Evaluation of safety and efficacy of traditional Cree medicinal plants

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Abstract

This thesis evaluated traditional medicines used by the Cree First Nation of Eeyou Istchee (James Bay Region, Quebec, Canada) for safety and efficacy in the context of Type 2 diabetes and Alzheimer's disease. Drug metabolism and absorption were evaluated using whole cell *in vitro* models: human liver microsomes and Caco-2 permeability assays, respectively. It was found that the 17 medicinal plant extracts had an effect on the cytochrome P450-mediated metabolism of one co-administered conventional antidiabetic therapy, repaglinide, while having no effect on another, gliclazide. Furthermore, 11 of the extracts were tested for absorption and had no effect on either conventional therapy.

One promising botanical, *Sarracenia purpurea* L. (Sarraceniaceae), was studied in detail. The phytochemistry of the traditional water preparation was compared to the more widely used ethanol extract. The concentrations of the 10 phenolic compounds present, as well as an active principle, the iridoid glycoside morroniside, were very similar between the two extracts, with generally slightly higher concentrations of phenolics in the ethanol extract as expected. However, two triterpenes, betulinic acid and ursolic acid, were 107 and 93 times more concentrated, respectively, in the ethanol extract compared to the water. Further analysis of safety of *S. purpurea* and morroniside was examined using Caco-2 cells, a model for first-pass metabolism, using 19K microarrays. Following 4-hour and/or 24-hour exposure to 100 µg/mL *S. purpurea* or 8.8 µg/mL morroniside, no transcript changes related to metabolism or drug transport were

significantly altered, thus further supporting its role as a generally safe botanical. However, when *S. purpurea* was examined in an *in vivo* model of Alzheimer's disease, the TgCRND8 (Tg) mice, it increased the frenetic behaviour observed in the Tg mice and further impaired learning and memory as examined by the Morris Water Maze. This impairment was associated with an increased level of the PC(O-16:0/2:0) lipid second messenger.

Taken together, these results suggest both potential safety and limitations of using Cree traditional medicines when co-administered with pharmaceuticals. While *in vitro* studies also suggested potential safety of *S. purpurea*, it impaired Tg mice *in vivo*. Furthermore, Cree elders clearly understand the limitations to their traditional therapy as they warned us not to use this plant medicine in a "weakened state".

Résumé

Cette thèse a évalué la sécurité et l'efficacité des médicaments traditionnels utilisés par la Première Nation Crie d'Eeyou Istchee (Baie James, Québec, Canada) dans le traitement du diabète de type 2 et de la maladie d'Alzheimer. La métabolisation et l'absorption de ces médicaments ont été évaluées en utilisant des modèles cellulaires *in vitro*: des microsomes hépatiques humains et des essais de perméabilité utilisant des cellules Caco-2, respectivement. Dix-sept des extraits de plantes médicinales ont eu un effet sur la voie métabolique du cytochrome P450 lorsque co-administré avec un traitement antidiabétique conventionnel, le répaglinide, et aucun effet sur l'autre, le gliclazide. De plus, 11 des extraits testés pour l'absorption n'ont eu aucun effet sur l'une ou l'autre des thérapies conventionnelles.

Une plante prometteuse, la *Sarracenia purpurea* L. (Sarraceniaceae), a été étudiée en détail. La phytochimie de la préparation traditionnelle dans l'eau a été comparée à celle extraite dans l'éthanol, qui est plus largement utilisé. La concentration de 10 composés phénoliques, ainsi que du principe actif, le morroniside, était très similaire entre les deux types d'extraction. Néanmoins, une légère augmentation des concentrations de composés phénoliques a été observée dans l'extrait d'éthanol. Pour ce qui est des triterpènes, l'acide bétulinique et l'acide ursolique, étaient respectivement 107 et 93 fois plus concentrés dans l'extrait d'éthanol par rapport à l'extrait d'eau. Une analyse plus approfondie de la sécurité de *S. purpurea* et du morroniside a été évaluée en utilisant des cellules Caco-2, un modèle pour le métabolisme du premier

passage, en utilisant des « microarrays » de 19K. Suite à une incubation de 4 et/ou 24 heures avec 100 µg/mL de *S. purpurea* ou 8,8 µg/mL de morroniside, aucune modification significative de transcriptions liée à la métabolisation ou au transport des médicaments a été observée, soutenant ainsi son rôle comme une plante généralement considérée sans danger. Toutefois, lorsque *S. purpurea* a été évalué dans un modèle *in vivo* de la maladie d'Alzheimer à l'aide des souris TgCRND8 (Tg), l'utilisation du labyrinthe d'eau de Morris a démontré une augmentation du comportement frénétique, ainsi que d'autres troubles d'apprentissage et de mémoire chez les souris Tg. Cette déficience pourrait être due à la présence accrue du second messager lipidique PC(O-16:0/2:0).

L'ensemble de ces résultats tend à démontrer la sécurité et les limitations potentielles de l'utilisation des médicaments traditionnels Cris lorsque co-administrés avec des produits pharmaceutiques. Bien que les études *in vitro* semblent démontrer la sécurité de *S. purpurea*, une détérioration de l'état de santé des souris modèles de la maladie d'Alzheimer a été observée *in vivo*. En outre, les aînés Cris connaissent les limitations de leur traitement traditionnel, puisqu'ils nous ont eux-mêmes avertis de ne pas utiliser cette préparation traditionnelle de la plante dans un « état de faiblesse ».

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Aβ – amyloid beta

ABCA2 – ATP-binding cassette, sub-family A (ABC1), member 2

ACC – acetyl Co-A carboxylase

ACN - acetonitrile

AD – Alzheimer's disease

AGE – advanced glycation endproducts

AMPK – adenosine-monophosphate-activated protein kinase

APP – amyloid precursor protein

ASI – adult social interaction

ATCC – American Type Culture Collection

AUC – area under the curve

BECN1 – beclin-1

CEC – 3-cyano-7-ethoxycoumarin

CEI – Cree of Eeyou Istchee

CIHR – Canadian Institutes of Health Research

CML – Nε-(carboxymethyl) lysine

CYP – cytochrome P450

DAD – diode array detector

DDI – drug-drug interactions

DFB – dibenzylfluorescein

DMEM – Dulbecco's Modified Eagle's Medium

DMSO – dimethyl sulfoxide

DPBS – Dulbecco's phosphate buffered saline

DPPH – diphenylpicrylhydrazyl

EPM – elevated plus maze

ESI – electrospray ionization

EST – expressed sequence tag

FBS – fetal bovine serum

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

GST – glutathione-S-transferase

GTT – glucose tolerance test

HLM – human liver microsomes

HPLC – high performance liquid chromatography

IPA – Ingenuity Pathway Analysis

ITT – insulin tolerance test

LC – liquid chromatography

LD – light dark

logP – partition coefficient

LPCAT – lysophosphatidylcholine acyltransferases

MBI – mechanism based inhibition

MeOH – methanol

MFC – 7-methoxy-4-(trifluoromethyl)-coumarin

MS – mass spectrometry

mTOR – mammalian target of rapamycin

MWM – Morris Water Maze

NADPH – nicotinamide adenine dinucleotide phosphate

NFκB – nuclear factor kappa B

NMR – nuclear magnetic resonance

nRT – negative RT control

OF – open field

PAF – platelet activating factor

PAF-AH – PAF-acetylhydrolase

PBS – phosphate buffered saline

PIS – precursor ion scan

PRKCD – protein kinase C, delta

qPCR – quantitative polymerase chain reaction

QR – quinone reductase

RAGE – receptor for advanced glycation endproducts

RIN – RNA integrity number

ROS – reactive oxygen species

RPLP0 – large ribosomal protein P0

RPM – revolutions per minute

RT – reverse transcription

SAM – significance analysis of microarray

SIM – selective ion monitoring

T2D – Type 2 diabetes

TAAM – Team in Aboriginal and Antidiabetic Medicines

TAP1 – transporter 1, ATP-binding cassette, sub-family B

TEER – transephithelial electrical resistance

Tg – TgCRND8 mice

UDPGA – uridine diphosphoglucuronic acid

UDPGT – UDP-glycosyltransferase

UV – ultra violet

Chapter 1: Introduction

1.1 Introduction to thesis

The Canadian Institutes of Health Research (CIHR) Team in Aboriginal and Antidiabetic Medicines (TAAM) was established to evaluate the safety and efficacy of the traditional Cree medicines used to treat symptoms of Type 2 diabetes (T2D) (Haddad et al., 2012) as a culturally appropriate approach to complementary health care in First Nation's communities. The research was divided among the six different labs from three different universities concentrating their efforts on 17 ethnobotanically identified traditional medicines. The contribution of this thesis was first to examine the safety of Cree traditional medicines in the context of their potential to cause drug interactions. A subsequent study focused on the safety and efficacy of a promising Cree antidiabetic plant *Sarracenia purpurea* L. in an animal model of Alzheimer's disease (AD).

1.2 Background and Literature Review

Diabetes in Cree of Eeyou Istchee

The prevalence of T2D in the Canadian population is about 6% (Public Health Agency of Canada, 2011), while this percentage is at least three to five times higher in the First Nations populations (Leduc et al., 2006). Therefore about 18-30% of the Aboriginal population has the disease, which is prevalent due to both genetic and environmental factors (Hegele and Bartlett, 2003; Hegele

et al., 1999; Young et al., 2000). The Aboriginal populations have become less physically active due to the advancement of technology and their food intake has drastically changed from a traditional diet to one with an increase in dietary fat and refined carbohydrates, both of which can lead to obesity and diabetes. Furthermore, studies show that there is very low compliance in the number of people that take conventional medicines (Young *et al*., 2000). In general, the First Nations populations prefer to take traditional medicines instead of conventional medicines, which is one reason why the low compliance is observed. Of particular interest to us is the Cree population of Eeyou Istchee (CEI) from Quebec, which has experienced this drastic increase in T2D prevalence. Particularly, Kuzmina et al. (2010) has now reported that more than one in five CEI adults has T2D. Because of the lack of success with conventional therapy, the Cree Health Board of Eeyou Istchee entered into collaboration with our university-based team, formally known as CIHR TAAM, to evaluate the safety and efficacy of traditional remedies as complementary therapies in T2D.

Cree pharmacopeia

An ethnobotanical survey was performed by Leduc et al. (2006) to examine which traditional medicines are used by the CEI to mitigate the symptoms of diabetes. Using a syndromic importance value methodology, which incorporates healer consensus as well as importance of symptoms, 17 species belonging to the Ericaceae, Pinaceae, Rosaceae, Betulaceae, Cupressaceae,

Salicaceae, Lycopodiaceae, and Sarraceniaceae families were prioritized for research. A list of the species can be found in Chapter 2, Table 2.1. The majority of the plant species were not previously explored for their antidiabetic potential with the exception of *Juniper communis* L., *Lycopodium clavatum* L. and *Vaccinium vitis-idaea* L. (Al-Mustafa and Al-Thunibat, 2008; Marles and Farnsworth, 1995; Sanchez de Medina et al., 1994), although they have been reported as medicinal plant species used by aboriginal populations of Canada (Arnason et al., 1981; Black, 1980; Leighton, 1985).

However, significant work done by the TAAM has now evaluated their antidiabetic potential, as well as potential mode of action. Spoor et al. (2006) and Harbilas et al. (2009) evaluated the crude extracts of all 17 plants for their *in vitro* antidiabetic activity via glucose uptake in both skeletal muscle cells (C2C12) and adipocytes (3T3-L1), as well as adipogenesis, also in the 3T3-L1 cells. Furthermore, glucose-simulated insulin secretion by pancreatic β cells and glucose toxicity and deprivation in PC-12 cells were also studied, as well as the ability of the extracts to scavenge free radicals in a diphenylpicrylhydrazyl (DPPH) assay. Fraser et al. (2007) further evaluated their antioxidant activity in three separate antioxidant assays and Nachar et al. (2013) examined their role in hepatic glucose homeostasis. Their ability to inhibit the formation of advanced glycation endproducts (AGEs), a common side effect of diabetes contributing to vascular complications, was also examined (Harris et al., 2011). While most of the extracts proved quite potent in one or more of the assays, *Alnus incana*, *Larix laricina*, *Sorbus decora*, *Populus balsamifera*, and *Vaccinium vitis-idaea* were

particularly potent and became the subject of further, more in depth, studies including bioassay-guided fractionation, as well as *in vivo* work (Beaulieu et al., 2010; Eid et al., 2010; Harbilas et al., 2012a; Harbilas et al., 2012b, 2013; Martineau et al., 2010b; Martineau et al., 2010c; Shang et al., 2012; Vianna et al., 2009). *Sarracenia purpurea* was also identified as one of the key plants requiring further study and is the focus of this thesis.

Sarracenia purpurea

Little is known about *Sarracenia purpurea* L. (Sarraceniaceae) (Figure 1.1), the pitcher plant, Ayigadash in Cree, and its medicinal properties in the scientific literature today. It is a plant readily found in bogs and peatlands, both nitrogen-poor environments, throughout North America (Ellison et al., 2012). Thus, in order to obtain the necessary nitrogen, as well as other essential nutrients, it has evolved a symbiotic relationship with microbes that live in its pitcher-forming leaves (Gray et al., 2012; Karagatzides et al., 2009). These are then able to break down and help absorb the nutrients from insects that have become trapped in the carnivorous plant's leaves.

Its first recorded medicinal uses date back to the 19th century, when both Miles (1862) and Grant (1864) reported it as a potent smallpox therapy. This activity was recently investigated by Arndt et al. (2012) who showed that *S. purpurea* acts on early viral transcription, thus inhibiting pox replication. Furthermore, Sarapin® (High Chemical Company, Levittown, PA, USA), a suspension of powdered pitcher plant in an alkaline solution, has been used for

Figure 1.1: *Sarracenia purpurea* (Northern Quebec; Photo credit: Cory Harris)

many years as an analgesic to relieve pain of neuralgic origin (Manchikanti et al., 2001; Rask, 1984). However, no previous antidiabetic activity has been reported for the plant until the team's investigation.

Spoor et al. (2006) reported an insulinomimetic activity of *S. purpurea* in C2C12 skeletal muscle cells at a concentration of 100 µg/mL. At this concentration, the basal uptake of glucose was increased by 47% and the response was greater than that observed with 400 µM metformin used as a positive control. A 200 µg/mL concentration was also able to increase insulin-independent glucose uptake in differentiated 3T3-L1 adipocytes, although not to as great extent. This activity was later shown to be due to the activation of the adenosine-monophosphate-activated protein kinase (AMPK) (Martineau et al., 2010a), as demonstrated by increased phosphorylation of AMPK and acetyl Co-A carboxylase (ACC), while having no effect on Akt, as well as causing mitochondrial uncoupling. This effect is most likely mediated through quercetin-3-O-galactoside, isorhamnetin-3-O-glucoside and kaempferol-3-O-(6"-caffeoylglucoside), as these compounds were identified by bioassay-guided fractionation (Muhammad et al., 2012).

Interestingly, quercetin-3-O-galactoside was also identified as an activator of the AMPK pathway in *Vaccinium vitis-idaea* (Eid et al., 2010) and both, *Vaccinium vitis-idaea* and quercetin-3-O-galactoside, were also shown to be potent inhibitors of the formation of AGEs (Beaulieu et al., 2010). A major complication with diabetes is the formation of diabetic neuropathy, which can be observed through the formation of AGEs (Xu et al., 2006). AGEs are formed

when there is an increase in blood glucose levels, as observed in diabetes, and this formation is irreversible causing the accumulation of AGEs in the blood as well as tissues (Xu et al., 2006; Yokozawa et al., 2008). Thus, *S. purpurea*'s ability to inhibit Nε-(carboxymethyl) lysine (CML) (Harris et al., 2011), one of the most important AGEs, that is able to interact with the AGE receptor and activate the NFκB pathway, as well as cause other inflammatory events (Ramasamy et al., 2006), might be mediated by quercetin-3-O-galactoside.

Furthermore, *S. purpurea* was the only plant out of the 17 to exhibit neuroprotective activity in states of both high and low glucose as examined in PC12 cells, a model of diabetic peripheral neuropathy, at a concentration of 30 μg/mL (Spoor et al., 2006). This activity was associated with the presence of both morroniside and again quercetin-3-O-galactoside, although the activity of the pure compounds was lower than that of the crude extract (Harris et al., 2012). Interestingly, morroniside has been isolated as an active principle from a Traditional Chinese Medicine, Corni Fructus (*Cornus officinalis* SIEB. *et* ZUCC.) that has been shown to be neuroprotective, as well as protective against renal and hepatic injury (Park et al., 2011; Wang et al., 2010; Xu et al., 2006; Yokozawa et al., 2010; Yokozawa et al., 2008). Thus, it is likely that the presence of these phytochemicals contributes to *S. purpurea*'s antidiabetic activity observed *in vitro*.

Adverse reactions mediated by cytochrome P450s

As part of the TAAM, one of the main objectives of the pharmacopeia evaluation is to evaluate safety issues and the possibility of drug interactions (Haddad et al., 2012). By having T2D, these populations are more prone to other diseases such as coronary artery disease (Hegele and Bartlett, 2003). Consequently, most patients also use conventional therapy to treat secondary problems associated with T2D. One of the safety issues then is the possibility of adverse reactions.

Adverse reactions are an important risk inherent to taking drugs and related health care products, which is why both physician and pharmacist intensively evaluate drugs when a patient is prescribed a combination of medications. Unfortunately, because phytomedicines come from natural sources, the public tends to have a preconceived notion that they are safe and patients often fail to mention that they are taking them when consulting a health care professional (Walker and Donovan, 1999). In contrast, herbal medications do have the potential to interact with each other and conventional drugs, making this a serious problem. This interaction is particularly serious when it affects the disposition of the substance, such as altering the metabolic breakdown of the drug, which is in large part performed by the cytochrome P450 (CYP450) family of metabolic enzymes (Bailey and Dresser, 2004). These enzymes are involved in Phase I drug metabolism, which involves the modification of xenobiotics (compounds foreign to normal intermediary metabolism) to make them more hydrophilic (Figure 1.2). The CYP450 family comprises more than 50 P450

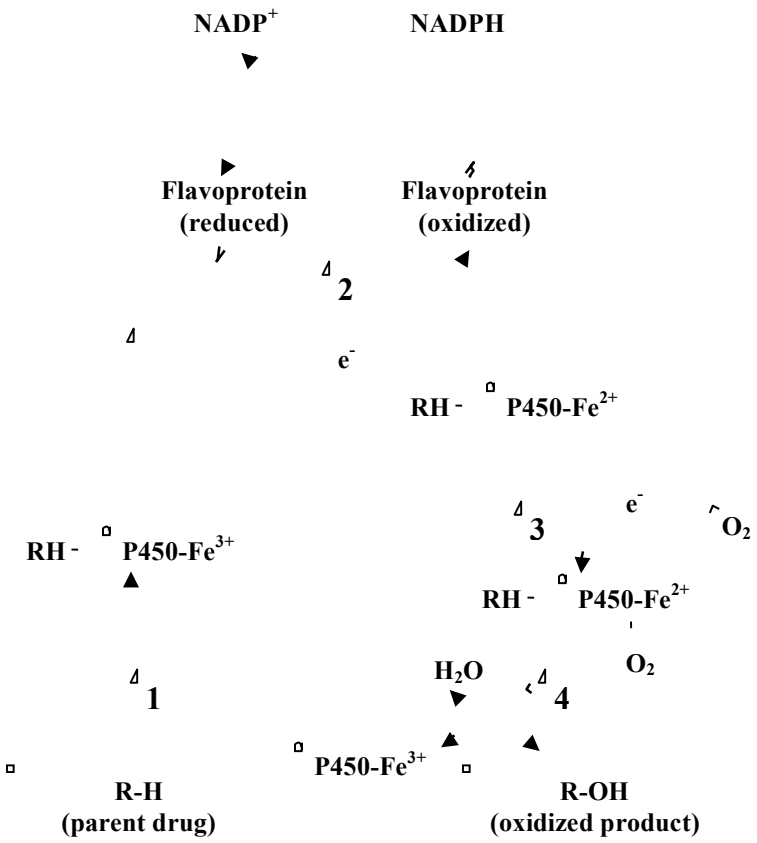

Figure 1.2: Drug oxidation by cytochrome P450s (Budzinski, 2003).

genes, which are subdivided into at least 17 families (Omari and Murry, 2007). The CYP1, CYP2 and CYP3 families are chiefly involved in the metabolism of xenobiotics. A compound may be a substrate for more than one P450, and inhibiting one isoform may lead to shunting through additional pathways potentially changing the safety and efficacy profile for the product. The potential interaction of xenobiotics with most of the remaining P450s is unknown.

In particular, CYP3A4 is responsible for the oxidoreductive metabolism of at least 60% of all drugs (Bailey and Dresser, 2004). This isozyme plays a key role both in first-pass metabolism, which occurs in the intestine before the drug reaches the liver, as well as hepatic metabolism. When CYP3A4 is disregulated, this has serious consequences on the bioavailability of the drug. This is particularly important if its activity or expression is upregulated, so that the bioavailability of the drug decreases and can lead to suboptimal drug concentration in the body. This is the case with St. John's Wort, which has been shown to induce CYP3A4 function (Durr et al., 2000). Since many HIV therapies, such as indinavir, are metabolized by hepatic CYP3A4, prolonged use of St. John's Wort has been shown to decrease its bioavailability and hence increase the viral load (Piscitelli et al., 2000).

Furthermore, if CYP3A4 is downregulated or inhibited, bioavailability increases which can lead to severe toxicity. An example is grapefruit juice which inhibits intestinal CYP3A4 function (Kato, 2008). It has been shown that taking grapefruit juice with therapies metabolized by enteric CYP3A4 can result in an

increase in bioavailability of the drug to potentially toxic levels (Bailey and Dresser, 2004; Bailey et al., 1998; Glaeser et al., 2007).

Disregulation can occur at the level of gene expression, or enzymatic level and can involve CYP450 family members directly or indirectly by altering the expression levels of genes responsible for their regulation, such as the nuclear receptors *CAR* and *PXR* (constitutive androstane receptor and pregnane X receptor) (Waxman, 1999). CAR is responsible for the gene expression of the *CYP2* family members, while PXR is responsible for the *CYP3A* family, although some cross-expression has been observed with some xenobiotics (Waxman, 1999). Furthermore, P-glycoprotein, an efflux transporter, is important in drug absorption because it can transport xenobiotics back into the intestinal lumen and thereby reduce bioavailability (Pfrunder et al., 2003). The *ABCB1* gene is responsible for the expression of P-glycoprotein (P-gp), which in turn has been shown to be regulated by PXR.

It is possible that botanical substances disregulate these key genes, because the plants have evolved secondary metabolites in order to be resistant against microbes and insects (Harborne, 2000) and possess compounds designed to inhibit CYP450 in order to potentiate their defences. Therefore it is important to look at the expression of the *CYP450* genes, as well as related genes, in order to determine the effect that botanical substances have on them. Antidiabetic drugs, such as sulphonylureas, thiazolidinediones and meglitinide derivatives are metabolized by the CYP family, more particularly CYP1A1/2, CYP2C8, CYP2C9 and CYP3A4 (Kirchheiner et al., 2005; Scheen, 2005). As

well, drugs used to treat coronary artery disease such as HMG-CoA reductase inhibitors, commonly known as statins, are also metabolized by this family of metabolic enzymes (Scheen, 2005). By knowing which genes are disregulated by the Cree botanicals, we should be able to predict possible drug interactions. Consequently, health care providers and healers could avoid interactions leading to hypoglycaemia in the case of CYP inhibitors or having the hyperglycaemia persist with CYP inducers, as well as other possible interactions with other medications.

In vitro models of studying adverse reactions

One of the ways to study possible drug interactions *in vitro* is to use recombinant cytochrome P450 proteins in a fluorometric plate assay and examine the possible inhibition of the extract on the enzyme (Budzinski, 2003; Budzinski et al., 2007; Foster et al., 2001). This work has already been completed with the 17 Cree extracts. Tam et al. (2009) demonstrated that several Cree extracts, such as *Rhododendron groenlandicum*, *Sorbus decora* and *Kalmia angustifolia,* at a concentration of 10 µg/mL, are strong inhibitors of many of the CYP isoforms. Furthermore, *Alnus incana*, *S. purpurea* and *Lycopodium clavatum*, at 20 µg/mL, were potent mechanism based inhibitors (MBI) of CYP3A4 (Tam et al., 2011).

The relevance of using human liver microsomes to examine modification of oseltamivir, an important influenza pro-drug medication Tamiflu, when co-administered with other therapies, as well as Cree medicines, has also been

examined (Liu et al., 2010a). It was reported that most of the traditional Cree medicines altered the metabolism of oseltamivir by inhibiting the production of one or more of its metabolites. However, the alteration of other antidiabetic medicines by Cree extracts has not been previously examined.

The importance of first-pass metabolism is clear from the discussion above and an appropriate experimental *in vitro* model for first-pass metabolism has been developed using Caco-2 cells (Pfrunder et al., 2003; Watabe et al., 2003). Particularly, the Caco-2 monolayers induced with 1α, 25-dihydroxyvitamin D3 can be used to model absorption and fate of test pharmaceuticals alone and in the presence of other drugs or plant extracts (Engman et al., 2001; Hubatsch et al., 2007; Schmiedlin-Ren et al., 1997). This has previously been done by Aiba et al. (2005) who used Caco-2 monolayers to examine the co-administration of digoxin, a heart medication, with ouabain, a Na^+, K^+-ATPase inhibitor. It was found that 100 μM concentration of ouabain decreased cellular drug accumulation but had little effect on transport of digoxin.

Furthermore, gene expression of the *CYP*s, as well as drug transporters, has also been examined in the Caco-2 cells (de Waard et al., 2008). Gene analysis can be done through the use of real-time PCR technology, which examines only particular gene transcripts (Guo et al., 2011) or through a more global analysis of all transcript changes via the use of microarray technology (Altamirano-Dimas et al., 2007). In recent years, microarray technology has been used to evaluate both safety and potential mechanism of action of various plants and their active constituents (Altamirano-Dimas et al., 2007; Jensen et al.,

2006a; Jensen et al., 2006b; Moon et al., 2007; Paruthiyil et al., 2009; Ren et al., 2013). Therefore, Caco-2 cells are a good whole cell *in vitro* model to study both transport as well as transcription changes at the first-pass metabolism level, and microarrays can be used to evaluate those changes.

T2D and AD

T2D is associated with glucotoxicity, neuroinflammation, and the formation of AGEs, and these symptoms have also been associated with AD (Craft, 2007, 2009; Guglielmotto et al., 2012; Kojro and Postina, 2009). The pathological characteristics of AD are neurofibrillary tangles as a result of hyperphosphorylated tau and aberrant processing of the amyloid precursor protein (APP) by β and γ-secretase into amyloid β (Aβ) peptides (Goedert and Spillantini, 2006). Particularly, a 42 amino acid protein, $A\beta_{42}$, has been classified as the most toxic and has been associated with neurodegeneration and cognitive impairment (Cleary et al., 2005; Selkoe, 2002). It has been suggested that presence of AGEs, as seen in T2D, upregulates *BACE-1* expression, which is responsible for β-secretase production, via activation of the NFκB signalling pathway (Guglielmotto et al., 2012). Furthermore, the NFκB pathway is very pro-inflammatory thus leading to the activation of other factors contributing to both diseases such as reactive oxygen species (ROS) (Morgan and Liu, 2011). NFκB has also been previously identified as a potential contributor to AD (Tan et al., 2008). Furthermore, insulin resistance, in the first three years of onset, has been shown to be associated with an elevated risk of AD (Schrijvers et al., 2010).

