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In vitro inhibition of metabolism but not transport of gliclazide and repaglinide by Cree medicinal plant extracts

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ABSTRACT

Ethnopharmacological relevance: Interactions between conventional drug and traditional medicine therapies may potentially affect drug efficacy and increase the potential for adverse reactions. Cree traditional healing is holistic and patients may use medicinal plants simultaneously with the conventional drugs. However, there is limited information that these medicinal plants may interact with drugs and additional mechanistic information is required. In this study, extracts from traditionally used Cree botanicals were assessed for their potential interaction that could alter the disposition of two blood glucose lowering drugs, gliclazide (Diamicron) and repaglinide (Gluconorm) though inhibition of either metabolism or transport across cell membranes.

Materials and methods: The effect of 17 extracts on metabolism was examined in a human liver microsome assay by HPLC and individual cytochrome P450s 2C9, 2C19, 2C8 and 3A4 in a microplate fluorometric assay. Gliclazide, rhaponticin and its aglycone derivative, rhapontigenin were also examined in the fluorometric assay. The effect on transport was examined with 11 extracts using the intestinal epithelial Caco-2 differentiated cell monolayer model at times up to 180 min.

Results: Both blood glucose lowering medications, gliclazide and repaglinide traversed the Caco-2 monolayer in a time-dependent manner that was not affected by the Cree plant extracts. Incubation of the Cree plant extracts inhibited CYP2C9, 2C19, 2C8 and 3A4-mediated metabolism, and the formation of four repaglinide metabolites: M4, *m/z* 451-A, *m/z* 451-B and the glucuronide of repaglinide in the human liver microsome assay. Gliclazide caused no significant inhibition. Likewise, rhaponticin had little effect on the enzymes causing changes of less than 10% with an exception of 17% inhibition of CYP2C19. By contrast, the aglycone rhapontigenin showed the greatest effects on all CYP-mediated metabolism. Its inhibition ranged from a mean of 58% CYP3A4 inhibition to 89% inhibition of CYP2C9. While rhaponticin and the aglycone did not show significant effects on repaglinide metabolism, they demonstrated inhibition of gliclazide metabolism. The aglycone significantly affected levels of gliclazide and its metabolites.

Conclusion: These studies demonstrate that the Cree plant extracts examined have the potential *in vitro* to cause drug interactions through effects on key metabolic enzymes.

1. Introduction

Cree traditional healing for Canadian aboriginal populations, such as the Cree of Eeyou Istchee (CEI), who inhabit the James Bay area in northern Quebec, is holistic and may use medicinal plants simultaneously with conventional pharmaceuticals. Diabetes is one of the greatest health concerns in this population as the prevalence of Type 2 diabetes mellitus is now two to five times greater than that of the general Canadian population (Kuzmina et al., 2010). In collaboration with the James Bay Cree healers and Elders, it was demonstrated that many of these Cree plants have effectiveness in diabetes assays (Harbilas et al., 2009; Spoor

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et al., 2006) and in animal trials (Harbilas et al., 2012a, 2012b, 2013).

Reports of notable drug interactions with grapefruit juice, St. John's Wort, as well as other natural products and substances have demonstrated that there exists a major concern about the safe use of traditional remedies, including Cree medicinal plants, which may have consequences for the patient and the health care system with increased risk of adverse events, hospitalization and related costs (Bailey and Dresser, 2004; Eagling et al., 1999; Foster et al., 2001; Pal and Mitra, 2006; Rengelshausen et al., 2005; Wenk et al., 2004; Xu et al., 2008). Often, this interaction occurs at the level of drug metabolism mediated mainly by the cytochrome P450 (CYP) family of enzymes as they are largely responsible for xenobiotic Phase I metabolism. Consequently, interference with their activity can result in issues of safety and efficacy with the drugs. Inhibition of a CYP enzyme may inhibit drug metabolism leading to an increase in plasma concentration beyond the drug's therapeutic range. For pharmacologically active drugs, this may cause an accumulation of the drug to toxic levels. Meanwhile, inactive pro-drugs or plant conjugated substances which require biotransformation to the active form may remain inactive and thus, ineffective. While most of the Cree extracts examined previously showed low to moderate potential for inhibition, several were ranked as strong inhibitors, and some were mechanism-based inactivators that could affect the bioavailability and pharmacokinetics of traditional and conventional drugs leading to toxicity or inefficacy (Tam et al., 2009, 2011). These previous studies, however, only dealt with *in vitro* enzyme inhibition, particularly addressing the P450 family of enzymes.

In this study, extracts of 17 plants identified by the healers (Fraser et al., 2007; Leduc et al., 2006) were assessed for their mechanistic potential for drug–drug interaction through the inhibition of individual metabolic enzymes, such as cytochrome P450s 2C9, 2C19, 2C8 and 3A4 and those present in a 50 donor mixed pool of human liver microsomes. Moreover, given the awareness that transport of these substances into the plasma may be the most critical factor in drug safety, the Caco-2 model with 1α, 25-dihydroxyvitamin D3 induction was used to model absorption and fate of test substances alone and in the presence of these extracts (Engman et al., 2001; Hubatsch et al., 2007; Schmiedlin-Ren et al., 1997). Finally, the effects of these medicinal plants were examined on activity of two representative conventional blood glucose lowering drugs, repaglinide and gliclazide. Repaglinide is known to be metabolized by CYP2C8 and CYP3A4 (Bidstrup et al., 2003). Gliclazide is metabolized primarily by CYP2C9 and CYP2C19 (Elliot et al., 2007) into several known inactive metabolites (Oida et al., 1985). Thus, if CYP2C9/2C19 activity was affected it could result in safety problems related to drug concentration. For example, rifampin, a known effecter of CYP2C9, when co-administered with gliclazide decreased the gliclazide plasma concentration by 70% with reduction in half-life by about 6.2 h (Park et al., 2003). Overall, this interaction caused a decrease in gliclazide glucose-lowering activity. Conversely, a rat interaction study with gliclazide and pravastatin, a known inhibitor of CYP2C9 and 3A4, or with gemfibrozil, a known substrate for CYP2C9, increased the hypoglycaemic effect of gliclazide probably due to decreased metabolism causing an increase in plasma levels of active drug (Sultanpur et al., 2010). We further extended this study to examine the effect of two of the major constituents present in tamarack (larch) (*Larix laricina*) (Shang et al., 2012), known in Cree as Watnagan, which is used as a traditional medicine across Canada to treat many conditions including jaundice, asthma, and tuberculosis (MacKinnon et al., 2009). In a previous study conducted by our research team, *Larix laricina* showed significant adipogenic activity in 3T3-L1 cells, where rhapontigenin and rhaponticin were found to be in part responsible for that activity (Shang et al., 2012). Thus, we investigated the *in vitro* interaction between rhaponticin and its derivative rhapontigenin on gliclazide to further establish the potential interactions that may occur in the Cree community. Rhaponticin is a glycoside stilbene compound and is also employed in Asian medicine for the treatment of pain, inflammation, allergies, and its antidiabetic properties (Chen et al., 2009; Choi et al., 2006; Matsuda et al., 2001). The glycoside is present in much higher concentration in *Larix laricina* than its aglycone, rhapontigenin, which is believed to be the active form of the molecule after the absorption step (Shang et al., 2012).

