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Cree traditional medicine and type 2 diabetes: An ethnobotanical and pharmacological study

par

Charles Leduc

Département de pharmacologie
Faculté de médecine

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Ce mémoire intitulé:

Identification and pharmacological evaluation of Cree medicinal plants with potential use for the treatment of type 2 diabetes

présenté par:

Charles Leduc

a été évalué par un jury composé des personnes suivantes:

Jacques De Champlain, PhD
président-rapporteur

Pierre Haddad, PhD
directeur de recherche

Alain Cuerrier, PhD
co-directeur

Timothy Johns, PhD
membre du jury

RÉSUMÉ

Le diabète de type 2 (DT2) est un problème important chez la nation Cris de la Baie James, tel qu'indiqué par une prévalence trois fois plus élevée que celle de la population canadienne non-autochtone. Cette prévalence continue d'augmenter due en partie à une difficulté d'observance des thérapies modernes. Dans le but de développer un traitement mieux adapté à la culture Cris, nous avons évalué l'efficacité pharmacologique de la médicine traditionelle Cris dans le contexte du DT2. Une étude ethnobotanique effectuée à Mistissini (QC) a identifié 18 espèces de plantes recommandées par les guérisseurs Cris pour traiter une quinzaine de symptômes reliés au diabète. Les extraits éthanoliques des 8 espèces les plus prometteuses ont été criblés à l'aide de bioessais de transport de glucose *in vitro* (basal et stimulé à l'insuline) chez des lignées de cellules musculaires (C2C12) et d'adipocytes (3T3). Toutes les espèces ont stimulé le transport de glucose dans au moins une des conditions testées. Les trois espèces les plus efficaces ont ensuite été évaluées *in vivo* chez des souris normales et diabétiques (streptozotocine et/ou nutrionnel). Aucune des espèces n'a démontré un effet anti-diabétique *in vivo*, mais ces résultats demeurent incertains car la metformine n'a pas été efficace dans ces modèles non plus. Ces plantes démontrent donc un fort potentiel thérapeutique *in vitro* qui restent à être confirmé dans des conditions *in vivo* plus optimales.

MOTS CLÉS: diabète, Nation Cris, plantes médicinales, ethnobotanique quantitative, transport de glucose, C2C12, 3T3, streptozotocine, obésité induite par la diète.

Abstract

Type 2 diabetes (T2D) is a major health problem among the Cree Nation of James Bay, illustrated by a three-fold higher prevalence of this syndrome than non-aborginal Canadians. This prevalence continues to rise due in part to a difficulty of compliance with modern medical therapy. In order to develop a therapy more in harmony with Cree culture, we evaluated the pharmacological efficacy of Cree traditional medicine in the context of T2D. An ethnobotanical survey carried out in Mistissini (QC) identified 18 plant species recommended by Cree Elders for 15 symptoms related to T2D. Ethanol extracts of the 8 most promising species were screened using *in vitro* glucose uptake assays (with or without insulin) in muscle cells (C2C12) and adipocytes (3T3). All species stimulated glucose uptake in at least one of the conditions tested. The three most active species were then evaluated *in vivo* in normal and diabetic (streptozotocin and/or nutrionally-induced) mice. None of the extracts showed an antidiabetic effect in vivo, yet these results remain inconclusive since metformin was not entirely effective in the models tested either. Thus, the plants tested exhibited a significant therapeutic potential *in vitro*, which remains to be confirmed under more optimal *in vivo* conditions.

Keywords: diabetes, Cree Nation, medicinal plants, quantitative ethnobotany, glucose uptake, C2C12, 3T3, streptozotocin, diet-induced obesity.

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List of abbreviations

AAC = Area above the curve

AMP = Adenosine monophosphate

AMPK= AMP-activated protein kinase

ATP = Adenosine triphosphate

AUC = Area under the curve

BG = Blood glucose

BW = Body weight

CEI = Cree of Eeyou Istchee

CTL = Control

DMEM= Dulbecco's modified eagle medium

DMSO = Dimethyl sulfoxide

FBS = Fetal bovine serum

FFA = Free fatty acid

GIR = Glucose infusion rate

GLUT4 = Facilitative glucose transporter 4

GRC = Glucose response curve

HEC = Hyperinsulinaemic-euglycaemic clamp

HF = High fat

HOMA = Homeostasis model assessment

HS = Horse serum

IGF = insulin–like growth factor

ITT = Insulin tolerance test

Kg = glucose disappearance constant

OGTT = Oral glucose tolerance test

PG= Plasma glucose

PPAR = Peroxisome proliferator activated receptor

SIV = Syndromic importance value

STZ = streptozotocin

T2D = Type 2 diabetes

TNF = Tumour necrosis factor

Dedication

This thesis is dedicated to the Cree Nation of Eeyou Istchee and in particular to the people of Mistissini (Quebec) who welcomed me into their community, and to the Cree Elders and Healers who shared their knowledge of traditional medicine with our research team.

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Foreword

The personal motivation behind the undertaking of this M.Sc. thesis springs from two main interests: 1) a sincere fascination with the relationship between people and plants as it relates to the traditional practice of the healer; 2) a desire to combine the study of ethnobotany and pharmacology as a means of validating, preserving and contributing to the practice of traditional medicine by aboriginal peoples today. In its formative years, the study of ethnobotany consisted mainly of compiling and publishing lists of plants used by indigenous communities. Although this practice contributed to the preservation of rapidly vanishing body of traditional knowledge, it did little to address or solve the various socio-economic, political, or medical issues being faced by the stewards of this knowledge. The modern practice of ethnobotany and ethnopharmacology has evolved to attend to this problem, and with the Convention on Biological Diversity of 2001 (http://www.biodiv.org/default.shtml), indigenous communities worldwide are gaining well-warranted rights over the knowledge of their ancestors and the political leverage to affect positive social change within their communities.

In this context, the greater project encompassing this M.Sc. thesis aims from the outset to address a serious health issue within an indigenous Nation, by combining the knowledge and skills of traditional healers and scientific researchers in a manner that will benefit both parties equally. Although benefit sharing is not a new concept in ethnopharmacological research, it most often involves two parties, a pharmaceutical company and an indigenous community, with the return of a portion of capital gains (to the participating indigenous community) accrued from the sale of a modern pharmaceutical drug. This is not the case for the project elaborated herein, which sets itself apart in the field of natural product research in several ways. First, the end goal is to develop a standardized plant extract to be used by the participating indigenous communities, and not a conventional drug sold commercially. Second, the project is entirely funded by public grants and does not involve the participation of any pharmaceutical company. Third, the project is truly collaborative in nature, involving the participation of researchers from 4 scientific domains (ethnobotany, phytochemistry, nutrition, and pharmacology), clinicians and social workers from both outside and within the native communities, Native band chiefs and their band council, and Native Elders and Healers and their council. Finally, the project is

designed to involve the approval and input of indigenous partners not only to identify medicinal plants but also throughout the entire progression of the project.

The framework for this thesis can be summarized by the following 3 points: 1) The Cree Nation of James Bay suffer from an extremely high prevalence of Type 2 Diabetes; 2) It has been shown that the Cree Nation and other Canadian Native Nations have a wealth of ethnomedicinal knowledge; 3) The Cree Nation of James Bay has agreed to collaborate with our research team to identify medicinal plants and evaluate their potential use as anti-diabetic therapies. The underlying hypotheses for this thesis are the following: 1) Elders and Healers from the Cree Nation of James Bay possess and upkeep knowledge of Cree traditional medicine that is applicable to the symptoms of Type 2 Diabetes; 2) Extracts from one or more of the plants identified by Cree Elders and Healers will demonstrate a significant anti-diabetic potential when screened *in vitro*; 3) Extracts from one or more of the plants showing a significant anti-diabetic potential *in vitro* will also demonstrate a significant anti-diabetic effect *in vivo*.

This study is the first of its kind to identify and pharmacologically evaluate medicinal plants used within Cree traditional medicine in the context of Type 2 Diabetes. It proposes and implements a novel approach to quantitative ethnobotany geared towards multi-factorial syndromes such as diabetes, and uses well-established pharmacological screening assays and animal models to effectively assess the anti-diabetic potential of a number of poorly-studied plant species, none of which have been previously studied in this context. It is hoped that the work presented here will help to advance of the study of medicinal plants and diabetes, while contributing to the development of a sustainable and culturally acceptable plant-based therapy for Type 2 Diabetes in Cree communities.

Chapter 1: *Introduction*

1.1 Introduction

There are currently over 171 million people worldwide who have diabetes, and this figure is expected to more than double in the next 30 years [1]. In Canada, diabetes is a significant health problem with a prevalence of 4.8% among people aged over 20 years, representing an annual economic burden of approximately CAD$1.6 billion [2]. The significance of this disease is most pronounced in Canada's aboriginal population, wherein the age-standardized prevalence is 3 to 5 times that of the general population [2]. Moreover, aboriginal patients with diabetes have a greater chance of mortality due to overloaded medical facilities and a difficulty of compliance with modern medical treatment [3].

Diabetes present in the Native Canadian population is almost entirely of type 2 [4, 5]. The adoption of a sedentary lifestyle and the consumption of non-traditional foods are thought to be the major underlying causes of the epidemic [3, 6, 7]. Genetic causes also are thought to play a significant role in this population. Although single gene polymorphisms correlated to Type 2 diabetes (T2D) have been identified in the Oji-Cree Nation of northern Ontario [8], the ensemble of genetic data suggests that hereditary involvement is polygenic in nature [9]. It has also been suggested that fetal malnutrition and perturbations in the interuterine environment play a pivotal role in intitiating T2D in the Native North American population [10]. This hypothesis has been correlated to the high prevalence of gestational diabetes in this population [11], and is supported by two related findings: 1) First degree relatives of T2D patients have a 3-fold greater chance of developing the disease than non-relatives [12]; 2) Individuals with low ($\leq$ 6.5 lbs) birth weights are 10 times more likely to develop T2D than those with higher ($\geq$ 9.5 lbs) birth weights [13]. The combined effects of these early metabolic disruptions followed by relatively high caloric intake and sedentarism place this population at a particularly high risk of developing T2D.

The Cree are a member of the Algonkian language family and represent the largest aboriginal group in Canada, numbering over 72,000 individuals across the country [14]. The Cree of Eeyou Istchee (CEI) represent a subpopulation of the greater Cree Nation having a total population of approximately 13,500 people. They are present in 9 communities spread out between the 49^{th} and 55^{th} parallels in the province of Quebec, Canada [15]. Over the past decade, the age-adjusted prevalence of diabetes among the CEI has risen from 6.6% to 17.7% for people aged over 20

years [4, 5, 16]. This dramatic rise in diabetes, coupled with a high risk of obesity [17] and the second highest prevalence of gestational diabetes reported in an aboriginal group worldwide, highlights the urgent need for effective prevention and treatment strategies for T2D [18].

Despite the implementation of dietary intervention strategies and the presence of numerous educational programs, the prevalence of T2D continues to increase among the CEI [3, 4, 6, 19]. This is largely due to the fact that most management strategies are designed by non-aboriginal professionals and there is an overall lack of cultural appropriateness and awareness [6]. Consequently, there is a growing need to develop novel approaches towards the management and prevention of diabetes, particularly ones that are in harmony with aboriginal people's culture and lifestyle. In this context, it has been shown that despite access to conventional medical facilities, the use of and dependence upon complementary and alternative medicine by many minority populations continues to thrive [20, 21]. Thus, it is not surprising that numerous publications over the last two decades have underlined the importance of exploring traditional medicine as a potential source of effective anti-diabetic remedies [3, 22-26].

Worldwide, over 1200 species of plants have been recorded as traditional medicine for diabetes [23]. Although most of these species have not undergone rigorous scientific evaluation, over 80% of those tested show some degree of antidiabetic activity [23]. With such a wealth of ethnomedicinal data, it is not surprising that a number of drugs currently used to treat diabetes are historically derived from plant or fungal material. These include metformin (derived from *Galega officinalis L.*) [24], acarbose (derived from *Actinoplances* spp.) [23], and 4-hydroxyisoleucine (currently undergoing clinical trials, derived from *Trigonella foenum-graecum* L.) [27]. However, the lack of convincing clinical data still hinders the large scale acceptance of plant- based therapies for diabetes. A recent systematic review confirms that although most plant remedies marketed for diabetes are safe, only 2 species have sufficient evidence for efficacy, namely *Coccinia indica* Wight & Arn. ("ivy gourd") and *Panax quinquefolius* var. *americanus* Raf. ("American ginseng") [28]. Other species which have shown promising preliminary clinical results are *Gymnema sylvestre* (Retz.) Schult., *Aloe vera* Mill., *Momordica charantia* L. [28, 29], *Trigonella foenum-graecum* L., *Allium sativum* L., *Glycine max* Merr.

[29], and *Amorphophallus konjac* K.Koch [30].

It is thus clear that plants have an important role to play in the treatment of T2D, both in present day therapy and in years to come. It is also evident that the Cree Nation have and maintain a wealth of ethnomedicinal knowledge [31-41]. However, no work has been done to examine the antidiabetic potential of Cree medicinal plants. Thus, with a growing need for culturally-appropriate therapies for T2D, a multidisciplinary project was created to investigate the antidiabetic properties of Cree medicinal plants. The project's ultimate mandate is to work with the Cree Nation in order to re-integrate a pharmacologically- validated antidiabetic plant therapy into Cree communities.

The M.Sc. thesis presented here, completed within the context of the aforementioned project, is composed of three main sections: 1) Ethnobotany; 2) *In vitro* pharmacology; 3) *In vivo* pharmacology. The first consists of an ethnobotanical study carried out to identify medicinal plant species within the Cree pharmacopoeia that have potential antidiabetic properties. The second evaluates the anti-diabetic activity of the identified species through a series of diabetes specific *in vitro* assays. The final section consists of confirming the anti-diabetic effect of the most promising plant species *in vivo*. One chapter is dedicated to each section which is organized into general subsections as follows: Introduction/foreword, material and methods, results, discussion, and conclusion. The final chapter is a summary conclusion which relates the three main sections of the thesis while providing suggestions for future studies.

1.2 **Background**

1.2.1 Glucose transport in humans

Glucose metabolism is a complex balancing act involving the tight control of numerous catabolic (lipolysis, glycolysis, glycogenolysis) and anabolic (lipogenesis, gluconeogenesis, glycogenesis) processes [42]. Although glucose metabolism occurs in all cells, glucose homeostasis is largely regulated by three main tissues: liver, pancreas, and skeletal muscle [43]. Combined with adipose tissue, the latter accounts for over 85% of whole body glucose disposal, and these two tissues are directly involved in insulin stimulated glucose uptake, the main rate-limiting step for glucose metabolism [44-47]. They are also the two tissues chosen for the *in vitro* screening of medicinal plants in this thesis. For these reasons, the following background

discussion will focus mainly on muscle and adipose tissue, while recognizing that both the pancreas and the liver play equally crucial roles in glucose metabolism

As mentioned above, it has now been clearly shown that glucose transport across the plasma membrane is the main rate-limiting step for glucose metabolism [46, 47]. This process occurs in all mammalian tissues via proteins called glucose transporters, of which the first was cloned in 1985 [48, 49]. Since then, a total of 8 glucose transporters have been identified with varying distribution throughout the body (Table 1.1) [50]. Among this family, only GLUT4 has been shown to be highly insulin sensitive, with expression restricted to muscle cells and adipocytes [51]. Two pathways of insulin stimulated glucose transport have been identified, one of which seems to be unique to the adipose tissue (Figure 1.1) [52].

Activation of GLUT4 translocation is not, however, restricted to insulin (or insulin mimicking/ insulin-like growth factors) dependent pathways. In skeletal muscle, muscle contraction/exercise and hypoxia both lead to increased glucose transport through translocation of GLUT4 to the membrane (Figure 1.2) [53, 54]. In fact, insulin in conjunction with contractile activity stimulates GLUT4 translocation to a greater extent than either stimulus alone [55]. The key protein of this insulin-independent pathway is thought to be AMP-activated protein kinase (AMPK), whose activity rapidly increases during exercise in response to changes in the ratio of ATP:AMP [56, 57]. Direct pharmacological stimulation of AMPK in skeletal muscle strips of T2 diabetic subjects has confirmed that activation of this protein results in increased glucose transport and cell surface GLUT4 content [58]. Although AMPK is also present in adipocytes, its role in glucose transport seems to be negligible [59]. Rather it seems that insulin-independent GLUT4 translocation in adipocytes can be stimulated by a pathway unrelated to AMPK [59], which might be related to nitric oxide, yet the exact mechanism remains to be elucidated [60]

1.2.2 Type 2 diabetes

Type 2 Diabetes (T2D), previously known as non-insulin-dependent-diabetes or adult-onset diabetes, is a syndrome characterized by disorders of insulin action and insulin secretion, either of which may be the predominant feature [61]. In adults, the clinical diagnosis of the disease is based on the glycaemia of an individual in both the fasted state and 2 hours following a fasted oral glucose tolerance test (OGTT) of 75 g glucose: fasted venous blood glucose $\geq$ 110 mg/dL (~ 6 mM); post-OGTT venous

Table 1.1. Mammalian glucose transporters and there distribution and function. Reproduced from Ducluzeau et al (2002), with permission.

Name	Tissue distribution	Functions
GLUT1	Wide distribution, abundant in red blood cells, endothelial cells and tissue culture cell lines	Basal glucose uptake in many cells (incl. insulin sensitive cells), transport in growing cells and across blood-brain barrier
GLUT2	Limited to pancreatic β cells, hepatocytes, intestine, kidney	Glucose-sensing in β cells, high capacity transport, trans-epithelial transport, major transporter in liver
GLUT3	Wide distribution in humans, limited to brain in some species	Basal transport, uptake from cerebral fluid
GLUT4	Largely expressed in insulin-responsive tissues of skeletal and cardiac muscle, and adipose	Insulin-sensitive glucose uptake, vital in postprandial glucose disposal
GLUT5	Primarily intestine, small amounts in adipose, muscle, brain, kidney	Absorption of fructose in intestine
GLUT7	Gluconeogenic tissues: hepatocytes	Release of glucose from gluconeogenesis from ER lumen
GLUT8	Blastocyst, possibly other tissues	Insulin-stimulated glucose uptake into blastocyst and possible other tissues lacking GLUT4
GLUTX1	High in testis, moderate in central nervous system, low in insulin responsive tissues	? Sequestered intracellularly therefore may play a role in regulatable glucose uptake

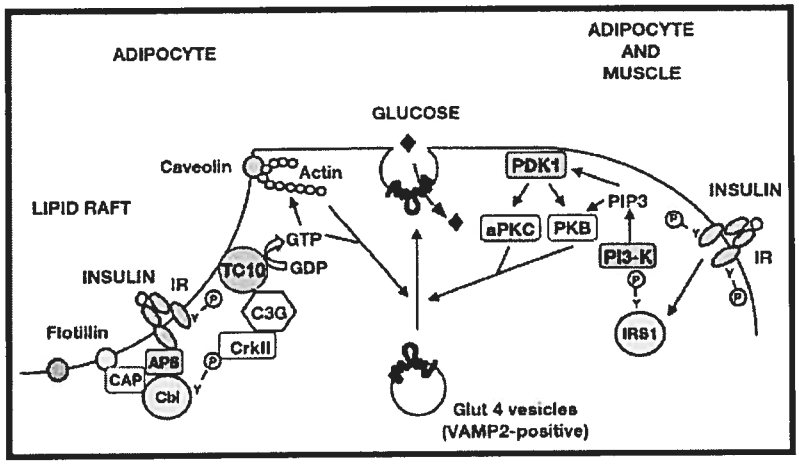

Figure 1.1. Insulin-dependent signalling pathways of glucose transport in the adipocyte and muscle cell. Reproduced from Gual et al, (2003) with permission.

