RESEARCH ARTICLE

Stimulation of AMP-activated protein kinase and enhancement of basal glucose uptake in muscle cells by quercetin and quercetin glycosides, active principles of the antidiabetic medicinal plant *Vaccinium vitis-idaea*

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Several medicinal plants that stimulate glucose uptake in skeletal muscle cells were identified from among species used by the Cree of Eeyou Istchee of northern Quebec to treat symptoms of diabetes. This study aimed to elucidate the mechanism of action of one of these products, the berries of *Vaccinium vitis idaea*, as well as to isolate and identify its active constituents using a classical bioassay-guided fractionation approach. Western immunoblot analysis in C2C12 muscle cells revealed that the ethanol extract of the berries stimulated the insulin-independent AMP-activated protein kinase (AMPK) pathway. The extract mildly inhibited ADP-stimulated oxygen consumption in isolated mitochondria, an effect consistent with metabolic stress and the ensuing stimulation of AMPK. This mechanism is highly analogous to that of Metformin. Fractionation guided by glucose uptake activity resulted in the isolation of ten compounds. The two most active, quercetin-3-*O*-glycosides, enhanced glucose uptake by 38–59% (50 μM; 18 h treatment) in the absence of insulin. Quercetin aglycone, a minor constituent, stimulated uptake by 37%. The quercetin glycosides and the aglycone stimulated the AMPK pathway at concentrations of 25–100 μM, but only the aglycone inhibited ATP synthase in isolated mitochondria (by 34 and 79% at 25 and 100 μM, respectively). This discrepancy suggests that the activity of the glycosides may require hydrolysis to the aglycone form. These findings indicate that quercetin and quercetin 3-*O*-glycosides are responsible for the antidiabetic activity of *V. vitis* crude berry extract mediated by AMPK. These common plant products may thus have potential applications for the prevention and treatment of insulin resistance and other metabolic diseases.

Keywords:
Aboriginal populations of North America / Bioassay-guided fractionation / Flavonoids / Mitochondria / Traditional medicine

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Abbreviations: ACC, acetyl-coA carboxylase; **AICAR,** aminoimidazole carboxamide ribonucleotide; **AMPK,** AMP-activated protein kinase; **APCI,** atmospheric pressure chemical ionization; **FC,** functional capacity; **FCCP,** carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; **KPB,** Krebs-phosphate buffer; **RASOC,** rate of ADP-stimulated O2 consumption; **RBOC,** the rate of basal oxygen consumption

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1 Introduction

Aboriginal populations the world over are particularly at risk for developing type II diabetes mellitus. The same genetic attributes that have favored the survival of these populations in harsh environments have now turned into a liability, increasing susceptibility to metabolic diseases when a sedentary lifestyle and a calorie-dense diet are adopted [1, 2]. The incidence rate of diabetes in these populations is often accompanied by a disproportionately high rate of diabetic complications, including nephropathy, retinopathy, and peripheral neuropathy, a phenomenon attributed to low adherence to modern anti-diabetic medications [1, 3]. As it is these complications more than diabetes itself that contribute to a decrease in quality of life and to important social costs, there is an imperative to develop treatment options that are well-adapted from a cultural perspective in order to ensure adherence.

One approach is to identify efficacious treatments for diabetes within the traditional pharmacopeia of the affected populations and to promote the integration of such products into the diet. This is the approach that our research team has been using in order to address this issue in Canadian native populations, specifically the Cree of Eeyou Istchee (Northeastern James Bay area of the Canadian province of Quebec), a population experiencing one of the highest rates of diabetes in Canada [4–9]. In collaboration with the Cree of Eeyou Istchee, we have conducted two ethnobotanical surveys and identified 17 medicinal plant species that are traditionally used to treat symptoms related to diabetes [10, 11]. Two bioactivity-screening projects for antidiabetic properties using cell-based assays have revealed that over half of the 17 species enhance glucose uptake in skeletal muscle cells [12, 13]. Seven species identified through a survey of the community of Mistissini [10] and found to promote glucose uptake [12] were recently studied together in an attempt to elucidate their mode of action [14]. This study concluded that, in all cases, activity involved the AMP-activated protein kinase (AMPK) pathway, a well-recognized therapeutic target for metabolic diseases and mediator of the effects of Metformin [15, 16]. Moreover, the activation of AMPK was related to a transient disruption of mitochondrial energy tranduction, a mechanism analogous to that of Metformin [17]. Although the active principles were not identified, such effects on mitochondrial function were observed to be consistent with the anti-microbial role of many plant metabolites [18].

This study focuses on the glucose-uptake-enhancing effects of the berries of *Vaccinium vitis idaea*, also known as Mountain cranberry or lingonberry, a medicinal plant product used in the communities of Whapmagoostui and Mistissini to treat frequent urination and a number of other symptoms of diabetes [10]. This product was the most active to emerge from our second bioactivity-screening project [13]. Various members of the *Vaccinium* genus, including lowbush blueberry (*V. angustifolium*), American cranberry (*V. macrocarpon*) and European bilberry (*V. myrtillus*), are traditionally used for the treatment of diabetes by several cultures throughout the world [19]. The goal of this study was to test the hypothesis that the enhancement of glucose uptake by *V. vitis idaea* berry extract is mediated by a mechanism similar to that of the boreal forest medicinal plant species studied previously [14] and to simultaneously elucidate the active principles of this medicinal species using our expertise in the phytochemistry of *Ericacea* [20, 21]. We conclude that quercetin and certain glycosides of this well-studied and widely distributed flavonoid [22] transiently inhibit mitochondrial ATPsynthase, leading to the activation of AMPK, and propose that quercetin and quercetin glycosides are responsible for the anti-diabetic activity of *V. vitis* and perhaps of other species of this genus.

2 Materials and methods

2.1 Plant material and extraction

Berries of *V. vitis idaea* L. (*V. vitis)* were collected in Whapmagoostui, Que., Canada, and kept at −20°C until use. Plant material was authenticated by a taxonomist (A. Cuerrier, Montreal Botanical Garden, Montreal, Que., Canada) and voucher specimens were deposited at the Montreal Botanical Garden herbarium (voucher Whap04-21). In total 800 g of the berries were freeze-dried (Super Moduylo freeze dryer; Thermo Fisher, Ottawa, Ont., Canada) to yield 114 g of dry material. The dry material was then extracted three times for 24 h with ten volumes of 80% ethanol on a mechanical shaker and then filtered under vacuum using Whatman 1 paper. The supernatants were combined and dried using a rotary evaporator (RE 500; Yamato Scientific, Tokyo, Japan) followed by lyophilization. Preliminary phytochemical characterization of *V. vitis* berry crude extract in the form of extract yield, total phenolic content and identification of a small number of marker compounds, have been reported in an earlier study [13]. Markers include catechin, para-coumaric acid, cyanidin glycosides, and quercetin glycosides. The freeze-dried ethanol extract was reconstituted in water (15% w/v) and extracted in a separatory funnel with equal volume of ethyl acetate to yield an ethyl acetate soluble fraction. The aqueous solution remaining after ethyl acetate extraction was freeze-dried and kept for bioactivity screening. Crude extract and fractions were solubilized in DMSO at 200 mg/mL, aliquoted, and stored at −20°C until bioactivity testing. Isolates were similarly prepared and used at a final concentration of 100 mM. Quercetin and quercetin-3-*O*-glucoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercetin-3-*O*-galactoside was purchased from Indofine Chemical (Hillsborough, NJ, USA). Pure compounds were reconstituted to a concentration of 100 mM in DMSO, aliquoted, and stored frozen.

