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Bioassay-guided fractionation of *Larix laricina* du Roi, and antidiabetic potentials of ethanol and hot water extracts of seventeen medicinal plants from the traditional pharmacopeia of the James Bay Cree

par
Nan Shang

Département de Pharmacologie
Faculté de médicine

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Bioassay-guided fractionation of *Larix laricina* du Roi, and antidiabetic potentials of ethanol and hot water extracts of seventeen medicinal plants from the traditional pharmacopeia of the James Bay Cree

Présentée par :
Nan Shang

a été évaluée par un jury composé des personnes suivantes :

Dre Hélène Girouard, président-rapporteur
Dr Pierre S. Haddad, directeur de recherche
Dr Raynald Bergeron, membre du jury
Dre Tatjana Stevanovic Janezic, examinateur externe
Dre Christine Des Rosiers, Représentante du doyen de la FESP

Résumé

Nous avons utilisé une approche ethnobotanique pour identifier des espèces de plantes utilisées par les Cris afin de traiter les symptômes du diabète de type 2. *Larix laricina* du Roi *(L. laricina)* a récemment été identifiée comme une des meilleures plantes qui a stimulé le transport de glucose dans les cellules C2C12 et fortement potentialisé la différenciation des 3T3-L1 en indiquant une sensibilité potentiellement accrue à l'insuline. Ensuite, ces études de criblage ont été effectuées sur des extraits éthanolique (EE) en utilisant une série de bioessais *in vitro*. Cependant, les préparations traditionnelles des plantes sont souvent faites avec l'eau chaude. Le but de cette thèse de doctorat était d'isoler les principes actifs de *L. laricina* par un fractionnement guidé par l'adipogenèse; d'évaluer et de comparer l'activité et les mécanismes antidiabétiques des EE et des extraits aqueux (HWE) de ces 17 plantes.

Pour le fractionnement de *L. laricina*, on a isolé plusieurs composés connus et identifié un nouveau composé actif cycloartane triterpene, qui a amélioré fortement l'adipogenèse et a été responsable en partie de l'activité adipogénique (potentiellement similaire à l'effet sensibilisateur à l'insuline des glitazone) de l'extrait éthanolique issu de l'écorce de *L. laricina*.

Pour le métabolisme lipidique, nos résultats ont confirmé que 10 parmi les 17 EE ont augmenté la différenciation des adipocytes alors que 2 extraits seulement l'ont inhibée. Les HWE ont montré une faible activité adipogénique ou antiadipogénique. Les EE de *R. groenlandicum* et *K. angustifolia* ont le PPAR γ (peroxisome proliferator-activated receptor γ), le SREBP-1 (sterol regulatory element binding protein-1) et le C/EBP (CCAAT-enhancer binding proteins) α, alors que ceux de *P. balsamifera* et *A. incana* les ont inhibés. L'effet inhibiteur de *P. balsamifera* a également été prouvé d'avoir impliqué l'activation de la protéine kinase activée par l'AMP (AMPK). Les EE et HWE de *R. groenlandicum* ont stimulé les mêmes facteurs de transcription alors que les extraits aqueux d'autres plantes sélectionnées ont perdu ces effets en comparaison avec leurs extraits éthanoliques respectifs. L'analyse phytochimique a également identifié le groupe des espèces actives et inactives, notamment lorsque les espèces ont été séparées par famille de plante.

Finalement concernant l'homéostasie de glucose, nos résultats ont confirmé que plusieurs EE ont stimulé le transport de glucose musculaire et inhibé l'activité de la glucose-6-phosphatase (G6Pase) hépatique. Certains des HWE ont partiellement ou complètement perdu ces activités antidiabétiques par rapport aux EE, tandis qu'une seule plante (*R.groenlandicum*) a juste conservé un potentiel similaire entre les EE et HWE dans les deux essais. Dans les cellules musculaires, les EE de *R.groenlandicum, A. incana et S. purpurea* ont stimulé le transport de glucose en activant la voie de signalisation de l'AMPK et en augmentant le niveau d'expression des GLUT4. En comparaison avec les EE, les HWE de *R.groenlandicum* ont montré des activités similaires; les HWE de *A. incana* ont complètement perdu leur effet sur tous les paramètres étudiés; les HWE de *S. purpurea* ont activé la voie de l'insuline au lieu de celle de l'AMPK pour augmenter le transport de glucose. Dans les cellules H4IIE, les EE et HWE des 5 plantes ont activé la voie de l'AMPK, et en plus les EE et HWE de 2 plantes ont activé la voie de l'insuline. La quercétine-3-O-galactoside et la quercétine 3-O-α-L-arabinopyranoside ont été identifiées comme des composés ayant un fort potentiel antidiabétique et donc responsables de l'activité biologique des plantes HWE actifs avec le transport du glucose.

En conclusion, on a isolé plusieurs composés connus et identifié un nouveau triterpène actif à partir du fractionnement de *L. laricina*. Nous avons fourni également une preuve directe pour l'évaluation et la comparaison d'une action analogue à l'insuline ou insulino-sensibilisateur des EE et HWE de plantes médicinales Cris au niveau de muscle, de foie et de tissus adipeux. Une partie de leur action peut être liée à la stimulation des voies de signalisation intracellulaire insulino-dépendante et non-insulino-dépendante, ainsi que l'activation de PPARγ. Nos résultats indiquent que les espèces de plantes, les tissus ou les cellules cibles, ainsi que les méthodes d'extraction sont tous des déterminants significatifs de l'activité biologique de plantes médicinales Cris sur le métabolisme glucidique et lipidique.

Mots-clés: Le diabète de type 2, l'AMPK, l'Akt, le PPAR γ, le SREBP-1, le C / EBP α, la G6Pase, la médecine traditionnelle, l'homéostasie du lipidique et du glucose.

Abstract

We have used a collaborative ethnobotanical approach to identify plant species used by the Cree of Eeyou Istchee (CEI) to treat symptoms of type 2 diabetes. Several screening studies were performed on 17 species identified in a survey of the Cree Nation. Firstly, *Larix laricina* du Roi *(L. laricina)* was recently identified as one of the top plants, which stimulated glucose uptake in C2C12 muscle cells and strongly potentiated the differentiation of 3T3-L1 pre-adipocytes suggesting enhanced insulin sensitivity. Secondly, these screening studies were performed on ethanol extracts (EE) using an *in vitro* bioassay platform, however, traditional preparations are often based on hot water. So the purpose of this PhD thesis was to isolate the active principles from *L. laricina* through adipogenesis-guided fractionation, and to evaluate and compare the antidiabetic activity and mechanisms of EE and hot water extracts (HWE) of these 17 Cree plants.

For the fractionation of *L. laricina*, we isolated several known compounds and identified a new active cycloartane triterpene, which strongly enhanced adipogenesis in 3T3-L1 cells and was responsible partly for the adipogenic (potentially glitazone-like insulin sensitizing) activity of the ethanol extract of the bark of *L. laricina*.

In the adipocyte lipid metabolism course, the results confirmed that 10 of the 17 EE stimulated adipocyte differentiation and adipogenesis, whereas 2 had inhibitory effects. Corresponding HWE exhibited partial or complete loss of such adipogenic or anti-adipogenic activity. *R. groenlandicum* and *K. angustifolia* EEs activated Peroxisome proliferator-activated receptor γ (PPAR γ), sterol regulatory element binding protein-1 (SREBP-1) and CCAAT-enhancer binding protein (C/EBP) α, whereas *P. balsamifera* and *A. incana* decreased these transcription factors. *P. balsamifera*'s inhibitory effect was also found to involve AMP-activated protein kinase (AMPK) activation. *R. groenlandicum* HWE and EE stimulated similar transcription factors, but HWE of other selected plants lost such effects compared to their respective EE. Phytochemical analysis also uncovered clustering of active versus inactive species, notably when species were segregated by plant family.

The results showed that several EE stimulated muscle glucose uptake and inhibited hepatic glucose-6-phosphatase (G6Pase) activity. Some of the HWE

partially or completely lost these antidiabetic activities in comparison to EE; while one plant (*R.groenlandicum*) retained similar potential between EE and HWE in both assays. In C2C12 muscle cells, EE of *R.groenlandicum, A. incana and S. purpurea* stimulated glucose uptake by activating AMPK pathway and increasing GLUT4 expression level. In comparison to EE, HWE of *R.groenlandicum* exhibited similar activities; HWE of *A. incana* completely lost its effect on all parameters; interestingly, HWE of *S. purpurea* activated insulin pathway instead of AMPK pathway to increase glucose uptake. In the H4IIE cells, all selected 5 plants HWE and EE activated AMPK pathway, and in addition, 2 plants EE and HWE also activated insulin pathways. Quercetin-3-O-galactoside and quercetin 3-O-α-L-arabinopyranoside were identified as potential candidates to be responsible for the biological activity of the active HWE plants in the glucose transport assay.

In conclusion, we isolated several known compounds and identified a new active triterpene from fractionation of *L. laricina*. We also provide direct evidence evaluating and comparing of an insulin-like or insulin-sensitizing action of EE and HWE of Cree medicinal plants at the level of muscle, liver and adipose tissue. Part of their actions may be related to stimulation of insulin-dependent and insulin-independent intracellular signaling pathways, as well as to PPARγ activation. The results indicate that plant species, target tissues or cells, as well as extraction methods, are all significant determinants of the biological activity of Cree medicinal plants on glucose and lipid metabolism.

Keywords:

type 2 diabetes, AMPK, Akt, PPAR γ, SREBP-1, C/EBP α, G6Pase, traditional medicine, lipid and glucose homeostasis,

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List of abbreviations

ACC: acetyl-CoA carboxylase

A.balsamea: Abies balsamea (L.) Mill.

A.incana: Alnus incana subsp. rugosa (Du Roi) R.T. Clausen

ACE: angiotensin converting enzyme

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

AMPKK: AMPK kinase

APS: adaptor protein

ARB: angiotensin II receptor antagonist

ATGL: adipose triglyceride lipase

ATP: adenosine triphosphate

BMI: Body mass index

C/EBP: CCAAT-enhancer-binding protein

CAM: complementary and alternative medicines

CaMKKβ: Ca^{2+}-calmodulin-dependent kinase kinase β

cAMP: cyclic adenosine monophosphate

CAP: Cbl-associated protein

CAPE: caffeic acid phenethyl ester

CDA: Canadian Diabetes Association

CEI: Cree of Eeyou Istchee

CPT-1: carnitine palmitoyltransferase-I

DEX: dexamethasone

DGAT: diacylglycerol acyltransferase

DPP4: dipeptidyl peptidase 4

EE: ethanol extracts

F6P: fructose 6-phosphate

FABP4 or aP2: fatty acid-binding protein 4 or adipocyte protein 2

FAS: fatty acid synthase

FATP: fatty acid transport protein

FFA: free fatty acid

Foxo-1: Forkhead box o protein

G.hispidula: Gaultheria hispidula (L.) Muhl.

G6Pase: glucose-6-phosphatase

GC: gas chromatography

GDM: gestational diabetes mellitus

GDP: guanosine diphosphate

GEF: guanyl nucleotide exchange factor

GLP-1: glucagon like peptide- 1

GLP-1R: GLP-1 receptor

GLUT: glucose transporters

GPAT: glycerol 3-phosphate acyltransferases

GPCR: G-protein-coupled receptors

Grb2: growth factor binding protein 2

GS: glycogen synthase

GSK-3: glycogen synthase kinase 3

HbA1C: glycated hemoglobin

HNF4α: hepatic nuclear factor 4α

HPLC: high performance liquid chromatography

HSL: hormone sensitive lipase

HWE: hot water extracts

IBMX: 3-isobutyl-1-methylxanthine

IDF: International Diabetes Federation

IFG: Impaired fasting glucose

IGF: Insulin-like growth factor

IGT: impaired glucose tolerance

IKK: IkB kinase

IL: interleukin

IR: insulin resistance

IRS: insulin receptor substrate

J.communis: Juniperus communis L.

JNK1: c-Jun N-terminal kinase

K.augustifolia: Kalmia angustifolia L.

L. laricena: Larix laricina Du Roi (K.Koch)

L.clavatum: Lycopodium clavatum L.

LKB1: liver kinase B 1

LPA: lysophosphatidate

LC: liquid chromatography

LPL: Lipoprotein lipase

MAG: monoacylglycerol pathway

MAPK: mitogen-activated protein kinase

MCP-1: monocyte chemo-attractant protein-1

MEK: MAP kinase-kinase

MetS: metabolic syndrome

MGL: monoglyceride lipase

MO25: mouse protein 25

MODY: maturity onset diabetes of the young

MS: mass spectrometry

mTOR: mammalian target of rapamycin

NAFLD: non-alcoholic fatty liver disease

OGTT: oral glucose tolerance test

OHA: oral hypoglycemia agent

PA: phosphatidate

PAP: phosphatidate phosphatases

P.balsamifera: Populus balsamifera L.

P.banksiana: Pinus banksiana Lamb.

P.glauca: Picea glauca (Moench) Voss

Pref-1: preadipocyte factor-1

P.mariana: Picea mariana (P. Mill.) BSP

PDK: PI-dependent kinase

PEPCK: phosphoenolpyruvate carboxykinase

PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-α

PI3K: phosphatidylinositol-3-kinase

PIP2: phosphatidylinositol 4,5-bisphosphate

PIP3: phosphatidylinositol 3,4,5-triphosphate

PKA: cAMP-dependent protein kinase A

PKB: protein kinase B

PLC: phospholipase C

PPAR γ: peroxisome proliferator-activated receptor γ

PPRE: peroxisome proliferate response element

PTB: phosphotyrosine binding

R.groenlandicum: Rhododendron groenlandicum (Oeder) Kron and Judd

R.tomentosum: Rhododendron tomentosum (Stokes) Harmaja subsp.subarcticum (Harmaja)G.Wallace

RXR: retinoid X-receptor

QTOF: quadrupole-time-of-flight

S.decora: Sorbus decora (Sarg.) C.K.Schneid.

S.planifolia: Salix planifolia Pursh

S.purpurea: Sarracenia purpurea L.

SGLT: sodium-dependent glucose co-transporter

SH2: Src homology 2 domain

SLC: solute carrier

SOCS: suppressor-of-cytokine-signalling

SoHo: sorbin homology

SOS: son of sevenless

SREBP-1: sterol regulatory element-binding proteins-1

STRAD: Ste20-related adaptor

SUR: sulfonylurea receptor

T1D: type 1 diabetes

T2D: type 2 diabetes

TCA cycle: tricarboxylic acid cycle

TGs: triglycerides

TM: traditional medicines (TM)

TNF-α: tumor necrosis factor- α

TOF: time-of-flight

TZD: thiazolidinedione

UPLC: ultra performance liquid chromatography

V.vitis-idaea: Vaccinium vitis-idaea L.

VAT: visceral adipose tissue

WC: waist circumference

To my family

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Chapter 1: Introduction

1.Introduction

Type 2 diabetes (T2D) is a growing global health problem. A significant increase in prevalence is expected from 171 million people affected in 2000 to a possible 366 million in 2030 (Wild et al., 2004). According to WHO, 347 million people worldwide now have diabetes, and an estimated 3.4 million people died from consequences of hyperglycemia in 2004 (WHO, 2011).

The prevalence of T2D is especially high among some Canadian First Nations (Ayach and Korda, 2010). The age-adjusted prevalence of disease in Cree of Eeyou Istchee (CEI) of northern Quebec reached 29% in 2009 or 3 to 5 times higher compared with the rest of the Canadian population (Kuzmina et al., 2010). And the their rate of diabetic complications is also disproportional to the rate of diabetes (Young et al., 2000).

This serous problem is most likely caused by factors such as sedentary lifestyle and non-traditional diet (Hegele, 2001), and a genetic predisposition towards obesity (Neel, 1999; Skyler, 2004). The difficulty to comply with modern medical treatment also contributes (Young et al., 2000). Therefore, new strategies are needed to address this specialized issue and to provide culturally acceptable alternative medicines (Brassard et al., 1993).

We have used a collaborative community-based ethnobotanical approach to identify several plant species, which are used by the Cree for treating a variety of symptoms of diabetes (Leduc et al., 2006). Several screening studies were performed on 17 species identified in a survey of the Cree Nation (Harbilas et al., 2009; Harris et al., 2011b; Nachar et al., 2013; Nistor Baldea et al., 2010; Spoor et al., 2006). Firstly, *Larix laricina* Du Roi (K.Koch) (*L. laricina*) was recently identified as one of the top plants, which stimulated glucose uptake in C2C12 muscle cells and strongly potentiated the differentiation of 3T3-L1 pre-adipocytes indicating potentially enhanced insulin sensitivity (Spoor et al., 2006). Secondly, the screening studies for these 17 plants were performed on ethanol extracts (EE) using an *in vitro* bioassay platform, however, traditional preparations are often based on hot water.

So the purpose of this PhD thesis was to isolate the active principles from *L. laricina* through adipogenesis-guided fractionation; to evaluate and compare the

antidiabetic activity and mechanisms of action of ethanol and hot water extracts of the 17 Cree plants, as well as to analyze the metabolites in these plants based on their activity.

1.1 Energy homeostasis

The main goal of the metabolic processes is to provide the required and proper amounts of energy to the body. Overall, there are two groups of metabolic processes: energy consuming or anabolic (gluconeogenesis, glycogenesis, lipogenesis, protein synthesis) and energy producing or catabolic (glycolysis, glycogenolysis, lipolysis, proteolysis) metabolisms. When energy is needed, it can be derived from the oxidation or breakdown of fat, protein or carbohydrates. When there is extra energy, the body can store carbohydrate in the form of glycogen and fat in the form of triglycerides (TGs).

1.2 Glucose homeostasis

Glucose is a monosaccharide and is the primary energy source, enabling the generation of adenosine triphosphate (ATP) following glycolysis. Although most tissues can also use fats and protein as an energy source, the brain mainly only use glucose (ketone bodies (Owen et al., 1967) provides a major alternative source during fasting).

The low blood concentrations of glucose (hypoglycemia) can causes seizures, loss of consciousness, and death; while long lasting high blood glucose levels (hyperglycemia), can result in blindness, renal failure, and vascular disease. Therefore, blood glucose needs to be maintained within a narrow range and this process for maintaining stable blood glucose is called glucose homeostasis.

There are several metabolisms for glucose homeostasis, including glycolysis (oxidation of glucose into ATP and energy); gluconeogenesis (endogenous glucose production in the liver and kidney); glycogenesis (the conversion of excess glucose into glycogen as storage energy); and glycogenolysis (the breakdown of glycogen and another supply of glucose besides gluconeogenesis).

The body can adjust blood glucose levels by a variety of cellular mechanisms, such as insulin and glucagon. When glucose levels increasing, insulin is released from pancreatic β cells in the islets of Langerhans to the blood. Then insulin could stimulate the uptake of glucose, and also promote storage in the body, especially in the liver, as glycogen (glycogenesis). Glucose transporters facilitate the uptake of glucose across the cell membrane into cells.

In contrast, low glucose levels could stimulate the secretion of glucagon from pancreatic α cells. To increase the blood glucose, glucagon promotes the conversion of glycogen (the storage form of glucose in the liver) to glucose (glycogenolysis). Epinephrine also causes the release of glucose into the blood (Sherwin and Sacca, 1984) when the sympathetic nervous system is activated. Glucose can also be generated from non-carbohydrate precursors including pyruvate and glycerol, by gluconeogenesis.

1.2.1 Glucose transporters

Because the lipid bilayer of the plasma membrane is impermeable to polar molecules, glucose is transported across the plasma membrane by glucose transporters. There are two different types of transporter proteins, sodium dependent glucose co-transporter (SGLT) and the facilitative glucose transporters (GLUT) (Mueckler, 1992).

Both types of transporters belong to families of the solute carrier (SLC) (Scheepers et al., 2004) gene series. The two most well known members of SGLT family are SGLT1 and SGLT2, which are members of the SLC5A gene family and are involved in intestinal glucose absorption and renal reabsorption (Gerardi-Laffin et al., 1993; Scheepers et al., 2004).

The human genome contains 14 members of the GLUT family (gene name SLC2A), which can be grouped into 3 classes. They differ in the functional characteristics, including substrate profiles, kinetic constants, and binding affinities as well as different tissue distribution and expression (Gould and Holman, 1993), for a complex and specific regulation of glucose uptake.

The widely distributed GLUT1, is targeted predominantly to the plasma membrane, indicating to mediate basal glucose transport in various cell types (Kozlovsky et al., 1997). On the other hand, GLUT4 is a glucose transporter expressed in several insulin-sensitive tissues (heart, skeletal muscle and adipose tissue). GLUT4 is the primary transporter expressed in the skeletal muscle (Zorzano et al., 2005), whereas GLUT1 account for only 5% of total expression of transporters. The newly synthesized GLUT4, present in the Golgi apparatus and the endoplasmic reticulum and undergo maturation and glycosylation. Normally, the GLUT4 is sequestered to the intracellular membrane compartments. In contrast, insulin, muscle contraction, and/or hypoxia increases GLUT4 expression at the cell surface by promoting the translocation of the transport protein from intracellular storage vesicles, resulting in an immediate 10- to 20-fold increase in glucose transport (Bryant et al., 2002).

Skeletal muscle cells exhibit insulin-stimulated glucose uptake that is dependent on the phosphatidylinositol-3-kinase (PI3K) pathway. However, enhanced glucose transport could also be induced by an insulin-independent mechanism, which could be activated by muscle contractions and hypoxia (Azevedo et al., 1995; Nesher et al., 1985; Wallberg-Henriksson and Holloszy, 1985). Evidence suggests that the AMP-activated protein kinase (AMPK) plays an important role in glucose transport stimulated by contraction (Hayashi et al., 1998). There are also other possible signal intermediates, including calcium, nitric oxide and others (Balon, 1998; Jessen and Goodyear, 2005; Wright et al., 2005).

1.2.2 Hepatic glucose production

The liver is capable of maintaining blood glucose homeostasis by glucose production and release (through gluconeogenesis and glycogenolysis), as well as glucose uptake/storage (through glycogenesis).

1.2.2.1 Gluconeogenesis and glycolysis

Glycolysis is the pathway, which converts the glucose molecule (six-carbon) into two molecules of the pyruvate (three carbon) and occurs virtually in all tissues. It

consists of two phases: a preparatory phase, where ATP is consumed and a pay-off phase where ATP is produced. During fasting, low-carbohydrate diets, or intense exercise, gluconeogenesis occurs, mainly in the liver and kidney. It is a process by which glucose is synthesized from non-carbohydrate carbon substrates such as pyruvate, glycerol, alanine and lactate. These substrates mainly come from the breakdown from amino acids and lactate, transported to the liver by the bloodstream. This pathway consists of a series of enzyme-catalyzed reactions and begins in the mitochondria or cytoplasm, dependent on the substrate being used (Nordlie et al., 1999). The process of gluconeogenesis uses most of the reactions of glycolysis reversely to this pathway to re-synthesize glucose.

In the first step in glycolysis, glucose is phosphorylated into glucose-6-phosphate (G6P) by hexokinases. For glycolysis, G6P is then rearranged into fructose 6-phosphate (F6P) by glucose phosphate isomerase and phosphofructokinase converts F6P into fructose-1,6-biphosphate, which eventually leads to the formation of phosphoenolpyruvate (PEP). Pyruvate kinase then converts PEP into pyruvate.

Glycolysis process continues as the one of the two methods: aerobic metabolism of glucose or anaerobic glycolysis. If there is oxygen, the process continues into aerobic respiration, where pyruvate is further metabolized to acetyl-CoA and CO_2 in the mitochondria in a process called oxidative decarboxylation (Voet and Voet, 2003). Then acetyl-CoA could enter the tricarboxylic acid cycle (TCA cycle), or the Krebs cycle and get completely oxidized into CO_2, water. The anaerobic glycolysis usually occurs when there is no oxygen. In this case, pyruvate is converted to lactate, by lactate dehydrogenase.

One common substrate for gluconeogenesis is lactate, which is produced in the muscle in the absence of oxygen. The lactate is released to the blood, and then transported into the liver and is converted to pyruvate, then into glucose, which goes back into the bloodstream and returns to the muscle. This is termed the “Cori cycle” (Garrett and Grisham, 2010). Gluconeogenesis begins with the formation of oxaloacetate by the carboxylation of pyruvate in the mitochondria. Oxaloacetate is decarboxylated and then phosphorylated to form PEP using the enzyme phosphoenolpyruvate carboxykinase (PEPCK) in cytosol. This is the first

rate-limiting step in gluconeogenesis. The next steps are the same as reversed glycolysis, however fructose-1,6-bisphosphatase (another rate-limiting enzyme) converts fructose-1,6-bisphosphate to F6P. Finally, glucose is produced when G6P is hydrolyzed by glucose-6-phosphatase (G6Pase), which is the last rate-limiting enzyme (Barthel et al., 2001) (Figure 1).

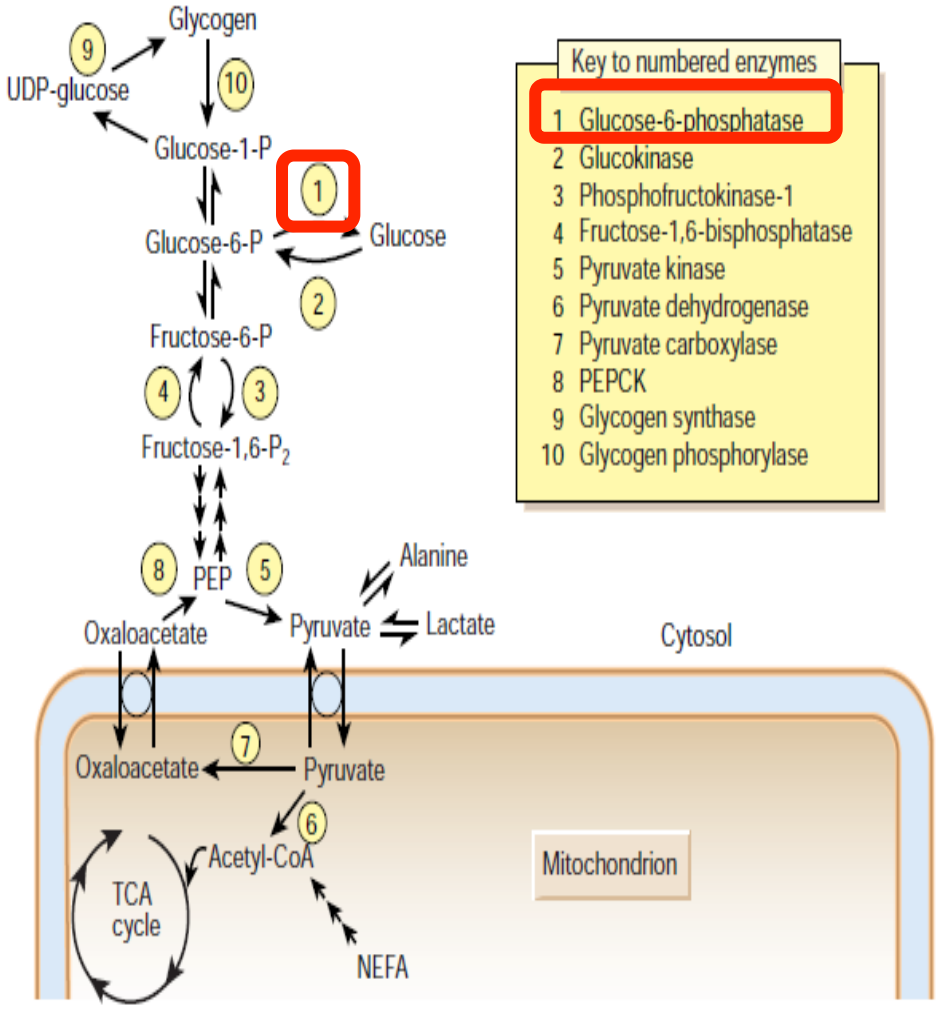

Figure 1: Glucose metabolism in liver (Moller, 2001).

1.2.2.2 Glycogenolysis and glycogenesis

Glycogen is a highly branched polymeric, formed by basic monomer glucose. Glycogenesis is the process of glycogen synthesis glucose storage. Glucose is metabolized to G6P by hexokinase or glucokinase (Vinuela et al., 1963), and then converted to glucose-1-phosphate by phosphoglucomutase. Through UDP-glucose pyrophosphorylase, glucose-1-phosphate then is converted into UDP-glucose. Glycogen synthase (GS) then catalyzes the reaction between UDP-glucose and a pre-existing glycogen primer, forming a chain (Voet and Voet, 2003). Then glycogen branching enzyme transfers the glucan chains into branches and glycogen (Voet and Voet, 2003).

The first step is the conversion from glycogen to glucose-1-phosphate by glycogen phosphorylase and glucose-1-phosphate is then converted to G6P by phosphoglucomutase (Voet and Voet, 2003). In hepatocytes, the phosphate group of G6P is finally removed by G6Pse (Nordlie et al., 1999), which is not presenting in muscle. The free glucose exits through glucose transporters of the cell into the bloodstream for uptake by other cells.

These hepatic functions are under tight metabolic control by hormones such as insulin, glucagon and epinephrine. In the fasting state, glucagon (released when blood glucose levels are low) binding to a G protein coupled-receptor triggers phosphorylation of enzymes and regulatory proteins by cAMP-dependent protein kinase (Protein Kinase A, PKA) (Wang et al., 2012). This results in an inhibition of glycolysis and concurrent stimulation of gluconeogenesis in the liver, providing the glucose needed for the brain and other tissues. Conversely, in the fed state, secreted insulin can suppress glucose production in liver cells by the inhibition of gluconeogenesis and glycogenolysis and induce glycogenesis.

The transcriptional coactivator peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) functions as a central regulator of gluconeogenesis by binding to several factors, including hepatic nuclear factor 4α (HNF4α) (Rhee et al., 2003) and a transcription factor called forkhead box o protein (Foxo-1) (Puigserver et al., 2003), lying upstream of several genes encoding gluconeogenic enzymes. Foxo1 plays an important role in the control of several key gluconeogenic enzymes,

including G6Pase and PEPCK. One mechanism by which insulin signaling suppresses gluconeogenesis is through phosphorylation of Foxo-1 by Akt and its subsequent exclusion from the nucleus, thus inactivating the transcription of these rate-controlling enzymes of gluconeogenesis (Hall et al., 2000).

For the induction of glycogenesis, insulin activates Akt resulting in the dephosphorylation and activation of GS through the phosphorylation and inhibition of glycogen synthase kinase-3 (GSK-3) (Ciaraldi et al., 2010; Ciaraldi et al., 2007; Nikoulina et al., 2002).

1.3 Lipid metabolism

Lipids are hydrophobic compounds, insoluble in aqueous solutions and soluble in organic solvents. Triglycerides (TGs) mainly stored in adipose tissue, for energy storage in humans. In non-adipose tissues, such as skeletal muscle, excessive TGs are associated with insulin resistance and can lead to T2D.

Foods travel to the stomach and are digested into compounds, including TGs, protein and glucose. TGs are emulsified by bile salt to facilitate the hydrolyzation to monoglycerides, FFAs and glycerol by pancreatic lipase (Mattson and Beck, 1955) in the small intestinal. Then the formations of micelles with bile salt facilitate the transport and delivery of monoglycerides, FFAs to the intestinal epithelial cells, where they are absorbed through the simple diffuse.

After entering the epithelial cell, the FFAs and monoglycerides are taken up by the endoplasmic reticulum, and here they are mainly re-esterified into new TGs. TGs are then packaged with apoprotein into blood soluble chylomicrons (CMs). CMs are excreted and travel through the bloodstream into adipocyte for storage or to muscle cells to oxidation as fuel for energy.

Adipose tissue stays in a dynamic state with continuous synthesis and breakdown of TGs. When energy is required, free fatty acids (FFAs) are released into the blood by cleaving off the glycerol from TGs, a process called lipolysis. The oxidation of fatty acids provides 9 kcal/g while the breakdown of glucose and proteins provide 4 kcal/g. In contrast, lipogenesis is a process that stores extra energy.

1.3.1 Lipogenesis

Lipogenesis is a metabolic process that converts carbohydrates to fatty acids and synthesizes TGs through the reaction of FFAs with glycerol, in adipose tissue and non-adipose tissue, such as liver.