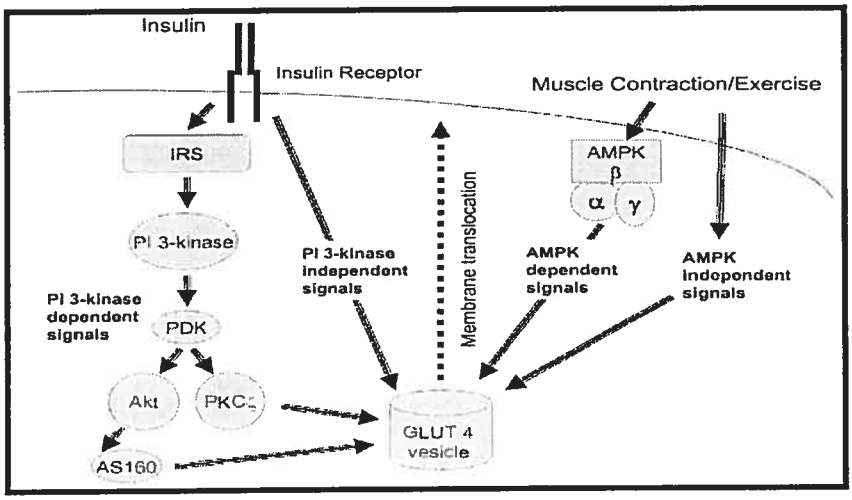

Figure 1.2. Insulin dependent and independent signalling pathways of glucose transport in muscle cells. Reproduced from Krook et al (2004) with permission.

blood glucose ≥ 180 mg/dL (~10 mM). This diagnosis is often prompted by a variety of symptoms associated with the syndrome, the most apparent of which are increased thirst and urination, recurrent infections, fatigue, unexplained weight loss, blurring of vision and drowsiness. Left untreated, the severity of the symptoms increases, leading to hypertension, renal failure, amputation, loss of eyesight, coma and death [61].

It is becoming increasingly evident that the clinical manifestation of T2D in obese subjects is preceded by a heterogeneous metabolic disorder called the metabolic syndrome or syndrome X. According to the most recent definition, an individual is considered to have the metabolic syndrome when they present 3 or more of the following symptoms: Central obesity (waist circumference > 102 cm [male], > 88 cm [female]); Hypertriglyceridaemia (triglycerides ≥ 1.7 mmol/L); Low HDL cholesterol (<1.0 mmol/L [male], <1.3 mmol/L [female]); Hypertension (blood pressure ≥135/85 mm Hg); Glucose intolerance (fasting plasma glucose ≥ 110 mg/dL) [62]. The ensemble of these metabolic abnormalities place individuals with the metabolic syndrome at an elevated risk of T2D and cardiovascular disease [63, 64]. Given the high prevalence of T2D within Native Canadian populations [65], it is not surprising that recent studies have shown a particularly high prevalence of metabolic syndrome in these populations as well [66, 67].

1.2.3 Insulin resistance

A prominent and unifying theme in the metabolic syndrome and T2D is insulin resistance. Indeed, the metabolic syndrome was initially termed the insulin resistance syndrome by some research groups [68], and is still commonly referred to by this name. The underlying causes of this abnormality are multifactorial with the main regulators being genetics, adipokines, and circulating free fatty acids (FFA), with the latter being the major contributor [69, 70]. The specific role of genetics in insulin resistance remains somewhat of a black box, yet it is becoming clear that it most likely involves polymorphisms in multiple genes encoding for proteins involved in insulin signalling, insulin secretion and intermediary metabolism [71].

Adipokines, hormones secreted by adipocytes, play a crucial role (both para- and autocrine) in regulating metabolism beyond their ability to regulate glucose levels [72]. With respect to insulin resistance, 3 adipokines are particularly important: tumour necrosis factor-α (TNFα), leptin, and adiponectin. TNFα is increased in obese subjects and has been shown to induce insulin resistance by reducing insulin receptor

kinase activity [73]. Leptin is the main hormone controlling appetite, and acts primarily on the central nervous system to inhibit food intake and increase energy expenditure [70]. Insulin resistance is a main characteristic of leptin deficiency or resistance, such as present in genetic models of obese mice (*ob/ob, db/db, KKAy*), and leptin resistance in itself has been proposed as one of the key initial promoters of the metabolic syndrome [74]. Like leptin, adiponectin is another hormone shown to be deficient in obese subjects [75]. Recent evidence shows that adiponectin has profound influence on metabolism illustrated by its antidiabetic, antiatherosclerotic and anti-inflammatory properties [76]. Its anti-diabetic activity is linked to its ability to decrease plasma FFAs and triglyceride content in muscle cells and fat, increase FFA oxidation, and increase the ability of insulin to suppress hepatic glucose production [77, 78]. Antagonizing these effects are the circulating FFAs, which are found in high concentration in insulin resistant individuals, [69, 70]. High FFAs are associated with a multitude of deleterious effects including inhibition of glucose uptake, glycogen synthesis and glucose oxidation, and increase in hepatic glucose output [79]. They have also been shown to decrease insulin secretion via their lipotoxic effects on pancreatic β cells [80]. As insulin resistance sets in, insulin's ability to inhibit lypolysis decreases concomitantly, allowing for an unregulated increase in circulating FFAs [69].

1.2.4 Therapy

Considering the modern origins of T2D, it has been suggested that the most effective therapy for this syndrome would be a return to a more traditional lifestyle and diet – an approach cleverly termed by one group as the "Paleolithic prescription" [81]. Indeed, both dietary restriction and exercise have been shown to increase insulin sensitivity [82, 83], and for this reason they are the modalities most commonly recommended for the treatment of T2D [84]. However, in a society which has become increasingly dependent upon, and expecting of, pharmacological fixes to health problems, compliance with lifestyle modification programs have only had modest success [85]. This is especially true for the Cree of Northern Quebec, although the underlying reasons for reduced compliance differ (see section 1.1 Introduction).

For these reasons, there is a strong reliance on anti-diabetic drugs, and the pharmaceutical industry has responded accordingly. In the last decade, the number of

classes of anti-hyperglycemic agents approved by the United States for T2D has increased from two (insulin, insulin secretagogues) to five (addition of biguanides, α-glucosidase inhibitors, thiazolidinediones), while choices within each class have increased as well [86]. Since the use of insulin is well known and its mechanisms of action have been already introduced (section *1.2.3 Insulin resistance*), the following discussion will focus on the other 4 classes of treatment, all of which are taken orally.

Within the class of insulin secretagogues, there are numerous sulfonylureas (Chlorpropamide, Glimepiride, Glipizide, Glyburide, Tolazamide, Tolbutamide) and two non-sulfonylurea compounds (Nateglinide, Repaglinide). These compounds increase the secretion of insulin from the β-cells of the pancreas by binding to a component of ATP-dependent potassium channels [87]. Since they act directly on the pancreas, their use is limited to patients with a sufficiently large and functional β-cell mass. Although they are still used for T2D, their popularity has decreased due to a multitude of factors related to the patient (non-compliance, weight gain), therapy (desensitization of β-cells), and features of the disease itself (escalating insulin resistance and deficiency) [88, 89].

Among the two biguanide drugs developed for diabetes (phenformin, metformin) only metformin remains in clinical use. Metformin's principal anti-hyperglycamic effect is due to a potentiation of insulin's suppressive effects on hepatic glucose production [90]. It also has been shown to decrease weight gain, circulating levels of insulin, and low density lipoprotein [91]. Although the mechanism of action is not yet fully understood, metformin's main site of action is thought to be AMPK, a key cellular regulator of glucose metabolism (see section *1.2.1 Glucose transport in humans*) [92]. It does not cause overt hypoglycaemia, as can sulfonylureas, and is widely used as first-line therapy or in combination with a secretagogue or a thiazolidinedione [86].

There are three α-glucosidase inhibitors: Acarbose, miglitol, and voglibose. These drugs decrease postprandial hyperglycaemia by impairing the action of enzymes that digest complex carbohydrates in the gut [93]. These drugs can be used in monotherapy, but they are commonly combined with insulin or sulfonylureas [94]. Their use is somewhat limited due to gastro-intestinal secondary effects such as bloating, flatulence and diarrhea, as well as possible hepatic injury following chronic treatment [94, 95].

Thiazolidinediones are the most recently developed class of ant-diabetic

drugs, with two drugs currently on the market: rosiglitazone and pioglitazone. These drugs enhance the effect of insulin without increasing pancreatic secretion of insulin. They do so mainly by binding to the peroxisome proliferator-activated receptor-γ (PPARγ) in adipocytes, thereby increasing the expression of numerous insulin-dependent genes, including GLUT 4 [96]. Thiazolidinediones are generally very well tolerated [97], however weight gain is a common side effect [98].

Despite the large number of anti-diabetic drugs, acting via a variety of different mechanisms, T2D as treated presently leads to severe medical consequences [86]. In an attempt to better control the complex symptomatology of T2D, many pharmaceutical companies have marketed drug combinations such as Metaglip (glipizide + metformin), Glucovance (glyburide + metformin), and Avandamet (rosiglitazone + metformin). While the industry has been developing new combinations and formulations, the general public and family physicians has been increasingly exploring alternative therapies for T2D[29]. A review of the literature generated a list of approximately 10 plants which show clinical proof or promise of anti-diabetic efficacy, although the number of species currently used worldwide for diabetes greatly exceeds this number (see section 1.1 Introduction). With increased public demand for alternative treatments, it is likely that research in this area will continue to grow. It is hoped that projects such as the one presented in this thesis will contribute to the growing body of knowledge on anti-diabetic plants, while taking an initial step towards a T2D therapy more readily acceptable to the Cree Nation of Eeyou Istchee.

Chapter 2: *Ethnobotany*

2.1. **Introduction**

The objective of this study was to identify medicinal plants that have potential antidiabetic properties within the Cree pharmacopoeia. This study represents the first stage of a collaborative project aiming to identify and pharmacologically validate Cree medicinal plants that demonstrate an antidiabetic potential so that they may be integrated into dietary intervention strategies in collaboration with the Cree Board of Health and Social Services of James Bay and the local Public Health department. Rather than performing an exhaustive survey of the plants used by the Cree of Eeyou Istchee, our research team focused on one community to develop an experimental method suitable to the greater objective of this project. In so doing, this study will provide general ethnobotanical data on a historically understudied population [34, 40], illustrate the ethnobotanical applicability of several quantitative tools, and generate a prioritized list of plant species for further pharmacological investigation.

2.2 **Material and methods**

2.2.1 Study site

Interviews were conducted with Cree Elders in the community of Mistissini, located along the southern border of the CEI territory (48 51' N and 72 12' W; elevation of 121.90 m). This community was chosen due to a limited ethnobotanical coverage and a willingness to collaborate in the research project. It is located within the eastern boreal forest of Canada, a region characterized by an open lichen woodland which progresses to denser spruce forest as the crown closure becomes greater [99]. This is a relatively harsh climate with an average precipitation of 900 mm, an average daily temperature of 1.4 °C, and only three months of the year where the average temperature is above 15 °C. Dominant tree species include *Picea glauca* (Moench) Voss (white spruce) and *Abies balsamea* (L.) Mill. (balsam fir) in the dense upland forest; stands of *Pinus banksiana* Lamb. (jack pine) on drier sites; *Populus tremuloides* Michx. (trembling aspen), *Betula papyrifera* Marsh.(paper birch); *Alnus incana* subsp. *rugosa* (Du Roi) R.T.Clausen (speckled alder) in fire cleared patches; and *Picea mariana* BSP (black spruce) and *Larix laricina* K.Koch (tamarack) in lowland bogs [99].

2.2.2. Interviews

Since diabetes is a complex multifactorial disease and a relatively new phenomenon among the Cree population, the ethnobotanical approach used was based on the numerous symptoms and complications of the disease. Hence, a questionnaire was developed that addressed 15 symptoms of T2D, rather than diabetes per se. The choice of these symptoms was validated from previous ethnobotanical literature and a number of endocrinologists and diabetes scientists [24, 100, 101]. This approach has the dual advantage of obtaining a large amount of pertinent ethnomedicinal knowledge, while being suitable to the Cree herbalist's role of treating ailments with easily discernable symptoms [41].

A total of 34 Elders from 23 households participated in the study. Our objective was to interview a large number of Elders to maximize the number of species cited while providing a sufficient sample size to determine the degree of consensus among informants. Allowing couples to be interviewed together reduced the interview sample size, yet it allowed for a more comfortable environment for the informants during the interview session. The Elders who participated were identified by community members as those who were most knowledgeable in Cree traditional medicine. The age of the informants ranged from 60 to 97 years, with an average of 75.5 years. Of the 34 Elders interviewed, 12 had T2D. Interviews took place in the Cree language in the informant's home with the help of an interpreter. Informants were aware of their right to refuse to answer specific questions, to stop the interview at any time, or to simply decline the interview altogether. Interview length ranged from 30 to 120 minutes.

Interviews began by asking informants about their understanding of the nature of diabetes and its causes. Informants were then asked if they knew of any traditional remedies that specifically treated diabetes. Following this, informants were presented with each of the following 15 symptoms and asked what traditional remedies they would use to treat the symptom: 1) arthritis/rheumatism; 2) frequent headaches; 3) back and/or kidney pain; 4) diarrhoea; 5) swelling and/or inflammation; 6) general weakness; 7) increased appetite; 8) heart and/or chest pain; 9) increased thirst; 10) abscesses and/or boils; 11) blurred vision; 12) increased urination; 13) foot numbness and/or foot sores; 14) slow healing infections; 15) sore and or swollen limbs. Questions were therefore semistructured [102].

2.2.3. Plant material

A total of 5 voucher specimens were collected for all plants cited during the interviews and their botanical identity was confirmed at the Montreal Botanical Gardens. One specimen is stored at the Marie-Victorin Herbarium (MT), another will be placed at the Cree Cultural Institute (Aanischaaukamikw) in Oujé-Bougoumou, while the others will be mounted and made available to the Cree School Board. Plant material was also collected for pharmacological analysis following the practice and spiritual guidelines proposed by the Elders.

2.2.4. Data analysis

Over the past quarter century, several quantitative methods for the analysis of ethnobotanical data have been proposed [21, 103-111]. Most methods are designed to establish the degree of consensus for the use of a plant within a cultural group, to evaluate the nature of ethnobotanical knowledge held by one cultural group in relation to the available flora, or to compare the patterns of plant use between cultural groups. The differences in our technique from those previously proposed result partly in the nature of our approach and the resulting data set. For example, most ethnobotanical surveys are based on open-ended interviews, wherein the scope of data generated is limited only by the amount of time available and the intellectual generosity of the informant. In our case, the ethnobotanical data generated from our survey is limited by our predetermined list of symptoms related to diabetes. Also, due to the reduced mobility of our informants, our interviews took place indoors which reduces the contribution of visual cues present in the forest. We have thus employed a novel method which is more suitable to this specific study. However, in the interest of comparison and to employ a more pluralistic approach, the quantitative technique proposed by Phillips and Gentry was also used [109].

In order to prioritize plant species for pharmacological investigation, three main parameters were taken into account: 1) number of different symptoms for which the plant was cited; 2) frequency of citation by different elders and 3) relative importance of symptoms for which the plant was cited. The latter refers to a relative weight of importance assigned to each symptom according to its degree of association to T2D. This procedure was accomplished by a team of 5 scientists which included clinical endocrinologists and professional researchers involved in type 2 diabetes research and treatment. The clinician/researcher evaluated each of the symptoms

using a scale of 1 to 4 as follows: 1= highly associated; 2 = moderately associated; 3 = weakly associated; 4 = Not at all associated. Each species was then assigned a Syndromic Importance Value (SIV) value which takes into account the three aforementioned parameters:

$$\text{SIV} = \frac{\left[\frac{\sum ws}{S}\right]+\left[\frac{\sum wf}{SF}\right]}{2} = \frac{\sum ws+\left[\frac{\sum wf}{F}\right]}{2}$$

where w = weight of the symptom,

s = the symptom contribution for the species

f= the frequency of citation for the species

S = the total number of symptoms used for the survey

F= the total number of interviews used in the survey

The weight of the symptom w, is the degree of association converted to a number between 0 and 1, where $\Sigma w = 1$. The symptom contribution s is either 1 or 0, based on the plant species being cited for the particular symptom or not, respectively, where $\Sigma s = S = 15$ in the case where the species is cited for all symptoms. The frequency of citation f refers to the total number of instances the plant was cited for one of the symptoms, where a maximum $\Sigma f = \Sigma F = 15*23 = 345$, if all informants were to cite the plant species for all 15 symptoms. Finally the equation is divided by two since the SIV represents an average value equally dependent on both frequency and symptom contribution.

An equivalent ranking system was applied to the symptoms themselves, based on the number of traditional remedies provided for each symptom, and the number of different species used for each symptom. When compared to the ranking performed by health professionals, this ranking, based on healer's traditional knowledge, will help illustrate the extent to which the results of the ethnobotanical survey is geared to the symptoms deemed to be most important. Ideally, the traditional knowledge displayed by the informants will be the greatest for the most important symptoms.

2.2.4.1 Correspondence Analysis

Correspondence analysis was performed in order to determine the degree of association between individual plant species and specific symptoms within the list. This analysis can be used to analyze two-dimensional contingency matrices. In such

matrices, the states of the first descriptor (symptoms) are compared to the states of the second descriptor (species). Data in each cell of the table are frequencies of citation. The results of these analyses are best visualized by a three-dimensional scatter diagram in which each species and symptom is represented by a point. A species will lie in proximity to a symptom if the following holds true: 1) the species had the highest frequency of citation for the symptom; and 2) the symptom had the highest frequency of citation for the particular species. All calculations were performed using STATISTICA 6.0 (Statsoft®).

2.2.4.2 Cluster Analysis (Euclidean distance using single linkage clustering)

Cluster analysis was used to examine the grouping of species as a function of the remedies for which they are employed. This analysis generates dendrograms which illustrate how closely the species are related to each other based on the symptoms for which they were cited. It is hypothesized that species within the same family will be grouped together based on the similarity of their phytochemical profile. Although these analyses are most commonly employed in plant systematics and ecology, they can be ethnobotanically useful in that they provide the researcher with a quantitative tool to examine the relationship between taxonomic groups based on their ethnopharmacological uses. All calculations were performed using STATISTICA 6.0 (Statsoft).

2.2.4.3 Literature Survey

Our ethnobotanical data and ranking were evaluated and compared to related scientific review articles by McCune and Johns [100, 101], as well as a subset of Cree-specific ethnobotanical data [34, 36, 37, 39-41]. In the case of the latter, we explored the literature for the number of diabetes-related symptoms reported for each species identified in this study. In cases where the total number of contributing informants was unspecified, each ethnobotanical reference was considered as equivalent to one informant. The species were then ranked as explained above (section *2.2.4)*, based solely on the literature survey. In order to compare our results with those stemming from the literature, 3 species had to be discarded, since their identity was not confirmed by the informants. These taxa were *Populus sp.* (probably *P. balsamifera*), *Typha latifolia* and a lichen. The data matrix generated from the literature survey was tested against that of our experimental data using the Mantel test

[112], based on the null hypotheses that the two data matrices being compared are no more similar than two matrices randomly generated. Matrices were constructed based on the presence/absence of cited species for each symptom, with symptoms as rows and species as columns. Analysis was then accomplished by the permutation of symmetric distance matrices including double zeros (Euclidean distances, 10,000 permutations). The applicability of these analyses to plant systematics can be further examined by consulting Cuerrier et al. [113, 114]. Matrix correlation was further explored by using an algorithm developed by J. Podani (Eötvös University, Hungary) which calculates the Manhattan differences between raw matrices via the permutation of rows, columns, or both. The Podani algorithm, which is based on the same hypothesis as the Mantel test, allows for the computation of Manhattan differences without relying on prior modification of raw matrices into distance or similarity matrices. Correlation between the original and literature-based ranking orders was determined by Pearson's Product Moment correlation, which amounts to doing a Spearman correlation test, since the data are already ranked.

In these comparisons, there is an inherent bias which stems from the decision to consider only those species which were identified from the ethnobotanical survey. However, this decision is appropriate for two main reasons. First, the Cree in Canada inhabit an area of approximately 1.4 million hectares [115], covering a wide range of ecozones which harbour unique floras which could not be quantitatively compared in their totality. The second point relates to the final goal of this project, which is to reintegrate a pharmacologically validated and phytochemically standardized extract of a plant mentioned by the Cree community of Mistissini.

Finally, it should be noted that, during the interview process, Cree Elders often referred to related species interchangeably and did not distinguish between certain species within the same genus. Taxa in which this issue was noted were *Sorbus* (*S. americana* Marshall, *S. decora* C.K.Schneid.), *Alnus* (*A. incana* subsp. *rugosa* (Du Roi) R.T.Clausen, *A. viridis* DC. ssp. *crispa*), *Vaccinium* (*V. angustifolium* Aiton, *V. myrtilloides* Michx., *V. boreale* I.V.Hall & Aalders), and *Salix* (over 18 species).These findings correspond with earlier work involving Cree healers and other Nations across Canada [36].