2.2 Cell culture

C2C12 murine skeletal myoblasts and H4IIE murine hepatocytes were obtained from the American Type Cell Collection (Manassas, VA, USA). Cell culture media were purchased from Invitrogen Life Technologies (Burlington, Ont., Canada) unless otherwise noted. Other reagents were purchased from Sigma-Aldrich (Oakville, Ont., Canada) unless otherwise noted. C2C12 myoblasts were cultured in 6- or 12-well plates in high-glucose DMEM supplemented with 10% fetal bovine serum, 10% horse serum and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL) at 37°C in a 5% CO_2 atmosphere. After 80% confluence, myoblasts were differentiated into myotubes in DMEM supplemented with 2% horse serum and antibiotics for exactly 7 days, resulting in the fusion of all cells into multinucleated myotubes. H4IIE hepatocytes were grown in 6-well plates in DMEM supplemented with 10% fetal bovine serum until fully confluent and experiments were performed 1–3 days later. Treatments were initiated 18 h prior to glucose uptake or signaling experiments. Aliquots of crude extract and fractions were diluted in differentiation medium at 1:1000 for a final DMSO concentration of 0.1% and a final extract or fraction concentration of 200 μg/mL. The crude extract concentration of 200 μg/mL was previously used for bioactivity screening and determined to be non-cytotoxic [13]. Aliquots of isolates or pure compounds were diluted in differentiation medium at 1:1000 for a final concentration of 100 μM. To obtain, concentrations of 50 and 25 μM, original aliquots were diluted at 1:2000 and 1:4000, respectively, and DMSO was added to maintain final concentration at 0.1% in all conditions.

2.3 Glucose uptake assay

The effects of plant products on the rate of uptake of glucose by differentiated C2C12 skeletal myotubes were assessed with a ^{3}H-deoxyglucose uptake assay as described previously [12, 13, 23, 24]. Briefly, treatments or vehicle alone were applied for 18 h to 6-day differentiated cells. Following the treatment period, cells were rinsed twice with Krebs-phosphate buffer (KPB; 20 mM HEPES, 4.05 mM Na_2HPO_4, 0.95 mM NaH_2PO_4, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 5 mM glucose) at 37°C and allowed to equilibrate in this buffer for 30 min at 37°C. During this time, insulin (100 nM) was added to some wells. Following this, cells were washed twice with glucose-free KPB at 37°C, and 0.5 μCi/mL 2-deoxy-D-[1-^{3}H]-glucose (TRK-383, Amersham Biosciences, Buckinghamshire, UK) in this same buffer was applied for exactly 10 min at 37°C. Cells were then placed on ice and rapidly washed three times with ice-cold KPB, and lysed with 0.1 M NaOH for 30 min. The lysate was added to 4 mL of liquid scintillation cocktail (Ready-Gel 586601; Beckman Coulter, Fullerton, CA, USA) and radioactivity was measured in a liquid scintillation counter (LKB Wallac 1219; Perkin-Elmer, Woodbridge, Ont., Canada).

2.4 Western immunoblot

The effects of plant products on the insulin and AMPK signaling pathways of C2C12 muscle cells or H4IIE hepatocytes were assessed by western immunoblot. Treatments or vehicle alone were applied for 18 h to 6-day differentiated C2C12 cells or to post-confluent H4IIE cells. Thirty minutes prior to the end of the treatment, insulin (100 nM) or aminoimidazole carboxamide ribonucleotide (AICAR; 1 mM) were added to some vehicle-treated wells as positive controls. Following treatment, cells were placed on ice and washed three times with ice-cold PBS (8.1 mM $NaHPO_4$, 1.5 mM KH_2PO_4, pH 7.4, 137 mM NaCl, and 2.7 mM KCl) and lysed in 250 μL of lysis buffer (25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 0.5 mM EDTA, 1% Triton-X-100, 1% sodium deoxycholate, and 0.1% SDS) containing a commercial cocktail of protease inhibitors (Complete Mini; Roche, Mannheim, Germany) supplemented with 1 mM phenylmethanesulfonyl fluoride, and a cocktail of phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride). Lysates were scraped into microcentrifuge tubes, kept on ice for 30 min with periodical vortexing, then centrifuged at 600 × *g* for 10 min. Supernatants were decanted and stored at −80°C until analysis. Protein content was determined by the bicinchoninic acid method (Thermo Scientific Pierce, Rockford, IL, USA) standardized to bovine serum albumin. Lysates were diluted to a concentration of 1.0 μg/μL total protein and boiled for 5 min in reducing sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue). Briefly, 100 μL of each sample were separated on 10% polyacrylamide full-size gels and transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, and 137 mM NaCl) containing 0.1% Tween-20. The blots were then incubated overnight at 4°C on a mechanical shaker in blocking buffer with phospho- or pan-specific antibodies against Akt or acetyl-coA carboxylase (ACC) at 1:1000 (Cell Signaling Technologies, Danvers, MA, USA). Membranes were washed five times with Tris-buffered saline Tween-20 followed by a 1.5 h incubation at ambient temperature with horseradish-peroxidase-conjugated secondary antibodies diluted 1:100 000 (Jackson Immunoresearch, Cedarlane Laboratories, Hornby, Ont., Canada). Revelation was performed using the enhanced chemiluminescence method and blue-light-sensitive film (Amersham Biosciences). Experiments were repeated on three different passages of cells, each passage containing all conditions in parallel. All samples from a given passage were separated and transferred simultaneously to a single membrane. Quantification of the integrated density of bands was performed using a flatbed scanner (ScanJet 6100; Hewlett Packard, Palo Alto, CA, USA) and NIH Image 1.63 software (National Institutes of Health, Bethesda, MD, USA).