Fatty acids synthesis builds up by the addition of two carbon units in the cytoplasm staring with acetyl-CoA, through fatty acid synthase (FAS) (Chirala and Wakil, 2004). After lipogenesis, the TGs are packaged as lipoprotein and secreted by the liver or could store energy in adipose tissue for long term.

FFAs and glycerol can be released from TGs of CMs and very-low-density lipoproteins (VLDLs) by lipoprotein lipase (LPL) (Eckel, 1989; Wood et al., 2011) secreted by adipocytes on the wall of blood vessel. After entering into the adipocytes, FFAs can be stored as TGs again in the adipose tissue (lipogenesis).

FFAs are then transported across the membrane into the cytoplasm of the cell. Through two main pathways (glycerol-3-phosphate pathway and monoacylglycerol pathway (MAG)) (Shi and Burn, 2004), as shown in Figure 2.

Glycerol-3-phosphate is esterified by glycerol 3-phosphate acyltransferases (GPAT) (Monroy et al., 1973; Wendel et al., 2009) producing 1-acylglycerol 3-phosphate (lysophosphatidate, LPA). 1-Acylglycerol-3-phosphate acyltransferase (AGPAT) (Cattaneo et al., 2012) catalyzes the esterification of LPA forming phosphatidate (PA). The phosphatidate phosphatases (PAP) then dephosphorylate PA producing 1,2-diacylglycerol (DAG), which can then be used to synthesize TGs. DAG can also be generated via the monoacylglycerol acyltransferase (MGAT) pathway through the acylation of MAG. The last step of the lipogenesis is catalyzed by the enzyme diacylglycerol acyltransferase (DGAT) (Harris et al., 2011a) at the endoplasmic reticulum to form an ester bond.

1.3.2 Lipolysis

Lipolysis occurs during various conditions, including stress, exercise, and fasting, and uncontrolled diabetes mellitus. Several hormones (e.g., glucagon and epinephrine) increase cyclic adenosine monophosphate (cAMP) levels, which induce PKA to phosphorylate hormone sensitive lipase (HSL), thus activating it (Shi and

Burn, 2004). Released TGs are then catabolized by HSL, adipose triglyceride lipase (ATGL), and monoglyceride lipase (MGL) to generate FFAs and glycerol, as shown in Figure 2. Lipolysis products serve for energy production, cellular signaling, or lipid synthesis.

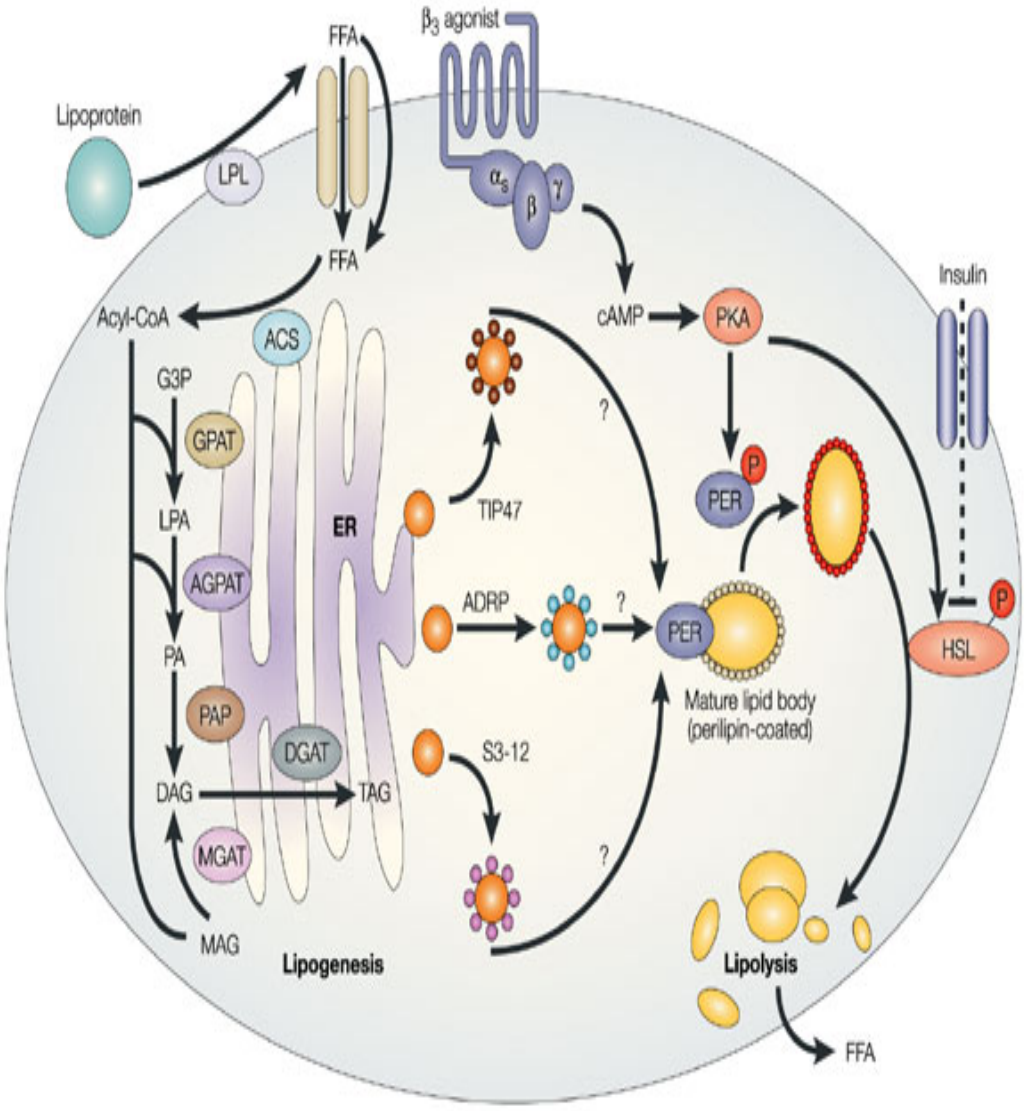

Figure 2: Lipogenesis and lipolysis (Shi and Burn, 2004).

1.3.3 Adipogenesis

Two distinct phases of adipogenesis are identified: determination of adipocytes from multipotent stem cells, (such as mesenchymal stem cells (MESCs)) and, terminal differentiation of pre-adipocytes into mature adipocytes.

For *in vitro* bioassays, preadipocytes, such as 3T3-L1 cells, growth arrest (near confluence) are firstly required to start cell differentiation. After growth arrest at confluence, preadipocytes receive an appropriate mitogenic and adipogenic signals to continue differentiation, such as a mix of inducers, including insulin, dexamethasone (DEX), as well as 3-isobutyl-1-methylxanthine (IBMX). These inducers activate the insulin, glucocorticoid and cAMP-signaling pathways, respectively (Feve, 2005).

After induction, grow-arrested cells undergo several rounds of DNA replication and cell division known as mitotic clonal expansion, which is accompanied by induction of CCAAT-enhancer-binding protein (C/EBP) β and C/EBP δ (Tang and Lane, 2012).

Growth arrest and clonal expansion are then followed by early and late differentiation. C/EBP and peroxisome proliferator-activated receptor γ (PPAR γ) are induced during early adipocyte differentiation.

Preadipocyte factor-1 (Pref-1), a recently described preadipocyte protein, was reported to be involved in maintaining the preadipocyte phenotype with high expression in preadipocyte and not detectable in mature adipocyte (Smas et al., 1997; Smas and Sul, 1993).

Then during the late differentiation, adipocytes markedly increase lipogenesis and acquire sensitivity to insulin, with increased expression of C/EBP α, sterol regulatory element-binding proteins-1 (SREBP-1) and PPAR γ (Loftus and Lane, 1997). These central transcriptional regulators of adipogenesis drive several adipocyte-specific gene expressions.

PPAR γ can be thought of as a “master” gene regulator of adipocyte differentiation, being both necessary and sufficient to drive conversion of fibroblastic precursors into fat cells (Tontonoz et al., 1994). Thus preadipocytes lose their fibroblastic morphology, increase lipogenesis and accumulate triglycerides, finally

become mature adipocytes and start to express characteristic adipocyte markers, such as fatty acid-binding protein 4 or adipocyte protein 2 (FABP4 or aP2), fatty acid transport protein (FATP) (Chui et al., 2005; Frohnert et al., 1999; Motojima et al., 1998) to promote FA and glycerol transport (Kishida et al., 2001), acetyl-CoA carboxylase (ACC), FAS (for FFA synthesis and lipogenesis) (Castelein et al., 1994; Jitrapakdee et al., 2005), and mitochondrial biogenesis and PGC-1α and carnitine palmitoyltransferase-1 (CPT-1, mediates the transport of fatty acids across the membrane for oxidation) for FFA oxidation (Bogacka et al., 2005a; Bogacka et al., 2005b).

1.4 Insulin receptor and insulin pathway

The insulin receptor is a heterotetrameric transmembrane protein receptor composed of two α subunits and two β subunits, linked by disulphide bonds. It belongs to the tyrosine kinase receptor family. Insulin or insulin-like growth factor (IGF) binds to the extracellular α subunits and brings them closer to trigger the conformational changes (Saltiel and Kahn, 2001). This enables ATP binding to the β-subunits and facilitates autophosphorylation (intrinsic tyrosine kinase activity) of various tyrosine residues and activation of the extrinsic tyrosine kinase activity of the receptor.

This autophosphorylation creates several binding sites in the receptor and promotes the recruitment of phosphotyrosine binding (PTB) domain containing substrate proteins, such as Src, Grb2, Shc, Gab-1, Cbl, APS, and the insulin receptor substrate (IRS) proteins (Kimura et al., 2002). The subsequent tyrosine phosphorylation and activation of these substrates enables them to working as docking sites for downstream effectors with Src homology 2 domain (SH2) (Kimura et al., 2002). This triggers three signaling pathways: the mitogen-activated protein kinase (MAPK) pathway, the PI3K pathway, and the CAP/Cbl/ TC10 pathways, which involving in mediating the variety of responses to insulin (Saltiel and Kahn, 2001).

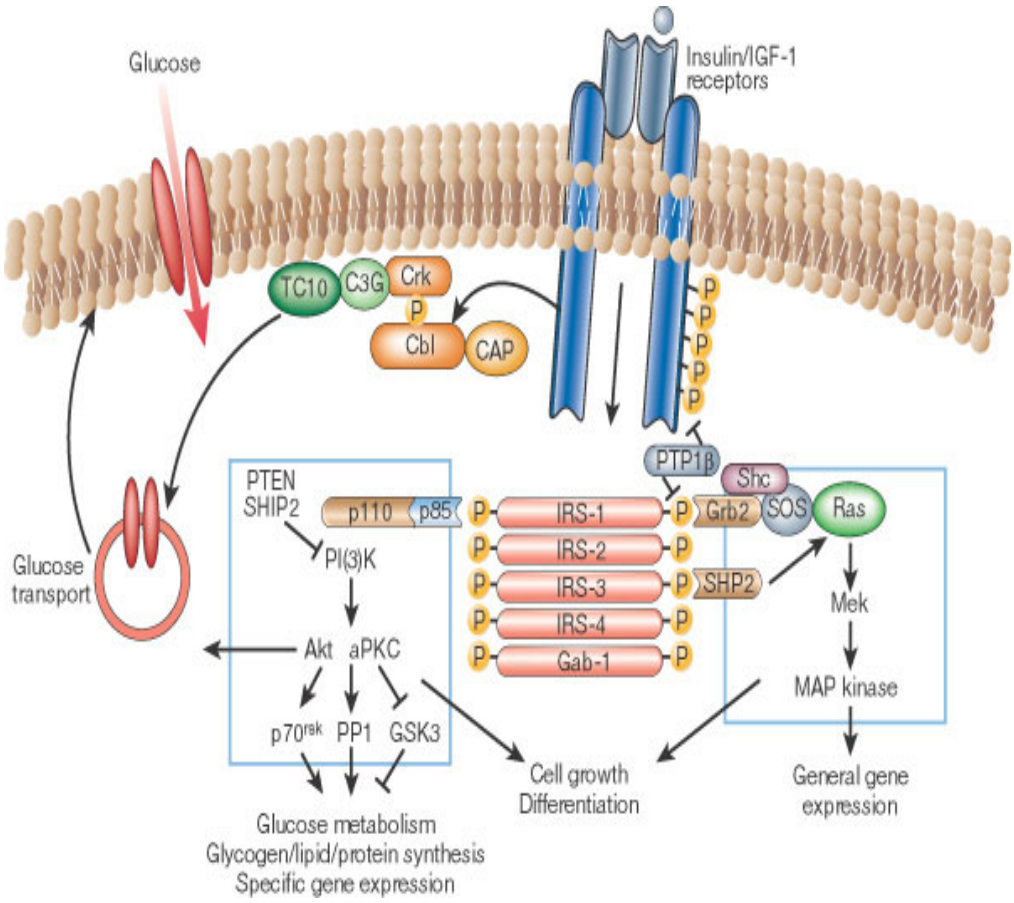

Figure 3: Insulin signaling pathways (Saltiel and Kahn, 2001).

1.4.1 PI3K pathway

The PI3K pathway plays a central role in the metabolic actions of insulin and controls gluconeogenesis, glycogen and protein synthesis as well as glucose transport through GLUT4 translocation (Kido et al., 2001). It consists of a p110 catalytic subunit and a SH2 domains containing p85 regulatory subunit. Binding through the SH2 domain to IRS-1 leads to the phosphorylation and activation of PI3K. After activation, PI3K can convert the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). The increase in intracellular PIP3 leads to activation of the PI-dependent kinase-1 and -2 (PDK1 and PDK2), which phosphorylate the protein kinase B (PKB or /Akt) on amino acid residues threonine 308 and serine 473 (Hill et al., 2002; Stokoe et al., 1997), leading

to its activation. Akt has been shown to mediate actions of insulin on GSK-3, the mammalian target of rapamycin (mTOR) and others such as PEPCK, GLUT4. PI3K/PDK1 also phosphorylate and activate atypical isoforms of PKC zeta and PKC delta (Chou et al., 1998; Le Good et al., 1998).

1.4.2 MAPK pathway

The MAPK pathway is involved in mitogenic cellular responses, cell growth and survival. Insulin triggers the MAPK pathway upon translocation and binding of cytosolic adaptor protein growth factor receptor binding protein 2 (Grb2) to phosphorylated Shc or IRS proteins via its SH2 domain. Grb2, through its SH3 domain, is then able to bind to mammalian son of sevenless (SOS), a guanyl nucleotide exchange factor (GEF) that catalyzes the exchange of guanosine diphosphate (GDP) for GTP on Ras (a small GTP binding protein), thus resulting in activation of Ras (Vanderkuur et al., 1997). Upon activation, Ras binds the protein kinase Raf, recruiting Raf to the plasma membrane, which results in Raf activation (Avruch et al., 2001). Ras-Raf then activates MAP kinase-kinase (MEK) and MAP kinases ERK1 and ERK 2 (Vanderkuur et al., 1997). The activated MAPKs enter the nucleus and phosphorylate various nuclear transcription factors to elicit their actions.

1.4.3 CAP/Cbl/ TC10 pathways

This signalling cascade is initiated when the activated insulin receptor recruits and activates the adaptor proteins (APS) binding Cbl, Cbl and Cbl-associated protein (CAP) (Onnockx et al., 2009). CAP belongs to a family of adapter proteins with common SH3 domains and sorbin homology (SoHo) domain. Upon phosphorylation, the Cbl–CAP complex translocates to lipid raft domains (in the plasma membrane), by the interaction of the SoHo domain in CAP with the flotillin protein (Baumann et al., 2000) (Chang et al., 2004). Translocation of Cbl then recruits the adapter protein Crk and the GEF C3G to the membrane, where C3G catalyses the exchange of GTP in place of GDP on G protein TC10 (Chang et al., 2004). Once activated, GTP-bound TC10 provides another signal to the GLUT4 protein and promotes its translocations to the membrane, in parallel with the activation of the PI3K pathway.

1.5 Other mechanisms involved in energy metabolism:

1.5.1 AMPK pathway

AMPK is a "metabolic master switch", which regulates whole-body and cellular energy metabolism, activated in condition of intracellular ATP depletion, including fasting, heat shock, hypoxia and exercise. The activation of AMPK regulates many downstream pathways to inhibit energy-consuming anabolic pathways (such as fatty acid and cholesterol synthesis, gluconeogenesis, protein synthesis and insulin secretion from β-cell) and accelerate ATP-producing catabolic ones (such as glucose uptake, fatty acid uptake and oxidation and mitochondrial biogenesis), as shown in Figure 4 (Hardie, 2011; Towler and Hardie, 2007).

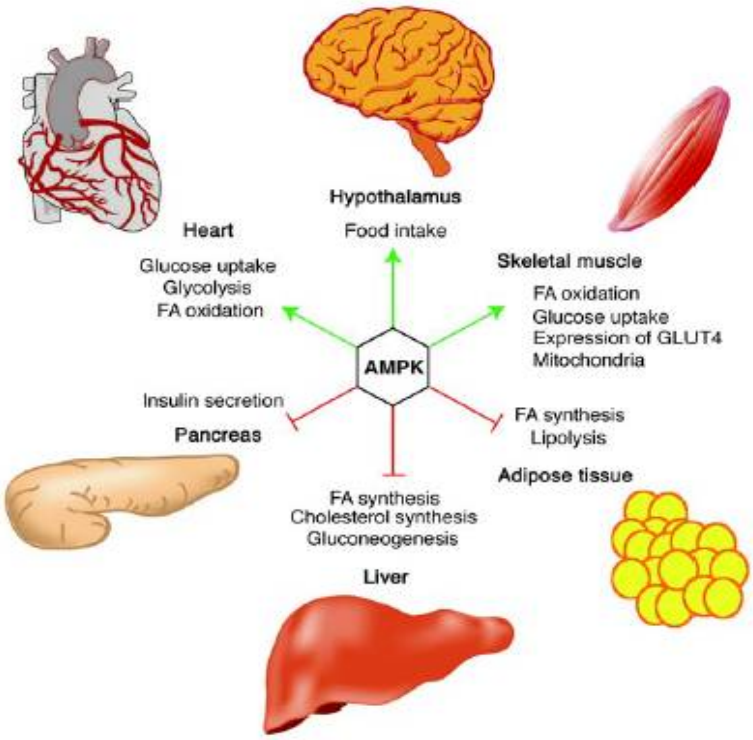

Figure 4: AMPK functions involved in energy metabolism (Hardie, 2004).

AMPK is a heterotrimeric protein, composed of a catalytic (α) and two non-catalytic (β and γ) subunits (Xiao et al., 2011). When the AMP to ATP ratio increases, AMP binds to both Bateman domains and leads to a conformational

change in the γ subunit, which exposes the catalytic domain of the α subunit. Moreover, as an allosteric activator, AMP also could make AMPK a poorer substrate for dephosphorylation.

AMPK becomes activated when phosphorylated at threonine 172 by an upstream AMPK kinase (AMPKK) and inactivated by dephosphorylation (Oakhill et al., 2011; Xiao et al., 2011). One kinase activator of AMPK is Ca^{2+}-calmodulin-dependent kinase kinase β (CaMKKβ) (Oakhill et al., 2011), which responses to increased calcium and is primarily expressed in the brain. The serine/threonine liver kinase B 1 (LKB1) is required for the activation of AMPK (Oakhill et al., 2011). The active LKB1 is actually a complex of three proteins, including LKB1, Ste20-related adaptor (STRAD) and mouse protein 25 (MO25). Phosphorylation of AMPK by LKB1 also occurs on T172. LKB1 is widely expressed, and it is the primary AMPK-regulating kinase. AMPK could also be activated by receptors that are coupled to phospholipase C-γ (PLC γ) and by adipokines such as adiponectin and leptin (Carling et al., 2008).

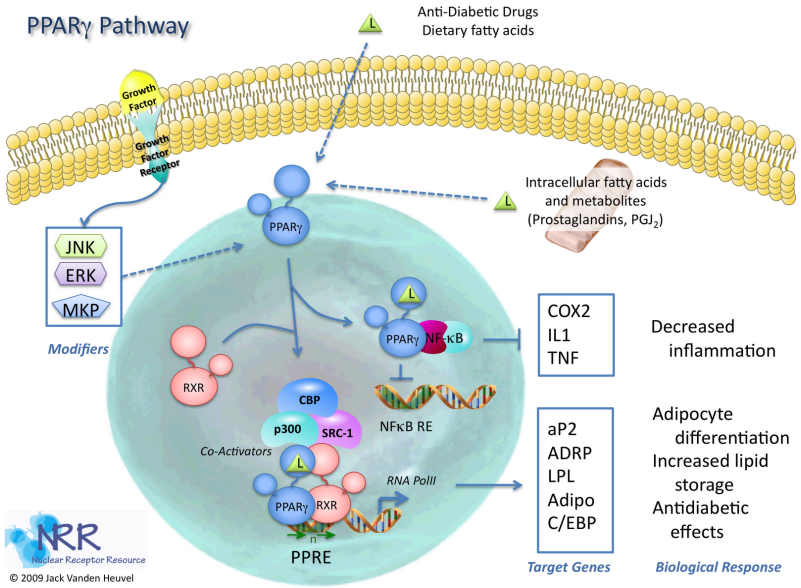

Figure 5: PPAR γ activation and mechanisms involved in glucose and lipid metabolism. Adapted form http://inflammablog4.blogspot.ca/2011/05/ppar-gamma-pathway-i-included-this.html

1.5.2 PPAR γ and regulation of lipid homeostasis

When PPAR γ is bound with a ligand or other agonist, it becomes active and complexes with another transcription factor; namely, retinoid X-receptor (RXR) (Chandra et al., 2008). The complex PPAR-γ-RXR, with several co-activators is then bound to a specific DNA motif, known as peroxisome proliferate response element (PPREs) in the promoters of target gene (Lenhard, 2001), as shown in Figure 5.

Activation of PPAR γ regulates a variety of adipocyte genes involved in virtually all pathways of lipid metabolism to increase FFAs influx to adipocyte and storage as TGs: LPL to promote FFAs release from circulating lipoproteins (Schoonjans et al., 1996), low density lipoprotein (LDL) receptor, fatty acid-binding protein 4 or adipocyte protein 2 (FABP4 or aP2), CD36, fatty acid transport protein (FATP) (Chui et al., 2005; Frohnert et al., 1999; Motojima et al., 1998) to promote FA and glycerol transport (Kishida et al., 2001), PEPCK to enhance glyceroneogenesis (Tontonoz et al., 1995), acetyl-CoA carboxylase (ACC), FAS (for FFA synthesis and lipogenesis) (Castelein et al., 1994; Jitrapakdee et al., 2005), and mitochondrial biogenesis and PGC-1α and carnitine palmitoyltransferase-1 (CPT-1, mediates the transport of fatty acids across the membrane for oxidation) for FFA oxidation (Bogacka et al., 2005a; Bogacka et al., 2005b).

PPAR γ activation functions as an “adipose remodeling factor” that could redistribute lipids from non adipose tissue and insulin-resistant adipocytes into differentiated and insulin-sensitive adipocytes by enhancing adipogenesis, regulating adipokines, increasing adiponectin, decreasing leptin, tumor necrosis factor-α (TNF-α) and FFAs and exerting insulin-sensitizing effects (Bermudez et al., 2010; Lenhard, 2001) as shown in Figure 6. The typical adipocyte remodeling induced by PPAR-γ agonist involves an increased number of small insulin-sensitive adipocytes and fewer large insulin-resistant adipocytes (Bergman et al., 2006; Laplante et al., 2003).

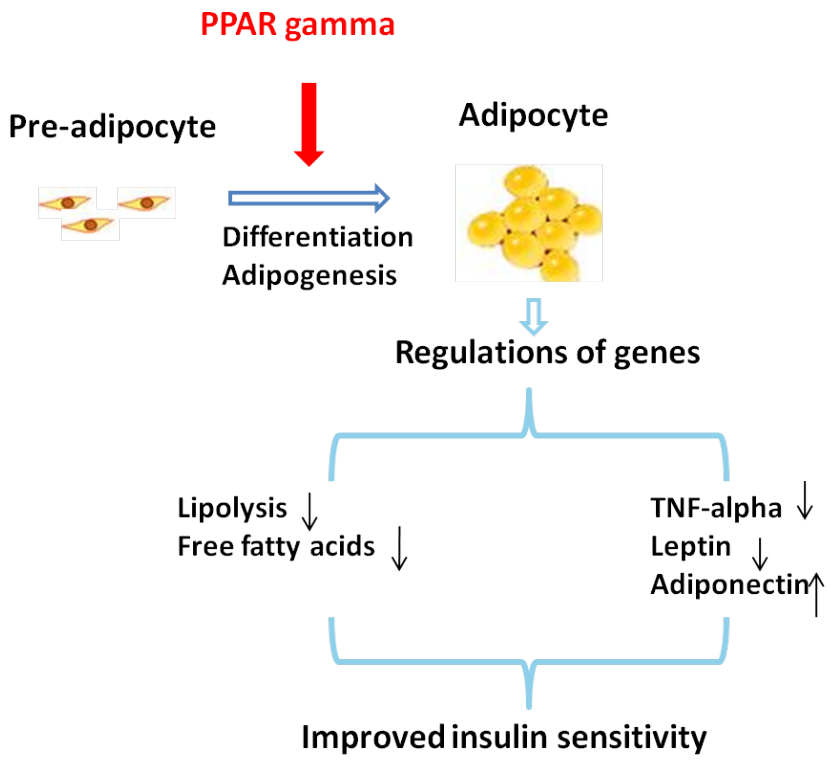

Figure 6: PPAR γ activation and improved insulin sensitivity.

1.6 Metabolic syndrome and obesity

1.6.1 Metabolic syndrome

Metabolic syndrome (MetS) in an individual reflects the clustering of multiple metabolic abnormalities, including central obesity, T2D, dyslipidemia, hypertension, and non-alcoholic fatty liver disease (NAFLD). The definition of the MetS determined by the International Diabetes Federation (IDF) includes central obesity (increased waist circumference (WC) or waist-to-hip ratio of over 102 cm and 0.90 for males, and of over 88 cm and 0.85 for females respectively) and at least 2 of the following: increased fasting serum glucose (over 5.6 mmol/L), TGs (over 150 mg/dL), blood pressure (over 130/ 85 mm Hg), or a low HDL cholesterol concentration (under 40 mg/dL in males, under 50 mg/dL in females) (Alberti et al.,

2005). Other MetS definitions have been put forth by the National Cholesterol Education Program-Adult Treatment Panel III (ATPIII) (Grundy et al., 2005) and the WHO (Alberti and Zimmet, 1998), who both imparted similar risks to MetS for developing cardiovascular disease and diabetes.

1.6.2 Obesity

Obesity is defined as excessive fat accumulation, which may impair health. Body mass index (BMI) is commonly used to determine obesity in adults, which is defined as a person's weight divided by the square of the height (kg/m2) (WHO, 2012). The individual with BMI greater than 30 is considered to be obese (WHO, 2012). Obesity, particularly central or visceral obesity, is associated with several diseases, including cardiovascular disease, diabetes and NAFLD.

Indeed, many investigations demonstrated that excess in visceral adipose tissue (VAT) or abdominal adipose tissue (known as central obesity), but not that in subcutaneous adipose tissue, was characterized with adipocyte dysfunction and significantly correlated with insulin resistance, T2D, and cardiovascular disease (Banerji et al., 1999; Banerji et al., 1997; Fujimoto et al., 1999; Pouliot et al., 1992).

1.6.3 Adipocyte dysfunction, inflammation and insulin resistance

The importance of adipose tissue is confirmed by observations made in lipoatrophic diabetes in mice and human (Gavrilova et al., 2000) (Sovik et al., 1996). Such “individuals” have no white fat tissue (Reitman and Gavrilova, 2000). They also suffer from severe metabolic disorders (Sasaki et al., 1992), including insulin resistance, hyperglycemia, hyperlipidemia, and fatty livers. Therefore, having a proper quantity of functional adipose tissues is crucial (Gavrilova et al., 2000); too many (obesity) or too less (lipoatrophy) adipose tissue impairing energy homeostasis and insulin sensitivity.

As we discussed in lipid metabolism in chapter 1.3, adipocytes store energy as triglyceride (lipogenesis and adipogenesis) and release fatty acids (lipolysis) to the blood or to be used for energy through oxidation. Human adipocytes can grow up to 20 fold in diameter and several thousand-fold in volume (Jernas et al., 2006).

However when there is too much excess energy, adipocytes become too large (hypertrophy), and hypoxia occurs (Kabon et al., 2004; Ye et al., 2007) due to inadequate blood flow (West et al., 1987) in the expanded adipose tissue

Stressed and hypertrophied adipocytes secrete monocyte chemo-attractant protein-1 (MCP-1) and other chemo-attractants to increase macrophage infiltration (Harman-Boehm et al., 2007) into adipose tissue. Adipose tissue of lean subjects usually consists of less than 10% macrophages, whereas in obese patients, macrophage content can rise as high as 50% (Weisberg et al., 2003).

Infiltrating macrophage recruitment in turn results in adipocytes producing more pro-inflammatory adipokines and cytokines, such as TNFα (Hotamisligil et al., 1993). This low-grade chronic inflammation decreases energy storage and increases lipolysis (Xu et al., 2003). The excess FFAs (Horowitz and Klein, 2000) and altered adipokines result in the accumulation of lipids in non-adipose tissue, including the liver and muscle, causing lipotoxicity and impairing the normal metabolism and insulin sensitivity.

1.6.4 Adipose tissue and adipokines

Adipose tissue is not only the main site for energy storage and a master regulator of whole-body lipid flux (by lipogenesis and fatty acid releasing and oxidation), but also functions as an endocrine organ. It modulates both glucose and lipid metabolism in other tissues by secreting a number of adipokines, including adiponectin, leptin, resistin, TNF-α, interleukin (IL)-1, and IL-6.

Leptin plays a key role in inhibiting energy intake and promoting energy expenditure by acting on the receptors in the central nervous system. Humans and mice lacking leptin demonstrate obesity. Obesity and T2D are often associated with leptin deficiency or resistance, deregulating satiety signals (Considine et al., 1996), and are characterized by an elevated leptin-to-adiponectin ratio.

Adiponectin (also called Acrp30 or adipoQ) is a fat cell-derived peptide and modulates glucose and fatty acid handling (Diez and Iglesias, 2003). Adiponectin is an insulin-sensitizing hormone with antidiabetic, anti-inflammatory and anti-atherosclerotic properties (Diez and Iglesias, 2003; Ukkola O, 2002). Acute treatment

of mice with adiponectin decreases insulin resistance, plasma FFAs and the TGs content of muscle and liver, while increasing expression of genes involved in fatty-acid oxidation and energy expenditure (Yamauchi et al., 2001).

Inflammatory cytokines such as TNF-α are also adipocyte derived. The serum concentration of TNF-α is increased in obesity and is associated with chronic and low-grade inflammation of metabolic disease. TNF-α could induce insulin resistance by phosphorylation of IRS-1 at serine sites (Krogh-Madsen et al., 2006).

1.7 Diabetes mellitus

1.7.1 Diabetes definition and diagnosis

As one of the chronic metabolic disease, diabetes mellitus is characterized by hyperglycemia. It might be caused by from impaired insulin secretion and/or insulin resistance (IR); the later being determined by impaired sensitivity of insulin in target organs (adipose tissue, liver, and muscle) (DeFronzo and Ferrannini, 1991).

According to WHO, diabetes is diagnosed when fasting plasma glucose levels reach higher than 7.0 mmol/L (126 mg/dL) or when plasma glucose reaches higher than 11.1 mmol/L (200 mg/dL) 2 hours after an oral glucose tolerance test (OGTT) (WHO, 1999). There are also two pre-diabetic states. Impaired fasting glucose (IFG) is recognized when fasting glucose levels range from 6.1 to 6.9 mmol/L (110 to 125 mg/dL), whereas impaired glucose tolerance (IGT) is characterized by plasma glucose levels between 7.8 mmol/ L to 11.1 mmol/L (140 mg/dL to 200 mg/dL) 2 hours after OGTT (WHO, 1999).