2. Experimental

2.1. Materials and sample preparation

The 17 Cree plant species identified in Table 1 were harvested in the Eastern James Bay region of Quebec, Canada following instructions given by the healers and Elders of the community. Plants were identified by Dr. A. Cuerrier and voucher specimens were deposited at the Marie-Victorin herbarium at the Montreal Botanical Garden, Montreal, Quebec, Canada (Leduc et al., 2006). Plants were subsequently extracted with ethanol, lyophilized,

Table 1
List of the 17 Cree antidiabetic plant extracts examined in this study.

Species [Voucher no.]	Common name	Cree name	Family
Abies balsamea (L.) Mill.[a] [Mis03-1]	Balsam fir	Innasht	Pinaceae
Alnus incana ssp. *rugosa* (Du Roi) Clausen[a] [Mis03-4]	Speckled alder	Atushpi	Betulaceae
Gaultheria hispidula (L.) Muhl. [Mis03-7]	Creeping snowberry	Pieuminaan	Ericaceae
Juniperus communis L. [Whap04-6]	Ground juniper	Kakachiiminatuk	Cupressaceae
Kalmia angustifolia L.[a] [Mis03-30]	Sheep laurel	Uishichipukw	Ericaceae
Larix laricina K.Koch[a] [Mis03-12]	Tamarack	Watnagan	Pinaceae
Lycopodium clavatum L. [Mis03-43]	Common clubmoss	Pashtnahoagin	Lycopodiaceae
Picea glauca (Moench) Voss. [Whap04-12]	White spruce	Minhikw	Pinaceae
Picea mariana (Mill.) BSP.[a] [Mis03-71]	Black spruce	Innahtikw	Pinaceae
Pinus banksiana Lamb.[a] [Mis03-14]	Jack pine	Ushchishk	Pinaceae
Populus balsamifera L. [Mis03-49]	Balsam poplar	Mitus	Salicaceae
Rhododendron groenlandicum (Oeder) Kron Judd[a] [Mis03-2]	Labrador tea	Kachichepukw	Ericaceae
Rhododendron tomentosum Harmaja[a] [Whap04-33]	Northern Labrador tea	Weeshichbuksh	Ericaceae
Salix planifolia Pursh [Whap04-37]	Tealeaf willow	Pieuatikw	Salicaceae
Sarracenia purpurea L.[a] [Mis03-5]	Pitcher plant	Ayigadash	Sarraceniaceae
Sorbus decora (Sarg.) Schneid.[a] [Mis03-9]	Showy mountain ash	Mushkuminanatikw	Rosaceae
Vaccinium vitis-idaea L.[a] [Whap04-21]	Mountain cranberry	Wishichimna	Ericaceae

[a] Extracts tested in the transport study.

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and analyzed for phytochemical markers as previously described (Spoor et al., 2006). The plant extracts used in the absorption assays were solubilised in 80% ethanol at a stock concentration of 100 mg/mL, and stored at −20 °C prior to use. The plant extracts used in the hepatosome assays were solubilised in 100% methanol at a stock concentration of 5 mg/mL and used within 1 week.

2.1.1. Chemicals and reagents

All solvents for HPLC analysis were optimal grade including methanol (MeOH) and acetonitrile (ACN) (Fisher Scientific, Ottawa, ON, Canada). Dibenzylfluorescein (DBF), 3-cyano-7-ethoxycoumarin (CEC), 7-methoxy-4-(trifluoromethyl)-coumarin (MFC), human liver microsomes (HLM) and microsomes derived from Baculovirus infected insect cells expressing CYP3A4 or CYP2C8/9/19 and CYP reductase were purchased from BD Biosciences (Mississauga, ON, Canada). Gliclazide, repaglinide, nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), uridine diphosphoglucuronic acid (UDPGA), alamethicin, sulfaphenazole, tranylcypromine and β-glucosidase were purchased from Sigma-Aldrich (Oakville, ON, Canada). Ketoconazole was purchased from Calbiochem (Gibbstown, NJ, USA). Rhaponticin and rhapontigenin were donated by J. T. Arnason, extracted from tamarack larch collected from wild sources as noted above. A stock concentration of 1 mg/mL of each compound was prepared in 100% MeOH. It was further diluted in water to have a final well concentration less than 1% MeOH. Since the quantity of rhapontigenin was limited to conduct further experimental work, additional material was prepared through an enzymatic degradation from rhaponticin.

2.1.2. Enzymatic synthesis of rhapontigenin

A reaction mixture was prepared by first adding 10 mg rhaponticin to 32 mL of sodium acetate buffer (pH of 5.0) in a 50 mL conical bottom plastic tube. The solution was sonicated for 10 min and then 16 mL of 1 mg/mL β-glucosidase solution in sodium acetate buffer was added. The solution was incubated at 37 °C in a shaking incubator for 15 h and afterwards extracted with CH_2Cl_2. The organic fraction was collected, dried with sodium sulfate and the CH_2Cl_2 was evaporated in a vacuum rotary evaporator. The remaining product was redissolved in chloroform and then allowed to dry at room temperature for two days. The conversion to rhapontigenin was confirmed by HPLC, and ^1H and ^{13}C NMR. A stock concentration of 1 mg/mL in 100% MeOH was prepared. It was further diluted in water to have a final well concentration less than 1% MeOH.