2.5 Respiration of isolated liver mitochondria

The effects of the crude extract and of selected isolates on the function of mitochondria were assessed by oxygraphy. Mitochondria were isolated from the liver of male Wistar rats as *per* the method of Johnson and Lardy [25]. Surgery, isolation of mitochondria, and measurement of oxygen consumption were performed as described previously [26]. All animal manipulations were sanctioned by the animal ethics committee of the Université de Montréal and respected the guidelines from the Canadian Council for the Care and Protection of Animals. Briefly, rats obtained from Charles River (St. Constant, Que., Canada) and weighing between 200 and 225 g were anesthetized and laparotomized. The portal vein was cannulated and the hepatic artery and the infrahepatic inferior vena cava were ligated. The liver was flushed with 100 mL of Krebs-Henseleit buffer (25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, pH 7.4, 154 mM NaCl, 4.8 mM KCl, 2.1 mM $CaCl_2$, and 1.2 mM $MgSO_4$) at 22°C prior to excision. In total 2 g of tissue were homogenized on ice using a Teflon potter homogenizer in ice-cold isolation buffer (10 mM Tris, pH 7.2, 250 mM sucrose, and 1 mM EGTA). The homogenate was centrifuged at $600 \times g$ for 10 min at 4°C in order to remove cellular fragments and the resulting supernatant was centrifuged at $12\,000 \times g$ for 5 min at 4°C. The pellet was delicately washed once with this same buffer and re-centrifuged. The pellet was then washed once with EGTA-free buffer and again re-centrifuged. The final pellet, containing viable mitochondria, was suspended in EGTA-free isolation buffer and kept on ice. Protein content of the mitochondrial preparation was determined by Lowry protein assay. O_2 consumption was measured at 25°C in a Hansatech Oxygraph apparatus (Norfolk, UK) with a 1 mL reaction chamber, as described previously [26]. Briefly, 1 mg of mitochondrial protein was added to respiration buffer (5 mM KH_2PO_4, pH 7.2, 250 mM sucrose (ultra pure), 5 mM $MgCl_2$, 1 mM EGTA, and 2 μM of the complex I inhibitor rotenone) at 25°C in the reaction chamber, for a final volume of 990 μL. Mitochondrial respiration was initiated by the injection of 6 mM (final concentration) of the complex II substrate succinate, and the rate of basal oxygen consumption *per* milligram mitochondrial protein (the rate of basal oxygen consumption (RBOC) or state 4 respiration) was determined. In total 1 μL of 1000× concentrated plant extract or 1 μL of DMSO was then injected and its effect on RBOC was assessed. Basal respiration was allowed to proceed for at least 30 additional seconds. Oxidative phosphorylation (state 3 respiration) was induced by the addition of 200 μM (final concentration) ADP and the rate of ADP-stimulated O_2 consumption (RASOC) *per* milligram mitochondrial protein (RASOC) was determined. Extracts were tested in three different experimental sessions, with at least two replicate experiments *per* mitochondrial preparation. DMSO-vehicle control experiments were conducted at the beginning and end of each experimental session in order to establish the session-normal RBOC and RASOC and to ensure no loss in mitochondrial viability over the duration of the session, typically less than 4 h from the end of the isolation protocol. DMSO was confirmed to have no effect on the basal rate of O_2 consumption. The effect of each plant extract was evaluated as: (i) the increase in the RBOC (a measure of the magnitude of the uncoupling effect); (ii) the decrease in functional capacity (FC) *per* milligram protein (a measure of the magnitude of the uncoupling effect plus any additional inhibitory effect), where FC was defined as the difference of the RASOC (maximal functional rate of consumption) and the RBOC (rate of consumption driven by proton leak and not contributing to ATP synthesis). Calculations were as follows: the average FC *per* milligram protein of the vehicle control experiments for a given session was calculated by subtracting the average RBOC from the average RASOC. For (i) above, the absolute increase in RBOC measured in a given experiment was expressed as a percentage of the average control FC for the session. For (ii) above, the FC measured in a given experiment was expressed as a percentage of the average control FC for the session to give the percentage residual FC.

2.6 Assay of cell culture rate of acidification

A spectrophotometric assay of change in cell culture medium pH over time was developed based on similar assays [27, 28]. The assay medium consisted of Dulbecco's PBS containing Phenol Red as a pH indicator and modified for reduced buffering capacity while keeping other ion concentrations within physiological range (modified Dulbecco's PBS (mD-PBS) 1.5 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 137 mM NaCl, 25 mM glucose, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, Phenol Red 0.1 mM, and deionized ultra-filtered water). This formulation resulted in a pH of 7.1, which was adjusted to 7.2 at ambient temperature with NaOH immediately prior to the assay using an Accumet pH meter with calomel electrode (Fisher Scientific). Absorbance of 100 μL samples of medium transferred to 96-well plates (Sarstedt, Montreal, Que., Canada) was measured at ambient temperature at 530 and 450 nm using a Wallac Victor 2 plate reader (Perkin-Elmer, St. Laurent, Que., Canada) and the ratio of A 530/A 450 was calculated. The relationship between pH and the log of this ratio was observed to be linear over the range of pH 6.4–7.2 (Fig. 1A) and was modeled with the following function: $pH = 0.765 \times \ln(A\,530/A\,450) + 7.61$ ($R^2 = 0.99$). The buffering capacity of mD-PBS was determined to be linear and equal to 1.075 mM equivalents *per* pH units between pH 6.3 and 7.1. Experiments were performed on 7-day differentiated C2C12 muscle cells and on 1-day post-confluent H4IIE liver cells grown in 12-well plates. On the day of the experiment, cells were gently rinsed twice with mD-PBS, and then allowed to equilibrate in exactly 1.0 mL of mD-PBS for 30 min at 37°C

in a humidified air atmosphere. The assay was started by gently mixing pre-warmed 3× concentrated treatments in a 500 μL volume of mD-PBS to the 1.0 mL volume of mD-PBS already present, for a final volume of exactly 1.5 mL and treatments at their final working concentration. After the rapid addition of treatments to all the wells of a single plate, an initial 100 μL sample of medium, corresponding to time 0, was transferred to microtiter plate for spectrophotometric analysis. Cells were then incubated at 37°C in a humidified air atmosphere for the duration of the experiment. At times 20, 40, 60, 120, 180, and 240 min, plates were stirred and a 100 μL sample of medium was transferred to a microtiter plate for analysis. Calculations of rate of acidification and cumulative secretion of acid equivalents over time accounted for the decreasing experimental volume with each sampling. As DMSO was observed to stimulate acidification, as noted by others [29], quercetin was solubilized in ethanol (final vehicle concentration of 0.08%). Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma-Aldrich) solubilized in ethanol was used at 5 μM as a positive control. Results were expressed as cumulative secretion of acid equivalents (micromoles) for four to five replicates *per* condition *per* time point.