1.7.2 Diabetes classification

Diabetes is classified into four categories: type 1 diabetes; type 2 diabetes; type 3 or others specific types; and type 4, gestational diabetes (ADA, 2006, 2011). They differ in terms of their causes and in their distribution in different populations. However, each type of diabetes will result in insufficient production and/or action of insulin to reduce blood glucose and will lead to hyperglycemia.

1.7.2.1 Type 1 diabetes (T1D)

This form of diabetes, previously called insulin-dependent diabetes or juvenile diabetes, accounts for only 5-10% of cases and the majority of which are found in children (ADA, 2011). T1D is caused by β cell destruction and absolute deficiency of insulin secretion, involving cellular mediated autoimmune or idiopathic origins (Skyler, 2011).

Most patients are otherwise healthy and not obese when onset occurs. Irregular hyperglycemia and ketoacidosis sometimes accompany newly recognized or untreated T1D. This type of diabetes is treated with exogenous insulin therapy, along with lifestyle management.

1.7.2.2 Type 2 diabetes (T2D)

T2D, formerly referred to as insulin-independent diabetes, is the most common type and accounts for 90-95% of all the diabetes cases (ADA, 2011). It is characterized by insulin resistance associated with relative (rather than absolute) insulin deficiency. Most patients with T2D are obese, and others who are not obese may have metabolic disorders and increased abdominal fat.

This category goes undiagnosed for a long period of time because it develops gradually and the degree of hyperglycemia may be not sufficient to cause clinical symptoms in the early stage. The risk of developing T2D increases with obesity, age and lack of physical activity (Ansari, 2009; Colberg et al., 2010). Most patients need lifestyle modification as well as oral hypoglycemic drugs to control blood glucose. Insulin therapy may be not required initially but regularly occurs when β cell mass becomes insufficient as described further below.

1.7.2.3 Type 3 diabetes

Others specific types of diabetes are less common and can result from a variety of conditions, including specific genetic defects, endocrinopathies, drugs, infections, and other illnesses. For example, maturity onset diabetes of the young (MODY) might be caused by monogenetic defects (ADA, 2011). MODY 3, MODY 2 and MODY 1 are the most common forms of MODY, which are caused by

mutation of HNF 4A, glucokinase and HNF 1 A genes respectively (Bell and Polonsky, 2001; Fajans, 1990).

1.7.2.4 Type 4 diabetes

Gestational diabetes mellitus (GDM) is diabetes with hyperglycemia, which is first recognized during pregnancy. It increases the risk of complications for both mother and neonate. It occurs in about 2%–10% of all pregnancies and is associated with the placental hormones being implicated in the development of insulin resistance (Desoye and Hauguel-de Mouzon, 2007). While GDM may go away after pregnancy, women who have had GDM have great risks of developing diabetes (mainly T2D) later in life (Kim et al., 2007; Oubré et al., 1997).

1.7.3 Pathogenesis of T2D

A variety of pathogenic processes are believed to be involved in the development of T2D. They range from insulin resistance (IR) in the complex pathways of the hormone action, to β cell failure in the pancreas with consequent insulin deficiency.

T2D is considered to be a chronic and progressive disease. In the early stages of T2D, the abnormal high blood glucose is compensated by increased insulin production and secretion by β cells, generating hyperinsulinemia and termed β cell compensation. As the disease progresses, β cells become unable to secret enough insulin for the body's needs and β cells failure occurs. This failure includes loss of β cells mass due to apoptosis (Butler et al., 2003) and secretory defect. Several factors are involved, such as genetic determinants, chronic inflammation, glucotoxicity and lipotoxicity (Buchanan, 2003).

Insulin resistance is characterized with an impaired biologic response to either exogenous or endogenous insulin. So in IR patients, insulin fails to promote peripheral glucose utilization through glucose uptake (Utriainen et al., 1998) in muscle and adipose tissue; unable to suppress hepatic glucose production (Vaag et al., 1995); and fails to stimulate TGs production and to decrease lipolysis thereby resulting more FFAs efflux from adipocytes (Kelsey et al., 2013).

The majority of T2D patients are overweight or obese; however not all obese persons have insulin resistance. About 50% of overweight and 30% of obese adults are metabolically healthy in the USA (Wildman et al., 2008); that is, they do not have diabetes or exhibit disturbances in glucose and lipid metabolism. In reverse, such metabolic abnormalities occur in 23.5 % of people with normal body weights. So it is now commonly accepted that chronic and low-grade inflammation associated with visceral obesity (bad fat or abdominal fat, more details discussed in 1.6.2 and 1.6.3) are involved in insulin resistance and T2D.

The cellular and molecular mechanisms underlying IR are still not fully understood. Several molecular mechanisms may contribute to IR, specifically in insulin responsive tissues such as liver, muscle and adipose tissue. These include decreases in 1) insulin receptor expression and tyrosine kinase activity; 2) the expression and phosphorylation of IRS-1/2; and 3) PI3K activity. It is reported that decreased expression of IRS-1 protein and especially increased IRS-1 serine phosphorylation is associated with IR (Anai et al., 1998; Goodyear et al., 1995; Kerouz et al., 1997; Saad et al., 1992; Stephens et al., 1997). The serine phosphorylation of IRS proteins reduces the ability of IRS proteins to attract PI3K. This impairs downstream effectors (Aguirre et al., 2002) and can also accelerate the degradation of the IRS-1 protein (Shah et al., 2004). Several serine/threonine kinases have been shown to phosphorylate IRS-1 within various inflammatory signalling pathways, such as c-Jun N-terminal kinase (JNK1), IκB kinase-β (IKK-β), or PKC. Furthermore, suppressor-of-cytokine-signalling (SOCS) proteins, upregulated by the cytokine IL-6, interfere with insulin signaling by promoting the degradation of IRS-1 and IRS-2 (Wellen and Hotamisligil, 2003).

1.7.4 Diabetes symptoms and complications

Symptoms of marked hyperglycemia include polyuria, polydipsia, and polyphagia accompanied by weight loss as well as blurred vision and others. Acute, consequences of uncontrolled diabetes are hyperglycemia with ketoacidosis and the nonketotic hyperosmolar syndrome (Kitabchi et al., 2001; Peden et al., 1984), which are two acute complications of diabetes that can result in increased morbidity and

mortality. Diabetes ketoacidosis is characterized by hyperglycemia, metabolic acidosis, especially high level of total body ketone, while hyperglycemia hyperosmolar syndrome is characterized by hyperglycemia and high osmolality without ketoacidosis (Gerich et al., 1971).

Long-term chronic complications of diabetes are divided into microvascular and macrovascular. Microvascular complications include retinopathy, nephropathy and neuropathy. These are recognized as the three major complications of diabetes and increase the risk of blindness, kidney failure or diabetic foot (ulcers and amputations) (ADA, 2011). Macrovascular complications include cardiovascular disease, cerebrovascular disease as well as peripheral vascular disease, leading to increased mortality and morbidity rates in T2D (ADA, 2011).

In the Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines (CIHR-ATTM) project (described further below), the following 15 symptoms and complications were used to ask informants about traditional remedies that would have beneficial potential for T2D: arthritis/rheumatism; frequent headaches; back and/or kidney pain; diarrhea; swelling and/or inflammation; general weakness; increased appetite; heart and/or chest pain; increased thirst; abscesses and/or boils; blurred vision; increased urination; foot numbness and/or foot sores; slow healing infections; and sore and/or swollen limbs (Leduc et al., 2006).

1.7.5 Diabetes treatment

Optimal glycemic control is the fundament and the main goal for the management of diabetes (Cade, 2008). For poorly controlled diabetes, excess glucose molecules enter into the red blood cells and attach to the hemoglobin to form glycated hemoglobin (A1C) through nonenzymatic reactions. A buildup of A1C stays for the lifespan of a red blood cell, so reflects blood glucose control for the past 2 to 3 months (Gonen et al., 1977). A1C test is considered to be a gold standard for long-term glycemic control (Nathan et al., 1984). The A1C concentration of 7.0% or less is recommended as a target for all patients with diabetes to reduce the risk of complications; and a target A1C concentration of 6.5% or less in T2D to further

reduce the risk of nephropathy if it can be safely achieved (Diabetes Care, 2009; Genuth et al., 2003).

According to Canadian Diabetes Association (CDA) (Canadian Diabetes Association Clinical Practice Guidelines Committee, 2008), individuals may require lifestyle modifications, as well as up to five groups of drugs to treat T2D and to prevent MetS and T2D associated complication, especially to reduce cardiovascular risk. These include: 1) oral hypoglycemia agents (OHAs); 2) insulin replacement therapy in more advanced stages; 3) lipid-lowering drugs (primarily statins) to reduce LDL-cholesterol, which is more susceptible to oxidation even at a normal level; 4) low-dose aspirin to prevent thrombosis; and 5) antihypertensive drugs, such as angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor antagonists (ARBs) to control blood pressure. Many also resort to some alternative medicines (Crawford, 2009; John et al., 2003; Vuksan et al., 2008).

1.7.5.1 Lifestyle interventions

Nonpharmacologic therapy, including a healthy diet, physical exercise, weight loss, smoke cessation and alcohol reduction, remains a critical component in the treatment of diabetes and should be superior to other treatment. It was reported that a 7% body weight loss in human subjects with T2D resulted in 59±21 % improvement in insulin sensitivity (Hays et al., 2008).

The optimal rate of weight loss is 1 to 2 kg/month and the modest weight loss of 5 to 10% of initial body weight (Goldstein, 1992) could substantially improve glycemic control, hypertension and dyslipidemia. It is recommended by CDA (Canadian Diabetes Association Clinical Practice Guidelines Committee, 2008) that Patients with diabetes should take aerobic exercise for at least 150 min per week (with no more than 2 consecutive days without exercise) and also be encouraged to perform resistance exercise 3 times per week.

1.7.5.2 Oral hypoglycemia agents

T2D can be delayed or prevented with lifestyle interventions or medication. But because lifestyle modifications are hard to instate and adhere to (Kravitz et al.,

1993; Kurtz, 1990), pharmacological treatment is often necessary to treat T2D and to achieve optimal glycemic control. OHAs can be used either alone, or combined with other OHAs or insulin. There are various classes of OHAs (such as α-glucosidase inhibitors, biguanides, insulin secretagogues, insulin sensitizers and others) now available to target different factors (Table 1) (Krentz and Bailey, 2005; Moller, 2001).

1.7.5.2.1 α -Glucosidase inhibitors

These drugs (Acarbose and Miglitol) competitively inhibit enzymes such as α-glucosidase in the intestinal brush border. The latter is responsible for the breakdown of oligosaccharides and disaccharides into monosaccharides suitable for absorption (Tundis et al., 2010). α-Glucosidase inhibitors thus delay the intestinal absorption of glucose and lower postprandial glucose levels. They are very useful when combination with other OHAs. The main side effects of these drugs are gastrointestinal upset, including diarrhea and flatulence.

Drug class	Molecular target	Site(s) of action	Adverse events
Insulin	Insulin receptor	Liver, muscle, fat	Hypoglycaemia, weight gain
Sulphonylureas (e.g. glibenclamide) plus nateglinide and repaglinide	SU receptor/ K^+ ATP channel	Pancreatic β-cell	Hypoglycaemia, weight gain
Metformin — biguanides	Unknown	Liver (muscle)	Gastrointestinal disturbances, lactic acidosis
Acarbose	α-glucosidase	Intestine	Gastrointestinal disturbances
Pioglitazone, rosiglitazone (thiazolidinediones)	PPARγ	Fat, muscle, liver	Weight gain, oedema, anaemia

Table 1: Current key therapeutic agents for type 2 diabetes (Moller, 2001).

1.7.5.2.2 Biguanides

There were several biguanides available in the market 30 years ago. However, all but metformin were discontinued due to the high risk of lactic acidosis (Bolzano, 1978). The mechanisms by which metformin exerts its antihyperglycemic effects are still not fully understood.

Its major action in patients with diabetes is to reduce body glucose production by suppressing hepatic gluconeogenesis (Kirpichnikov et al., 2002). Secondly, metformin enhances glucose uptake in skeletal muscle partly by stimulating the translocation of GLUT4 to the membrane (Lee et al., 2011). Moreover, metformin increases peripheral insulin sensitivity (Li et al., 2011; Tamura et al., 2008), increases fatty acid oxidation, reduces triglyceride levels (Collier et al., 2006), and decreases absorption of glucose from the gastrointestinal tract (Grzybowska et al., 2011; Wilcock and Bailey, 1991). The insulin pathway, AMPK activation, as well as other AMPK-independent mechanisms are involved in these therapeutic effects (Foretz et al., 2010).

Metformin is recommended as the first-line drug of choice for the treatment of T2D, in particular, in obese patients. It is also the only OHAs to show cardiovascular benefit in diabetes (Tankova, 2003). The most common adverse effect of metformin is gastrointestinal upset.

1.7.5.2.3 Insulin secretagogues

To stimulate insulin secretion, sulfonylureas (Gliclazide, glimepiride, glyburide) bind to the sulfonylurea receptor (SUR) on the surface of pancreatic β cells, which are coupled to subunits of an ATP sensitive potassium channel (Kir6.2) (Proks et al., 2002). The binding of a sulfonylurea to the sulfonylurea receptor results in the closure of the potassium channels, the depolarization of the cell membrane, and the opening of voltage-dependent calcium channels (Proks et al., 2002). The influx of calcium causes microtubules to contract and the exocytosis of insulin from secretory granules (Winkler and Gero, 2011). Non-sulfonylureas secretagogues (Meglitinides) have similar effects to sulfonylureas but bind to the receptor at a different site (Ojima et al., 2004). Hypoglycemia and weight gain are probably the main side effects for

these insulin secretagogues. The second generations of sulfonylureas are more effective and less associated with hypoglycemia than the first generations. They are useful only in T2D and cannot be used in T1D, since they work by stimulating endogenous release of insulin. But it also needs to concern the potential acceleration of β cell failure occurring as a result of β cell decompensation as described earlier.

1.7.5.2.4 Insulin sensitizers

Thiazolidinediones (TZDs, including troglitazone, rosiglitazone and pioglitazone) function as ligands for the PPAR γ, to improve insulin sensitivity. Through PPAR γ activation, TZDs improve insulin sensitivity partly by enhancing differentiation of pre-adipocyte to adipocyte to improve lipogenesis and avoid lipotoxicity in non-adipose tissue. They also reduce lipolysis and hence FFAs in circulation. Finally, they alter cytokine and adipokines secretion (Bermudez et al., 2010) from adipose tissue (notably, by decreasing TNF-α and leptin, and increasing adiponectin).

The major side effects of TZDs include water retention (Tang et al., 2003), edema, congestive heart failure (Hernandez et al., 2011; Toprani and Fonseca, 2011) and weight gain (Fonseca et al., 2013). Although the latter side effect appears counter productive (obesity being a prominent risk factor for T2D), beneficial fat distribution changes occur since there is less visceral or abdominal fat and more subcutaneous fat. Troglitazone was withdrawn due to high risk of hepatic damage and failure (Faich and Moseley, 2001; Graham et al., 2003); pioglitazone has been withdrawn in some countries due to high risk of bladder cancer (Lewis et al., 2011). Rosiglitazone was found to increase cardiovascular disease (Graham et al., 2010), therefore has been withdrawn in Europe and is used in significant restrictions in the US.

1.7.5.2.5 Others

Incretins are gastrointestinal peptide hormones and the short-lived activities of native incretins such as glucagon-like peptide 1 (GLP-1) are due to the rapid degradation by the dipeptidyl peptidase 4 (DPP4) enzyme. Unlike other secretagogues acting on the potassium channel, GLP-1 stimulates pancreas insulin

secretion by promoting proinsulin gene expression (Drucker et al., 1987) and β cells proliferations through GLP-1 receptor (GLP-1R). In addition to this, GLP-1 has also multi actions to treat T2D including, inhibiting glucagon secretion and reducing gastric emptying and thus induce satiety after nutrient ingestion (Ahren, 1998; Ahren and Schmitz, 2004) (shown in Figure 7). This prompted the development of GLP-1 analogues (exenatide and liraglutide) and DPP4-selective inhibitors (sitagliptin, vildagliptin, and linagliptin) (Behme et al., 2003; Doupis and Veves, 2008; Nauck et al., 2011) to address hyperglycemia.

Beyond the GLP-1 analogues and DPP4 inhibitors, there are other potential targets: SGLT2 (Idris and Donnelly, 2009); G-protein-coupled receptors (GPCRs) and partial agonists of PPAR γ as well as selective PPAR α/γ modulators. Indeed, canagliflozin (or Invokana) is the first in class SGLT2 targeting drug approved by the FDA (Traynor, 2013) this March.

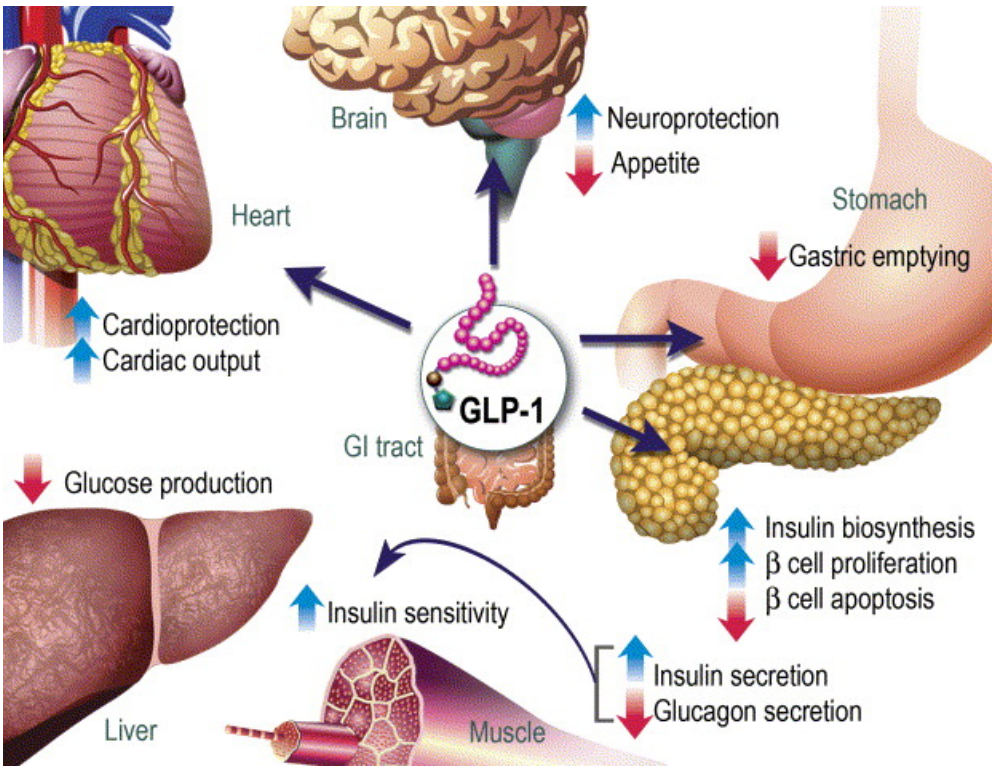

Figure 7: Direct actions of GLP-1 on pancreas, heart, stomach, brain and indirect actions on liver and muscle. (Drucker, 2006)

1.7.5.3 Insulin therapy

Since T2D progresses over time, many patients need to add insulin when their blood glucose is no longer well controlled by OHAs. There are various types of injectable insulin, which are classified into rapid acting, short acting, immediate acting and long acting insulin. Blood glucose levels need to be measured and monitored daily (often at mealtime) to adjust the doses of insulin therapy.

1.7.5.4 Complementary and alternative medicines

Dissatisfaction with the high costs and side effects of conventional pharmaceuticals has resulted in increasingly patients seeking for various complementary and alternative medicines (CAM) with antihyperglycemic effect. In both the developing countries, those are Asia, Africa, Latin America and the Middle East and some industrialized nations, Canada, France, Germany and Italy for instance, 70–90% of the populations rely on traditional medicines (TM) as primary health care to address their health-care concerns (Robinson and Zhang, 2011).

There are many plant species that have been identified worldwide as having antidiabetic potential. Examples of some widely used herbal medicines with antidiabetic potentials are *Trigonella foenum graecum* (Fenugreek) (Gupta et al., 2001); *Momordica charantia* (bitter melon)(John et al., 2003); *Cinnamomum cassia* (Chinese cinnamon) (Crawford, 2009; Khan et al., 2003); *Aloe vera* (Bunyapraphatsara et al., 1996) and *Ginseng* (Sotaniemi et al., 1995; Vuksan et al., 2008). A number of drugs currently used to treat diabetes were derived from plant or fungal material. These include metformin (derived from *Galega officinalis* L. (Oubré et al., 1997)), acarbose (derived from *Actinoplances* spp.) (Lee and Egelkrout, 1998; Lee et al., 1997).

As we discussed previously, Canadian Aboriginals, such as the CEI, exhibit a much higher prevalence of T2D than the general populations. Their age-adjusted prevalence of disease reached 29% in 2009 or 3 to 5 times higher (Kuzmina et al., 2010) compared with the rest of the Canadian population. They also suffer from higher rate of diabetic complications (Young et al., 2000). This serious problem has been related to a sedentary lifestyle and non-traditional diet (Hegele, 2001), a genetic

predisposition towards obesity (Neel, 1999; Skyler, 2004), as well as cultural disconnect (Young et al., 2000) of the modern or western pharmaceutical therapies described above.

For centuries, the aboriginal peoples relied on traditional healing for many diseases, knowledge that was verbally taught and handed down from generation to generation (Young et al., 2000). Medicinal plants played an important role in this holistic approach. Although some of this knowledge was lost, anthropologists documented the use and administration of many plant species belonging to aboriginal medicine.

For responding to this primary health care needs of the CEI and to search for culturally adapted T2D therapies, in 2013, CIHR Team in Aboriginal Antidiabetic Medicines (CIHR-TAAM) was instated. It was a multipartite project researching the antidiabetic effects of plants used by aboriginals. This project especially required a highly coordinated effort between three parts, including the CEI, research scientists, and health care professionals. The multi-disciplinary research team assembled for this project possessed considerable expertise in pharmacology, phytochemistry, and in nutrition, human clinical as well as health systems research.

An ethnobotanical approach (Fraser et al., 2007) was used to identify medicinal plants, based on several symptoms related to diabetes. Seventeen plant species were evaluated in several screening studies (Harbilas et al., 2009; Harris et al., 2011b; Nachar et al., 2013; Nistor Baldea et al., 2010; Spoor et al., 2006) using a variety of *in vitro* bioassays to assess their antidiabetic potentials. As explained earlier in terms of the clinical consequences of IR, bioassays were selected for primary (those likely to result in a reduction in blood glucose) and for secondary (diabetes complications) antidiabetic potential. Hence, among primary antidiabetic bioassays, the potential to increase glucose uptake in muscle cells, the inhibition of hepatic glucose production, the effects on adipogenesis, and those on insulin secretion in pancreatic β cells were evaluated (Haddad et al., 2012; Harbilas et al., 2009; Nachar et al., 2013; Spoor et al., 2006), whereas secondary bioassays such as for antioxidant capacities were also carried out.

1.8 Secondary metabolites and bioactive compounds

Secondary metabolites are a group of non-essential compounds that play a key role in the wellbeing of plants. They are involved in defense against ultraviolet radiation or aggression by parasites, as well as contributing to flavours and colors of plants. Several natural bioactive compounds have been discovered for their potential health benefit effects related to the prevention and treatment of chronic disorders such as cardiovascular disease, cancer, inflammatory and metabolic diseases including diabetes and obesity (Adisakwattana et al., 2009; Ishikawa et al., 2007; Wahle et al., 2010; Welsch et al., 1989). They are grouped into classes such as phenolics, terpenoids, alkaloids, and glycosides, based on similar structures or their plant origin.

1.8.1 Natural Phenolics

Phenolics are compounds with one or more aromatic rings containing at least one hydroxyl group covalently bonded to the aromatic ring. They are widely distributed and are one of the most abundant secondary metabolites of plants. There are more than 8,000 known phenolics, ranging from simple molecules to highly polymerized substances such as tannins. Natural phenolics include several subclasses such as flavonoids, phenolics acids, tannins and the less common stilbenes and lignans (Crozier et al., 2009).

1.8.1.1 Flavonoids

Flavonoids are one of the most important polyphenols in plants and our diets. All flavonoids share the basic C6-C3-C6 structural skeleton, consisting of two aromatic C6 rings and a heterocyclic ring that contains one oxygen atom (Crozier et al., 2009) (Figure 8). Studies show that flavonoids have strong antioxidant, anti-inflammatory, anti-allergic, anti-microbial and anti-cancer activities, as well as antidiabetic potentials (Crozier et al., 2009). They have been classified into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins, according to the oxidation state of the central carbon ring.

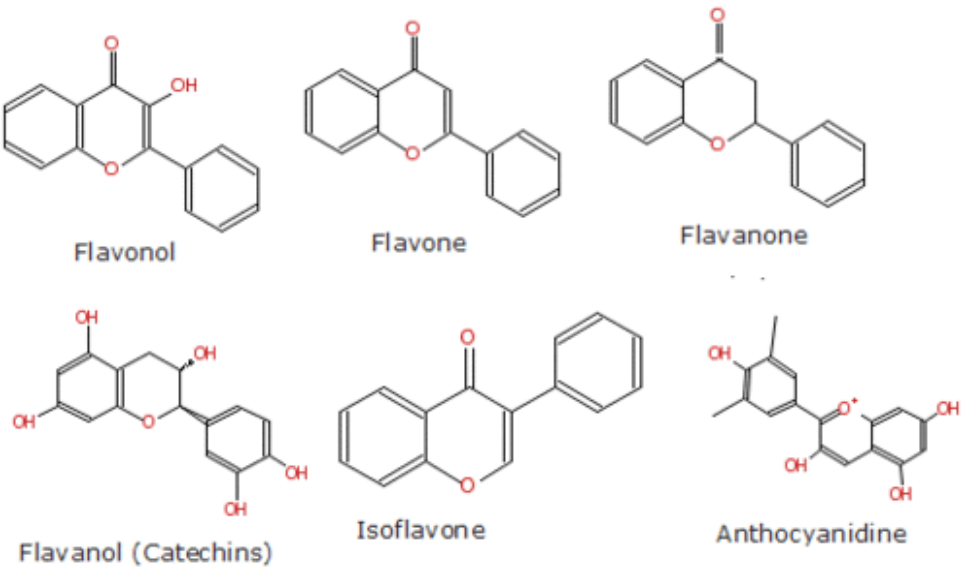

Figure 8: Structures of flavonoids.

1.8.1.2 Phenolic acids

Phenolic acids can be divided into two classes: derivatives of benzoic acid such as gallic acid, and derivatives of hydroxycinnamic acid such as caffeic, coumaric and ferulic acids. Caffeic acid is one of the most abundant phenolic acids in many fruits and vegetables. Besides their well-known antioxidant properties, several studies showed that phenolic acids also have antidiabetic potentials. Chlorogenic acid was reported to enhance glucose uptake in time- and dose-dependent manner in myotubes (Prabhakar and Doble, 2009). A series of phenolic acids, including caffeic acid, chlorogenic acid (ester of caffeic acid) and *p*-coumaric acid exhibited α-glucosidase inhibitory activity, thus indicating a potential to reduce intestinal glucose absorption (Adisakwattana et al., 2009; Ishikawa et al., 2007; Welsch et al., 1989).

Caffeic acid phenethyl ester (CAPE) and other derivatives were confirmed to stimulate glucose uptake and uncouple isolated mitochondria. Additionally, their activity required that there is no strongly ionized group in the compound (Eid et al., 2010).

1.8.1.3 Tannins

Tannins are polyphenols ubiquitously present in plants and diets. They have attracted attention recently for their beneficial properties to human health, which include antioxidant (Barreiros et al., 2000), immunomodulatory (Lin et al., 2002), antidiabetic (Klein et al., 2007; Li et al., 2005) and cardioprotective activities (Bagchi et al., 2003).

Tannins are divided into two groups: hydrolysable tannins and condensed tannins. The former are polyphenolic acids esterified to a carbohydrate core, made up mostly of glucose, with phenolic groups such as gallic acid for gallotannins or ellagic acid for ellagitannins. Condensed tannins, like proanthocyanidinson, are polymers of covalently bonded flavonols with high molecular weights of up to 23 900 Da.

One particular type of condensed tannin, are procyanidins, which are oligomers or polymers (2 to 50, or more) of flavan-3-ols (mostly catechins or epicatechins) units linked by carbon-carbon bonding (Santos-Buelga, 2000).

1.8.1.4 Stilbenes

Stilbenes are a small group of phenolics characterized by a 1,2-diphenylethylene backbone. Resveratrol has been extensively studied and has been shown to possess potent life extension (Howitz et al., 2003), cancer prevention (Jang et al., 1997; Sheth et al., 2012). Rhapontigenin (an analog of resveratrol), rhaponticin (the glycosylated form of rhapontigenin) and piceatannol were shown to have anti-allergic and anti-oxidant activities (Matsuda et al., 2001; Park et al., 2002). Rhapontigenin has also been shown to have antidiabetic activity by inhibiting mammalian intestinal α-glucosidase activity (Suresh Babu et al., 2004). These stilbenoids, but not resveratrol were identified in *L. laricina* (Figure 9) (Shang et al., 2012). See Chapter 2 for further details about its isolation and biological activity.

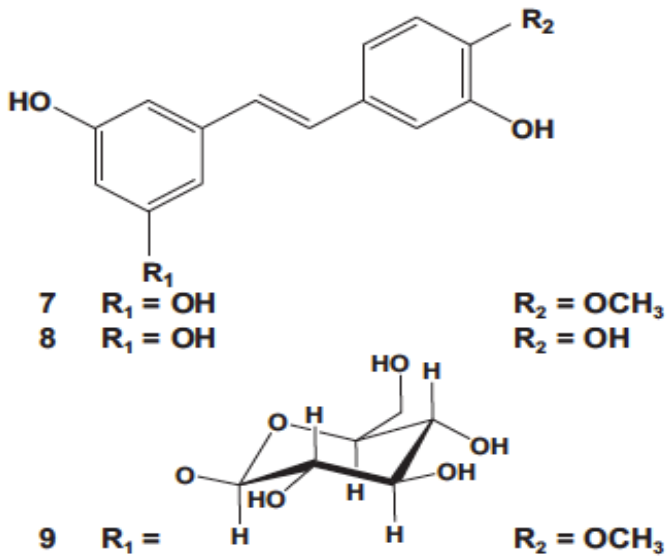

Figure 9: Structure of rhapontigenin (**7**), piceatannol (**8**), rhaponticin (**9**) identified in *Larix laricina* (Shang et al., 2012).

1.8.1.5 Lignans

Lignans are another major polymer polyphenols compounds that are distributed in a large number of plants and foods, such as flax and sesame seeds. They represent a major class of phytoestrogens; other classes including isoflavones, coumestans and stilbenes (Gencel et al., 2012). Only a few of lignans (such as seco-isolariciresinol, matairesinol, pinoresinol and lariciresinol) can be converted into the absorbable enterolignans by the intestinal microflora.

Lignans possess several biological activities, associated with decreasing the risk of breast cancers (Dai et al., 2002; Pietinen et al., 2001) as well as cardiovascular diseases (Vanharanta et al., 2003). Two derivates of lariciresinol were isolated in *L.laricina* and are further presented in Chapter 2 (Shang et al., 2012).

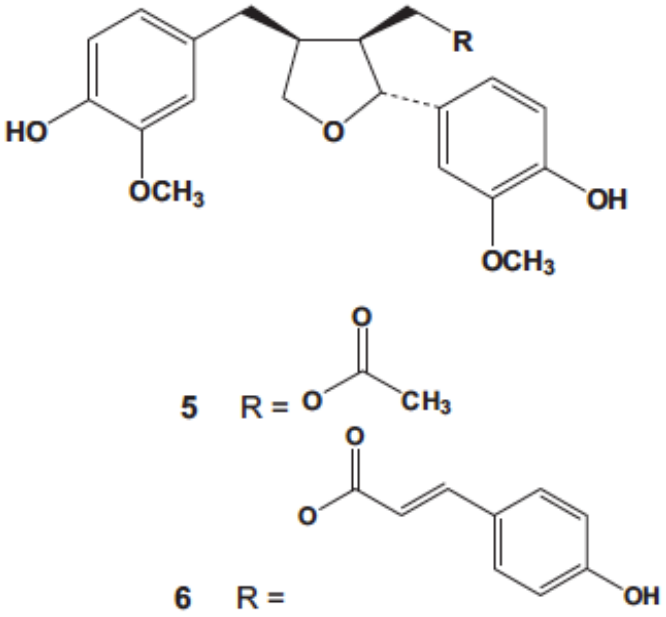

Figure 10: Structures of (+)-lariciresinol-3-acetate (**5**) and (+)-lariciresinol-9'-*p*-coumarate (**6**) found in *Larix laricina*. (Shang et al., 2012)

1.8.2 Terpenes

Terpenes and terpenoids (the modified triterpenes) are biosynthetically derived from a branched isoprene (C5H8). According to the numbers of the units, they have been further subdivided into: hemiterpene (C5); monoterpene (C10); sesquiterpene (C15); diterpenes (C20); sesterterpene (C25); triterpenes (C30) and higher terpenoids with more units (Turner et al., 1999).