2.2. Caco-2 absorption assay

The C2BBe1 cell line, a clone of the Caco-2 colorectal adenocarcinoma cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 1% penicillin/streptomycin, and 0.01 mg/mL human transferrin. These cell culture reagents were purchased from Life Technologies (Burlington, ON, Canada). C2BBe1 cell suspension was then seeded onto 6-well Transwell membrane inserts (0.4 μm pore, 4.67 cm^2, polyester filter; Corning Costar Co., NY, USA) at a concentration of 5×10^5 cells per well, followed by an exchange of apical media within 16 h (Hubatsch et al., 2007). Media in the apical and basolateral chambers was exchanged every second day and 72 h prior to the start of the absorption assay. CYP3A4 expression was induced in the cells by 72 h prior treatment with 0.25 μM 1α,25-dihydroxyvitamin D3 (Sigma-Aldrich, Oakville, ON, Canada) in DMEM (Schmiedlin-Ren et al., 1997).

Absorption experiments were completed on the differentiated monolayers between 21 and 23 days after seeding. Transwell chambers were rinsed and equilibrated in PBS for 20 min at 37 °C on a rocker at low speed. Transepithelial electrical resistance (TEER) values were measured with a Millicell ERS-2 (Millipore, MA, USA) to ascertain monolayer integrity. Only monolayers with TEER values greater than 196 Ω cm^2 (Hubatsch et al., 2007) were used in the absorption assay. Integrity and distribution of the monolayer was further verified using immunofluorescence for ZO-1 antibody (Life Technologies, Burlington, ON, Canada), staining for tight junctions (results not shown).

Blood glucose lowering drugs, repaglinide and gliclazide were diluted in phosphate buffered saline (PBS), pH 7.2 (Life Technologies, Burlington, ON, Canada), to a final concentration of 50 μg/mL. Stock concentrations of solubilised plant extracts were further diluted to a working concentration of 100 μg/mL. In preliminary studies, this concentration was shown to be non-cytotoxic, and believed to cause no physical damage to cells. Donor solutions were also prepared with combinations of plant extracts and each of the blood glucose lowering drugs to the same final concentrations in PBS. Buffer or donor solution was placed into both basolateral and apical chambers; 2.6 mL of PBS was placed into the basolateral (receiver) chamber, and 1.5 mL of donor solution was placed in the apical chamber. Chambers were incubated at 37 °C on a rocker at low speed, and 200 μL samples were removed from the basolateral chamber at 0.25, 0.5, 1, 2, and 3 h following loading. After each sample was removed, an equal volume of PBS was added to the basolateral chamber to maintain hydrostatic pressure. At the final time point, 200 μL samples were also removed from the apical chambers to determine recovery. Samples collected from basolateral and apical chambers were mixed with an equal volume of 100% methanol, filtered with PTFE filters (0.2 μm pore; Chromatographic Specialties Inc., Brockville, ON, Canada). Each assay was performed in triplicate and results were analyzed for percent absorption of the blood glucose lowering drug across the Caco-2 monolayer, as defined by basolateral peak area divided by the initial apical peak area. Results were analyzed through a two-way ANOVA with a Bonferroni *post-hoc* test.

2.3. Human liver microsome-mediated metabolism

Human liver microsomes (HLM), 50 donor mixed pool (BD Biosciences, Mississauga, ON, Canada; Cat # 452156) were thawed in a 37 °C water bath, and then placed on ice until required. Medicinal plant extract at 50 μg/mL, control vehicle or 10 or 20 μg/mL of rhaponticin or rhapontigenin was used in an incubation system containing 100 mM phosphate buffer solution (pH 7.4), 21.6 μM NADPH, 40 μM $MgCl_2$, 44.2 μM repaglinide and 2 mg/mL HLM at a final volume of 500 μL. The reaction mixture was incubated for 90 min in a 37 °C shaking incubator set at 200 rpm. All reaction mixtures were stopped by the addition of 500 μL of ethyl acetate and the organic layers were extracted twice and combined. The solvents were then evaporated with a SpeedVac and the residues were re-dissolved in 500 μL of MeOH. The samples were filtered into HPLC vials using 0.2 μm PTFE filters and analyzed by HPLC. Methanol was used as vehicle control and ketoconazole as positive control at 0.4 μM.

2.3.1. UDP-glucuronosyltransferase-mediated metabolism

HLM were thawed in a 37 °C water bath, and then placed on ice until required. A 5 μL aliquot of a 5 mg/mL plant extract or control vehicle was added to 50 μL of 50 mM Tris buffer (pH 7.5), 12.7 μM alamethicin, 2 mM of UDGPA, 22.1 μM of repaglinide, 100 μM of $MgCl_2$ and 2 mg/mL HLM at a final volume of 500 μL. The reaction mixture was incubated for 40 min in a 37 °C shaking incubator set

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at 200 rpm and stopped with 250 μL of ice cold CH_3CN. The reaction mixture was filtered through a 0.2 μm PTFE filter before HPLC analysis. Methanol was used as a vehicle control. Results were reported as percent inhibition.

2.4. Cytochrome P450 inhibition assay

The assays were performed in triplicate in 96-well plates with white walls and clear, flat bottoms under red-colored light to minimize the exposure of photosensitive material to fluorescent light. The fluorescence was measured using a Cytofluor 4000 Fluorescence Measurement System (Applied Biosystems, Foster City, CA). For CYP3A4, a volume of 10 μL of the test compound, 10 nM CYP3A4, 1 μM DBF (dissolved in acetonitrile) and 0.6 mM NADPH were incubated in 0.19 M phosphate buffer solution (buffer, pH 7.4) at a final volume of 200 μL for 20 min. The initial and final fluorescence was read at 485 nm excitation and 530 nm emission with a gain of 50. The alcohol extracts were diluted five-fold in water prior to testing. The positive inhibitor used was 1.9 μM ketoconazole dissolved in MeOH.