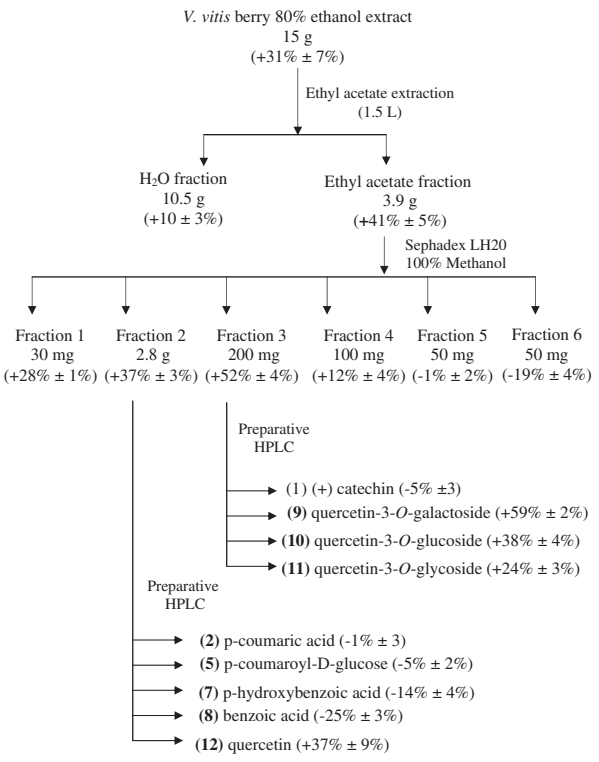

Figure 1. Phytochemical fractionation of *V. vitis* berry extract guided by muscle cell glucose-uptake activity. Values in brackets represent activity expressed as percentage change in the rate of basal glucose uptake relative to the vehicle control (0.1% DMSO) following an 18 h treatment with respective fractions at 200 μg/mL or isolates (Fig. 2 and Table 1) at 100 μM.

2.7 Cytosolic ATP assay

Total cytosolic ATP was measured in cell lysates by luminescence using the ATPlite assay kit (Perkin-Elmer, Waltham, MA, USA), as *per* the manufacturer's protocol. Briefly, C2C12 myotubes in 24-well plates or H4IIE hepatocytes in 96-well plates were treated in parallel for 1, 3, or 6 h with extract or DMSO. FCCP was used at 5 μM as a positive control. Results were expressed in % ATP content of vehicle-treated wells for one to two experiments of three to four replicates *per* condition *per* time point.

2.8 Statistical analysis

Results are reported as means ± SEM, with the number of replicates and number of independent experiments indicated. Data were analyzed by one-way analysis of variance with a Fisher *post hoc* test or by *t*-test when appropriate using StatView software (SAS Institute, Cary, NC, USA). Statistical significance was set at $p \leq 0.05$.

2.9 Fractionation, isolation and identification

Fractionation of the ethyl acetate soluble fraction of *V. vitis* berry ethanol extract is shown in Fig. 1. Gel filtration chromatography of the ethyl acetate soluble fraction was performed using Sephadex LH-20 (Pharmacia, Uppsala, Sweden) as a stationary phase. Sephadex LH-20 (110 g) was soaked in methanol and loaded on a glass column (25 × 105 cm). The mobile phase (methanol 100%) was delivered by an HPLC pump (model 9012; Varian, Mississauga, Ont., Canada) at a flow rate of 3 mL/min. In total, 150 mL fractions were collected using an automated collector (Dynamax FC-4; Varian). Fractions were analyzed and pooled based on the similarity of their HPLC profiles (Fig. 1) and tested for the stimulation of glucose uptake in an *in vitro* bioassay as described in Section 2.3 at a concentration of 200 μg/mL.

The isolation and purification of compounds from subfractions of ethyl acetate soluble fraction of *V. vitis* was achieved on a 1200 series preparative HPLC system (Agilent Technologies, Santa Clara, CA, USA), equipped with an autosampler with a 2 mL loop, a binary pump (flow rate range 5–100 mL/min), DAD and a fraction collector. A Gemini C18 reversed phase column (4.6 × 250 mm, particle size 10 μm) (Phenomenex, Torrance, CA, USA) was used to monitor the fractionation process and for scaling up for the isolation of the compounds from target fractions on a preparative scale Gemini C18 reversed phase column (21.2 × 250 mm, particle size 10 μm) (Phenomenex). Preparative scale isolation of the most active fractions was achieved by using a binary solvent system of solvent A (0.05% aqueous trifluoroacetic acid) and solvent B (100% ACN). The gradient elution program afforded a total of nine compounds from the two fractions.

LC-MS analysis of the crude *V. vitis* berry extract, its fractions, and the isolated compounds was performed on an HPLC-DAD-atmospheric pressure chemical ionization (APCI)-MSD system (Agilent Technologies, model 1100) which consisted of an autosampler with a 100 μL loop, a quaternary pump (maximum pressure, 400 bar), a column thermostat, a DAD and APCI-MS. The separations were achieved on an YMC-ODS-AM, 100 mm × 4.6 mm id, particle size 5 μm (YMC, Kyoto, Japan). The mobile-phase system consisted of water (solvent A) and ACN (solvent B). The optimized elution conditions were a linear gradient of 5–100% B in 35 min, the column was washed for 5 min at 100% B, brought back to starting mobile-phase composition in 0.1 min and equilibrated for 7 min before next injection. The HPLC separations were monitored at 290, 325, and 520 nm.

Mass spectrometric characterization was performed in both positive and negative ionization modes. For positive ionization mode, the optimized spray chamber conditions were: drying gas flow rate of 6 L/min, nebulizer pressure of 40 psig, drying gas temperature of 300°C, vaporizer temperature of 400°C, capillary voltage of 3000 V, and corona current of 3 μA. For negative ionization mode, the conditions were: drying gas flow rate of 6 L/min, nebulizer pressure of 60 psig, drying gas temperature of 350°C, vaporizer temperature of 400°C, capillary voltage of −3000 V, and corona current of 15 μA. APCI was conducted at 300°C with the vaporizer at 400°C; nebulizer pressure, 40 psig; nitrogen (drying gas) flow rate, 6 L/min; fragmentation voltage, 20 V; capillary voltage, 3000 V; corona current, 3 μA. The MS was operated in scan mode within 100–800 amu with fragmentation voltages of 20 and −160 V for positive and negative ionization, respectively.

The identification of the isolates was achieved by: (i) the comparison of UV absorption spectra against those from a custom metabolomics library consisting of 140 pure reference phenolic compounds [20]; (ii) co-chromatography with reference standards; (iii) the confirmation of the presence of characteristic ions; (iv) the comparison of the recorded 1H and 13C-NMR spectra (Avance 400 MHz NMR spectrometer; Bruker BioSpin, Billerica, MA, USA) with published spectra. Isolates were quantified by generating five-point linear calibration curves on the basis of area under the peaks recorded at: 325 nm, reference off, bandwidth 4 for phenolics; 290 nm, reference off, bandwidth 4 for procyanidins and catechins; 520 nm, reference off, bandwidth 4, for anthocyanins.

3 Results

3.1 *V. vitis* berry extract stimulates glucose uptake in C2C12 myotubes

An 18 h treatment with 200 μg/mL of the crude ethanol extract of *V. vitis* berries stimulated glucose uptake by 31 ± 7%, $n = 6$ (Fig. 1). These results are comparable to those reported in the previous screening study in which an earlier collection of the same species was tested [13]. This stimulation of muscle cell glucose uptake was quantitatively similar to that obtained after 15 min treatment with 100 nM insulin (positive control; data not shown).