A new terpenoid, determined to be 23-oxo-3-hydroxycycloart-24-en-26-oic acid, as well as several diterpenes were identified in *L. laricina* (Figure 11), as detailed in Chapter 2 (Shang et al., 2012). Ginsenosides are steroid-like triterpenoids found exclusively in Ginseng. Ginsenosides have multiple targets in cells and a multitude of therapeutic effects, e.g. being used in inflammatory diseases, cancer and neurodegenerative disorders and diabetes.

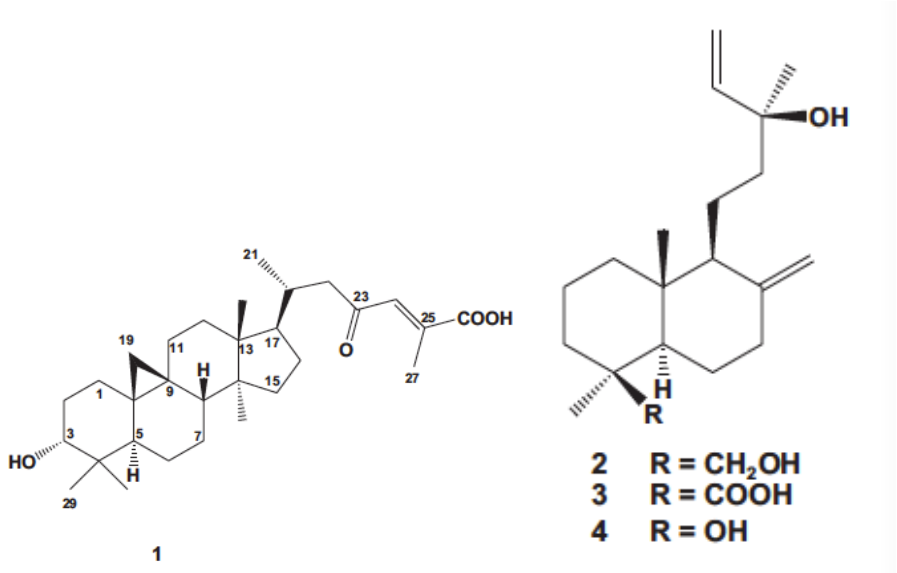

Figure 11: Structure of the new terpenoid as 23-oxo-3α-hydroxycycloart-24-en-26-oic acid (compound 1) and three diterpenes 13-epitorulosol (compound 2) and 13-epicupressic acid (compound 3), 19-norlabda-8 (17), 14-dien-4,13-diol (compound 4) found in *Larix laricina*. (Shang et al., 2012)

1.8.3 Metabolites analysis

Individual plant species contain thousands of metabolites. Metabolomics is a comprehensive analysis for small molecules in a biological system, such as cell, tissue, organ and plants. It has been applied in various research areas. . Wide variety of extraction, separation, detection and the statistical analyses techniques are currently under use to study plant compounds.

These include separation methods like gas chromatography (GC) and liquid chromatography (LC) and detection methods like mass spectrometry (MS) and nuclear magnetic resonance (Griffin, 2003; James et al., 2013; Negi et al., 2012; Trethewey et al., 1999). Coupling separation and detection methods, like LC-MS, is preferred to explore changes in metabolite profiles at qualitative and quantitative levels.

High performance liquid chromatography (HPLC) is a powerful separation technique to separate compounds with a wide range of polarity with high resolution.

Majority of HPLC applications are covered under reversed phase chromatography, with non-polar stationary phase and an aqueous, polar mobile phase. Application of ultra performance liquid chromatography (UPLC) is a recent advancement which has considerably further improved chromatographic resolution of overlapping metabolites and the sensitivity of MS detection (Wu et al., 2006).

A mass spectrometer is typically composed of three major parts: an ion source (like electrospray ionization (ESI) to convert metabolites into ions), a mass analyzer, and a detector. The main function of the mass analyzer is to separate these ionized molecules according to the mass-to-charge (m/z) ratios. There are a number of mass analyzers available now, include quadrupoles, time-of-flight (TOF)， magnetic sector, ion-trap and fourier transformation ion cyclotron. Tandem (MS-MS) mass spectrometers have more than one analyzer, like quadrupole-time-of-flight (QTOF) (Thurman et al., 2013), can be used for structural identification and quantitative studies. UPLC coupled to MS, especially with a high mass resolution such as a QTOF MS (Abu-Reidah et al., 2013), is a powerful approach to separate, detect, and analyze metabolites present in plant extracts.

Then the raw LC-MS data is converted into a peak list with m/z and retention time by different software before the statistic analysis, aiming to detect the peaks whose intensity levels are significantly altered between distinct groups.

In multivariate analyses, categorized as unsupervised principal component analysis (PCA) as well as supervised orthogonal partial least squares discriminant analysis (OPLS-DA). As one projection method, PCA could be used to reduce and summarize the variability of the matrix of peaks data form LC-MS. PCA could show a good overview of all the variables in score plots, with two new variables. Observations those near to each other are probably similar; while those who are far away from each other are different.

OPLS-DA is a maximum separation projection method used for classification study and biomarker identification (Boccard and Rutledge, 2013), especially from the S-plot. These usually lie in the top or bottom extremities of the S-plot, and are characterized by sufficiently important statistical differences to render them potential biomarkers. Biomarker identification is accomplished by performing a match of

elemental composition and / or monoisotopic mass with entries in online databases including METLIN, ChemSpider, PlantMetaboliteNetwork, ADC labs, NIST.

1.9 Objectives of the study

17 CEI medicinal plants have been identified through a comprehensive approach by our group as candidate antidiabetic plants used by the CEI First Nation to treat various symptoms of T2D. Among them, *L. laricina* was recently identified as one of the top plants. It stimulated glucose uptake in C2C12 muscle cells and strongly potentiated the differentiation of 3T3-L1 pre-adipocytes indicating potentially enhanced insulin sensitivity (Spoor et al., 2006). The thesis firstly aimed at isolating putative antidiabetic active principles responsible for the potentiation of adipogenesis of the extract of the inner bark of *L. laricina* by using a bioassay-guided fractionation approach.

Secondly, all screening studies performed thus far by the CIHR-TAAM have focused on ethanol extracts (EE) using an *in vitro* bioassay platform. However, traditional preparations are often based on hot water. This thesis thus also aimed to evaluate and compare the antidiabetic potentials of ethanol and water extracts of these plants using the same three *in vitro* bioassays, namely adipogenesis in 3T3-L1 cells, glucose uptake in C2C12 myocytes and glucose production in H4IIE hepatocytes. The possible mechanisms involved in the regulation of glucose and lipid metabolism was also assessed for a selected subset of Boreal forest plant species.

Chapter 2: Article 1

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Role of each co-author belonging to this manuscript

I performed the pharmacological experimental work, data analysis and wrote the paper.

José A. Guerrero-Analco contributed to the isolation and identification of the pure compounds.

Dr Lina Musallam, contributed to conceptual input and correcting of the articles.

Asim Muhammad, Brendan Walshe-Roussel and Dr. Ammar Saleem contributed to the preparation of the plant species in the lab of Dr. John T. Arnason.

Dr. Alain Cuerrier contributed in the characterization of the plant species

Dr. Pierre S. Haddad, my supervisor, contributed conceptual, and intellectual input as well as correcting the article.

Adipogenic constituents from the bark of *Larix laricina* du Roi (K. Koch; Pinaceae), an important medicinal plant used traditionally by the Cree of Eeyou Istchee (Quebec, Canada) for the treatment of type 2 diabetes symptoms

Nan Shang[a,b,1], José A. Guerrero-Analco[b,c,1], Lina Musallam[a,b], Ammar Saleem[b,c], Asim Muhammad[b,c], Brendan Walshe-Roussel[b,c], Alain Cuerrier[b,d], John T. Arnason[b,c,*], Pierre S. Haddad[a,b,*]

[a] Natural Health Products and Metabolic Disease Laboratory, Department of Pharmacology, Faculty of Medicine, Université de Montréal, Montreal, QC H3T 1J4, Canada

[b] Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines and Montreal Diabetes Research Center, Montreal, QC H3T 1J4, Canada

[c] Center for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON K1N6N5, Canada

[d] Jardin botanique de Montréal, Institut de recherche en biologie végétale, Université de Montréal, Montréal, QC H1X 2B2, Canada

[1] Authors contributed equally to the manuscript.

Addresses for correspondence

**Pierre S. Haddad, PhD
Department of Pharmacology
Université de Montréal
P.O. Box 6128, Station Centre-Ville
Montréal, Québec, Canada H3C 3J7

*John T. Arnason, PhD
Department of Biological Sciences
University of Ottawa
30, Marie-Curie Street
Ottawa, Ontario, Canada

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Abstract

Ethnopharmacological relevance: Diabetes is a growing epidemic worldwide, especially among indigenous populations. *Larix laricina* was identified through an ethnobotanical survey as a traditional medicine used by Healers and Elders of the Cree of Eeyou Istchee of northern Quebec to treat symptoms of diabetes and subsequent *in vitro* screening confirmed its potential. **Materials and Methods:** We used a bioassay-guided fractionation approach to isolate the active principles responsible for the adipogenic activity of the organic extract (80% EtOH) of the bark of *L. laricina*. Post-confluent 3T3-L1 cells were differentiated in the presence or absence of the crude, fractions or isolates of *L. laricina* for 7 days, then Triglycerides content was measured using AdipoRed reagent. **Results:** We identified a new cycloartane triterpene (compound **1**), which strongly enhanced adipogenesis in 3T3-L1 cells with an EC_{50} of 7.7 μM. It is responsible for two thirds of the activity of the active fraction of *L. laricina*. The structure of compound **1** was established on the basis of spectroscopic methods (IR, HREIMS, 1D and 2D NMR) as 23-oxo-3*α*-hydroxycycloart-24-en-26-oic acid. We also identified several known compounds, including three labdane-type diterpenes (compounds **2**-**4**), two tetrahydrofuran-type lignans (compounds **5-6**), three stilbenes (compounds **7-9**), and taxifolin (compound **10**). Compound **2** (13-epitorulosol) also potentiated adipogenesis (EC_{50} 8.2 μM) and this is the first report of a biological activity for this compound. **Conclusions:** This is the first report of putative antidiabetic principles isolated from *L. laricina*, therefore increasing the interest in medicinal plants from the Cree pharmacopeia.

1. Introduction

Type 2 diabetes (T2D), a chronic metabolic disorder characterized by impaired insulin secretion and insulin sensitivity, has become a worldwide epidemic according to WHO (Roglic and Unwin, 2010; Shaw et al., 2010; Whiting et al., 2010; Wild et al., 2004). T2D is more pronounced among indigenous populations, as is the case in the Cree Nation of Eeyou Istchee (CEI) of northern Quebec, where the age-adjusted prevalence of disease reached 29% in 2009 (Kuzmina et al., 2010). The burden of T2D and the ensuing deleterious complications (cardiovascular, retinopathy and nephropathy) prompted the search for culturally adapted treatment alternatives for these aboriginal populations.

Larix laricina (Watnagan, Tamarack, mélèze) is a highly respected plant used by many First Nations throughout Canada as a culturally important medicine to treat several ailments, including cuts, burns, coughs, stomach pain, sores (mouth, skin and throat), heart problems and frostbite (Holmes, 1884; Leighton, 1985; Marles et al., 2000; Marshall et al., 2006; Marshall et al., 1989; Siegfried, 1994). The bark was especially identified for the treatment of sores, frequent urination, poultice on infected wounds and to heal the insides by Aboriginals of Eastern Canada (Arnason et al., 1981; Black, 1980; Holmes, 1884). Through a quantitative ethnobotanical study of traditional medicines conducted by our team in collaboration with Healers and Elders of four CEI communities, the inner bark *L. laricina* was recently identified as one of the top eight plants for the treatment of symptoms of diabetes [namely frequent urination, blurry vision, slow healing wounds, etc.] (Leduc et al., 2006). The extract of this plant was tested in a comprehensive platform of *in vitro* bioassays designed to detect potential antidiabetic biological activities (Haddad et al., 2012). *L. laricina* stimulated glucose uptake in C2C12 muscle cells and strongly potentiated the differentiation of 3T3-L1 pre-adipocytes indicating enhanced insulin sensitivity (Spoor et al., 2006). The present study aims at isolating putative antidiabetic active principles responsible for the potentiation of adipogenesis of an organic extract of the inner bark of *L. laricina* by using a bioassay-guided fractionation approach.

2. Materials and Methods

2.1 Cell lines, chemicals, biochemical and equipment

2.1.1Bioassay:

The pre-adipocyte 3T3-L1 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). Dexamethasone (DXM), bovine pancreatic insulin, 3-isobutyl-1-methylxanthine (IBMX), dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Rosiglitazone came from Alexis Biochemicals (Hornby, ON). Dubelcco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and bovine calf serum (BCS) were from Wisent Inc (St-Bruno, QC). Triglyceride content was measured using Adipored reagent purchased from Lonza Walkersville Inc (Walkersville, MD).

2.1.2 Phytochemistry:

The preparative scale isolation of the phytochemicals was undertaken on a reverse phase Gemini Axia column 250 × 21.2 mm I.D., particle size 10 microns (Phenomenex Inc., Torrance, CA, USA) using a 30 minute linear gradient of 20 to 80% of CH_3CN in water at the flow rate of 31.5 mL/min, at the monitoring wavelength of 210 nm, band width 4, reference off. The Agilent 1200 Series preparative HPLC system (Agilent Technologies, Montreal, QC, Canada) consisted of a binary pump (flow rate range 5-100 mL/min), an autosampler with a 2mL loop, a diode array detector with a flow cell (path length 3 mm and maximum pressure limit 120 bars) and a fraction collector (40 mL collection tubes). IR spectra were recorded using a Shimadzu 8400-S FT/IR spectrometer. Optical rotations were assessed on a Perkin-Elmer 241 digital polarimeter. NMR spectra were obtained from a Bruker Avance 400 spectrometer (Bruker, Biospin Corporation, Billerica, MA, USA) in C_5D_5N, either at 400 MHz (^1H) or 100 MHz (^{13}C), using tetramethylsilane (TMS) as an internal standard. EIMS and HREIMS were obtained on a Kratos Concept IImass spectrometer (Kartos Analytical, Chestnut Ridge, NY, USA). Open column chromatography was carried out on silica gel 60 (70-230 mesh; Merck & Co., Whitehouse Station, NJ, USA). TLC analyses were performed on silica gel 60 F_{254}

plates (Merck & Co, Whitehouse Station, NJ, USA), and visualization of the plates was carried out using a ceric sulfate (10%) solution in H_2SO_4.

2.2 Plant material

Stem bark (1.5 Kg) of *L. laricina* was collected in Mistissini, Quebec, in August 2008 and identified by Dr. Alain Cuerrier, a taxonomist at the Plant Biology Research Institute, Université de Montréal. A voucher specimen (MIS0312) was deposited at the Marie-Victorin Herbarium (MT) of the same Institute.

2.3 Extraction and Isolation

Dried and shredded bark (1.5 Kg) was extracted with 15.0 L of 80% EtOH: 20% water during 24 h at room temperature. The supernatant was removed and the pellet was re-extracted with 8.0 L of 80% EtOH: 20% water. The pooled supernatants were evaporated in vacuo to yield 430 g of a dark red residue (crude extract, LLE). Part of the dried crude extract (100 g) was then chromatographed in a glass column (600 mm × 100 mm I.D.) packed with silica gel (2.0 Kg) eluting with hexanes-ethyl acetate (EtOAc) (1:0→0:1) and EtOAc-methanol (MeOH) (1:0→0:1) to yield a total of one hundred and forty fractions (250 ml each), which were combined on the basis of their TLC profiles. After solvent evaporation and combination of the fractions, sixteen primary fractions (LLE1 – LLE16) were obtained. From fraction LLE10 (1.0 g) eluted with EtOAc (100%), 600 mg of rhaponticin (compound **9**) spontaneously precipitated. The structure of each compound is illustrated in Figure 1.

From fraction LLE1 (20.0 g) eluted with hexanes-EtOAc (9:1), 15 mg of 19-norlabda-8(17),14-dien-4,13-diol (compound **4**) were obtained by reversed phase preparative scale HPLC using the same analytical condition as described above. Fraction LLE-2 (9.0 g) eluted with hexanes-EtOAc (8:2), afforded 25 mg of 13-epitorulosol (compound **2**) and 15 mg of 13-epicupressic acid (compound **3**), which were obtained by preparative scale. Fraction LLE4 (4.0 g) eluted with hexanes-EtOAc (4:6) was subjected to the same purification HPLC procedure yielding 8 mg of (+)-lariciresinol-3-acetate (compound **5**), 12 mg of (+)-lariciresinol-9'-p-coumarate (compound **6**), 10 mg of rhapontigenin (compound **7**) and 15 mg of piceatannol (compound **8**). From fraction LLE5 (1.0 g), eluted with hexanes-EtOAc (3:7), 20 mg of the new compound 23-oxo-3*α*--hydroxycycloart-24-en-26-oic acid

(compound **1**) were obtained by the same preparative scale HPLC methodology. In addition to compound **1**, compounds **4** to **7** were also obtained from fraction LLE5 as well as 15 mg of taxifolin (compound **10**).

2.4 Identification of 23-oxo-3*α*-hydroxycycloart-24-en-26-oic acid (1)

Compound 1 was determined to be a novel chemical entity. The purified compound presented as a beige amorphous powder, with the following optical properties: $[\alpha]^{25}_{D}$ +34.5° (c 1.09, MeOH). UV spectrum (MeOH) yielded two peaks: λ_{max} 205 and 215 nm. Infrared spectroscopy (KBr) yielded the following values: ν_{max}3350, 2920, 1705 (br), 1675, 1375, 1220 and 1146 cm^{-1}. For mass spectroscopy, the following results were obtained: EIMS m/z 470 $[M]^+$(8), 452 $[M\text{-}H_2O]^+$(16), 431 $[M - CO_2 + H]^+$(25); HREIMS m/z 471.3936 $[M + H]^+$ (calcd. for $C_{30}H_{47}O_4$: 471.8368). For ^{13}C NMR (100 MHz, Pyr-d_5), the following signals were displayed δ 15.0 (q, C-27), 18.2 (q, C-18), 19.5 (q, C-30), 19.6 (q, C-21), 19.8 (q, C-9), 21.4 (t, C-6), 21.7 (q, C-28), 26.1 (t, C-11), 26.5 (t, C-16), 26.9 (q, C-29), 27.1 (s, C-10), 28.1 (t, C-7), 28.5 (t, C-19), 29.7 (t, C-2), 29.8 (t, C-1), 33.1 (t, C-12), 33.5 (d, C-20), 35.6 (t, C-15), 40.1 (s, C-4), 41.1 (d, C-5), 45.6 (s, C-13), 48.1 (d, C-8), 49.3 (s, C-14), 52.3 (t, C-22), 52.6 (d, C-17), 76.0 (d, C-3), 132.9 (d, C-24), 142.2 (s, C-25), 170.5 (s, C-26), 202.7 (s, C-23); ^1H NMR (400 MHz, Pyr-d_5) δ1.90 (2H, m, H-1a, H-1b), 1.28 (2H, m, H-2a, H-2b), 3.69 (H, t, J = 2.4 Hz, H-3β),2.23 (H, m, H-5),0.75 (H, m, H-6a), 1.04 (H, m, H-6b), 1.05 (H, m, H-7a),2.3 (H, m, H-7b), 1.65 (H, m, H-8), 1.14 (2H, m, H-11a, H-11b), 1.58 (2H, m, H-12a, H-12b), 1.28 (2H, m, H-15a, H-15b),1.90 (H, m, H-16a), 1.98 (H, m, H-16b),1.64 (H, m, H-17),1.04 (3H, s, H_3-18),0.32 (H, d, J = 3.4 Hz, H-19a),0.48 (H, d, J = 3.4 Hz, H-19b),1.53 (H, m, H-20),1.02 (3H, d, J = 6.0 Hz, H_3-21),2.36 (H, dd, J = 15.6, 9.8 Hz, H-22a),2.73 (H, dd, J = 15.6, 2.8 Hz, H-22b), 7.57 (H, d, J = 1.2 Hz, H-24), 2.57 (3H, d, J = 1.2 Hz, H_3-27), 0.96 (3H, s, H_3-28), 1.2 (3H, s, H_3-29), 0.89 (3H, s, H_3-30).

2.5 Cell culture

Pre-adipocyte 3T3-L1 cells were grown to confluency in DMEM containing 10% FBS (proliferation medium). 24 h post-confluence (day 0), cell were induced to

differentiate with high-glucose DMEM supplemented with 10 % FBS, 1 μM dexamethasone, 250 μM 3-isobutylmethylxanthine (IBMX) and 500 nM insulin. After 48 h, the media was replaced with DMEM containing 10 % FBS and 500 nM Insulin. Cells were differentiated for a total of 7 days with media change every 2 days. Plant extracts and fractions (25 μg/ml), compounds (0.5 to 100 μM) as well as rosiglitazone (10 μM; inducer of differentiation) were dissolved in DMSO (0.1 % final concentration), and added to the cells at day 0 of differentiation and replenished at every medium change thereafter.

2.6 Adipogenesis

Adipogenesis was assessed in the well-characterized 3T3 L1 cell model by measuring the accumulation of triglycerides (TG) upon differentiation (at day 7), using the AdipoRed reagent according to the manufacturer's instructions as described previously with minor modifications (Harbilas et al., 2009; Spoor et al., 2006). Briefly, after washing each well twice with Phosphate-Buffered Saline (PBS: 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, and 2.68 mM KCl; pH 7.4), 2 mL of PBS containing 60 μL of AdipoRed reagent were added to each well and incubated for 15 minutes at room temperature. AdipoRed becomes fluorescent when partitioned in a hydrophobic compartment. The fluorescence of each well was measured by fluorimeter (Wallac Victor2; Perkin-Elmer, St-Laurent, Qc) at 485 nm excitation and 572 nm emission wavelengths. The results were reported as percentage of the vehicle control (0.1 % DMSO).

2.7 Statistical analysis

Intracellular TG content results are presented as mean ± SEM of 3 independent experiments, each performed in triplicate. Statistical calculations and EC_{50} (calculated using sigmoidal dose-response (variable slope) analysis was performed with Prism GraphPad software. Differences between groups were analyzed by one-way analysis of variance (ANOVA). A p value below 0.05 was considered statistically significant.

3. Results

3.1 Adipogenic activity of *L. laricina* fractions and compounds

The crude 80% EtOH extract of *L. laricina* bark (LLE) at a concentration of 25 μg/mL increased the accumulation of TG in 3T3-L1 cells by approximately 2.5 fold, when compared to the DMSO vehicle control (Figure 2A). The sixteen primary fractions (LLE-1-LLE-16) obtained from silica gel column chromatography of LLE were also assessed alongside the parent extract. Also tested at 25 μg/ml, fractions LLE-1 as well as LLE-3 to LLE-7 significantly increased intracellular TG up to 5.4 fold as compared to vehicle control (0.1% DMSO; Figure 2A). More specifically, fractions LLE-1 (317 ± 71 %), LLE-3 (367 ± 35 %), LLE-4 (401 ± 53 %), and LLE-5 (538 ± 46 %) demonstrated significantly stronger adipogenic activity than the crude extract (238 ± 32 %; $p \leq 0.05$; Figure 2A).

Compounds **1** to **8** and **10** were isolated from fractions LLE-1 to LLE-5 after preparative scale isolation (Figure 1). These isolates were: a new cycloartane triterpenoid identified as 23-oxo-3*α*-hydroxycycloart-24-en-26-oic acid (compound **1**); three labdane diterpenes: 13-epitorulosol (compound **2**) 13-epicupressic (compound **3**) and one 4-hydroxy-nor-diterpene: 19-norlabda-8(17),14-dien-4,13-diol (compound **4**); two lignans: (+)-lariciresinol-3-acetate (compound **5**) and (+)-lariciresinol-9'-*p*-coumarate (compound **6**); two stilbenoids: rhapontigenin (compound **7**), piceatannol (compound **8**), and one flavonoid taxifolin (compound **10**). In addition, significant amounts of rhaponticin (compound **9**) were obtained from the fraction LLE-10. The known compounds **2** to **10** were identified by comparison of their spectroscopic data with those already reported in the literature. (Caputo et al., 1973; Chen et al., 2009; Kashiwada et al., 1984; Markham, 1976; Mills, 1973; Nair and Von Rudloff, 1959; Pacheco et al., 2009; Pichette et al., 2006; Rajasekhar and Subbaraju, 2000; Xue et al., 2004; Xie et al., 2003; Yang et al., 2005). The identification of the new compound (compound **1**) was conducted by 1D NMR (^{1}H, ^{13}C, DEPT) and 2D NMR (NOESY, COSY, HMQC) analysis, as described further below.

The compounds were tested at varying concentrations ranging from 0.5 to 25 μg/mL (0.5-100 μM) on 3T3-L1 cells to determine their adipogenic effect. As illustrated in Figure 2B, most isolates did not show significant potentiation of adipogenesis as compared to vehicle when tested at concentrations up to 10 μg/ml. Only the new compound **1** (23-oxo-3α-hydroxycycloart-24-en-26-oic acid) and compound **2** (13-epitorulosol) caused significant increases in adipogenesis with physiological dose-response relationships yielding EC_{50} values of 7.7 μM (3.6 μg/mL) and 8.2 μM (2.5 μg/mL) respectively (Table 1). Indeed, the maximal potentiating effect of compound **1** (453± 30 %; 10 μg/ml) was significantly higher than that of the crude extract (305 ± 23 %; 25 μg/mL; $p \leq 0.01$), while not significantly different from that of the active fraction LLE-5 (555 ± 36 %; 25 μg/ml; not significant (ns); Figure 2B). 3T3-L1 cells treated with 10 μg/mL of compound **2** potentiated adipogenesis by 275 ± 40 % ($p \leq 0.001$ as compared to DMSO; Figure 2B), which almost reached the crude extract level (305 ± 23 %; 25 μg/mL; ns), however it caused cell death and detachment at high concentrations, as determined morphologically (25 μg/mL; data not shown). The remaining 8 compounds did not display any toxicity to 3T3-L1 adipocytes at any of the concentrations tested. Noteworthy, compound **7** (rhapontigenin) significantly potentiated adipogenesis of 3T3-L1 cells at high concentration only (337 ± 36 %; 25 μg/ml; $p \leq 0.001$; data not shown), with an EC_{50} of 145 μM (37.5 μg/mL).

3.2 Chemical identification of compound 1

Compound **1** was obtained as a white amorphous powder. A molecular formula of $C_{30}H_{46}O_4$ was assigned for this compound by HREIMS, which was corroborated by ^{13}C NMR data recorded in pyridine d-5. Its IR spectrum showed a broad carbonyl (1705 cm^{-1}) band, an alpha beta unsaturated double bond (1675 cm^{-1}) and hydroxyl group associated band (3350 cm^{-1}). The ^{1}H NMR spectrum of **1** indicated the presence of a pair of upfield doublets at δ0.32 (H, d, J = 3.4 Hz, H-19a) and 0.48 (H, d, J = 3.4 Hz, H-19b), characteristic of cycloartane triterpenoids (Li et al., 2012). The ^{1}H NMR spectrum of **1** also displayed four angular methyls at δ_H 18.2 (s, H_3-18), 19.5 (s, H_3-30), 21.7 (s, H_3-28) and 26.9 (s, H_3-29), a methyl doublet at δ_H

19.6 (d, J = 6.0 Hz, H_3-21), and an olefinic methyl doublet at δ_H 19.6 (d, J = 1.2 Hz, H_3-27). The ^{13}C NMR spectrum of **1** showed two signals belonging to its carbonyls: an enone at δ_C 202.3 (C-23), and a carboxyl at δ_C 171.5 (C-26). Comparisons of the ^{13}C NMR spectra data with those of cycloartanes, particularly the 3-oxo-23-hydroxycycloart-24-en-26-oic acid isolated from *Mangifera indica* (Anjaneyulu et al., 1989; Anjaneyulu et al., 1999) and the 23-dioxo-cycloart-24-en-26-oic acid isolated from *Pseudolarix amabilis* (Jayasuriya et al., 2005) indicated that **1** is 23-oxo-3-hydroxycycloart-24-en-26-oic acid, a new cycloartane triterpene derivative. The analysis of the coupling constant values for H-3 (δ_H 3.69, t, J = 2.4 Hz) indicated the alpha configuration for the hydroxyl group in C-3. The structural assignment of compound **1** (see Experimental part) was confirmed by the exhaustive analysis of their 1D (^1H, ^{13}C, DEPT) and 2D (NOESY, COSY, HMQC) NMR spectrums and the key HMBC correlations of H_3-27 to C-24, C-25, and C-26; H-24 to C-23, C-26, and C-27; H_3-21 to C-17, C-20, and C-21; H_2-22 to C-20 and C-23; H_3-29 to C-3, C-4, and C-5; H_3-18 to C-12, C-13, C-14, and C-17; H_3-30 to C-8, C-13, C-14, and C-15; and H_3-28 to C-3, C-4, and C-5 in **1** (Figure3).

4. Discussion

Larix laricina du Roi (K. Koch; Pinaceae) is a medicinal plant identified by our group as a candidate antidiabetic plant used by the CEI First Nation to treat various symptoms of Type 2 diabetes (Leduc et al., 2006). It was notably found to significantly enhance adipogenesis in differentiating 3T3-L1 adipocytes in a screening study using *in vitro* cell based bioassays (Spoor et al., 2006). In the present study, we used the same cell line to carry out bioassay-guided fractionation in order to identify potential active principles of the plant.

The results of a silica gel column chromatography showed that fractions LLE-1, LLE-3, LLE-4 and LLE-5 exhibited the largest increases of 3T3-L1 cell adipogenesis, the most active one (LLE-5) reaching more than double the activity of the crude LLE and some two-thirds of the activity of the reference drug rosiglitazone. Nine compounds were then isolated from these active fractions and two (compounds **1** and **2**) were found to be most active; displaying physiological dose-response curves.

We also calculated the expected activity of each compound according to their mass yield and their absolute concentration in LLE-5 (Table 1). Compounds **3** through **7** displayed weak adipogenic activity, which was not statistically or physiologically significant (data not shown). However, taking into account these weak activities, we could hypothesize that a synergistic relationship might exist between these compounds that could contribute to the activity of the crude extract LLE and to the potentiating effect found in fraction LLE-5. This warrants further investigation. Interestingly, the expected activity of the new triterpenoid (compound **1**) at the concentration found in LLE-5 (12.78 μM) was estimated at 351%; this accounts for almost 63% of LLE-5 adipogenic activity.

Although compounds **2** and **7** were previously identified (Chen et al., 2009; Pichette et al., 2006), this is the first report of such antidiabetic activity. Indeed, there is currently no reported biological activity for 13-epitorulosol (compound **2**). On the other hand, rhapontigenin, a natural analogue of resveratrol with known antioxidant and anti-cancer activities (Roberti et al., 2003; Roupe et al., 2005), has been shown to

inhibit mammalian intestinal α-glucosidase activity (responsible for first step in glucose degradation and therefore absorption (Suresh Babu et al., 2004). Here, we report a glitazone-like insulin sensitizing activity for rhapontigenin, albeit at high concentrations.