Therefore, T2D has been identified as a risk factor for AD (Nicolls, 2004; Qiu et al., 2009; Schrijvers et al., 2010) and some have even referred to AD as “type 3 diabetes” (de la Monte and Wands, 2008). The exact mechanism by which the two diseases are related, however, still remains to be elucidated, as is the link between tau and Aβ. Herrup (2010) has suggested that a separate “change of state” occurs that alters the metabolic state towards one of neurodegeneration and dementia and a modified model linking the disease states is shown in Figure 1.3. It is known that diabetes and AD contribute to a potent inflammatory response; however, the mechanism by which that translates to a memory and learning impairment is not understood. Thus, therapies used to treat T2D have been used to explore their effect on memory loss and the learning impairment associated with both diseases.

Use of T2D therapies to improve cognitive function

Cognitive function in animal models can be studied using the Morris Water Maze (MWM) test (Morris, 1981) developed to study rats with hippocampal lesions (Morris et al., 1982). The test has now become the standard for examining spatial learning and memory in *in vivo* models of disease, as it employs a water-filled pool with a hidden platform and spatial cues spread around the room. Therapies used to treat T2D have shown promise in improving cognitive function, as evaluated by MWM, in models of T2D. For example, Pathan et al. (2008) used a 5 mg/kg daily dose of rosiglitazone, an insulin sensitizer, for seven days prior to behaviour testing to improve spatial learning

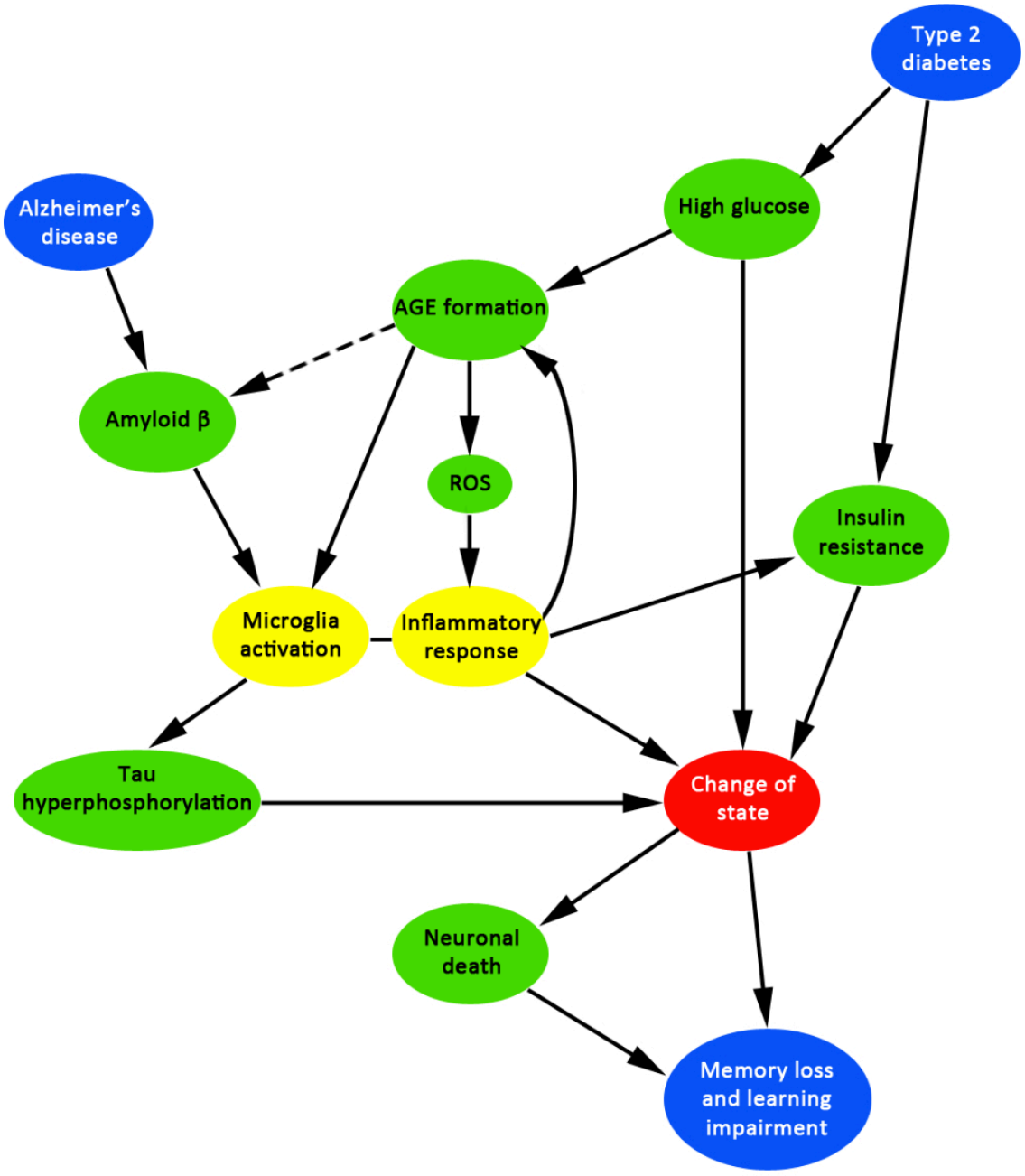

Figure 1.3: Proposed mechanism linking Alzheimer's disease and Type 2 diabetes based on a model by Herrup (2010). AGE – advanced glycation endproducts, ROS – reactive oxygen species

and memory in rats fed a high fat diet for four weeks. Liu et al. (2010b) used pioglitazone, another insulin sensitizer, at a concentration of 10 mg/kg b.w. per day for 12 weeks, to see if it could combat the deficit induced by a 10% fructose solution. Pioglitazone was able to significantly reduce the learning deficit as seen by a reduced escape latency in the MWM and this correlated with receptor for advanced glycation endproducts (RAGE) inhibition through blockage of NFκB activation. Furthermore, a traditional antidiabetic therapy from Ayurvedic medicine, *Clitorea ternatea* (ethanol extract), was successfully used to improve spatial learning and memory in streptozotocin treated rats, after a treatment of both 200 mg/kg b.w. daily and 400 mg/kg b.w. daily for two weeks (Talpate et al., 2012). Quercetin and ursolic acid have also shown positive benefits in these models (Bhutada et al., 2010a; Lu et al., 2011; Lu et al., 2007). Furthermore, aged garlic has also been successful in improving cognitive deficits in a mouse model of AD, the TgCRND8 mice (Chauhan and Sandoval, 2007). However, none of the antidiabetic Cree traditional therapies have ever been studied in this context.

TgCRND8, a mouse model of AD

TgCRND8 mice were developed at the University of Toronto and are now one of the model animals used to study AD (Chishti et al., 2001). They contain the human *APP* gene containing the Swedish and Indiana mutations (KM670/671NL + V717F) under the control of the hamster prion promoter which leads to accumulation of Aβ_{42} and cognitive deficits as early as at three months

of age. Thus, this model allows for study of the disease in a much shorter time frame.

1.3 Rationale and Objectives

The objectives of this thesis were guided by our lab's role in evaluating safety and efficacy of the traditional Cree plants as outlined by the TAAM and described in greater detail by Haddad et al. (2012). More specifically, the evaluation of the interaction of Cree medicines with conventional therapies was a primary safety concern addressed here with all of the ethnobotanically identified plants and in detail with *S. purpurea*. The efficacy of other traditional antidiabetic medicines in improving cognitive deficits observed in mouse models of T2D suggested the study of *S. purpurea* in the TgCRND8 mouse model of AD.

As a first step in safety evaluation, the first objective was to determine the potential of all 17 Cree traditional medicines identified in the ethnobotanical survey by Leduc et al. (2006) to alter *in vitro* transport and metabolism of two blood glucose lowering pharmaceuticals, repaglinide and gliclazide (Chapter 2). This study was undertaken to determine the potential for drug interactions. Subsequently a more focused evaluation was undertaken on one of the most promising traditional medicines, *S. purpurea*, in several objectives. Thus, the second objective of this thesis was to characterize the phytochemical composition of the traditionally used water extract and ethanol extract mandated by pharmacological assay protocols of *S. purpurea* (Chapter 3). This objective provided information on bioactive components and their variation by preparation

method. The third objective was to examine potential for *S. purpurea* extracts, and its pure active principle, morroniside, to cause transcriptional changes to the *CYP* family of genes, as well as drug transporters (Chapter 4). This objective also provided information on possible drug interactions. The last objective, examined in Chapter 5, was to study the potential of *S. purpurea* to alter the transition from pre-symptomatic to symptomatic state in an *in vivo* model of AD, TgCRND8 mice, and explore a potential mode of action of the plant. This objective provided information on both safety and efficacy, *in vivo*. Taken together, the overarching hypothesis of this thesis was that the Cree have an understanding of how their traditional medicines work (efficacy) and any potential limitations of their applications (safety). It was expected that this traditional knowledge by the Cree would correspond with the scientific findings on safety and efficacy.

Chapter 2

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

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Statement of author contribution

CC, JTA and BCF conceived and designed this study. Plant identification and collection was undertaken by AC. JAGA isolated rhaponticin and rhapontigenin. The Caco-2 absorption assays were performed by CC and AF with analytical help from RL. Analysis of metabolites was performed by RL with assistance from AS. SAMS performed the rhaponticin and rhapontigenin assays. SALB contributed critical equipment to the experiments. PSH is the principal investigator of the CIHR TAAM project. CC, JTA and BCF wrote the manuscript, with RL, SALB, PSH and AC contributing to final manuscript submission.

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2.1 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 three 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 et al., 2006) and in animal trials (Harbilas et al., 2012a; Harbilas et al., 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 of the healers' top used plants (Fraser et al., 2007; Leduc et al., 2006) were assessed for their mechanistic potential for drug-drug interaction (DDI) 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 hrs (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). This study was further extended 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 antidiabetic study conducted by our research team, *L. 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, the *in vitro* interaction between rhaponticin and its derivative rhapontigenin on gliclazide was investigated 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 *L. laricina* than its aglycone, rhapontigenin, which is believed to be the active form of the molecule after the absorption step (Shang et al., 2012).

2.2 Materials and Methods

2.2.1 Materials and sample preparation

The 17 Cree medicinal plant species identified in Table 2.1 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). Voucher numbers were previously reported (Fraser et al., 2007; Tam et al., 2009). Plants were subsequently extracted with ethanol, lyophilized, and analysed 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 one week.

Table 2.1: 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

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. The concentrations of reagents used in each assay were guided by previous work and laboratory standard operating procedures, as well as the limitations of analytical instruments used.

Enzymatic synthesis of rhapontigenin

A reaction mixture was prepared by first adding rhaponticin (10 mg) 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 hrs and afterwards extracted with dichloromethane (CH_2Cl_2). The organic fraction was collected, dried with sodium sulphate 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 ^{1}H 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.

2.2.2 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 six-well Transwell membrane inserts (0.4 μm pore, 4.67 cm^2, polyester filter; Corning Costar Co.,

NY, USA) at a concentration of 5 x 10^5 cells per well, followed by an exchange of apical media within 16 hrs (Hubatsch et al., 2007). Media in the apical and basolateral chambers was exchanged every second day. Prior to the start of the absorption assay, CYP3A4 expression was induced in the cells by treatment with 0.25 µM 1α,25-dihydroxyvitamin D3 (Sigma-Aldrich, Oakville, ON, Canada) in DMEM (Schmiedlin-Ren et al., 1997) for 72 hrs, since Caco-2 cells typically express low levels of CYP3A4.

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 (Cat# 339188, 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 hrs 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% MeOH, filtered with PTFE filters (0.2 µm pore; Chromatographic Specialties Inc., Brockville, ON, Canada). Each assay was performed in triplicate and results were analysed 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 analysed through a two-way ANOVA with a Bonferroni *post-hoc* test.

2.2.3 HLM-mediated metabolism

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 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.

2.2.4 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 the medicinal plant extract (5 mg/mL) 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 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. MeOH was used as a vehicle control. Results were reported as percent inhibition.

2.2.5 CYP 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

fluorescent light to photosensitive material. 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.

2.2.6 CYP-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 37ºC 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 analysed by HPLC.

2.2.7 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 x 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) with 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.

2.2.8 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 HPLC (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.

2.3 Results

2.3.1 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 (Figures 2.1 and 2.2). The maximum mean absorption observed at 3 hrs 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 (Figure 2.1), the time course with extracts present was not significantly different from that of gliclazide alone. When co-administration of extracts with repaglinide was examined (Figure 2.2), each of the 11 extracts showed a time-dependent increase in concentration of repaglinide, but most

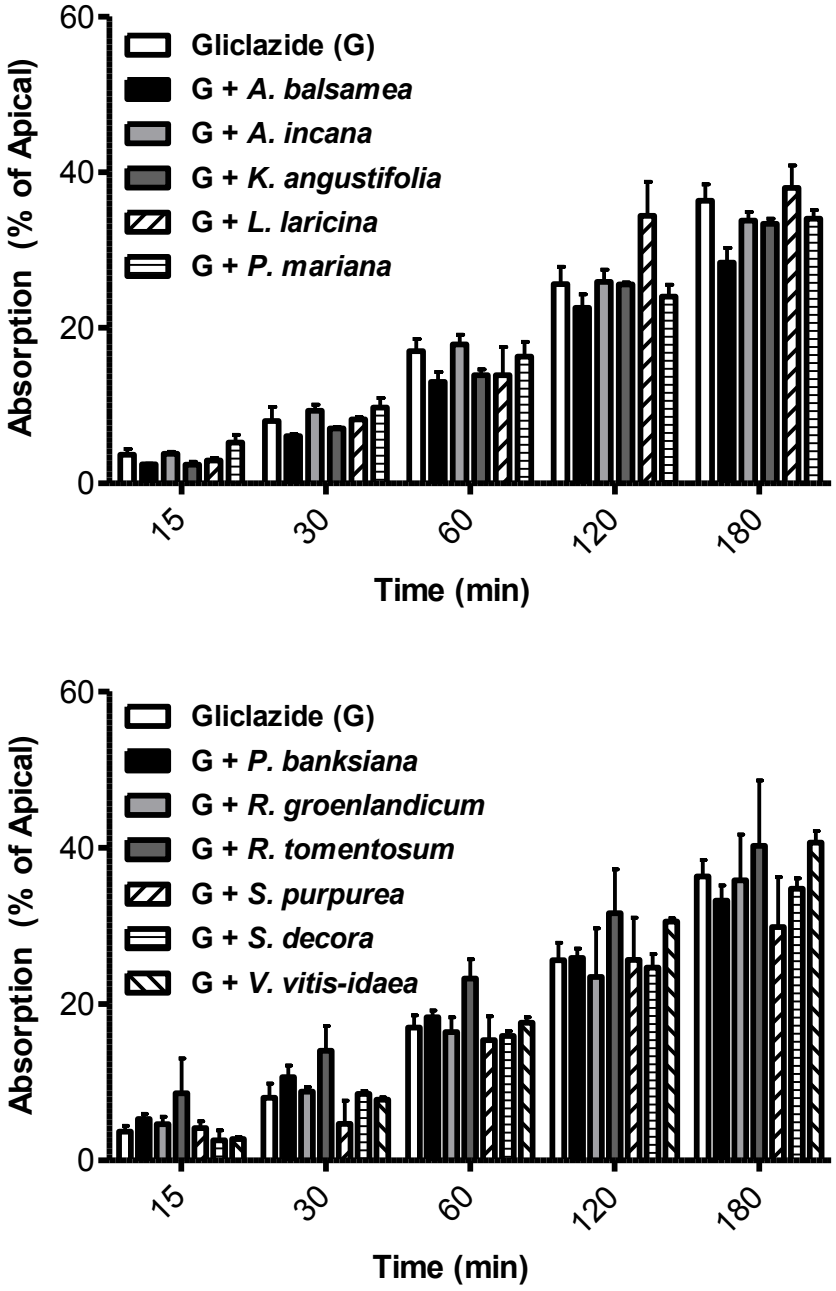


Figure 2.1: Absorption of gliclazide (50 µg/mL) alone and in the presence of Cree plant extracts at a concentration of 100 µg/mL through the intestinal epithelial Caco-2 monolayer. Results are mean percent absorption ± SEM (n=3).

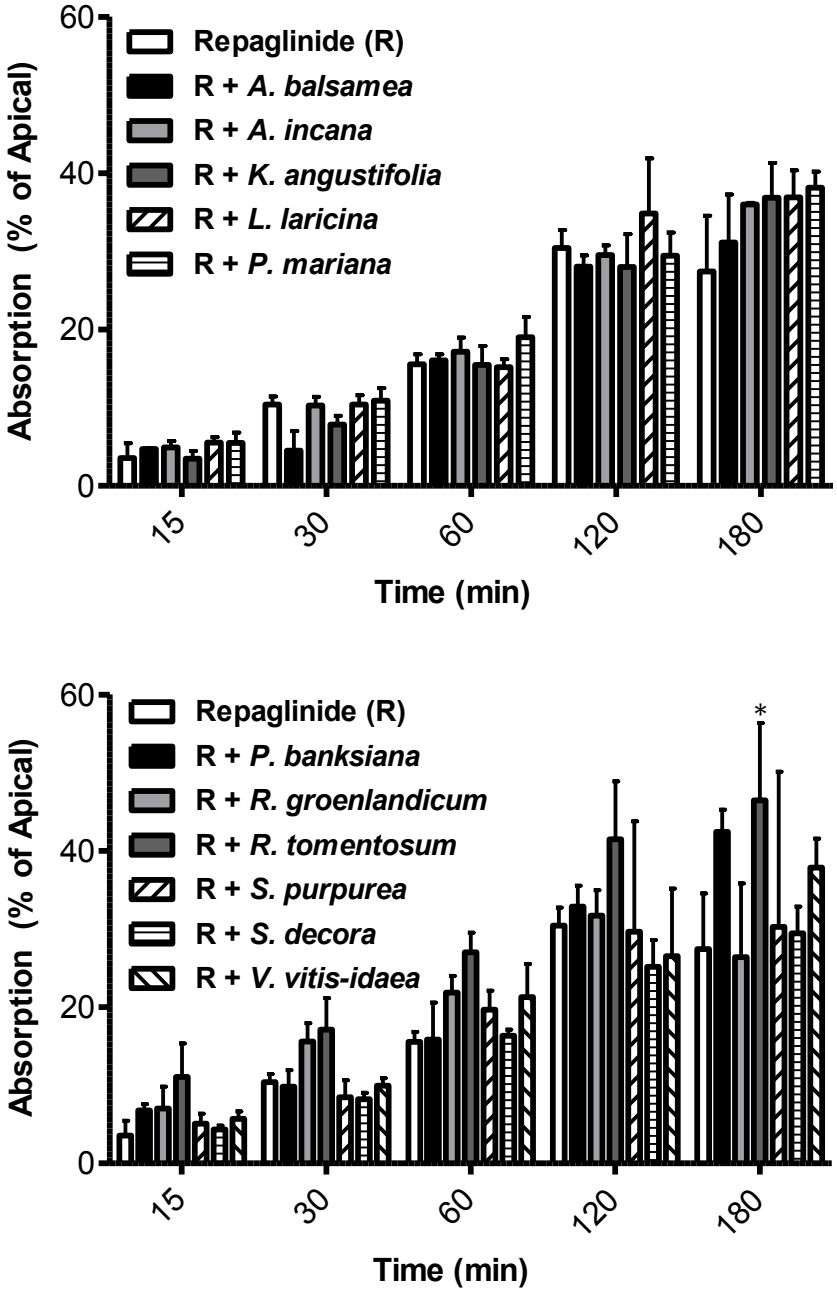


Figure 2.2: Absorption of repaglinide (50 µg/mL) alone and in the presence of Cree plant extracts at a concentration of 100 µg/mL through the intestinal epithelial Caco-2 monolayer. Results are mean percent absorption ± SEM (n=3). *p<0.05

results were not significantly different from repaglinide alone. Only *R. tomentosum* showed a significant difference ($p<0.05$) towards increasing the percent of repaglinide absorbed.

2.3.2 HLM-mediated metabolism of repaglinide

Four metabolites of repaglinide with distinct retention times were detected by HPLC (Figure 2.3). 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 (Figure 2.4A). The most potent M4 inhibitors, as demonstrated by more than 80% inhibition were *A. balsamea*, *A. incana*, *J. communis*, *K. angustifolia*, *L. laricina*, *P. mariana*, *P. banksiana*, *R. groenlandicum*, *R. tomentosum*, *S. planifolia* and *S. decora*. Moderate inhibitors, as demonstrated by inhibition between 50-80% were *G. hispidula*, *P. glauca*, *P. balsamifera* and *S. purpurea*, while only two extracts were mild inhibitors (<50%) *L. clavatum* and *V. vitis-idaea*. Ketoconazole (0.4 μM), a potent CYP3A4 inhibitor, had no effect on the production of M4, since this metabolite is primarily produced via CYP2C8 (Gan et al., 2010).

The extracts of *A. balsamea*, *A. incana*, *K. angustifolia*, *L. laricina*, *P. mariana*, *P. banksiana*, *R. groenlandicum*, *R. tomentosum*, *S. planifolia*, and *S. decora* were more potent inhibitors of the m/z 451-A and m/z 451-B metabolites (Figures 2.4B and C). *G. hispidula*, *J. communis*, *L. clavatum*, *P. balsamifera*,

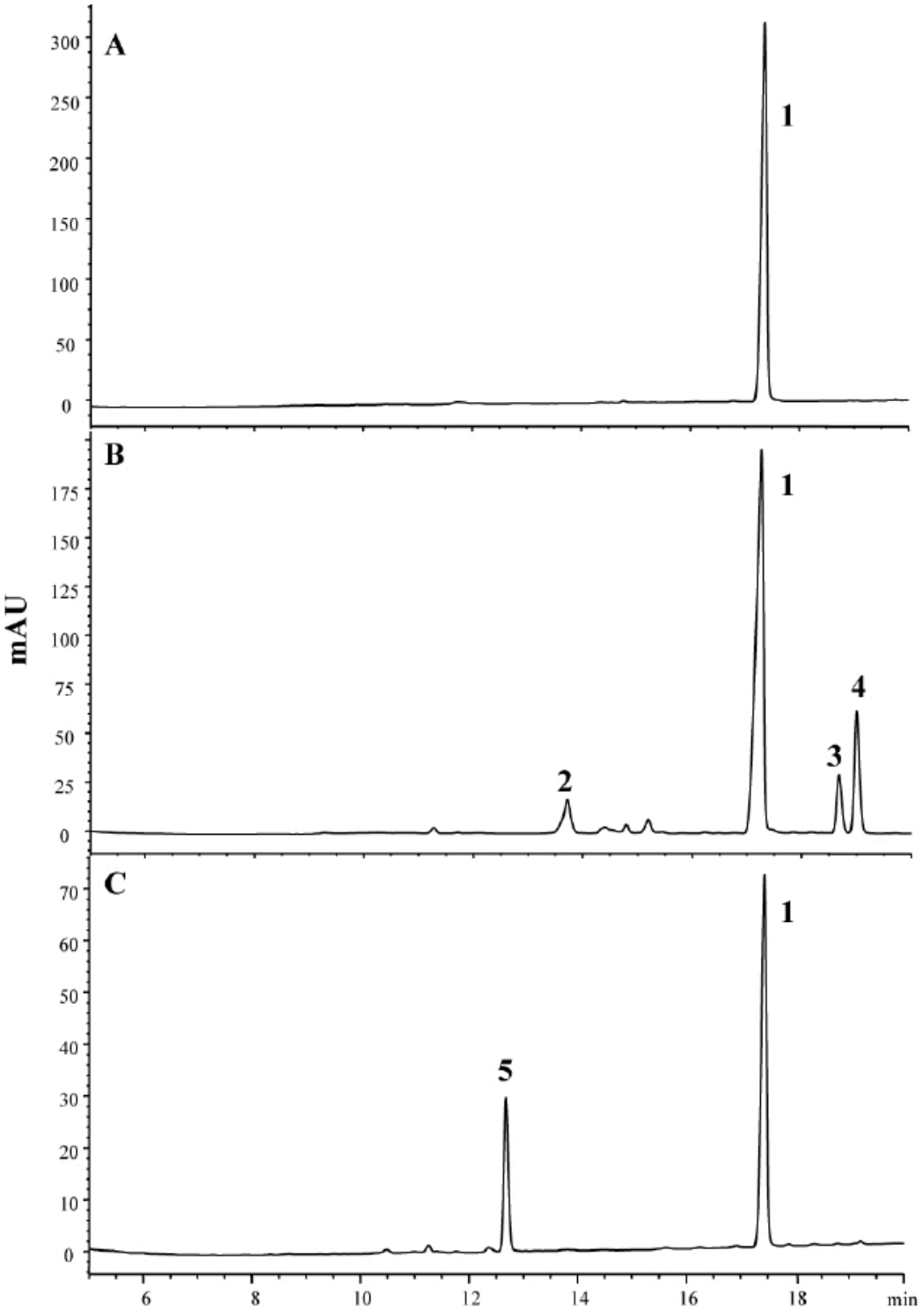
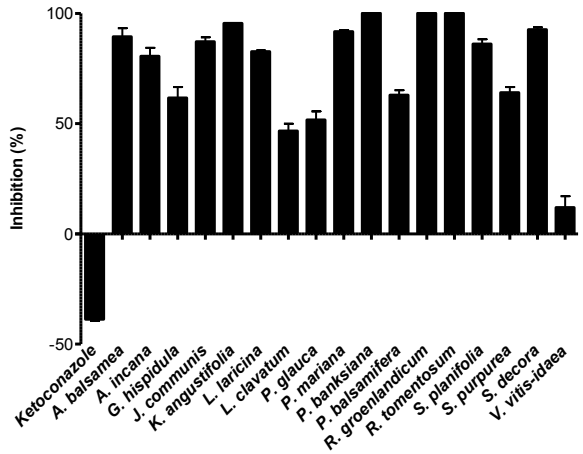


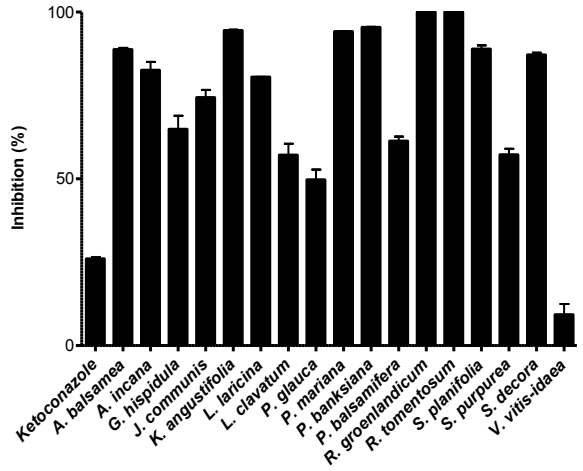
Figure 2.3: A representative HPLC trace of human liver microsome-mediated metabolisms of repaglinide. (A) Repaglinide standard. (B) CYP2C8 and CYP3A4 metabolites. (C) UDP-glucosyltransferase metabolite. (**1**) repaglinide, (**2**) repaglinide M4, (**3**) m/z 451-A, (**4**) m/z 451-B and (**5**) glucuronide metabolite.