3.2 Bioassay guided fractionation, isolation, and identification of active principles

In order to identify its active principles, the *V. vitis* berry extract was fractionated using a multi-step approach guided by the enhancement of glucose uptake activity in C2C12 cells treated 18 h. The fractionation scheme and activity results obtained at every step are shown in Fig. 1. All fractions were tested at 200 μg/mL and all isolates at 100 μM. The crude ethanol extract was first fractionated into ethyl acetate-soluble and -insoluble fractions. Only the ethyl acetate-soluble fraction showed a significant stimulation of glucose uptake (41 ± 5% above DMSO; $n = 3$) and was selected for further fractionation on a Sephadex LH20 column.

This yielded six subfractions pooled according to similar HPLC profiles (see Section 2). Of these, subfractions 2 and 3 showed significantly higher stimulation of glucose uptake than the original *V. vitis* berry extract and other fractions (37 ± 3% and 52 ± 4%, respectively; $n = 6$). These two fractions were selected for further fractionation.

Using preparative HPLC chromatographic fractionation, five compounds were isolated from subfraction 2 (Figs. 2 and 3; Table 1): *p*-coumaroyl-D-glucose; *p*-hydroxybenzoic acid; *p*-coumaric acid; benzoic acid; quercetin. Of these, only quercetin stimulated uptake when tested at 100 μM (37 ± 9%; $n = 6$).

Finally, eight compounds were identified from subfraction 3: quercetin-3-*O*-galactoside; quercetin-3-*O*-glucoside; an unidentified quercetin-3-*O*-glycoside; catechin; epicatechin; cyanidin-glucoside; cyanidin-galactoside (Figs. 2 and 3; Table 1). The first five of these compounds were isolated and the three quercetin-3-*O*-glycosides were found to induce a significant enhancement of glucose uptake at 100 μM (59 ± 2%, 38 ± 4%, and 24 ± 3%, respectively; $n = 6$). Cyanidin glycosides were also tested and found to be inactive (results not shown).

The identity of the purified compounds was confirmed by a combination of LC-MS and NMR and by comparison of their physicochemical properties with those reported in the literature [30] or with those of reference compounds. The predominant phenolic acid present in the berries was *p*-coumaric acid (33.8 ± 0.6 μg/g dry weight). The predominant flavonols present in the berries were quercetin-3-*O*-glucoside (19.2 ± 1.2 μg/g dry weight) and quercetin-3-*O*-galactoside (15.9 ± 0.4 μg/g dry weight) (Table 1).

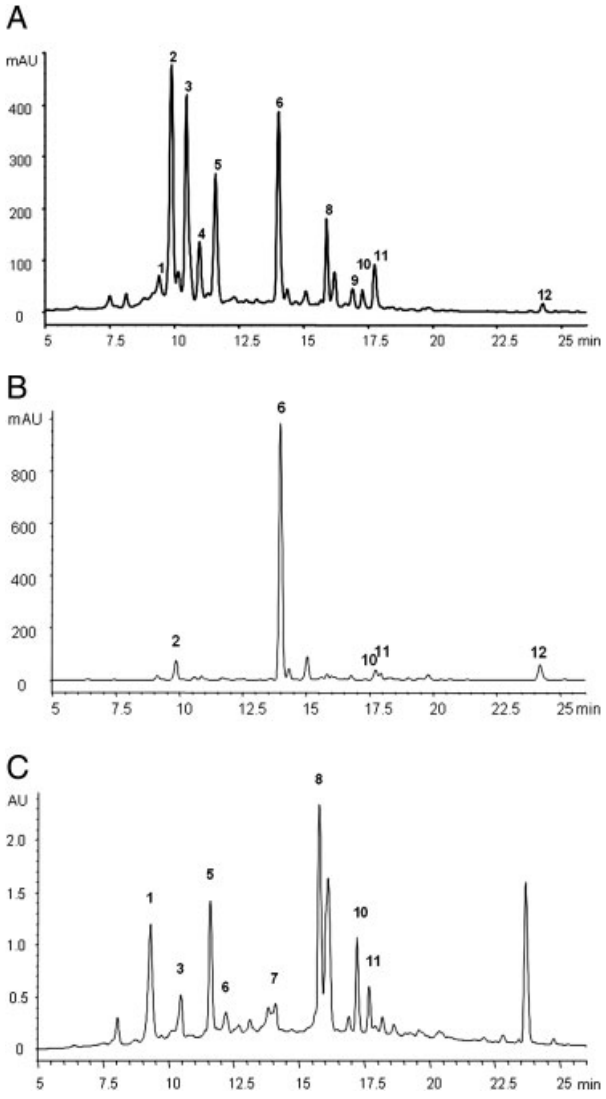

Figure 2. HPLC chromatograms of *V. vitis* berry crude extract (A), subfractions 2 (B) and 3 (C) of its ethyl acetate-soluble fraction. Absorbance at 325 and 520 nm (milliabsorbance units) is plotted against retention time (in min). Twelve constituent compounds were identified using a metabolomics approach, as described in Section 2.1. The identity of these peaks is listed in Table 1.

Table 1. Yield of *V. vitis* berry extract constituents

	Compound	Content (μg/g dry weight of berries)
1	(+)-Catechin	2.8±0.7
2	*p*-Coumaric acid	33.8±0.6
3	Cyanadin-glucoside	30.4±0.7
4	Cyanadin galactoside	34.4±0.3
5	*p*-coumaroyl-D-glucoside	22.5±0.4
6	Epicatechin	3.8±1.4
7	*p*-Hydroxybenzoic acid	3.6±0.2
8	Benzoic acid	35.1±0.2
9	Quercetin-3-*O*-galactoside	15.9±0.4
10	Quercetin-3-*O*-glucoside	19.2±1.2
11	Unidentified quercetin-3-*O*-glycoside	21.9±1.3
12	Quercetin	2.3±0.3

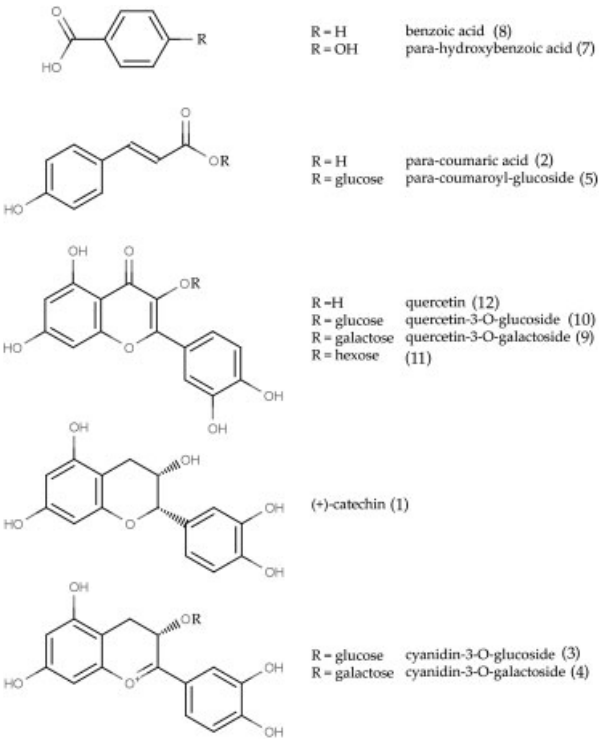

Figure 3. Chemical structures of the 12 isolated constituents *V. vitis* berry ethanol extract.