Finally, no antidiabetic activity was reported for compound **3** (13-epicupressic acid), compound **4** (19-norlabda-8(17),14-dien-4,13-diol), compound **5** (lariciresinol-3-acetate), compound **6** (lariciresinol-coumarate), or compound **10** (taxifolin).

In conclusion, we have identified one major active principle responsible for the effect of L. laricina on adipogenesis. The compound is a previously unidentified cycloartane triterpenoid, 23-oxo-3*α*-hydroxycycloart-24-en-26-oic acid, which strongly potentiates adipogenesis with an EC_{50} of 7.7 μM. In addition, we report a new biological activity for 13-epitorulosol (EC_{50} 8.2 μM) and rhapontigenin (at high concentrations). Further investigation is needed to determine the possible synergistic activity between compound **3** (13-epicupressic acid), compound **5** (lariciresinol-3-acetate) and compound **6** (lariciresinol-coumarate) at the concentration present in the most active fraction (LLE-5).

Finally, *L. laricina* was the object of previous pharmacological studies. One study identified anti-tumor active principles [labdane-type diterpenes (Pichette et al., 2006)], while others studied the effect of the environment on phytochemicals components of *L. laricina* [volatiles (Nair and Von Rudloff, 1959; Powell and Raffa, 1999; Von Rudloff, 1987), phenolics (Niemann, 1969; Niemann and Bekooy, 1971) and diterpenes (Mills, 1973)]. Hence, ours is the first study that focuses on the identification of the active principles responsible for the adipogenic effect as putative antidiabetic mechanism of *L. laricina*, as used by traditional Healers in Canadian Aboriginal populations. In support of this, we have recently shown that *L. laricina* decreases hyperglycemia and insulin resistance in vivo, using the diet-induced obese mouse model (Harbilas et al., 2012). In addition to the expected terperenes, our study has identified compounds from other chemical classes including lignans, phenolics and stilbenes. This is interesting and of significance due the fact that *L. laricina* belongs to Pinaceae family, which has been mainly used for timber, pulp and paper.

Indeed, very little work was done on its biological effects and only a few reports focus on their essential oils as predominant class of compounds.

The identification of such active compounds of *L. laricina* paves the way to developing methods for the quality control/quality assurance of traditional preparations that could be used alongside modern pharmaceuticals to provide culturally adapted treatment options for Cree diabetics. Likewise, methods are being developed to measure *L. laricina* bioactives in biological fluids in order to enable pharmacokinetic studies of Cree Traditional Medicine, alone and in combination with diabetes drugs. Our study thus provides an important piece to the puzzle of an evidence-based approach to favor the inclusion of Cree Healing Ways into diabetes care.

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7. Figure legends

Figure 1: Chemical structure of compounds **1-8** isolated from *L. laricina*.

A new cycloartane triterpenoid identified as 23-oxo-3α-hydroxycycloart-24-en-26-oic acid (compound **1**); three labdane diterpenes: 13-epitorulosol (compound **2**) and epicupressic acid (compound **3**), 19-norlabda-8 (17), 14-dien-4,13-diol (compound **4**); two lignans: (+)-lariciresinol-3-acetate (compound **5**) and (+)-lariciresinol-9'-p-coumarate (compound **6**); three stilbenoids: rhapontigenin (compound **7**), piceatannol (compound **8**), rhaponticin (compound **9**) and taxifolin (compound **10**).

Figure 2: The adipogenic activity of *L. laricina* crude extract, fractions and compounds.

A) Preadipocytes (3T3-L1 cells) were induced to differentiate in the presence of *L. laricina* crude extract (LLE; 25 μg/mL), fractions (LLE-1-LLE-16; 25 μg/mL) or rosiglitazone (inducer of differentiation; 10 μM). DMSO 0.1% was used as vehicle control. Following 7 days of differentiation, cells were washed and the intracellular content of triglycerides (TG) was determined as detailed in Materials and Methods Section. * denotes statistically significant from vehicle control ($p \leq 0.05$). $ denotes statistically significant from crude extract LLE ($p \leq 0.05$). B). Preadipocytes were treated with rosiglitazone (10 μM), LLE (25 μg/mL), LLE-5 (25 μg/mL) and 8 of the isolated compounds (10 μg/mL) for 7 days then TG content was determined. ** denotes statistically significant from vehicle control ($p \leq 0.01$). *** denotes statistically significant from vehicle control ($p \leq 0.001$).

Figure 3: Key HMBC (H→C) correlations of compound.

Table 1: EC_{50}, activity and yields of *L. laricina* compounds

ID	Compound name	EC_{50}[a] (μM)	Maximal activity[b] (% of control)	Yield in LLE (mg/g dry weight)	Yield in LLE-5 (mg/g dry weight)	Conc in LLE-5 (μM)	Expected activity in LLE-5 (% of control)
1	Compound **1**	7.7	372	13.04	240.23	12.78	351
2	13-Epitorulosol	8.2	271	207.81	31.59	2.57	116
3	13-Epicupressic acid	NE	259[c]	143.00	346.69	2.70	153
5	Lariciresinol-3-acetate	34.3	228[c]	7.54	230.40	14.30	141
6	Lariciresinol-coumarate	NE	235[c]	5.18	247.65	12.20	155
7	Rhapontigenin	145	337[c]	35.50	146.62	14.20	132
8	Piceatannol	4.1	172	0.40	1.16	0.12	100
10	Taxifolin	10.5	133	5.40	146.23	12.00	110

ID 1: Compound **1**, 23-oxo-3α-hydroxycycloart-24-en-26-oic acid.

ID 4: Compound **4**, 19-norlabda-8(17),14-dien-4,13-diol was not isolated in enough amounts to be tested.

ID 9: Compound **9**, rhaponticin was mainly isolated from LLE-10.

NE, not estimated.

[a] EC_{50} as estimated by sigmoidal dose–response (variable slop) analysis using Prism GraphPad software.

[b] Maximal activity as estimated by sigmoidal dose–response (variable slop) analysis using Prism GraphPad software.

[c] Observed activity at highest concentration tested.

Figure 1

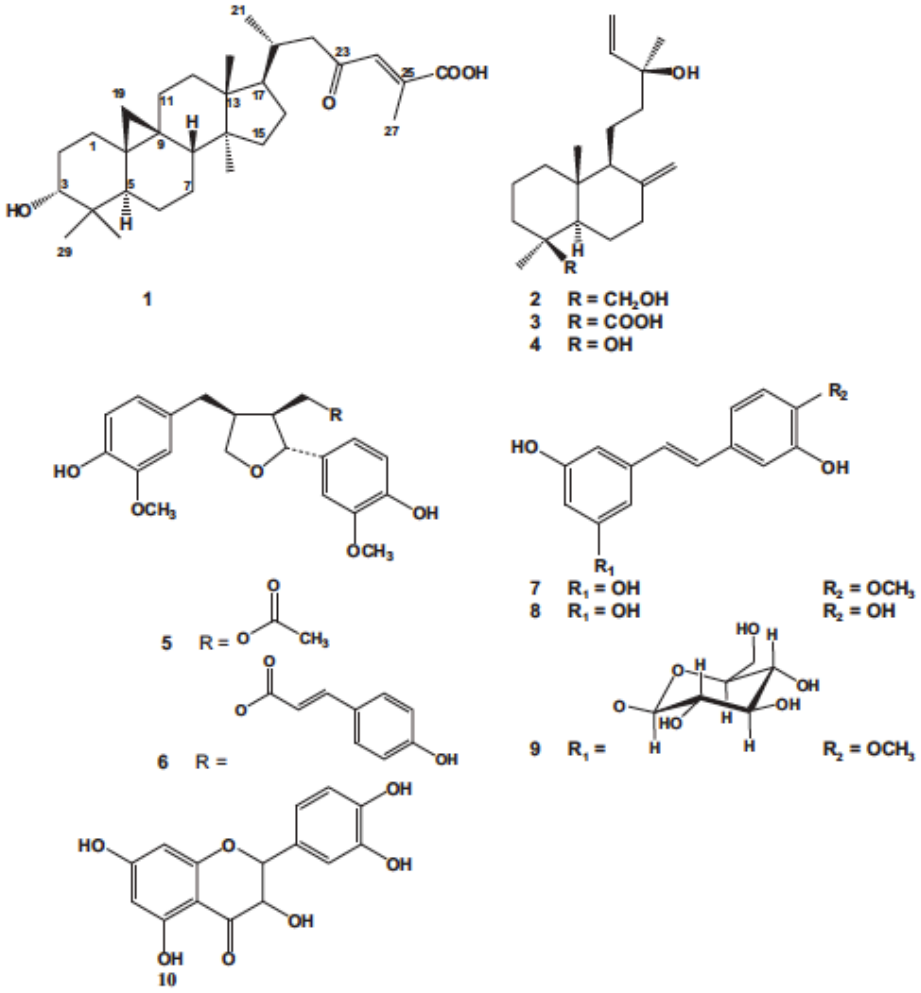
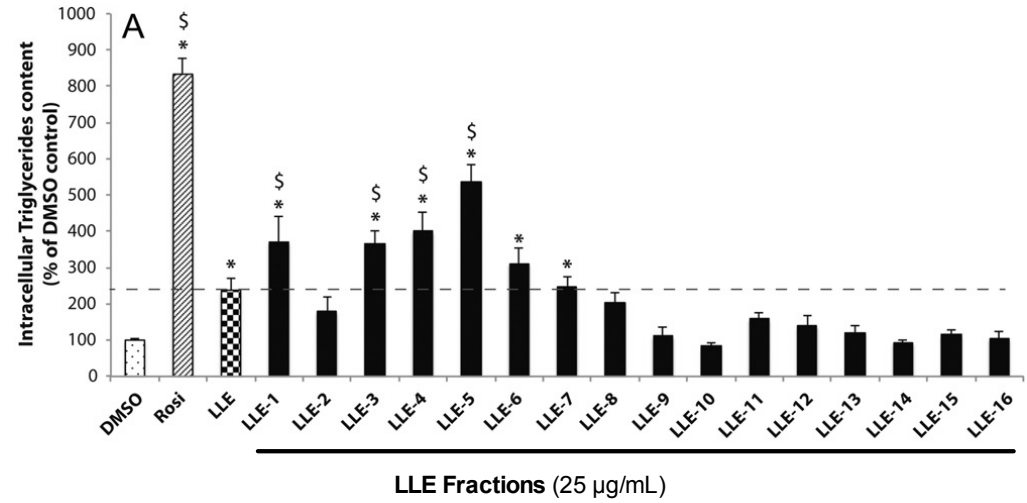

1
2 R = CH2OH
3 R = COOH
4 R = OH
5 R =
6 R =
7 R1 = OH R2 = OCH3
8 R1 = OH R2 = OH
9 R1 = R2 = OCH3
10

Figure 2


A
Intracellular Triglycerides content (% of DMSO control)
1000
900
800
700
600
500
400
300
200
100
0
DMSO
Rosi
LLE
LLE-1
LLE-2
LLE-3
LLE-4
LLE-5
LLE-6
LLE-7
LLE-8
LLE-9
LLE-10
LLE-11
LLE-12
LLE-13
LLE-14
LLE-15
LLE-16
LLE Fractions (25 µg/mL)

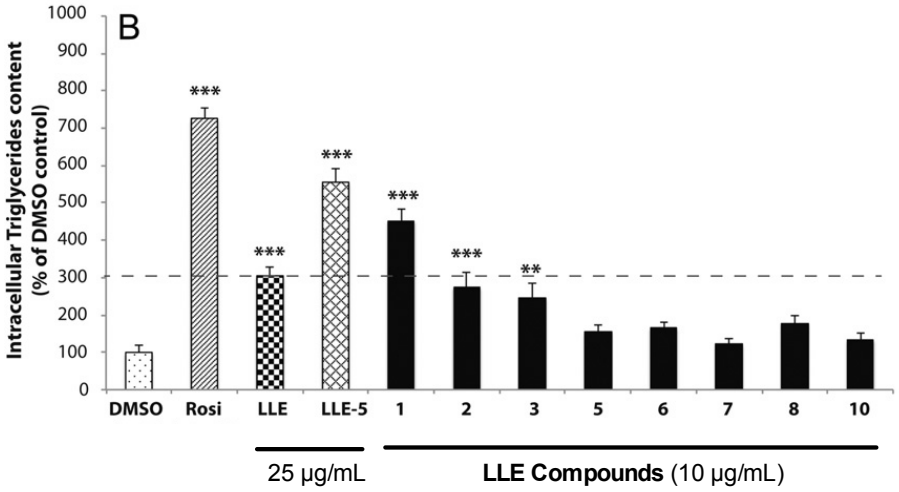
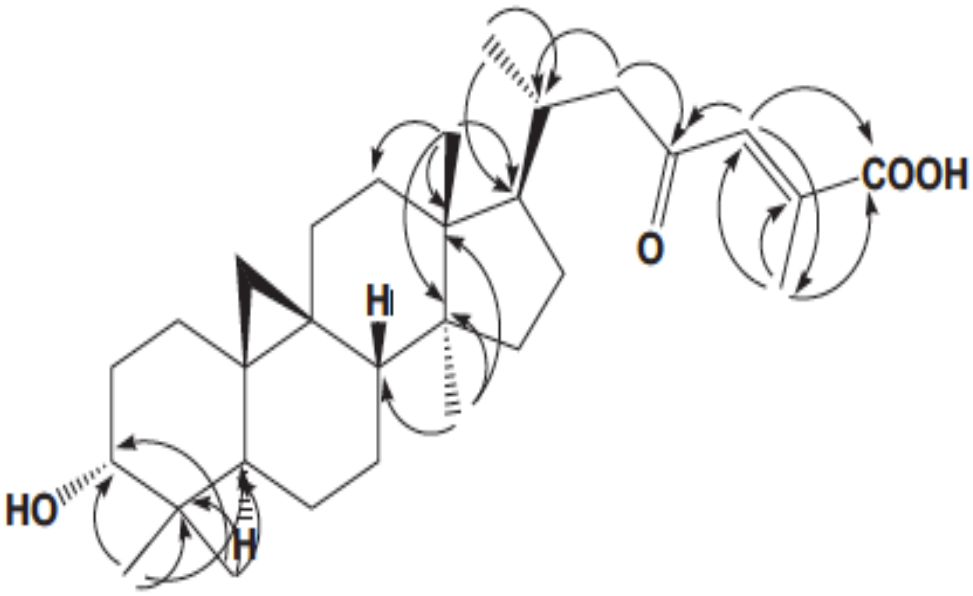

B
Intracellular Triglycerides content (% of DMSO control)
1000
900
800
700
600
500
400
300
200
100
0
DMSO
Rosi
LLE
LLE-5
1
2
3
5
6
7
8
10
25 µg/mL
LLE Compounds (10 µg/mL)

Figure 3



Chapter 3: Article 2

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Role of each co-author belonging to this manuscript

I performed the experimental work, data analysis and wrote the paper.

Dr Lina Musallam, contributed to conceptual input.

Brendan Walshe-Roussel contributed to the preparation of the plant species in the lab of Dr. John T. Arnason.

Dr. Ammar Saleem helped with the phytochemical analysis of the plant species.

Dr. Alain Cuerrier contributed in the characterization of the plant species.

Dr. Alaa Badawi contributed to provide funding to this project.

Dr. Pierre S. Haddad, my supervisor, contributed conceptual, intellectual and moral input as well as correcting the article.

Comparative cellular and molecular effects of ethanol and hot water extracts of several Canadian medicinal plants of Cree Eeyou Istchee on adipocyte lipid homeostasis

Nan Shang [1,2,3,4,5], Lina Musallam[1,4,5], Ammar Saleem[4,6], Brendan Walshe-Roussel[4,6], Alaa Badawi [7], Alain Cuerrier[4,8], John T. Arnason[4,6], Pierre S. Haddad[1,4,5]

[1]Natural Health Products and Metabolic Disease Laboratory, Department of pharmacology, Faculty of Medicine, Université de Montréal, Montreal, Quebec, Canada;

[2]Department of Hepatology and Gastroenterology, Qilu Hospital, Shandong University, Jinan, PR China;

[3]Department of Geriatrics, Qilu Hospital, Shandong University, Jinan, PR China

[4]Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines and Montreal Diabetes Research Center, Montreal, Quebec, Canada;

[5]Institute of Nutraceuticals and Functional Foods, Université Laval, Quebec, Quebec, G1V 0A6, Canada;

[6]Center for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, Ontario, K1N 6N5, Canada;

[7]Office for Biotechnology, Genomics and Population Health, Public Health Agency of Canada, Toronto, Ontario, M5V 3L7, Canada;

[8]Jardin botanique de Montréal. Institut de recherche en biologie végétale, Université de Montréal, Montréal, Quebec, H1X 2B2, Canada.

Addresses for correspondence

Pierre S. Haddad, PhD

Department of Pharmacology

Université de Montréal

P.O. Box 6128, Station Centre-Ville

Montréal, Québec, Canada

H3C 3J7

Key words:

3T3-L1 adipocytes; adipogenesis; adipocyte differentiation; diabetes; obesity; natural health products; Aboriginal traditional medicine.

Abbreviations:

AMPK, AMP-activated protein kinase;

C/EBP, CCAAT-enhancer binding proteins;

MetS, Metabolic syndrome;

PPAR, peroxisome proliferator-activated receptor;

SREBP-1, sterol-regulatory element-binding protein-1;

T2D, Type 2 diabetes;

TZDs, thiazolidinedione.

Abstract

Aim: We aimed to evaluate and compare the antidiabetic and antiobesity potential of 17 Cree plants' ethanol extracts (EE) and hot water extracts (HWE) on lipid homeostasis *in vitro* as well as to assess underlying molecular mechanisms.

Materials and methods: Post-confluent 3T3-L1 cells were differentiated in the presence of each of the extracts for 7 days and intracellular triglyceride content was measured. Peroxisome proliferator-activated receptor (PPAR) γ, sterol-regulatory element-binding protein-1 (SREBP-1) and CCAAT-enhancer binding proteins (C/EBP) α, key transcription factors involved in adipocyte differentiation, were also assessed by Western blot analysis. Both EE and HWE were characterized by UPLC-QTOF/MS and the data subjected to multivariate analysis using MassLynx and MarkerLynx software.

Results: Our results confirmed that 10 of the 17 EE potentiated adipocyte differentiation and adipogenesis, whereas 2 inhibited the same. Corresponding HWE exhibited partial or complete loss of such adipogenic or anti-adipogenic activity. *R. groenlandicum* and *K. angustifolia* EEs increased the expressions of PPARγ, SREBP-1 and C/EBPα whereas *P. balsamifera* and *A. incana* decreased them. *P. balsamifera*'s inhibitory effect was also found to involve AMP-activated protein kinase (AMPK) activation. *R. groenlandicum* HWE and EE stimulated the expression of these transcription factors but HWE of other selected plants lost such effects compared to their respective EE. Multivariate analysis uncovered clustering of active versus inactive species, notably when species were segregated by plant family.

Conclusion: These studies indicate that the method of extraction is a significant determinant of the biological activity of Cree medicinal plants on 3T3-L1 adipogenesis.

1. Introduction

Metabolic syndrome (MetS) in an individual reflects the clustering of multiple metabolic abnormalities and is related to the continuum between obesity (especially visceral in origin) and Type 2 diabetes (T2D). The latter conditions are currently considered to be worldwide epidemics. For instance, about 1 in 5 Canadian adults have MetS [1], 1 in 10 adults of the world's population is now obese [2], and 346 million people worldwide have diabetes [3]. In particular, T2D and obesity are more pronounced among indigenous peoples than in the rest of the population. This is the case in the Cree Nation of Eeyou Istchee (CEI) of northern Quebec, where the age-adjusted prevalence of T2D reached 29% in 2009 and where the prevalence of obesity and overweight is also 2-3 times higher than in the general Canadian population [4].

In order to respond to the primary health care needs of the CEI and to search for culturally adapted T2D therapies, our team (CIHR-TAAM) conducted a number of quantitative ethnobotanical studies to identify culturally relevant anti-diabetic treatments [4, 5]. Seventeen plant species were evaluated in two screening studies using a variety of *in vitro* bioassays to assess their anti-diabetic potentials. We found that several CEI plant extracts strongly enhanced the differentiation and adipogenesis of 3T3-L1 pre-adipocytes (thus potentially enhancing insulin sensitivity). Unexpectedly, two extracts displayed a potent anti-obesity potential *in vitro* by effectively preventing the differentiation [6, 7]. Adipogenesis is highly complex and numerous key factors are involved in the regulation of transcription, such as peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT-enhancer binding proteins α (C/EBP α) and sterol-regulatory element-binding protein-1 (SREBP-1) [8-11].

In these studies, plant materials were extracted with 80% ethanol at room temperature, which is a well-established phytochemical approach to enhance the extraction of bioactive phenolic secondary metabolites. However, such an approach does not correspond to traditional methods used by Aboriginals, which often rely on hot water as an extraction solvent. It is expected that ethanol (EE) and hot water

extracts (HWE) may contain different levels and/or combinations of molecules and thus exert different antidiabetic biological activities. Therefore, the present study aimed to evaluate and compare the adipogenic or anti-adipogenic activities of Cree plant EE and HWE in 3T3-L1 cells, as well as to begin elucidating the possible underlying mechanisms responsible for the activities *in vitro*.

2. Materials and Methods

2.1 Plant material and extraction

The 17 Cree medicinal plant species identified as being relevant to the treatment of symptoms of diabetes [4] are listed in Table 1. The specific organ part tested and the concentrations used are also presented in accordance with previous studies [4]. These samples were collected in a culturally respectful manner in two areas of the CEI territory in northern Quebec, Canada. Dr. Alain Cuerrier, taxonomist at the Montreal Botanical Garden, ascertained the botanical identity of the plant species. Voucher specimens were deposited in the Marie-Victorin herbarium of the Montreal Botanical Garden in Montreal, Quebec, Canada. The collected plant samples were air dried and then sent to University of Ottawa, where they were cleaned and separated into plant organ parts. Plant material was ground using a Wiley Mill (Arthur H. Thomas, Swedesboro, USA) and a filter with a 2-millimetre mesh-size. EE were prepared by extracting ground material in 80% ethanol twice for 24 h on a mechanical shaker. Following vacuum filtration with Whatman paper, EE were dried using a rotary evaporator at 40°C followed by lyophilization. HWE were prepared by boiling ground material for 75 minutes. Following vacuum filtration with Whatman paper, HWE were dried using a spray dryer. All dried extracts were conserved at 4 °C in a desiccator and protected from light.

2.2 Phytochemical characterization of extracts and metabolites analysis

Chromatography was carried out on Acquity BEH C18 column (1.7μm 2.1 × 100 mm) connected with a VanGuard Pre-column 2.1 × 5 mm using an Acquity UPLC^{TM} system with the column temperature at 50 °C and sample temperature at 10 °C. The mobile phase consisted of (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid (Fisher Optima LC-MS). The gradient conditions of the mobile phase were: 0-1 min 5% A isocratic, 1-6 min linear gradient 5-50% B, 6-8 min 50-95%B, 8.01-10 min 5% A isocratic (total run time 10 min). The flow rate was 0.5 mL/min, and 1 μL of sample was injection followed by a strong

wash 200 µL (90% acetonitrile+10% water) and weak wash 600 µL (10% acetonitrile+90% water).

Mass spectrometric analysis was performed using a Waters Synergy Quantitatvie Time of Flight mass spectrometer (QTOF/MS) equipped with an electrospray ionization (ESI) interface (Xevo G2, Waters Inc.). The ESI source was operated in positive ionization mode with source temperature of 120 °C, desolvation temperature of 400 °C, Cone gas (N2) flow of 50 L/hr, and desolvation gas (N2) flow of 1195 L/hr. Leucine-enkephalin was used as the lock mass generating an [M+ H]+ ion (m/z 556. 2615). The optimal conditions used for MSe analysis were as follows: mass range 100-1500 Da, function 1 CE, 6V, function 2 CER 10-30V, cone voltage 20 V, scan time 0.1 sec. System was calibrated with, sodium formate and the data were acquired and processed with MassLynx (version 4.1) and MarkerLynx (version 8.03) software with principal component analysis (PCA). The retention times and the protonated masses were generated at a noise threshold of 500 counts and no smoothing was applied. Pareto scaling was applied to generate the score plots. To maximize class discrimination and biomarkers, the data were further analyzed using the orthogonal partial least square discriminant analysis (OPLS-DA) method. S-plots were calculated to visualize the relationship between covariance and correlation within the OPLS-DA results. Variables that had significant contributions to discrimination between groups were considered as potential biomarkers.

2.3 Cell culture

Pre-adipocyte 3T3-L1 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). Dexamethasone (DXM), bovine pancreatic insulin, 3-isobutyl-1-methylxanthine (IBMX), dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Rosiglitazone came from Alexis Biochemicals (Hornby, ON). Dubelcco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and bovine calf serum (BCS) were from Wisent Inc (St-Bruno, QC). Pre-adipocyte 3T3-L1 cells were cultured in high-glucose DMEM supplemented with 10% BCS and penicillin-streptomycin antibiotics in a 5% CO_2 atmosphere at 37°C. Cells were passaged at about 80% confluence or grown to full

confluence for differentiation experiments. On day 0 (24 h post-confluence), cells were induced to differentiate with high-glucose DMEM supplemented with 10 % FBS, 1 μM dexamethasone (DXM), 250 μM 3-isobutylmethylxanthine (IBMX), and 500 nM insulin. After 48 h, the media was replaced with DMEM containing 10 % FBS and 500 nM Insulin. Cells were differentiated for a total of 7 days with media change every 2 days. Plant extracts (mg/ml) at previously determined optimal concentrations [4], as well as positive control rosiglitazone (10 μM), were dissolved in DMSO (0.1 % final concentration) and added to the cells at day 0 of differentiation and replenished at every medium change thereafter. Cells treated with vehicle (0.1% DMSO) in proliferation medium for the entire process served as a negative (undifferentiated) control and cells treated with 0.1% DMSO in differentiation medium for this same duration served as the vehicle control.

2.4 Adipogenesis assay and assessments of intracellular triglycerides

Adipogenesis was assessed in the well-characterized 3T3-L1 cell model by measuring the accumulation of triglycerides (TGs) upon differentiation (at day 7), using the AdipoRed reagent (Lonza Walkersville Inc, Walkersville, MD) according to the manufacturer's instructions, as described previously with minor modifications [7]. Briefly, after washing each well twice with Phosphate-Buffered Saline (PBS: 8.1 mM Na_2HPO4, 1.47 mM KH_2PO_4, 137 mM NaCl, and 2.68 mM KCl; pH 7.4), 1 ml of PBS containing 30 μl of AdipoRed reagent were added to each well of a 24-well plate and incubated for 15 min at room temperature. AdipoRed becomes fluorescent when partitioned in a hydrophobic compartment. The fluorescence of each well was measured using a fluorimeter (Wallac Victor2; Perkin-Elmer, St-Laurent, Qc) at 485 nm excitation wavelength and 572 nm emission wavelength. For each experiment, the mean fluorescence value from the negative undifferentiated control condition was considered as background and was subtracted from all other fluorescence readings. The results were reported as a percentage of the vehicle control.

2.5 Western blot

3T3-L1 cells were placed on ice and washed 3 times with ice-cold PBS and lysed in RIPA lysis buffer (50mM Hepes, 150mM NaCl, 5mM EGTA, 2mM $MgCl_2.6H_2O$, 5% glycerol, 1% Trition X-100, 0.1% SDS, 1% Na-deoxycholate, 2mM phenylmethanesulfonyl fluoride (PMSF), 10mM NaF, 100uM Na-orthovanadate, 1mM Na-pyrophosphate and one tablet of commercial cocktail of protease inhibitors (Complete Mini; Roche, Mannheim, Germany)) at pH 7.4. Protein content was determined by the bicinchoninic acid (BCA) method (Thermo Scientific Pierce, Rockford, IL, USA) standardized to bovine serum albumin (BSA). The proteins of each sample were boiled for 5 minutes, separated in 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% non-fat milk in TBST (200 mM Tris base, 1.37m NaCl and 0.1% Tween-20) pH 7.6 for 1 hour and then incubated with primary antibody (PPAR γ (E-8), Santa Cruz, 1:500; C/EBP α (14AA), Santa Cruz, 1:500; SREBP-1 (H160), Santa Cruz, 1:500; Phospho-AMPKα (Thr172), Cell Signaling, 1:500; AMPKα, Cell Signaling, 1:1000 or β-actin, Cell Signaling, 1: 1000) overnight at 4 ° C. Membranes were washed with TBST followed by a 1h incubation at room temperature with horseradish-peroxidase-conjugated secondary antibodies (anti-rabbit, Jackson, 1:20000 or anti-mouse IgG, Cell Signaling, 1:2000). The bands were detected by enhanced chemiluminescence (ECL) method with ECL Plus (Perkin Elmer, Woodbridge, Canada). All experiments were conducted on three different cell preparations. All samples from a given passage were separated and transferred simultaneously to a single membrane. Quantification of the integrated density of bands was performed using NIH Image J 1.45s software (National Institutes of Health, Bethesda, MD, USA).

2.6 Statistical analysis

Intracellular TG content results are presented as mean ± SEM of 3 independent experiments, each performed in triplicate. Statistical calculations were performed with Prism GraphPad software. We used one-way analysis of variance (ANOVA) and post hoc Dunnett's test to assess differences between vehicle controls and stimulating, inactive plant extracts (EE or HWE) as well as between vehicle

controls and inhibiting plant extracts (EE or HWE), respectively. Differences between ethanol and water extracts of all plants were analyzed by two-way analysis of variance (ANOVA).

3. Results

3.1 Effects of ethanol and hot water extracts on adipogenesis and adipocyte differentiation

Extracts were tested and compared for adipogenic activity in 3T3-L1 preadipocytes treated continuously with optimal concentrations throughout a 7-day differentiation period. At the end of the differentiation, intracellular content of TG was assessed fluorescently in cells. TG content was significantly increased by 5.7-fold by treatment with 10 μM rosiglitazone (positive drug control) when compared with vehicle control.

TG content was significantly stimulated by 2- to 5-fold by treatment with the EE of 10 out of the 17 plants (namely, *A. balsamea, P. glauca, R. groenlandicum, R. tomentosum, L. laricina, K. angustifolia, V. vitis-idaea, P. banksiana, L. clavatum, and P. mariana*; Figure 1). Out of this subset of plants, the HWE of 3 species (*A. balsamea, P. glauca* and *R. groenlandicum*) had lower activity but still significantly stimulated adipogenesis. However, for the other stimulating species, the effects of their EE in 3T3-L1 cells were completely lost when HWE were used. In contrast to these species, EE of *P. balsamifera* and *A. incana* could inhibit adipogenesis in 3T3-L1 cells to 0.3% and 15% compared with vehicle control, respectively. As seen with several species having positive adipogenic activity, *P. balsamifera* and *A. incana* completely lost their action (inhibitory, in this case) on adipogenesis when the water extracts were used instead.

3.2 Effect of selected extracts on the expression of PPAR γ, C/EBP α and SREBP-1

Five plants were selected for further in-depth pharmacological mechanism analysis; namely, *R. groenlandicum, K. angustifolia, S. purpurea, A. incana* and *P. balsamifera*. The selection process sought to compare species with adipogenic or anti-adipogenic potentials as well as species with varying or stable activities between EE and HWE.

PPAR γ, C/EBP α and SREBP-1 are three key transcription factors involved in the regulation of adipocyte differentiation. As shown in Figure 2A, EE of *R. groenlandicum* and *K. angustifolia*, which stimulated 3T3-L1 differentiation and adipogenesis, also increased PPAR γ, C/EBP α and SREBP-1 levels. The HWE of *R. groenlandicum*, which had a lesser albeit significant effect on adipogenesis than its EE, also maintained its activation of the same transcription factors. *K. angustifolia* HWE, which completely lost its potentiating effect on adipogenesis as compared to its EE, was also ineffective in modulating PPAR γ, C/EBP α and SREBP-1.

The inhibition of adipocyte differentiation and adipogenesis is a potential therapeutic target for obesity. EE of *P. balsamifera* and *A. incana*, which inhibited adipogenesis, were found to successfully decrease PPAR γ and SREBP-1 levels (Figure 2). However, only the EE of *P. balsamifera*, but not that of *A. incana*, could also successfully decrease C/EBP α levels. On the other hand, the HWE of *P. balsamifera* and *A. incana* lost all of these effects. Neither the EE nor the HWE of *S. purpurea* could activate the three transcription factors, consistent with the plant's lack of effect on adipogenesis.