Table 2.1. Alphabetical list of species mentioned during the survey including plant family, Cree name, plant organ(s) used, number of informants who cited the species, and the number of symptoms for which the species was used. B = inner bark; C = cones; F = fruit; L= leaves; R = roots; S= shoots; W=whole plant.

Species	Family	Cree name	Organ	Informants	Symptoms
Abies balsamea (L.) Mill.	Pinaceae	Inaasht	B	14	5
Alnus incana subsp. *rugosa* (Du Roi) R.T.Clausen	Betulaceae	Atushpi	B	6	4
Gaultheria hispidula (L.) Muhl.	Ericaceae	Pieuminaan	F	2	1
Kalmia angustifolia L.	Ericaceae	Uishichipukw	L	2	2
Larix laricina K. Koch	Pinaceae	Watnagan	B	18	6
Rhododendron groenlandicum (Oeder) Kron & Judd	Ericaceae	Kachichepukw	L	12	10
Lichen sp.	Lichenophyta	N/A	W	1	1
Lycopodium clavatum L.	Lycopodiaceae	Pashtnahoagin	S	3	2
Picea glauca (Moench) Voss	Pinaceae	Minhikw	B	2	1
Picea mariana BSP	Pinaceae	Inaahtikw	C	8	5
Pinus banksiana Lamb.	Pinaceae	Ushchishk	C	4	2
Populus sp.	Salicaceae	N/A	B	1	1
Salix sp.	Salicaceae	Pieuatikw	B	3	4
Sarracenia purpurea L.	Sarraceniaceae	Ayigadash	W	7	2
Sorbus decora C.K.Schneid.	Rosaceae	Mushkuminanatikw	B	5	6
Typha latifolia L.	Typhaceae	N/A	R	1	1
Vaccinium angustifolium Aiton	Ericaceae	Minshe	R, F	3	2
Vaccinium vitis-idaea L.	Ericaceae	Wishichimna	F	1	1

2.3 Results and Discussion

2.3.1 Survey results and species ranking

A total of 18 species were cited during the course of our study (Table 2.1). These span a range of 8 plant families, the most cited being Pinaceae and Ericaceae, with 4 and 5 species cited respectively. Of the 18 species cited in the study, 3 species, *Populus sp., Typha sp,* and a lichen could not be confirmed by dried or live specimens with the informants due to unforseen circumstances. However, the informant's taxonomical descriptions lead the authors to conclude that these unconfirmed species are most likely the taxa listed. Although these species were excluded from the species ranking and literature review, this information was retained in our original list due to its potential use in subsequent ethnobotanical research.

Ranking of the species according to our described method yielded a prioritized list of species (Figure 2.1). The top three species, *Rhododendron groenlandicum* (Oeder) Kron & Judd, *Larix laricina* K. Koch, and *Abies balsamea* (L.) Mill., owe their rank mainly to their frequency of citation, which was almost double that of the other species. Due mainly to its high symptom contribution (10 symptoms cited out of 15), *R. groenlandicum* was ranked in the top position, even though it's frequency of citation was less than two thirds that of *L. laricina* K. Koch. Symptom ranking plays an important role in the ranking of *Picea mariana* Britton, Sterns & Poggenb. above the equally cited *Sorbus decora* C.K.Schneid, and the latter is placed before *Alnus incana* subsp. *rugosa* (Du Roi) R.T.Clausen due to a higher symptom contribution, despite having a lower frequency of citation. Results from McCune & Johns (2003) further underline the role of symptoms in species ranking, as it was found that a species' antioxidant properties were positively correlated with the number of symptoms for which it was cited. While the top 6 species demonstrate convincing data, SIVs remain low beyond *Salix sp.*, since both the average frequency of citation and symptom contribution drop to below one quarter that of the previous species.

Comparison of our method with that described by Phillips and Gentry (1993b) illustrates a general consensus between the two ranking systems, with the exception of *Sarracenia purpurea* L. and *Kalmia angustifolia* L.. This is due to two main

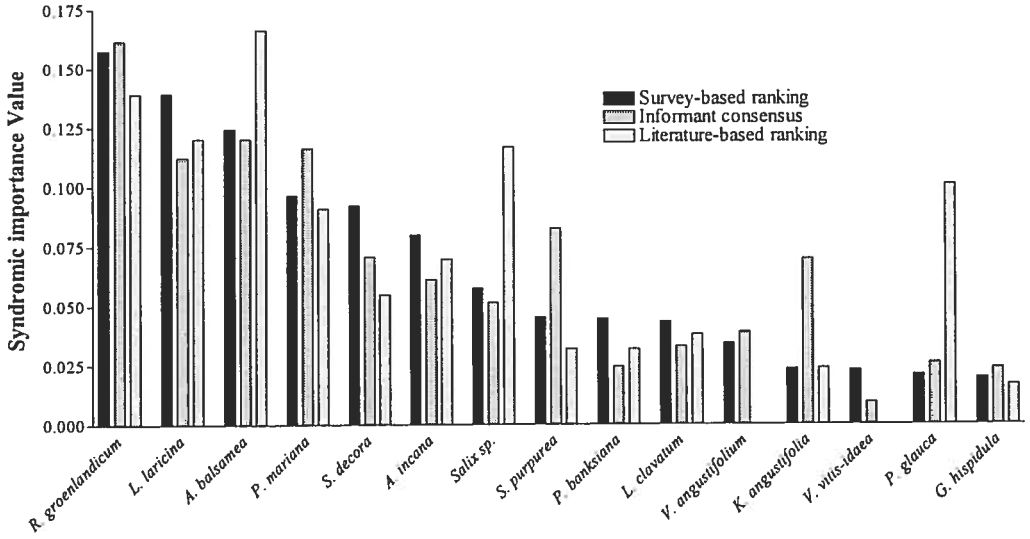

Figure 2.1. Ranking of plant species according to the Syndromic Importance Value (SIV) as compared to Phillips and Gentry's informant consensus and SIVs generated from Cree-specific literature. The species are listed from left to right according to the original survey-based SIV. A general correlation can be noted between the three ranking methods.

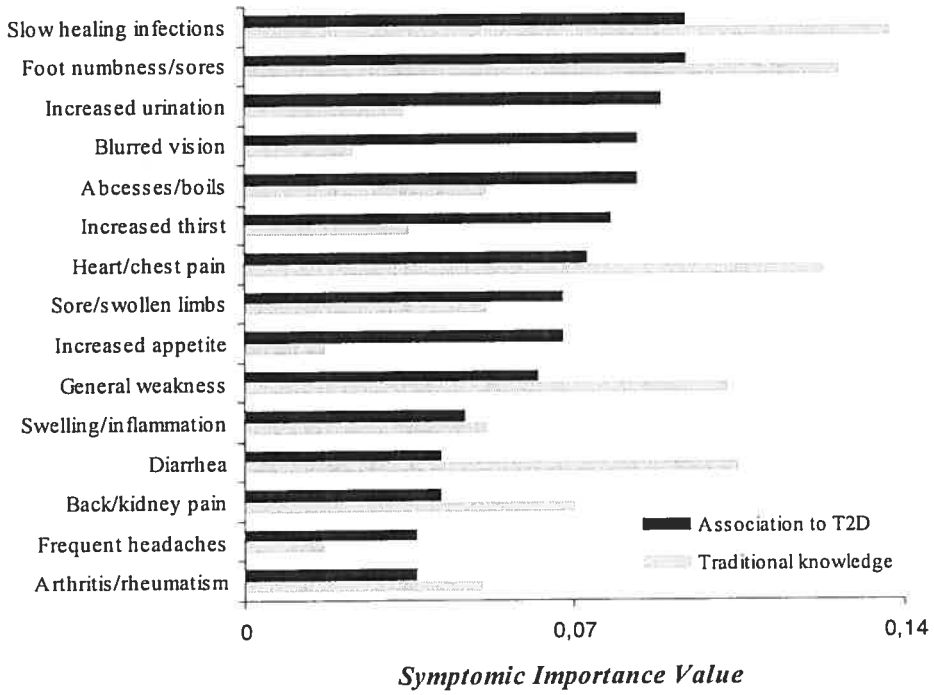

Figure 2.2. Ranking of the 15 symptoms used during the survey according to their association to Type 2 Diabetes and according to the amount of traditional knowledge held for that symptom. The two methods of ranking demonstrate a strong correlation for the first two symptoms.

differences between our method and that proposed by Phillips and Gentry (1993b). First, the latter method does not favour one use over another as does our method. Second, prior to generating an overall use value, Informant Consensus takes into account the use value of single species by an informant within the scope of their individual knowledge. Thus, differences become increasingly apparent when species are highly used by individual informants for symptoms which have a low specificity to T2D, as is the case with the two aforementioned species.

2.3.2 Relevance of traditional knowledge

The variability in the information gathered during the interviews was fairly low, as the number of remedies and diversity of species shared by each Elder did not differ based on age or the presence of T2D.

A comparison of the symptom ranking based on the clinician/scientists and that based on the amount of traditional knowledge held for that symptom, (Figure 2.2) illustrates that the informants were most knowledgeable for the two highest ranked symptoms on our list: slow healing infections and foot sores. This concordance between the first two positions in each list demonstrates the efficacy and relevance of the ethnobotanical data recorded, seeing as these two symptoms represent approximately 35% of treatments cited in our study. However, these results are not surprising since these symptoms require the use of anti-microbial plant extracts, which were primordial prior to the advent of modern antibiotics and whose use is well documented by native peoples of North America [38, 116].

2.3.3 Cluster Analysis

There seems to be no symptom-based correlation between members of the same plant family (Figure 2.3). More detailed information on the active principles of each species might demonstrate that the groupings shown have a common phytochemical signature. It seems more probable, however, that phenolic compounds, and more particularly flavonoids, play an important role in the purported antidiabetic activity of these plants. Flavonoids are known for their antioxidant activity and oxidative stress plays a major role in numerous complications of T2D [100, 117, 118]. Given the ubiquitous nature of these compounds, a lack of phylogenetic clustering is not surprising [119, 120]. Another complicating factor is the relative

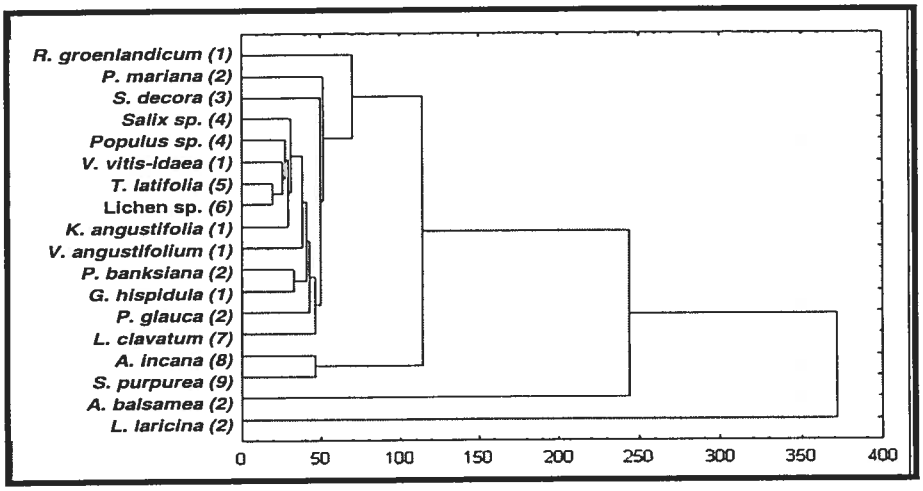

Figure 2.3. Dendogram resulting from a single linkage cluster analysis of plant species based on Euclidean distances. Species closely linked by the dendrogram have a strong index of correlation based on the symptoms for which they were mentioned. Resulting clusters have no phylogenetic relationship. 1=Ericaceae; 2=Pinaceae; 3=Rosaceae; 4=Salicaceae; 5=Typhaceae; 6=Lichenophyta; 7=Lycopodiaceae; 8=Betulaceae; 9=Sarraceniaceae.

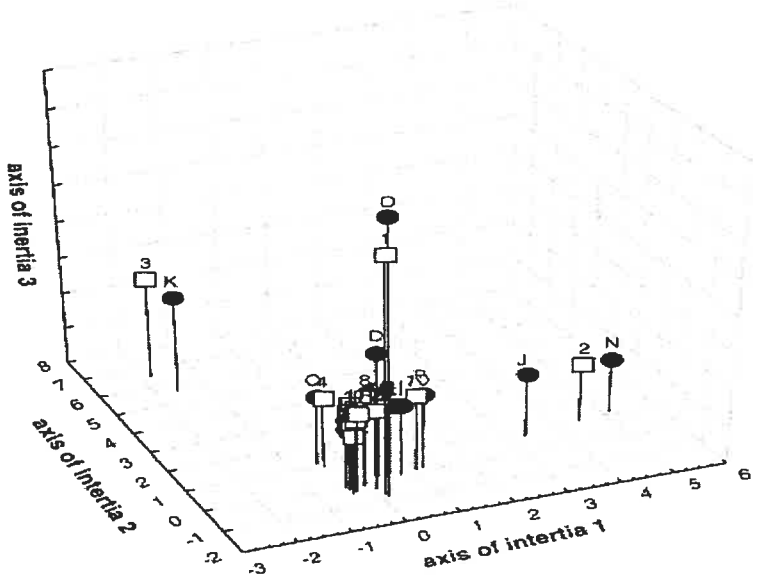

Figure 2.4. Scatterplot resulting from correspondence analysis of plant species (•) and symptoms (□) showing the first three dimensions. Symptoms and species located in proximity to one another are deemed to be highly associated. Interpretation of scatter plot yielded 3 symptom-species associations (Table 2). Species legend: A=*L. groenlandicum;* B=*L. laricina;* C=*A. balsamea;* D=*P. mariana;* E=*S. decora;* F=*A. rugosa;* G=*Salix sp.;* H=*S. purpurea;* I=*P. banksiana;* J=*L. clavatum;* K=*V. angustifolium;* L=*Populus sp.;* M=*K. angustifolia;* N: *V. vitis idaea;* O=*P. glauca;* P=*G. hispidula;* Q=*Typha sp;* R=Lichen. Symptom legend: 1=Increased thirst; 2=Increased urination; 3=Increased appetite; 4=General weakness; 5=Slow healing infections; 6=Blurred vision; 7=Foot numbness/sores; 8=Diarrhea; 9=Arthritis/ rheumatism; 10=Heart/chest pain; 11=Swelling/inflammation; 12=Sore/swollen limbs; 13=Frequent headaches; 14=Back/kidney pain; 15=Abcesses/boils.

broad nature of many of the symptoms used. This allowed for a greater capture of possible plant remedies, yet it is likely that treatment for the same symptom was accomplished by plants having different modes of action and hence different phytochemicals, a possibility supported by the wide array of plant-derived active principles used for diabetes [22, 23, 121]. The limitations of such analysis would be reduced using a larger data set based on more specific symptoms or disorders, and coupled with phytochemical data for the species cited. Nonetheless, this study demonstrates the applicability of cluster analysis to the study of plant taxonomy and phytochemistry in the context of ethnopharmacology. The cluster analysis performed by McCune & Johns (2003) yielded similar results as their groupings were artificial as far as phylogenetic relationships are concerned.

2.3.4 Correspondence analysis

A comparison in two and three dimensions of the perceptual maps resulting from the correspondence analysis (Figure 2.4) yielded the 3 species-symptom associations (Table 2.2). With respect to the other species, these 3 species are deemed to be more closely associated to their corresponding symptom than to the other 14 symptoms in the list. Symptoms with a high knowledge-based ranking and species with elevated syndromic importance value lie within the dense cluster of points in the scatter plot, since these rankings emphasize association to a variety of different species or symptoms, respectively. Thus, for the purpose of this study, this analysis serves as a rapid screen to isolate those species which would have little relevance to the treatment of the complex of symptoms associated with T2D. However, given a larger ethnobotanical data set, which includes plant taxa linked to predefined symptoms or ailment categories, correspondence analysis can be employed to generate perceptual maps which can be rapidly interpreted to determine the degree of association between taxa and ailments. This type of analysis would be of great use for a specialist wishing to choose a few species for detailed study in their specific field.

2.3.5 Literature survey

In their antioxidant screening of plants from the North American boreal forest, McCune and Johns (2002) selected plant species based on the fact that they were cited in the literature for three or more symptoms of diabetes. Of the 21 species

Table 2.2 Species-symptom associations obtained by comparing two and three dimensional perceptual maps (Figure 2.4) resulting from correspondence analysis of our contingency table.

Species	Symptom
V. angustifolium (K)	Increased appetite (3)
V. vitis-idaea (N)	Increased urination (2)
P. glauca (O)	Increased thirst (1)

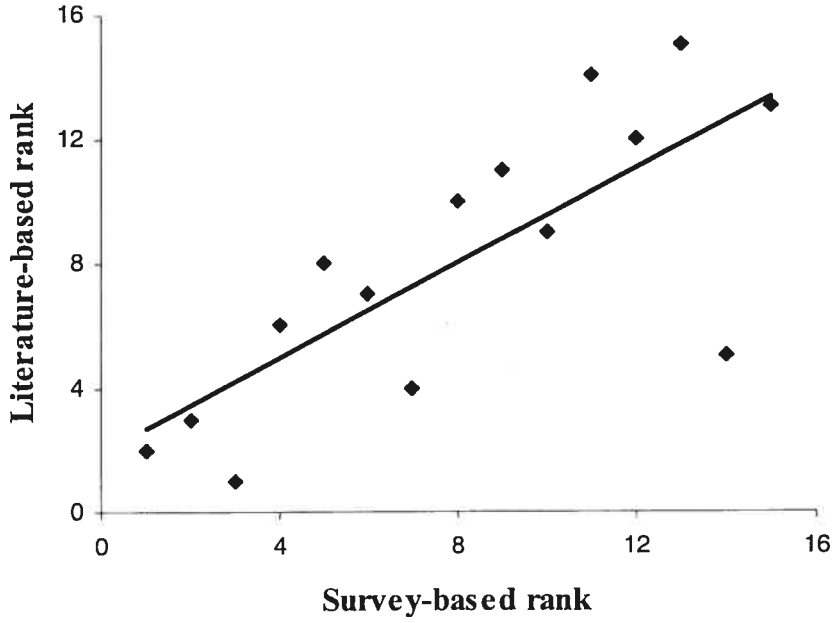

Figure 2.5. Regression of species ranking order (Table 3) based on the ethnobotanical survey (x axis) and review of Cree specific literature (y axis). Linear relationship demonstrates correlation (r^2= 0.5733, p<0.01) according to the Pearson test.

Table 2.3. Ranking order according to the Syndromic Importance Value of each species. The order based on the ethnobotanical survey is compared to the order obtained based on a review of the Cree-specific literature.

Species	Survey-based ranking	Literature-based ranking
R. groenlandicum	1	2
L. laricina	2	3
A. balsamea	3	1
P. mariana	4	6
S. decora	5	8
A. incana ssp rugosa	6	7
Salix sp.	7	4
S. purpurea	8	10
P. banksiana	9	11
L. clavatum	10	9
V. angustifolium	11	14
K. angustifolia	12	12
V. vitis-idaea	13	15
P. glauca	14	5
G. hispidula	15	13

Table 2.4. Summary of permutation tests and resulting r and p values confirming the significance of matrix correlation of raw data with literature-based data. Each test is based on 10,000 permutations.

Test	Source data	*r*	*p*
Mantel	Symmetric distance matrices	0.2595	0.0128
Podani algorithm	Raw matrices: permutation of columns & rows	N/A	<0.0001
Podani algorithm	Raw matrices: permutation of columns only	N/A	0.0022
Podani algorithm	Raw matrices: permutation of rows only	N/A	0.0023

selected in their study, 6 species are common to our list: *R. groenlandicum, A. balsamea, P. mariana, S. americana, K. angustifolia,* and *P. glauca.* It is worth noting that 4 of these common plants figure within the top five species according to our ranking. Moreover, all six common species were found to have a greater free radical scavenging ability than market produce [101].