3.3 *V. vitis* crude extract and its actives increase activity of the AMPK signaling pathway not of the insulin receptor pathway in C2C12 myotubes

To understand the mechanism mediating the effect of *V. vitis* berry extract on skeletal muscle cell glucose uptake, we evaluated the activity of the two main signaling pathways that regulate rate of glucose uptake in this cell-type: the insulin-receptor pathway and the AMPK pathway. Following an 18 h treatment in C2C12 cells, there was no indication of increased phosphorylation of Akt (Fig. 4A), a marker of the former pathway. Stimulation with 100 nM insulin for 30 min produced a clear activation of this enzyme. In contrast, treatment with the extract increased the phosphorylation of the AMPK effector ACC (Fig. 4B). AICAR, an AMP mimetic and known activator of AMPK signaling, served as a positive control and also greatly enhanced activation ACC. Concordant with the activity of the crude extract, treatment of C2C12 cells for 18 h with 50 or 100 μM quercetin, quercetin-3-*O*-galactoside or quercetin-3-*O*-glucoside did not increase phosphorylation of Akt (Fig. 4A) but increased phosphorylation of ACC (Fig. 4B). Total content of ACC was not significantly altered by any treatment.

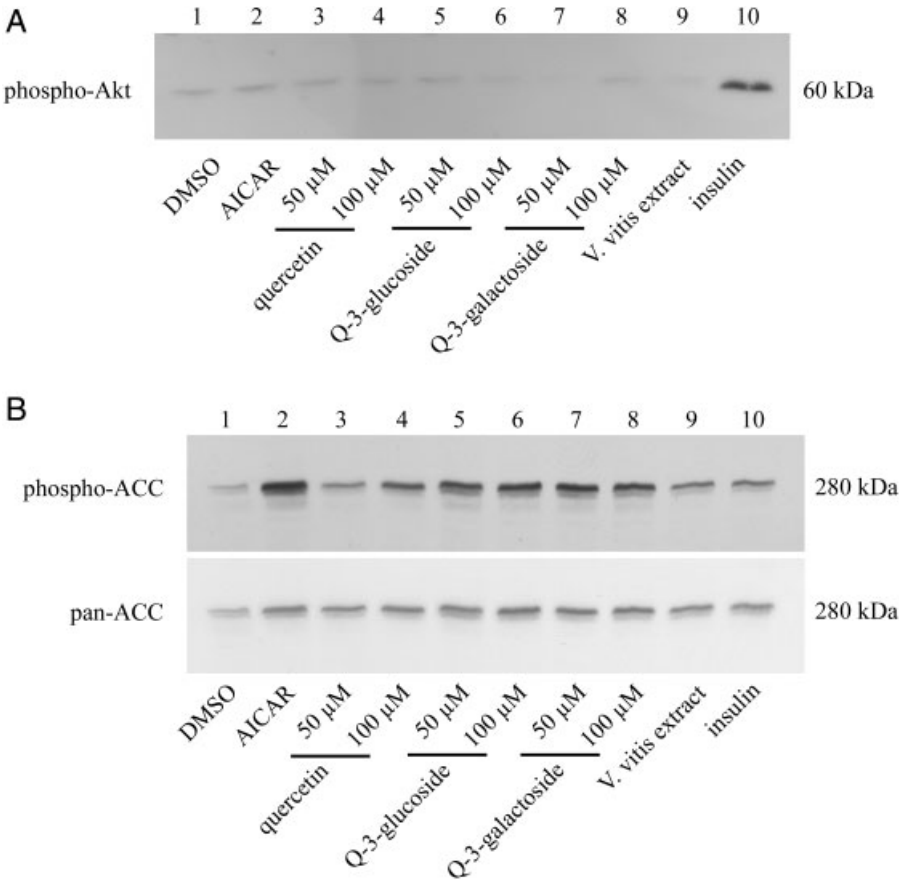

Figure 4. *V. vitis* berry extract and its active principles stimulate the AMPK signaling pathway but not the insulin receptor pathway. C2C12 skeletal muscle cells were treated for 18 h with either 0.1% DMSO (vehicle), 200 μg/mL of *V. vitis* berry extract, or 50 and 100 μM of quercetin, quercetin-3-*O*-glucoside or quercetin-3-*O*-galactoside. Phosphorylation of the insulin receptor pathway marker Akt (A) and of the AMPK effector ACC was measured by western immunoblot. Insulin (100 nM) and AICAR (2 mM) applied for 30 min served as positive controls.

3.4 *V. Vitis* berry extract and quercetin, but not quercetin glycosides, inhibit respiration in isolated mitochondria

AMPK is highly sensitive to metabolic stress such as can occur when energy transduction is disrupted. To test whether *V. vitis* berry extract and its active principles may have caused such a disruption, we assessed the effect of these products on respiration of isolated mitochondria. Succinate-supported rates of basal and ADP-stimulated oxygen consumption were measured in rat liver mitochondria treated with vehicle, 200 μg/mL crude extract, or 25–100 μM of quercetin or quercetin glycosides. The crude extract had no stimulatory effect on the rate of basal O_2 consumption but induced a mild inhibitory effect on the rate ADP-stimulated O_2 consumption (Fig. 5), reducing the capacity for ATP synthesis by $9 \pm 3\%$. This pattern of disruption of mitochondrial function is consistent with an inhibition of ATP synthase. The quercetin aglycone produced a similar, but more pronounced inhibitory effect: at 25 and 100 μM, capacity was inhibited by $40 \pm 10\%$ and $85 \pm 5\%$, respectively (Fig. 6A). The quercetin glycosides had much less effect than the

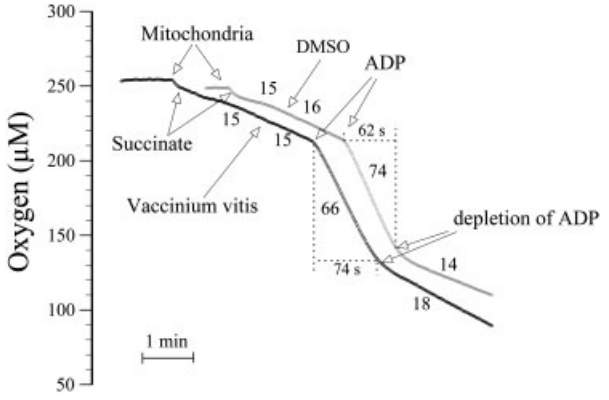

Figure 5. The *V. vitis* berry extract induces a mild instantaneous inhibition of respiration in isolated rat liver mitochondria, as illustrated by a representative oxygen consumption tracing. Mitochondria (1 mg mitochondrial protein) were treated with vehicle (0.1% DMSO) or 200 μg/mL of extract and the rates of succinate-supported basal and ADP-stimulated oxygen consumption were measured. As compared to control, extract-treated mitochondria exhibited an unchanged rate of basal oxygen consumption but a mildly inhibited rate of ADP-stimulated oxygen consumption. Values represent rate of consumption in nmol O/min/mg protein. Experiments were repeated in three different mitochondrial preparations.