As suspected from the above results, there were strong and significant positive correlations between the selected plants' abilities to modulate PPAR γ, C/EBP α and SREBP-1 levels and their adipogenic activity (R^2>0.8, $p < 0.05$, Figure 2E, F and G.)

3.3 The ethanol extract of *P. balsamifera* inhibits adipogenesis partly via activating AMPK

We also investigated the AMPK pathway, which has been shown to inactivate PPAR γ transcriptional activity and to inhibit adipocyte differentiation [12]. Metformin, a known activator of AMPK signaling, served as a positive control and greatly enhanced the activation and phosphorylation of AMPK (Figure 3A). Of the selected five plant species, only treatment with the EE of *P. balsamifera* significantly increased the phosphorylation of the AMPK (Fig. 3B).

3.4 Metabolites analysis

Using the optimal UPLC–QTOF/MS conditions described above, representative fingerprints for all HWE and EE of 17 plants were processed. Overall, more than 4000 metabolites were detected from these extracts within the chromatographic retention time range of 0.4-6.5 min. In order to better visualize the subtle similarities and differences among these complex data, multivariate analysis, PCA and OPLS-DA were used to classify the phenotypes of phytochemical metabolites.

Using an unbiased PCA approach, all HWE and EE of the 17 plants clustered together (data not shown). Moreover, such analysis did not allow any clear distinction between the active species (stimulating or inhibiting adipogenesis) and inactive ones (having no effect on adipogenesis).

We then carried out more specific PCA approaches guided by bioactivity, notably examining results for HWE and EE samples separately. For HWE, the PCA process did not show any discriminating characteristics when adipogenic (stimulating) and antiadipogenic (inhibiting) plants were grouped with inactive species (data not illustrated). This held true even if an additional segregation of plants was made on the basis of plant families.

Similarly, when all EE samples were analyzed together (stimulating, inhibiting and inactive species), the PCA process did not show any clustering (data not illustrated). However, clear clustering of active species could be observed as shown in Figure 4. In the case of species stimulating adipogenesis, clear clustering of active and inactive species was obtained and more obviously when plants were segregated according to their affiliation with the Ericaceae (*R. gromenlandicum, R. tomentosum, and K. augusfolia*; Figure 4A and 4B) or Pinaceae (*A. balsamea, L. laricina, P. mariana, P. banksiana*; Figure 4A and 4C) plant families.

A S-plot was then generated to determine plant metabolites that could significantly contribute to discriminating species. These usually lie in the top or bottom extremities of the S-plot, and are characterized by sufficiently important statistical differences to render them potential biomarkers for the biological activity studied. As shown in Figure 5C and 5E, there were several potential markers.

4. Discussion

Using ethnobotanical surveys, our group identified 17 CEI medicinal plants as candidate antidiabetic species used by the CEI First Nations to treat various symptoms of Type 2 diabetes [13-18]. We found that several CEI plants (ethanol extracts) strongly potentiated the differentiation and adipogenesis of 3T3-L1 pre-adipocytes (associated with enhanced insulin sensitivity, a hallmark of the thiazolidinedione (TZDs) class of antidiabetic medications) and two extracts displayed a potent anti-obesity potential *in vitro* by effectively inhibiting the differentiation [6, 7]. In the present study, we used the same bioassay to evaluate and compare the antidiabetic potentials of ethanol and water extracts of these plants as well as potential underlying mechanisms *in vitro*.

Generally in line with previous observations [4, 5], our results showed that 10 of the 17 plants EE stimulated adipogenesis, hence indicating their antidiabetic potential. Out of these plants, only 3 HWE (*R. groenlandicum A. balsamea,* and *P. glauca*) still significantly stimulated adipogenesis, albeit at a lower level than their EE counterparts. However, this does not imply that the traditional method of preparation and usage is not efficient. Firstly, for the purpose of this study, it was important, in a pharmacological perspective, to compare equal concentrations of raw ethanol and water extracts. Hence, our results agree with the generally accepted contention that the phytochemical approach by ethanol extraction is more efficient at concentrating bioactive plant components (notably, phenolic compounds) than the traditional hot water extraction. This suggests that phenolic compounds may underlie the biological activity of the active EE samples and this is consistent with our findings on a number of the concerned species [19-21].

Secondly numerous parameters might affect the quality of extractions carried out using hot water, such as extraction time, temperature, and water quality. Traditional medicinal plant preparations of Aboriginal healers might thus differ from the HWE conditions used in this study and could yield more concentrated solutions. Further studies should therefore compare crude plant extracts prepared by Cree healers with those prepared in the laboratory over a wider range of concentrations.

Thirdly, the analysis of phytochemical metabolite profiles for the 17 plants extracted by either ethanol or hot water support our interpretations. Overall, more than 4000 metabolites were detected from these extracts by UPLC-QTOF. The PCA scores take into consideration both the quality and quantity of plant metabolites/biomarkers. At first view, the clustering together of the EE and HWE of all species in the initial unbiased PCA process may be seen to indicate that the selected antidiabetic Boreal plant species have no discriminating characteristics that can relate to their effects on the adipogenesis of 3T3-L1 cells. However, the fact that we have two extracts of 17 plants with varying composition and biological activities that include stimulating, inactive (neutral) and inhibiting actions limits the discriminating capacity of the otherwise powerful PCA process. We thus used two approaches to "guide" the PCA. Firstly, we segregated HWE or EE extracts and found no discriminating characteristics when all plants species were included. We then selected active stimulating species and combined them specifically with inactive ones, thereby excluding inhibitory species. In doing so, we successfully uncovered two strongly discriminating relationships among the EE. Both discriminating conditions were obtained when active plant species were grouped according to their belonging to either the Ericaceae or Pinaceae plant families (after removal of outlier species, *V. vitis-idaea* and *P. glauca* respectively) in PCA and OPLS-DA. Such results strongly support the contention that these plant families can be characterized by common phytochemical profiles that can explain, at least in part, the capacity of member species to stimulate the differentiation of pre-adipocytes in culture. Although clearly beyond the scope of the present work, further in-depth discriminant analyses will be correlated with additional phytochemical assessments to elucidate and identify plant metabolites that could then gain the status of adipogenic stimulating biomarkers at the plant species or family levels.

Finally, the present studies sought to gain further insight into the mechanisms of action underlying the activity of CEI plants on adipogenesis. We selected five plant species that represented the full spectrum of conditions that we sought to study. *R. groenlandicum* was a plant that strongly stimulated adipogenesis (as previously observed [22]) and whose HWE maintained a significant level of activity. Likewise,

the UPLC total ion chromatographic (TIC) profiles of its EE and HWE showed strong similarities. In contrast, *K. angustifolia* lost its adipogenic potential when HWE was used instead of EE, despite relatively similar phytochemical profiles. *S. sarracenia* was selected as a "neutral" plant that showed no adipogenic or anti-adipogenic activity and whose EE and HWE UPLC profiles showed good similarity. Finally, we selected both inhibiting species. Indeed, although the HWE of both plants lost inhibitory potential, the UPLC TIC profile was quite similar to its corresponding EE profile for *A. incana* but not for *P. balsamifera*. We thus examined several transcription factors that regulate a variety of genes and represent key elements involved in the process of adipocyte differentiation and lipogenesis, namely PPAR γ, C/EBP α and SREBP -1[8, 9]. PPAR γ agonists function as 'adipose remodeling factors' that could redistribute lipids from non adipose tissue and insulin-resistant adipocytes into insulin-sensitive adipocytes by enhancing adipogenesis, regulating adipokines, decreasing free fatty acids and exerting insulin-sensitizing effects. In this study, we confirmed that several selected stimulators of adipogenesis activated PPAR γ, as well as other transcription factors. In fact, strong correlations were obtained between the expression of each transcription factor and the adipogenic potential of each plant extract. Our results thus further support the use of *R. groenlandicum* and *K. angustifolium* as potential insulin sensitising plants that could complement modern pharmaceuticals and represent culturally adapted additions to help Cree diabetics manage their disease. As mentioned above, traditional preparations of these plants need to be compared with EE, which exerted the strongest effects in this regard, as compared with laboratory HWE.

Conversely, the inhibition of adipogenesis is also a potential therapeutic target of antiobesity and ensuing antidiabetic therapies [23, 24]. Numerous plant extracts and naturally occurring compounds are known to inhibit adipogenesis, and a variety of mechanisms by which this is mediated have been identified [25-27]. The EE of two species (*P. balsamifera* and *A. incana*) inhibited adipogenesis *in vitro* and the EE of *P. balsamifera* also showed antidiabetic and antiobesity potential and improved insulin sensitivity in a mouse model of diet-induced obesity *in vivo* without fat redistribution to ectopic sites [28]. In the present study, we found that both species

inhibited adipogenesis partly through decreasing the expression of the same key transcription factors, namely PPAR γ and SREBP-1 or C/EBP α. Since our previous reports highlighted the fact that the two plants acted at different steps in the adipogenesis program [20, 29], our results suggest that these sites of action appear to lie upstream of the three transcription factors studied. We further tested another important pathway regulating adipogenesis and confirmed that *P. balsamifera*, but not *A. incana*, stimulated the phosphorylation of AMPK, a key energy sensor. In fact, a number of natural compounds, such as genistein and curcumin, demonstrated an anti-adipogenic effect by activating AMPK [12, 30, 31]. The fact that the HWE of both *P. balsamifera* and *A. incana* lost anti-adipogenic activity, despite the variable changes in their respective metabolite profiles, will require further in-depth study through PCA and additional chemical analysis.

In summary, we evaluated and compared the adipogenic/anti-adipogenic potentials of EE and HWE of 17 plants as well as the possible molecular mechanisms responsible for such activities in 3T3-L1 cells. We confirmed that the method of extraction is a significant determinant of the biological activity of several medicinal plants. UPLC-QTOF TIC profiles and corresponding principal component analysis enabled us to segregate the metabolomes of active plant species from the inactive ones, in particular when stimulating species were further grouped based on plant family. This powerful approach enabled us to uncover similarities and differences of the chemical profiles of these plants. Mining such PCA results offers an interesting avenue to identify potential bioactive plant metabolites. Our work and its future applications can help to develop novel as well as culturally relevant plant-based therapeutic approaches against insulin resistance and obesity that target pre-adipocytes and adipocytes directly. Studies comparing different plant extracts in other insulin sensitive tissues, such as liver and muscle, can complement this. Finally, our studies can serve as quality control tools to foster reliable and effective plant-based treatments, using the information relative to the content of active ingredients and/or the biological activity.

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7. Figure legends

Figure 1: Effects of extracts on rate of adipogenesis. The effects of extracts on rate of adipogenesis were assessed by TG content at the end of a 7-day differentiation period with continuous treatment with vehicle (0.1% DMSO), EE or HWE extract (concentrations indicated in Table 1), or positive control (10 μM rosiglitazone). Results are means ± SE for 3 separate experiments, normalized to the vehicle-treated condition. # Denotes stimulator EE samples statistically significant from vehicle control ($p<0.05$), one-way ANOVA and post hoc Dunnett's test. $ Denotes HWE samples statistically significant from vehicle control ($p < 0.05$), one-way ANOVA and post hoc Dunnett's test. & Denotes inhibitory EE samples statistically significant from vehicle control ($p<0.05$), one-way ANOVA and post hoc Dunnett's test. * ($p<0.05$), **($p<0.01$) and *** ($p<0.001$) denote statistically significant between EE and HWE counterpart, Two-way ANOVA.

Figure 2: Effect of the selected extracts on expression of PPAR γ, C/EBP α and SREBP-1. 3T3-L1 cells were differentiated and treated for 7 days with either 0.1% DMSO (vehicle control), Rosiglitazone (10 μM, positive control), EE or HWE of the 5 selected plants. Expression of PPAR γ, C/EBP α and SREBP-1 were measured by western blot and representative samples are illustrated in Panel A. Western blot results for 3 separate experiments are also presented as means ± SE, normalized to the vehicle-treated condition (Panels B, C and D). # Denotes EE samples statistically significant from vehicle control ($p<0.05$), one-way ANOVA and post hoc Dunnett's test. $ Denotes HWE samples statistically significant from vehicle control ($p < 0.05$), one-way ANOVA and post hoc Dunnett's test. * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) denote statistically significant between EE and HWE counterpart, Two-way ANOVA. Correlations between the expression of PPAR γ, C/EBP α and SREBP-1 levels and the adipogenic activity are shown in panels E, F and G. Correlation results were analyzed by linear regression and the equations were $y = 163.6x - 47.39$, $y = 207.7x - 70.12$, $y = 245.6x - 98.96$ respectively.

Figure 3: EE of *P. balsamifera* stimulates the AMPK signaling pathway to inhibit adipogenesis. 3T3-L1 cells were differentiated and treated for 7 days with either 0.1% DMSO (vehicle control), Metformin (400 mM. positive control), EE or HWE of the 5 selected plants. Phosphorylation of AMPK was measured by western blot and representative samples are illustrated in Panel A. Western blot results for 3 separate experiments are also presented as means ± SE, normalized to the vehicle-treated condition (Panel B). # Denotes EE samples statistically significant from vehicle control ($p<0.05$), one-way ANOVA and post hoc Dunnett's test. *** ($p<0.001$) Significantly different between EE and HWE counterpart, Two-way ANOVA.

Figure 4: Metabolite analysis of selected ethanol extracts.

PCA scores plot of stimulators of ethanol extract for adipogenesis in Ericaceae family, Pinaceae family and the five inactive plants are shown in Panel A OPLS-DA scores plot and S-plot of stimulators of ethanol extract for adipogenesis in Ericaceae family (*R. gromenlandicum, R. tomentosum,* and *K. augusfolia*) and the five inactive plants are shown in Panel B and C. Likewise stimulators of ethanol extracts of species belonging to the Pinaceae family (*A. balsamea, L. laricina, P. mariana, P. banksiana*) are compared with inactive species in Panel D and E..

In PCA and OPLS-DA scores plots, t [1], t [2] and t [1] P and t [2] O are the new most important indices in summarizing the peak lists of the plants. In S-plot, it displays easily the observations that have a large absolute value of p (corr) [1] and a large absolute value of the coefficients. These are the observations that differentiate the most between the 2 groups

Table 1: List of investigated plant species and the concentrations of the extracts tested in 3T3-L1 cells

Species	Abbreviation	Plant part	Family	3T3-L1 cells µg/mL
Rhododendron groenlandicum (Oeder) Kron and Judd	*R.groenlandicum*	Leaves	Ericaceae	75
Abies balsamea (L.) Mill.	*A.balsamea*	Inner bark	Pinaceae	50
Larix laricina Du Roi (K.Koch)	*L.laricina*	Inner bark	Pinaceae	25
Picea mariana (P. Mill.) BSP	*P.mariana*	Cones	Pinaceae	10
Sorbus decora (Sarg.) C.K.Schneid.	*S.decora*	Inner bark	Rosaceau	15
Alnus incana subsp. rugosa (Du Roi) R.T. Clausen	*A.incana*	Inner bark	Betulaceau	50
Sarracenia purpurea L.	*S.purpurea*	Whole plant	Sarraceniaceau	100
Pinus banksiana Lamb.	*P.banksiana*	Cones	Pinaceae	15
Rhododendron tomentosum (Stokes) Harmaja subsp.subarcticum (Harmaja) G.Wallace	*R.tomentosum*	Leaves	Ericaceae	50
Kalmia angustifolia L.	*K.augustifolia*	Leaves	Ericaceae	50
Picea glauca (Moench) Voss	*P.glauca*	Leaves	Pinaceae	125
Juniperus communis L.	*J.communis*	Berries	Cupressaceau	5
Salix planifolia Pursh	*S.planifolia*	Inner bark	Saliaceau	25
Lycopodium clavatum L.	*L.clavatum*	Whole plant	Lycopodiaceau	100
Populus balsamifera L.	*P.balsamifera*	Inner bark	Saliaceau	100
Gaultheria hispidula (L.) Muhl.	*G.hispidula*	Leaves	Ericaceae	25
Vaccinium vitis-idaea L.	*V.vitis-idaea*	Berries	Ericaceae	200

Figure 1

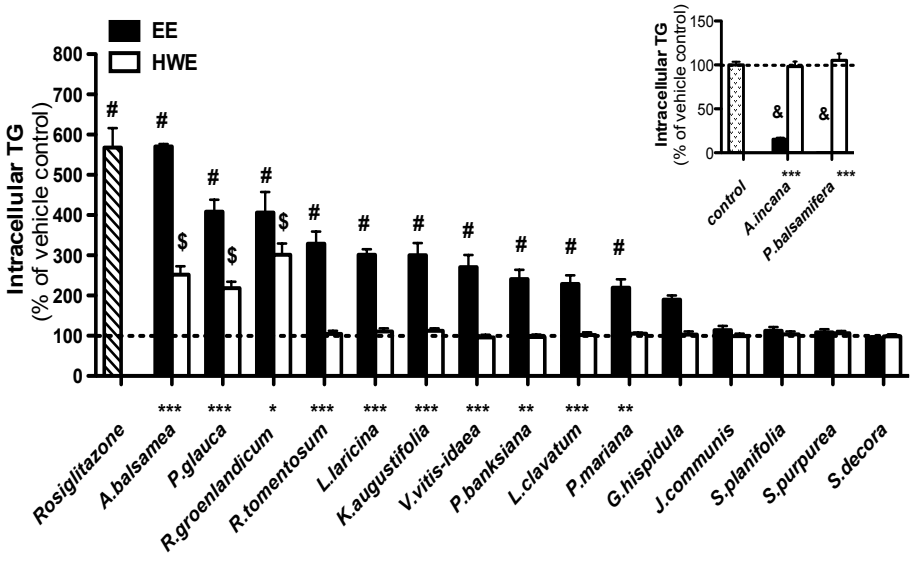

EE
HWE
Intracellular TG
(% of vehicle control)
800
700
600
500
400
300
200
100
0
Rosiglitazone
A.balsamea
P.glauca
R.groenlandicum
R.tomentosum
L.laricina
K.augustifolia
V.vitis-idaea
P.banksiana
L.clavatum
P.mariana
G.hispidula
J.communis
S.planifolia
S.purpurea
S.decora
Intracellular TG
(% of vehicle control)
150
100
50
0
control
A.incana
P.balsamifera

Figure 2

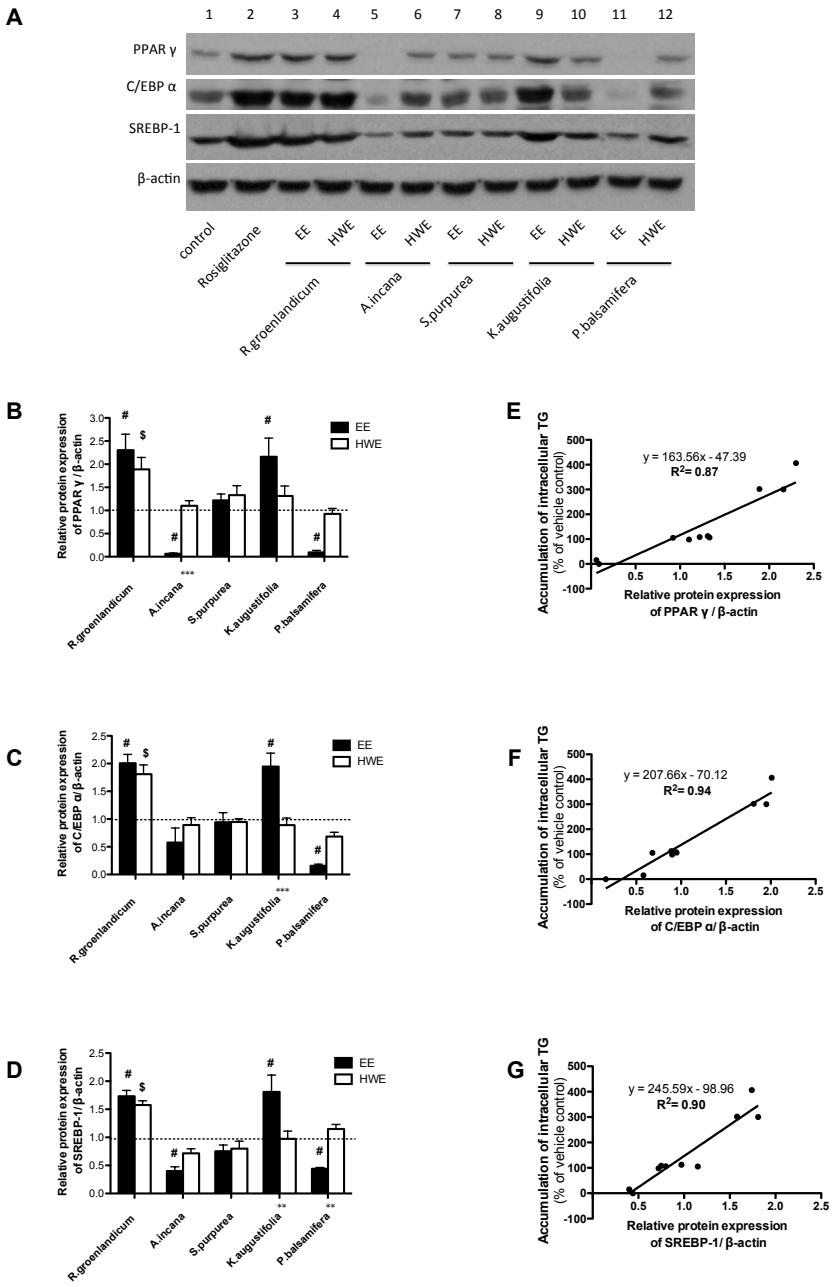

A
1 2 3 4 5 6 7 8 9 10 11 12
PPAR γ
C/EBP α
SREBP-1
β-actin
control
Rosiglitazone
EE
HWE
R.groenlandicum
A.incana
S.purpurea
K.augustifolia
P.balsamifera
B
Relative protein expression of PPAR γ / β-actin
C
Relative protein expression of C/EBP α/ β-actin
D
Relative protein expression of SREBP-1/ β-actin
E
y = 163.56x - 47.39
R²= 0.87
Accumulation of intracellular TG (% of vehicle control)
Relative protein expression of PPAR γ / β-actin
F
y = 207.66x - 70.12
R²= 0.94
Relative protein expression of C/EBP α/ β-actin
G
y = 245.59x - 98.96
R²= 0.90
Relative protein expression of SREBP-1/ β-actin

Figure 3

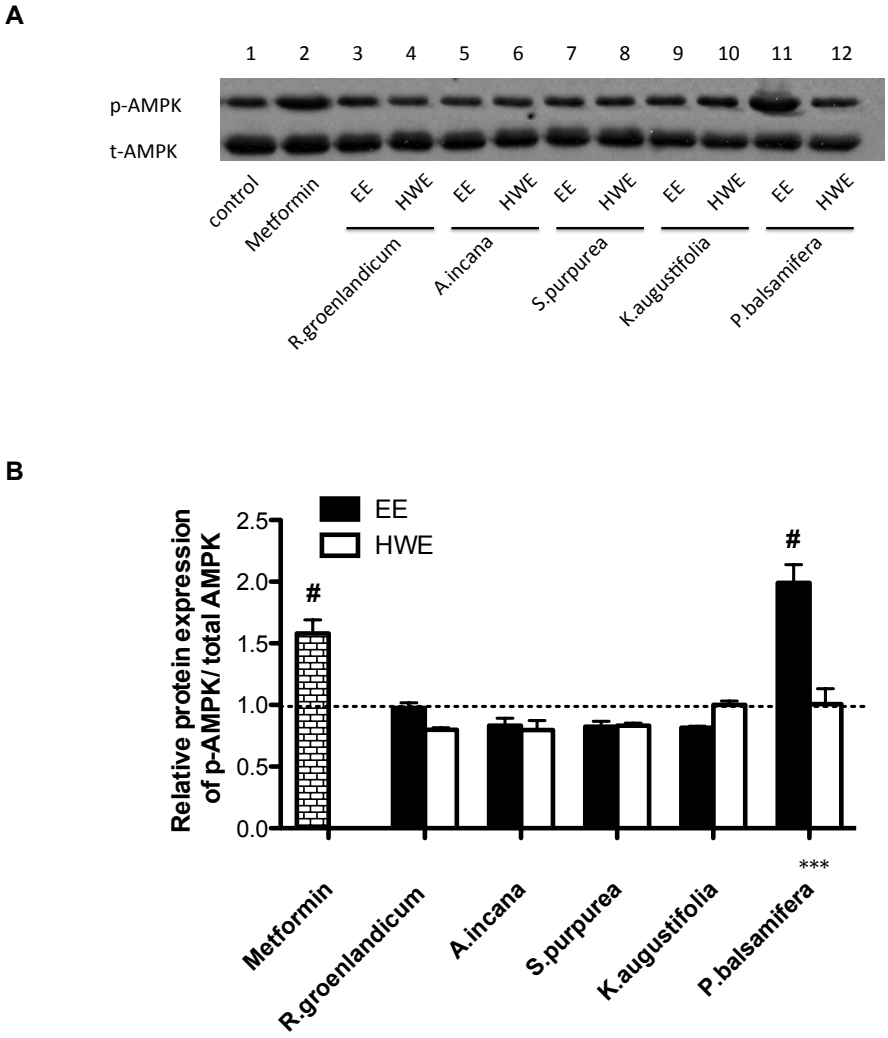
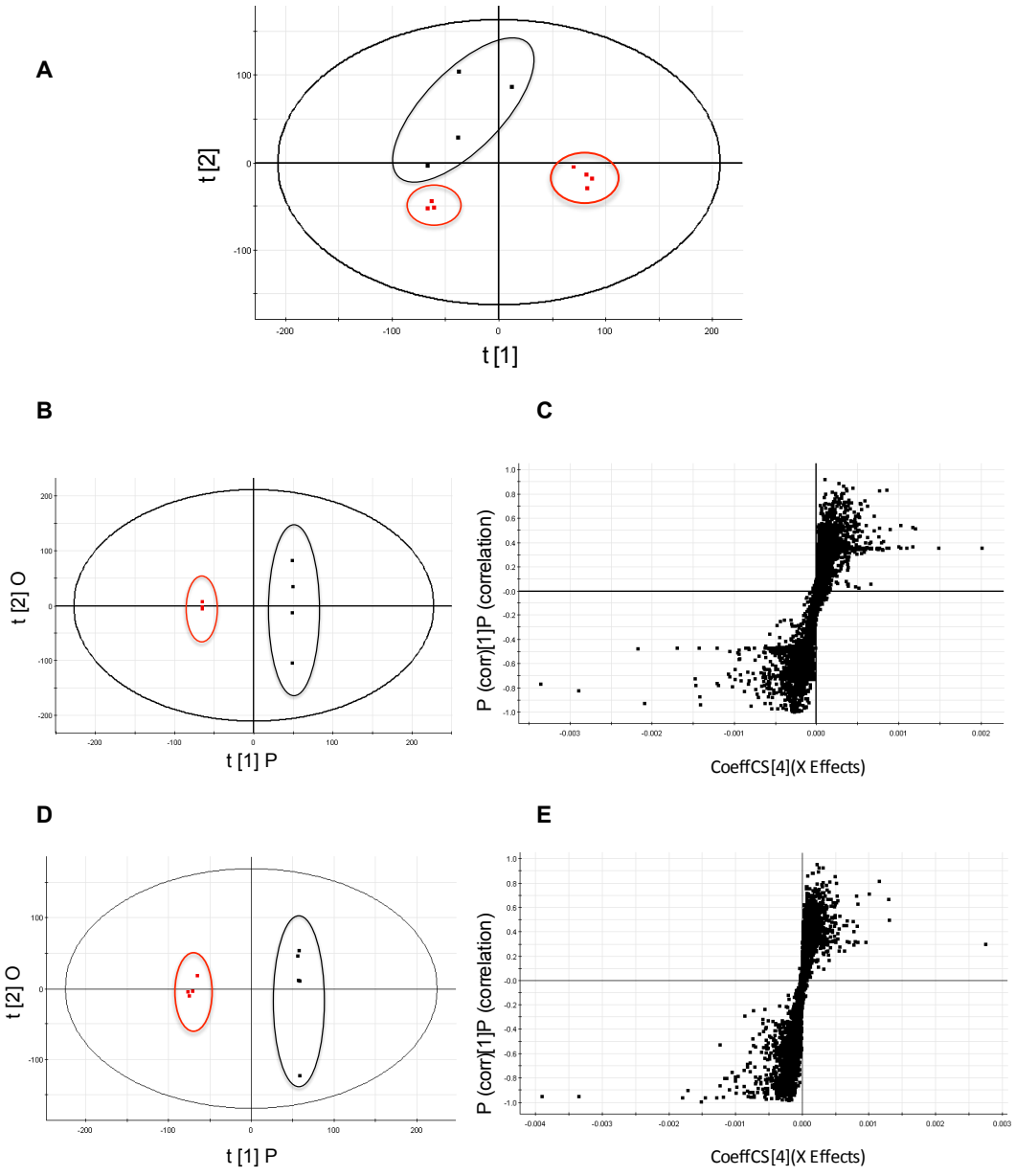

A
1 2 3 4 5 6 7 8 9 10 11 12
p-AMPK
t-AMPK
control
Metformin
EE
HWE
R.groenlandicum
A.incana
S.purpurea
K.augustifolia
P.balsamifera
B
EE
HWE
Relative protein expression of p-AMPK/ total AMPK
2.5
2.0
1.5
1.0
0.5
0.0
#
#
***
Metformin
R.groenlandicum
A.incana
S.purpurea
K.augustifolia
P.balsamifera

Figure 4


A
t [2]
t [1]
B
t [2] O
t [1] P
C
P (corr)[1]P (correlation)
CoeffCS[4](X Effects)
D
t [2] O
t [1] P
E
P (corr)[1]P (correlation)
CoeffCS[4](X Effects)

Chapter 4: Article 3

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Role of each co-author belonging to this manuscript

I performed the experimental work, data analysis and wrote the paper.

Dr Lina Musallam, contributed to conceptual input.

Brendan Walshe-Roussel contributed to the preparation of the plant species in the lab of Dr. John T. Arnason.

Dr. Ammar Saleem helped with the phytochemical analysis of the plant species.

Dr. Alain Cuerrier contributed in the characterization of the plant species.

Dr. Alaa Badawi contributed to provide funding to this project.

Dr. Pierre S. Haddad, my supervisor, contributed conceptual and intellectual input as well as correcting the article.

Comparative cellular and molecular effects of ethanol and hot water extracts of several Canadian medicinal plants of Cree Eeyou Istchee on glucose homeostasis

Nan Shang [1,2,3,4,5], Lina Musallam[1,4,5], Ammar Saleem[4,6], Brendan Walshe-Roussel[4,6], Alaa Badawi [7], Alain Cuerrier[4,8], John T. Arnason[4,6], Pierre S. Haddad[1,4,5]

[1]Natural Health Products and Metabolic Disease Laboratory, Department of pharmacology, Faculty of Medicine, Université de Montréal, Montreal, Quebec, Canada;

[2]Department of Hepatology and Gastroenterology, Qilu Hospital, Shandong University, Jinan, PR China;

[3]Department of Geriatrics, Qilu Hospital, Shandong University, Jinan, PR China

[4]Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicines and Montreal Diabetes Research Center, Montreal, Quebec, Canada;

[5]Institute of Nutraceuticals and Functional Foods, Université Laval, Quebec, Quebec, G1V 0A6, Canada;

[6]Center for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, Ontario, K1N 6N5, Canada;

[7]Office for Biotechnology, Genomics and Population Health, Public Health Agency of Canada, Toronto, Ontario, M5V 3L7, Canada;

[8]Jardin botanique de Montréal. Institut de recherche en biologie végétale, Université de Montréal, Montréal, Quebec, H1X 2B2, Canada.

Addresses for correspondence

Pierre S. Haddad, PhD

Department of Pharmacology

Université de Montréal

P.O. Box 6128, Station Centre-Ville

Montréal, Québec, Canada

H3C 3J7

Key words:

C2C12 cell; H4IIE cell; diabetes; glucose uptake; glucose production; Glucose-6-phosphatase; glucose transporter 4; AMP-activated protein kinase; Aboriginal traditional medicine.