While performing the literature survey, only 15 of the 18 species were considered since, as cited earlier, 3 species had not been ethnobotanically confirmed with informants. The review and analysis of Cree-specific literature yielded a species ranking similar to that based on our survey (Figure 2.1; Table 2.3). Two species, *Rhododendron groenlandicum* and *Sorbus scopulina*, which are among or closely related to the top five plants in our ranking, were cited specifically for the treatment of diabetes within the literature [39, 41]. Matrices generated from the survey and the literature review demonstrate significant similarity as determined by both Mantel test and the Podani algorithm (Table 2.4). Species ranking orders based on the literature and our survey were also found to be highly correlated (Pearson correlation r^2= 0.5733, p<0.01; Table 2.3; Figure 2.5). It can therefore be affirmed that for the 15 symptoms used in our survey, the same species are used for similar purposes. The correlation between the two data sets supports the validity of our data while demonstrating a certain degree of pharmacopoeial conservation among culturally related yet geographically separated Cree populations. Moreover, these results illustrate that the acceptance and applicability of a new standardized therapy would not be limited to one community but could rather be extended to other Cree communities across the country.

2.4 **Conclusion**

This study represents the first of its kind in exploring the antidiabetic potential of Cree traditional medicine by means of a targeted and quantitative ethnobotanical approach. Although it does not represent an exhaustive survey of the traditional pharmacopoeia of the Cree of Eeyou Istchee, the survey yielded a prioritized list of 18 species, which, along with their corresponding ethnopharmacological usage, also represent a significant contribution to the relatively poor body of knowledge of Eastern Cree ethnobotany.

The informants in our study displayed a thorough knowledge of traditional

remedies for the 15 symptoms in our list, particularly for the 2 symptoms deemed to be most closely associated to T2D. While there seemed to be no taxonomical basis for the ethnomedicinal use of each of the 18 species generated from our survey, cluster analysis proves to be a useful tool in studying the relationship between traditional ethnopharmacological usage within a phylogenetic context. Furthermore, when coupled with phytochemical profiling, ethnopharmacological data could aid researchers in focusing their attention on particular taxa. This study also illustrates how correspondence analysis can be applied to ethnobotanical data in linking plant species to specific symptoms. Finally, while the ultimate evaluation of the antidiabetic efficacy of these plants awaits pharmacological data, the supportive ethnobotanical data from Cree-specific sources coupled with the established antioxidant potential of 30% of the species in our survey, provides convincing support of our approach.

Making the link between a traditionally recognized disease state and a complex of symptoms established as a syndrome through modern medicine is a recurring stumbling block of ethnopharmacological work. Although the recognition of diabetes has been noted in India as early as 700 B.C.[24], there is no evidence to support this within the Cree of Eeyou Ischtee. The approach taken in this study, which combines frequency of citation with both the number and syndromic specificity of symptoms, offers a rapid quantitative method of identifying plant species with the highest potential to treat a syndrome which has been historically absent or traditionally regarded as unrelated symptoms. Such an approach, when coupled with a thorough review of relevant literature and appropriate analytical methods, provide the researcher with a prioritized list of species on which further pharmacological and phytochemical research can be based. The latter is currently being carried out by members of our research team while further ethnobotanical research is being undertaken with the collaboration of the Cree of Eeyou Istchee inhabiting coastal communities. By using a multi-disciplinary approach to medicinal plant selection, the validation of traditional remedies can be streamlined to develop sustainable alternative therapies for T2D in First Nations communities, which in turn will encourage a more integrated approach to healthcare based on both traditional and modern medicine.

Chapter 3: *In vitro pharmacology*

3.1 **Introduction**

3.1.1 Foreword

Based on the ethnobotanical study presented in Chapter 2, 18 plant species were found to be recommended by Cree Elders for one or more symptoms of T2D. To follow up on this study, the most promising plant species are to be screened for their anti-diabetic potential via a battery of *in vitro* screens. The primary screens, so called since they are directly related to glucose homeostasis, are: glucose uptake in muscle cells and adipocytes; insulin secretion in pancreatic β cells; and glucose output/glycogen synthesis in hepatocytes. These primary screens will be complemented by a series of assays related to secondary complications of T2D, which will test the extracts' general anti-oxidant properties, as well as their ability to protect a variety of tissues against toxicity induced by reactive oxygen species, hyperglycaemia, hyperlipidaemia, and other stressors.

The polyvalent nature of this approach allows for well-founded prioritization of plant species for further examination *in vivo*. This being said, the aforementioned primary screens are not "high-throughput", and for this reason only the 8 top-ranked species were chosen for initial *in vitro* screening, aiming for a promising effect in at least 1 species. This is in general agreement with previous ethnopharmacological studies, which show a success rate of at least 1 in 10 plant species identified through ethnobotanical surveys [122]. Screening such a small number of extracts may seem overly optimistic, however medicinal plant extracts have already undergone generations of rigorous "traditional screening", and have repeatedly shown greater activity than plants selected at random [123, 124]. Several pharmaceutical companies use a somewhat analogous "quality in, quality out" approach, which reduces the amount of compounds screened by applying more rigour at the input stage of drug discovery [125].

The screening of 8 extracts on all the assays mentioned above requires the collaboration of numerous laboratories, and is clearly beyond the scope of a M.Sc. thesis. Therefore, only the results of glucose uptake in C2C12 muscle cells and 3T3-L1 adipocytes will be presented in this chapter. *In vitro* glucose uptake has become a standard screen in anti-diabetic drug research [126], and is commonly used to evaluate natural products in these specific cell lines [127-132].

3.1.2 Background

The 8 species to be screened *in vitro* represent 5 plant families as follows: *Alnus incana* subsp. *rugosa* (Du Roi) R.T.Clausen (Betulaceae); *Rhododendron groenlandicum* (Oeder) Kron & Judd (Ericaceae); *Larix laricina* K. Koch, *Pinus banksiana* Lamb., *Picea mariana* BSP (Pinaceae); *Sarracenia purpurea* L. (Sarraceniaceae); *Sorbus decora* C.K.Schneid. (Rosaceae). For purposes of practicality, the two following adjustments in botanical nomenclature will be made: 1) *Rhododendron groenlandicum* (Oeder) Kron & Judd will be referred to by its basionym (the first name published for a biological taxon) *Ledum groenlandicum* Oeder since it is shorter, and still commonly recognized; 2) Since no two species are members of the same genus, only the genus name will be used as a shorthand to refer to each species, although this is not approved botanical practice.

Using NCBI's PubMed database, a review of the pertinent pharmacological literature revealed that none of the specific species to be screened have been previously examined for their anti-diabetic potential. In fact only 4 species have been studied in the context of human health: *A. incana* as a clinical immunotherapy for allergic rhinitis [133]; *A. balsamea* for its anti-tumour activity in several solid tumor cell lines [134]; *L. groenlandicum* for its antigenotoxic effect in *Drosophila melanogaster* ; *L. groenlandicum* and *L. laricina* for their inhibition of xanthine oxidase *in vitro* [135, 136]. The latter has an indirect relation to diabetes, as damage by xanthine oxidase has been associated with gout [136], which in turn has been linked to diabetes [137].

However, some interesting results have been reported for inter-generic (within the same genus) species of *Abies* and *Pinus*. For the former, the leaves of *Abies pindrow* Royle have been shown to increase insulin secretion in INS-1 cells [138]. The purported anti-diabetic effects of species in the genus *Pinus*, range from *in vitro* inhibition of α-amylase and α–glucosidase by *Pinus densiflora* Siebold & Zucc. {Virgili, 1998 #147, to the clinical reduction of hyperglycaemia [139, 140] and diabetic retinopathy [141] by *P. pinaster* Ait. (currently marketed for this purpose under the tradename Pycnogenol®).

Finally, when the literature search was expanded to the level of plant family, 2 families, Ericaceae and Rosaceae, were found to contain experimentally confirmed anti-diabetic species. In the case of Ericaceae both *Vaccinium myrtillus* L. (bilberry) and *Vaccinium macrocarpon* Ait. (cranberry) seem to improve dyslipidamia and/or its

effects *in vivo* [142, 143]. The family Rosaceae was found to contain 7 studied species, among which almonds (*Prunus dulcis* (Mill.) D.A.Webb) and chokeberry (*Aronia melanocarpa* (Michx.) Nutt. & Elliott) are the most well-known and studied. Although almonds have shown no clinical effect on glucose homeostasis [144], incorporating them into the diet has resulted in weight reduction [145] and favourable diet modifications [146] in obese subjects. Chokeberry has a hypoglycaemic effect in normal and alloxan diabetic mice [147], and this effect has been confirmed in patients with advanced T2D [148]. Other members of the Rosaceae which have shown a hypoglycaemic effect in STZ or alloxan diabetic rats are: *Rubus fructicosis* L. [149], *Rosa rugosa* Thunb. [150], *Poterium spinosum* L.[151], *Rubus ulmifolius* Schott [152], and *Potentilla fulgens* Wall. ex Hook.[153].

3.2 **Materials and methods**

3.2.1 Plant material and preparation

Plants were obtained from Mistissini, Quebec, as described in Chapter 2. Plant material was air-dried and then stored in darkness at room temperature. Materials were cleaned and ground to 40 mesh size with a Wiley mill or homogenized by polytron mixer. Extracts were prepared using 80% ethanol in order to extract a wide spectrum of secondary metabolites. Powdered plant materials were extracted with 10mL solvent/gram of material by shaking in darkness for 24 hours at 200 rpm. The extracts were filtered and the solids re-extracted with 5 mL solvent/gram and mixed for an additional 24 hours at 200 rpm. Filtered extracts were combined and solvent was removed by roto-evaporation and lyophilization. Dried extracts were stored in darkness at 4°C and reconstituted at desired concentrations for experimental use. Fenugreek (*Trigonella foenum-graecum* L.) seed was used as a positive control in 3T3s and was purchased from Lone Wolf Herb (Phippen, SK) and extracted as described above.

3.2.2 Materials

C2C12 murine skeletal myoblasts and 3T3-L1 murine pre-adipocytes were obtained from American Type Cell Collection (ATCC; Chicago, IL). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Wisent (Saint-Bruno, QC), while foetal bovine serum (FBS), horse serum, and antibiotics (penicillin – streptomycin) were obtained from Gibco (Grand Island, NY). Insulin, dimethyl sulfoxide (DMSO), 1, 1-Dimethylbiguanide hydrochloride (metformin), as well as all

reagents used in 3T3 culture medium were purchased from Sigma Aldrich (St-Louis, NJ). Liquid scintillation cocktail (Ready-Gel 586601) and 2-deoxy-D-[1-^{3}H] glucose (TRK-383) were obtained from Beckman Coulter (Fullerton, CA) and Amersham Biosciences (UK) respectively.

3.2.3 Cell culture

All cells were cultured at 37°C in a humidified 5% CO_2, 95% air atmosphere. C2C12 myoblasts were cultured in high glucose DMEM supplemented with 10% FBS, 10% HS, and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), until 75-80% confluent. Myoblasts were then differentiated into myotubes over 6 days in DMEM containing 2% HS. At the end of this period, all cells were multinucleated. 3T3-L1 cells were proliferated in DMEM containing 10% FBS and antibiotics. Upon confluence, differentiation was initiated by adding 0.25 nM isobutyl-methyl-xanthine, 1 µM dexamethasone and 0.5 mg/L insulin to this medium for two days. Differentiation was then continued in DMEM containing 10% FBS and 5 mg/L insulin for 10 days, at which point more than 90% of cells contained lipid droplets visible under low power magnification.

3.2.4 Cytotoxicity assay

Following day 3 of differentiation, C2C12 myotubes were maintained in their differentiation medium and exposed overnight to 0, 25, 50, 75, and 100 µg/mL of each of the 8 extracts. Extracts were solubilized in DMSO beforehand at 1000 fold their final concentration such that the percentage of DMSO in the medium was no greater than 0.1. Cell viability was assessed by 3-5 blinded individuals via visual qualification of the cells under 100 fold magnification. Evaluation was assessed using a predetermined scale ranging from 0 to 3 as follows: 0 (>50 % cell death); 1 (presence of cell death); 2 (irregular cell morphology in the absence of cell death); 3 (normal cell morphology). Cell morphology was studied in reference to healthy C2C12 cells using criteria on individual cells such as cell size, shape, granularity, and adhesion, as well as the quality, number, and size of myotubes present. Extracts which resulted in reduced cell quality with respect to control (0.1% DMSO), were re-evaluated at concentrations of 5, 10, and 20 µg/mL. Similarly, extracts which demonstrated no effect on cell quality were re-evaluated at concentrations of 125, 150, 175, and 200 µg/mL.

3.2.5 Glucose transport assay

Glucose transport was assessed in confluent, differentiated C2C12 myotubes and 3T3-L1 adipocytes (grown in 12-well culture plates) following short-term and long-term exposures to the extracts. For the long term exposure, cells were incubated approximately 18 h with extracts in DMEM containing 0.5% serum (HS for C2C12, FBS for 3T3-L1), followed by 3 h in serum-free medium with extracts. For the short term exposure, myotubes and adipocytes were incubated 18 h in DMEM containing 0.5% serum (HS for C2C12, FBS for 3T3-L1), 2 h in serum-free medium without extracts, and 1 h in serum-free medium with extracts. Cells were then rinsed twice with a Krebs phosphate buffer (20 mM HEPES, 5 mM PO_4, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose, 0.5% BSA) at 37°C and incubated at 37°C-5%CO_2 with 0, 1, or 100 nM insulin for 30 min in this buffer with or without extract. Cells were washed twice with a glucose-free Krebs buffer at 37°C and incubation was continued for exactly 10 min at 37°C in this buffer plus 0.5μCi/mL 2-deoxy-D-[1-^{3}H]glucose without extracts. Cells were then placed on ice and immediately washed three times with ice-cold Krebs buffer. Cells were then lysed with 0.1 M NaOH for 30 min and scraped. The lysate was added to 1 mL of liquid scintillation cocktail and the incorporated radioactivity was read in a scintillation counter. Metformin (400 μM) or fenugreek extract (75 μg/mL) were used as positive controls.

3.2.6 Statistics

Results from glucose uptake experiments were analyzed with a randomized complete block design analysis of variance (ANOVA) and Fisher protected least significant difference (PLSD), at the 95% confidence interval.

3.3 **Results**

3.3.1 Cytotoxicity assay

The mean score of cells treated with DMSO was 2.8 ± 0.04 (SEM). Based on this, plant extract concentrations which resulted in a mean score ≥ 2.8 were deemed non-toxic, and the highest non-toxic dose was used for the study (Table 3.1). The highest non-toxic dose of the extracts determined for C2C12s were then evaluated in 3T3s and all extracts were found to be non-toxic in this cell line as well.

3.3.2 Glucose uptake (Table 3.1; Figure 3.1)

Supraphysiological insulin concentration (100 nM) increased glucose uptake by 25-40% in C2C12 myotubes and by 150-300% in 3T3 adipocytes (Figure 3.1). All extracts significantly increased glucose uptake in at least one of the conditions tested, as did positive controls Metformin and Fenugreek (Figure 3.1). The significant effects and their magnitude are summarized in Table 3.1. In general, the greatest effects were seen following chronic exposure in the basal state (0nM insulin), although there were two cases, *Alnus* and *Picea*, where increases in uptake in 3T3s occurred only in the presence of insulin. Inhibition by certain extracts also occurred, and was strongest on insulin-stimulated uptake and was observed only following acute exposure. However, all inhibitory effects were followed by stimulatory effects in the long term, as seen for *Abies* and *Ledum* in C2C12s and *Sarracenia* in 3T3s. In chronic experiments the greatest increases in uptake occurred following incubation with *Sarracenia* and *Sorbus* in C2C12s, and *Abies*, *Ledum* and *Sarracenia* in 3T3s. Only half the extracts had an acute effect, with *Alnus*, *Larix* and *Sarracenia* acting in C2C12s, and *Pinus* in 3T3s.

3.4 **Discussion**

3.4.1 Effects of insulin and positive controls on glucose uptake

Resistance to insulin stimulated glucose uptake is a major pathological feature of T2D and the related metabolic disorder Syndrome X [154, 155]. Increasing insulin sensitivity in peripheral tissue has thus become a key area of research in the development of new anti-diabetic drugs, with the biguanide metformin remaining a gold standard drug [156, 157]. For this reason it is crucial to test the effect of the extracts in both basal and insulin stimulated environments. In C2C12s, the effect of 100 nM insulin on glucose uptake in our study (Figure 3.1) is in agreement with, if not slightly higher than, previous studies which show an increase of approximately 20% at the same concentration [158]. In 3T3s however the effect of maximal insulin observed in our study is slightly lower than published results in which 100 nM insulin increases glucose uptake from 400-650% [129, 159]. Personal experience has shown that response to insulin is quite variable between batches and generations of cells, and thus these results are not surprising. Our 3T3 still demonstrated a dose-dependent response to insulin, and the fact that they might have a reduced sensitivity to insulin might actually improve the relevance of this assay with respect to T2D, and increase

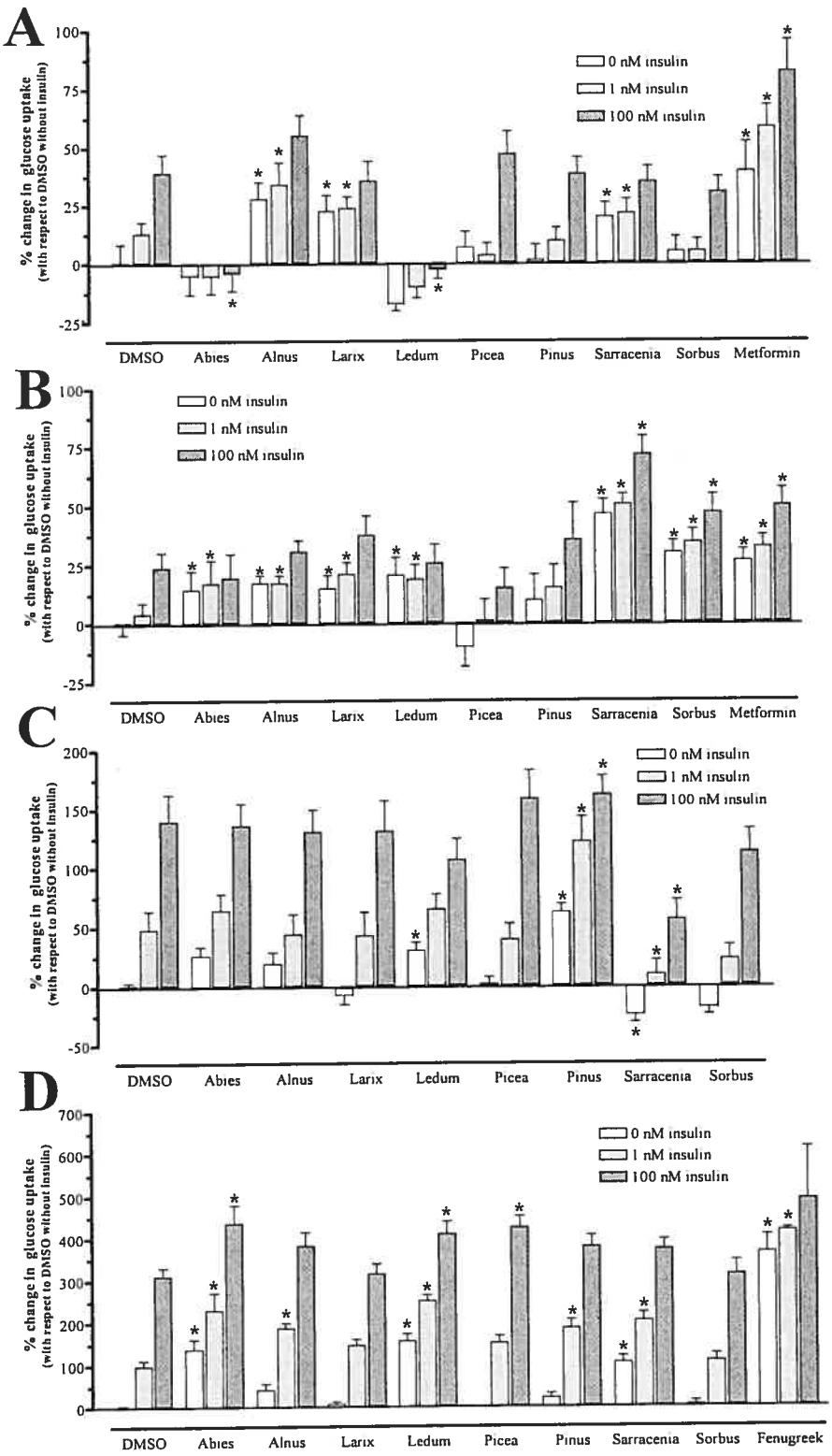

Figure 3.1. Effect of plant extracts on glucose uptake in C2C12 myotubes (A, B) and 3T3-L1 adipocytes (C, D), following a 1 hr (A, C) or 18 hr (B, D) pre-incubation period with extract. Values (± SEM) are expressed relative to vehicle DMSO, at 0 nM insulin. * = Value significantly different to DMSO at the same concentration of insulin ($p<0.05$; n=4-8)

Table 3.1. Percent change (relative to DMSO with corresponding insulin concentration) in glucose uptake following acute (1hr) and chronic (18 hr) incubation with extract. Only significant effects (± SEM) are shown ($p<0.05$, n=4-8). NS = Not Significant; NT = Not Tested.