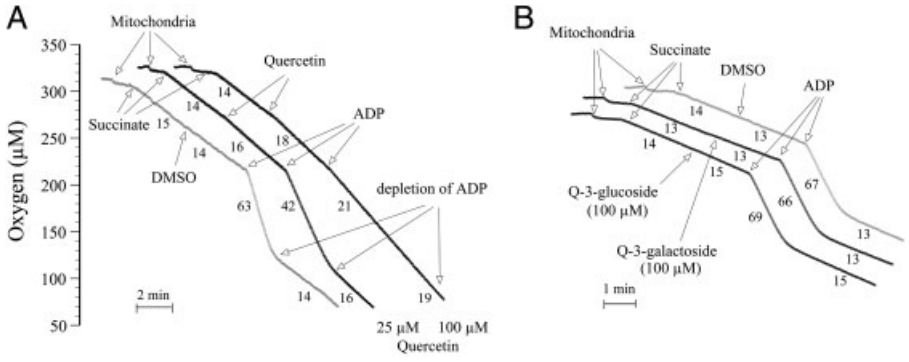

Figure 6. Quercetin (A), but not quercetin-3-*O*-glycosides (B), induces an important instantaneous and dose-dependent inhibition of respiration in rat liver mitochondria, as illustrated by representative oxygen consumption tracings. Values represent rate of consumption in nmol O/min/mg protein. Experiments were repeated in three different mitochondrial preparations.

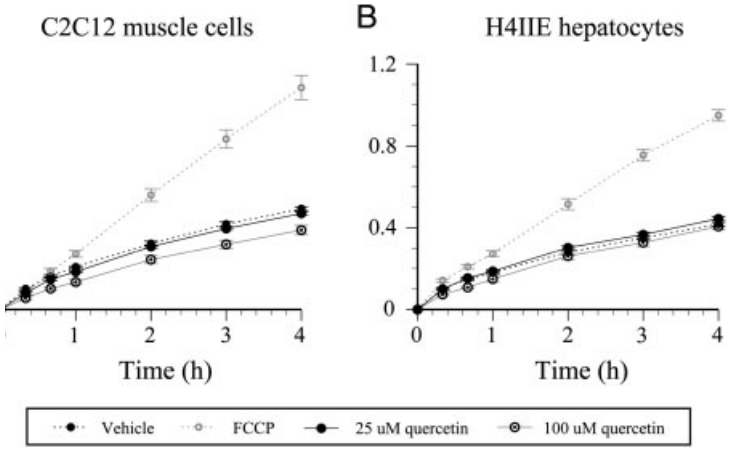

Figure 7. Quercetin does not increase the rate of secretion of acid equivalents by C2C12 (A) or H4IIE (B) cells. Acidification of the cell medium. The pH of the culture medium was assessed with a Phenol Red-based spectrophotometric assay at several time points. Change in pH was expressed as the cumulative secretion of acid equivalents. FCCP (5 μM) was used as a positive control. Quercetin treatment was not significantly different from vehicle (0.08% ethanol) in either cell line and at either concentration (25 and 100 μM). Data are mean ± SEM for two experiments of four to five replicates *per* condition *per* time point.

aglycone, only decreasing capacity by 3–7% at 100 μM (Fig. 6B).

3.5 Quercetin does not increase the rate of secretion of acid equivalents or reduce intracellular ATP

Since quercetin powerfully inhibited respiration in isolated mitochondria, it was hypothesized that it would induce a compensatory increase in flux through anaerobic glycolysis and therefore an increase in the rate of secretion of acid equivalents. To test this, the pH of the culture medium of H4IIE hepatocytes and C2C12 muscle cells was spectrophotometrically assessed at several time points over a 4 h treatment with quercetin. Quercetin at either 25 or 100 μM did not significantly influence the rate of acidification of the medium of C2C12 or H4IIE cells (Fig. 7). In contrast, the positive control uncoupling compound FCCP greatly enhanced this rate.

Similarly, it was pertinent to verify if the metabolic stress induced by quercetin would negatively impact the intracellular ATP concentration. Again, neither 25 nor 100 μM of quercetin induced a drop in ATP in H4IIE hepatocytes over a 6-h period (Fig. 8); instead, cellular ATP was paradoxically increased after 3 h of treatment. FCCP used as a positive control transiently decreased content of ATP in H4IIE hepatocytes after 1 h of treatment.

4 Discussion

Aboriginal populations worldwide are susceptible to metabolic disorders related to lifestyle changes. Indeed, the incidence of obesity and diabetes in these populations is the highest in the world [1, 7, 31]. When this predisposition is coupled with a cultural disconnection with modern pharmaceuticals, the rate of diabetic complications and the associated social costs can become staggering. In an effort to remedy the situation in Canadian aboriginal populations facing these problems, our team has been working towards identifying safe and efficacious alternative treatment options for diabetes based on these populations' own traditional medicine and associated pharmacopeia. In collaboration with the Cree of Eeyou Istchee (James Bay area of Que., Canada), we have used a novel ethnobotanical approach [10] to identify relevant medicinal plant species used to treat symptoms of diabetes. Follow-up studies screening the antidiabetic activity of extracts of these species have revealed eight products capable of enhancing glucose uptake in skeletal muscle cells [12, 13].

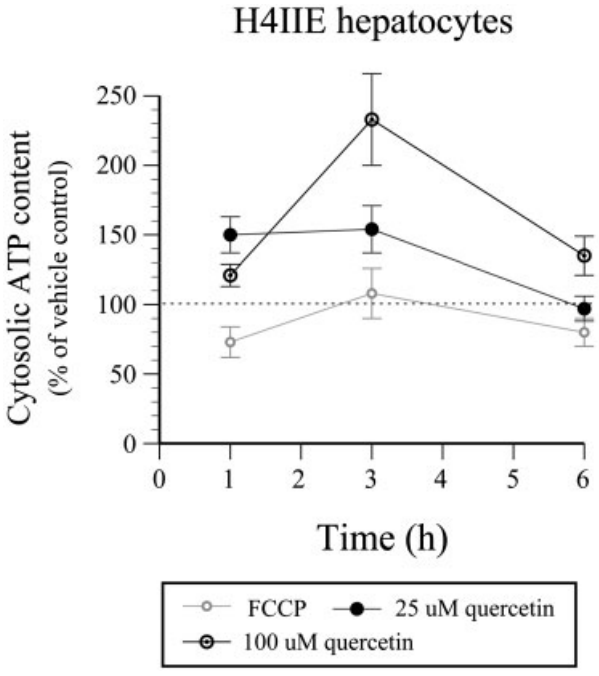

Figure 8. Quercetin does not reduce intracellular ATP concentration in H4IIE hepatocytes. Cytosolic ATP content was measured in H4IIE hepatocytes using a luminescent ATP assay. FCCP (5 μM) was used a positive control. Quercetin treatment was not significantly different from vehicle (0.1% DMSO) at either concentration (25 and 100 μM). Data are expressed as mean ± SEM of two experiments of four to five replicates *per* condition *per* time point.