Figure 2.4: 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.

A.


100
50
0
-50
Inhibition (%)
Ketoconazole
A. balsamea
A. incana
G. hispidula
J. communis
K. angustifolia
L. laricina
L. clavatum
P. glauca
P. mariana
P. banksiana
P. balsamifera
R. groenlandicum
R. tomentosum
S. planifolia
S. purpurea
S. decora
V. vitis-idaea

B.


100
50
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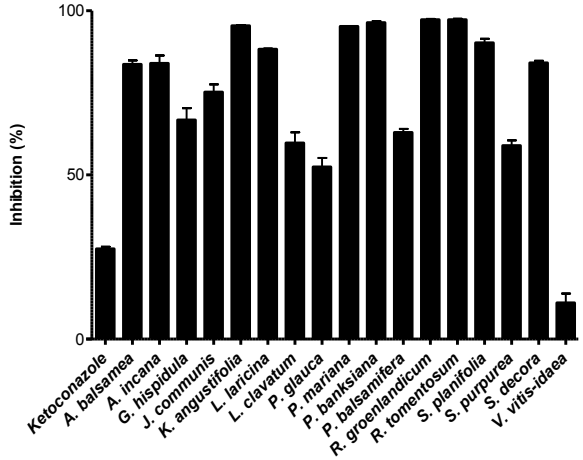
Inhibition (%)
Ketoconazole
A. balsamea
A. incana
G. hispidula
J. communis
K. angustifolia
L. laricina
L. clavatum
P. glauca
P. mariana
P. banksiana
P. balsamifera
R. groenlandicum
R. tomentosum
S. planifolia
S. purpurea
S. decora
V. vitis-idaea

C.


100
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Inhibition (%)
Ketoconazole
A. balsamea
A. incana
G. hispidula
J. communis
K. angustifolia
L. laricina
L. clavatum
P. glauca
P. mariana
P. banksiana
P. balsamifera
R. groenlandicum
R. tomentosum
S. planifolia
S. purpurea
S. decora
V. vitis-idaea

and *S. purpurea* were moderate inhibitors of both metabolites. *V. vitis-idaea* was only a mild inhibitor of both metabolites. *P. 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, as these metabolites are produced via CYP3A4 (Gan et al., 2010).

The plant extracts at 50 µg/mL were not potent inhibitors of glucuronidation (Figure 2.5). Only *A. balsamea*, *K. angustifolia*, *L. clavatum*, *R. groenlandicum*, *R. tomentosum*, and *S. decora* showed inhibition greater than 30%. *G. hispidula*, *P. balsamifera*, and *V. vitis-idaea* exhibited no inhibition at all.

2.3.3 *CYP-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 (Figure 2.6). 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 five major (M1 to M5) metabolites (Figure 2.7). 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, rhapontigenin at 10 µg/mL showed greater inhibitory effects, ranging from a mean of 58% CYP3A4 inhibition to 89% inhibition of CYP2C9. The positive controls used, ketoconazole (1.9 µM),

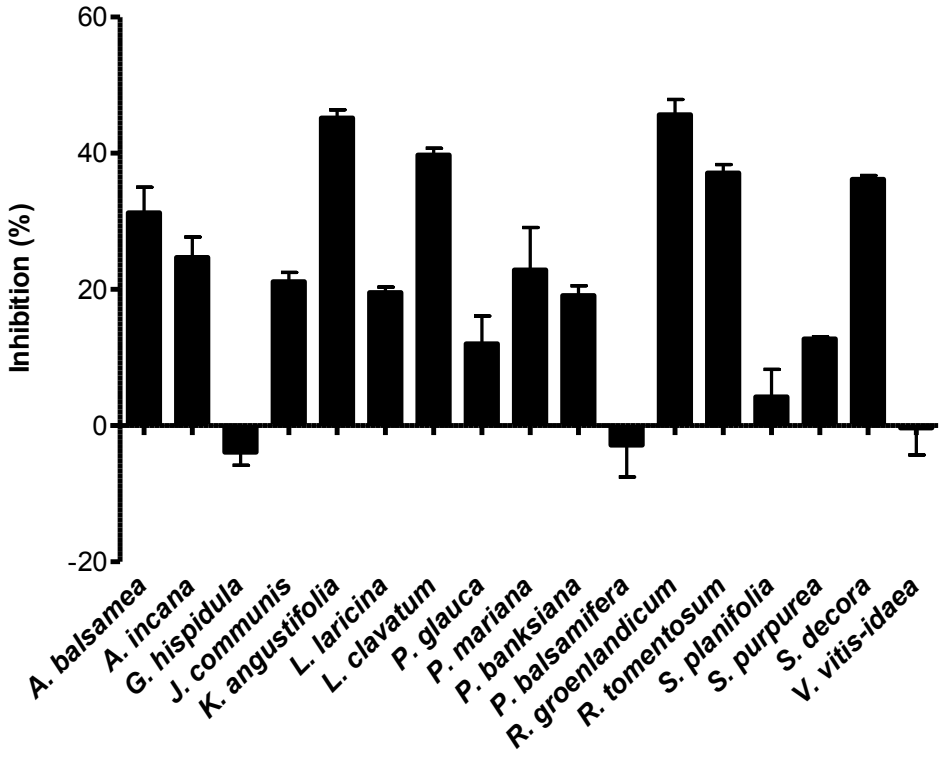

Figure 2.5: Inhibition of UDP-glucuronosyltransferase-mediated metabolism of repaglinide (10 µg/mL) by Cree plant extracts at a concentration of 50 µg/mL.

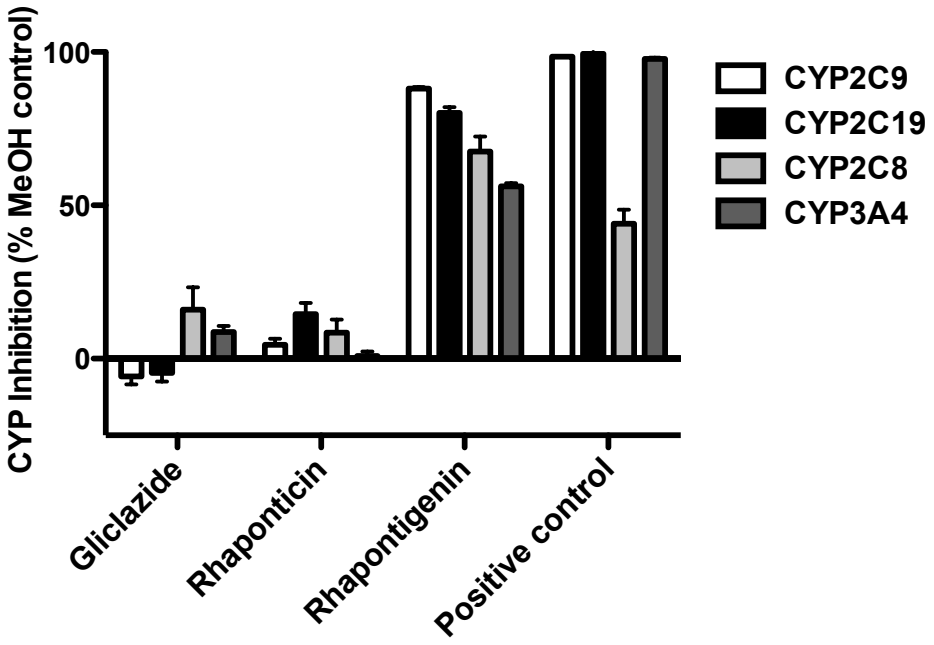

Figure 2.6: Effect of gliclazide, rhaponticin and its aglycone derivative, rhapontigenin (10 µg/mL) on CYP2C9-, 2C19-, 2C8- and 3A4-mediated metabolism of a marker substance in a 20 min or 60 min microtitre fluorometric assay. The results were expressed relative to a MeOH vehicle control as mean CYP 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).

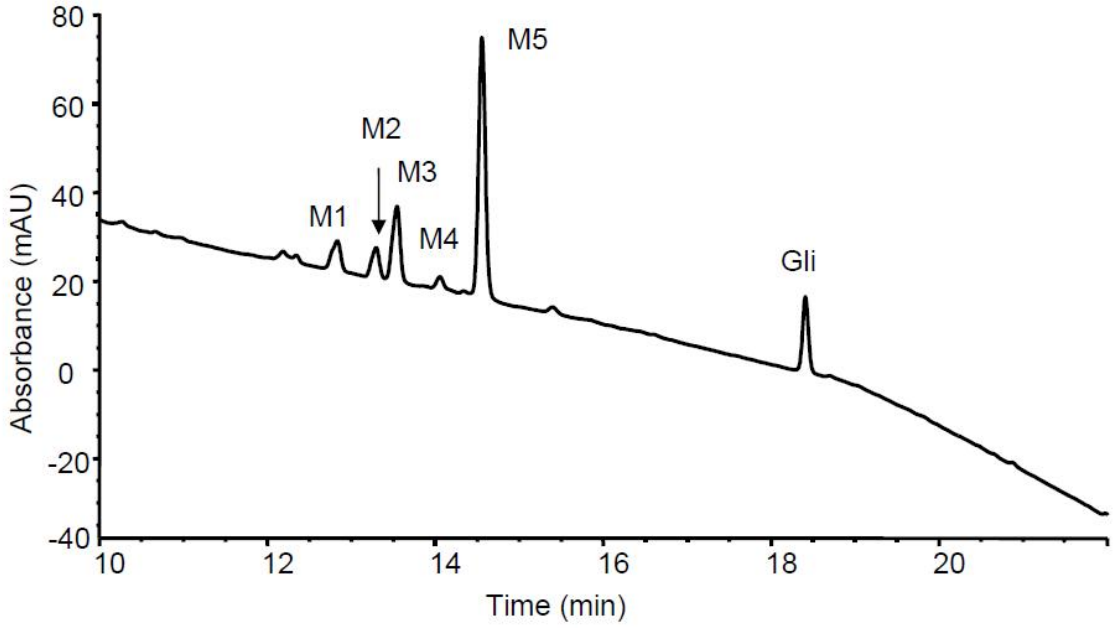

Figure 2.7: HPLC trace of gliclazide (Gli, 10 µg/mL) and metabolites following 90 min metabolism by CYP2C19. The metabolites (M1-M5; eluted at min 12.8, 13.3, 13.5, 14.1 and 14.6) and Gli eluted at 18.4 min.

sulfaphenazole (100 μM) and tranylcypromine (100 μM), completely inhibited enzyme activity of CYP3A4, 2C9 and 2C19 respectively and ketoconazole (10 μM) 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 (Figure 2.8). 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 2.2). Rhaponticin and rhapontigenin had a marked effect on the CYP2C19 mediated-metabolism of gliclazide (Table 2.3) as seen by the increased ratio of gliclazide and decreased ratio of the major metabolite.

2.4 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 hypoglycemic 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

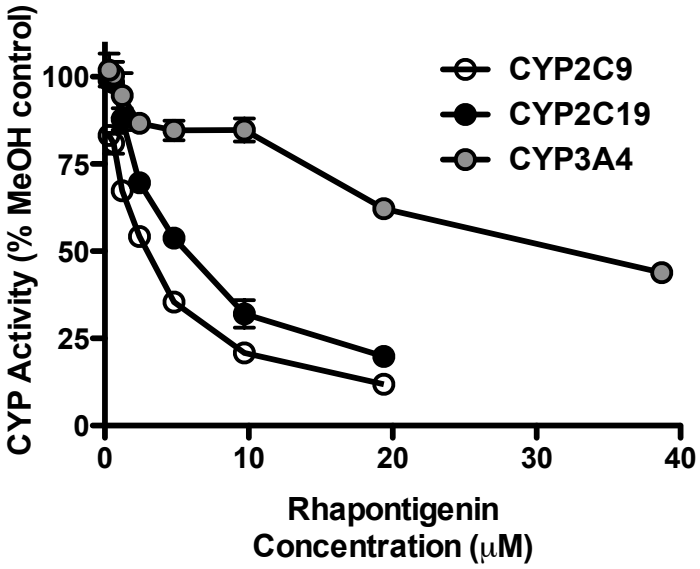

Figure 2.8: Inhibition effect of various concentrations of rhapontigenin on CYP2C9-, 2C19- and 3A4-mediated metabolism. The results were expressed relative to a MeOH vehicle control as mean CYP activity.

Table 2.2: 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).

	Relative Ratios			
Compound	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 2.3: 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

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 *S. decora*, *L. laricina*, and *A. balsamea*, boreal forest species previously shown to be strong inhibitors of both CYP2C9 and CYP2C19 (Tam et al., 2009). Likewise, *S. decora* was a very potent inhibitor of CYP2C8 and *R. groenlandicum* and *P. 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 upregulation of metabolic enzymes or transport proteins. The net outcome of this complex relationship is 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 *R. tomentosum*, whose extract significantly increased the transepithelial 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, all known metabolites of repaglinide in the HLM–mediated biotransformation assay were not detected (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 produced in this microsome assay. 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 *A. balsamea*, *A. incana*, *J. communis*, *K. angustifolia*, *L. laricina*, *P. mariana*, *P. banksiana*, *R. groenlandicum*, *R. tomentosum*, *S. planifolia* and *S. decora*. The extracts were more potent inhibitors than the positive control, ketoconazole. *A.*

balsamea, *A. incana*, *K. angustifolia*, *L. laricina*, *P. mariana*, *P. banksiana*, *R. groenlandicum*, *R. tomentosum*, *S. planifolia*, and *S. decora* were potent inhibitors of both m/z 451-A and B metabolites. *V. 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 CYP-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 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 some 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, these results suggest that *L. laricina* 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 the 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.

The extracts of selected putative antidiabetic medicinal plants of 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 do 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 warrant clinical evaluation of these Cree medicinal plants, particularly tamarack, that may be taken by patients simultaneously with oral hypoglycaemics and other drugs.

Chapter 3

Phytochemical comparison of the water and ethanol leaf extracts of *Sarracenia purpurea* L.

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Statement of author contribution

CC, BWR and JTA conceived and designed this study. Plant identification and collection was undertaken by AC. AM isolated taxifolin-3-O-glucoside. CC and BWR performed the phytochemical analysis with analytical help from RL and AS. PSH is the principal investigator of the CIHR TAAM project and BCF and JTA contributed to manuscript preparation.

3.1 Introduction

Traditional medicine continues to be an important part of the culture for the Cree First Nation of Eeyou Istchee in Northern Quebec. They use plants to treat the symptoms and causes of many diseases, and traditionally prepare all their plant extracts as decoctions (boiling water extracts). In 2003, the Cree Health Board initiated a research project in collaboration with six university laboratories, named the CIHR Team in Aboriginal Antidiabetic Medicines (CIHR-TAAM) (http://www.taam-emaad.umontreal.ca/), to evaluate the safety and efficacy of culturally relevant medicines used to treat symptoms of Type 2 diabetes (Fraser et al., 2007; Leduc et al., 2006). This disease is particularly important as the prevalence of diabetes is greater than 20% in the Cree adult population (Kuzmina et al., 2010) and compliance with conventional therapies is low. As an experienced medicinal plant group, the CIHR-TAAM normally uses ethanolic extracts, which are better optimized for many pharmacological studies, with the exception of immunomodulatory studies.

It is well known that different solvents are able to extract different families of phytochemicals, hence varying the activity of the extracts (Soares et al., 2013; Wang and Weller, 2006; Zarnowski and Suzuki, 2004). Alcohol extracts are preferred to water extracts in many pharmacological assays, as the bioactive secondary metabolites are better extracted than with water. Hot water tends to extract other biological components (Spigno et al., 2007), such as polysaccharides and denatured proteins, which may interfere with some assays

(Sharma et al., 2006). However, the phytochemistry of the two types of plant extracts is very seldom compared in medicinal plant research.

One of the main plant species identified by the Cree for its antidiabetic property was *Sarracenia purpurea* L. (Sarraceniaceae), the pitcher plant, also known as Ayigadash in Cree (Leduc et al., 2006). It is a carnivorous plant found to inhabit bogs and other nitrogen-poor environments (Ellison et al., 2012). A quantitative ethnobotanical survey by Leduc et al. (2006), showed that *S. purpurea* was among the top eight plant species used for the symptoms of diabetes by the Cree, and it was identified as one of the priority plant species for the TAAM to study. Therefore, a robust phytochemical comparison of the traditional water and the ethanol extract was necessary. The purpose of this work was to identify known phytochemical markers (Figure 3.1) in the water and ethanol extracts and to quantify the differences.

3.2 Materials and Methods

3.2.1 Chemicals and Standards

The standards (+)-catechin, (-)-epicatechin, luteolin-7-O-glucoside, kaempferol-3-O-rutinoside, betulinic acid, ursolic acid, and rutin were all purchased from Extrasynthese (Genay Cedex, France). Quercetin-3-O-galactoside, quercetin-3-O-glucoside, and myricetin were purchased from Sigma Aldrich (Oakville, ON, Canada). Morroniside was purchased from AvaChem Scientific (San Antonio, TX, USA) and taxifolin-3-O-glucoside was isolated in-house and its structure and purity was verified through its ultra violet (UV)

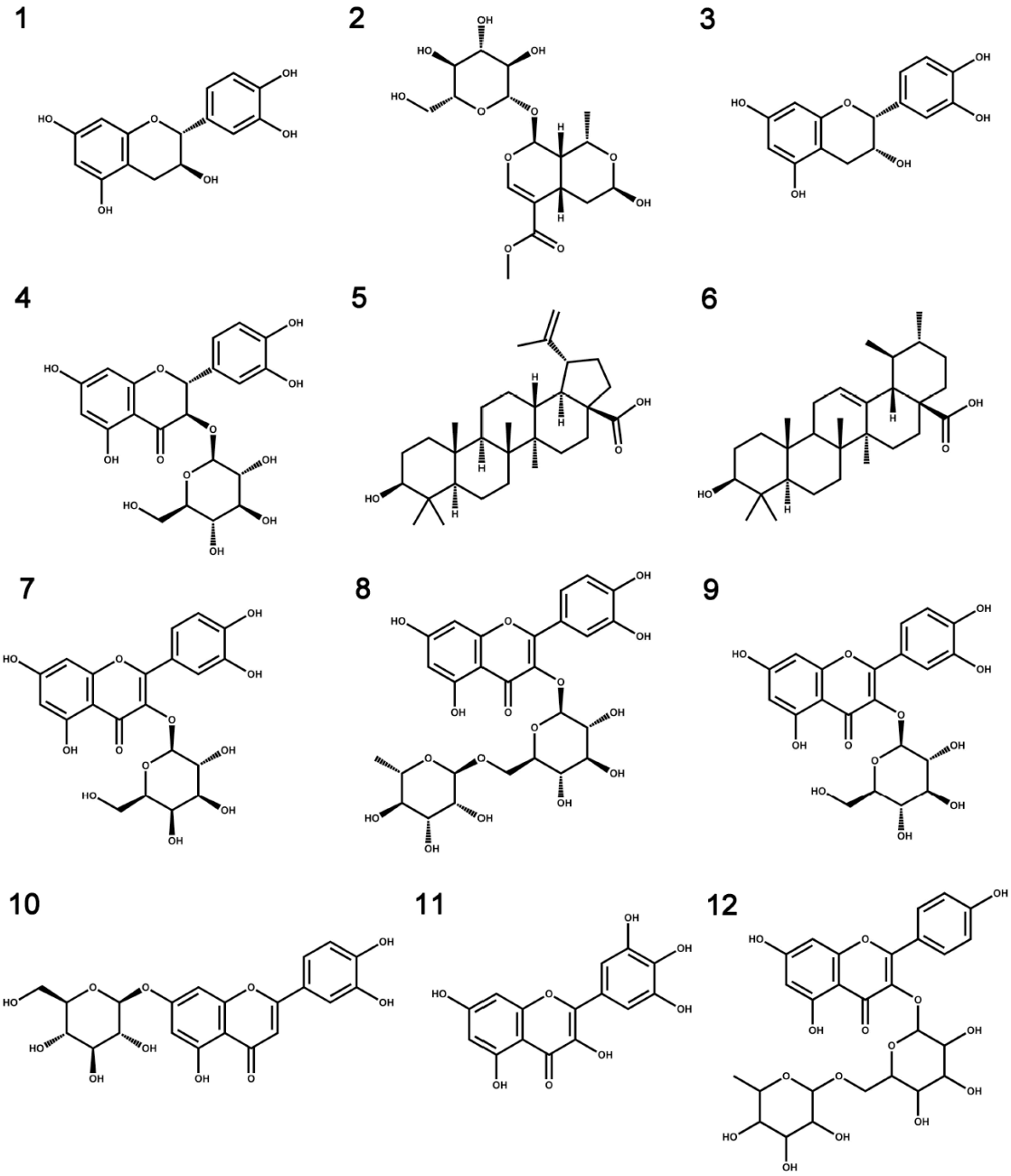

Figure 3.1: Chemical structure of compounds in *Sarracenia purpurea*. **1**. (+)-catechin; **2**. morroniside; **3**. (-)-epicatechin; **4**. taxifolin-3-O-glucoside; **5**. betulinic acid; **6**. ursolic acid; **7**. quercetin-3-O-galactoside; **8**. rutin; **9**. quercetin-3-O-glucoside; **10**. luteolin-7-O-glucoside; **11**. myricetin; **12**. kaempferol-3-O-rutinoside

spectrum, mass spectrometry (MS) and nuclear magnetic resonance (NMR) (Muhammad et al., 2012). All solvents were LC-MS grade and purchased from Fisher Scientific (Ottawa, ON, Canada).

3.2.2 Plant extracts

S. purpurea L. leaves were collected in a bog in the Eastern James Bay Region in Quebec, Canada according to healer's and elder's instructions and under permit from the TAAM – Cree Nation Intellectual Property agreement. This agreement assigns majority rights to the Cree. Its identity was confirmed by Dr. A. Cuerrier from the Montreal Botanical Garden and a voucher specimen #2003-05 was deposited at the Marie-Victorin herbarium at the Montreal Botanical Garden (Fraser et al., 2007; Leduc et al., 2006). The pitchers of the plant were cut open and cleaned of any potential insects or debris and dried in an electric food dehydrator (Nesco/American Harvest, Two Rivers, WI, USA) at 40˚C. The leaves were then ground to a powder using a Thomas Wiley Mill (1 mm filter) (Thomas Scientific, Swedesboro, NJ, USA). Subsequently, two different types of extracts were prepared.

The ethanol extract was prepared by using 80% ethanol in a ratio of 10 mL per gram of dry plant material. The extract was then separated from the plant residue using a Whatman qualitative no.1 filter (11 µm pore size) (Whatman plc, Maidstone, Kent, UK) using a vacuum filtration system. The residue was then extracted two more times. The filtered extracts were then combined and the ethanol evaporated using a rotary evaporator at 40°C and residual water

removed through lyophilisation. The water extract was prepared by gently boiling the plant material in distilled water for one hour in a ratio of 10 mL per gram of dry plant material. The extract was then separated from the plant residue as above and dried using a spray dryer. Both the ethanol and water extracts were ground with a mortar and pestle.

3.2.3 Ultra performance liquid chromatography – electrospray ionization – mass spectrometry (UPLC-ESI-MS)

The identification and quantification of the 12 standards found in the water and ethanol extracts was performed using a Shimadzu UPLC-MS system (Mandel Scientific Company Inc, Guelph, ON, Canada) containing LC30AD pumps, a CTO20a column oven, a SIL-30AC autosampler and a LCMS-2020 mass spectrometer using ESI mode. Two methods had to be employed, method A for the triterpenes, betulinic acid and ursolic acid, and method B for the 10 other compounds. Briefly for method A, the separation of the compounds was carried out on a Phenomenex Kinetex C18 column (2.6 µm particle size, 100 mm x 2.1 mm; Phenomenex Inc., Torrence, CA, USA) using an isocratic elution method with 62.5% acetonitrile (ACN) for 5 min. The flow was set to 0.4 mL/min with a column temperature of 55°C. Method B consisted of using the above column with a gradient elution method with 0.1% formic acid (solvent A) and 70% ACN with 30% MeOH and 0.1% formic acid (solvent B). The gradient was initially set at 86% A to 14% B for 5.5 min, followed by 65% A to 35% B for 2 min. The column was then washed for 2 min with 100% of solvent B and then solvent

concentration was returned to initial condition. The flow was set to 0.4 mL/min with a column temperature of 55°C.

Peaks within the extracts were identified based on a comparison with commercially purchased purified standards or isolated compounds based on the retention time and mass data. Table 3.1 summarizes the retention times and the ions used for the selective ion monitoring (SIM) of the standards. Quantification was performed using a standard curve composed of dilutions of the purified standards and calculating the amount of compound in each extract based on the curve. In order to account for any instrumental variation, each sample was injected and quantified in triplicate.

3.2.4 Analysis

Statistical analysis

Data was analysed using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA) using a multiple t-test comparing the concentration of each marker compound between the extracts. A false discovery rate was set to 1%.

Determination of the partition coefficient (logP)

The partition coefficient logP, a measure of lipophilicity, was evaluated using ChemBioDraw Ultra 13.0 software (Perkin Elmer, Waltham, MA, USA)

Table 3.1: List of identification parameters for the twelve standards used in the quantification of marker compounds in the leaves of *Sarracenia purpurea* using UPLC-ESI-MS. Compounds are numbered in order of retention time.

Peak	Compound	Separation method	Ionization mode	Retention time (min)	Ion detected (m/z)
1	(+)-catechin	B	Positive	1.65	291 ($M+H^+$)
2	Morroniside	B	Positive	1.83	429 ($M+Na^+$)
3	(-)-epicatechin	B	Positive	2.12	291 ($M+H^+$)
4	Taxifolin-3-O-glucoside	B	Negative	2.49	465 ($M-H^+$)
5	Betulinic acid	A	Negative	3.38	455 ($M-H^+$)
6	Ursolic acid	A	Negative	3.62	455 ($M-H^+$)
7	Quercetin-3-O-galactoside	B	Positive	4.88	487 ($M+H^+$)
8	Rutin	B	Negative	5.12	609 ($M-H^+$)
9	Quercetin-3-O-glucoside	B	Positive	5.35	487 ($M+H^+$)
10	Luteolin-7-O-glucoside	B	Positive	5.67	449 ($M+H^+$)
11	Myricetin	B	Positive	7.32	319 ($M+H^+$)
12	Kaempferol-3-O-rutinoside	B	Negative	7.64	593 ($M-H^+$)

3.3 Results

The water extraction produced a powder at a yield of 20.6%, while the ethanol extraction produced a resinous/glassy extract at a yield of 36.9%. Two different UPLC-ESI-MS methods used to analyze the ethanol and water extracts of the leaves of *S. purpurea* provided good resolution (Figure 3.2) of the 12 targeted compounds previously identified in *S. purpurea* (Figure 3.1). In the ethanol extract, the peaks representing betulinic acid (**5**), ursolic acid (**6**), morroniside (**2**), (-)-epicatechin (**3**), taxifolin-3-O-glucoside (**4**) and quercetin-3-O-galactoside (**7**) were present in high enough quantities to be visible on the chromatogram. Their identification in the extracts was achieved based on selective ion monitoring for particular mass over charge (m/z) and retention times (Table 3.1) compared to pure standards. In the water extract, the same compounds were identifiable, however at a lower concentration.