		C2C12 myotubes						3T3-L1 adypocytes					
		Acute			Chronic			Acute			Chronic		
	[Extract]	Insulin (nM)			Insulin (nM)			Insulin (nM)			Insulin (nM)		
Species	(µg/mL)	0	1	100	0	1	100	0	1	100	0	1	100
Abies	50	NS	NS	-31 ± 6	+14 ± 8	+11 ± 7	NS	NS	NS	NS	+137 ± 23	+67 ± 14	+31 ± 11
Alnus	50	+28 ± 7	+34 ± 9	NS	+17 ± 3	+12 ± 2	NS	NS	NS	NS	NS	+45 ± 6	NS
Larix	25	+22 ± 7	+24 ± 5	NS	+15 ± 6	+15 ± 2	NS	NS	NS	NS	NS	NS	NS
Ledum	75	NS	NS	-30 ± 3	+21 ± 8	+13 ± 5	NS	+30 ± 7	NS	NS	+155 ± 17	+83 ± 14	+24 ± 7
Picea	10	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	+28 ± 7
Pinus	15	NS	NS	NS	NS	NS	NS	+62 ± 7	+52 ± 9	+12 ± 7	NS	+45 ± 10	NS
Sarracenia	100	+20 ± 6	+21 ± 6	NS	+46 ± 6	+43 ± 3	+36 ± 4	-25 ± 6	-26 ± 4	-35 ± 4	+105 ± 15	+55 ± 14	NS
Sorbus	15	NS	NS	NS	+30 ± 5	+28 ± 5	+17 ± 8	NS	NS	NS	NS	NS	NS
Metformin	6.6E-02	43 ± 13	+63 ± 10	+34 ± 10	+26 ± 5	+27 ± 3	+21 ± 2	NT	NT	NT	NT	NT	NT
Fenugreek	75	NT	NT	NT	NT	NT	NT	NT	NT	NT	+212 ± 89	+83 ± 62	NS

our chances in identifying insulin sensitizing extracts. Effects of plants and positive control were greater in 3T3s than in C2C12s, and this is most likely due to the fact that cellular components involved in glucose uptake are constitutively more sensitive in adypocytes than in muscle cells [160], as illustrated by the effect of insulin in these cell types.

Metformin caused a significant increase in both basal and insulin stimulated glucose uptake, by 30-60% and 20-27% following acute and chronic exposure respectively. These results are in the same range as a previous study in C2C12s, which demonstrated a 20% increase following a 24 hr exposure to metformin [158]. In this study, metformin increased only basal uptake, while in our study it increased both basal and insulin-stimulated uptake. It is now known that metformin's main site of action in skeletal muscles is the AMP-activated protein kinase (AMPK), an enzyme which stimulates glucose uptake independently of insulin [161]. AMPK's main stimulatory mechanism is through GLUT4 translocation [58, 162], however recent studies have shown it increases GLUT1 activation as well [58, 163]. Although studies have yet to confirm AMPK activation by metformin in the C2C12 cell line, activation of this pathway would explain the relatively conserved increase in glucose transport by metformin in the presence or absence of insulin, seen in our study. However, since GLUT 4 translocation is the rate limiting step of insulin-stimulated glucose uptake [164], and that both insulin and AMPK pathways recruit GLUT4 transporters, the combined effect of elevated insulin and metformin might saturate this cellular mechanism, leading to a slight decrease in the magnitude of effect as insulin concentrations increase.

3.4.2 Effects of the extracts on glucose uptake

3.4.2.1 Basal vs. insulin stimulated uptake

Although the greatest increases in uptake were seen in the absence of insulin, most extracts which stimulated basal uptake also increased it in the presence of insulin. In these cases, there was a general decrease in the magnitude of the effect as insulin concentration increased, as seen with metformin, indicative of a possible saturation of the cellular uptake mechanism. Evidence of increased uptake only in the presence of insulin was seen only in 3T3s, following chronic exposure to *Alnus* or *Picea*. In these cases it can be said that a true insulin-sensitizing activity is taking place, where the extract directly or indirectly increases the effect of insulin. This is

somewhat of a unique result since the effect of both biguanides and thiazolidinediones, drugs said to be insulin-sensitizing, is an increase in both basal and insulin-stimulated glucose uptake [165, 166]. The effect could be the result of any number of insulin dependent mechanisms such as increased insulin receptor binding or decreased dissociation, or accentuated/prolonged phosphorylation or activation of one or more proteins in the signalling cascade: notably, insulin receptor substrates, phosphoinositide 3-kinase, and protein kinase B.

In all other cases, where both basal and insulin-stimulated uptake was increased, differentiation between an insulin-independent and insulin-dependent mechanism is difficult to conclude. However, as explained for metformin, if the effect of the extract is truly insulin-sensitizing, then the magnitude of the effect should be greater in the presence than in the absence of insulin, which, apart from *Alnus* and *Picea* in 3T3s, was not seen in our study. Insulin-independent mechanisms of glucose uptake are numerous and will be explored in the following two sections as hypotheses for differential effects based on incubation time and cell type. One of these, common to both cell types, could offer a general basis for increased basal uptake: increased glucose uptake in response to hyperosmotic stress [52]. It has been shown that hyperosmolarity mimics insulin responses such as GLUT4 translocation and glucose transport. Regardless of the nature of the compounds, the elevated solute content in extracts would create an environment higher in osmolarity than the vehicle DMSO alone. A pre-incubation in a hyperosmotic environment could increase the number of GLUT 4s in the cell membrane, and directly increase glucose uptake. However, the degree of osmolarity used in these studies was much greater than would result from extract doses of 10-100 μg/mL [167, 168]. Moreover, were hyperosmotic stress to be a generalized mechanism, we would expect extracts used at higher concentrations to result in the largest increases, which is not the case in our study.

3.4.2.2 Short term vs long term uptake

In some cases, extracts that increased glucose uptake following chronic exposure, had no effect or decreased uptake following acute exposure. Examples of the former are *Abies* and *Ledum* in C2C12s and *Sarracenia* in 3T3s, while decreased uptake was observed for *Sorbus* in C2C12s and *Abies* in 3T3s. The lack of sustained inhibition in chronic experiments demonstrates that this acute effect is only transitory, and that increased exposure time actually reverses the effect. The cocktail of

compounds in crude extracts opens the possibility that inhibition and stimulation are caused by different compounds and that antagonistic compounds are short-lived, eventually allowing for the effect of stimulatory compounds with a longer half-life to occur. Another possibility is that these extracts operate by increasing oxidative stress, a process shown to cause a transient decrease in glucose transport followed by an increase, for instance after 6 hr of exposure in L6 muscle cells [169]. It is known that some antibiotics operate by submitting pathogens to oxidative environments [170, 171], and a similar mechanism might underlie the antibiotic uses (see Chapter 2) for some of the plants tested here. Indeed, this might be particularly probable for *Abies*, which is used as an anti-microbial by the Cree (Chapter 2, data not shown), and whose reported anti-tumour activity is thought to be due to the production of reactive oxygen species [134]

Plants showing increases in uptake only in chronic experiments, without any significant effects over the short term, might operate by increasing transcription of glucose transport related genes, or by directly stimulating proteins in the insulin signalling cascade. An example of the former is the PPARγ agonist rosiglitazone that increase glucose uptake after a minimum of 6hr pre-incubation [166]. As concerns the second possibility, it has been shown that constitutively activated Akt increases glucose uptake in a hormone dependent manner in L6 muscle cells and 3T3 adipocytes, but this requires a substantially longer time than direct agonism of the insulin receptor [172].

3.4.2.3 Differential effects in 3T3 and C2C12 cells

Yet another interesting pattern witnessed in our study was the notably cell-specific effects of extracts like *Sorbus* and *Larix* (effects on C2C12s only) and/or *Picea* and *Pinus* (effects on 3T3s only). Despite having a common role as glucose "sinks" in the body, skeletal muscle and adipose tissue have unique form and function. By exploring the subtle differences in these two cell types, extract driven discrepancies in glucose transport become more easily justifiable.

Although insulin-dependent glucose uptake mechanisms seem to be conserved between skeletal muscle cells and adipocytes [58], new evidence suggests that the recently identified PI3-K independent insulin stimulated glucose uptake mechanism, dependent on the G protein TC10, might be unique to adipocytes (Figure 1.2)[52]. This putative cell-specific pathway, combined with subtle differences in cellular

components and morphology could have a strong influence on glucose transport in response to exogenous compounds, and help to explain the variation in response from one cell type to another.

Two well-studied sites of action for anti-diabetic compounds, the nuclear receptor PPAR and the cytosolic protein AMPK, have numerous isoforms whose expression differs according to cell type. While the γ-isoform of PPAR, targeted by the thiazolidinediones, is the dominant isoform of the receptor in adipocytes, it is minimally expressed in skeletal muscle, with β/δ isoform predominating [96]. As for AMPK, it has been shown that, while adipocytes express α_1- and α_2-isoforms of the catalytic subunit equally [173, 174], the $\acute{a}_2$-isoform predominates in skeletal muscle [175]. Other pertinent differences between these cell types are there ability to produce and/or respond to circulating growth factors or hormones such as the insulin-like-growth- factor-1 (IGF-1) and adiponectin. Although IGF-1 is expressed and secreted in both cell types, it is unlikely that it has any direct effects on adipocytes since they lack functional type 1 IGF receptors [176]. Conversely, adiponectin increases glucose uptake in both cell types, but is produced exclusively in adipocytes [159, 177], opening the possibility of an autocrine effect *in vitro*. Moreover, adiponectin acts through both insulin-dependent and independent mechanisms in skeletal muscle, while mainly through the latter in adipocytes [159]. Hence, while an extract-derived IGF-I agonist would have an effect on C2C12s instead of 3T3s, the opposite would be true for a compound which increased production and secretion of adiponectin.

The intrinsic form and function of the two cell types can also directly or indirectly affect glucose uptake. It is well-known that muscle contraction results in an acute stimulation of glucose uptake [178-182]. Since cultured C2C12s are known to contract *in vitro* [183, 184], and that some crude plant extracts have been shown to stimulate muscle contraction [185, 186], it is possible that some of our extracts act in a similar manner. Moreover, the numerous sodium, potassium, and calcium channels, which occur in high density in skeletal muscle cells, are all known to be targets of natural products [187]. Another well known target of natural products is actin [188-190], which is known to play a vital role in GLUT4 translocation [191, 192]. Compared to the long cytoskeletal stress fibers in muscle cells, adipocyte cytoskeletons are characterized by cortical actin, which points to cell-specific differences in the mechanism of GLUT4 translocation [172].

3.5 Conclusion

Without a thorough examination of the mechanism(s) of action of each of the extracts, no definitive conclusions about their cellular targets can be made. Although such detailed mechanistic studies will be a pivotal part of this project in the future, the purpose of the present study was to screen crude extracts for there ability to stimulate glucose transport in muscle cells and adipocytes. As a primary screen, this study was also aimed at identifying those extracts which showed the most therapeutic promise for further study *in vivo*.

All 8 extracts tested showed a glucose uptake enhancing effect in at least one condition tested. However, the magnitude and duration of the effect, as well as its target tissue were markedly different from one extract to another. It is upon these differences that a prioritization of species was made. This being said, the author acknowledges that prioritization in an ideal situation would be made based on results from glucose uptake as well as insulin secretion and hepatic glycogen production/glucose output. Indeed, extracts from the 8 plants tested here were also screened for their ability to stimulate insulin secretion in a line of β-cells in the lab of one of our collaborators (Dr. Mark Prentki, Department of Nutrition, Université de Montréal). Preliminary results indicate that none of the plants stimulate insulin secretion (data not shown). These results, combined with logistical restraints limiting our screening capacity, led to the establishment of species priority based on one main assay. Since glucose uptake is a pivotal step in insulin action, and that a resistance to insulin stimulated glucose uptake is a hallmark of T2D [58, 155, 172], this assay was used as a foundation for our initial decisions.

Therapy of T2D is achieved through chronic medication [86], and thus a long-lasting pharmacological effect is more desirable than a transient one. In this respect, the results following chronic exposure were more heavily weighted than those following acute exposure. If we choose the top 3 ranking plants in each cell type based on the magnitude of their effect following chronic exposure only, we obtain the following: *Sarracenia*, *Sorbus*, and *Ledum* in C2C12s, and *Ledum*, *Abies*, and *Sarracenia* in 3T3s. The next logical step was to favour results from one cell type over another. The two most commonly used oral anti-diabetic drugs, metformin (biguanide) and troziglitazone (thiazolidinedione), increase glucose uptake by targetting principally the skeletal muscle and the adipose tissue respectively [193]. However, for multiple reasons, metformin remains the standard choice for first line

treatment. One of these reasons is that thiazolidinediones, due to increased insulin sensitivity in adipocytes, cause patients to gain weight [98]. Thus it seems more preferable to have a drug which targets either both muscle and adipose tissue or muscle cells only. With this in mind, the magnitude of the effect in C2C12s following chronic exposure became the major factor in the prioritization of species.

Taking all the aforementioned factors into consideration, we chose three species with promising therapeutic potential: *Sarraccenia*, *Sorbus* and *Ledum*. However, it is only through the use of normal and diabetic *in vivo* models, that the anti-diabetic effect of these three species can be confirmed.

Chapter 4: *In vivo pharmacology*

Part 1: Streptozotocin-induced diabetic mice

4.1.1 **Introduction**

4.1.1.1 Foreword

From the study presented in Chapter 2, it was shown that among the 8 plant extracts screened, 3 showed particular promise: *Sarracenia, Sorbus,* and *Ledum.* Progression from *in vitro* screening to *in vivo* validation of physiological effect is common in pharmaceutical [125] and natural product [194, 195] research alike, and this is the approach we adopted here. Since this was a pilot *in vivo* study, we restricted testing to only the 2 most promising species, *Sarracenia* and *Sorbus*, which had their greatest effect on basal glucose transport. Our main goal here is to confirm a hypoglycaemic effect *in vivo* via a simple and rapid diabetic model, while simultaneously observing the effect of the extracts in normal mice. The hypothesis is that the extracts will, in a chronic and/or acute fashion, reduce fasting plasma glucose, improve glucose tolerance (reduce peak glycaemia and the area under the glucose response curve), and increase insulin sensitivity (increase the post-insulin drop in insulin and increase the percent area above the insulin response curve).

4.1.1.2 Animal model

The choice of species for *in vivo* experimentation was made primarily on the basis of quantity of extract required and cost. The distance of the plant sourcing site coupled with seasonal restrictions (lack of plant material in the winter) limited the amount of raw plant material at our disposal. Hence, the use of a small species such as the mouse was more appropriate than other widely used species such as the rat. Moreover, the mouse as an experimental model is indicated over larger ones particularly in cases where detailed characterization of specific compounds is not required, which is precisely the situation in the case of this study [196].

The streptozotocin (STZ) – induced diabetic model is a relatively rapid and inexpensive method to screen compounds for their hypoglycaemic activity [121, 197]. It is the most widely used experimental model of diabetes and murine STZ models have been extensively employed to confirm anti-diabetic activity of a variety of plant species extracts [198-209]. STZ is a broad spectrum antibiotic isolated from *Streptomyces achromogenes.* Its use as a diabetogenic agent is related to its potent genotoxic properties, as it is known to cause a selective necrosis of β- cells through DNA alkylation as well as through hydroxyl radical damage [210, 211].

By varying the dose and posology of STZ, as well as the age and strain of

animal, experimenters can achieve complete or partial β-cell destruction, resulting in a diabetic state resembling Type 1 or Type 2 diabetes respectively [197, 205]. In our case we opted for the former, since it is quicker, less variable and more appropriately designed to test extracts which primarily stimulate basal glucose uptake. Hence, we expect those extracts that stimulated the uptake of glucose by muscle and/or fat *in vitro* to have a similar effect *in vivo*, resulting in an alleviation of the marked hyperglycamia which characterizes this model.

4.1.2 **Materials and Methods**

4.1.2.1 Materials

Male CD-1 mice were purchased from Charles River (Montreal, Canada) at 9 weeks of age. Standard rodent chow, was obtained from Harlan Teklad (Madison, WI). Streptozotocin (Zanosar®) was obtained from Pharmacia Canada (Mississauga, Ontario). Glucose (dextrose) and 1, 1-Dimethylbiguanide hydrochloride (Metformin) were purchased from Fisher Scientific (Fairlawn, NJ) and Sigma Aldrich (St-Louis, MO) respectively. Human insulin (Humulin R®) was obtained from Eli Lilly Canada Inc. (Toronto, Ontario). Heparinized micro-hematocrit capillary tubes were purchased from Fisher Scientific (Pittsburgh, PA), and glucose was measured using Autokit Glucose CII from Wako Chemicals USA Inc. (Richmond, VA). Syringes and needles (25G ⅝ and/or 26G ½) were purchased from Becton Dickinson and Company (Franklin Lanes, NJ). Sodium chloride solution (0.9%) was obtained from Baxter Corporation (Toronto, Ontario).

4.1.2.2 Extracts

Ethanolic extracts were prepared as indicated in section 2.

4.1.2.3 Model development and experimental groups

Animals were kept in a temperature controlled room with a 12-hour light/dark cycle. The mice were housed 4 animals per cage for the first week upon arrival, after which they were placed in separate cages (1 animal/cage) 2 weeks prior to the experiment. Ten days prior to the start of daily dosing, 32 mice were fasted overnight and injected (intra-peritoneal) with 150 mg/kg streptozotocin (STZ), while the remaining 19 mice were left untouched. One week following the STZ injection, the 32 mice were separated into 4 groups based on the blood glucose in the daytime un-fasted state. The 19 other mice were randomly assigned to one of three groups. The 7

experimental groups (n=7-9) are as follows: 1) "N-Control": normal mice administered 10 mL/kg/day water; 2) "N-*Sarracenia*": normal mice administered 100 mg/kg/day ethanolic extract of *Sarracenia purpurea* (whole plant); 3) "N-*Sorbus*": normal mice administered 100 mg/kg/day ethanolic extract of *Sorbus decora* (inner bark); 4)"STZ-Control": STZ injected mice administered 10 mL/kg/day water; 5) "STZ-Metformin": STZ injected mice administered 200 mg/kg/day metformin; 6) "STZ-*Sarracenia*": STZ injected mice administered 100 mg/kg/day ethanolic extract of *Sarracenia purpurea* (whole plant); 7) "STZ-*Sorbus*": STZ injected mice administered 100 mg/kg/day ethanolic extract of *Sorbus decora* (inner bark). During the last week (week 4), plant extract doses were doubled such that all groups received the listed dose bi-daily, with the exception of the metformin group which received half of the listed dose bi-daily. All extracts and metformin were administered in distilled water via intragastric gavage at a volume of 10 mL/kg. The experiment was performed over a period of 4 weeks, and animals were familiarized to testing by daily handling and gavage with water during the week preceding the experiment.

4.1.2.4 Plasma glucose (PG)

Blood sampling was performed by saphenous vein puncture as described previously [212]. To examine the chronic effect of the treatments, PG was monitored once a week from week 0-4 following a 3 hour fast. The acute effect of the treatments on the PG of the STZ groups was examined following 3 weeks of treatment. Mice were administered their daily dose of treatment and glycaemia was taken at 0, 60, and 120 minutes following gavage.