Similar methods to the CYP3A4 inhibition assay were used for CYP2C9*1, CYP2C19*1 and CYP2C8. For CYP2C9*1, the mixture included Tris buffer (pH of 7.4), 10 nM CYP2C9*1, 100 μM 7-MFC (dissolved in acetonitrile) and 100 μM sulfaphenazole as the positive control. The initial and final fluorescence was read at 409 nm excitation and 530 nm emission with a gain of 80, with an incubation time of 60 min. For CYP2C19*1, the mixture included 10 nM CYP2C19*1, 25 μM CEC (dissolved in acetonitrile) and 100 μM tranylcypromine as the positive control. The initial and final fluorescence was read at 409 nm excitation and 460 nm emission with a gain of 65, with an incubation time of 60 min. For CYP2C8, the mixture included 20 nM CYP2C8, 2 μM DBF and 10 μM ketoconazole as the positive control. The initial and final fluorescence was read at 485 nm excitation and 530 nm emission with a gain of 60, with an incubation time of 60 min.

Initial fluorescence was subtracted from respective final fluorescence for the calculations. The percent inhibition of each extract was calculated relative to the CYP activity with the water vehicle control.

2.5. Cytochrome P450-mediated metabolism

A reaction mixture was prepared containing 30 μL of CYP2C19, 2.42 mM NADPH, 6 mM $MgCl_2$ and 70 μL Tris buffer (pH 7.4) at a final volume of 500 μL. Gliclazide at 10 μg/mL was tested alone or with 20 μg/mL of rhaponticin or rhapontigenin. The reaction was incubated at 37 °C in a shaking incubator set at 200 rpm for 90 min and then stopped by adding 500 μL of ACN. The mixture was then centrifuged and the supernatant was filtered through a 0.2 μm PTFE filter and analyzed by HPLC.

2.6. High performance liquid chromatography (HPLC-DAD)

A 10 μL aliquot of the prepared samples was injected into a Phenomenex Synergi MaxRP column (4 μm particle size, 250 mm × 2 mm; Phenomenex, Torrence, CA, USA) in an Agilent 1100 Series HPLC system with a diode array detector (DAD). The elution method initially had a ratio of 0.1% acetic acid:acetonitrile (ACN; 95:5 v/v) and a gradient change to a ratio of 40:60 at 15 min. The column was washed with 100% ACN at 20 min, returned to initial conditions at 25.1 min and re-equilibrated for 5 min. Flow rate was set at 0.4 mL/min, and column temperature was 55 °C. A wavelength of 245 nm was used to monitor repaglinide and any metabolites. Gliclazide separation was completed with an initial ratio of 95:5 for 15 min followed by gradient changes to 60:40 (v/v) for 5 min and then 0:100 (v/v) at 20 min, at a column temperature of 55 °C, a flow rate of 0.4 mL/min at 235 nm.

2.7. LC/MS/MS of metabolites

LC/MS/MS was used to identify the metabolites of repaglinide. Analysis was undertaken by injecting 5 μL of incubation matrix into an Agilent 1200 series high performance liquid chromatography (Agilent Technologies, Santa Ana, CA, USA) with an AB SCIEX 3200 QTRAP® triple quadrupole/ion trap mass spectrometer System (AB SCIEX, Foster City, CA, USA) using the above HPLC conditions. The mass spectrometer was operated with electrospray ionization (ESI) in positive mode using a scanning mass range of 50–600 amu.

3. Results

3.1. Caco-2 absorption

Both blood glucose lowering medications, gliclazide and repaglinide at 50 μg/mL, were absorbed through the Caco-2 cell membrane into the receiver chamber in a time-dependent manner (Figs. 1 and 2). The maximum mean absorption observed at 3 h was 36.3 ± 2.2% for gliclazide and 27.4 ± 7.1% for repaglinide. When gliclazide was co-administered with 100 μg/mL of 11 of the extracts (Fig. 1), the time course with extracts present was not significantly different from that of gliclazide alone. When co-administration of extracts with repaglinide was examined (Fig. 2), each of the 11 extracts showed a time-dependent increase in concentration of repaglinide, but most results were not

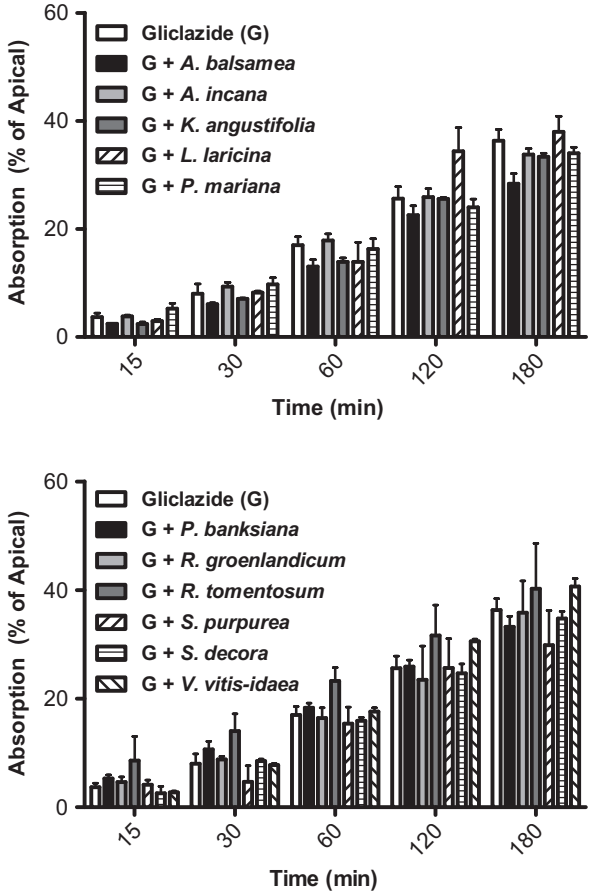

Fig. 1. Absorption of gliclazide (50 μg/mL) alone and in the presence of Cree plant extracts at a concentration of 100 μg/mL through the intestinal epithelial Caco-2 monolayer. Results are mean percent absorption ± SEM (n=3).