As shown in Figure 3.2, the relative abundance of the phytochemicals varied between the water and ethanol extracts. This was quantified in Table 3.2, where the amount of (+)-catechin (**1**) and morroniside (**2**) was significantly greater in the water extract compared to the ethanol extract ($p<0.001$). The concentration ratio (EtOH:H_2O) for the two marker compounds was 0.43 and 0.83, respectively. Thus **2** was slightly less concentrated in ethanol. Conversely, the phenolic compounds (-)-epicatechin (**3**), taxifolin-3-O-glucoside (**4**), quercetin-3-O-galactoside (**7**), and quercetin-3-O-glucoside (**9**) were present in significantly higher quantities ($p<0.001$ and $p<0.01$ for **9**) in the ethanol extract compared to the water extract with EtOH:H_2O concentration ratios of 1.64, 2.32,

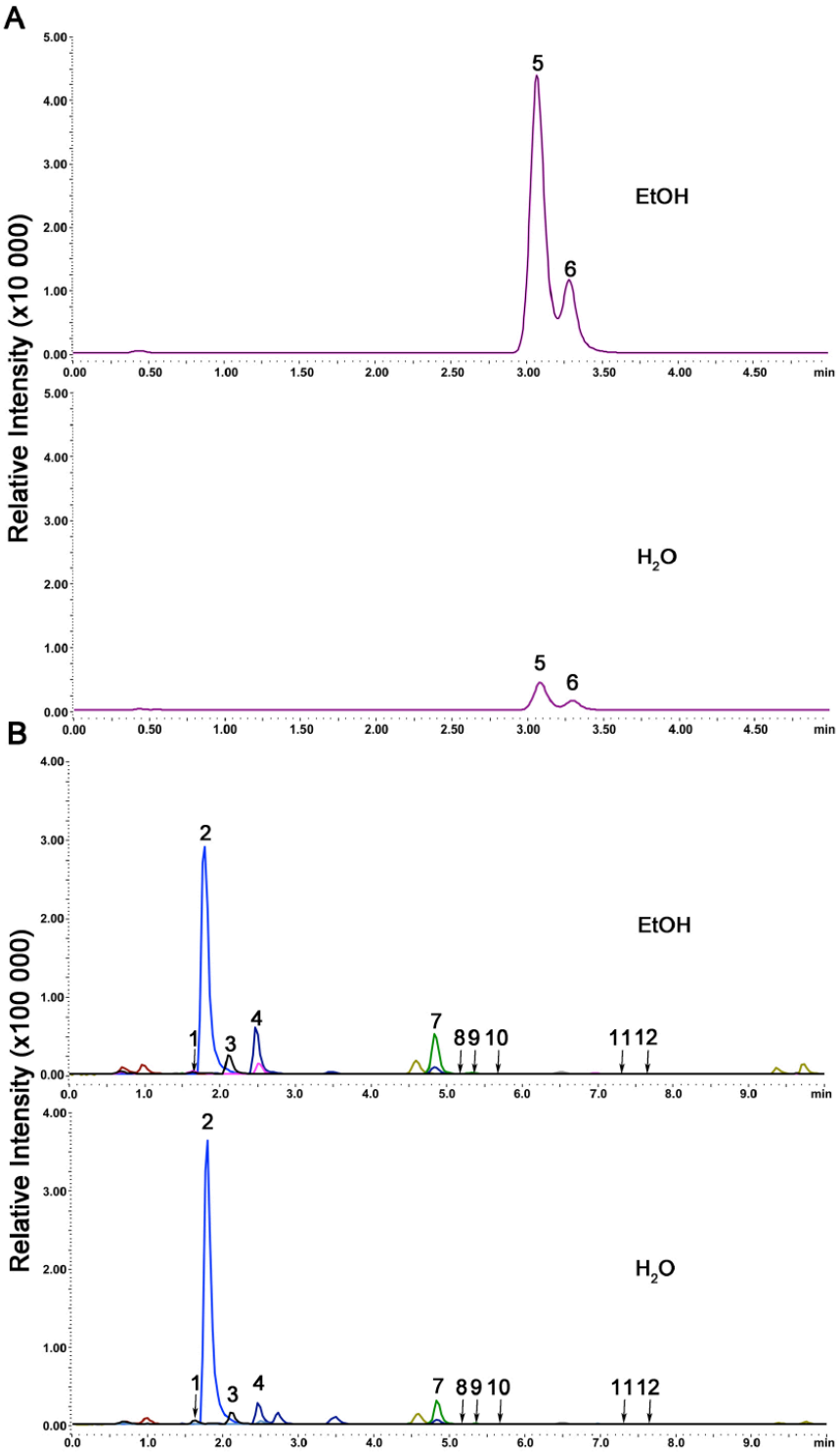

Figure 3.2: UPLC-MS-ESI chromatograms for the ethanol (EtOH) and water (H_2O) extracts analyzed via method A for triterpenes (A) and method B for the non-terpene compounds (B). The relative concentration differences of (+)-catechin (**1**), morroniside (**2**), (-)-epicatechin (**3**), taxifolin-3-O-glucoside (**4**), betulinic acid (**5**), ursolic acid (**6**) and quercetin-3-O-galactoside (**7**), rutin (**8**), quercetin-3-O-glucoside (**9**), luteolin-7-O-glucoside (**10**), myricetin (**11**), kaempferol-3-O-rutinoside (**12**) can be seen.

Table 3.2: Quantification of phytochemical compounds in ethanol and water extracts of *Sarracenia purpurea* using UPLC-ESI-MS. The concentration of each compound in each extract is presented in mg/g. Means of three injections and SEM (in brackets) are presented. Concentration ratio between the ethanol and water extract is also presented.

Peak	Compound	Ethanol extract (SEM) (mg/g)	Water extract (SEM) (mg/g)	[Ratio] (EtOH:H_2O)
1	(+)-catechin[a]	0.497 (0.033)	1.145 (0.011)	0.43
2	Morroniside[a]	106.169 (0.887)	127.824 (0.232)	0.83
3	(-)-epicatechin[a]	6.226 (0.053)	3.786 (0.025)	1.64
4	Taxifolin-3-O-glucoside[a]	90.659 (0.433)	39.094 (0.138)	2.32
5	Betulinic acid[a]	4.074 (0.036)	0.038 (0.001)	107.21
6	Ursolic acid[a]	1.769 (0.046)	0.019 (0.001)	93.11
7	Quercetin-3-O-galactoside[a]	34.833 (0.734)	20.784 (0.261)	1.67
8	Rutin	Trace[b]	Trace	--
9	Quercetin-3-O-glucoside[c]	1.398 (0.041)	1.118 (0.030)	1.25
10	Luteolin-7-O-glucoside	n.d. [d]	n.d.	--
11	Myricetin	n.d.	n.d.	--
12	Kaempferol-3-O-rutinoside	Trace	Trace	--

[a] significant difference between water and ethanol extracts ($p<0.001$)
[b] detected in trace amounts
[c] significant difference between water and ethanol extracts ($p<0.01$)
[d] n.d. Not detected

1.67, 1.25, respectively. The triterpenes, betulinic acid (**5**) and ursolic acid (**6**), were also more abundant in the ethanol extract ($p<0.001$), and here the magnitude of the difference was much greater as they were about 107 and 93 times more abundant in the ethanol extract, respectively. Kaempferol-3-O-rutinoside (**12**) and rutin (**8**), minor compounds in *S. purpurea*, were detected in trace amounts and below the limit of accurate quantitation. On the other hand, luteolin-7-O-glucoside (**10**) and myricetin (**11**) were not detected in the samples analyzed.

Evaluation of lipophilicity as determined by logP value can be found in Table 3.3. The majority of the compounds had a logP value between -2.28 and 1.5. This included all the phenolics and the iridoid glycoside, morroniside (**2**). The two triterpenes, betulinic acid (**5**) and ursolic acid (**6**) had significantly higher logP values of 7.38 and 7.33, respectively.

3.4 Discussion

This study was the first comparing the phytochemical content of *S. purpurea* extracts prepared in two different ways. The water extraction used in this study was consistent with the traditional preparation used by the Cree of Eeyou Istchee whereas the ethanol extract is preferentially used in most pharmacological studies examining the activity of medicinal plants. This comparison was crucial for the Cree community, as they wanted to know that the phytochemicals present and active in our assays were also the same ones present in their traditional preparation. It was shown here that the 80% ethanol

Table 3.3: Lipophilicity (LogP) values for the 12 compounds.

Peak	Compound	LogP
1	(+)-catechin[a]	1.5
2	Morroniside[a]	-1.77
3	(-)-epicatechin[a]	1.5
4	Taxifolin-3-O-glucoside[a]	-1.16
5	Betulinic acid[a]	7.38
6	Ursolic acid[a]	7.33
7	Quercetin-3-O-galactoside[a]	-1.39
8	Rutin	-2.28
9	Quercetin-3-O-glucoside[c]	-1.39
10	Luteolin-7-O-glucoside	-0.33
11	Myricetin	-0.04
12	Kaempferol-3-O-rutinoside	-1.89

extract generally results in significantly higher quantities of phenolics present, as well as the triterpenes. The only exceptions were the two most polar compounds present (+)-catechin (**1**) and morroniside (**2**).

This result was not surprising as higher quantities of phenolics have been consistently seen by other groups in alcohol extracts (Adedapo et al., 2011; Lapornik et al., 2005; Spigno et al., 2007). In fact, this is why alcohol extracts are preferred in lab bioassays since higher phenolic content results in higher antioxidant activity. Alcohols, being less polar solvents, are able to break down plant cell walls more efficiently and hence extract the necessary components (Lapornik et al., 2005). However, pure alcohol extracts are less efficient than alcohol/water mixtures (Sultana et al., 2009). The 80% ethanol/water mixture was optimized previously in the laboratory for phenolic extraction (Bergeron et al., 2000; Harris et al., 2007). Also, it is understandable that (+)-catechin (**1**) and morroniside (**2**) were present in significantly higher quantities in the water extract. They are the two most polar marker compounds examined, as can be seen by their early retention time, and hence more readily extracted in the more polar solvent (Lapornik et al., 2005).

Although significantly different between ethanol and water ($p<0.05$), the quantity of morroniside was only 1.2 times higher in the water extract as compared to the ethanol. Other groups have shown that morroniside has very potent antidiabetic activity (Park et al., 2011; Wang et al., 2010; Yokozawa et al., 2010), but our group, through bioassay-guided fractionation, has also shown that the neuroprotective activity of *S. purpurea* can be mediated through quercetin-3-

O-galactoside (Harris et al., 2012). An extract concentrated in the two compounds is therefore more biologically active.

Furthermore, Nan Shang's work in Dr. Haddad's lab (unpublished results) has shown that the ethanol extract was slightly more active than the water one in its antidiabetic activity, as demonstrated by glucose transport assays in muscle cells. However, it was the only extract that activated the AMPK pathway (Nan Shang, unpublished results), previously shown to be an active mechanism for *S. purpurea* (Martineau et al., 2010a; Spoor et al., 2006). A reason for this difference could be the extremely high quantity of betulinic acid and/or ursolic acid present in the 80% ethanol extract, where betulinic acid has been shown by Quan et al. (2013) to activate AMPK and the activation by ursolic acid has been shown by many groups (He et al., 2013; Son et al., 2013; Zheng et al., 2012). However, bioassay-guided fractionation of the extract by Muhammad et al. (2012), using this particular C2C12 mouse muscle cell assay, did not reveal betulinic acid or ursolic acid as a contributor. In fact, quercetin-3-O-galactoside was again a key active principle.

In terms of potential drug discovery, quercetin-3-O-galactoside, as well as the other phenolics, are more favourable options as their logP value, a measure of lipophilicity, is much lower than five. According to Lipinski's Rule of Five, compounds with logP values higher than five are less likely to be absorbed (Lipinski et al., 2001). Therefore, betulinic acid or ursolic acid, with logP greater than seven, are less likely to be biologically relevant options. However, discrimination of compounds based on their logP values is not ideal, as it does

not apply for compounds that are actively transported (Leeson, 2012). Furthermore, preliminary *in vitro* and in *vivo* work has already shown that ursolic acid and betulinic acid exhibit some antidiabetic activity (de Melo et al., 2009; Jang et al., 2010; Jang et al., 2009; Jayaprakasam et al., 2006; Oh et al., 2007; Zhang et al., 2006). Thus, it would be interesting to further explore the potential antidiabetic mechanism of betulinic acid and ursolic acid in more detail and determine why they are not the active principles in our assays.

An ethanol extract is generally preferred because water is able to extract other cellular components not limited to secondary metabolites and hence dilute the potential actives (Sharma et al., 2006; Spigno et al., 2007). This could then translate to a decreased, altered, or no activity in an *in vitro* assay. An exception to this preference would be in immunological studies, where pro-inflammatory polysaccharides may be necessary (Walshe-Roussel, 2013). This has been particularly important in the case of *Echinacea* (Singh, 2010) or ginseng (Assinewe et al., 2002), where the water extract has been shown to be quite pro-inflammatory in comparison to the ethanol one.

Although previously identified as minor components of *S. purpurea* (Rapinski, 2013*)*, luteolin-7-O-glucoside and myricetin were not detected in our extracts. Previous phytochemical analysis of the leaves of this species showed that these two compounds were present in trace quantities. As the contribution of these two compounds to the overall extract is fairly minor, the seasonal and geographic variability in phytochemical content may account for these compounds not being present in the plant material used in this study.

For the phytochemical markers that have been quantified in this study, a similar phytochemical composition for the two extracts can be seen and the phytochemicals responsible for the antidiabetic action in the traditional Cree medicine, as prepared by a water extract, are present and in same or similar quantities in the ethanol extract. This allows the use of a more standardized extract for future studies.

Chapter 4

Evaluation by microarray of the potential safety of *Sarracenia purpurea* L. (Sarraceniaceae) a traditional medicine used by the Cree of Eeyou Istchee

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Statement of author contribution

CC, JTA and BCF conceived and designed this study. Plant identification and collection was undertaken by AC. Morroniside was isolated by AM. The microarray experiments were performed by CC with assistance from CM. Microarray analysis was performed by JN and AB. The real-time validation experiments were performed by CC. SALB contributed critical equipment to the experiments. PSH is the principal investigator of the CIHR TAAM project and BCF and JTA contributed to manuscript preparation.

4.1 Introduction

The Cree of Eeyou Istchee (CEI) of James Bay in Northern Quebec are experiencing a rate of Type 2 diabetes (T2D) which is three to five times higher than that of the general Canadian population. More than one Cree adult in five has been diagnosed with diabetes (Kuzmina et al., 2010). This rate is high due to both environmental and genetic factors but is further compounded by low compliance with conventional medicines (Hegele and Bartlett, 2003; Young et al., 2000). The transition from a traditional diet and life on the land to a modern diet and sedentary lifestyle is a major contributing cause. To address the need for compliance, the Cree Health Board initiated a research project to examine the safety and efficacy of culturally relevant medicines and plant products in collaboration with the CIHR Team in Aboriginal Antidiabetic Medicines. An ethnobotanical survey was performed in 2003 to identify traditional medicines used to treat symptoms of T2D (Fraser et al., 2007; Leduc et al., 2006). One of the top eight plants identified was pitcher plant, *Sarracenia purpurea*, where leaves were used for treatment.

Sarracenia purpurea L. (Sarraceniaceae), the pitcher plant, Ayigadash in Cree, is a carnivorous plant found throughout North America that grows near nitrogen-poor environments such as bogs and peatlands (Ellison et al., 2012). Its leaves form pitchers that allow rain water to collect and with the help of variety of bacteria are able to break down and absorb the necessary nutrients from insects that have become trapped (Gray et al., 2012). Initial screening of *S. purpurea* by our collaborators discovered its potent insulinomimetic activity on glucose uptake

in muscle cells, even higher than that of the common antidiabetic therapy metformin (Spoor et al., 2006). Furthermore, it was shown to be very neuroprotective in states of both high and low glucose toxicity in PC12 cells. Recently, it has been shown that *S. purpurea* mediates its activity through a metformin-like mechanism by activating the adenosine-monophosphate-activated protein kinase (AMPK) by disrupting mitochondrial energy transduction (Martineau et al., 2010a). The AMPK pathway has been shown to be highly involved in regulation of metabolism, autophagy, and cell growth making it a very potent target for metabolic disease (Misra, 2008; Rautou et al., 2010; Shackelford and Shaw, 2009; Viollet et al., 2009; Zoncu et al., 2011). Thus, this further supports *S. purpurea*'s role as a traditionally relevant antidiabetic therapy.

S. purpurea contains numerous active compounds, many of which have been isolated through bioassay-guided fractionation (Harris et al., 2012; Muhammad et al., 2012). One of the active principles is morroniside (**1**), an iridoid glycoside, responsible for at least some of the antidiabetic activity, such as protection from cytotoxicity in the presence of glucose toxicity (Harris et al., 2012), observed with *S. purpurea*. Morroniside has also been isolated by other groups from *Cornus officinalis*, a traditional Chinese medicine, and shown to possess potent antioxidant, antiapoptotic and antidiabetic activities both *in vitro* and *in vivo* (Park et al., 2011; Wang et al., 2010; Yokozawa et al., 2010).

Parallel to evaluation of the traditional medicine *S. purpurea* for its efficacy, it was also important to consider safety. Traditional medicines can potentially contribute to adverse reactions as patients not only take one or more

of these medicines, but due to an increase in prevalence of other diseases, such as coronary artery disease, are more likely to be treated by other therapies as well (Hegele and Bartlett, 2003). A common way these interactions are manifested is by either inhibition or upregulation of the cytochrome P450 family of metabolic enzymes responsible for breakdown of xenobiotics (Bailey and Dresser, 2004). Any disregulation to the CYPs has the potential to cause adverse effects either by increasing the load of another drug and hence increasing toxicity or not providing the drug in high enough concentration as to render it ineffective (Bailey and Dresser, 2004; Ernst et al., 1999; Foster et al., 2001; Moore et al., 2000; Obach, 2000; Pal and Mitra, 2006). Work done by our group has already suggested that the CEI traditional medicines have the potential for such interactions by inhibiting members of the CYP family in enzyme assays (Tam et al., 2009, 2011). However, some plants work by disrupting *CYP* transcription, which has not been evaluated for *S. purpurea* or any other Cree medicinal plant.

The objective of this work was to first examine possible transcript changes to the *CYP* family of genes using a 19K human cDNA microarray in Caco-2 intestinal cells, which are a model of first-pass metabolism (Pfrunder et al., 2003; Watabe et al., 2003), in response to a *S. purpurea* extract and an active principle, morroniside. A second objective was to determine if any transcript changes observed could potentially explain the antidiabetic mechanism of action of the plant. Cells were treated with *S. purpurea* extract for 4 and 24 hrs, as well as the pure compound **1** for 4 hrs to determine their effects.

4.2 Materials and Methods

4.2.1 Materials and Sample Preparation

S. purpurea leaves were harvested in a bog in Eastern James Region, Quebec, Canada following directives by the healers and elders of the community. The plant was identified by Dr. A. Cuerrier and a voucher specimen # 2003-05 was deposited at the Marie-Victorin herbarium at the Montreal Botanical Gardens, Montreal, Quebec, Canada (Fraser et al., 2007; Leduc et al., 2006). It was subsequently extracted with ethanol, lyophilized, and analysed for phytochemical markers as previously described (Muhammad et al., 2012; Muhammad et al., 2013), as well as discussed in chapter 3. The crude extract was dissolved in dimethyl sulfoxide (DMSO) (Cat# D2650, Sigma-Aldrich, Oakville, ON, Canada) at a concentration of 100 mg/mL and filter sterilized with RC 0.20 nm filters (Cat# 431222, Corning Costar Co., NY, USA) to obtain a sterile stock.

The pure compound morroniside (Figure 4.1) was isolated and identified by one of us (AM) from *S. purpurea* using column chromatography and spectroscopic methods (Muhammad et al., 2012; Muhammad et al., 2013). It was dissolved in DMSO at a concentration of 10 mg/mL and filter sterilized with RC 0.20 nm filters to obtain a sterile stock.

4.2.2 Cell Culture

The C2BBe1 cell line, a clone of the Caco-2 colorectal adenocarcinoma cell line was obtained from the American Type Culture Collection (Cat# CRL-

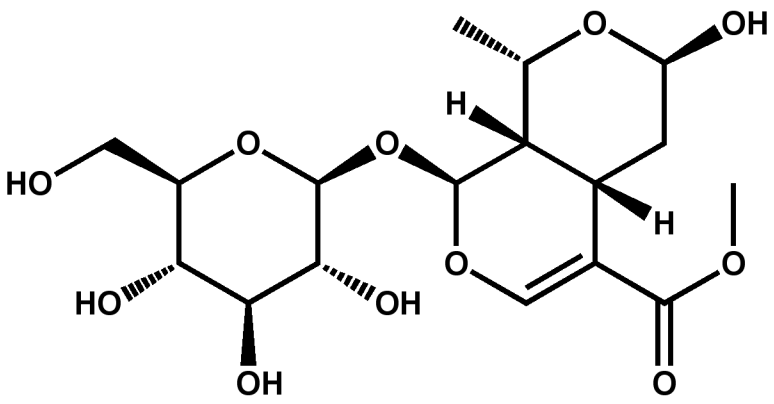


Figure 4.1: Structure of morroniside (**1**)**.**

2102, Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum, 100 units/mL of penicillin-streptomycin, 0.01 mg/mL lyophilized human transferrin and 4 mM L-glutamine. All cell culture reagents were purchased from Life Technologies (Burlington, ON, Canada). Cell culture was performed under sterile conditions and cultures were grown in a humidified incubator at 37°C with 5% CO_2.

Media was added to the cell culture every second day until the cells formed an 80 to 100% confluent monolayer across the bottom of the flask. At this point the flask was trypsinized and split. To split the flask, the media was removed and the cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS). The DPBS was then removed and discarded and 0.25% Trypsin-EDTA was added. The cells were trypsinized at 37°C until most of the cells were detached or for a maximum of 10 min. Media was then added to the flask to deactivate the 0.25% Trypsin-EDTA. The detached cells and media were spun down at 1000 rpm for 5 min. The supernatant was then decanted and the cells were re-suspended in media.

Caco-2 plant exposure

The cells were counted with a haemocytometer and plated at a concentration of 300 000 cells/well in a 24-well plate (Cat#3526, Corning Costar Co., NY, USA) with a final volume of 500 µL. The cells were allowed 24 hrs to attach to the plate before the media was changed and the treatment was added. The experimental cells were treated with 100 µg/mL of *S. purpurea* extract in

0.1% DMSO or 8.8 µg/mL morroniside in 0.1% DMSO and control cells were treated with 0.1% DMSO. After 4 or 24 hrs of incubation, the RNA was extracted. Twelve control and 12 treated samples were generated per experiment.

4.2.3 RNA Extraction

RNA extractions were performed according to protocol using the Qiagen RNeasy kit (Cat# 74106). The Qiashredder (Cat# 79654) was used to homogenize the cells and an on-column DNase treatment with the RNase-Free DNase Set (Cat# 79254) was used to remove DNA contamination. The kit and additional components were purchased from Qiagen (Toronto, ON, Canada).

The RNA was eluted into 30 µL of RNase free water and immediately stored at -80°C.

RNA Quantity and Quality

Before experiments were performed on the sample, the quality and quantity of the RNA was measured. The quantity of RNA was measured using the NanoDrop ND-1000 UV-Vis Spectrophotometer to measure absorbance at 260 nm and 280 nm. The quality of RNA was determined as per included protocol with the Agilent 2100 Bioanalyzer using the RNA 600 Nano LabChip Kit (Cat#5065-4476, Agilent Technologies, Mississauga, ON, Canada). The 2100 Expert software analyzed the results of the run to produce an electropherogram

and a gel-like image for each RNA sample. All samples had an RNA Integrity Number (RIN) higher than 9.

4.2.4 Microarrays

Human 19K cDNA microarrays were obtained from the University Health Network Microarray Centre (Array# H19K, Toronto, ON, Canada). The 12 control samples were pooled to produce a single control pool (CTRL). The treated samples were pooled into four pools (TRT1, TRT2, TRT3, TRT4), with three samples each. These RNA pools were used for the microarray experiments as per the 3DNA Array 900 MPX protocol; the Cy3 Kit (Cat# W599130, Genisphere, Hatfield, PA, USA) and the Cy5 Kit (Cat# W599140, Genisphere, Hatfield, PA, USA) were used. Two of the treated samples were labelled with Cy3 and the two remaining treated samples were labelled with Cy5, with the control being labelled with the alternative tag for each slide. The slides were also treated with Cot-1 DNA (Cat# 15279-011, Life Technologies, Burlington, ON, Canada) in the prehybridization step to minimize background. The microarray slides were read with the Scanarray 5000XL (Packard Biochip Technologies, Erie, PA, USA) using Scanarray Express software and analyzed with Quantarray software.

Microarray Analysis

Data was analyzed by our collaborators at the Public Health Agency of Canada. Data was converted from the format output used by the Scanarray scanner (Packard Biochip Technologies, Erie, PA, USA) to the .mev format used

by the TIGR's TM4 suite of array analysis tools (http://www.tm4.org/) using an in-house Microsoft Excel script. Raw data was normalized using Lowess and in-slide SD normalizations via the Ginkgo program (http://pfgrc.jcvi.org/index.php/bioinformatics/ginkgo.html), and missing values were removed. Data was imported into TM4's MeV array analysis program for further analysis. Appropriate dye-swap experiments were used to accommodate any biases, which could occur using a single dye. These results were treated as replicates within MeV. Analyses were performed using the Significance Analysis of Microarray (SAM) algorithm (Tusher et al., 2001), and a list of differentially expressed genes was generated using a false discovery rate of 1.5%. Fold change values were transformed using $\log_2$.

4.2.5 Real-time quantitative PCR

Reverse transcription to cDNA

Each RNA pool (CTRL, TRT1, TRT2, TRT3, TRT4) was converted to cDNA using Superscript II RNAse H RT with buffer and DTT (Cat# 18064-014, Life Technologies, Burlington, ON, Canada). Briefly, a 2 µg sample of RNA was combined with 1 µL of pdN6 random primers (Cat# C1181, Promega Corporation, Madison, WI, USA) and NF-H_2O to make a 12 µL reaction. The samples were incubated at 70°C for 10 min followed by 4°C for 2 min in the Biometra PCR Thermocycler (Biometra, Goettingen, Germany). A 7 µL aliquot of a mastermix consisting of 4 µL of First Strand Buffer, 2 µL DTT (0.1 M) and 1 µL dNTPs (10 mM) was added to each tube, and each sample further incubated at

37°C for 2 min then cooled to 25°C again using the Biometra Thermocycler. A 1 µL of Superscript RT was then added to the reaction and the tubes further incubated at 25°C for 10 min; 42°C for 60 min; 50°C for 30 min and 4°C for infinity. A negative RT control (nRT) was also ran at the same time consisting of 2 µg of each RNA sample and a 1 µL of NF-H_2O instead of the RT enzyme, as well as a no template control consisting of NF-H_2O instead of RNA. Furthermore, a Reference cDNA was also created using 2 µg of Stratagene QPCR Reference Total RNA (Cat# 750500, Lot# 0006153539, Cedarlane, Burlington, ON, Canada). The cDNA samples were stored at -80°C.