4.1.2.5 Oral Glucose Tolerance Test (OGTT)

Following 4 weeks of treatment, glucose tolerance was assessed in the normal mice ("N-") via an OGTT following a 3 hour fast. Blood samples during the OGTT were taken from the tail vein. One hour (t=-60 minutes) prior to oral administration of glucose (2 g/kg body weight) (t=0 minutes), blood glucose was measured and mice were administered their daily dose of treatment. Blood glucose was then measured at 0, 30, 60 and 120 minutes after glucose load. Area under the curve (AUC) was measured using fasting glycaemia as a baseline, as described previously [213]. AUC was calculated from both -60 to 120 minutes (AUC(-60)) and 0-120 minutes (AUC(0)), with glycaemia at initial time point serving as baseline. The reasons for the two modes of measurements are explained in more detail in section 4.1.3.

4.1.2.6 Intra-peritoneal Insulin Tolerance Test (ITT)

An insulin tolerance test (ITT) was performed on the STZ groups after 4 weeks of treatment. Mice were well fed *ad libitum* prior to the ITT. One hour (t=-60 minutes) prior to intra-peritoneal administration of insulin (7.5 U/kg body weight) (t=0 minutes), blood glucose was measured and mice were administered their daily dose of treatment. Blood glucose was then measured at 0, 60 and 120 minutes after insulin load. The magnitude of response to insulin was measured as the % area above the curve (%AAC) from -60 – 120 min (%AAC(-60)) and from 0-120 min (%AAC(0)), as described previously [214]. The %AAC is essentially the inverse of the AUC, expressed as a percentage of the total rectangular area above and below the curve. The area is calculated for each mouse and is delineated in the top left corner by the x and y coordinates of the PG at -60 min (in the case of %AAC(-60), or 0 min in the case of %AAC(0)), and of the PG at 120 min in the bottom right corner.

4.1.2.7 Statistics

Analysis of the effect of the treatments on the progression of body weight and PG over time as well as of OGTT and ITT response curves, was performed via a 2-way repeated measure analysis of variance (ANOVA) at the 95% confidence interval. If the treatment was found to have a significant effect over time ($p<0.05$), a one-way ANOVA was performed along with a Fisher PLSD (protected least significant difference) at each time point (95% confidence interval) to determine where the main differences occured. Analysis of the AUC and %AAC was also performed with a one-way ANOVA along with a Fisher PLSD at the 95% confidence interval. The normality of the data's distribution and the equality of the variances between groups were assessed using the Kolmogorov-Smirnov normality test and Bartlett's test for homogeneity, respectively. Abnormally distributed data was analyzed with a Kruskal-Wallis test. In the case of normally distributed data with heterogeneous variances, Welch's test was used along with Games-Howell or Scheffé post hoc tests. All statistics were performed using Statview ® 5.0.1.

4.1.3 **Results**

4.1.3.1 Body weight and plasma glucose (Figure 4.1.1)

At the start of the experiment, 10 days following the injection of STZ, STZ-treated mice presented a greater than 2-fold increase in plasma glucose concentration and an inferior weight (12%) compared to controls (Figure. 4.1.1 - A(i) and (ii)).

Despite a transient decrease in the STZ-group glycaemia during the first week, the magnitude of the difference in both parameters continued to increase over the course of the experiment, reaching 3.5-fold for PG and 14% for BW. Chronic treatment with metformin had no overall effect on fasting glycaemia or BW, yet seemed to cause a slight loss of weight from wk 3 to wk 4 (Figure. 4.1 - A(i) and (ii)).

For the normal mice, body weight was relatively homogeneous, with the *Sorbus* group remaining slightly below the control by week 4 (Figure 4.1.1 - B(ii)). Both extract treated groups maintained a mean glycaemia greater than control during the first 2 weeks, yet. this difference was lost after 3 weeks of treatment, as treatment groups decreased to control levels, making an overall treatment effect which just barely escaped significance (p=0.0537). Although the treatments did not reduce plasma glucose compared to the control group, all groups underwent a decrease in mean plasma glycaemia, ranging from 40% in the control group to 16% in the *Sorbus* group.

Within the STZ treated mice, extracts had no effect on PG or BW as compared to control. As with the normal mice, the BW of the *Sorbus* group remained slightly below control contributing to a treatment effect on BW which almost achieved significance (p=0.0575). However, this was mostly due to differences between the two treatment groups and not between the treatment groups and the control (Fisher PLSD: p<0.05 between STZ-*Sorbus* and STZ-*Sarracenia*; p>0.1 between STZ-CTL and either group). Although *Sorbus* seemed to have a modest hypoglycaemic effect at weeks 1 and 3, these effects were only transient, and too variable (SD>150 mg/dL at wk 3 for STZ-*Sorbus* group) to result in an overall treatment effect (p=0.1972). The *Sarracenia* group, however, despite starting with and maintaining a higher BW during the first few weeks, eventually decreased to control levels by week 4.

4.1.3.2 Oral glucose tolerance test (OGTT) (Figure 4.1.2)

All groups demonstrated a slight increase in glycaemia following the initial blood sampling/gavage. Although there were no significant differences in AUC, mean glycaemia did vary between groups throughout the test, resulting in a significant treatment effect (p=0.0351). One way ANOVA performed at each time point indicates where the main differences occurred. In general, extract treated groups tended to increase mean glycaemia, despite having distinct glucose response curves (GRCs). The N-*Sorbus* GRC has a steep rise in glycemia following glucose load, a

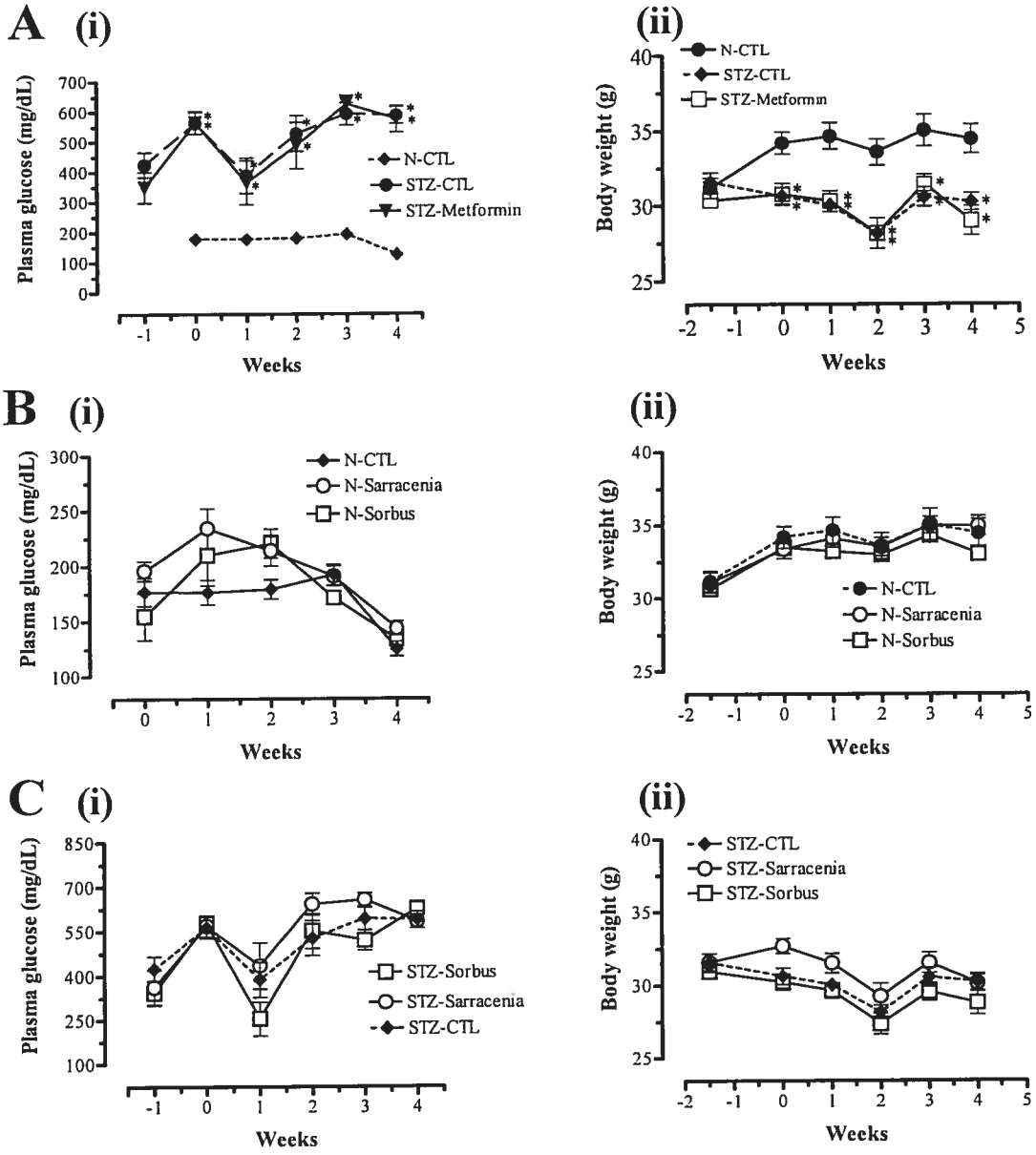

Figure. 4.1.1. Plasma glucose (i) and body weight (ii) over the course of the study: (A) Effects of STZ and STZ+Metformin; (B) Effects of extracts on normal mice; (C) Effects of extracts on STZ-diabetic mice. Values displayed ± SEM. * = Value significantly different from N-CTL (A,B) or STZ-CTL (C) ($p<0.05$) . [Note that 1 mM = 18 mg/dL glucose; 100 mg/dL ≈ 5.6 mM; 250 mg/dL ≈ 14 mM; 500 mg/dL ≈ 28 mM glucose]

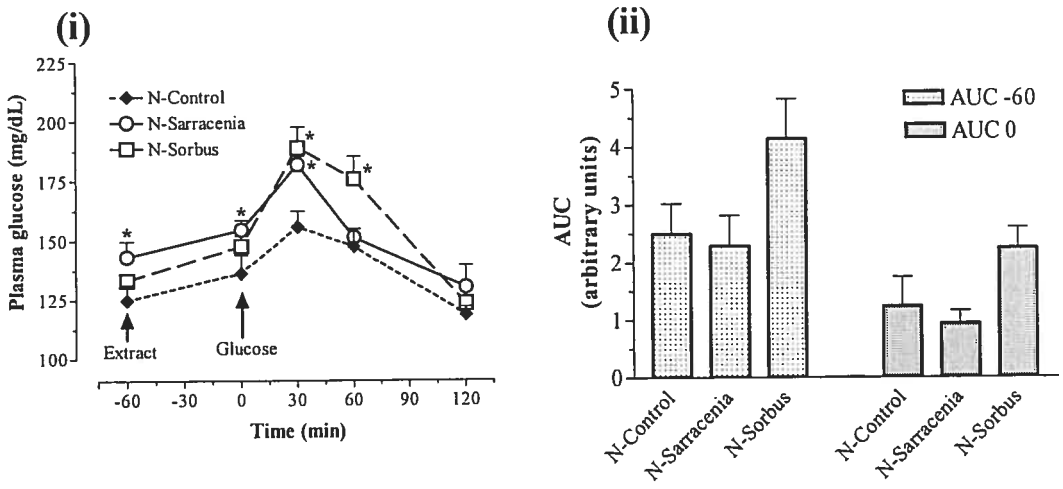

Figure 4.1.2. Oral glucose tolerance test (OGTT) (i), and corresponding area under the curve (AUC) (ii) from -60 to 120 min (AUC-60) and from 0 to 120 min (AUC 0), performed on normal mice. Extract was delivered one hour prior to glucose load. Values displayed +SEM. * = Value significantly different from N-Control, $p < 0.05$.

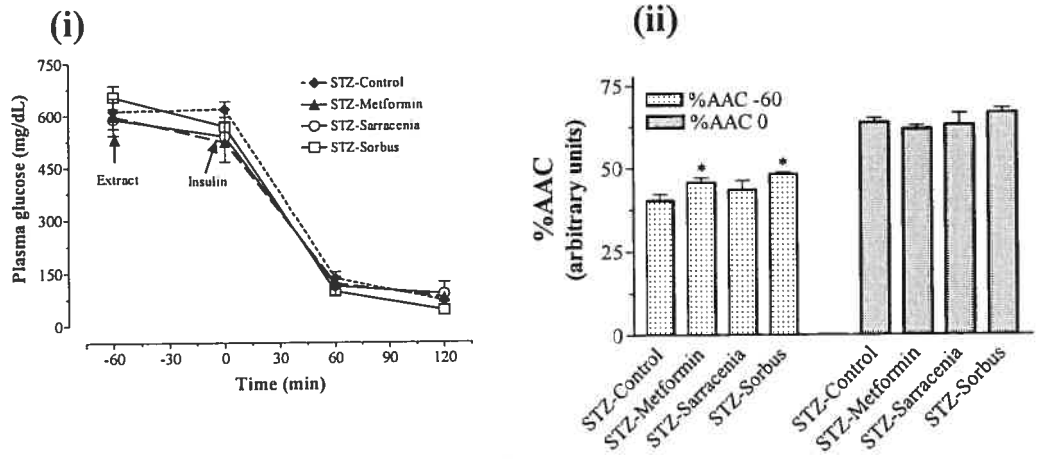

Figure 4.1.3. Insulin tolerance test (ITT) (i) and corresponding percent area above the curve (%AAC) (ii) from -60 to 120 min (%AAC-60) and from 0 to 120 min (%AAC 0)., performed on STZ-treated mice. Extract/drug was delivered one hour prior to insulin bolus. Values displayed ± SEM (i), +SEM (ii). * = Value significantly different from STZ-Control, $p < 0.05$.

slow return to baseline (30 -120 min), resulting in increases of 65% and 80% in AUC (-60) and AUC (0), respectively. In contrast, the N-*Sarracenia* GRC, has a pre-peak slope resembling that of the control group, followed by a rapid re-establishment of glycemia at 60 minutes, resulting in a slightly decreased AUC(-60)(-8%) and AUC(0)(-24%). However, in the case of both experimental groups, these changes in AUC were not statisitically significant. It should be noted that due to the nature of our experiment, AUC (-60) is indicative of not only the treatment's effect on glucose tolerance, but also its short term effect on PG during the hour preceding glucose administration. The reason for presenting both here is that PG at t = 0 min is not truly a fasted glycaemia since extracts were administered one hour prior. The results of AUC (-60) should therefore be interpreted with caution, paying particular attention to concordance with the AUC (0) and the GRC. Nonetheless, a decrease in AUC(-60) is of great interest since it is indicative of either a hypoglycaemic effect or an increase in glucose tolerance, both of which are positive effects. Moreover, a non-significant decrease in glycaemia from -60 to 0 min, might lead to a significant decrease in AUC (-60) or increase %AAC (-60) in the case of the ITT.

4.1.3.3 Insulin tolerance test (ITT) (Figure 4.1.3)

During the hour preceding insulin delivery at 0 min, the glycaemia of all treatment groups (except STZ-CTL) decreased. However, only that of the *Sarracenia* group was significantly lower than control. Although the mean glycaemia of the metformin group was the lowest at t = 0 min, the difference did not reach significance due to high within-group variability. Treatment with metformin did, however, significantly increase the %AAC (-60) (12% increase), as did treatment with *Sorbus* (20% increase). Similar to the OGTT AUC, results of %AAC (-60) should be interpreted alongside %AAC (0) and the ITT curve.

4.1.4 **Discussion**

Following STZ injection, the high variability in glycaemic response, the decrease in mean BW, and the transient decrease in PG seen in our study are all well documented effects of STZ in animal models [126, 211]. Previous studies using a single high-dose (100-200mg/kg IP STZ) murine model achieved similar control results, citing minimum blood glycaemia (BG) of 300-350 mg /dL and mean BG exceeding 400 mg/dL at 7-10 days following STZ injection [198-202, 203]. Note that

for the purpose of discussion PG will be compared with BG and *vice-versa*, with the understanding that PG is generally 10-15% higher than BG [215].

The elevated mean glycaemia of the STZ treated mice is indicative of severe destruction of pancreatic β cells, which was expected given the initial dose of STZ. The excessively high hyperglycaemia should allow subtle effects on glucose metabolism to materialize in detectable changes in PG since there a spread of at least 200 mg/dL between normal PG values and that of the STZ mice. However, this feature of the model also makes it almost impossible to restore normoglycaemia [197]. Although one month of daily treatment with metformin has been previously shown to decrease hyperglycaemia in STZ treated mice, the model used was much less severe (60 mg/kg/day for 3 days), and resulting mean glycemia of the STZ-control group was approximately 40% less than in our study [209]. In another study using high dose STZ (160 mg/kg) in rats, metformin had no effect on glycaemia or glucose tolerance [216]. However, the increase in %AAC (-60) in the metformin group ITT is indicative of an acute hypoglycaemic effect, possibly related to increased glucose uptake. Lack of a chronic effect is most likely due to metformin's reliance on endogenous insulin for optimum effect. Indeed, it is also well known that optimum clinical efficacy of metformin requires some β-cell function [193]. With this in mind, one might have expected metformin to have a significant effect during the ITT. One possible explanation is that the bolus of insulin used simply saturated cellular uptake of glucose allowing no room for improvement by metformin. Although an insulin bolus of 1 U/kg is commonly employed [217], this quantity might saturate glucose transport in our model.

Neither extract examined had a promising effect on long term PG, glucose tolerance, or insulin sensitivity. The hypoglycaemic trend of the *Sorbus* groups at week 1 and later at week 3 was only transient, and was completely lost when the dose was doubled during the last week. In fact, *Sorbus* slightly exacerbated the effects the effects of STZ on BW (Figure 4.1.1-C(ii))) and decreased glucose tolerance in normal mice. Effects on the latter are reflected by the GRC and AUC of the *Sorbus* group during the OGTT (Figure 4.1.2-(i)). One hour following the glucose load, the PG of the N-*Sorbus* group remained almost 20% higher than the other two groups, resulting in almost significant ($p=0.069$) increase in AUC(-60), possibly indicating a decreased sensitivity to insulin. However, there was no indication of the latter during the ITT, with results tending towards the contrary. The significant increase of %AAC (-60) in

the STZ-*Sorbus* group is mostly due to a modest short term hypoglycaemic effect from -60-0 min since no significant difference is present for %AAC(0). As for BW in STZ treated mice, it is difficult to determine if the effect of *Sorbus* is related to anomalies in glucose metabolism, since the nature of this model is not conducive to GTTs, due to the lack of insulin production in these animals. However, the positive correlation between weight loss and decreased glycemic control has been noted in type 1 diabetic humans [218] and it is not unlikely that a similar correlation exist in the N-*Sorbus* group.

Unlike the *Sorbus* groups, *Sarracenia* treated mice presented no indication of reduced glucose tolerance. Rather, the contrary could be argued. Even though N-*Sarracenia* mice had significantly higher mean PG at -60, 0 and 30 min of the OGTT, a rapid post-peak glucose disposal re-established PG to control levels at 60 minutes, and resulted in a mean AUC slightly lower than that of the control. This effect on the post-peak PG might have been more clearly shown by the use of a different method based on the calculation of the glucose disappearance constant (Kg). The Kg is determined bythe rate at which glucose disappears from the blood stream. However, the use of this methodology was not possible under our experimental conditions. Indeed, accurate calculation of the Kg is commonly practiced during IV-GTTs, since its accuracy is dependent on numerous sampling of PG/BG immediately following delivery of glucose bolus [219-221].

The seemingly improved glucose disposal in the N-*Sarracenia* group is not, however, supported by the significantly higher fasting PG of this group after 4 weeks of treatment. However, this is most likely due to a higher PG at week 0, since the N-*Sarracenia* group, like the N-control group, had significant within-group decrease in PG by week 4 (Figure 4.1.1: D(i)). This suggests that the effect of *Sarracenia* on PG of normal mice might only be detected in the post-prandial state, and/or that the effect of the extract is of only short duration and can be followed by increase in PG between extract doses. In the STZ treated mice, *Sarracenia* significantly reduced PG prior to the ITT. This reinforces the hypothesis of a post prandial specific effect since animals were fed *ad libitum* prior to delivery of the extract at -60 of the ITT.