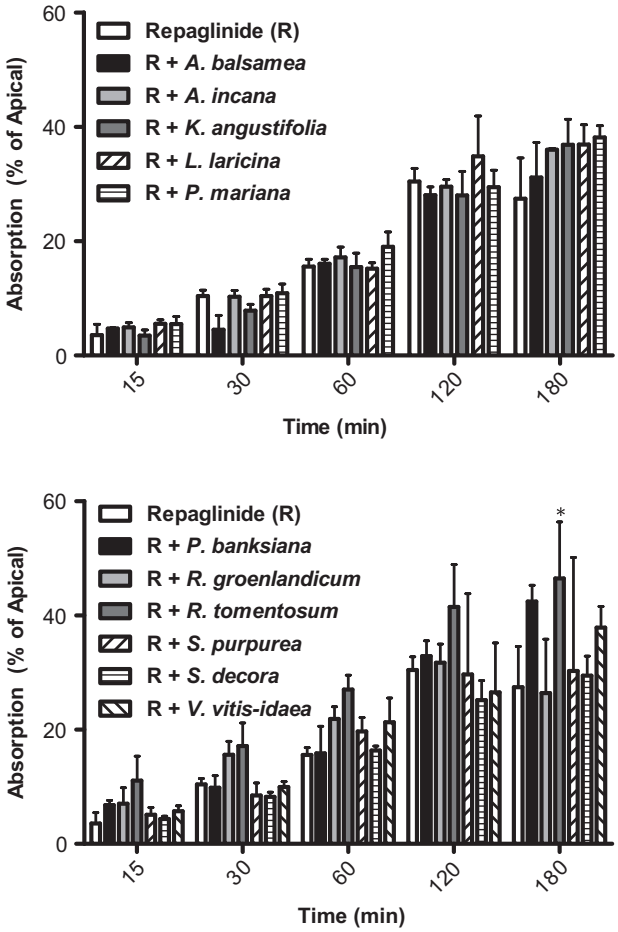

Fig. 2. Absorption of repaglinide (50 μg/mL) alone and in the presence of Cree plant extracts at a concentration of 100 μg/mL through the intestinal epithelial Caco-2 monolayer. Results are mean percent absorption ± SEM ($n=3$).

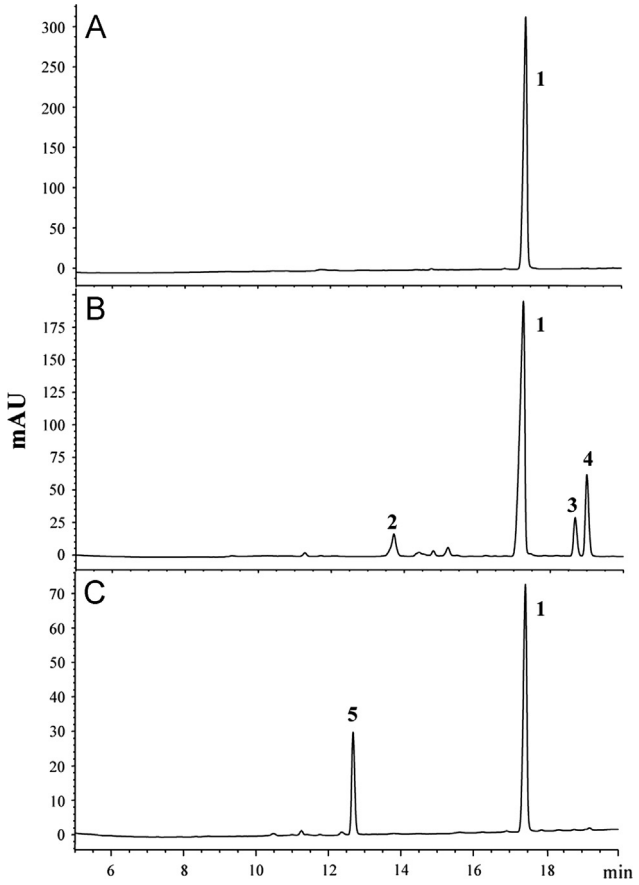

Fig. 3. A representative HPLC trace of human liver microsome-mediated metabolisms of repaglinide. (A) Repaglinide standard. (B) CYP2C8 and CYP3A4 metabolites. (C) UDP-glucosyltransferase metabolite. (**1**) repaglinide, (**2**) repaglinide M4, (**3**) *m/z* 451-A, (**4**) *m/z* 451-B and (**5**) glucuronide metabolite.

significantly different from repaglinide alone. Only *Rhododendron tomentosum* showed a significant difference ($p<0.05$) towards increasing the percent of repaglinide absorbed.

3.2. Human liver microsome-mediated metabolism of repaglinide

Four metabolites of repaglinide with distinct retention times were detected by HPLC (Fig. 3). The identity of these known metabolites was confirmed through QTRAP analysis as: (**2**) repaglinide M4, (**3**) *m/z* 451-A, (**4**) *m/z* 451-B and (**5**) glucuronide metabolite (Gan et al., 2010). The individual co-administration of the 17 extracts had an inhibitory effect on the formation of the repaglinide metabolite M4 to a different extent (Fig. 4A). The most potent M4 inhibitors, as demonstrated by more than 80% inhibition were *Abies balsamea*, *Alnus incana*, *Juniperus communis*, *Kalmia angustifolia*, *Larix laricina*, *Picea mariana*, *Pinus banksiana*, *Rhododendron groenlandicum*, *Rhododendron tomentosum*, *Salix planifolia* and *Sorbus decora*. Moderate inhibitors, as demonstrated by inhibition between 50% and 80% were *Gaultheria hispidula*, *Picea glauca*, *Populus balsamifera* and *Sarracenia purpurea*, while only two extracts were mild inhibitors (<50%) *Lycopodium clavatum* and *Vaccinium vitis-idaea*. Ketoconazole (0.4 μM), a potent CYP3A4 inhibitor, had no effect on the production of M4.

The extracts of *Abies balsamea*, *Alnus incana*, *Kalmia angustifolia*, *Larix laricina*, *Picea mariana*, *Pinus banksiana*, *Rhododendron groenlandicum*, *Rhododendron tomentosum*, *Salix planifolia*, and *Sorbus decora* were more potent inhibitors of the *m/z* 451-A and *m/z* 451-B metabolites (Fig. 4B and C). *Gaultheria hispidula*, *Juniperus communis*, *Lycopodium clavatum*, *Populus balsamifera*, and *Sarracenia purpurea* were moderate inhibitors of both metabolites. *Vaccinium vitis-idaea* was only a mild inhibitor of both metabolites. *Picea glauca* was a moderate inhibitor of *m/z* 451-B and a mild inhibitor of *m/z* 451-A. Ketoconazole had an inhibitory effect of about 30% on the formation of each metabolite.