Efficacy of genomic DNA removal

The cDNA was verified for genomic contamination using an in lab GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protocol. Briefly, a 1 µL sample of each RT reaction including controls was combined with 24 µL of a mastermix containing 18 µL of NF-H2O, 2.5 µL of 10X Titanium Buffer (Clontech, Mountain View, CA, USA), 2 µL dNTPs (10 mM), 0.5 µL each of forward and reverse GAPDH primers, and 0.5 µL of Titanium Taq DNA Polymerase (Cat# 639209, Clontech, Mountain View, CA, USA). The sequence of the forward primer was 5'-TGG TGC TGA GTA TGT CGT GGA GT-3' and of the reverse was 5'-AGT CCT CTG AGT GGC AGT GAT GG-3'. A no template control consisting of NF-H_2O instead of RT reaction product was also run. The samples were run on a Bio-Rad PCR thermocycler (Bio-Rad, Mississauga, ON, Canada) using the following cycling conditions: 94°C for 5 min; followed by 35 cycles of 94°C for 25

s, 59°C for 50 s and 72°C for 105 s; then 1 cycle of 72°C for 7 min and 4°C for infinity. The products of the reactions were visualized with ethidium bromide on a 1% agarose gel (results not shown).

Real-time quantitative PCR (qPCR)

The qPCR was performed using Applied Biosystems' Taqman Gene Expression Assays (Life Technologies, Burlington, ON, Canada) on a Stratagene Mx3005P qPCR machine (Agilent Technologies, Mississauga, ON, Canada). The assays used were the following: *mTOR* (*FRAP1*), mammalian target of rapamycin – Hs00234508_m1; *BECN1*, beclin 1 – Hs00186838_m1; *TAP1*, transporter 1, ATP-binding cassette, sub-family B – Hs00388675_m1; *ABCA2*, ATP-binding cassette, sub-family A (ABC1), member 2 – Hs00242232_m1; *PRKCD*, protein kinase C delta – Hs01090047_m1; *CYP2D6*, cytochrome P450 2D6 – Hs02576168_g1 all labelled with a FAM tag and *RPLP0*, large ribosomal protein P0 – 4326314E used as a housekeeping gene labelled with VIC. The reaction tubes consisted of 5 µL of sample of interest with 15 µL of mastermix consisting of 10 µL of Taqman Gene Expression Master Mix (Cat# 4369016, Life Technologies, Burlington, ON, Canada), 4 µL NF-H_2O and 1 µL of the respective Taqman assay. The thermocycler conditions were as follows: 50°C for 2 min; 95°C for 10 min; followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The cycle threshold (Ct) value was measured at the end of each cycle based on fluorescence using the FAM/SYBR and VIC/HEX filters.

Initially, the linear range of each Taqman assay was determined using the Reference cDNA using a two-fold standard curve starting at 1/12.5 going to 1/200, representing 40 to 2.5 ng of RNA, with each dilution ran in duplicate. The Ct values were graphed against log[RNA] in GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA) and an R^2 value obtained based on the best three or more points. It was determined that a 1/25 dilution of the samples would be ideal to show an increase or a decrease in fold change. Then for each gene of interest a standard curve consisting of the Reference cDNA was ran again with the actual cDNA samples (CTRL, TRT1, TRT2, TRT3, TRT4) diluted 1/25 ran in triplicate. The nRT of each pool as well as no template controls were also run. The standard curve was again plotted in GraphPad Prism to determine an equation of the line from which the log[RNA] concentration of the sample was determined. For each gene of interest for each pool, the log[RNA] concentration was determined and normalized to the respective log[RNA] for the housekeeping *RPLP0* gene. The values were converted to [RNA] in ng and a fold change determined for each TRT pool normalized to the CTRL pool.

Three-fold qPCR changes

The protocol for qPCR was the same as described above except the samples of interest were a 1/25 sample dilution and a 1/75 dilution of the CTRL pool run in triplicate for *mTOR* and a 1/25 sample dilution and a 1/8.3 sample dilution run in triplicate for *BECN1*. The results were converted to [RNA] as described above and graphed using GraphPad Prism 6.0.

4.3 Results

Following 24-hr treatment with 100 µg/mL of *S. purpurea*, out of 19 200 possible cDNAs at an FDR stringency of 1.5%, a total of 8 transcripts were found to be significantly upregulated and 54 significantly downregulated, of which 12 transcripts could not be identified (Figure 4.2). A similar number of altered genes was seen following a 4-hr 100 µg/mL *S. purpurea* exposure (Figure 4.3), where a total number of 62 transcripts were deregulated, however the distribution pattern differed with 32 upregulated and 30 downregulated, where 13 could not be identified. The pure compound **1**, 4-hr exposure, produced the greatest number of observable changes as 137 transcripts were upregulated and 25 were downregulated, where 40 could not be identified (Figure 4.4). The pattern observed with the four separate arrays for each experiment was similar for each transcript, hence displaying good reproducibility within the four replicates.

Further analysis of the data using Ingenuity software examined the shared changes between the experiments. The number of transcripts altered that was shared between the different exposures/treatments was quite low (Figure 4.5). Only one transcript was altered within all three experiments, *RSBN1L* (round spermatid basic protein-1 like) (Table 4.1), and the direction of the change was variable over time showing a downregulation within the 4-hr exposure for both *S. purpurea* and **1**, and an upregulation following 24-hr exposure. This was consistent for the other comparisons of the 24-hr experiment with either *S. purpurea* or **1** at 4 hrs, where the direction of the change was always the inverse. The limited number of overlaps was also surprising where only two transcripts

Figure 4.2: Upregulated and downregulated genes following treatment with 100 µg/mL *Sarracenia purpurea* for 24 hours compared to 0.1% DMSO control. Four discrete biological replicates are presented in these results. Each replicate is a combination of three separate wells of cells.

Figure 4.3: Upregulated and downregulated genes following treatment with 100 µg/mL *Sarracenia purpurea* for 4 hours compared to 0.1% DMSO control. Four discrete biological replicates are presented in these results. Each replicate is a combination of three separate wells of cells.

Figure 4.4: Upregulated and downregulated genes following treatment with 8.8 µg/mL morroniside for 4 hours compared to 0.1% DMSO control. Four discrete biological replicates are presented in these results. Each replicate is a combination of three separate wells of cells.


ARRAY 1
ARRAY 2
ARRAY 3
ARRAY 4
-3.0
0.0
3.0
Transcribed locus
TGFA - Transforming growth factor, alpha
DENND2A - DENN/MADD domain containing 2A
USP1 - Ubiquitin specific peptidase 1
NA
FURIN - Furin (paired basic amino acid cleaving enzyme)
PTGS1 - Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
MED15 - Mediator complex subunit 15
EPSTI1 - Epithelial stromal interaction 1 (breast)
SMARCB1 - SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
LMAN2 - Lectin, mannose-binding 2
NUDT14 - Nudix (nucleoside diphosphate linked moiety X)-type motif 14
Transcribed locus
NA
COBRA1 - Cofactor of BRCA1
AGRN - Agrin
NA
H19 - H19, imprinted maternally expressed transcript (non-protein coding)
Transcribed locus
NMRAL1 - NmrA-like family domain containing 1
TNFAIP3 - Tumor necrosis factor, alpha-induced protein 3
RAB31 - RAB31, member RAS oncogene family
ENG - Endoglin
ALPP - Alkaline phosphatase, placental (Regan isozyme)
TMPO - Thymopoietin
C8orf4 - Chromosome 8 open reading frame 4
RPS7 - Ribosomal protein S7
ETNK1 - Ethanolamine kinase 1
KIF13B - Kinesin family member 13B
ZNF83 - Zinc finger protein 83
CTSA - Cathepsin A
H19 - H19, imprinted maternally expressed transcript (non-protein coding)
KIF2A - Kinesin heavy chain member 2A
GPR137 - G protein-coupled receptor 137
SRA1 - Steroid receptor RNA activator 1
SLC9A5 - Solute carrier family 9 (sodium/hydrogen exchanger), member 5
PDIA3 - Protein disulfide isomerase family A, member 3
ASPSCR1 - Alveolar soft part sarcoma chromosome region, candidate 1
ATP2B4 - ATPase, Ca++ transporting, plasma membrane 4
DDX39 - DEAD (Asp-Glu-Ala-Asp) box polypeptide 39
ETF1 - Eukaryotic translation termination factor 1
MYH10 - Myosin, heavy chain 10, non-muscle
SOCS3 - Suppressor of cytokine signaling 3
CIR - CBF1 interacting corepressor
B3GALT6 - UDP-Gal:betaGal beta 1,3-galactosyltransferase polypeptide 6
PSG11 - Pregnancy specific beta-1-glycoprotein 11
DYNLL1 - Dynein, light chain, LC8-type 1
TLN2 - Talin 2
RSPO2 - R-spondin 2 homolog (Xenopus laevis)
Transcribed locus
LAGE3 - L antigen family, member 3
RCVRN - Recoverin
SSH3 - Slingshot homolog 3 (Drosophila)
CYR61 - Cysteine-rich, angiogenic inducer, 61
NA
Transcribed locus
ELAVL3 - ELAV (embryonic lethal, abnormal vision, Drosophila)-like 3 (Hu antigen C)
PTPRG - Protein tyrosine phosphatase, receptor type, G
Transcribed locus
PGP - Phosphoglycolate phosphatase
ANKS1A - Ankyrin repeat and sterile alpha motif domain containing 1A
MAP1S - Microtubule-associated protein 1S
MRNA; cDNA DKFZp686B07124 (from clone DKFZp686B07124)
SDK1 - Sidekick homolog 1, cell adhesion molecule (chicken)
RABGGTA - Rab geranylgeranyltransferase, alpha subunit
NA
RTBDN - Retbindin
NA
MRPS12 - Mitochondrial ribosomal protein S12
TOX - Thymocyte selection-associated high mobility group box
THTPA - Thiamine triphosphatase
Transcribed locus
RRP1 - Ribosomal RNA processing 1 homolog (S. cerevisiae)
SERPINF2 - Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2
CASZ1 - Castor zinc finger 1
Mar-02 - Membrane-associated ring finger (C3HC4) 2
DPF3 - D4, zinc and double PHD fingers, family 3
Transcribed locus
CPNE2 - Copine II


ARRAY 1
ARRAY 2
ARRAY 3
ARRAY 4
-3.0
0.0
3.0
TBC1D9 - TBC1 domain family, member 9 (with GRAM domain)
FXYD1 - FXYD domain containing ion transport regulator 1
IREB2 - Iron-responsive element binding protein 2
ELAVL3 - ELAV (embryonic lethal, abnormal vision, Drosophila)-like 3 (Hu antigen C)
CDNA FLJ37032 fis, clone BRACE2011265
COL6A2 - Collagen, type VI, alpha 2
PVRL4 - Poliovirus receptor-related 4
AIPL1 - Aryl hydrocarbon receptor interacting protein-like 1
IGF2 - Insulin-like growth factor 2 (somatomedin A)
Transcribed locus
SDC1 - Syndecan 1
GNAT1 - Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1
Transcribed locus
VPS35 - Vacuolar protein sorting 35 homolog (S. cerevisiae)
NCOA2 - Nuclear receptor coactivator 2
DNAJC12 - DnaJ (Hsp40) homolog, subfamily C, member 12
AMZ2 - Archaelysin family metallopeptidase 2
MFGE8 - Milk fat globule-EGF factor 8 protein
LIMS1 - LIM and senescent cell antigen-like domains 1
PCSK1N - Proprotein convertase subtilisin/kexin type 1 inhibitor
HIST1H1C - Histone cluster 1, H1c
MAP4 - Microtubule-associated protein 4
VAV2 - Vav 2 guanine nucleotide exchange factor
ZFP36 - Zinc finger protein 36, C3H type, homolog (mouse)
PES1 - Pescadillo homolog 1, containing BRCT domain (zebrafish)
NA
PPP1R3F - Protein phosphatase 1, regulatory (inhibitor) subunit 3F
NR2F6 - Nuclear receptor subfamily 2, group F, member 6
MGC70870 - C-terminal binding protein 2 pseudogene
TRAPPC1 - Trafficking protein particle complex 1
NA
NA
AKAP8L - A kinase (PRKA) anchor protein 8-like
PRSS1 - Protease, serine, 1 (trypsin 1)
RP11-345P4.4 - Similar to solute carrier family 35, member E2
NXPH2 - Neurexophilin 2
ST3GAL2 - ST3 beta-galactoside alpha-2,3-sialyltransferase 2
ZNF385B - Zinc finger protein 385B
NA
AKAP13 - A-kinase anchor protein 13 isoform 2, A-kinase anchor protein 13 isoform 1
ST6GALNAC4 - ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 4
CTSD - Cathepsin D
AP1B1 - Adaptor-related protein complex 1, beta 1 subunit
SRM - Spermidine synthase
MRPS30 - Mitochondrial ribosomal protein S30
ZNF585B - Upstream of 5' of Zinc finger protein 585B and downstream of 3'Zinc finger protein 585A
NA
H2AFZ - H2A histone family, member Z
TOX - Thymocyte selection-associated high mobility group box
ZNF335 - Zinc finger protein 335
Transcribed locus
NALCN - Sodium leak channel, non-selective
HEY1 - Hairy/enhancer-of-split related with YRPW motif 1
BAG5 - BCL2-associated athanogene 5
CITED4 - Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4
CDNA clone IMAGE:5275948
NA
NA 343179
RSBN1L - Round spermatid basic protein 1-like
SLC39A9 - Solute carrier family 39 (zinc transporter), member 9
FOXO1 - Forkhead box O1
TFAP2B - Transcription factor AP-2 beta (activating enhancer binding protein 2 beta)
MMP2 - Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
PSMA7 - Proteasome (prosome, macropain) subunit, alpha type, 7
MRNA; cDNA DKFZp434G1972 (from clone DKFZp434G1972)
CNTNAP2 - Contactin associated protein-like 2
SET - SET nuclear oncogene
TSPAN5 - Transcribed locus, similar to transmembrane 4 superfamily member 9
SNED1 - Sushi, nidogen and EGF-like domains 1
MTF2 - Metal response element binding transcription factor 2
ZNF24 - Zinc finger protein 24
C1orf26 - Chromosome 1 open reading frame 26
SOS1 - Son of sevenless homolog 1 (Drosophila)
MGRN1 - Mahogunin, ring finger 1
GTF2H1 - General transcription factor IIH, polypeptide 1, 62kDa
ZNF532 - Zinc finger protein 532
DEPDC1B - DEP domain containing 1B
RPL36AL - Ribosomal protein L36a-like
SRRM1 - Serine/arginine repetitive matrix 1
WDR25 - WD repeat domain 25
SNX10 - Sorting nexin 10
SF1 - Splicing factor 1
NA

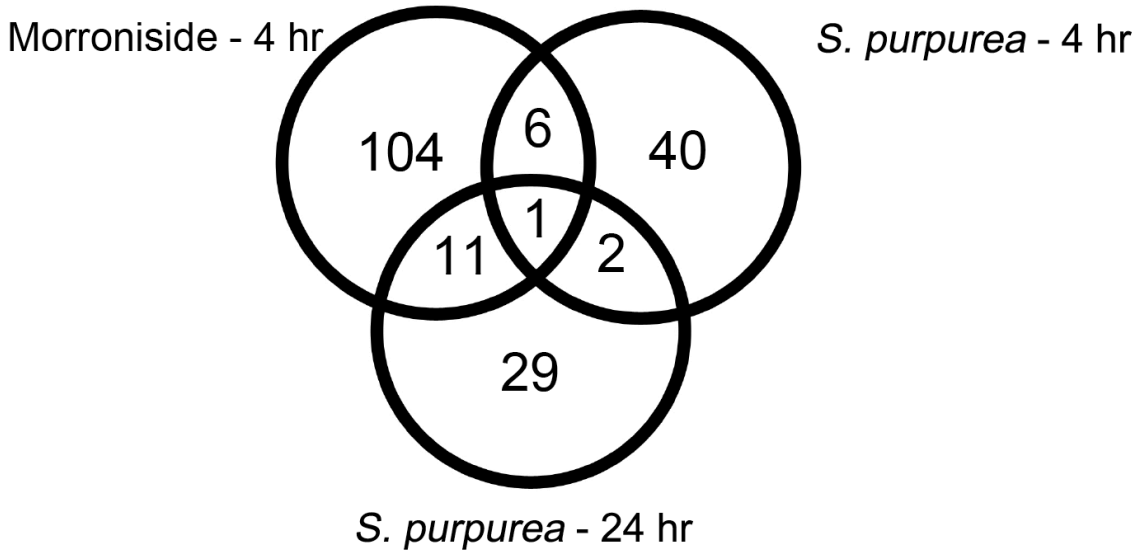


Figure 4.5: Overlap of shared genes that are deregulated between the three different experimental conditions with either morroniside or *Sarracenia purpurea.*

Table 4.1: List of altered genes that are represented in comparisons between the three different experiments with *Sarracenia purpurea* and morroniside (**1**). Red represents genes that are consistently upregulated across specified conditions, Green represents genes that are consistently downregulated across specified conditions, Black represents no consensus. Analysis performed using Ingenuity software.

ALL	*S. purpurea* 4-hr and 24-hr	*S. purpurea* 4-hr and **1** 4-hr	*S. purpurea* 24-hr and **1** 4-hr
RSBN1L	*ECHS1*	*EPSTI1*	*B3GALT6*
	UNC5A	*H2AFZ*	*BAG5*
		PES1	*CITED4*
		RABGGTA	*CTSD*
		SNX10	*ELAVL3*
		TFAP2B	*MAP1S*
			MYH10
			PDIA3
			TMPO
			TOX
			VAV2

were shared between the 4-hr and the 24-hr incubation with *S. purpurea* and 11 transcript changes between the 4-hr **1** and 24-hr *S. purpurea*. A comparison between *S. purpurea* and **1** at 4 hrs yielded six transcripts that were altered in the same direction, thus suggesting that the particular effect was mediated in part by the pure compound, however the transcripts do not belong to a particular canonical pathway and are therefore not limited in their scope. A further examination of all the shared altered genes suggested changes in cellular development and cell assembly and organization, thus proposing an overall effect on the cell that may not be representative of the action of the plant. What was most surprising, with all the transcript changes observed, was that none of them belonged to the CYP family of metabolic proteins, nor to other metabolic enzymes such as GST (glutathione-S-transferase) or QR (quinone reductase), nor to any major drug transporters such as the ABC transporters involved in xenobiotic metabolism.

To see whether or not the overall observed changes belonged to any particular cellular process, all three array experiments were examined. However, the focus of the analysis became on the 24-hr exposure, as all transcript changes were greater than two-fold as opposed to the 4-hr experiments, where only two transcripts for *S. purpurea* (including one unidentified) and five for morroniside (including two unidentified) were modified greater than two-fold. Upon further pathway analysis, some key transcripts involved in mTOR signalling were identified, where *mTOR* (*FRAP1*) itself was significantly downregulated by a 2.6-fold change following 24-hr exposure and beclin-1 (*BECN1*) was significantly

upregulated by a 3.1 fold change. These transcript changes were verified through the use of quantitative real-time PCR (Table 4.2). As can be seen, no change was observed for both *mTOR* and *BECN1* with qPCR. Furthermore, additional genes were examined by qPCR, *ABCA2*, *PRKCD*, *TAP1* all significantly altered on the microarray, with changes greater than two-fold, and *CYP2D6*, one of the major CYP metabolizing enzymes and not altered on the array, to see if the microarray could be validated (Table 4.2). None of the transcripts were altered in the qPCR experiment.

In order to ensure that the qPCR technology was working correctly, an experiment was performed to see whether or not a three-fold upregulation or a three-fold downregulation could actually be observed. As can be seen in Figure 4.6A, when 1/3 of the amount of cDNA was used, a three-fold decrease in the *mTOR* gene expression was detected, and when three times the amount of starting material was used with *BECN1* Taqman assay, a corresponding three-fold increase was noted (Figure 4.6B).

4.4 Discussion

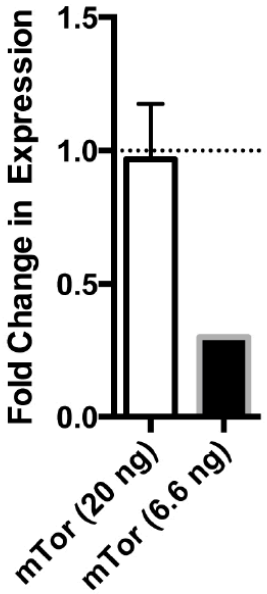
While traditional Cree medicinal plants have been shown to have potent antidiabetic activity both *in vitro* (Harbilas et al., 2009; Spoor et al., 2006) and *in vivo* (Harbilas et al., 2012a; Harbilas et al., 2012b, 2013) their potential safety has only been evaluated in the context of potential drug interactions mediated by inhibitory effects on CYP enzymes using an *in vitro* assay (Tam et al., 2009, 2011). This present study was the first examining the effect of one of the most

Table 4.2: List of genes verified by qPCR using Taqman Gene Expression Assays for the 24-hour exposure of C2Bbe1 cells to 100 µg/mL *Sarracenia purpurea*.

Gene	RefSeq ID	Microarray fold change	qPCR fold change
ABCA2	NM_001606	-3.7	n.c.[a]
BECN1	NM_003766	3.1	n.c.
CYP2D6	NM_000106	n.c.	n.c.
FRAP1	NM_004958	-2.6	n.c.
PRKCD	NM_006254	3.2	n.c.
TAP1	NM_000593	-2.9	n.c.

[a] n.c. no change

A



B

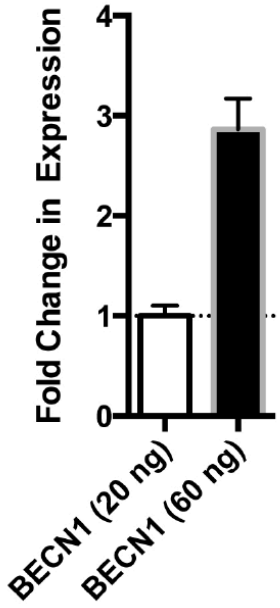


Figure 4.6: Three-fold change in expression as determined by qPCR. (A) A 1/25 dilution and a 1/75 dilution of CTRL pool from the 24-hour *Sarracenia purpurea* exposure experiment were run in a qPCR reaction using the *mTOR* gene expression assay. (B) A 1/25 dilution and a 1/8.3 dilution of the CTRL pool from the 24-hour *S. purpurea* exposure experiment were run in a qPCR reaction using the *BECN1* gene expression assay. Each dilution was run in triplicate. Mean + SEM are presented.

important traditional medicines used by the Cree, *S. purpurea*, and one of its components, morroniside, on potential disregulation in cell culture of a wide range of cytochrome P450 genes, as well as other transporters important for disposition of xenobiotics through the use of microarray technology. By using an array with a wide variety of expressed sequence tags (ESTs), an untargeted approach was used to capture information on almost any change occurring. Therefore it was possible to further look at other effects including potential beneficial mode of action of the plant.

Using a 19K cDNA microarray and a cell line responsible for first-pass metabolism, no significant changes to *CYP1B1*, *2C8*, *2D6*, *3A4*, *3A5*, *4A11*, *4B1*, *4F12*, *11A1*, *19A1*, *24A1*, and *26A1* in any of the three treatments (*S. purpurea* for 4 or 24 hrs, morroniside **(1)** for 4 hrs) were observed. This was further verified by using qPCR technology and the TaqMan Gene Expression Assay system, which has been shown to have high correlation with microarray data and is a robust method (Canales et al., 2006), for the *CYP2D6* probe set and again no significant change was observed. This was somewhat surprising as many plants have evolved secondary metabolites in order to be resistant against microbes and insects (Harborne, 2000) and often possess compounds that inhibit CYP450 in order to potentiate their defences. Although mostly described on a protein level (Guo et al., 2012; Kaneko et al., 2013; Krizkova et al., 2009; Sergent et al., 2009), these changes also occur on a transcript level (de Waard et al., 2008; El-Readi et al., 2013; Guo et al., 2011; Jensen et al., 2006a; Vrba et al., 2012). Furthermore, other reports have demonstrated changes to genes

important for xenobiotic disposition such as ABC transporters and Phase II metabolizing enzymes in response to plant extracts, including but not limited to GSTs and QRs (Bermudez-Soto et al., 2007; de Waard et al., 2008; Gerhauser et al., 1997) all present on this array but not significantly altered. Another important family of transcripts involved in conjugation of xenobiotics, UDP-glucuronosyltransferases (UDPGTs), found to be altered by others (de Waard et al., 2008), unfortunately were not spotted on this array. Moreover, the International Transporter Consortium et al. (2010) has recently recognized other key transporter families important in drug disposition, such as the solute carrier superfamily, where over 200 ESTs were spotted on this array, but none were significantly altered. Since *S. purpurea* extracts showed minimal inhibition of the CYPs in enzyme assays (Tam et al., 2009, 2011) and now no significant deregulation in the CYP transcripts, as well as to other important xenobiotic metabolizing transcripts is shown, these results suggest no major activity in the extract and purified compound with respect to metabolism and clearance of xenobiotics. This was demonstrated at a concentration higher or equal to that used by others when examining plant extracts (Budzinski et al., 2007; El-Readi et al., 2013; Raucy, 2003).

Using Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com), further examination of the significantly altered transcript changes revealed that the deregulated genes did not belong to a particular pathway or network. Furthermore, the overlap in transcript changes was very limited, where only *RSBNL1* (round spermatid basic protein 1 like protein) was

deregulated in all three experiments. RSBNL1 belongs to the family of round spermatid basic protein 1, whose function still remains unknown. What was evident, however, was the biphasic nature of the plant extracts, where the common deregulated genes between 4 and 24-hr treatments were always in the opposite direction. This has previously been reported, as plant extracts tend to produce these biphasic effects (Jensen et al., 2006a; Muhammad et al., 2012). Furthermore, only a limited number of transcript changes were shared between **1** and *S. purpurea* at 4 hrs, thus suggesting that the action of *S. purpurea* may be mediated by **1** at that time point for those transcripts. However, even looking at the limited number of altered transcripts shared, no single pathway was implicated, thus suggesting multiple mechanisms of action for the plant extract. This is not surprising given the large number of bioactive compounds in *S. purpurea* (Muhammad et al., 2013) and the observation that other medicinal plants such as St John's Wort also show multiple modes of action due to presence of several bioactive constituents (Butterweck, 2003).