4.1.5 **Conclusion**

Proper interpretation of our results can only be accomplished within the particular context of natural product research for diabetes therapy. Lack of

pharmacokinetic data on these extracts posed a particular methodological challenge, which in turn can complicate interpretation of results. Frequency, quantity, and method of extract administration remain difficult to justify without access to substantial prior experimental data. Indeed, to perform a GTT, natural product researchers administer their extracts orally, intravenously or intra-peritoneally anywhere from 0, 30, 60, or 240 min prior to the glucose load [208, 222, 223]. Still others incorporate the extracts into the food or water (or both), and ignore pre-administration altogether [201]. Moreover, some extracts have proven ineffective when pre-administered, yet effective when co-administered with glucose for an OGTT [223]. Of primary concern are the rate of absorption from the gut and the half-life of the compounds once they are in circulation. Since the plants studied here are traditionally taken as herbal teas, it can be assumed that compounds of interest are absorbed in the intestinal tract, thus making the choice to deliver them orally justifiable by logic as well as by information in pertinent literature [126]. Yet even within the oral administration route, one must choose between administration by diet or gavage, which can significantly affect the outcome since the latter reduces the actual amount of time the target organ is exposed to the drug or metabolite [224]. Similarly, although the choice of a 60 min pre-administration time is completely justifiable based on previously published works, it may have no direct relevance on the pharmacokinetics of the specific plant species studied here.

Nonetheless, by studying the effects of extracts on a combination of basal and physiological tests on both the short and long-term, natural products researchers can still glean valuable insight into their biological activity, despite the void in pharmacological data. This is precisely what we have done here. We set out to perform a rapid assessment of the *in vivo* hypoglycaemic activity of two extracts shown to increase glucose transport *in vitro*. Had the extracts tested shown dramatic and sustained decreases in PG or increases in glucose tolerance, detailed pharmacodynamic and pharmacokinetic investigation would be a logical and warranted progression. However, lack of such robust effects in this study is not sufficiently conclusive to discontinue research on these extracts.

Due to the complex aetiology of diabetes mellitus and the aforementioned methodological complications, no definitive conclusion about the anti-diabetic potential of the extracts tested here can be made based on the STZ-mouse model used in this study. This is reinforced by the inability of our positive control metformin to

significantly reduce fasting PG or improve insulin sensitivity in this model. The short term hypoglycaemic effect and rapid post-peak glucose disposal of the *Sarracenia* treated mice remain particularly interesting results, worthy of further investigation. This could be done by evaluating the glucose disappearance through an IV-GTT or a hyperinsulinaemic-euglycaemic clamp in the same or similar model. Also, taking into consideration the widely recognized anti-oxidant power of plants, it might also be interesting to examine the extracts' ability to reduce or prevent the oxidant-induced diabetogenic effects of STZ (note that the direct link between STZ and the induction of oxidative stress is still under debate [225, 226]). Within the context of this project, however, it remains appropriate to pursue investigation of these and other promising extracts in an animal model more closely resembling T2D in the Cree population, such as diet-induced obese and diabetic mice.

Chapter 4: *In vivo pharmacology*

Part 2: Diet-induced diabetic mice

4.2.1 **Introduction**

4.2.1.1 Foreword

In the study presented in section 4.1, extracts from *Sarracenia* and *Sorbus* were tested on normal and STZ-treated diabetic mice. Although neither extract improved glucose tolerance or insulin sensitivity, administration of *Sarracenia* resulted in a short term decrease in the PG of fasting STZ mice, and showed signs of an increased rate of glucose disposal in normal mice following peak PG of the OGTT. However, the overall variability and extreme hyperglycaemia of the STZ model made it impossible to make any definitive conclusions about the *in vivo* anti-diabetic effect of these two plant species.

In this study we re-examine the effects these two extracts and a third promising extract, *Ledum*, in a diet-induced model of diabetes. The lack of extract efficacy in the STZ model does not preclude inefficacy in another model such as the diet-induced obese mouse, as has been shown in previous studies [208]. Moreover, the diabetic state induced by diet related obesity is strikingly different from the STZ model [211], and more closely resembles the etiology of T2D among the Cree [7] and other populations worldwide afflicted by this disease [227, 228].

4.2.1.2 Animal model

The diet induced obese C57BL6 mouse is a well characterized model of T2D, and is actively used for natural product research [229, 230]. It has been shown that successful development of diabetes is most heavily related to fat content in the diet, with diets containing greater than 50% fat being most effective [231, 232]. The C57BL6 mouse, genetically predisposed to obesity [233], gains weight as early as 2 weeks on a HF diet, and is used experimentally for diabetes research anywhere from 4 weeks to 11 months [137, 231, 233-236]. Metformin was chosen as a positive control as it remains the drug of choice for patients with T2D [156, 157, 237, 238], and it has been shown to improve insulin sensitivity and glucose tolerance in this model [239, 240].

4.2.2 **Materials and Methods**

4.2.2.1 Materials

Male C57BL/6 mice were purchased from Charles River (Montreal, Canada) at 7-8 weeks of age. High-carbohydrate (24.48%) / high-fat (59.36 %) (HF) diet was

obtained from Bio-Serv (Product # F3282, Frenchtown, NJ). Blood glucose (BG) was measured using Glucometer Elite XL (Bayer, Toronto, Ontario). Plasma insulin was determined using Ultrasensitive mouse insulin ELISA from ALPCO Diagnostics (Windham, NH). Heparin sodium and sodium pentobarbital (Somnotol®) were purchased from Leo Pharma Inc. (Thornhill, Ontario) and MTC Pharmaceutical (Cambridge, Ontario), respectively. Sourcing of the following materials is outlined in section 4.1.2.1: Standard rodent chow, glucose, metformin, capillary tubes, syringes, needles, and human insulin.

Intramedic® polyethylene (PE) tubing was obtained from Becton Dickinson (Sparks, MD). Silastic® silicone (SS) tubing was purchased from Dow Corning (Midland, MI). Masterflex® silicone (MS) tubing and Cole Parmer barbed T-connectors were obtained from Labcor (Montreal, QC). Homeothermic blankets and infusion pumps were obtained from Harvard Apparatus (Holliston, MA). Micro-Renathane (MR) catheter tubing was obtained from Braintree Scientific (Braintree, MA.). Industrial glucose analyzers and the syringe pipette (Syringepet®) were purchased fromYellow Springs Instruments (Yellow Springs, OH). Pressure transducers were obtained from Digi-Med (Louisville, KY).

4.2.2.2 Extracts:

Extracts were prepared as described in section 3.3.1

4.2.2.3 Housing and diet

Animals were kept in a temperature controlled room with a 12-hour light/dark cycle. The mice were housed 5 animals per cage for the first 8 weeks, after which they were placed in separate cages (1 animal/cage) for the remainder of the experiment. They were allowed *ad libitum* access to one of two diets as follows: 8 animals were fed standard rodent chow, and 40 were fed a diet high in fat and carbohydrates (HF diet – see *4.2.2.1 Materials*). All diets contained 16% protein. Animals were maintained on these diets for 10 weeks prior to the treatment with extracts. Body weight was monitored weekly and blood glucose was determined after 8 weeks of HF feeding following a 3 hour fast to verify the progression of the model. Blood sampling for purposes of monitoring glycemia was performed by saphenous vein puncture as described previously [212].

4.2.2.4 Experimental groups

The study consisted of 6 experimental groups: 1 group fed on chow and 5 groups fed on the HF diet. The 5 treatment groups were standardized to have equal mean (and variance in) body weight and blood glycaemia at the start of treatment period. This was based on the body weight and 12-hr fasted blood glycaemia taken 1 week prior to the onset of treatment (at 9 wks of diet). The 6 treatment groups (n=8) were as follows: 1) "Chow": 8 mice maintained on standard rodent chow and administered 10 mL/kg/day water; 2) "HF-CTL": mice maintained on the HF diet and administered 10 mL/kg/day water; 3) "HF-Metformin": mice maintained on the HF diet and administered 200 mg/kg/day Metformin; 4) "HF-*Sarracenia*": mice maintained on the HF diet and administered 200 mg/kg/day ethanolic extract of *Sarracenia* (whole plant); 5) "HF-*Sorbus*": mice maintained on the HF diet and administered 200mg/kg/day ethanolic extract of *Sorbus* (whole plant); 6) "HF-*Ledum*": mice maintained on the HF diet and administered 200mg/kg/day ethanolic extract of *Ledum groenlandicum* (leaf). All extracts and metformin were administered in distilled water via intragastric gavage at a volume of 10 mL/kg. Dosing with treatments occurred on a bi-daily basis, with half of the listed concentration being delivered in the morning, and the other half in the afternoon. The experiment was performed over a period of 6-8 weeks, and animals were familiarized to testing by daily handling and gavage with water during the week preceding the onset of the experiment.

4.2.2.5 Oral Glucose Tolerance Test (OGTT)

On the first day of chronic treatment, as well as after 1, 2, 4, and 5 week(s) of dosing, glucose tolerance was assessed via an OGTT. Prior to the OGTT mice were fasted for 6 hours. One hour (t=-60 minutes) prior to oral administration of glucose (3 g/kg body weight) (t=0 minutes), blood glucose was measured and mice were administered their daily dose of treatment. Blood glucose was then measured at 0, 30, 60 and 120 minutes after glucose load. The OGTT performed after 5 weeks of treatment also included blood glucose (BG) monitoring at 15 minutes following glucose load. Area under the curve (AUC) was calculated as described in section 4.1.2.5.

4.2.2.6 Intra-peritoneal Insulin Tolerance Test (ITT)

An insulin tolerance test was performed following 3 weeks of daily dosing. Prior to the ITT mice were fasted for 2 hours. One hour (t=-60 minutes) prior to intra-peritoneal administration of insulin (1 U/kg body weight) (t=0 minutes), blood glucose was measured and mice were administered their daily dose of treatment. Blood glucose was then measured at 0, 30, 60 and 120 minutes after insulin load. Percent area above the curve (%AAC) was calculated as described in section 4.1.2.6.

4.2.2.7 Basal glycaemia and insulinaemia

The effect of treatment on basal BG was assessed using weekly measurements taken one hour prior to OGTTs, on 6 hour fasted animals, as described above. On days 15-18 of daily dosing, animals were fasted overnight (14-17 hrs) and blood glucose and plasma insulin were determined to assess insulin resistance and β-cell function via the computer program based homeostasis model assessment (HOMA) as described previously [241, 242].

4.2.2.8 Hyperinsulinemic-euglycemic clamp (HEC)

HEC experiments were performed after 6-8 weeks of treatment. Prior to the clamp experiment, 2 hour fasted mice were anesthetized by intraperitoneal administration of pentobarbitol (65 mg/kg). Body temperature was monitored by a rectal probe and kept at 37° C by means of a heated surgical table and overhead lamp. The right carotid artery (PE tubing #10) and jugular vein (MR tubing #025) were cannulated and connected via a sleeve (MF tubing #13 with SS tubing at junctions) to form a loop. Midway along the sleeve, a T junction (1/16 inch) is inserted with its open end attached to a pressure transducer via PE tubing (PE20). The result is an arterial-venous blood sampling shunt (AV shunt) that allows blood to flow uninterrupted from the artery to the vein [243, 244]. Blood samples were taken by puncturing the arterial side of the shunt while pharmacological agents were infused into the venous side of the shunt. By clamping the silicon sleeve on the venous side of the shunt, it is possible to measure arterial pressure. The trachea was cannulated using MR (#40) tubing, allowing the animal to breathe spontaneously. Glucose and insulin infusion lines were inserted into the silicon sleeve on the jugular supplement line and were controlled by an infusion pump. Animals were heparinized throughout the experiment (100 U/kg/min in insulin solution) to prevent clotting in the AV shunt. Upon completion of the surgery, the animals were allowed to stabilize for a minimum

of 30 min, at which time a blood sample (25 µL) was taken and analyzed for blood glucose concentration using a glucose analyzer. Blood sampling continued every 5 min until three successive stable measurements were taken. The mean of these three samples was used as the basal glucose level.

Following the establishment of a stable basal glucose level, insulin (in 0.9% saline) was infused at 5 mg/kg/min. Blood continued to be sampled every 5 min and glucose was variably infused to maintain a blood glycemia of 150 mg/dL. The experiment lasted for 120 minutes, and glucose infusion rates (GIRs) during the last 30 minutes were recorded for comparison between groups. Anesthesia was adequately maintained throughout the experiment by supplemental pentobarbitol (6.5 mg/kg) if animals responded to a tail/toe pinch.

4.2.2.9 Statistics

All statistics were performed as described in the Methods of section *4.1.2.7*.

4.2.3 **Results**

Please note that since we are looking at the effects of plants in DIO mice, all data is represented and analyzed in reference to the HF-Control Group.

4.2.3.1 Basal glycaemia and body weight (Figure 4.2.1)

At the beginning of the study, following 10 weeks of HF diet, there was no difference in the mean fasting BG between Chow and HF-CTL groups, despite a large difference in weight. Body weight progression was significantly different between HF-Control, HF-Metformin and Chow groups (p=0.0113; repeated measure ANOVA), with the latter remaining significantly lower than the other groups at week 4 and 5. Body weight and BG of HF-Control and extract fed groups increased over the course of the experiment. Neither the extracts nor metformin had a significant effect on BW or BG as compared to HF-Control over the course of the study.

4.2.3.2 Glucose Tolerance Test (Figure 4.2.2)

At both the beginning and end of the study GRCs of HF-Control and HF-Metformin groups were significantly different ($p<0.0001$; repeated measure ANOVA). The GRC of the Chow group was characterized by a significantly lower peak, and a faster return to baseline than than the HF-Control. Treatment with metformin had a strong hypoglycaemic effect from -60 to 0 min and completely

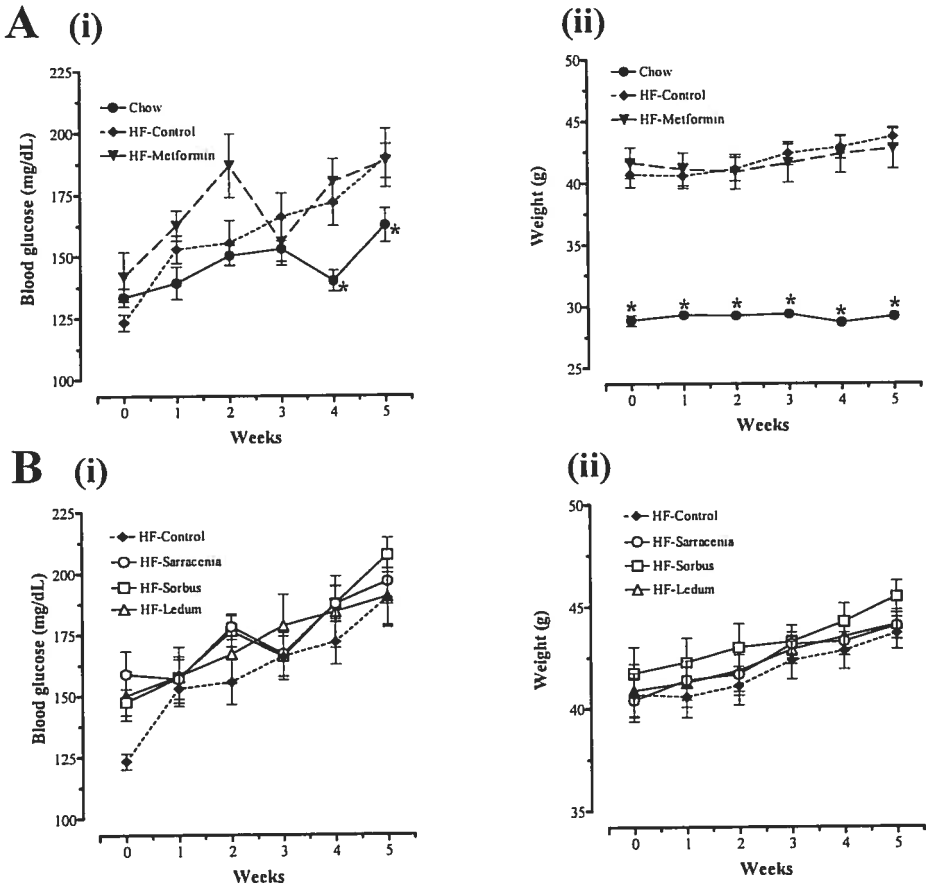

Figure 4.2.1. Blood glucose (i) and body weight (ii) over the course of the study: (A) Effects of HF diet and HF diet + metformin; (B) Effects of extracts on diet induced obese mice. At 0 weeks, mice have been fed HF diet or standard chow for 10 weeks. Values displayed ± SEM. * = Value significantly different from HF-CTL, $p<0.05$.

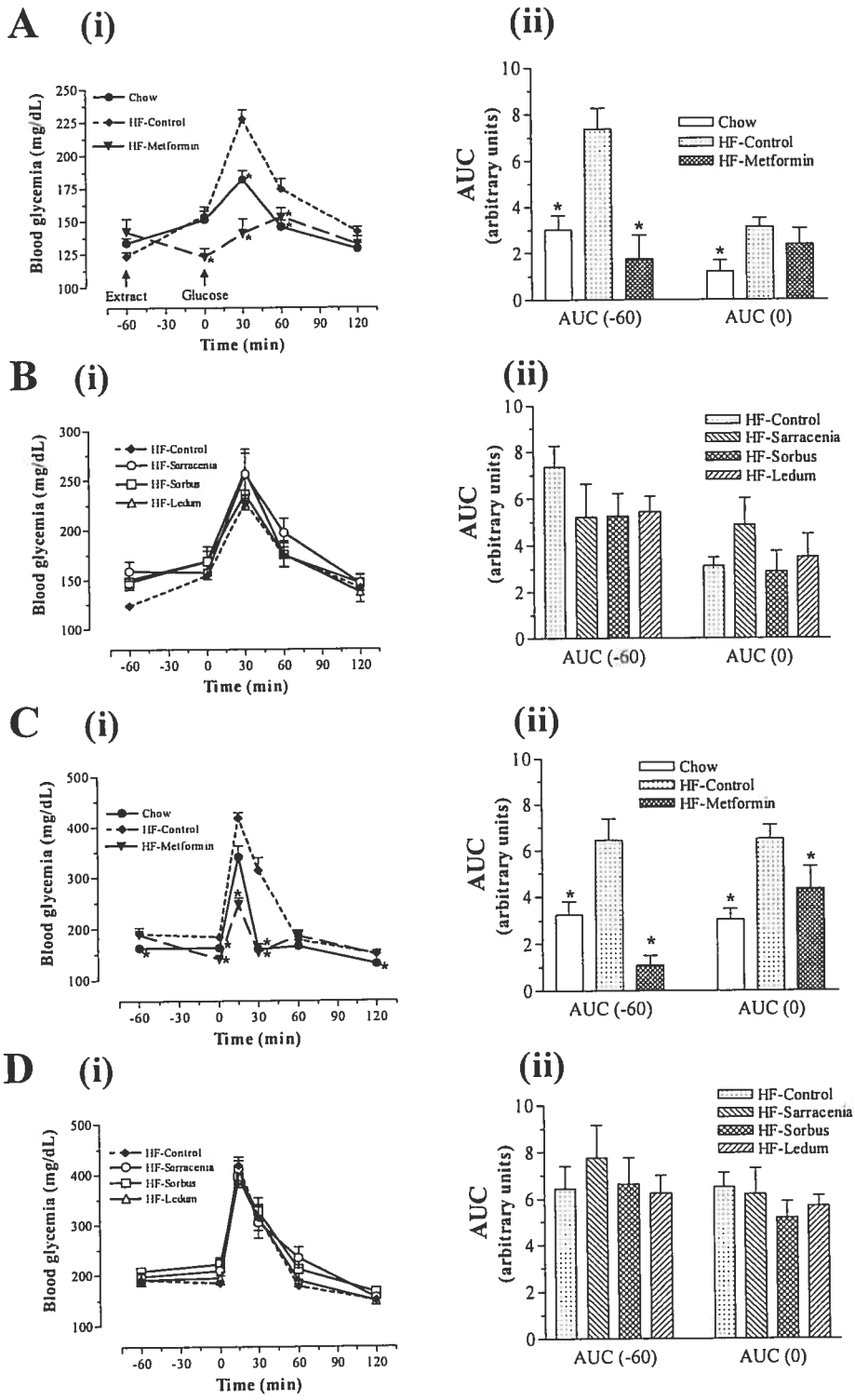

Figure 4.2.2. Oral glucose tolerance test (OGTT) (i), and corresponding area under the curve (AUC) (ii) from -60 to 120 min (AUC-60) and from 0 to 120 min (AUC 0), performed at week 0 (A, B) and week 5 (C,D). Chow, HF-Control and HF-Metformin (A, C) are presented separately from extracts (B, D). Extract was delivered one hour prior to glucose load. Values displayed + SEM * = Value significantly different from HF-Control, $p < 0.05$.

prevented a peak at 30 minutes. Both Chow and HF-Metformin group had lower AUC(-60), yet significance was maintained only in the Chow group for the AUC(0). The markedly low BG of the HF-Metformin group at 0 min, is most likely the cause of the lack of significant effect of metformin for the AUC(0) since BG remains above this point even at 120 min. There were no significant differences in the GRCs or overall AUC between extract-treated and control groups. OGTTs were performed at weeks 1, 2 and 4, with similar results (data not shown).

