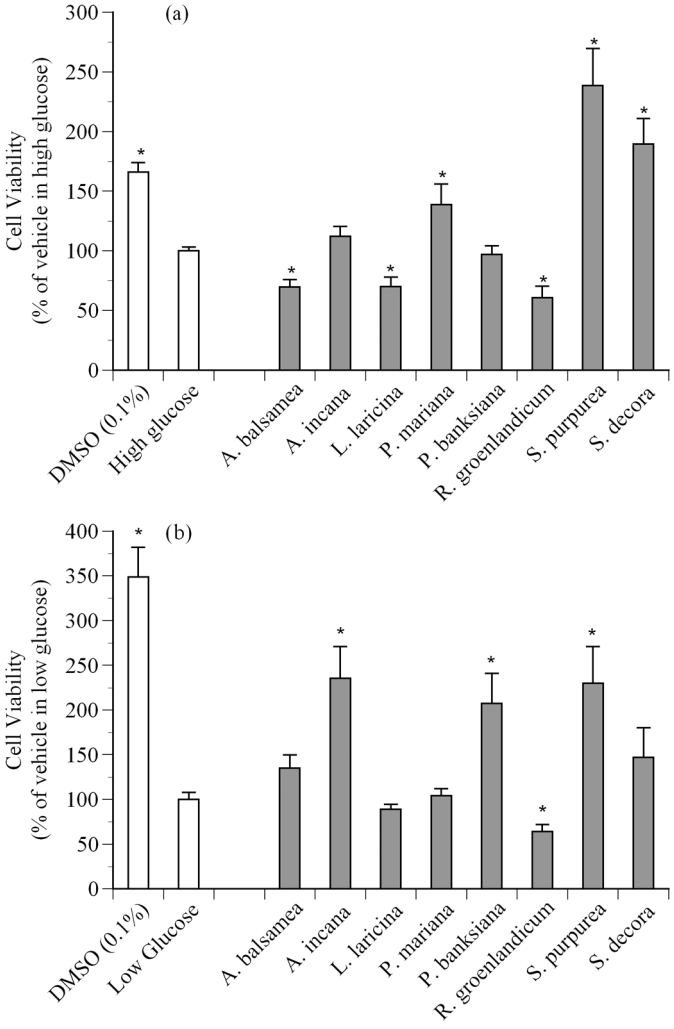
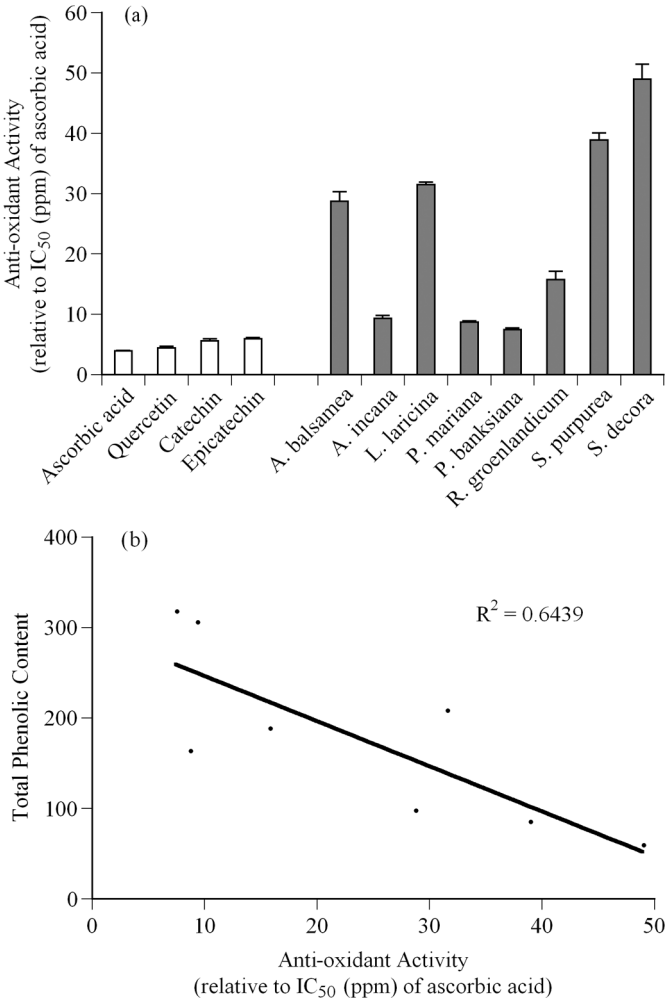