Another objective of this study was to examine if any potential mode of action of the plant could be observed, albeit cautiously, as the Caco-2 model is important for safety but not necessarily diabetic involvement. The analysis was concentrated on the 24-hr *S. purpurea* exposure because morroniside 4-hr exposure produced over 100 transcript changes, thus potentially leading to more undesirable effects. Since plants generally possess a wide variety of phytochemicals, they can act together to cancel the negative effects produced by one compound. The use of a plant extract was also more relevant to validating

this Cree traditional medicine. The 24-hr exposure was then chosen over the 4-hr for further study, as transcriptional changes generally take time (Spoor et al., 2006), the Cree take the medicines over an extended period of time, and usually groups looking at transcriptional changes expose the cells for 24 hrs or more (de Waard et al., 2008; Gonzalez-Sarrias et al., 2009).

Interestingly, two key transcripts were found to be altered by the 24-hr treatment with *S. purpurea*, *BECN-1* and *FRAP1* coding for beclin-1 and mTOR, respectively. These two genes are part of the mTOR signalling pathway, which corresponds to the previously published work by our group of the role of *S. purpurea* in altering the AMPK pathway (Martineau et al., 2010a). However, this could not be verified using qPCR. Therefore, mRNA changes in *TAP1*, *PRKCD* and *ABCA2* were selected for verification (Table 4.2). These three were chosen, as they were some of the most significantly deregulated genes, *TAP1* and *ABCA2* being downregulated and *PRKCD* being upregulated. As well, they were the most interesting from the safety and diabetes standpoints as high presence of TAP1 has been shown to be involved in drug resistance (Xu et al., 2013b), PRKCD has been shown to be important for insulin sensitivity (Bezy et al., 2011) and ABCA2 shown to be important for lipid function and metabolism, although primarily in the brain (Wolf et al., 2012). Again, the observed fold changes detected by microarray could not be replicated, which is not unusual as discrepancies between microarray and qPCR results have been reported in the past, both in Caco-2 experiments (Bermudez-Soto et al., 2007) and not (Lopes et al., 2010). However, three-fold differences, when either three times the amount

or 1/3 of the starting material was used, were observed, confirming successful qPCR methodology. Furthermore, the primer sets were verified to be amplifying the same transcripts as those on the cDNA microarray. Two main reasons for the observed differences are inherent to the two different technologies used. One is the way the cDNA was prepared. In the array preparation, random hexamers and oligo dT primers were used, while for the qPCR only random hexamers were used. Thus, the difference in priming methods may be responsible for this observed difference (Morey et al., 2006; Shippy et al., 2004). A more confounding factor still is the fact that the probe sequences for the array and the qPCR were not the same. This led to different target locations between the two methods and this has long been recognized as one of the key issues with validation (Canales et al., 2006; Etienne et al., 2004; Morey et al., 2006). Unfortunately, primer and probe sets for the exact location were not available and if ever further validation was required, they would have to be designed and synthesized *de novo*.

S. purpurea's mechanism of action does not appear to be through global transcriptional changes as demonstrated by this work. It further supports work done by our group that the mechanism of action is on the protein level (Martineau et al., 2010a) and in particular tissues. Therefore, future work should further examine the mechanism of action of the plant, particularly concentrating on the mTOR signalling pathway and its possible role in combating diabetes. Moreover, from a safety standpoint, the plant does not appear to alter the cytochrome P450 family of metabolic enzymes, in addition to other xenobiotic metabolizing

enzymes and transporters, on a transcriptional level as seen in the microarray and qPCR providing further evidence to its overall potential safety.

Chapter 5

A Cree traditional medicinal plant, *Sarracenia purpurea* L., alters learning and memory in TgCRND8 mice likely by increasing levels of PC(O-16:0/2:0) PAF

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Statement of author contribution

CC, JTA and SALB conceived and designed this study. Treatment and behavioural paradigms were performed by CC and FA. Lipid isolation was performed by CC and HX, with HX running the samples on the QTRAP. MWG contributed control male animals and APB contributed control female animals. All data analysis was performed by CC. PSH is the principal investigator of the CIHR TAAM project and BCF, JTA and SALB contributed to manuscript preparation.

5.1 Introduction

Two major molecular hallmarks of Alzheimer's disease (AD) are neurofibrillary tangles that result from hyperphosphorylated tau and aberrant processing of the amyloid precursor protein (APP) into amyloid β (Aβ) peptides (Goedert and Spillantini, 2006). The most toxic of the produced peptides is $A\beta_{42}$, which has been associated with neurodegeneration and cognitive impairment (Cleary et al., 2005; Selkoe, 2002). However, the link between tau and Aβ still needs to be elucidated and Herrup (2010) has suggested that a separate "change of state" occurs that alters the metabolic state required for conversion from a pre-symptomatic to symptomatic state in AD progression. One metabolic indicator is insulin resistance. Recently, AD has been referred to as "type 3 diabetes" (de la Monte and Wands, 2008), because Type 2 diabetes (T2D) has been identified as a risk factor for AD (Nicolls, 2004; Qiu et al., 2009; Schrijvers et al., 2010). Particularly, insulin resistance, but only in the first three years of onset, is associated with an elevated risk of AD (Schrijvers et al., 2010). Glucotoxicity, formation of advanced glycation endproducts (AGEs), as well as neuroinflammation, all present with T2D, are also associated with AD (Craft, 2007, 2009; Guglielmotto et al., 2012; Kojro and Postina, 2009). Thus, the aim of this work was to test whether traditional medicines designed to reduce these pre-diabetic pathologies might affect AD progression.

Our CIHR Team in Aboriginal Antidiabetic Medicines (TAAM) has worked with Cree of Eeyou Istchee (CEI) of James Bay in Northern Quebec to identify culturally relevant therapies to provide complementary therapies for T2D (Fraser

et al., 2007; Leduc et al., 2006) as prevalence of the disease is 3-5 times greater than in the general population of Canada (Kuzmina et al., 2010). This is further compounded by low compliance, although genetic and environmental factors are also present that contribute to T2D prevalence in this population (Hegele and Bartlett, 2003; Young et al., 2000). An ethnobotanical survey identified 17 plants used to treat symptoms of T2D, with one of the top plants being *Sarracenia purpurea* (Fraser et al., 2007; Leduc et al., 2006).

S. purpurea L. (Sarraceniaceae), the pitcher plant, can be found in bogs and peatlands, both nitrogen-poor environments, and obtains necessary nutrients from insects, as it is a carnivorous plant (Ellison et al., 2012). CEI healers identified this plant as highly effective at treating T2D symptoms (Leduc et al., 2006), but one that should not be used when the patient was “in a weakened mental state” (personal communication, CIHR TAAM group meeting, Mistissini, QC, August 16, 2010). Initial work on the plant by TAAM has shown its potent insulinomimetic activity on glucose uptake in muscle cells, and its neuroprotective ability in states of both high and low glucose toxicity in PC12 cells (Harris et al., 2012; Spoor et al., 2006). Furthermore, the mechanism of action has been shown to be similar to that of metformin, a common antidiabetic therapy, by activating the adenosine-monophosphate-activated protein kinase (AMPK) by disrupting mitochondrial energy transduction (Martineau et al., 2010a). The AMPK pathway is an important target for metabolic disease as it is highly involved in regulation of metabolism, autophagy, and cell growth (Misra, 2008; Rautou et al., 2010; Shackelford and Shaw, 2009; Viollet et al., 2009;

Zoncu et al., 2011). A phytochemical characterization of the plant, in chapter 3 and by Harris et al. (2012) and Muhammad et al. (2013), has identified morroniside, quercetin-3-O-galactoside, ursolic acid and betulinic acid as key phytochemicals present in the extract used.

Since *S. purpurea* has been shown to be neuroprotective *in vitro* and since quercetin and ursolic acid, as well as metformin itself, have all been shown to improve cognitive function *in vivo* (Bhutada et al., 2010a; Bhutada et al., 2010b; Lu et al., 2011; Lu et al., 2007; Pintana et al., 2012), we asked whether *S. purpurea* ethanolic extract would confer the same protection to a mouse model of AD. TgCRND8 mice were used, a model of AD on a mixed hybrid C57BL/6 X C3H background (Chishti et al., 2001), backcrossed in the Bennett laboratory for five generations to a C57BL/6 strain. On this genetic background, TgCRND8 mice transition from a pre-symptomatic state to a symptomatic state with respect to learning and memory impairment by six months of age (Wang et al., 2013). The effect of *S. purpurea* on peripheral glucose and insulin levels, and behavioural indices of anxiety, and learning and memory in these mice (Tg) and their NonTg littermates was evaluated. To provide mechanistic insight, the potential of *S. purpurea* treatment to alter defining changes in critical phospholipid species, for example the platelet activating factor (PAF) PC(O-16:0/2:0), previously shown to impact learning and memory in human condition and mouse models of AD (Ryan et al., 2009), was also assessed. The goal of this study was to establish whether behavioural changes in mice could be linked

to a metabolic change of state in central alkylacylglycerophosphocholine metabolism associated with AD pathology.

5.2 Materials and Methods

5.2.1 Materials and Sample Preparation

S. purpurea leaves were collected in the Eastern James Region of Quebec, Canada (Leduc et al., 2006), following directives of community healers and elders and according to the research and ethical agreement established as part of the Canadian Institutes of Health Research (CIHR) Team in Aboriginal Antidiabetic Medicines (CIHR-TAAM, CTP-79855). Plant identity was confirmed by Dr. A. Cuerrier (Institut de recherche en biologie végétale, Montreal Botanical Gardens) and a voucher specimen # 2003-05 was deposited at the Marie-Victorin herbarium at the Montreal Botanical Gardens, Montreal, Quebec, Canada (Fraser et al., 2007; Leduc et al., 2006). They were subsequently extracted with ethanol, lyophilized, and analyzed for phytochemical markers as previously described in chapter 3 and by our group (Harris et al., 2012; Muhammad et al., 2012; Muhammad et al., 2013).

Chemicals and Reagents

Chloroform (Cat#C298-4), 2-propanol (Cat#A416-4) and methanol (Cat#A412P-4) were all ACS grade and purchased from Fisher Scientific (Ottawa, ON). Water (Cat#9831-03) and acetonitrile with 0.1% formic acid (Cat#9832-03) were LC-MS grade purchased from VWR (Mississauga, ON).

5.2.2 Animals

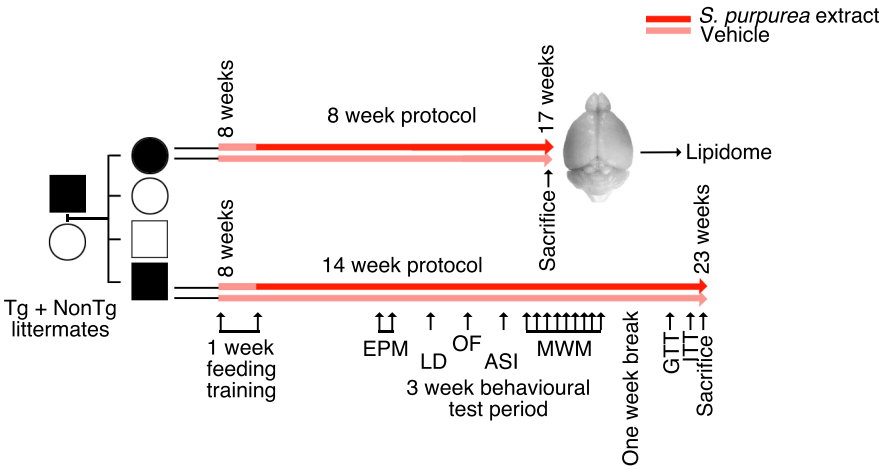
TgCRND8 mice (Tg) on a mixed C57BL/6 X C3H hybrid background, expressing the Swedish and Indiana mutations (KM670/671NL + V717F) in the human *APP* gene under the control of the hamster prion promoter (Chishti et al., 2001) were kindly donated by Dr. Fraser (University of Toronto). They were then backcrossed five generations to a C57BL/6 lineage (Charles River, Wilmington MA, USA) to obtain an N5 generation. Heterozygote Tg and congenic wild-type (NonTg) littermates were maintained in our breeding facility by mating NonTg females with Tg males. A total of 48 mice were used in this study, 36 males (Tg, n=15; NonTg, n=21) and 12 females (Tg and NonTg, n=6). To verify the Tg genotype, genomic DNA was isolated from biopsied tails and used in a PCR reaction to verify the presence of a 1 kb amplicon in the Tg mice. The PCR conditions were as follows: 94°C for 3 min, followed by 35 cycles of 20 s at 94°C, 20 s at 68°C, and 90 s at 72°C, followed by one cycle of 73°C for 7 min. The primer sequences were as follows: Forward – 5'-GGC CGC GGA GAA ATG AAG AAA CGC CAA GCG CCG TGA CT-3', Reverse – 5'-TGT CCA AGA TGC AGC AGA ACG GCT ACG AAA A-3'. Furthermore, template integrity was also validated by PCR for the platelet-activating factor receptor and the presence of a 289 kb amplicon in all animals. The PCR conditions were as follows: 95°C for 10 min, followed by 30 cycles of 20 s at 94°C, 20 s at 65°C, and 50 s at 70°C. The primer sequences were as follows: Forward – 5'-TAT GGC TGA CCT GCT CTT CCT GAT-3', Reverse – 5'-TAT TGG GCA CTA GGT TGG TGG AGT-3'. All animal manipulations were performed in strict accordance with the ethical

guidelines for experimentation established by the Canadian Council for Animal Care and with the approval of the University of Ottawa Animal Care Committee for the ethical treatment of experimental animals, protocol # BMI-134.

Treatment paradigm

Two-month-old Tg and NonTg littermates (both male and female) were treated orally with *S. purpurea* extract at 250 mg/kg b.w./day suspended in 25% sweetened condensed milk (President's Choice, Brampton, ON) (Cayer et al., 2013) (Figure 5.1). Animals were trained to drink the sweetened condensed milk vehicle directly from a 1 cc syringe while the appropriate volume was delivered slowly and continuously by the handling investigator for one week prior to assignment to treatment (*S. purpurea*) or vehicle (25% sweetened condensed milk) groups. Control animals received handling 2-3 times per week without oral treatment. Mice were singly-housed with *ad libitum* access to food and water. Following 60 days of treatment, four-month-old females (n=3 per genotype/treatment) were euthanized by lethal injection of euthanyl (Bimeda-MTC Animal Health Ins., Cambridge, ON) prepared in sterile water to a final concentration of 65 mg/mL, and their temporal cortex dissected, immediately flash frozen in liquid nitrogen and stored at -80°C for further lipid analysis. Males underwent a three week behavioural paradigm with continuous treatment and were sacrificed at 5.5 months of age with a lethal injection of euthanyl, as described above, and transcardial perfusion with 10 mM phosphate buffered saline (PBS; 10 mM sodium phosphate and 154 mM NaCl) followed by 3.7%

Figure 5.1: Treatment and behavioural paradigms. Two-month old Tg and NonTg littermates were treated with *S. purpurea* extract at 250 mg/kg b.w./day emulsified in 25% sweetened condensed milk following a one week acclimatization period to the milk. Female mice were treated for eight weeks followed by sacrifice via euthanyl injection and a temporal cortex dissection. The cortex was then extracted for lipids and analyzed using LC-ESI-MS/MS. Male mice were treated for eight weeks followed by a behavioural paradigm, while undergoing constant treatment. The behavioural paradigm consisted of the elevated plus maze (EPM), light dark (LD), open field (OF), adult social interaction (ASI) and a nine-day Morris Water Maze (MWM) test. The animals were allowed a one-week break before exposure to a glucose tolerance test (GTT). The insulin tolerance test (ITT) was performed on the following day. Animals were sacrificed via euthanyl injections and transcardial perfusion.


S. purpurea extract
Vehicle
8 weeks
8 week protocol
17 weeks
Lipidome
Sacrifice
8 weeks
14 week protocol
23 weeks
Tg + NonTg
littermates
1 week
feeding
training
EPM
LD
OF
ASI
MWM
3 week behavioural
test period
One week break
GTT
ITT
Sacrifice

paraformaldehyde in 10 mM PBS. The brains were removed, post-fixed for 24 hr, and cryoprotected in 20% sucrose. The behavioural paradigm is described below.

5.2.3 Behavioural paradigm

The male Tg and NonTg animals were exposed to a variety of behavioural tests examining anxiety and learning and memory in the order described below (Figure 5.1). Testing was performed at the University of Ottawa Behaviour Core. Prior to each test, mice were acclimatized to the testing room for one hour with white noise present at 70 dB and maintained for the duration of each test.

Elevated plus maze

Animals were tested in an elevated four-arm maze raised approximately 1 m off the floor, which consisted of having two arms, measuring 5 cm by 60 cm, crossed perpendicularly. One arm had open platforms, while the other had walls approximately 14.5 cm high. The place where the two arms met was open and the light level in that spot measured 50 lux. The mouse was placed in the centre of the maze and allowed to freely explore for 10 min. Movement was recorded with a camera mounted above the maze and tracked using EthoVision 8.0 software (Noldus Information Technology, Leesburg, VA, USA). Percent time spent in open and closed arms was calculated using the software, as well as the number of open and closed arm entries.

Light dark

The testing chamber measured 20.5 cm x 20.5 cm x 20.5 cm and was divided into two equal compartments. One compartment was white and open (light), while the other compartment was fully enclosed (dark). An opening in the dark compartment (transition zone) allowed for the mouse to freely move between zones during the 10-min test. This box was illuminated with 390 lux white illumination and enclosed in another box equipped with a fan. Mouse movements were tracked using infrared transmitters and receivers positioned within the chamber. A series of X and Y coordinates were then established to determine movements, as failure by a receiver to obtain a signal implied animal presence. The coordinates were then evaluated using Activity Monitor software (Med Associates Inc, St. Albans, VT, USA) for percent time spent in each zone and the number of entries into each zone.

Open field

The white testing chamber measured 44 cm x 44 cm x 44 cm with an open top and the mouse was allowed to explore for a period of 10 min. A camera was placed 1.75 m above the box and mouse movement tracked and analyzed by EthoVision 8.0 software (Noldus Information Technology, Leesburg, VA, USA). Light level above the box was 600 lux.

Adult social interaction

Social interaction was tested in the same chamber as employed in the open field test with the addition of a rectangular mesh cage (5.5 cm x 9.6 cm) centred 17 cm from one wall. This cage allowed for the confined placement of another animal, a social target. Test animals were placed first in the front left corner and allowed to explore the box and the cage for 5 min. The mouse was then removed and the social target animal was added in the cage (sex, genotype and age matched). The experimental mouse was then placed back in the box and allowed to explore the box and interact with the social target for another 5 min. This test was performed under red light. Mouse movement was recorded through the use of a camera placed above the box and the data tracked and analyzed by EthoVision 8.0 software (Noldus Information Technology, Leesburg, VA, USA).

Morris Water Maze

The apparatus consisted of a blue plastic pool measuring 127 cm in diameter and a depth of 42 cm (internal measurements). The pool was filled with water, rendered opaque white with water-soluble nontoxic paint, to a depth of 1 cm above the 10 cm clear escape platform located in the back right quadrant of the pool. Light level was set to 150 lux and water temperature maintained at 21°C. Visual cues consisted of a square and an “X” located within visual range of the swimming mice at front and left walls of the test room (2.98 m x 3.97 m x 2.62 m), respectively. Mice were tested over a period of eight consecutive days,

with each test day consisting of four separate trials, 20 min apart, from four different starting locations. Their swim pattern was tracked and analyzed by EthoVision 8.0 software (Noldus Information Technology, Leesburg, VA, USA). Each trial lasted until the animal found the platform or for a maximum of 60 s. Mice that failed to find the platform in the 60-s time frame were guided to the submerged platform, allowed to orient itself for 5 s, and then removed from the pool. On day 9, probe day, the platform was removed and the mice were allowed to search the pool for the missing platform for a period of 60 s.

Path efficacy

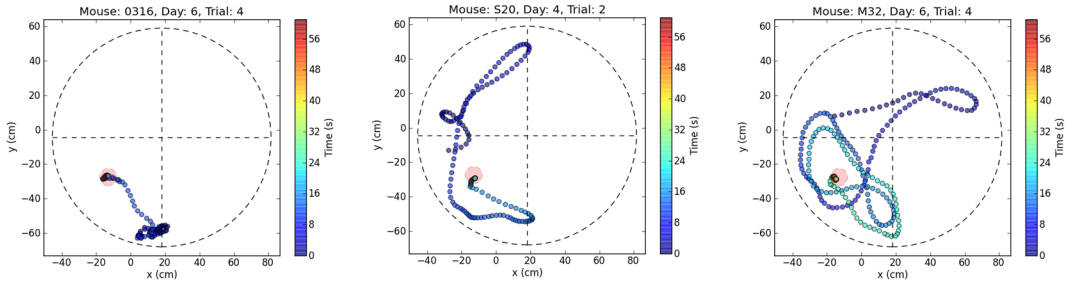
Following MWM data acquisition, path efficacy was calculated for each trial to reflect distance travelled by the mouse over the course of the trial. Data was expressed as a ratio of the direct distance to the platform from the drop-off location over the actual path length taken by the mouse (Nagy, 2013). Daily averages were then calculated and plotted.

Search strategy

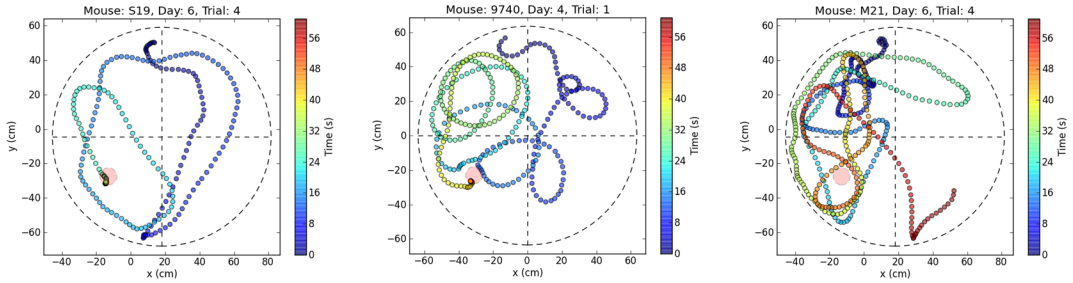
The apparent search strategy utilized by each mouse was determined based on previous work done by Janus (2004) and Brody and Holtzman (2006). Briefly, the swimming track of each mouse for each trial for each day was analyzed and assigned to one of the three major search strategies as defined by Janus (2004) (Figure 5.2). Briefly, a spatial search strategy was one defined by a direct swim path to the quadrant containing the escape platform (Figure 5.2A).

Figure 5.2: Search strategy categories as adapted from Janus (2004). A spatial search strategy was one defined by a direct swim path to the quadrant containing the escape platform (A). A systematic, non-spatial, strategy was defined by systematic scanning of the interior portion of the tank either without bias to any particular quadrant or focused on the incorrect quadrant (B). A repetitive looping strategy was defined by circular patterns of swimming at a fixed distance from the wall or in tight circles in a general directional movement (C).

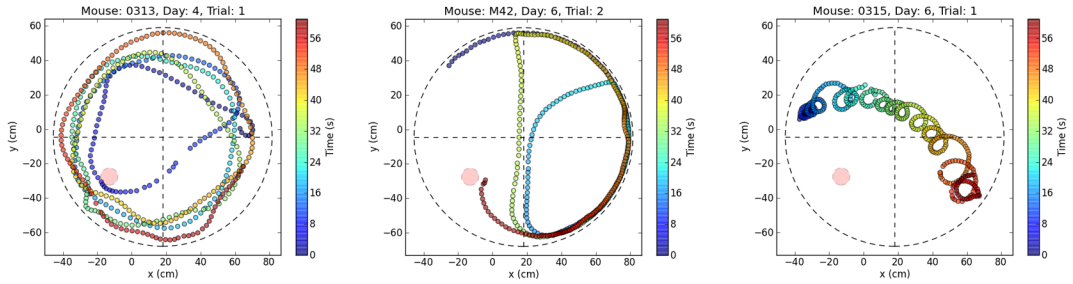
A. Spatial



B. Non-spatial, systematic



C. Repetitive Looping



A systematic, non-spatial, strategy was defined by systematic scanning of the interior portion of the tank either without bias to any particular quadrant or focused on the incorrect quadrant (Figure 5.2B). A repetitive looping strategy was defined by circular patterns of swimming at a fixed distance from the wall or in tight circles in a general directional movement (Figure 5.2C). Two investigators blind to the genotype/treatment of the mouse independently scored each trial. The data was then compared between the investigators to reach a consensus. The average incidence (%) for each strategy over the first four days, and also the last four days, was then plotted for each genotype/treatment.

5.2.4 Glucose tolerance and insulin tolerance tests

Glucose tolerance (GTT) and insulin tolerance tests (ITT) were performed based on a protocol established by Fujimoto et al. (2010). For the GTT, following a 16-hr fast, basal glucose level was measured using an Accu-Chek Nano glucometer (Roche Diagnostics, Indianapolis, IN, USA). Mice were then administered with 2 g dextrose/kg b.w. i.p. and their peripheral glucose levels monitored for 3 hrs. For the ITT, following a 4-hr fast, basal glucose level was measured and mice were treated with 0.71 U human recombinant insulin (Cat# I9278-5mL, Lot# 050M8401, Sigma Aldrich, Oakville, ON) per kg b.w. i.p. Mouse glucose levels were monitored for a total of 3 hrs.

5.2.5 Lipid extraction and analysis

The phosphocholine second messenger lipids were extracted from the hippocampus and temporal cortex samples of female mice according to a modified Bligh Dyer lipid extraction procedure previously published (Bonin et al., 2004; Xu et al., 2013a). Briefly, tissue was homogenized in 1 mL of acidified methanol (2% acetic acid in methanol) using a tissue grinder. PC(13:0/0:0) (Cat#855476, Avanti Polar Lipids Inc., Alabaster, AL, USA) (0.413 nmol) standard was added to control for extraction efficiency. The homogenate was transferred to a glass collection tube containing 3.2 mL of 0.1M sodium acetate. The homogenization tubes were subsequently washed three times with 1 mL acidified methanol and the liquid transferred to the glass collection tubes. Lipids were extracted by adding 3.8 mL of chloroform to the collection tube and centrifuging for 2 min at 2 000 rpm at 4°C. The bottom organic phase was transferred to another glass tube for retention, and the remaining aqueous phase was back-extracted three more times with 2 mL of chloroform. Each back-extraction was centrifuged and the organic phase retained. Chloroform was evaporated from the samples under nitrogen gas, and lipids were resuspended in 300 µL of absolute ethanol. Samples were then aliquoted into three 100 µL aliquots in amber glass vials under a N_2 atmosphere and stored at -80°C.