At five weeks, blood sampling at 15 minutes post glucose load allowed for the capture of a well-defined peak in BG, which had been lacking in the metformin group when BG was sampled at only 30 minute intervals at week 0. As with the OGTT at week 0, metformin had a hypoglycaemic effect from -60 to 0 min, and significantly improved AUC (-60). Moreover, metformin's improvement on glucose tolerance was statistically conserved for AUC(0) (-32%), despite a lower BG baseline at 0 min. By week 5, the Chow group had a lower fasting BG compared to HF-control, indicated by a lower pre and post OGTT BG. As expected, this group was also significantly more tolerant of the glucose load than the HF-Control group, indicated by a lower GRC peak (-18%) and BG (-50%) at 60 min, and a decrease of approximately 50% in both AUC(-60) and AUC(0). Treatment with extracts had no significant effect in glucose tolerance (p=0.6566; repeated measure ANOVA).

4.2.3.3 Insulin tolerance test (Figure 4.2.3)

During the hour preceding the delivery of the insulin bolus, BG increased in the HF–control group, decreased in the HF-metformin group, and remained unchanged in the Chow group. This led to significantly different insulin response curves (p=0.0279; repeated measure ANOVA), and different mean BGs at the moment of insulin delivery. The increase in %AAC (-60) in the Chow and HF-metformin groups is mostly due to a pre–insulin bolus effect, since no significant differences are present in %AAC(0). As for the plant extracts, no significant differences were found in BG or %AAC, despite a 45-60 % higher mean %AAC-(-60) in plant treatments as compared to control.

4.2.3.4 Homeostasis model assessment (HOMA) *and hyperinsulinemic-euglycemic clamp* (HEC) (Table 4.1)

After an overnight fast, there was no significant difference in BG between

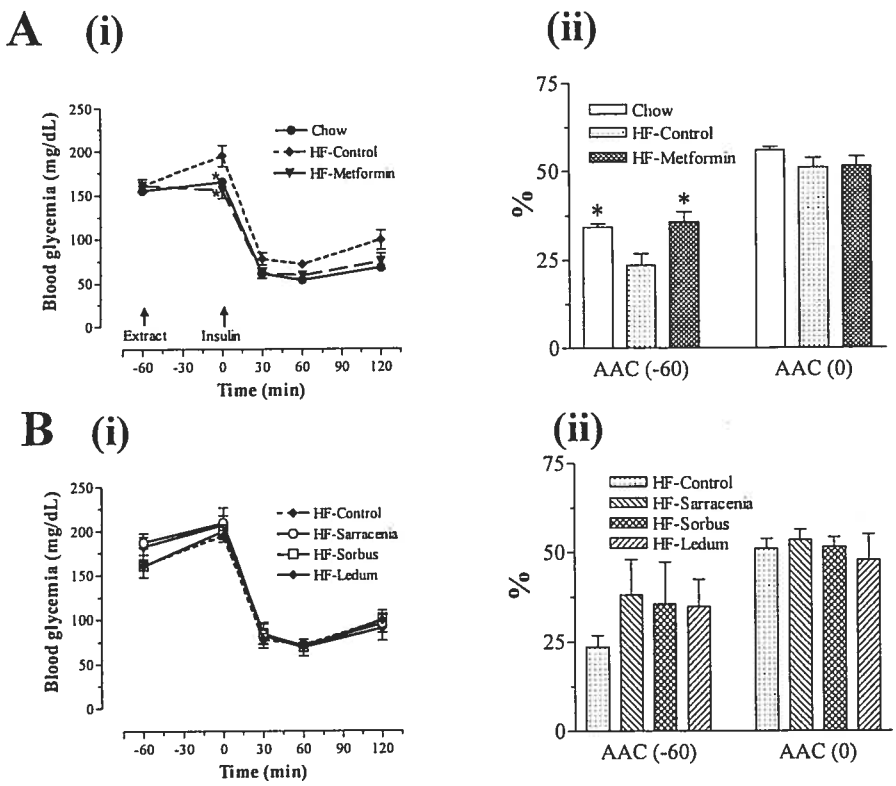

Figure 4.2.3. Insulin tolerance test (ITT) (i) and corresponding percent area above the curve (%AAC) (ii) from -60 to 120 min (%AAC-60) and from 0 to 120 min (%AAC 0)., performed after 3 weeks of treatment. Extract/drug was delivered one hour prior to insulin bolus. Chow, HF-Control and HF-Metformin (A, C) are presented separately from extracts (B, D). Values displayed ± SEM (i), +SEM (ii).* = Value significantly different from HF-Control, $p < 0.05$.

Table 4.2.1. Blood glucose (n=8), plasma insulin (n=5-8), and corresponding insulin resistance values (n=5-8) following an overnight fast, 2 weeks into the study. Glucose infusion rates (n=4-7) from hyperinsulinaemic-euglycaemic clamp performed following 6-8 weeks of treatment. Values displayed ± SEM. * = Value significantly different from HF-Control.

Group	Blood glucose (mg/dL)	Plasma insulin (pmol/L)	Insulin resistance (HOMA scale)	Glucose infusion rate (mg/kg/min)
Chow	89.6 ± 8.1	35.8 ± 3.9*	0.68 ± 0.08*	46.1 ± 4.7*
HF-Control	102.8 ± 4.9	70.7 ± 14.0	1.34 ± 0.24	19.8 ± 4.1
HF-Metformin	100.9 ± 4.7	58.3 ± 7.4	1.12 ± 0.15	30.5 ± 2.5*
HF-Sarracenia	115.3 ± 5.5	114.4 ± 23.5	2.22 ± 0.45	13.9 ± 1.8
HF-Sorbus	114.0 ± 5.4	144.1 ± 28.2*	2.77 ± 0.54*	11.2 ± 1.7
HF-Ledum	114.8 ± 5.5	130.2 ± 29.0	2.52 ± 0.56	19.8 ± 1.2

groups. Plasma insulin (PI), however, was approximately 50% lower in the Chow group, and was 2-fold higher in the HF-*Sorbus* group compared to HF-Control. Metformin slightly decreased PI, while plant extracts increased basal insulinaemia and reduced insulin sensitivity (%S) as calculated by the HOMA. Compared to control, glucose infusion rates (GIR) during the HEC seem to correlate well with HOMA-IR values, with the Chow and metformin groups having higher mean GIRs and lower IR values, and plant groups having equivalent or lower GIRs and slightly higher IR values.

4.2.4 **Discussion**

The nature of the DIO diabetic C57BL6 mouse is highly analogous to obesity onset T2D in humans [245], and as such was an appropriate choice for this study. However, the model is known for its inherent variability [197, 235], and elevated sensitivity to stress [233], which present significant logistical challenges to the researcher. In the present study, variability played a considerable role in analysing results by increasing the standard error of the mean (SEM) and threshold values for significance. This is particularly evident in the lack of significant differences in the ITT results (Figure 4.2.3), or OGTT results at week 0 (Figure 4.2.2). The use of fasting to impose a standardized post-prandial state in animals reduces within-group variability in BG, but also reduces between-group variability, making subtle effects more difficult to detect. This is illustrated by a previous study on this model, which demonstrated no significant increase in fasting BG after 7 weeks of HF diet [235]. Indeed, while HF mice already showed a marked reduction in glucose tolerance (Figure 4.2.2) at week 0, it took another 3 weeks for differences in fasting BG to reach significance as compared to lean controls (Figure 4.2.1).

The lack of large differences in the fasting BG of HF mice suggests that our model is more one of impaired glucose tolerance than of T2D *per se*. This is reinforced by the normo-glycaemia and hyper-insulinaemia of the mice as measured 2 weeks into the study (Table 4.2.1), which are in direct accordance with clinical features of pre-diabetic obese individuals with impaired glucose tolerance [12, 157]. Keeping this in mind, our study showed no significant effect of metformin or extracts on fasting BG or insulin tolerance over 5 weeks of treatment. These results disagree with two previous studies in this model [239, 240], which demonstrated a decrease in BG and increased ITT-measured insulin sensitivity following a 8-14 day treatment

with metformin. In the case of Xu et al (2002), it was not indicated if BG was measured in the fasted state, making results difficult to compare. Discrepancies with the other study [240] are most likely due to a 2-fold higher dose (400mg/kg), a shorter fast (3 hour), and a smaller insulin bolus (0.5U/kg). Indeed, although an insulin bolus of 1 U/kg is commonly employed [217], this quantity might saturate glucose transport in our model. Quantities of 0.5-0.75 U/kg, also commonly used [232, 246], might allow researchers to detect subtle changes in insulin sensitivity more effectively. Moreover, BG measurements in our study were taken just prior to the first daily administration of the extract, in order to capture the chronic effect of the treatment. As a result, BG samples were taken up to 18hrs after the last administration of drug, creating ample time for drug effect to disappear.

Over the short term, metformin did have a significant glucose-lowering effect prior to OGTTs, an anti-hyperglycaemic effect during OGTTs, and an increased GIR during the HEC. Metformin has been clinically shown to increase GIRs in HEC performed on humans [12], and its inhibitory effect on hepatic glucose output [247] most likely plays a principal role in mice submitted to prolonged hyper-insulinaemia (as is the case during a HEC). During the OGTT, however, metformin's effect was probably due to an increase in systemic glucose disposal by stimulation of peripheral glucose uptake, as observed previously in clinical settings [248]. Other effects of metformin, such as the stabilisation of BW and modest decreases in basal plasma insulin, are in accordance with the litterature [88, 193]. The loss of an anti-diabetic effect over the long-term is most likely due to the short half-life of the drug (2-5 hours in humans) [90], and prolonged time periods (up to 18 hours) between drug administration and BG sampling.

Despite careful equilibration of treatment groups (based on fasted BG 3 days prior to the start of the experiment), all three plant treatment groups were above HF-Controls at week 0. By week 1 all groups had similar mean BG levels, and it is unlikely that all three groups consisted of individuals with BG levels higher than those of the HF-control group. However, this possibility cannot be excluded, and it may have had a considerable effect on the outcome of the experiment. For example, were the entire BG progression curve (Figure 4.2.1) of the HF-*Ledum* group to be displaced so that to the BG at week 0 was equivalent to that of the HF-Control group, BG of the *Ledum* group would remain consistently below the Control group for the

duration of the study. A similar case could be made for the HF-*Sorbus* group during the OGTT at week 0.

All three plant treated groups also had higher basal plasma insulin levels, reaching significance in the HF-*Sorbus* group. Previous studies with mice have shown plasma insulin to be a more sensitive indicator of changes in glucose metabolism than BG [233, 246], sometimes showing significant changes in response to treatment without corresponding changes in BG [229]. Increases in plasma insulin of this magnitude are not sufficient to have a significant effect on BG, especially in an insulin resistant model. Also, it is difficult to say if this increase is also present on the short term, since PI levels were not measured during OGTTs due to logistical difficulties of the procedure [232]. It is possible that, over a longer period of treatment, plasma insulin levels might continue to increase, eventually leading to a decrease in fasting BG. In the case of the HF-*Ledum* group, this might indeed be the case, since PI increased while insulin sensitivity as measured by the HEC remained equivalent to control. Yet this is unlikely for the *Sarracenia* and *Sorbus* groups since increased plasma insulin was also tied to increased insulin resistance as indicated by lower GIRs and higher HOMA-IR values.

4.2.5 **Conlusion**

The HF regime used here induced a marked increase in weight, and induced a significant resistance to insulin compared to the standard chow diet. The HF animals were clearly obese and glucose intolerant, yet they were not necessarily representative of an advanced T2D since their fasting BG remained only slightly above or equal to lean animals after a 6 hour and overnight fast, respectively. Metformin demonstrated a significant hypoglycaemic and insulin-sensitizing effect on the short term, yet failed to cause a decrease in fasting BG after 5 weeks of treatment. On the other hand, neither an acute nor a chronic effect on glucose homeostasis was witnessed as a result of treatment with any of the three extracts. All three extracts did however increase plasma insulin, reaching significance in the *Sorbus* group. It would be interesting to determine the nature of this increase (central or peripheral), and the onset and duration of its action. The use of thorough time-course measurements of blood glucose and insulin, as well as detailed examination of β-cell morphology might help to answer some of these questions.

The DIO model is now a well established model of Type 2 diabetes and, as shown above, the methodology used here does not differ greatly from currently published diabetes research. However, despite the development and use of an obese and insulin resistant model, the lack of a chronic effect of our positive control metformin is indicative of an important methodological problem. It thus remains impossible to make any definitive conclusions about the efficacy of the plant extracts tested here. As with the STZ study, this study is also subject to numerous uncertainties underlying the proper methodological parameters of frequency, quantity, and method of extract administration (section 4.1.5).

The use of a less variable model with a fasting BG markedly greater than normal, would be more conducive to detecting subtle changes in glucose tolerance and insulin sensitivity. This might be achieved by simply increasing sample size, decreasing fasting time, or by model modification such as increasing pre-study exposure to diet [229, 233]. Direct modification of the model such as combining the HF diet with low levels of STZ [249], or the use of genetically-induced obese models (*db/db, ob/ob, KK/Ay*) should also be considered. Additionally, future studies should attempt increasing frequency of dosing and consider alternative methods of administration, such as incorporating the study treatment into the food, which would increase the exposure of the target tissues to the extract or its metabolites [224, 229, 230]. Together these changes would improve the relevance of the model, reduce variability, and increase our ability to capture changes in glucose metabolism - allowing for more robust conclusions about the anti-diabetic effect of the extracts tested.

Chapter 5: *Conclusion*

This study is the first of its kind to identify Cree medicinal plants used for the symptoms of T2D, and to evaluate their anti-diabetic effect *in vitro* and *in vivo*. Results from the ethnobotanical survey showed that Cree Elders and Healers in Mistissini (Qc.) maintain a thorough knowledge of traditional medicine which is applicable to the broad symptomatology of T2D. It was also shown how the prioritization of species can be catered to multi-factorial syndromes such as diabetes by combining easily defined symptoms with a robust mathematical formula and appropriate multivariate analysis.

Primary *in vitro* screening of the 8 most promising plant species demonstrated that all species increased glucose uptake in muscle cells and/or adipocytes following acute and/or chronic exposure. Evaluation of the glucose uptake data revealed that three extracts, *Sarracenia*, *Sorbus* and *Ledum*, showed particular promise when the chronic effect on muscle cells was favored. In fact, the magnitude of effect of *Sarracenia* and *Sorbus* under the latter conditions was equal to or greater than the well-known anti-diabetic drug metformin However, when these three promising extracts were evaluated *in vivo*, there was no marked effect on basal glycaemia, insulinaemia, or glucose and insulin tolerance. These results beg the following fundamental question: Why doesn't *in vivo* data correlate with *in vitro* data?

To answer this question, one must put into question the methodology of both primary screening and *in vivo* confirmation. An appropriate evaluation of the success of an assay is the effect of the positive control, which in this case was metformin. Metformin has become a gold standard in diabetes research [132, 153, 248-253] and thus the choice of this drug as a positive control both *in vitro* and *in vivo* was clearly a suitable one. However, close examination of the literature revealed that metformin does require some endogenous pancreatic function to have an effect [193], making it an inappropriate choice in the high dose STZ-treated diabetic mice. In the HF model however, metformin has been shown to work acutely and chronicly, which was not the case in our study. Although we did witness a positive acute effect in DIO mice, mean fasted glycaemia was not reduced after 5 weeks of 200mg/kg metformin Thus in both STZ and HF models we did not have an effective and reliable positive control, which diminishes the dependability and interpretability of our results. Indeed, despite the lack of significant anti-diabetic effect of the treatments tested, we cannot conclude about the efficacy of the extracts.

One key challenge in the area of natural product research is methodological design of plant screening assays. Virtually nothing is known about the stability, solubility, absorption, metabolism, mechanism, and duration of action of the extracts tested in these assays. In an attempt to capture the physiological response to our treatments, we tested their effect in both acute and chronic conditions. *In vitro*, this corresponded to 1hr (acute) and 18hrs (chronic) of pre-incubation. The approach was similar *in vivo*, where the acute and chronic effect were evaluated 1 hr and 18hrs following the last dose, respectively. *In vivo*, however, pharmacokinetics are clearly more complex, as delivery and elimination of a product involves the coordination of numerous tissues, and experimentation is fraught with extraneous sources of variability such as stress. Indeed, when testing *in vitro,* extracts are placed directly in contact with cells, bypassing the effects of metabolism and bioavailability which occur *in vivo*. In both cases however, we examined physiological parameters during 2 restricted windows in time. *In vitro*, we managed to choose appropriately, as the effect of every extract was captured under one of the two conditions. *In vivo*, it is quite possible that the mice experienced significant responses to the treatments, yet these effects occurred prandially, post-prandially, or somewhere between 2 and 18 hours following administration. Indeed, our protocol adhered to a 6 hr fast, which may be just enough time for a post-prandial effect to disappear.

Lack of positive *in vivo* results also invites a re-evaluation of the screening approach itself. Numerous groups in natural product and anti-diabetic plant research prefer to screen plants directly *in vivo*, with follow-up studies *in vitro* [122, 126, 196, 254, 255]. Given the limitations of *in vitro* screening, few researchers would disagree that the immediate confirmation of *in vivo* biological activity would be ideal. However, animal experimentation comes with its own set of limitations, the greatest of which are increased cost and duration of study. No less important are the ethical issues tied to studies involving animals. Nowadays, animal experimentation protocols are evaluated under strict scrutiny and such studies are generally justifiable only on the basis of preliminary *in vitro* data [256]. These restraints, combined with recent advances in cell-culture based assays, make *in vitro* screening an attractive (if not necessary) option in natural product research, even within the field of anti-diabetic plant research [194, 195, 197].

It seems thus fairly clear that failure to confirm the anti-diabetic effect of the 3 extracts *in vivo* was due to methodological issues rather than an intrinsic lack of

pharmacological activity. Currently the 8 highest ranked plant species are being screened in numerous other complementary *in vitro* assays (section 3.1) in our lab and those of our collaborators. The totality of the results generated by these assays, including glucose uptake, will provide a broad scale profile of the anti-diabetic potential of each plant, and allow for a more well-informed choice as to which plants to further investigate. Also underway in our laboratory is the breeding and characterization of the "KKAy" mouse, a genetically-induced model of T2D. Mice heterozygous for the Ay allele become obese and eventually diabetic, a process thought to be due to a reduction in hypothalamic norepinephrine and dopamine [257]. Although these mice will become obese on a standard chow diet, the process can be accelerated when they are fed a HF diet like the one used in this study- leading more rapidly to an advanced stage T2D [258]. In the context of this project, this model has a dual advantage: 1) it is considerably cheaper since the colony is housed at the Université de Montréal; 2) Unlike the DIO C57/BL6 mice used in this study, an advanced-stage diabetes can be achieved relatively rapidly in KKAy mice, which is accompanied by a hyperglycaemia which is maintained under fasting conditions. A cheaper and "more diabetic" model such as this one will allow for more flexibility and a greater ability to detect subtle changes in glucose homeostasis following treatment.

Our preliminary results from glucose uptake in muscle cells and adipocytes yielded positive results at a frequency and magnitude much greater than expected. These results remain extremely promising and pertinent to our search for anti-diabetic plant extracts. Moreover, they represent an initial confirmation of the Cree traditional knowledge and the novel ethnobotanical approach used to identify the most promising plants. Though the pilot *in vivo* studies were unsuccessful, they identified the need for protocol modification in some key areas. These changes could include: the development of a model of T2D with a fasting hyperglycaemia; decreasing the fasting time prior to blood sampling; changing the mode of extract administration; increasing the dose and frequency of extract. These changes, combined with *in vitro* assays on insulin secretion, PPARγ activation, and cytoprotection, will complement our current body of knowledge and increase our ability to detect, confirm, and harness the antidiabetic potential of Cree medicinal plants.

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