The plant extracts at 50 μg/mL were not potent inhibitors of glucuronidation (Fig. 5). Only *Abies balsamea*, *Kalmia angustifolia*, *Lycopodium clavatum*, *Rhododendron groenlandicum*, *Rhododendron tomentosum*, and *Sorbus decora* showed inhibition greater than 30%. *Gaultheria hispidula*, *Populus balsamifera*, and *Vaccinium vitis-idaea* exhibited no inhibition.

3.3. Cytochrome P450-mediated metabolism of gliclazide

Gliclazide, rhaponticin and its aglycone derivative, rhapontigenin, were examined for their effect on CYP-mediated metabolism in the fluorometric microplate assay (Fig. 6). Gliclazide under these study conditions did not inhibit CYP2C9 and 2C19 activity. The gliclazide inhibition values for CYP3A4 and 2C8 were low at 9% and 16%, respectively. However, gliclazide was readily metabolized by CYP2C19 to at least 5 major (M1 to M5) metabolites (Fig. 7). Metabolite M5 was tentatively identified on the basis of its mass spectral properties as methylhydroxygliclazide. Rhaponticin, at 10 μg/mL, was found to have little inhibitory effect, causing changes of less than 10% inhibition with an exception of 17% inhibition of CYP2C19. By contrast, 10 μg/mL rhapontigenin showed greater inhibitory effects, ranging from a mean of 58% CYP3A4 inhibition to 89% inhibition of CYP2C9. The positive

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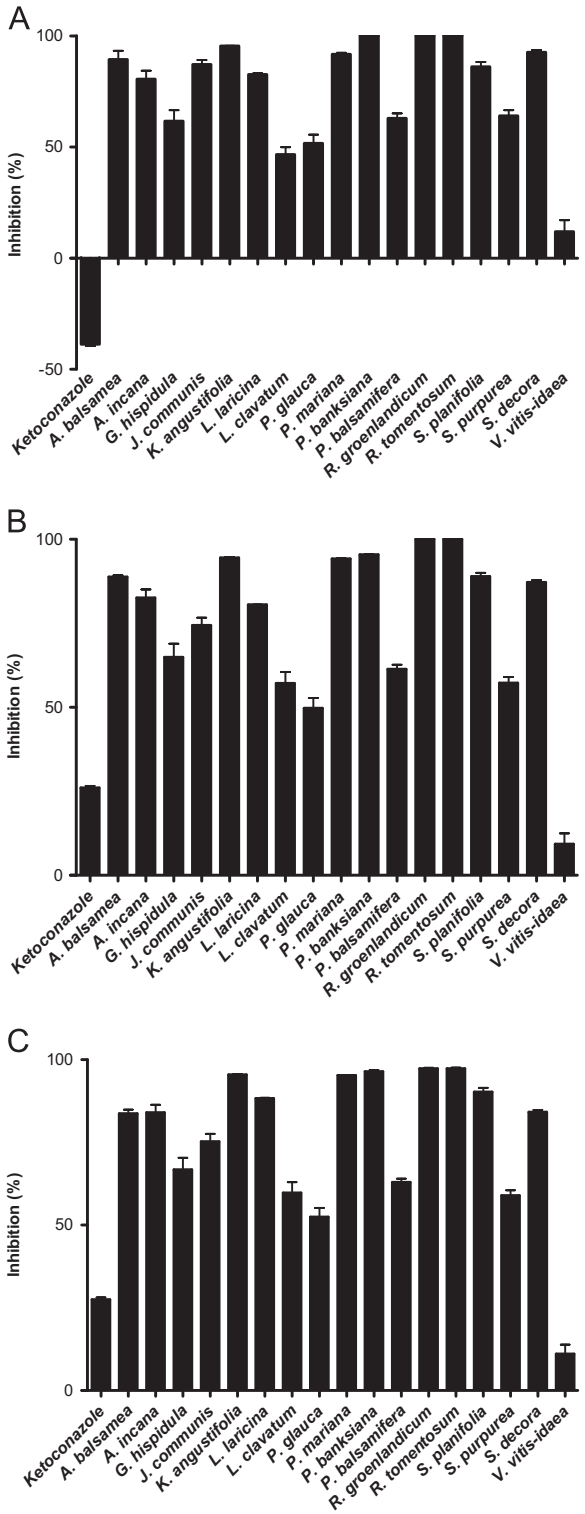

Fig. 4. Metabolism of repaglinide (20 μg/mL) following co-incubation with Cree medicinal plant extracts at a concentration of 50 μg/mL by human liver microsomes. (A) Percent inhibition of repaglinide M4 metabolite. (B) Percent inhibition of *m/z* 451-A metabolite. (C) Percent inhibition of *m/z* 451-B metabolite. Ketoconazole (0.4 μM) was the positive control.

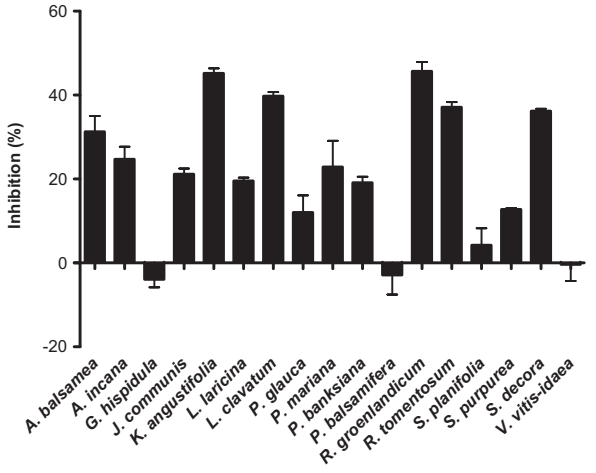

Fig. 5. Inhibition of UDP-glucuronosyltransferase-mediated metabolism of repaglinide (10 μg/mL) by Cree plant extracts at a concentration of 50 μg/mL.