Abbreviations:

ACC, acetyl-coA carboxylase;

AICAR, aminoimidazole carboxamide ribonucleotide;

AMPK, AMP-activated protein kinase;

GLUT4, glucose transporter 4;

GU, glucose uptake;

G6Pase, Glucose-6-phosphatase.

Abstract

Aim: Our Team previously identified 17 Cree medicinal plants having antidiabetic potential. We aimed to evaluate and compare the biological activity and molecular mechanisms of the 17 Cree plants' ethanol extracts (EE) and hot water extracts (HWE) on glucose homeostasis *in vitro* as well as to assess the relationships between phytochemical metabolites and biological phenotype.

Materials and methods: Two main bioassays routinely applied in our laboratory were used: 1) stimulation of muscle glucose transport by measuring ^{3}H-2-deoxyglucose uptake in C2C12 myoblasts, and 2) inhibition of hepatic glucose production by measuring inhibition of glucose-6-phosphatase activity (G6Pase) in H4IIE cells. We also investigated the phosphorylation of Akt and AMP-activated protein kinase (AMPK) for the selected plants by Western blot analysis. Both EE and HWE were characterized by UPLC-QTOF/MS and the data subjected to metabolites analysis using principal component analysis and discriminant analysis.

Results: Our results confirmed that several EE stimulated muscle glucose uptake and inhibited hepatic G6Pase activity. Some of the HWE partially or completely lost these antidiabetic activities in comparison to EE. Only one plant (*R. groenlandicum*) retained similar potential between EE and HWE in both assays. In C2C12 muscle cells, EE of *R. groenlandicum, A. incana and S. purpurea* stimulated glucose uptake by activating AMPK pathway and increasing GLUT4 expression level. In comparison to EE, HWE of *R. groenlandicum* exhibited similar activities; HWE of *A. incana* completely lost its effect on all parameters; interestingly, HWE of *S. purpurea* activated insulin pathway instead of AMPK pathway to increase glucose uptake. In the liver, for a subset of 5 plants, HWE and EE activated AMPK pathway whereas the EE and HWE of *S. purpurea and K. angustifolia* also activated insulin pathways. Quercetin-3-O-galactoside and quercetin 3-O-α-L-arabinopyranoside, were identified as strong potential to be responsible for the biological activity of the active HWE plants with stimulation of glucose uptake.

Conclusion: These studies indicate that plant species, target tissues or cells, as well as extraction methods are all significant determinants of the biological activity of Cree medicinal plants on glucose metabolism.

1. Introduction

Type 2 diabetes (T2D) is characterized by impaired insulin secretion and/or insulin sensitivity. There are 346 million people worldwide having diabetes [1]. In particular, T2D is more pronounced among indigenous populations, such as the Cree of Eeyou Istchee (CEI) of northern Quebec, where the age-adjusted prevalence of T2D reached 29% in 2009 [2, 3]. The burden of T2D and the ensuing deleterious complications (cardiovascular, retinopathy and nephropathy) prompted the search for culturally adapted treatment alternatives for these aboriginal populations.

In order to identify such culturally adapted T2D alternative therapies and to respond to CEI primary health care needs, our team (CIHR-TAAM) conducted quantitative ethnobotanical studies [2]. Seventeen plant extracts were tested for their anti-diabetic potential in several screening studies through a variety of *in vitro* bioassays. Results showed that several extracts strongly potentiated glucose uptake (GU) in muscle cells and inhibited Glucose-6-Phosphatase (G6Pase) activity in H4IIE cells [4-7]. Indeed, the muscle is the principal tissue involved in postprandrial glucose disposal, accounting for about 80% of GU, occurring principally through glucose transporter 4 (GLUT4) [8]. On the other hand, G6Pase is the rate-limiting enzyme for the final step of gluconeogenesis and glycogenolysis, two pathways controlling hepatic glucose production (HGP) [9]. In T2D, unsuppressed HGP has been linked to the increased G6Pase activity [10].

In these previous studies, a well-established and standard phytochemical extraction method was used, based on 80% ethanol to prepare crude plant extracts. However, traditional preparations of medicinal plants rely on other methods, such as hot water extraction. The first aim of the present study was therefore to evaluate and compare the biological activity of ethanol and hot water extracts (EE and HWE, respectively) of the 17 identified putative antidiabetic plant species from CEI traditional pharmacopeia. Secondly, we also began examining the molecular mechanisms underlying the modulating action of a subset of plants on glucose metabolism using the same *in vitro* bioassays.

2. Materials and methods

2.1 Plant material and extraction

The 17 Cree medicinal plant species identified as being relevant to the treatment of symptoms of diabetes [2] are listed in Table 1. Table 1 also lists the specific organ part tested, in accordance with the ethnobotanical data [2] and the concentrations used in different cell lines. Plant samples were collected for each species in a culturally respectful manner in two areas of the CEI territory in northern Quebec, Canada. Dr. Alain Cuerrier, taxonomist at the Montreal Botanical Garden, ascertained the botanical identity of the plant species. Voucher specimens were deposited in the Marie-Victorin herbarium of the Montreal Botanical Garden in Montreal, Quebec, Canada. The collected plant samples were air dried and then sent to the University of Ottawa, where they were cleaned and separated into relevant plant organ parts. Plant material was ground using a Wiley Mill (Arthur H. Thomas, Swedesboro, USA) with a 2-millimetre filter. The generated plant powder was extracted in two ways: the first (standard phytochemical) method used 80% ethanol (10 mL/g dry material) and extraction carried out twice for 24 h on a mechanical shaker (herafter designated as EE); the second method (mimicking CEI traditional preparation) used boiling water for 75min (hereafter designated as HWE). In both cases, extracts were filtered with Whatman paper. Extracts were subsequently dried using a rotary evaporator followed by lyophilization. All lyophilized extracts were preserved at 4 °C in a desiccator and protected from light.

2.2 Phytochemical characterization of extracts and UPLC-QTOF/MS analysis

Chromatography was carried out on Acquity BEH C18 column (1.7μm 2.1 × 100 mm) connected with a VanGuard Pre-column 2.1 × 5 mm using an Acquity UPLC^{TM} system with the column temperature at 50 °C and sample temperature at 10 °C. The mobile phase consisted of (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid (Fisher Optima LC-MS). The gradient conditions of the mobile phase were: 0-1 min 5% A isocratic, 1-6 min linear gradient

5-50% B, 6-8 min 50-95%B, 8.01-10 min 5% A isocratic (total run time 10 min). The flow rate was 0.5 mL/min, and 1 µL of sample was injection followed by a strong wash 200 µL (90% acetonitrile+10% water) and weak wash 600 µL (10% acetonitrile+90% water).

Mass spectrometric analysis was performed using a Q-TOF mass spectrometer equipped with an electrospray ionization (ESI) interface (Xevo G2, Waters Inc.). The ESI source was operated in positive ionization mode with source temperature of 120 °C, desolvation temperature of 400 °C, Cone gas (N2) flow of 50 L/hr, and desolvation gas (N2) flow of 1195 L/hr. Leucine-enkephalin was used as the lock mass generating an [M+ H]+ ion (m/z 556. 2615). The optimal conditions used for MSe analysis were as follows: mass range 100-1500 Da, function 1 CE, 6V, function 2 CER 10-30V, cone voltage 20 V, scan time 0.1 sec. The system was calibrated with sodium formate and the data were acquired and processed with MassLynx (version 4.1) and MarkerLynx (version 8.03) software with principal component analysis (PCA). The retention times and the protonated masses were generated at a noise threshold of 500 counts and no smoothing was applied. Pareto scaling was applied to generate the score plots. To maximize class discrimination and biomarkers, the data were further analyzed using the orthogonal partial least square discriminant analysis (OPLS-DA) method. S-plots were calculated to visualize the relationship between covariance and correlation within the OPLS-DA results. Variables that had significant contributions to discrimination between groups were considered as potential biomarkers and subjected to further identification of the molecular formula.

2.3 Cell culture

The C2C12 murine skeletal myoblasts and H4IIE rat hepatoma cell lines were acquired from the American Type Culture Collection (ATCC), Manassas, USA. Cell culture media was purchased from Invitrogen Life Technologies (Burlington, Canada) and Wisent (St. Bruno, Canada). Other reagents were purchased from Sigma-Aldrich (Oakville, Canada), unless otherwise specified below. All cell lines were cultured in a humidified incubator in a 5% CO_2: 95% air atmosphere at 37°C.

The C2C12 myoblasts were proliferated in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), and penicillin–streptomycin antibiotics. The C2C12 cells were proliferated until 80% confluence and then were passaged or differentiated for a period of 7 days into myotubes in DMEM containing 2% HS and antibiotics. The H4IIE cells were grown in DMEM supplemented with 10% FBS and antibiotics. Hepatic glucose production assay was performed when H4IIE cells reached approximately 90% confluence.

2.4 Glucose uptake bioassay

The effects of plant extracts on the rate of uptake of glucose by differentiated C2C12 skeletal myotubes were assessed with a ^{3}H-deoxyglucose uptake assay as described previously [5, 6]. Briefly, cells were grown in 12-well plates to 80% confluence and then differentiated as described above. On day 6 of differentiation, cells were treated for 18 h with either vehicle control (0.1% DMSO) alone, with a positive control Metformin (400 μM) or with extracts (EE and HWE respectively) in vehicle at optimal concentrations, as shown in Table 1. After the treatment, cells were rinsed twice with warm Krebs phosphate buffer, (KPB, 20 mM Hepes, 4.05 mM Na_2HPO_4, 0.95 mM NaH_2PO_4, 136 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgSO_4$, 5 mM glucose, pH 7.4). A subset of vehicle wells were treated with 100 nM insulin in KPB for 30 min, as a second positive control. Cells were then rinsed twice in warm glucose-free KPB and incubated in this buffer containing 0.5 μCi/mL 2-deoxy- D-[1-3^{H}]-glucose (TRK-383, Amersham Biosciences, Baie d'Urfe´, Canada) for exactly 10 min at 37°C. Cells were then placed on ice and rapidly rinsed 3 times in ice-cold glucose-free KPB. Cells were lysed in 0.5 mL of 0.1 M NaOH for 30 min, scraped and rinsed twice by 0.5 mL water. The lysates, along with 1 mL of water, were each added to 4 mL of liquid scintillation cocktail (Ready-Gel 586601, Beckman Coulter Inc., Fullerton, USA), and the incorporated radioactivity was measured in a scintillation beta counter (LKB Wallac 1219; Perkin-Elmer, Woodbridge, Ontario, Canada). Protein content was determined by the bicinchoninic

acid (BCA) method (Thermo Scientific Pierce, Rockford, IL, USA) standardized to bovine serum albumin (BSA).

2.5 Hepatic Glucose Production and Glucose-6-phosphatase assay

The effects of plant extracts on HGP were assessed in H4IIE rat hepatoma cells with a Glucose-6-phosphatase (G6Pase) enzyme activity assay as described previously [7, 11]. Briefly, 90% confluence cells were treated for 18h with vehicle control, or insulin (100 nM), EE or HWE plants extracts (at optimal concentrations described in Table 1). After the treatment, cells were washed and lysed in 15 mM phosphate buffer containing 0.05% Triton and 1.3 mM phenol (pH = 6.5). Cells lysates were incubated in the presence or absence of 200 mM glucose-6-phosphate (G6P) buffer for 40 min at 37°C, where G6P serves as a substrate for endogenous G6Pase to yield glucose. Lysates of plant treated cells in the absence of G6P were used as negative controls. Quantification of the glucose generated in this reaction was measured using a Wako AutoKit Glucose (Wako Chemicals USA Inc., Richmond, VA, USA), according to the manufacturer's recommendations. This is a colorimetric assay based on the glucose oxidase method where samples are incubated for 5 min at 37°C with Wako kit color reagent (composed of Mutarotase, Glucose oxidase, Peroxidase, 4-Aminoantipyrine and Ascorbate oxidase), then absorbance is recorded at 505 nm. Protein content was determined using the BSA method. Results were presented as percent activity of vehicle control.

2.6 Western blot

The effects of plants on the insulin and AMP-activated protein kinase (AMPK) signalling pathways in C2C12 muscle cells or H4IIE hepatocytes were assessed by Western blot. EE and HWE of plants, Metformin (400 μM) or vehicle alone were applied for 18 h to 6-day differentiated C2C12 cells or to 90% confluent H4IIE cells. Insulin (100 nM, 30 minutes) or aminoimidazole carboxamide ribonucleotide (AICAR; 2mM, 2h) was added prior to the end of the treatment to some vehicle-treated wells to serve as positive controls for the insulin or AMPK pathways, respectively. At the end of experiments, cells were placed on ice, washed 3

times with ice-cold PBS and lysed in RIPA lysis buffer (50mM Hepes, 150mM NaCl, 5mM EGTA, 2mM $MgCl_2.6H_2O$, 5% glycerol, 1% Trition X-100, 0.1% SDS, 1% Na-deoxycholate, 2mM phenylmethanesulfonyl fluoride (PMSF), 10mM NaF, 100uM Na-orthovanadate, 1mM Na-pyrophosphate and 1 tablet of commercial cocktail of protease inhibitors (Complete Mini; Roche, Mannheim, Germany)) at pH 7.4. A sucrose buffer (20mM Tris, 250mM sucrose, 1mM EDTA, 2mM PMSF, 2 tablets of protease inhibitor cocktail, pH 7.4) was used instead of RIPA lysis buffer in order to measure the amount of GLUT4 in total cellular membrane protein [12, 13]. Protein content was determined by the bicinchoninic acid (BCA) method (Thermo Scientific Pierce, Rockford, IL, USA) standardized to bovine serum albumin (BSA). The proteins of each sample were boiled for 5 min, separated in 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% non-fat milk in TBST (200 mM Tris base, 1.37 mM NaCl and 0.1% Tween-20) pH 7.6 for 1 h and then incubated with primary antibody (GLUT4 (1F8), Cell Signalling, 1:1000; Phospho-Akt (Ser473), Cell Signalling, 1:500; Phospho-AMPKα (Thr172), Cell Signalling, 1:500; Akt, Cell Signalling, 1:1000; AMPKα, Cell Signalling, 1:1000 or β-actin, Cell Signalling, 1: 1000) overnight at 4°C. Membranes were washed with TBST followed by a 1h incubation at room temperature with horseradish-peroxidase-conjugated secondary antibodies (anti-rabbit, Jackson, 1:20000 or anti-mouse IgG, Cell Signalling, 1:2000). The bands were detected by enhanced chemiluminescence (ECL) method with ECL Plus (Perkin Elmer, Woodbridge, Canada). All experiments were conducted on 3 different cell preparations. All samples from a given passage were separated and transferred simultaneously to a single membrane. Quantification of the integrated density of bands was performed using NIH Image J 1.45s software (National Institutes of Health, Bethesda, MD, USA).

2.7 Statistical analysis:

Results are presented as mean ± SEM of 3 independent experiments. Statistical calculations were performed with Prism GraphPad software. Differences between EE (or HWE) and vehicle controls were analyzed by one-way analysis of

variance (ANOVA) and post hoc Dunnett's test. And differences between EE and HWE were analyzed by two-way analysis of variance (ANOVA).

3. Results

3.1 Stimulation of glucose uptake in myotubes by different plant extracts

Plant extracts were tested for enhancing glucose transport properties in an insulin-responsive and GLUT4-containing cell line, namely, C2C12 myoblasts [14, 15]. Metformin (400 μM, 18h) and insulin (100nM, 30 min), applied as positive controls, significantly stimulated glucose transport to 130% and 119% of DMSO vehicle values, respectively. Eight out of the 17 EE of plants (namely, *S. purpurea, S. decora, R. groenlandicum, R. tomentosum, A. incana, V. vitis-idaea, L. laricina and A. balsamea*) enhanced glucose uptake in C2C12 cells after 18 h incubation. Among these, *S. purpurea* showed the strongest potential (144% compared with vehicle control) (Figure 1). In the case of HWE, only 3 species (namely, *R. groenlandicum, S. purpurea, R. tomentosum*) also elicited significant increases in glucose uptake. Only the HWE of *R. groenlandicum* exhibited an effect as strong as its EE. The HWE of *S. purpurea* and *R. tomentosum* exhibited a decreased effectiveness compared to the stimulation of their EE. For other species (such as *A. incana*), their HWE completely lost their effect in C2C12 cells as compared to their EE counterparts.

3.2 Inhibition of hepatic glucose production and G6Pase activity of extracts

Insulin 100 nM, applied as a positive control to H4IIE liver cells, inhibited G6Pase activity by 62% compared with vehicle control (Figure 2). When the Cree plant extracts were tested, a statistically significant reduction in G6Pase activity (varying from -54% to -17% compared with vehicle control) was observed for both the EE and HWE of 9 species (*P. glauca, A. balsamea, P. balsamifera, R. groenlandicum, K. angustifolia, S. purpurea, A. incana, L. laricina, V. vitis-idaea*), as well as by the EE of one species (*S. decora*). Hence, the inhibitory potential of most active species was maintained at a near equivalent level when HWE was used instead of EE. In three cases (*P. glauca, P. balsamifera and A. incana*), HWE biological activity on G6Pase was significantly lower (-34%, -22%, -17% respectively) when

compared with the respective EE counterpart (-54%, -44%, -37% respectively), whereas for *S. decora* the inhibitory effect of its EE (-22%) was completely lost with its HWE.

3.3 Effects of selected CEI plant extracts on expression of GLUT4 and phosphorylation of AMPK and Akt in C2C12 myotubes

Five plants, namely *R. groenlandicum, K. angustifolia, S. purpurea, A. incana and P. balsamifera* were selected for further in-depth pharmacological analysis. The selection process sought to capture representative situations encountered, namely, species with or without biological activity in our bioassays, as well as species with varying or stable biological activities between their EE and HWE.

To begin understanding the mechanisms underlying the observed effect of extracts on skeletal muscle cell glucose uptake, we evaluated the total membrane protein expression of GLUT4, the insulin-dependent kinase, Akt, and the insulin-independent AMPK pathway (the latter two representing main signaling pathways regulating glucose uptake in this cell line). Insulin served as a positive control for the first pathway, whereas metformin and AICAR were applied as activators of AMPK signaling. At the end of treatments in C2C12 cells, there was a clear increase in the expression of GLUT4 with all three positive controls as compared with vehicle (2.8-fold, 2.7-fold and 2.3-fold increases for insulin, metformin and AICAR respectively, Figure 3A). *R. groenlandicum* represents a species whose HWE and EE had comparable effects on glucose uptake. Both its extracts also stimulated the expression of GLUT4 (2.0-fold for both HWE and EE, Figure 3A and B), as well as AMPK phosphorylation (3.6-fold and 3.5-fold respectively, Figure 3D and E) in a similar manner, whereas Akt was not affected by either extract. In contrast, *A. incana* represents a species whose HWE completely lost its potentiating effect on GU as compared with its EE. Consistent with this, *A. incana* HWE was without effect on C2C12 GLUT4 content and AMPK phosphorylation whereas its EE increased both parameters by 1.9-fold (Figure 3A and B) and 3.4-fold (Figure 3D and E), respectively. In fact, linear regression analysis clearly demonstrated that effects of

selected species on glucose uptake were tightly correlated with their corresponding impact on C2C12 GLUT4 content (R^2=0.80, $p < 0.05$; Figure 3C). A similar, albeit less tight, correlation was also observed between a given plant extract's ability to increase glucose uptake and to enhance AMPK phosphorylation (R^2=0.46, $p < 0.05$, Figure 3F).

On the other hand, *S. purpurea* was the specie that yielded interesting observations. As shown in Figure 1, its HWE significantly stimulated GU, although at lower levels than its EE. When underlying mechanisms were probed, GLUT4 expression results were consistent with GU results (EE greater than HWE). However, *S. purpurea* HWE was the only extract found to significantly enhance Akt phosphorylation (3.9-fold increase, $p < 0.05$; Figure 3G and H), yet was without effect on AMPK phosphorylation (Figure 3D and E). Conversely, the plant's EE stimulated AMPK phosphorylation (3.1-fold, $p < 0.05$; Figure 3D and E) without impacting Akt phosphorylation.

3.4 Effects of the selected extracts on phosphorylation of AMPK and Akt in H4IIE cells

We used the same positive controls and probed the same molecular parameters to determine the latters' implication in the selected plant extracts' actions on hepatic glucose production.

In H4IIE hepatocytes, the EE and HWE of all selected plant species increased AMPK phosphorylation with similar intensity (around 4-fold compared with vehicle, $p < 0.05$; Figure 4A and B). Despite this, linear regression analysis uncovered a statistically significant correlation between the capacity of a given plant extract to stimulate AMPK and its ability to inhibit G6Pase (R^2=0.48, $p < 0.05$, Figure 4C). In contrast, only *S. purpurea* and *K. angustifolia* were found capable of stimulating Akt phosphorylation (2-3-fold, $p < 0.05$; Figure 4D and E); this effect being comparable for their EE and HWE crude preparations. There was no clear correlation found between Akt phosphorylation and inhibition of hepatic G6Pase activity (data not illustrated).

3.5 Metabolites analysis

Using the optimal UPLC–QTOF/MS conditions described above, representative fingerprints for all HWE and EE of the 17 plants were obtained. Overall, more than 4000 metabolites were detected from these extracts by UPLC-QTOF. This generated a matrix of data comprising qualitative (relating to phytochemical compounds, notably retention time and accurate mass) and relative quantitative (signal intensities) components. Information on the biological activity of each plant species was also incorporated into the data matrix. The PCA process was then used to assess the relationships between phytochemical metabolites (profiles) and biological phenotype. Using an unbiased PCA approach including HWE and EE, as well as all species regardless of their biological phenotype (e.g. active versus inactive for a given bioassay), all 17 plants clustered together (data not shown). We then carried out more specific PCA approaches guided by the biological activity found in our bioassays (presence or absence of effects on muscle glucose uptake or hepatic G6Pase activity) for HWE and EE samples separately.

We first examined EE preparations and compared species with a stimulating action on GU with inactive ones using the PCA process, but no discriminating characteristics were observed (data not illustrated). Similarly, when inhibiting G6Pase EE or HWE samples were analyzed against corresponding inactive species, the PCA process did not show any clustering (data not shown).

In contrast, when the HWE preparations of the 17 plants were specifically analyzed taking into consideration their capacity or not to stimulate glucose uptake in C2C12 cells, a clear discrimination of species was obtained (Figure 5A and 5B). Indeed, plants stimulating glucose uptake, namely *R. groenlandicum*, *S. purpurea* and *R. tomentosum*, clustered together. Similarly, inactive species (*A. balsamea, L. laricina, S.decora, P. glauca, P. banksiana V. vitis,*) were found to group together (outliers *K. augusfolia* being excluded from the analysis).

A so-called S-plot was then generated to determine plant metabolites that could significantly contribute to discriminating species. These usually lie in the top or bottom extremities of the S-plot, and are characterized by sufficiently important

statistical differences to render them potential biomarkers for the biological activity studied. As shown in Figure 5C, two such markers were extracted and identified as the potential biomarkers from S-plots constructed following the OPLS-DA Figure 5B.

The UPLC–QTOF/MS analysis that was performed in positive ion mode indicated the retention time and accurate mass within 5 ppm accuracy, and provided the high voltage fragmentation data that was necessary for the structural identification of the biomarkers. We searched for the presumed molecular formula in the ChemSpider (http://www.chemspider.com/), Metlin (http://metlin.scripps.edu/) and other databases to confirm possible chemical compositions, and the high fragmentation signals were used to determine the potential structures of the ions. The two biomarkers were identified as Quercetin 3-O-α-L-arabinopyranoside (with retention time 3.09 min, accurate mass of $[M+H]^+$ 435.0931) and Quercetin-3-O-galactoside (with retention time 2.83min, accurate mass of $[M+H]^+$ 465.1034), respectively.

4. Discussion

Through ethnobotanical surveys, our group identified 17 CEI medicinal plants as candidate antidiabetic species used by the CEI First Nations to treat various symptoms of Type 2 diabetes [2, 9]. We found that several CEI plants' ethanol extracts strongly potentiated glucose transport in C2C12 cells, whereas another group of species significantly inhibited G6Pase in H4IIE liver cells, indicating a potential to increase peripheral glucose disposition and to suppress hepatic glucose production, respectively [5-7]. In the present study, we used the same bioassays to evaluate the biological activity of a more traditional-like HWE preparation and compare it with the standard phytochemical ethanol extraction procedure. We also sought to begin assessing the potential underlying molecular mechanisms in this *in vitro* system.

Generally in line with previous observations by our group [2, 4], our results showed that several EE of CEI plants enhanced glucose transport in C2C12 differentiated myoblasts. The present studies demonstrated that three species (both *Rhododendrons* and *S. purpurea*) completely or partially maintained their capacity to stimulate such glucose transport when more traditional HWE preparations were used. Five other species completely lost this biological activity in similar conditions. All inactive species remained inactive when HWE was used instead of EE. These results could be interpreted to mean that more traditional preparations are not as "good" as the classical ethanol extraction procedure used in phytochemistry. However, a number of considerations can argue differently. Firstly, EE and HWE extracts were tested at identical mg/mL concentrations of the crude extracts for purposes of proper pharmacological comparison. Since ethanol is known to be a better solvent than water to extract several bioactive plant molecules (notably of the large class of phenolic compounds), our observations could simply reflect the fact that certain bioactives were more concentrated in EE and hence were able to enhance biological activity. Future studies should use wide ranges of extract concentrations to better compare the pharmacological potential of EE and HWE plant preparations. Secondly, the laboratory method used to prepare the HWE mimicked procedures used by traditional CEI healers and was indeed adjusted after discussions with them.

However, traditional healers may use lake water instead of distilled water and different heating conditions than was used in the laboratory. Hence, future studies should compare laboratory HWE with true traditional preparations of CEI healers.

Nonetheless, the present results confirmed the soundness of our current and previous studies to assess the biological activity of putative antidiabetic CEI plant species. Indeed, we found that the capacity of both EE and HWE preparations of five representative species to increase glucose transport strongly correlated with their extracts' ability to increase GLUT4 protein content in C2C12 cells. Hence, the potential of the various extracts to enhance peripheral glucose disposition is related to their action on the major glucose transport protein of skeletal muscle. As importantly, a significant, albeit somewhat weaker, correlation was observed between increased glucose transport and AMPK phosphorylation for both EE and HWE preparations tested. This is in full agreement with what was described in our previous study on a subset of 8 CEI plants [16]. Indeed, we had shown that glucose uptake was increased by several species though the AMPK pathway rather than the insulin-dependent pathway involving Akt [16]. The insulin-independent AMPK signaling pathway is also the one through which the common oral hypoglycemic drug, metformin, increases glucose uptake in skeletal muscle and inhibits glucose production in liver while also restoring insulin sensitivity in these tissues [17, 18].

On the other hand, we made a very interesting observation concerning the species *Sarracenia purpurea* L., commonly known as the purple pitcher plant, which was among the top ranked species in the C2C12 glucose uptake bioassay. We found that the EE of *S. purpurea* triggered significant AMPK phosphorylation (activation), whereas its HWE counterpart was the only one of all species tested to activate Akt phosphorylation, which is related to the insulin pathway. In parallel, the capacity of *S. purpurea* HWE to stimulate glucose transport was lower than the corresponding EE. In previous studies, we had tested the effect of the same plant's EE in the same bioassay and in the presence or absence of physiological and pharmacological concentrations of insulin [5]. We found that EE of *S. purpurea* had a tendency to increase the glucose uptake induced by insulin, consistent with an action on the "insulin-sensitizing" AMPK pathway. In contrast, preliminary studies with *S.*

purpurea HWE showed similar results on C2C12 glucose uptake with or without insulin (N. Shang and P.S. Haddad, unpublished information). We also isolated several bioactive compounds from *S. purpurea* EE using C2C12 cells to guide a fractionation process and some of these compounds were also shown to activate AMPK [11, 16]. The present studies thus suggest that the HWE of *S. purpurea* may contain other compounds that activate instead the insulin-dependent pathway involving Akt. Future studies using a similar bioassay-guided fractionation approach with HWE may clarify this interpretation.

The second assay related to glucose homeostasis that we have used is based on the inhibition of G6Pase in H4IIE liver cells as an indicator of an antidiabetic potential to reduce hepatic glucose production. Our results clearly showed that several species had a significant inhibitory effect of G6Pase. Unlike the C2C12 bioassay, however, the inhibitory action of most HWE was similar to that of their EE counterparts, only three species showing lower activities and only one losing it completely with HWE. Likewise, all five of the selected subset of representative species enhanced AMPK phosphorylation and this was equivalent between the plants' respective EE and HWE. This is consistent with the reported effects of metformin and many natural products that inhibit hepatic glucose production in liver [19-21]. In addition, two plants extracts (*S. purpurea* and *K. angustifolia*) also activated insulin pathways by inducing Akt phosphorylation; this being true for both their respective EE and HWE. The differences in results comparing EE and HWE in C2C12 differentiated myoblasts and H4IIE hepatocytes demonstrate that in some cases, bioactive components (and combinations thereof) of CEI plants can be extracted with similar efficiency by the two extraction methods used.

Rhododendron groenlandicum, (known as *Ledum groenlandicum* or *Labrador tea*), is a good case for this point. It is a flowering plant in the subsection *Ledum* of the large *genus Rhododendron* in the *Ericaceae* family. Leaves and other parts have showed antioxidant and anti-inflammatory [22] activity, as well as benefits against cancer [23], asthma [24], rheumatism [25] and diseases of the kidney [24]. In the present studies, *R. groenlandicum* was the only specie whose EE and HWE

preparations were found to be active in both assays and to exert their anti-diabetic effect through similar molecular pathways.

R. groenlandicum was also one of three species stimulating glucose transport (alongside *S. purpurea* and *R. tomentosum*) whose HWE clustered in a way that was clearly separated from inactive species, when photochemical profiling and PCA process were applied to our data. Most importantly, the discriminant analysis uncovered significant differences in the chemical profiles of these plants. Notably, two biomarkers were identified as strong potential to be responsible for the biological activity of the active plants, due to their highest contribution to this separation of clustering. One of these, quercetin-3-O-galactoside, was previously identified by our team and reported to be one of the active principles capable of enhancing glucose transport in C2C12 through the activation of AMPK [11, 16]. On the other hand, the second putative antidiabetic compound, identified in the present report as quercetin 3-O-α-L-arabinopyranoside, has not been isolated in prior bioassay-guided fractionation studies by our group or associated with this biological activity by others. However, because of the labor-intensive and tedious characteristics of the bioassay-guided fractionation approach, only the most active subfractions were selected for the identification of active principles. Hence, other active metabolites may exist in the active species and quercetin 3-O-α-L-arabinopyranoside may thus contribute to the antidiabetic potential of some CEI plant species. Future studies will need to carefully assess the biological activity of this compound in the context of glucose metabolism and diabetes.

In summary, we evaluated and compared the potentials of the EE and HWE of 17 plants to modulate glucose homeostasis in muscle and liver cells, two major targets of insulin action. We also assessed the possible molecular mechanisms responsible for such activities *in vitro*. We confirmed that the different species, target tissues or cells (muscle or liver), as well as extraction methods (EE or HWE), are all significant determinants of the biological activity of Cree medicinal plants on glucose metabolism. By applying principal component analyses to mass spectrometric data obtained by UPLC-QTOF enabled us to partly segregate the metabolomes of active plant species from the inactive ones. This powerful approach offers an interesting

avenue to identify potential bioactive plant metabolites. Our work and its future applications can help to develop novel as well as culturally relevant plant-based therapeutic approaches against insulin resistance that target muscle and liver cells directly. Finally, our studies can serve as quality control tools to foster reliable and effective plant-based treatments, using the information relative to the content of active principles as well as that pertaining to biological activity.

5. Acknowledgements

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6. Reference

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7. Figure legends

Figure 1: Effects of extracts on muscle glucose transport. C2C12 skeletal muscle cells were treated with either 0.1% DMSO (vehicle), Metformin (400 μM), EE and HWE (at concentrations described in Table 1) for 18 h, or with insulin (100nM) for 30 min. Results represent means ± SE for 3 separate experiments, normalized to the vehicle-treated condition. # Denotes EE samples significantly different from vehicle control ($p<0.05$), one-way ANOVA and post hoc Dunnett's test. $ Denotes HWE samples significantly different from vehicle control ($p< 0.05$), one-way ANOVA and post hoc Dunnett's test. * ($p<0.05$), **($p<0.01$) and *** ($p<0.001$) denote significant differences between respective EE and HWE counterparts, two-way ANOVA.