Fig. 6. (*a*) Anti-oxidant activity determined by the reduction of DPPH with ascorbic acid as the reference anti-oxidant and (*b*) correlation between anti-oxidant activity and total phenolic content. Quercetin, catechin, and epicatechin are polyphenolic anti-oxidant standards. Results are expressed as the mean ± SE (n = 3–6).



Discussion

Several plant species belonging to the rich pharmacopoeia of the CEI have traditionally been used to treat various symptoms associated with diabetes (Leduc et al. 2005). While TIID is a relatively new health problem to emerge in the Cree population, these medicinal plants may nevertheless possess significant anti-diabetic activity. The purpose of the present study was to assess the anti-diabetic potential of 8 plants ranked highly in terms of their frequency of citation by Cree healers and elders and the number and importance of symptoms for which they were cited (Leduc et al. 2005). This study is part of a collaborative project with the CEI that aims to identify culturally adapted complementary treatments for diabetes, in light of a report on adherence to medicines in the CEI community of Mistissini, which concluded that cultural sensitivity is essential to the efficacy of prescribed treatments (Rideout and Menzies 1994). Consequently, a number of plant species were identified that possess significant anti-diabetic potential; these warrant additional research and may be useful if integrated into eventual CEI diabetes intervention programs.

Of the 8 plants investigated, 7 belong to plant families that have been previously studied for anti-diabetic activity (Table 4) or are the source of commercial pharmaceuticals (Schönlau and Rohdewald 2001). Sarraceniaceae is the sole plant family uncited in diabetes literature. However, *S. purpurea* was previously referenced as a medicinal plant used by the Cree to treat kidney and bladder complaints (Marles et al. 2000). While ethnobotany was used to identify the plant species relevant to this study, as well as the appropriate plant parts for investigation and their mode of collection, traditional methods of preparation and administration were not used because they are less pertinent during this first phase of evaluation of in vitro anti-diabetic potential. Instead, ethanolic extracts were prepared from all plant specimens, and the extracts were tested in cell-based bioassays at their maximal non-cytotoxic concentrations to fully assess anti-diabetic potential.

Table 4. References to anti-diabetic activity for plant families addressed in this study.

Plant family	Reference	Finding
Betulaceae	Marles and Farnsworth 1995	Antidiabetic activity in a normal animal model
Ericaceae	Cignarella et al. 1996	Lipid-lowering activity in models of rat dyslipidemia
	Pieroni et al. 2002	Antioxidant activity
Pinaceae	Liu et al. 2004	Hypoglycaemic activity with improved endothelial function
	Schönlau and Rohdewald 2001	Beneficial effects on diabetic retinopathy
	Virgili et al. 1998	Radical scavenging activity
Rosaceae	Cho et al. 2004	Reduced oxidative stress in streptozotocin-induced diabetic rats
	Jaceldo-Siegl et al. 2004	Nutritional study. Decreased risks for heart disease and diabetes
	Jouad et al. 2002	Hypoglycaemic activity in streptozotocin-induced diabetic and normal rats
	Lovejoy et al. 2002	Beneficial effects on serum lipids
	Maslov et al. 2002	Hypoglycaemic activity in streptozotocin-induced diabetic and normal rats
	Shani et al. 1970	Hypoglycaemic activity in alloxan-induced diabetic rats
	Syiem et al. 2002	Hypoglycaemic activity in alloxan-induced diabetic and normal mice