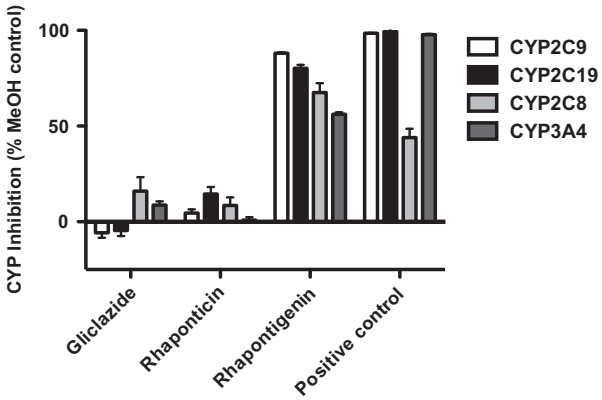

Fig. 6. Effect of gliclazide, rhaponticin and its aglycone derivative, rhapontigenin (10 μg/mL) on CYP2C9-, 2C19-, 2C8- and 3A4-mediated metabolism of a marker substance in a 20 min or 60 min microtitre fluorometric assay. The results were expressed relative to a MeOH vehicle control as mean CYP inhibition ± SEM. Controls: CYP2C9, sulfaphenazole (100 μM, $n=9$); CYP2C19, tranylcypromine (100 μM, $n=6$); and 3A4 and 2C8, ketoconazole (1.9 μM, $n=15$ for 3A4 and 10 μM, $n=3$ for 2C8).

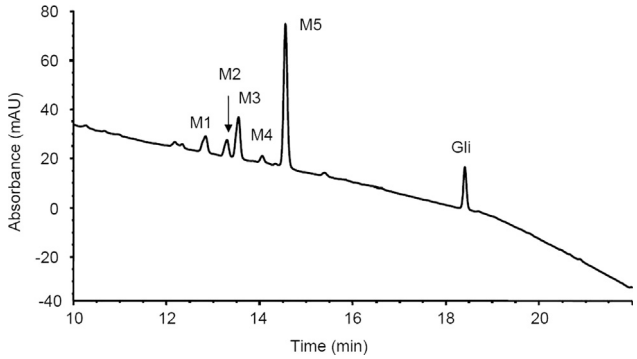

Fig. 7. HPLC trace of gliclazide (Gli, 10 μg/mL) and metabolites following 90 min metabolism by CYP2C19. The metabolites (M1-M5; eluted at min 12.8, 13.3, 13.5, 14.1 and 14.6) and Gli eluted at 18.4 min.

controls used, ketoconazole, sulfaphenazole and tranylcypromine completely inhibited enzyme activity of CYP3A4, 2C9 and 2C19 respectively and ketoconazole reduced activity of CYP2C8.

Varying concentrations of rhapontigenin (from 0.3 μM to 38.7 μM) were then tested to establish the 50% inhibitory concentration (IC_{50}) values for CYP2C9, 2C19 and 3A4 (Fig. 8). Rhapontigenin was a potent inhibitor of CYP2C9 with an IC_{50} value of 2.7 μM (0.7 μg/mL). The IC_{50} value was 7 μM (1.8 μg/mL) for CYP2C19 and 30 μM (7.7 μg/mL) for CYP3A4.

Rhaponticin and rhapontigenin had a minimal effect on HLM metabolism of repaglinide (Table 2). Rhaponticin and

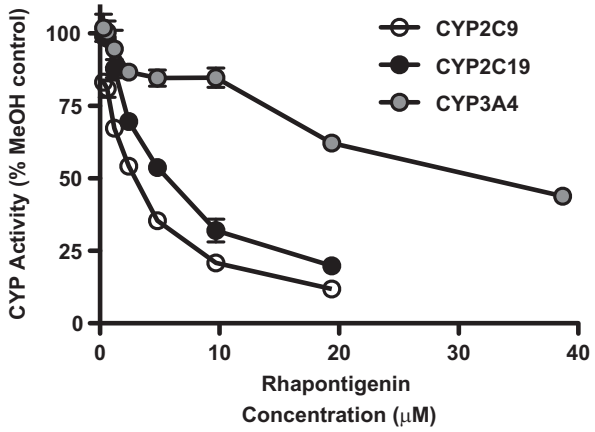

Fig. 8. Inhibition effect of various concentrations of rhapontigenin on CYP2C9-, 2C19- and 3A4-mediated metabolism. The results were expressed relative to a MeOH vehicle control as mean CYP activity.

Table 2
Effect of rhaponticin and rhapontigenin on repaglinide metabolism in human liver microsomes. The results are expressed as ratios of the metabolites and parent compound relative to the repaglinide alone control peak areas ($n=3$, ± SEM).

Compound	Relative ratios			
	M4	Repaglinide	*m/z* 451-A	*m/z* 451-B
Rhaponticin (47.6 μM)	0.89 ± 0.01	1.03 ± 0.06	0.98 ± 0.02	0.98 ± 0.01
Rhapontigenin (38.7 μM)	0.75 ± 0.10	1.10 ± 0.12	0.86 ± 0.08	0.88 ± 0.12
Rhapontigenin (77.4 μM)	0.85 ± 0.01	1.17 ± 0.03	0.92 ± 0.01	0.88 ± 0.01

Table 3
Effect of rhaponticin and rhapontigenin on CYP2C19-mediated metabolism of gliclazide. The results are expressed as ratios of the parent compound and the metabolite M5 relative to the gliclazide control peak area ($n=3$, ± SEM).

Compound	Metabolite M5	Gliclazide
Rhaponticin	0.79 ± 0.01	1.21 ± 0.13
Rhapontigenin	0.09 ± 0.01	1.9 ± 0.25

rhapontigenin had a marked effect on the CYP2C19 mediated-metabolism of gliclazide (Table 3) as seen by the increase ratio of gliclazide and decreased ratio of the major metabolite.