Lipid analysis was performed on an Agilent model 1100 capillary HPLC system (Palo Alto, CA, USA) connected to the QTRAP 5500 mass spectrometer according to Whitehead et al. (2007) with the following modifications. Briefly, a 5 µL lipid extraction sample was combined with 2.5 µL of deuterated lipid standard

mixture [1 ng/μL d4-PC(O-16:0/0:0), 1 ng/μL d4-PC(O-18:0/0:0), 0.5 ng/μL d4-PC(O-16:0/2:0 and 0.5 ng/μL d4-PC(O-18:0/2:0)] and 15.75 μL of 0.1% formic acid in H_2O in an Agilent 96-well sampling plate, covered with a preslit well cap and thermostated at 4°C. An Agilent 1100 autosampler was employed to introduce the sample onto a 75 μm × 100 mm column packed with ReproSil-Pur 120 C4, 5 μm beads (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) at a flow rate of 10 μL/min. Linear gradient of acetonitrile/isopropanol (5/2, containing 0.1% formic acid and 10 mM ammonium acetate) at a flow rate of 20 μL/min was used to achieve the separation of the glycerophospholipid species. Ionization was achieved using a 75 μm × 50 mm Picotip emitter (New Objective, Woburn, MA), which was interfaced with the mass spectrometer via electrospray ionization. Data were collected on the QTRAP 5500 mass spectrometer operated in positive ion mode with Analyst 1.5.1 (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Lipid species were further analyzed using a precursor ion scan for an MS/MS fragment with a mass to charge ratio (m/z) of 184.0, a diagnostic fragment of phosphocholine (Brugger et al., 1997).

The peak area for each lipid was analysed using Analyst software and normalised to the PC(13:0/0:0) peak area for each sample followed by normalization to tissue wet weight in mg.

5.2.6 Statistical analysis

All statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA, USA). Glucose and insulin tolerance tests, as well as

behavioural tests, were analysed using a two-way ANOVA or two-way repeated measures ANOVA, where necessary, followed by *post-hoc* Holm-Sidak test where required. Lipid changes were analysed using a one-way ANOVA, followed by *post-hoc* Holm-Sidak test where required. Unpaired t-tests against NonTg vehicle were also used. A p-value less than 0.05 was considered significant.

5.3 Results

5.3.1 Glucose and insulin tolerance

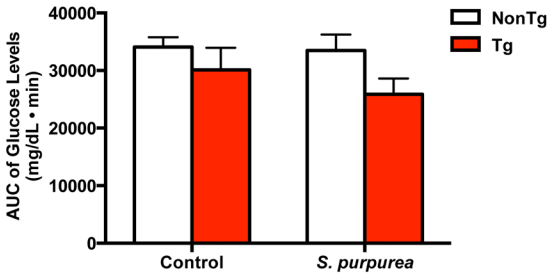
To assess glucose and insulin response of the Tg animals under control and treatment conditions, glucose and insulin tolerance tests were performed (Figure 5.3). No differences in glucose tolerance were detected between Tg and NonTg mice regardless of treatment (Figure 5.3A). Both control (including vehicle-treated) and *S. purpurea*–treated mice showed no difference in their area under the curve (AUC) glucose levels over the 180 min ($F(1,46) = 0.5602$). However, a difference in insulin tolerance was observed in the Tgs, particularly when treated with *S. purpurea* (Figure 5.3B). A main effect of genotype ($F(1,46)=7.379$, $p<0.01$) between the Tg and NonTgs was observed. Specifically this effect was seen with *S. purpurea*, where the Tgs had a significantly greater AUC than NonTgs ($p<0.05$, Holm-Sidak).

5.3.2 Behavioural measurements of anxiety and locomotion

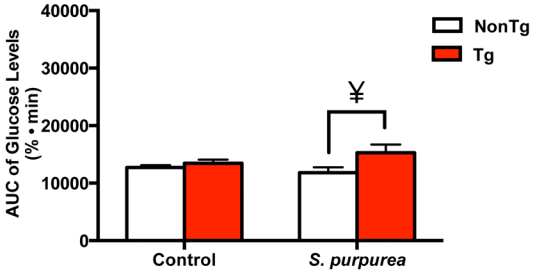
To assess how the insulin changes affect behavioural parameters of anxiety and locomotion in Tgs under vehicle and treated conditions, the animals

Figure 5.3: *S. purpurea* treatment has no effect on glucose tolerance, but impairs insulin response in TgCRND8 mice. (A) Area under the curve (AUC) of glucose levels following an i.p. injection of dextrose (2 g/kg). (B) AUC of glucose levels following an i.p. injection of 0.71 U recombinant insulin/kg. NonTg control, n=19, where n=9 control and n=10 vehicle; Tg control, n=15, where n=9 control and n=6 vehicle; NonTg *S. purpurea*, n=10; Tg *S. purpurea*, n=6. All data are presented as mean + SEM. Statistics were two-way ANOVA followed by *post-hoc* Holm-Sidak test. ¥$p<0.05$ relative to NonTg *S. purpurea*

A. Glucose Tolerance Test



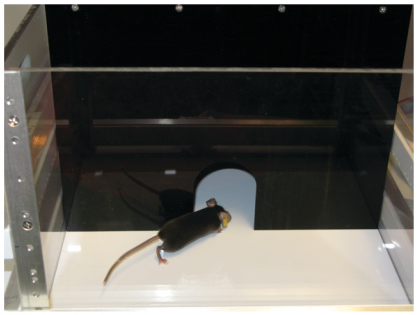
B. Insulin Tolerance Test



were exposed to four behaviour tests primarily exploring anxious behaviour. In the light dark test (Figure 5.4A) and the elevated plus maze (Figure 5.4B), Tg mice were less anxious regardless of treatment (vehicle-treated versus *S. purpurea*), as demonstrated by both the percent time spent in light zone (Figure 5.4C) ($F(1,30)=8.8$, $p<0.01$) and percent time spent in open arms (Figure 5.4E) ($F(1,30)=10$, $p<0.01$). The decrease in anxiety was not due to a motoric impairment, i.e. freezing, as the Tg mice had the same number of entries into the transition zone (Figure 5.4D) ($F(1,30)=0.1749$) and in fact, moved a lot more as demonstrated by the number of open arm entries (Figure 5.4F) ($F(1,30)=4.4$, $p<0.05$). Looking at both adult social interaction (Figure 5.5A) and open field (Figure 5.5D), Tgs appeared more exploratory regardless of treatment, where they spent a significantly higher amount of time in the interaction zone when no target was present (Figure 5.5B) ($F(1,30)=6.1$, $p<0.05$) and moved significantly more in the open field (Figure 5.5E) ($F(1,30)=17.11$, $p<0.001$). However, this behaviour was more frenetic than exploratory as the Tgs increased their velocity over time (Figure 5.5F) ($F(3,30)=5.746$, $p<0.01$). Moreover, the exploratory behaviour was eliminated in the presence of a more stressful environment, as measured by percent time spent in interaction when a social target animal was present (Figure 5.5C) ($F(1,30)=0.6908$) and percent time spent in the centre zone (Figure 5.5G) ($F(1,30)=1.314$).

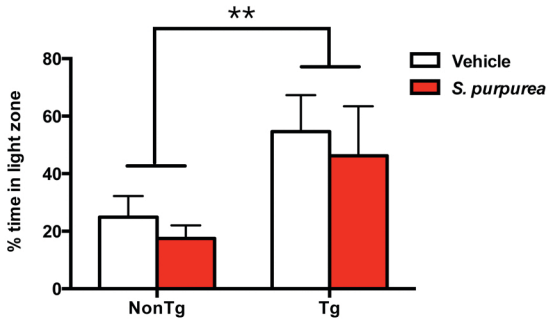
Figure 5.4: TgCRND8 mice are less anxious regardless of treatment and this decrease in anxiety is not due to a motoric impairment. (A) Light dark box experimental setup. (B) Elevated plus maze experimental setup. Anxiety measurement in the light dark box as reported by percent time spent in light zone (C) and number of entries in transition zone (D). Anxiety measurement in the elevated plus maze as reported by percent time spent in open arms (E) and number of open arm entries (F). NonTg vehicle, n=12; Tg vehicle, n=6; NonTg *S. purpurea*, n=10; Tg *S. purpurea*, n=6. All data are presented as mean + SEM. Statistics were two-way ANOVA followed by *post-hoc* Holm-Sidak test. $^{*}p<0.05$, $^{**}p<0.01$

A. Light Dark

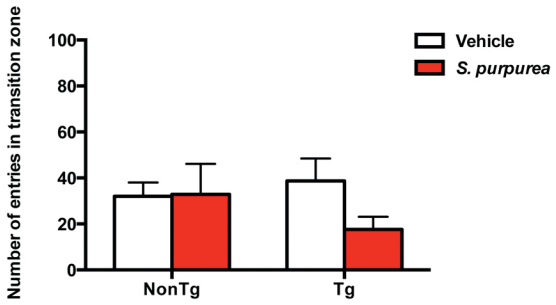


B. Elevated Plus Maze

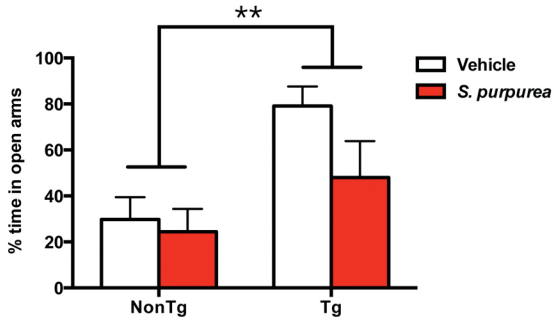
C. Light Dark



D. Light Dark



E. Elevated Plus Maze



F. Elevated Plus Maze

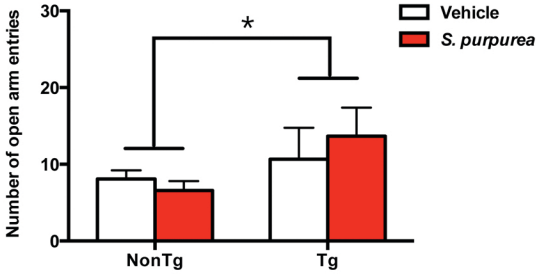
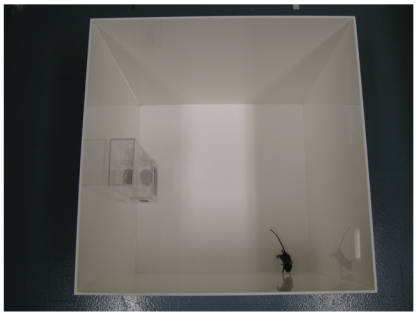
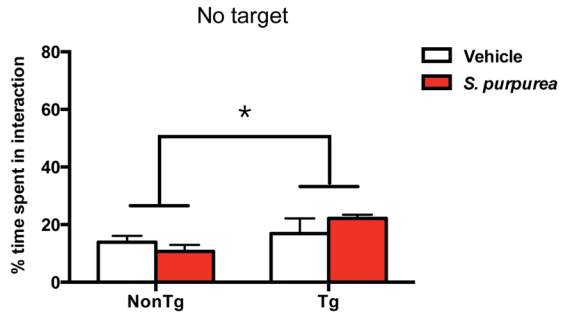


Figure 5.5: TgCRND8 mice are more frenetic regardless of treatment. (A) Adult social interaction experimental setup. Percent time spent in interaction when no social target is present (B) and when one is present (C). (D) Open field experimental setup. Distance moved (m) over the course of the open field test (E) and the mean velocity (m/min) over the 10 min time course (F). Percent time spent in the centre zone (G) during the open field test. NonTg vehicle, n=12; Tg vehicle, n=6; NonTg *S. purpurea*, n=10; Tg *S. purpurea*, n=6. All data are presented as mean + SEM, except for (F) where it is mean ± SEM. Statistics were two-way ANOVA followed by *post-hoc* Holm-Sidak test, except for mean velocity profiles (F) where the statistics were two-way repeated measures ANOVA followed by *post-hoc* Holm-Sidak test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

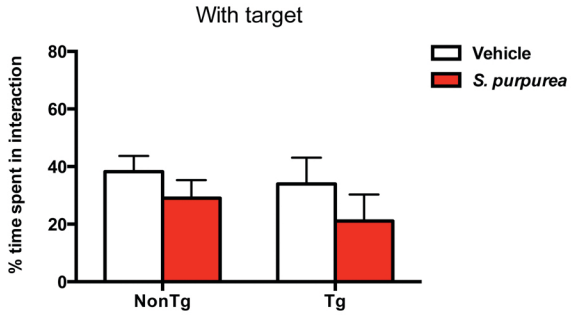
A. Adult Social Interaction



B. Adult Social Interaction

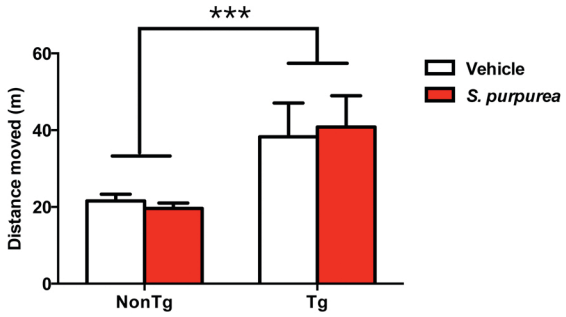


C. Adult Social Interaction

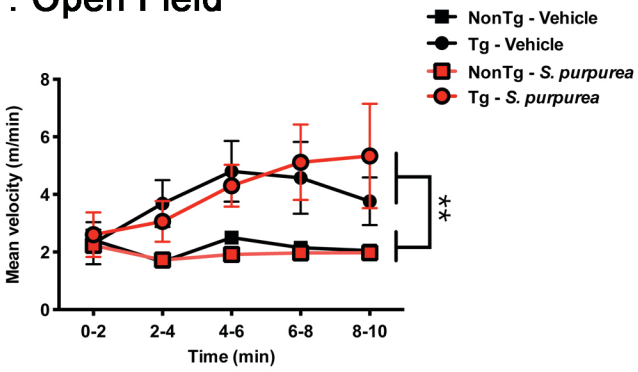


D. Open Field

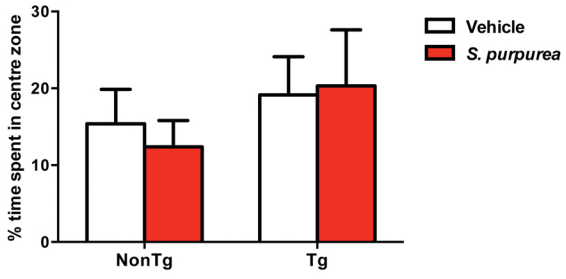
E. Open Field



F. Open Field



G. Open Field



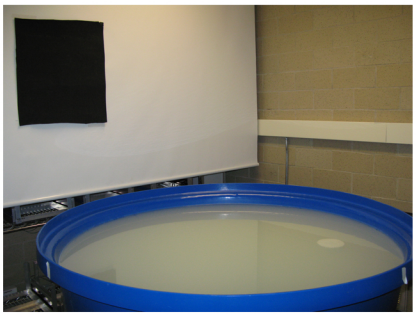
5.3.3 Effect of S. purpurea on learning and memory

To examine whether the insulin impairment seen with Tg *S. purpurea*-treated mice translated to a learning and memory deficit, the Morris Water Maze was used to assess this behaviour (Figure 5.6A). There was no effect of *S. purpurea* on escape latency in NonTg animals (Figure 5.6B) ($F(1,27)=0.3588$), where control animals included control and vehicle-treated animals. There was a main effect of genotype, where both Tg control (Figure 5.6C) ($F(1,32)=5.375$, $p<0.05$) and Tg *S. purpurea*-treated (Figure 5.6D) ($F(1,14)=6.021$, $p<0.05$) animals showed a learning and memory impairment as compared to their NonTg counterparts. This translated to a deficiency in path efficacy, where again a main effect of genotype could be observed for both Tg control (Figure 5.6F) ($F(1,32)=5.268$, $p<0.05$) and Tg *S. purpurea*-treated (Figure 5.6G) ($F(1,14)=19.26$, $p<0.001$) animals. *S. purpurea* had no effect on path efficacy in NonTg animals (Figure 5.6E) ($F(1,27)=0.1588$). However, the Tgs fed *S. purpurea* appeared to have a much lower path efficacy, only around 0.1, for the retention days (days 5-8), while Tg controls had a path efficacy around 0.2 (Figures 5.6F and G).

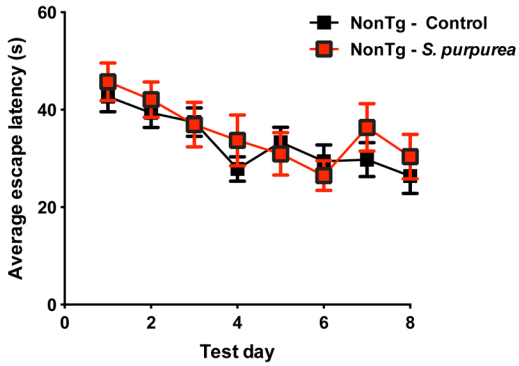
This further learning and memory impairment in Tg animals caused by *S. purpurea* resulted from a switch in search strategy during the retention days. As can be seen in Figure 5.7A for the acquisition days (days 1-4), Tg animals employed the spatial search strategy to a significantly lower extent than their NonTg counterparts regardless of treatment (interaction $F(6,36)=4.907$, $p<0.001$, Holm-Sidak $p<0.05$), while increasing the use of repetitive looping. There was no

Figure 5.6: TgCRND8 mice are impaired in the Morris Water Maze test examining spatial learning and memory with *S. purpurea* treated animals showing greater impairment. (A) Morris Water Maze experimental setup. Average escape latency (s) for NonTg control vs NonTg *S. purpurea* animals (B), NonTg control vs Tg control animals (C), and NonTg *S. purpurea* vs Tg. *S. purpurea* animals (D) over the course of eight days. A measure of path efficacy between NonTg control and NonTg *S. purpurea* animals (E), NonTg control vs Tg control (F) and NonTg *S. purpurea* vs Tg *S. purpurea* (G) animals over the course of eight days. NonTg control, n=19, where n=9 control and n=10 vehicle; Tg control, n=15, where n=9 control and n=6 vehicle; NonTg *S. purpurea*, n=10; Tg *S. purpurea*, n=6. All data are presented as mean ± SEM. Statistics were two-way repeated measures ANOVA. $^{*}p<0.05$ between NonTg control and Tg control, and between NonTg *S. purpurea* vs Tg *S. purpurea* $^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$

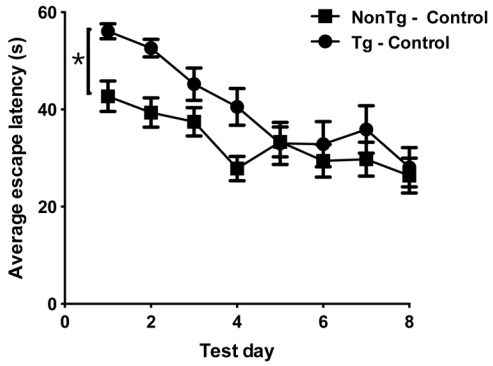
A. Morris Water Maze



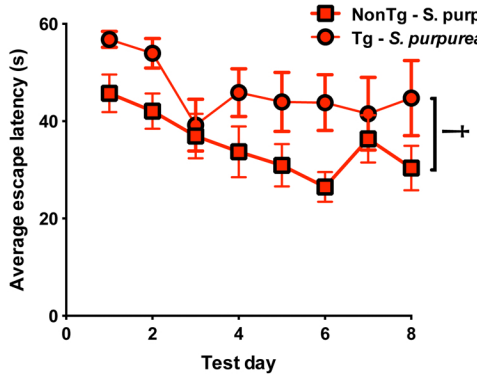
B. Escape Latency



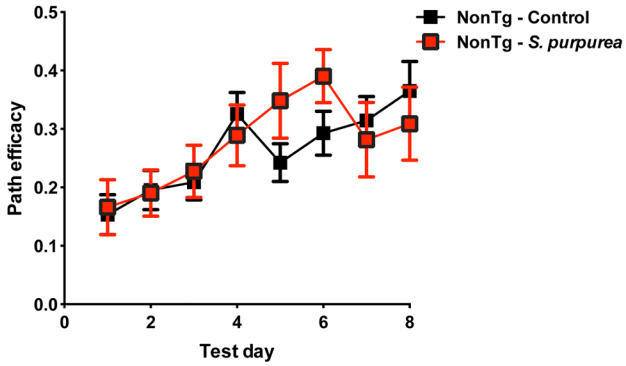
C. Escape Latency



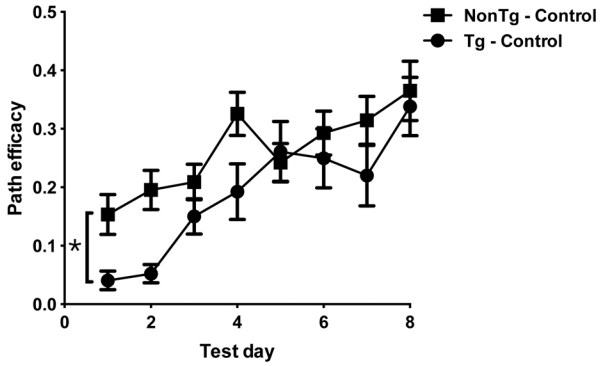
D. Escape Latency



E. Path Efficacy



F. Path Efficacy



G. Path Efficacy

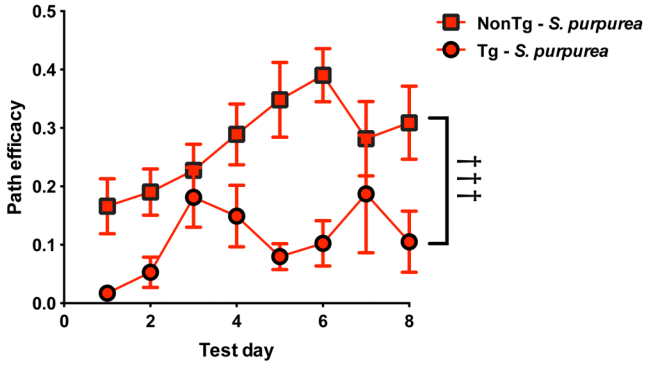
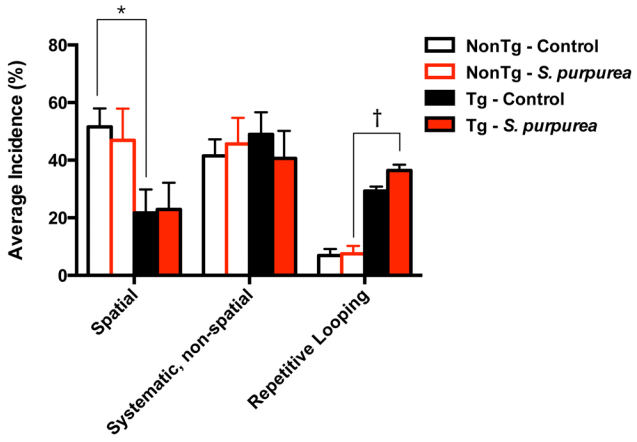
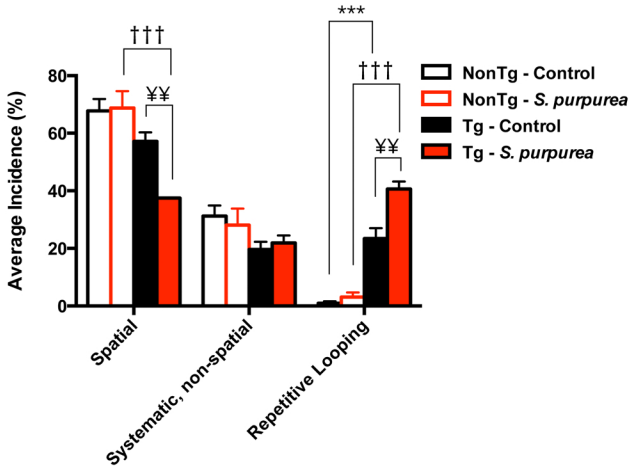


Figure 5.7: *S. purpurea* mildly increases learning and memory impairment in TgCRND8 mice due to a switch in strategy from spatial learning and memory to repetitive looping. Average percent incidence of each search strategy utilized between the first four days (acquisition) (A) and the last four days (retention) (B). Swimming distance (cm) over the course of the MWM (C), as well as velocity (cm/s) (D). NonTg control, n=19, where n=9 control and n=10 vehicle; Tg control, n=15, where n=9 control and n=6 vehicle; NonTg *S. purpurea*, n=10; Tg *S. purpurea*, n=6. All data are presented as mean + SEM. Statistics were two-way ANOVA followed by *post-hoc* Holm-Sidak test. $^{*}p<0.05$, $^{***}p<0.001$ or $^{****}p<0.0001$ between NonTg control and Tg control; $^{\dagger}p<0.05$ or $^{\dagger\dagger\dagger}p<0.001$ between NonTg *S. purpurea* vs Tg *S. purpurea*; $^{¥}p<0.05$ or $^{¥¥}p<0.01$ between Tg control vs Tg. *S. purpurea*.