TIID is a complex disease whose primary defects and secondary complications implicate several tissues. The thorough assessment of a product's anti-diabetic potential thereby requires numerous screening assays. Key among these is the assessment of positive effects on insulin action and insulin secretion. While none of the plant extracts improved insulin secretion or insulin production, all had positive effects on insulin action, as assessed by glucose uptake in muscle cells or fat cells. Interestingly, most effects were insulinomimetic rather than insulin-sensitizing, as observed by significant increases in insulin-independent glucose uptake. Such insulinomimetic activities in plant extracts are common (Adachi and Sakurai 2004; Pinent et al. 2004). Basal uptake was increased by as much as 47% (*S. purpurea*) in muscle cells and 155% (*R. groenlandicum*) in adipocytes. Half of the extracts acted within 1 h, which in some instances was transient and no longer observable after 18–21 h. Although differences in rapidity or duration of activity in this in vitro assay may provide information on mechanisms of action, they are not considered to be important for a comparative assessment of anti-diabetic potential at this early stage. Furthermore, certain effects on glucose transport were found to be cell-specific, observable in either muscle cells or adipocytes. Effects limited to muscle cells may represent an activation of the AMP-activated kinase pathway (Musi et al. 2002) and further studies are needed to test this hypothesis. Conversely, adipocyte-specific effects may involve the insulin-independent pathway unique to this tissue (Gual et al. 2003). In the evaluation of anti-diabetic potential, it seems reasonable to attribute more weight to the effects in skeletal muscle cells than in adipocytes, because muscle is responsible for the largest portion of glucose disposal. Stimulatory effects in both tissues may indicate a generalized insulinomimetic or insulin-sensitizing effect in all insulin-sensitive tissues.

PPARγ agonists, such as rosiglitazone and other members of the thiazolidinedione family, are known to increase the sensitivity of muscle and adipose tissue to insulin (Konrad et al. 2005). To detect glitazone-like activity, we screened for accelerated adipocyte differentiation (Tontonoz et al. 1995). Four of the extracts tested increased triglyceride content up to 8-fold. It is difficult to correlate results from this assay with results from the insulin sensitivity assay (glucose uptake), in part because effects mediated through PPAR receptors require transcriptional regulation that occurs over hours or days. Nevertheless, it is possible that some of the observed potentiation of insulin-dependent glucose uptake is due to glitazone-like activity and PPAR agonism.

An uncontrolled diabetic environment can be damaging to numerous tissues, affecting cellular function or causing apoptosis. Neuronal tissue is particularly sensitive to the hyperglycaemia that is characteristic of diabetes. In addition, neurons are sensitive to hypoglycaemia that can occur during insulin shock. Three extracts completely protected PC12 cells from glucose toxicity and 3 conferred partial cytoprotection against glucose deprivation. Interestingly, only 1 extract (*S. pupurea*) protected PC12 cells against both insults. In a hyperglycaemic environment, damage can be caused by glucose, but can also be caused by oxidative stress or by the glycation of proteins (Baynes 1991; Kikuchi et al. 2003). Therefore, a relationship between anti-oxidant activity, cytoprotection, and anti-diabetic potential could exist. Three extracts demonstrated high anti-oxidant activity, and a good correlation was observed between anti-oxidant activity and total phenolic content of extracts. However, only 1 extract with high anti-oxidant activity was observed to protect PC12 cells from glucose toxicity. Similarly, anti-oxidant activity could not predict effects on glucose transport.

In summary, this study demonstrates that all 8 plant extracts tested possess significant in vitro anti-diabetic activity, as established by a number of assays addressing insulin secretion, insulin action, and cytoprotection. While none of the extracts affected insulin secretion or production, every extract potentiated glucose transport to some extent and 5 were cytoprotective. These findings validate the novel ethnobotanical approach that was employed to identify these plant species (Leduc et al. 2005). Three plant species emerge from this study as especially promising candidates for in-depth analysis, including determination of the mechanisms of action and confirmation of the bioavailability of active molecules in vivo. *Sarracenia purpurea* extract appears to possess insulinomimetic activity, as supported by a rapid and insulin-independent effect on uptake in muscle and fat cells. This effect was quantitatively greater than that of 400 μmol/L of metformin. *Sarracenia purpurea* also conferred protection under both of the cytotoxic conditions tested. *Rhododendron groenlandicum* extract appears to possess insulinomimetic and glitazone-like activity. Finally, the

results for *P. mariana* extract are consistent with the presence of an insulin-sensitizer that could exert its effects through PPAR activation. In conclusion, there are several medicinal plants within the Cree pharmacopoeia that possess anti-diabetic activities measurable in vitro, and they may form the basis for future culturally adapted complementary approaches for the treatment of diabetes.

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