4. Discussion

In this study, 17 extracts of medicinal plants used by Cree healers for the treatment of diabetes-related symptoms (Fraser et al., 2007; Leduc et al., 2006) were assessed for their potential to interact with two blood glucose lowering drugs. Several Cree medicinal plants had been previously shown to be strong inhibitors of various cytochrome P450 isozymes in cell-free models (Tam et al., 2009). Hence, it was of interest to determine if the co-administration of these extracts would also alter disposition, the total effect of both metabolism and transport, of two oral hypoglycaemic drugs, gliclazide and repaglinide in cell-free and whole cell models. HLM were selected as these microsomes have a wide Phase I and II metabolic capacity. Vitamin D3 pre-treated Caco-2 cells were also chosen because they should have a greater expression of some metabolic enzymes and transport proteins, thereby increasing their relevance as a test model (Engman et al., 2001; Hubatsch et al., 2007; Schmiedlin-Ren et al., 1997). Together, these models provide a greater understanding of the overall disposition of the test substances. In particular, the interest was with *Sorbus decora*, *Larix laricina*, and *Abies balsamea*, boreal forest species previously shown to be strong inhibitors of both CYP2C9 and CYP2C19 (Tam et al., 2009). Likewise, *Sorbus decora* was a very potent inhibitor of CYP2C8 and *Rhododendron groenlandicum* and *Picea mariana* potent inhibitors of CYP3A4, both of which are important enzymes in the metabolism of repaglinide.

Baseline absorption of repaglinide and gliclazide through Caco-2 cell monolayers in the present studies was comparable to prior reports where bioavailability of repaglinide following oral administration was between 44.7% and 62.1% (Hatorp et al., 1998). The absorption of gliclazide was expected to vary depending on physiological or assay conditions, while being low overall (Amidon et al., 1995; Campbell et al., 1991). The experiment was designed to detect any significant interactions that may occur between the extracts and the two substances. Under the conditions used in this study with vitamin D3 pre-treated differentiated Caco-2 cells, none of the extracts had an effect on the recovery of either gliclazide or repaglinide in the basolateral chamber. In this whole cell model, disposition is affected by the relationship between the inhibition or up regulation of metabolic enzymes or transport proteins. The net outcome of this complex relationship was reflected by the total amount of substance and its metabolites present in the basolateral chamber. The fact that extracts of the selected Cree medicinal plants did not alter the overall disposition of these drugs may be attributed to several factors. Firstly, it is possible that the phytochemicals present in these extracts, mainly phenolics with some terpenes, have no significant effect on the transport or intestinal metabolism of either drug studied. Secondly, it is possible that plant compounds were not present in sufficiently high concentration to affect such drug metabolism or transport in the pre-treated Caco-2 cells. Nonetheless, these results suggest that Cree plants exhibit a general overall safety at the level of intestinal drug disposition, at least as assessed in an *in vitro* model. The only exception was observed with *Rhododendron tomentosum*, whose extract significantly increased the trans-epithelial transport of repaglinide. Further studies will be needed to determine which component(s) of the plant and which mechanism(s) of action are involved.

Under the present experimental conditions, not all known metabolites of repaglinide were detected in the HLM-mediated biotransformation assay (Bidstrup et al., 2003; Gan et al., 2010). However, the major metabolites M4, *m/z* 451-A and *m/z* 451-B, as well as the glucuronidation product were detected. It is noteworthy that most of the extracts examined showed potent inhibition of repaglinide metabolism. The production of the M4 metabolite, produced primarily through CYP2C8, was strongly inhibited by *Abies balsamea*, *Alnus incana*, *Juniperus communis*, *Kalmia angustifolia*, *Larix laricina*, *Picea mariana*, *Pinus banksiana*, *Rhododendron groenlandicum*, *Rhododendron tomentosum*, *Salix planifolia* and *Sorbus decora*. The extracts were more potent inhibitors than the positive control, ketoconazole. *Abies balsamea*, *Alnus incana*, *Kalmia angustifolia*, *Larix laricina*, *Picea mariana*, *Pinus banksiana*, *Rhododendron groenlandicum*, *Rhododendron tomentosum*, *Salix planifolia*, and *Sorbus decora* were potent inhibitors of both *m/z* 451-A and B metabolites. *Vaccinium vitis-idaea* was the least potent inhibitor of all three metabolites. The results of the present studies are generally consistent with those described for the inhibition of recombinant enzymes in cell-free assays (Tam et al., 2009, 2011). Hence, several Cree plant extracts continue to show a potential for pharmacokinetic interactions through an inhibition of cytochrome P450-dependent liver drug metabolism, this time in a more biologically relevant model provided by HLM. In addition, the extracts also exhibited an inhibitory effect on the

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production of the glucuronide metabolite of repaglinide, possibly indicating non-specific inhibition of other metabolic enzymes (Ito et al., 1998).

On the other hand, gliclazide was found to be a substrate for CYP2C19 but did not inhibit CYP-mediated metabolism in the test models used herein. Interestingly, the aglycone rhapontigenin had a strong inhibitory effect on CYP2C19-mediated metabolism of gliclazide. This supports previous studies that with some substances the glycone is less active than the aglycone. Hence, the possible presence of a glycone and its corresponding aglycone must be taken into consideration when determining the activity of a medicinal plant extract. Given that tamarack contains the glycone, the results suggest that *Larix laricina* preparation has the potential to enhance the hypoglycaemic effect of gliclazide if co-administered with the drug. Finally, several other factors should also be considered when extrapolating to a potential clinically relevant effect. Firstly, the test models used herein represent a significantly greater level of biological relevance and complexity than recombinant enzymes; yet they also have limitations. Secondly, traditional Cree plant preparations are based on hot water extracts rather than with ethanol, which is known to be a better solvent to concentrate plant compounds such as phenolics. Lastly, the plant extract and phytochemical concentrations used herein may be different from what can be obtained in a clinical setting.

5. Conclusions

The extracts of these selected medicinal plants from the Cree traditional pharmacopeia do affect hepatic metabolism *in vitro*, notably those affecting relevant oral hypoglycaemic drugs, without having any apparent effect on intestinal drug disposition. Although the present results raise a potential safety concern, *in vivo* and clinical evidence are needed to confirm that Cree plant preparations can alter the metabolic profile of the tested oral hypoglycaemic drugs. Indeed, the effects of the pure substances observed in the present studies, as well as those of unhydrolyzed boreal forest plant extracts, provide substantive evidence and a mechanistic basis that warrants clinical evaluation of these Cree medicinal plants, particularly tamarack, which may be taken by patients simultaneously with oral hypoglycaemics and other drugs.

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