The effects of all seven products to emerge from the first screening project [12] were found to be mediated by AMPK as a response to metabolic stress resulting from a disruption of mitochondrial energy transduction [14], a mechanism similar to that of the biguanide oral anti-hyperglycemic drug Metformin [17]. The purpose of this study was to evaluate whether the effects of a new product identified in our second screening study [13], the extract of the berries of *V. vitis idaea*, are also mediated by such a mechanism and to simultaneously isolate and identify the compounds responsible for this activity using our phytochemical expertise with *Ericacea* species [20, 21]. The identification of active compounds will be useful for standardizing the activity of different preparations of the plant product and may also provide insight into the activity of other small berries used for the treatment of diabetes in various parts of the world.

AMPK is recognized as an important therapeutic target for diabetes [15, 16]. Indeed, the effects of Metformin are mediated through this metabolic master enzyme and transducer of metabolic stress. Upon activation by an increase in the cellular ratio of AMP to ATP, AMPK serves to restore energy homeostasis by increasing flux through energy-producing pathways and decreasing energy-consuming processes [32]. Energy production is increased by simultaneous enhancement of uptake and oxidation of lipids and carbohydrates. Some more tissue-specific effects include the insulin-like inhibition of hepatic glucose output and the translocation of Glut-4 glucose transporters in skeletal muscle, activities that contribute to a systemic anti-hyperglycemic effect [17, 33–35]. In addition to acute actions for restoring energy homeostasis, the activation of AMPK produces long-term adaptive effects, such as increased capacity for substrate uptake and oxidation, that confer protection against future metabolic stresses [36–38].

Many plant products are known to activate AMPK [39–48], including compounds isolated from *Galega officinalis* and from which Metformin is derived [49, 50]. AMPK is not activated directly by these products, but rather as a consequence of the metabolic stress that they induce [39]. These compounds tend to be plant defensive metabolites that protect against microorganisms by disrupting well-conserved energy transduction pathways such as mitochondrial oxidative phosphorylation [18]. Several compounds of the flavonoid family are known to dissipate the mitochondrial proton gradient (*i.e.* uncoupling), while others have been shown to inhibit electron transport or ATPsynthase [18, 42, 51, 52]. Our recent study of the mechanism of action of medicinal plant products, in which we demonstrated that the extracts of seven species all acted through AMPK, revealed both uncoupling- and inhibitory-type activities [14]; interestingly, in most cases both types of disruption were observed concurrently, perhaps suggestive of a combination of active principles. In this study, the extract of *V. vitis* berries also induced an activation of AMPK that can be explained by a disruption of mitochondrial function. This disruption was observed to be purely of the inhibitory type, resulting in a mild decrease in the rate of ADP-stimulated oxygen consumption in isolated mitochondria, with no effect on the rate of basal consumption. As with species tested in our previous study, *V. vitis* berry extract did not stimulate the insulin-signaling pathway. These results reinforce the notion that disruption of energy transduction and subsequent activation of AMPK is a simple mechanism that may explain the activity of several antidiabetic plant products used by cultures throughout the world. It is a mechanism that likely requires less molecular specificity than the activation of the insulin receptor-signaling pathway. This pathway was found not to be stimulated by the extract of *V. vitis* berries, or by the plant products tested in our previous study.

Fractionation of *V. vitis* berry extract guided by muscle cell glucose uptake resulted in the isolation of quercetin-3-*O*-glycosides as main active principles. At 50 μM, these compounds enhanced basal glucose uptake by up to 59% following an 18 h treatment, an effect significantly greater than that of 100 nM insulin. These compounds were observed to increase the phosphorylation of ACC, thereby confirming that their mechanism of action was the same as that of the crude extract. However, unlike the crude extract, the quercetin glycosides failed to inhibit

mitochondrial respiration. In contrast, the aglycone of these compounds, a minor component of a less active fraction, was found to both stimulate the AMPK pathway and to potently inhibit the rate of ADP-stimulated oxygen consumption. Such an inhibitory effect of quercetin on ATP synthase has been reported by others and has recently been attributed to direct binding of quercetin to the F1-ATPase [53]. The sugar moiety of the glycosides reduces the lipophilicity of quercetin and may therefore prevent the compound from permeating the mitochondrial inner membrane. Indeed, it is widely accepted that flavonoids are often glycosylated in plants as a mechanism for facilitating their handling or sequestration. It is also possible that the hydroxyl group at position 3, replaced by the sugar moiety, is essential for the activity of quercetin. As the amount of quercetin aglycone contained in the extract is insufficient to account for the inhibition of mitochondrial respiration and the activation of AMPK, these findings suggest that quercetin-glycosides may be hydrolyzed to the aglycone form in order to become active. A less likely alternative is that quercetin glycosides may activate AMPK directly without inducing metabolic stress. In any case, the combined action of quercetin and its 3-*O*-glycosides appear to underlie the majority of the action of *V. vitis* on muscle cell glucose transport.

Quercetin does not appear to produce the dangerous side effects that can occur with powerful disruptors of oxidative phosphorylation. First, quercetin did not increase the rate of extracellular acidification, a marker of the contribution of anaerobic glycolysis to ATP synthesis. Second, quercetin did not decrease cytosolic ATP concentration following 1 or 3 h of treatment. Both observations support the notion that the metabolic stress induced is of low magnitude and short-lived, not affecting ATP concentration nor requiring a significant upregulation of glycolysis. Interestingly, ATP concentration was actually increased above normal by treatment with quercetin. This may be explained if the AMPK-derived signal for increased ATP synthesis through lipid and carbohydrate oxidation is longer-lived than the metabolic stress itself, resulting in an overshoot of ATP content. This ATP surfeit may also account for the observed tendency towards a paradoxically reduced rate of flux through anaerobic glycolysis. Together, these results can also be taken to indicate that quercetin is an easily metabolized compound, a conclusion supported by pharmacokinetic studies [22].

5 Concluding remarks

In summary, the results presented here demonstrate that quercetin and quercetin glycosides are active principles responsible for the enhancement of muscle cell glucose uptake by the extract of *V. vitis* berries. Quercetin and quercetin glycosides exert antidiabetic activity through the AMPK signaling pathway, activated as a response to the action of the quercetin aglycone on mitochondrial energy transduction. This mechanism concords with the anti-hyperglycemic activity of quercetin reported by others [54, 55]. Quercetin and quercetin glycosides are found in the berries of other members of the *Vaccinium* family used against diabetes and are likely to be active principles in these species as well. Preparation of *V. vitis* berries hold good potential for the treatment of diabetes in Canadian aboriginal populations.

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The authors have declared no conflict of interest.

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