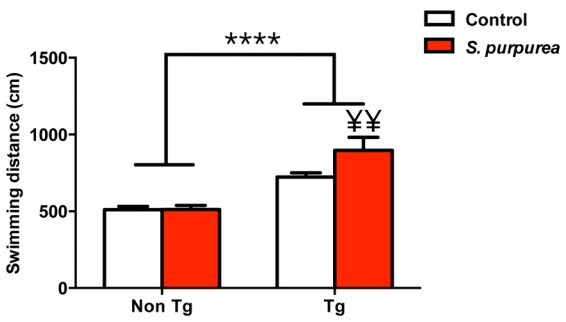
A. Search Strategy (Acquisition)



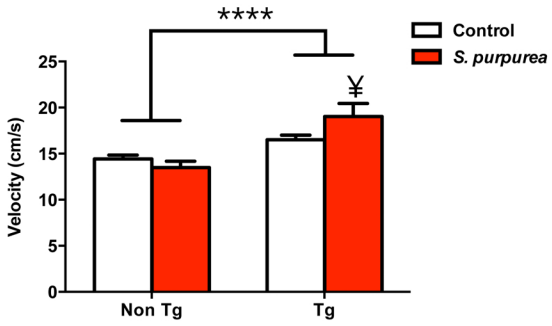
B. Search Strategy (Retention)



C. Morris Water Maze



D. Morris Water Maze

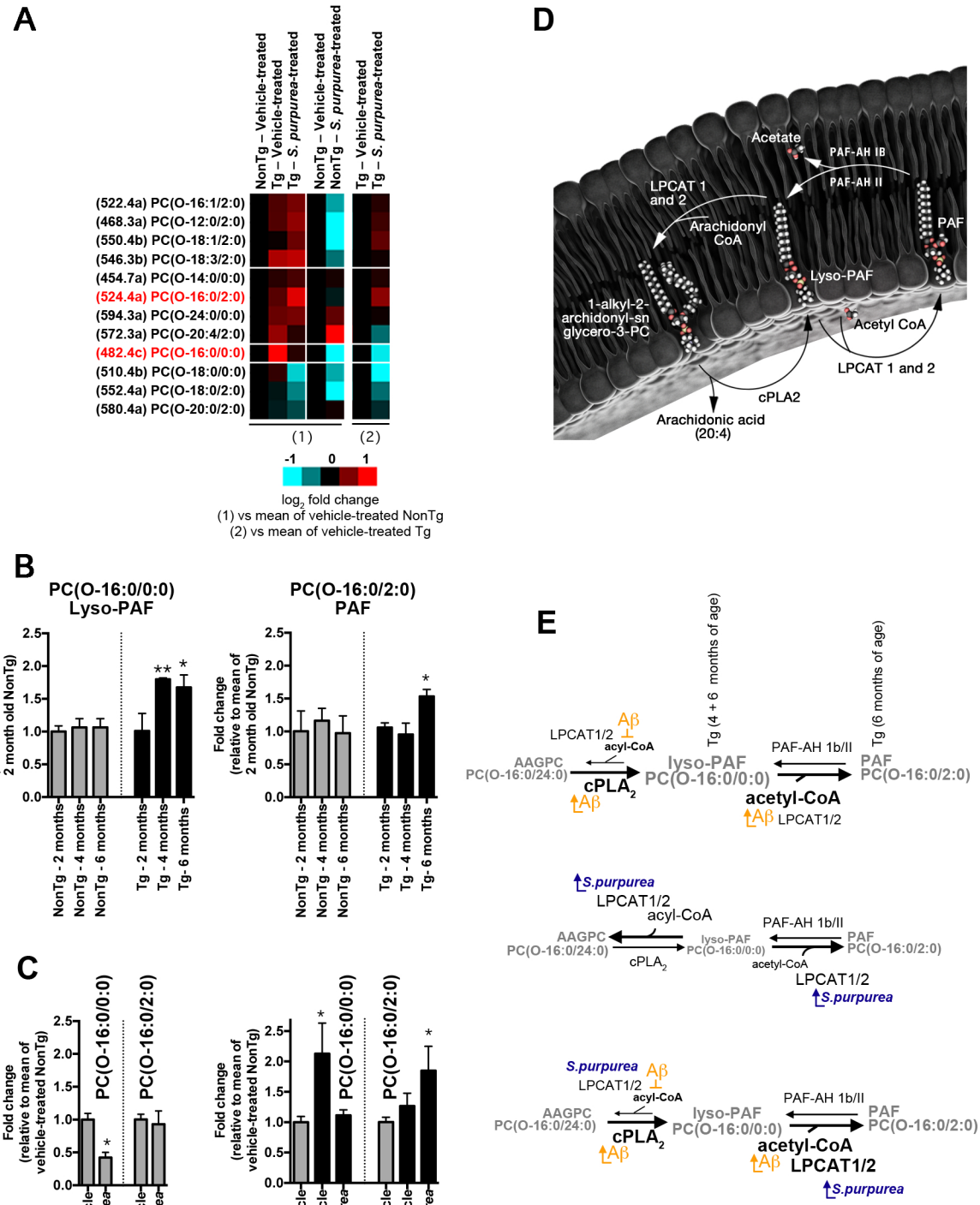


difference in the use of systematic, non-spatial strategy. However, during the retention days (Figure 5.7B), Tg control animals were not significantly different from NonTg controls in their use of spatial strategy, even though they still employed repetitive looping to a greater extent (interaction $F(6,36)=24.46$, $p<0.0001$, Holm-Sidak $p<0.001$). Conversely, *S. purpurea*-treated animals continued to employ spatial strategy to a lower extent than their NonTg counterparts (Holm-Sidak, $p<0.001$), while utilizing repetitive looping more (Holm-Sidak, $p<0.001$). Moreover, the *S. purpurea*-treated Tgs used significantly less spatial strategy (Holm-Sidak, $p<0.01$) than control Tgs and significantly more repetitive looping (Holm-Sidak, $p<0.01$). These differences in search strategy translated to a greater swimming distance for the mice (Figure 5.7C), where there was a main effect of genotype ($F(1,46)=71.91$, $p<0.0001$), since Tgs swam a greater distance, and the *S. purpurea*-treated Tgs even more so (Holm-Sidak, $p<0.01$). Furthermore, Tg animals also swam at a greater velocity (main effect of genotype, $F(1,46)=32.44$, $p<0.0001$) with *S. purpurea* further increasing this frenetic behaviour in the Tg animals (Holm-Sidak, $p<0.05$).

5.3.4 Lipid changes observed in the temporal cortex

To examine whether *S. purpurea* alters PAF metabolism in murine temporal cortex, changes in PAF second messengers (450-600 m/z) were profiled and a possible mode of action of the plant in Tg animals was examined. Comparing Tg animals to NonTg vehicle-treated animals (Figure 5.8A), the $\log_2$ fold changes of the majority of lipid species examined were increased in Tg

Figure 5.8: *S. purpurea* significantly increases levels of PC(O-16:0/2:0) in TgCRND8 mice, likely by increasing lysophosphatidylcholine acyltransferase (LPCAT) activity. (A) A heatmap showing clustering based on $\log_2$ fold change (1) versus mean of vehicle-treated NonTg and (2) versus mean of vehicle-treated Tg. Species in red were identified for further analysis. (B) Fold change differences (relative to mean of two-month-old NonTg) for control animals aged 2, 4, and 6 months of age for both PC(O-16:0/0:0) and PC(O-16:0/2:0). (C) Fold change differences (relative to mean of vehicle-treated NonTg) for vehicle and *S. purpurea*-treated animals for both PC(O-16:0/0:0) and PC(O-16:0/2:0). (D) A schematic representation showing the Land's cycle; breakdown of structural phosphatidylcholine lipids into platelet-activating factor (PAF) lipids. Modified from Bennett et al. (2013). (E) Possible mechanism of action of *S. purpurea* in the presence and absence of $A\beta_{42}$. n=3 for all animal groups. Data are presented as mean + SEM. Statistics were one-way ANOVA against NonTg vehicle followed by *post-hoc* Holm-Sidak test for (B). Statistics were one-way ANOVA for Tg vehicle against NonTg vehicle, as well as unpaired t-test between NonTg vehicle and *S. purpurea*, and NonTg vehicle vs Tg *S. purpurea* for (C). $^{*}p<0.05$, $^{**}p<0.01$


A
NonTg – Vehicle-treated
Tg – Vehicle-treated
Tg – S. purpurea-treated
NonTg – Vehicle-treated
NonTg – S. purpurea-treated
Tg – Vehicle-treated
Tg – S. purpurea-treated
(522.4a) PC(O-16:1/2:0)
(468.3a) PC(O-12:0/2:0)
(550.4b) PC(O-18:1/2:0)
(546.3b) PC(O-18:3/2:0)
(454.7a) PC(O-14:0/0:0)
(524.4a) PC(O-16:0/2:0)
(594.3a) PC(O-24:0/0:0)
(572.3a) PC(O-20:4/2:0)
(482.4c) PC(O-16:0/0:0)
(510.4b) PC(O-18:0/0:0)
(552.4a) PC(O-18:0/2:0)
(580.4a) PC(O-20:0/2:0)
(1)
(2)
-1
0
1
log2 fold change
(1) vs mean of vehicle-treated NonTg
(2) vs mean of vehicle-treated Tg
B
PC(O-16:0/0:0)
Lyso-PAF
PC(O-16:0/2:0)
PAF
Fold change (relative to mean of 2 month old NonTg)
NonTg - 2 months
NonTg - 4 months
NonTg - 6 months
Tg - 2 months
Tg - 4 months
Tg- 6 months
C
PC(O-16:0/0:0)
PC(O-16:0/2:0)
Fold change (relative to mean of vehicle-treated NonTg)
NonTg - Vehicle
NonTg - S. purpurea
Tg - Vehicle
Tg - S. purpurea
D
Acetate
PAF-AH IB
PAF-AH II
LPCAT 1 and 2
Arachidonyl CoA
PAF
Lyso-PAF
1-alkyl-2-archidonyl-sn glycero-3-PC
Acetyl CoA
LPCAT 1 and 2
cPLA2
Arachidonic acid (20:4)
E
Tg (4 + 6 months of age)
Tg (6 months of age)
LPCAT1/2
Aβ
acyl-CoA
AAGPC PC(O-16:0/24:0)
cPLA2
lyso-PAF PC(O-16:0/0:0)
PAF-AH 1b/II
PAF PC(O-16:0/2:0)
acetyl-CoA
S.purpurea

animals, regardless of treatment. Furthermore, looking at the effect of *S. purpurea* on the NonTg animals, a decrease in the majority of PAF species could be seen in the $\log_2$ ratio, while certain species were upregulated. This effect was not the same in the Tg treated animals, as a comparison between Tg vehicle-treated and *S. purpurea*-treated animals yielded more upregulated species than downregulated species. Most important of all the comparisons was the effect of both genotype and *S. purpurea* on PC(O-16:0/2:0) and PC(O-16:0/0:0), since previous work by Ryan et al. (2009) had shown an increase in the presence of PC(O-16:0/2:0) leads to neurotoxicity.

Examining the effect of age on NonTg and Tg animals (Figure 5.8B), it can be seen that there was no effect of age on NonTg animals for both PC(O-16:0/0:0) and PC(O-16:0/2:0). However, in the four-month and six-month-old animals, there was a significant increase in the presence of PC(O-16:0/0:0) in the Tg animals (Holm-Sidak, $p<0.05$) and in the presence of PC(O-16:0/2:0) in the six-month-old Tgs (Holm-Sidak, $p<0.05$). Moreover, when *S. purpurea* treatment was considered on four-month-old animals (Figure 5.8C), NonTg *S. purpurea*-treated animals had a significantly lower amount of PC(O-16:0/0:0) (unpaired t-test, $p<0.05$) compared to vehicle-treated NonTgs, while having no effect on PC(O-16:0/2:0). In contrast, Tg vehicle-treated animals had a higher amount of PC(O-16:0/0:0) compared to their NonTg counterparts ($F(3,12)=7.257$, $p<0.05$), with *S. purpurea* completely abolishing that effect. However, treatment with *S. purpurea* significantly increased the presence of PC(O-16:0/2:0) in the Tg animals compared to the NonTg vehicle-treated (unpaired t-test, $p<0.05$).

5.4 Discussion

This study has shown that this extract of *S. purpurea* has no measurable effect on behavioural indices of anxiety as measured by elevated plus maze, light dark, open field and adult social interaction tests. However, extract from this plant did enhance the learning and memory impairment of Tg mice, likely as a result of an increase in frenetic behaviour. This fast and energetic movement in a rather wild and uncontrolled way (Nesher et al., 2013) has previously been described for animals with an injured central nervous system or animals kept in captivity (Mason, 1991a, b), more particularly as a stereotypic behaviour without any obvious function and most commonly for behaviour in a home cage. It has also been linked to the "Behavioural and Psychological Symptoms of Dementia" described for humans, as AD patients are not only forgetful, but also exhibit periods of restlessness and wandering (Ambree et al., 2006). Musilli et al. (2013), Walker et al. (2011) and Ambree et al. (2006) have all reported an increase in locomotion for the TgCRND8 mice as measured by open field. However, this is the first time that this frenetic behaviour has been reported for learning and memory behavioural tests.

TgCRND8 mice are an early onset model of AD, with Aβ plaques apparent at about 12 weeks of age (Chishti et al., 2001). These plaques have been previously correlated to a deficit in learning and memory, as observed in the acquisition phase of the Morris Water Maze compared to their NonTg counterparts (Chishti et al., 2001). Here a similar reduction in learning and memory ability of Tg mice can be seen, and interestingly a further impairment in

the retention phase was elicited by *S. purpurea* treatment. The data presented here suggest that this impairment is caused by an alteration in the search strategy of these mice. Specifically, that these mice demonstrate repetitive looping, the least productive strategy, to a much higher extent.

To address the underlying mechanism of these behavioural changes, a targeted lipidomic approach was used to examine the effects of *S. purpurea* on the platelet activating factor (PAF) family of retrograde neurotransmitters. These were specifically selected as members of the PAF family act as key mediators of learning and memory (Chen and Bazan, 2005). It was found that levels of PC(O-16:0/0:0), but not PC(O-16:0/2:0), were significantly decreased in *S. purpurea*-treated NonTg animals. However, in Tg animals at four months of age, *S. purpurea* treatment led to an increase of PC(O-16:0/2:0) resulting in levels similar to Tg animals at six months of age. *S. purpurea* treatment also resulted in decreased levels of PC(O-16:0/0:0) in four-month-old Tg mice to levels similar to those in NonTg animals. This data together suggests a potential role *S. purpurea* plays in altering the Land's cycle (Figure 5.8D) (Bennett et al., 2013). The Land's cycle is a process where structural 1-alkyl-2-archidonyl-sn-glycero-3-phosphocholine lipids are converted to lyso-PAFs through $cPLA_2$, and are then further converted to PAFs by lysophosphatidylcholine acyltransferases 1 and 2 with a preference for the acetyl Co-A substrate. PAFs can then be broken down by PAF-acetylhydrolase (PAF-AH) to lyso-PAF and further converted back to structural lipids by LPCAT 1 and 2 with a substrate preference for acyl-CoA (Bennett et al., 2013). Interestingly, the presence of $A\beta_{42}$ both stimulates activity

of $cPLA_2$ (Figure 5.8E, top panel) in *in vitro* and *in vivo* experiments (Kriem et al., 2005; Sanchez-Mejia et al., 2008), and alters the preference of LPCAT 1/2 from acyl Co-A to the acetyl Co-A substrate (Ryan et al., 2009). These alterations result in higher levels of lyso-PAF and eventually PAF and are supported by the results seen in the aging animals here, as the $A\beta_{42}$ load increases with time. It is speculated that *S. purpurea* activates the LPCAT 1/2 activity with its more preferred substrate, acyl Co-A, when no $A\beta_{42}$ is present, leading to a decrease in lyso-PAF but not an increase in PC(O-16:0/0:0) (Figure 5.8E, middle panel). The structural lipids would thus be remodelled, though this remains to be experimentally verified. When $A\beta_{42}$ is present with *S. purpurea* treatment, *S. purpurea* may still act on LPCAT 1/2 (Figure 5.8E, bottom panel), but now has a different preferred substrate (acetyl Co-A) leading to an increase in PC(O-16:0/2:0). Taken together, *S. purpurea* further drives the production of PC(O-16:0/2:0), already seen with $A\beta_{42}$, leading to an increased impairment in learning and memory, as well as more frenetic behaviour.

Previous *in vitro* work suggested *S. purpurea* to be a good candidate to delay the symptomatic progression in Tg mice (Harris et al., 2012; Martineau et al., 2010a; Spoor et al., 2006), an assumption also supported by observed increases in cognitive ability with antidiabetic medicines (Liu et al., 2010b; Pintana et al., 2012), as well as phytochemicals found in *S. purpurea* (Bhutada et al., 2010a; Bhutada et al., 2010b; Lu et al., 2011; Lu et al., 2007). However, the Cree elders did caution against *S. purpurea* administration to individuals in a "weakened state". This weakened state could refer to individuals who are

already showing early signs of AD. There has been recent interest in the role AMPK plays in AD (Salminen et al., 2011). Particularly, Mairet-Coello et al. (2013) showed that AMPK induces $A\beta_{42}$ mediated synaptotoxicity both *in vitro* and *in vivo* in a transgenic mouse model similar to the one employed here (Mucke et al., 2000). However, both positive and negative effects of AMPK activation in AD have been extensively reported and reviewed in Salminen et al. (2011). Since *S. purpurea* is a potent activator of AMPK (Martineau et al., 2010a; Spoor et al., 2006), perhaps its dual effect can be explained by this differential response seen with AMPK activation. Although it is easy to speculate that AMPK activation plays a role in the activation of LPCAT 1/2, thus accounting for this biphasic nature dependent on presence of $A\beta_{42}$, a clear link between AMPK and LPCATs remains to be determined. Further work should concentrate on establishing (or debunking) such a connection. As well, *S. purpurea* should be evaluated in a model strictly focused on its antidiabetic role, without the presence of $A\beta_{42}$, in order to validate Cree traditional knowledge.

Chapter 6: General Discussion

6.1 Statement of Originality

The CIHR TAAM was established to work with the Cree Nation of Eeyou Istchee to determine the safety and efficacy of traditional medicines as complementary medicines to treat the symptoms of T2D. The purpose of this thesis was to examine the safety of 17 traditional medicines in specific assays and to advance safety evaluation of one promising traditional plant, *Sarracenia purpurea* L., which has not previously received attention in the published scientific literature.

The first study examined the potential for drug interactions by studying the effect of all 17 traditional medicines on the transport and metabolism of blood glucose lowering pharmaceuticals, gliclazide and repaglinide (Chapter 2). Caco-2 cells were used as a model of first-pass metabolism (Schmiedlin-Ren et al., 1997), and it was found that Cree medicinal plants had little to no effect on transport of either repaglinide and gliclazide, two widely prescribed blood glucose lowering drugs (Bidstrup et al., 2003; Elliot et al., 2007). However, they had an effect on metabolism of these drugs. Particularly, it was found that most of the Cree extracts had an effect on repaglinide metabolism *in vitro*, while rhaponticin and rhapontigenin, two key isolates from *Larix laricina* (Shang et al., 2012), had an effect on gliclazide metabolism. One of the conclusions of this section was that Cree medicine could lead to a change in the metabolism of the co-

administered pharmaceuticals, which may alter their efficacy. Therefore this should be critically examined in clinical work.

Concentrating on *S. purpurea*, an important first step was to characterize the traditional preparation of boiling water extraction (decoction), as well as the phytochemistry of the ethanol extract required by established protocols in the pharmacological assays. This was of great importance to the Cree and the focus of Chapter 3. This study found that generally the ethanol extract was able to concentrate the phenolic compounds present in the plant only slightly. However, two triterpenes, betulinic acid and ursolic acid were greatly enhanced in the ethanolic extract because of their high lipophilicity. Fortunately these terpenes were not isolated active principles, while the active principles responsible for the neuroprotection and antidiabetic action of the plant, morroniside and quercetin-3-O-galactoside (Harris et al., 2012; Muhammad et al., 2012; Muhammad et al., 2013), were present in near identical amounts in ethanol and water extracts. Thus, the use of ethanol extracts appears to provide a representative composition and activity of this plant. This was the first phytochemical comparison between the ethanol and water extract for any of the Cree plants.

S. purpurea's potential in altering genes involved in metabolism and transport, such as the *CYP* family of genes, *GST*, *QR*, and *ABC* transporters was also examined (Chapter 4). No changes in these particular genes were observed and this was verified with qPCR for *CYP2D6*. Furthermore, over 200 ESTs belonging to the solute carrier superfamily, coding for other xenobiotic transporters, were also not altered. Other genes were disregulated in the

microarray but these results could not be validated with real-time PCR as perfectly matched probe and primer sets do not exist for the genes that were selected for validation. Taken together, these results showed a small potential for drug interactions that would need to be validated by *in vivo* and clinical work. However, this was the first study examining the transcription of these genes with a Cree botanical in such an expanded fashion and with novel technology, such as the use of microarrays.

Lastly, *S. purpurea*'s neuroprotective mode of action was examined by utilizing TgCRND8 mice, a model of AD (Chishti et al., 2001), to see if the progression from pre-symptomatic to symptomatic state could be altered (Chapter 5). *S. purpurea* caused a greater cognitive deficit in the TgCRND8 mice, which is consistent with a warning from the Cree healers regarding this plant medicine. The healers recommended not using *S. purpurea* in individuals with a "weakened state". This study showed that *in vivo* the mice become more frenetic in behaviour with the traditional medicine treatment when weakened by the presence of the amyloid load. The finding also showed that this behavioural deficit can be associated with increases in a lipid signalling molecule, PC(O-16:0/2:0), thus suggesting a possible mode of action of the plant. This was the first study examining the potential mode of action of *S. purpurea in vivo* and a further confirmation of Cree knowledge of the boundaries of safe use of their medicines.

Combined, through the use of *in vitro* and *in vivo* work, the studies reported here on the safety of 17 Cree medicines, as well as both safety and

efficacy of one particular plant, *S. purpurea*, which was not previously explored in the literature. It is clear from the results obtained that the Cree have a great understanding of their medicine, both in terms of safety and efficacy, and greatly understand the limitations of this use.

6.2 Comparison with literature and a proposed model of action

Reports of using traditional plant preparations to treat symptoms of T2D exist in many cultures (Grover et al., 2002; Khan et al., 2012; Malviya et al., 2010). Even the early review of antidiabetic plants by Marles and Farnsworth (1995) showed that there were more than 1200 plants traditionally used in diabetes. Some of the medicines mentioned in these reviews include common plants such as *Trigonella foenum-graecum* (fenugreek), *Allium sativum* (garlic), *Curcuma longa* (tumeric) and *Panax ginseng* (ginseng). Some reports also include some medicines used by the Cree such as *Vaccinium angustifolium* and *Vaccinium vitis-idaea* (Beaulieu et al., 2010; Khan et al., 2012; McIntyre et al., 2009). The 15 remaining plants of the 17 plants identified by Cree healers in the Leduc et al. (2006) study are mostly unexplored in the antidiabetic literature. Therefore, most work published on the plants is done only by the TAAM team and that on *Sarracenia purpurea* is particularly limited. Compared to European traditional medicines, or Asian traditional medicines, the indigenous First Nation's pharmacopoeia has received very little attention in the scientific world. For this reason, these studies are pioneering investigation of North American traditional medicine. The comparison of the water and ethanol extract of *S. purpurea*

supported previous reports affirming that ethanol extracts should be the norm in most pharmacological studies (Lapornik et al., 2005) and it was particularly important for our work with the Cree, as this validated the work done by the TAAM team on the plants using ethanolic extracts. Similar to other groups who have studied water and ethanol extracts (Adedapo et al., 2011; Lapornik et al., 2005; Spigno et al., 2007), slightly higher concentrations of phenolics in ethanol extracts as compared to water were found here. There does not appear to be much literature on comparisons of terpenes, which were dramatically higher in this study, and no studies on the active principles morroniside and quercetin-3-O-galactoside, which in this study were present in both extracts at similar amounts. Clearly water and ethanol extraction can provide comparable active phytochemical profiles, but this needs to be monitored on a case by case basis since dramatic differences, such as seen with the terpenes here, can occur.

Further work reported in this thesis examining the role of drug interactions was also extremely important to the Cree Healer and Health Board as the Cree diabetics are receiving conventional antidiabetic drug therapy, as well other concomitant application of therapies is always an issue. Drug interactions pose a huge health and economic burden (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), therefore any potential for these interactions needed to be studied. Tam et al. (2009, 2011) have already shown that Cree traditional medicines have the potential to inhibit drug-metabolizing enzymes such as CYP3A4 and CYP2C9 *in vitro*. However, the relationship that those interactions

could have on concurrent administration of other antidiabetics had not been studied. Since grapefruit and St. John's Wort are two of the most notorious plants that cause severe drug interactions with other therapies, such as saquinavir for grapefruit (Eagling et al., 1999) and cyclosporine, theophylline and indinavir for St. John's Wort (Ernst, 1999; Nebel et al., 1999; Piscitelli et al., 2000), both on gene transcription and protein activity of both CYPs and transporters (Durr et al., 2000; Moore et al., 2000; Obach, 2000), it was important to study the potential for those interactions with the Cree plants. Since no impact of *S. purpurea* was found on transcription of *CYP*s as well as transporters in the Caco-2 whole cell model, and no impact of the Cree plants on transport of both gliclazide and repaglinide, these results may suggest potential safety of the Cree medicines used. However, the inhibition of the metabolism of repaglinide and gliclazide observed with medicinal plants is consistent with work already done by Tam et al. (2009, 2011), showing inhibition of CYP enzymes involved in drug metabolism. These results suggest an overdosing of the principle drug is possible due to this interaction. A study in a clinical setting is recommended.

Previous studies of the antidiabetic properties of *S. purpurea* by the TAAM demonstrated it as a potent neuroprotective plant, as well as insulinomimetic, mediating its activity through the AMPK pathway similar to metformin (Harris et al., 2012; Martineau et al., 2010a; Spoor et al., 2006). Pintana et al. (2012) has shown that metformin can be used to alleviate cognitive declines induced by high fat diet, and Pathan et al. (2008) and Liu et al. (2010b) have both used other antidiabetic therapies, rosiglitazone and pioglitazone, to alleviate cognitive

defects imposed by high fat diet and fructose drinking, respectively, in *in vivo* models. Furthermore, *Clitorea ternatea*, an Ayurvedic antidiabetic medicine, has been able to improve memory and learning in streptozotocin-induced diabetic rats (Talpate et al., 2012). Bhutada et al. (2010a) was also able to use quercetin, the aglycone of the glycosides found in *S. purpurea*, to improve cognitive function in a diabetes model, while Lu et al. (2007) and Lu et al. (2011) used ursolic acid to improve learning and memory. Moreover, aged garlic was used by Chauhan and Sandoval (2007) to improve learning and memory in TgCRND8 mice, the same mouse model as the one employed here. Therefore it was thought that *S. purpurea* might be an ideal treatment to improve cognitive function in the TgCRND8 mice. However, it was discovered that it hinders learning and memory by causing a switch in search strategy and by increasing levels of PC(O-16:0/2:0).

A proposed mechanism of action for *S. purpurea* was shown in Chapter 5 in Figure 5.6E, where *S. purpurea* may act on the Land's cycle and more particularly LPCAT 1/2 activity. It was proposed that in the nontransgenic state, *S. purpurea* was able to shuffle back the PC(O-16:0/0:0) species into structural lipids using acyl Co-A as a substrate, however, when $A\beta_{42}$ was present, the LPCAT 1/2 preferred the acetyl Co-A substrate and the amount of PC(O-16:0/2:0) produced was thus significantly increased. Ryan et al. (2009) has shown this shift for substrate specificity and more specifically the negative effects of PC(O-16:0/2:0). *S. purpurea* may simply enhance LPCAT 1/2 activity and the combination with $A\beta_{42}$ leads to this increase.

6.3 Future work

There still remains a great deal of unknowns with regards to Cree traditional knowledge, even though, this thesis attempts to shed some light on some aspects of safety and efficacy. Future safety work would ideally employ animal and clinical studies to determine the relevance of the results obtained *in vitro*. Particularly since the Cree currently use these medicines, population studies are needed that examine their concomitant use with conventional antidiabetic therapies in this patient population. Furthermore, an examination of the metabolic profile of the traditional medicines alone as well as in the combinations administered by the healers would also be required.

It is also suggested that further animal work to determine the antidiabetic efficacy of *S. purpurea* be employed similar to work already done on other Cree medicines (Harbilas et al., 2012a; Harbilas et al., 2012b, 2013). Using C57BL/6 mice, animals would be given a high fat diet for eight weeks to stimulate a diabetic phenotype, then have *S. purpurea* co-administered in the high fat diet at a dose of 250 mg/kg for an additional eight weeks. A variety of systemic parameters such as glucose, insulin, leptin, adiponectin, and triglyceride levels could then be measured. This would go to great lengths to understand Cree traditional knowledge, which is the main goal of the TAAM, and contribute to establishing an antidiabetic mechanism of action of the plant *in vivo*. Furthermore, a more thorough evaluation of the traditional preparation is also required both *in vitro* and *in vivo*, as it is clear from other studies that betulinic

acid and ursolic acid may be bioactive. Thus their relevance requires further study.

Since *S. purpurea* treatment of TgCRND8 mice led to an increase in PC(O-16:0/2:0), from a biochemical standpoint, it would also be interesting to explore the potential link, or lack thereof, between AMPK activation and LPCAT activity, as more studies now show the potential negative impact of AMPK activation in AD (Dash et al., 2006; Mairet-Coello et al., 2013; Salminen et al., 2011). Furthermore, the isolation of the compound(s) responsible for this action would also be beneficial.

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