Figure 2: Effects of extracts on hepatic Glucose-6-phosphatase activity. H4IIE rat hepatoma cells were treated for 18h with vehicle control, or insulin (100 nM), EE or HWE plants extracts (at concentrations as described in Table 1). Results represent means ± SE for 3 separate experiments, normalized to the vehicle-treated condition. # Denotes EE samples significantly different from vehicle control ($p<0.05$), one-way ANOVA and post hoc Dunnett's test. $ Denotes HWE samples significantly different from vehicle control ($p< 0.05$), one-way ANOVA and post hoc Dunnett's test. * ($p<0.05$), **($p<0.01$) and *** ($p<0.001$) denote significant differences between EE and HWE counterpart, Two-way ANOVA.

Figure 3: Effect of selected extracts on expression of GLUT4, Insulin and AMPK pathway in C2C12 cells. Cells were differentiated and treated for 18 h with either 0.1% DMSO (vehicle) or with EE and HWE of the 5 selected plants. Metformin (400 μM, 18h), AICAR (2mM, 2h) or insulin (100 nM, 30min) was applied as positive controls for the AMPK or insulin pathways, respectively. GLUT4 (A), phosphorylation of AMPK (D), phosphorylation of Akt (G) were measured by western blot and results (B, E, H) were expressed as means ± SE for 3 separate experiments, normalized to the vehicle-treated condition. # Denotes EE samples

significantly different from vehicle control (p<0.05), one-way ANOVA and post hoc Dunnett's test. $ Denotes HWE samples significantly different from vehicle control (p < 0.05), one-way ANOVA and post hoc Dunnett's test. * (p<0.05), **(p<0.01) and *** (p<0.001) denote significant differences between EE and HWE counterpart, two-way ANOVA. Correlation results (C, F) were analyzed by linear regression and the equations were y = 32.06x + 52.95 (R=0.80, p < 0.05), y = 11.602x + 84.825 (R=0.46, p < 0.05), respectively.

Figure 4: Effect of selected extracts on the modulation of insulin and AMPK pathway components in H4IIE hepatoma cells. The cells were treated for 18h with vehicle control, insulin (100 nM), EE or HWE plants extracts. Metformin (400 µM, 18h), AICAR (2mM, 2h) or insulin (100 nM, 18h) was used as positive controls for AMPK or insulin pathways, respectively. Phosphorylation of AMPK (A) and of Akt (D) were measured by western blot and results (B, E) expressed as means ± SE for 3 separate experiments, normalized to the vehicle-treated condition. # Denotes EE samples significantly different from vehicle control (p<0.05), one-way ANOVA and post hoc Dunnett's test. $ Denotes HWE samples significantly different from vehicle control (p< 0.05), one-way ANOVA and post hoc Dunnett's test. Correlation results (C) were analyzed by linear regression and the equation was y = -45.418x + 285.3 (R=0.48, p< 0.05).

Figure 5: Metabolites analysis of selected hot water extracts based on stimulating glucose uptake activity.

PCA scores (A), OPLS-DA scores (B) S-plot (C) of HWE stimulating glucose transport (*R. gromenlandicum*, *R. tomentosum*, and *S. purpurea*) compared with inactive ones (*K. augusfolia* was found to be outliers and were excluded from the process). The scores t [1], t [2] and t [1] P and t [2] O are the new most important indices in PCA and OPLS-DA in summarizing the peak lists of the plants. In S-plot, it displays easily the observations that have a large absolute value of p (corr) [1] and a large absolute value of the coefficients. These are the observations that differentiate the most between the 2 groups.

Table 1: List of investigated plant species and the concentrations of the extracts tested in C2C12 and H4IIE cells

Species	Abbreviation	Plant part	C2C12 cells µg/mL	H4II E cells µg/mL
Rhododendron groenlandicum (Oeder) Kron and Judd	*R.groenlandicum*	Leaves	75	50
Abies balsamea (L.) Mill.	*A.balsamea*	Inner bark	50	50
Larix laricina Du Roi (K.Koch)	*L.laricina*	Inner bark	25	25
Picea mariana (P. Mill.) BSP	*P.mariana*	Cones	10	10
Sorbus decora (Sarg.) C.K.Schneid.	*S.decora*	Inner bark	15	15
Alnus incana subsp. rugosa (Du Roi) R.T. Clausen	*A.incana*	Inner bark	50	50
Sarracenia purpurea L.	*S.purpurea*	Whole plant	100	25
Pinus banksiana Lamb.	*P.banksiana*	Cones	15	10
Rhododendron tomentosum (Stokes) Harmaja subsp.subarcticum (Harmaja) G.Wallace	*R.tomentosum*	Leaves	50	50
Kalmia angustifolia L.	*K.augustifolia*	Leaves	50	50
Picea glauca (Moench) Voss	*P.glauca*	Leaves	125	125
Juniperus communis L.	*J.communis*	Berries	5	3.75
Salix planifolia Pursh	*S.planifolia*	Inner bark	25	15
Lycopodium clavatum L.	*L.clavatum*	Whole plant	100	100
Populus balsamifera L.	*P.balsamifera*	Inner bark	100	100
Gaultheria hispidula (L.) Muhl.	*G.hispidula*	Leaves	25	25
Vaccinium vitis-idaea L.	*V.vitis-idaea*	Berries	200	200

Figure 1

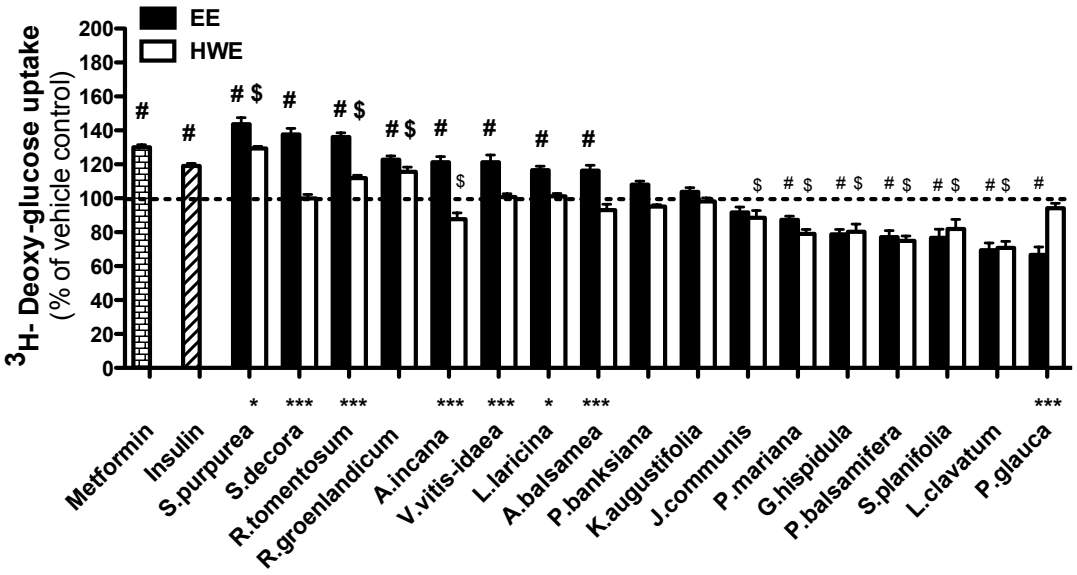

EE
HWE
^{3}H- Deoxy-glucose uptake
(% of vehicle control)
200
180
160
140
120
100
80
60
40
20
0
Metformin
Insulin
S.purpurea
S.decora
R.tomentosum
R.groenlandicum
A.incana
V.vitis-idaea
L.laricina
A.balsamea
P.banksiana
K.augustifolia
J.communis
P.mariana
G.hispidula
P.balsamifera
S.planifolia
L.clavatum
P.glauca

Figure 2

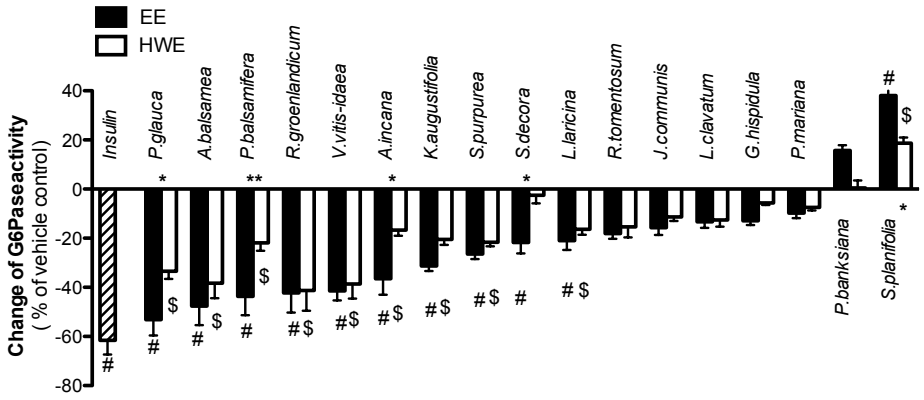

EE
HWE
Change of G6Paseactivity
(% of vehicle control)
40
20
0
-20
-40
-60
-80
Insulin
P.glauca
A.balsamea
P.balsamifera
R.groenlandicum
V.vitis-idaea
A.incana
K.augustifolia
S.purpurea
S.decora
L.laricina
R.tomentosum
J.communis
L.clavatum
G.hispidula
P.mariana
P.banksiana
S,planifolia

Figure 3

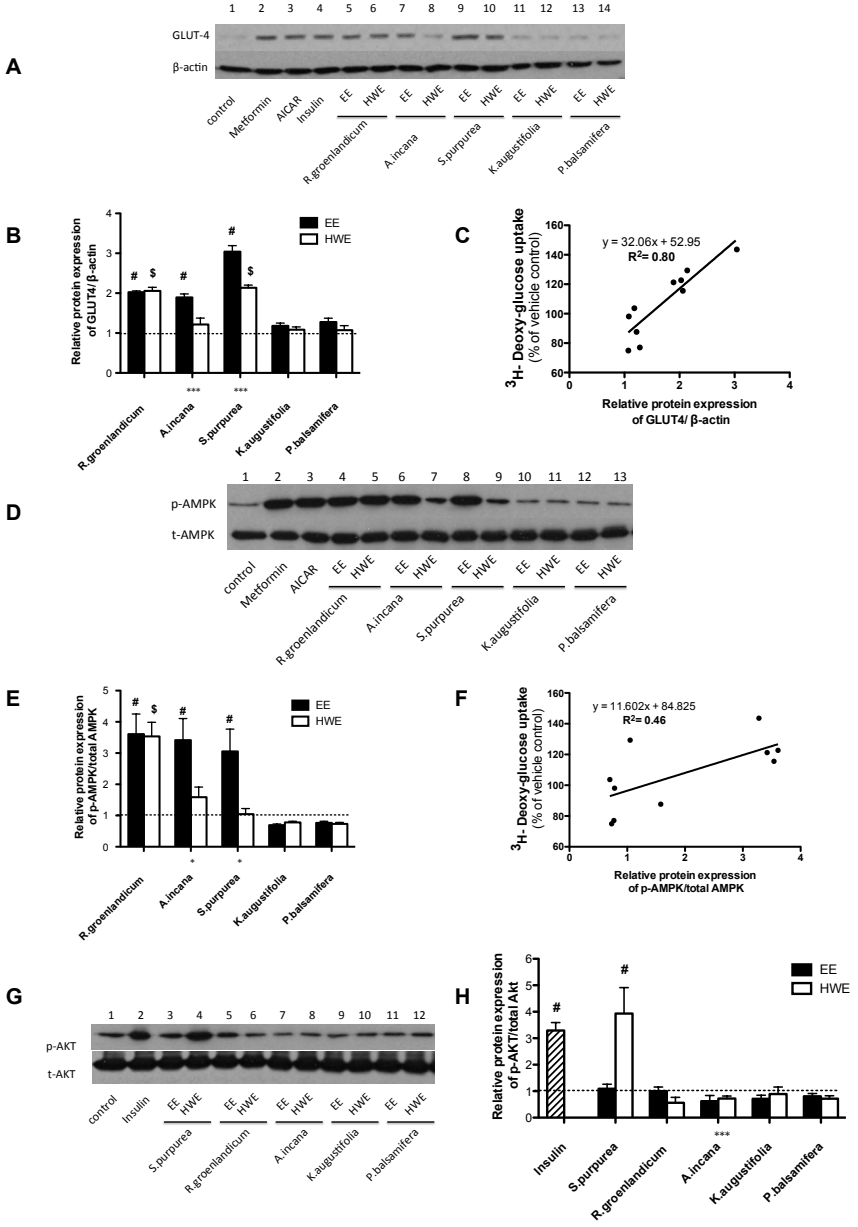

A
GLUT-4
β-actin
control
Metformin
AICAR
Insulin
EE
HWE
R.groenlandicum
A.incana
S.purpurea
K.augustifolia
P.balsamifera
B
Relative protein expression of GLUT4/ β-actin
C
y = 32.06x + 52.95
R²= 0.80
³H- Deoxy-glucose uptake (% of vehicle control)
Relative protein expression of GLUT4/ β-actin
D
p-AMPK
t-AMPK
E
Relative protein expression of p-AMPK/total AMPK
F
y = 11.602x + 84.825
R²= 0.46
³H- Deoxy-glucose uptake (% of vehicle control)
Relative protein expression of p-AMPK/total AMPK
G
p-AKT
t-AKT
H
Relative protein expression of p-AKT/total Akt

Figure 4

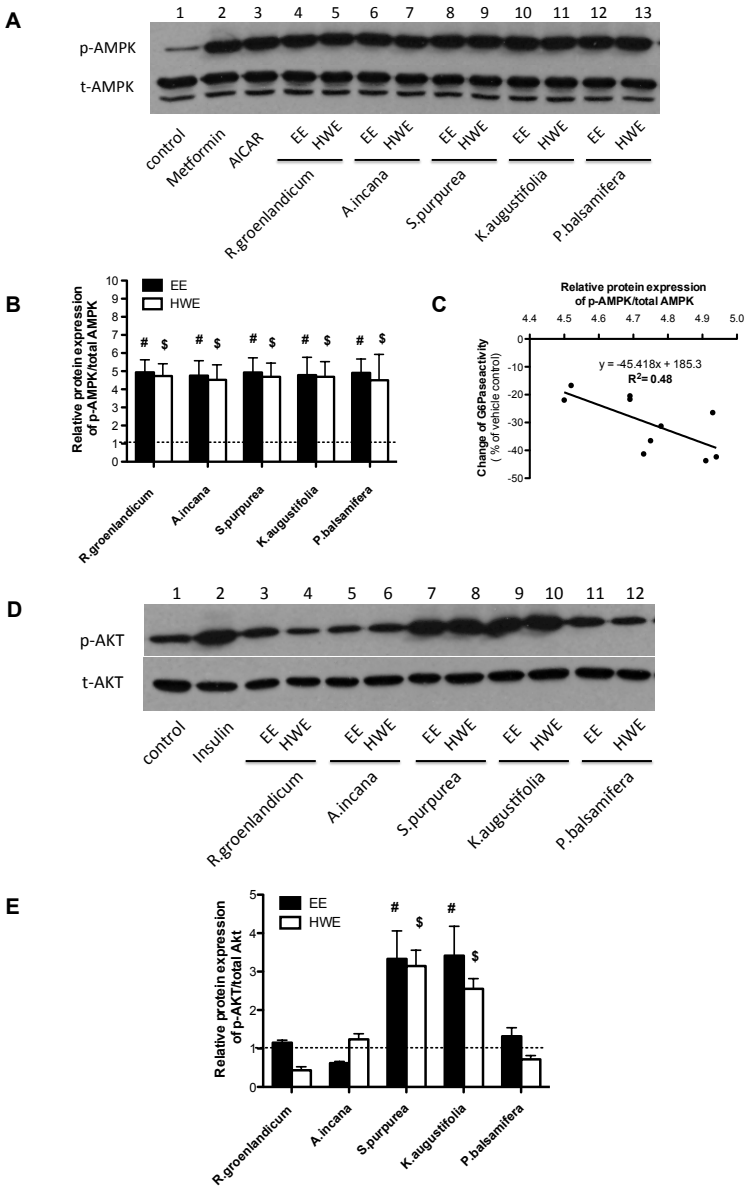
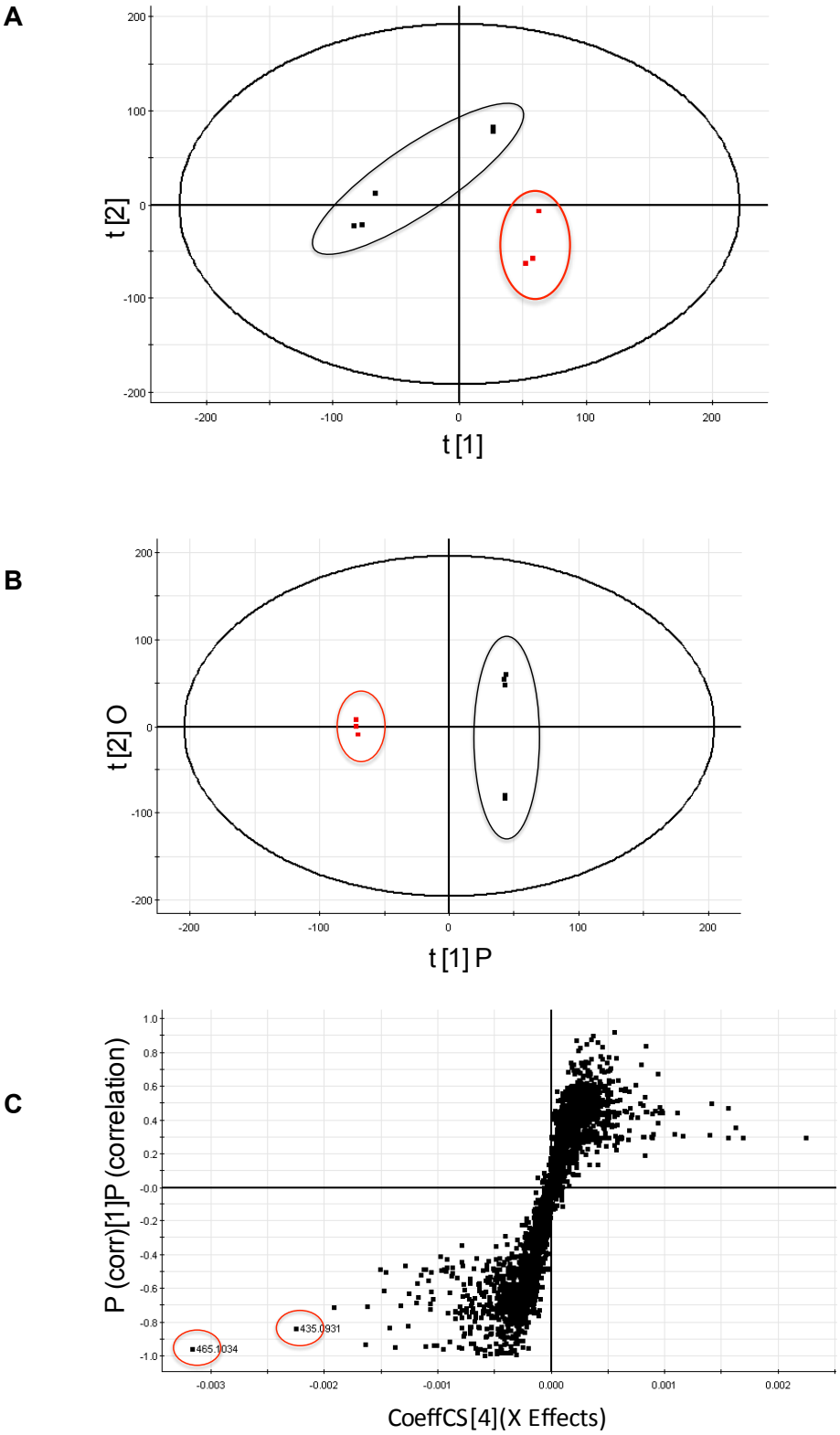

A
1 2 3 4 5 6 7 8 9 10 11 12 13
p-AMPK
t-AMPK
control
Metformin
AICAR
EE HWE EE HWE EE HWE EE HWE EE HWE
R.groenlandicum
A.incana
S.purpurea
K.augustifolia
P.balsamifera
B
EE
HWE
Relative protein expression of p-AMPK/total AMPK
C
Relative protein expression of p-AMPK/total AMPK
Change of G6Paseactivity (% of vehicle control)
y = -45.418x + 185.3
R2= 0.48
D
1 2 3 4 5 6 7 8 9 10 11 12
p-AKT
t-AKT
control
Insulin
E
Relative protein expression of p-AKT/total Akt

Figure 5


A
200
100
0
-100
-200
t [2]
-200
-100
0
100
200
t [1]
B
200
100
0
-100
-200
t [2] O
-200
-100
0
100
200
t [1] P
C
1.0
0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-1.0
P (corr)[1]P (correlation)
435.0931
465.1034
-0.003
-0.002
-0.001
0.000
0.001
0.002
CoeffCS[4](X Effects)

Chapter 5: General discussion

Metabolic syndrome (MetS) in an individual reflects the clustering of multiple metabolic abnormalities, including central obesity, T2D, dyslipidemia, hypertension, as well as NAFLD. MetS is currently considered to be a worldwide epidemic. For instance, about 1 in 5 Canadian adults have MetS (Riediger and Clara, 2011), 1 in 10 adults of the world's population is now obese (WHO, 2012), and 346 million people worldwide have diabetes (WHO, 2011).

In particular, T2D and obesity are more pronounced among indigenous peoples than in the rest of the population. This is the case in the CEI of northern Quebec, where the age-adjusted prevalence of T2D reached 29% in 2009 (Kuzmina et al., 2010) and where the prevalence of obesity and overweight is also 2-3 times higher than in the general Canadian population. They also suffer from higher rate of diabetic complications (Young et al., 2000). This serious problem has been related to a sedentary lifestyle and non-traditional diet (Hegele, 2001), a genetic predisposition towards obesity (Neel, 1999; Skyler, 2004), and especially due to cultural disconnect (Young et al., 2000) of the modern or western pharmaceutical therapies described above in the introduction.

Long before the birth of western medicines, the aboriginal peoples relied on traditional healing for many diseases, a knowledge that was verbally taught and handed down from generation to generation (Young et al., 2000). Medicinal plants played an important role in this holistic approach. Although some of this knowledge was lost, anthropologists documented the administration of many plant species belonging to aboriginal medicine. Over 1200 plants worldwide have been used as remedies for diabetes (Habeck, 2003; Marles and Farnsworth, 1995) and about 700 recipes and compounds have been scientifically evaluated for T2D treatment (Singh et al., 2011). Several of these medicinal plants are commonly recognized for their antidiabetic potentials and significant scientific evidence has been accumulated in support of this. These include *Trigonella foenum graecum* (Fenugreek) (Gupta et al., 2001); *Momordica charantia* (bitter melon)(John et al., 2003); *Cinnamomum cassia* (Chinese cinnamon) (Crawford, 2009; Khan et al., 2003); *Aloe vera*

(Bunyapraphatsara et al., 1996) and *Ginseng* (Sotaniemi et al., 1995; Vuksan et al., 2008).

For responding to the primary health care needs of the CEI and to search for culturally adapted T2D therapies, our team (CIHR-TAAM) was instated in 2003. It was a multipartite project researching the antidiabetic effects of plants used by Aboriginals in the Eastern James Bay area of Quebec, Canada. This project required a highly coordinated effort between three parts, including the CEI, research scientists, and health care professionals. Aide from traditional knowledge keepers and users, the multi-disciplinary research team assembled for this project possessed considerable expertise not only in pharmacology, but also in phytochemistry, human clinical as well as health systems research. A novel ethnobotanical approach (Leduc et al., 2006) was used to identify plants for treating several symptoms related to T2D disorder. 17 plant species were evaluated in several screening studies (Fraser et al., 2007; Harbilas et al., 2009; Harris et al., 2011b; Nachar et al., 2013; Nistor Baldea et al., 2010; Spoor et al., 2006) using a variety of *in vitro* bioassays to assess the antidiabetic potentials of the ethanol extracts.

5.1 General bioactive compounds and constituents from *L. laricina*

In a previous study, *L. laricina* potentiated adipogenesis in 3T3-L1 adipocytes (Spoor et al., 2006) acting like the TZD drug rosiglitazone, which is one of the OHAs of antidiabetic medication. It also demonstrated antidiabetic potential by increasing glucose uptake (comparable to another OHAs medication Metformin) (Spoor et al., 2006), activating AMPK in C2C12 myotubes and uncoupling mitochondria functions (Martineau et al., 2010a). A recent *in vivo* study also confirmed that *L. laricina* improves insulin resistance and decreases glycemia, using the diet-induced obese mouse model (Harbilas et al., 2012).

Medicinal plants contain numerous compounds and there are more than 200 pure compounds (Marles and Farnsworth, 1995) from plant sources reported to have antidiabetic potential. Thus it is important to use phytochemical methods to screen

and isolate bioactive secondary metabolites. Many kinds of natural products and constituents, belonging to chemical classes such as terpenoids, alkaloids, flavonoids, phenolics, and others, have shown antidiabetic potentials.

Bioassay-guided fractionation of the stem bark of *S. decora* (one of the Cree plants) resulted the isolation of 3 new pentacycle triterpenes. One of the triterpenes, identified as 23,28-dihydroxylupan-20(29)-ene-3β-caffeate, showed significant antidiabetic properties as determined by an increase in glucose uptake by C2C12 cells (Guerrero-Analco et al.). The triterpene dehydrotrametenolic acid isolated from dried sclerotia of *Poria cocos Wolf.* (Polyporaceae) was shown to have an anti-hyperglycemic effect *in vivo* as an insulin sensitizer (Sato et al., 2002). Twelve triterpenoid saponins have been isolated from the roots of *Gypsophila oldhamiana* and some showed significant inhibitory activities on α-glucosidase (Luo et al., 2008). *Corosolic acid (Glucosol)*, one triterpene isolated from the leaves of *Lagerstroemia speciosa* L. (Lythraceae), significant stimulated glucose transport and showed a significant reduction in the blood glucose levels at daily dosages of 32 and 48mg for 2 weeks in a randomized clinical trial involving T2D (Judy et al., 2003).

2 new pyrrolidine alkaloids, radicamines were isolated as inhibitors of α-glucosidase from *Lobelia chinensis Lour* (Campanulaceae) (Shibano et al., 2001). Alkaloids like canthin-6-one was isolated from the methanol root extract of *Aerva lanata Linn*. (Amaranthaceae), a plant used traditionally as an antihyperglycaemic in the Ayurvedic system of medicine. The compound is thought to contribute to the antihyperglycemia effects observed on streptozotocin-nicotinamide induced T2D in rats (Agrawal et al., 2013).

Several flavonoids were isolated from *V. vitis* (another of the CEI plants) through glucose uptake guided fractionation approach. The flavonoids quercetin and quercetin 3-O-glycosides might be responsible for the antidiabetic activity of *V. vitis* crude extract mediated by AMPK (Eid et al.). Glucose uptake activity guided fractionation of *Sarracenia purpurea* (pitcher plant, *S. purpurea*), one of the identified Cree plants, resulted in the isolation of 11 compounds, including a new phenolic glycoside and several flavonoid glycosides. Isorhamnetin-3-*O*–glucoside, kaempferol-3-*O*-(6″-caffeoylglucoside], and quercetin-3-*O*–galactoside strongly

potentiated glucose uptake (Muhammad et al., 2012). The new compound, namely 6′-*O* -caffeoylgoodyeroside, decreased hepatic glucose production by reducing G6Pase enzymatic activity, which also contributed to the antidiabetic potential of *S. purpurea* (Muhammad et al., 2012).

2 new phenolics, 7'-(3',4'-dihydroxyphenyl)-N-[(4 methoxyphenyl) ethyl] propenamide and 7'-(4'-hydroxy-3'-methoxyphenyl)-N-[(4-butylphenyl) ethyl] propenamide have been isolated from *Cuscuta reflexa Roxb* and showed strong α-glucosidase inhibitory activity with IC50 of 103.58 and 45.67 μM, respectively (Anis et al., 2002). Mangiferi was isolated from *Anemarrhena asphodeloides Bunge rhizome*. It improved hyperinsulinemia and, on insulin tolerance test, reduced blood glucose levels after oral administration for 3 weeks in mice (Miura et al., 2001).

As we discussed above, through bioassay-guided fractionation, our team isolated several compounds, belonging to terpenoids, alkaloids, flavonoids and phenolics from Boreal forest plants. These plants include *S. decora* (Guerrero-Analco et al.), *V. vitis* (Eid et al.), *S. purpurea* (Muhammad et al., 2012), *A. incana* and *P. balsamifera* (Martineau et al., 2010b). And the chapter 2 of this thesis is the first report of putative antidiabetic components isolated from *L. laricina*, increasing interest in the medical plants from the Cree pharmacopeia.

In that chapter, fractionation of *L. laricina* extract guided by adipogenesis in 3T3-L1 cells was carried out to identify putative bioactive compounds. This resulted in the isolation of a novel cycloartane triterpene (identified as 23-oxo-3*α*-hydroxycycloart-24-en-26-oic acid) and a known diterpene (13-epitorulosol) as the possible active principles responsible for the enhancement of adipogenesis. Along with other bioactive principles identified by the CIHR-TAAM, the identification of such active compounds of *L. laricina* can serve two main purposes. Firstly, they could also help develop methods for the quality control/quality assurance of traditional preparations that could be used alongside modern pharmaceuticals to provide culturally adapted treatment options for Cree diabetics. Secondly, although drug development was not a goal of the CIHR-TAAM initiative, novel putative antidiabetic compounds may serve as templates to generate new therapeutic avenues for T2D. If so, this should be done in respect of the research agreement that

intervenes between academic and Aboriginal partners. This agreement ensures that any joint intellectual property generated by the Team will be managed in such a way as to benefit Aboriginal communities, notably by adhering to international access and benefit sharing principles.

5.2 Antidiabetic activity and mechanisms of action of general plants and the CEI plants

Currently available therapies for T2D include insulin and various OHAs, such as α-glucosidase inhibitors, metformin, sulfonylureas and TZDs, which have a number of serious adverse effects. Generally speaking, the molecular and cellular targets of action of antidiabetic plants that have been investigated thus far also fall into one or more of these categories: α-glucosidase inhibitors, insulin secretagogues, insulin mimetics and insulin sensitizers.

As we discussed above, radicamines from *Lobelia chinensis Lour* (Campanulaceae) were shown to suppress α-glucosidase activity (intestinal glucose absorption) (Shibano et al., 2001). Two serotonin derivatives isolated from *Safflower* (Carthamus tinctorius) seed were shown to suppress α-glucosidase activity, stronger than the positive control acarbose (Takahashi and Miyazawa, 2012). Butyl-isobutyl-phthalate, an active compound of *Laminaria japonica*, showed the inhibitory effect on α-glucosidase activity *in vitro* (Bu et al., 2010).

Stimulation of insulin secretion constitute the most commonly reported mechanism of action of many antidiabetic plants, such as *Asparagus racemosus* (Hannan et al., 2007), *Ocimum sanctum* (Hannan et al., 2006), Trigonella *foenum graecum* (Fenugreek) (Vijayakumar et al., 2005) and *Aloe vera* (Ajabnoor, 1990; Rajasekaran et al., 2004). The hypoglycemic effect of *Aloe vera* and its bitter component might be mediated by stimulation of synthesis and/or release of insulin from the β cells of Langerhans (Ajabnoor, 1990). *Aloe vera* gel also resulted in significant reductions (Rajasekaran et al., 2004) in fasting blood glucose *in vivo*.

Insulin mimetics include plants that could activate one or multiple sites to enhance insulin signaling. *Cinnamomon cassia* (cinnamon) was shown to increase

tyrosine phosphorylation of insulin receptor and IRS-1 (Qin et al., 2003) *in vivo* in rats and *Nigella sativa* increased the phosphorylation of Akt in both C2C12 cells and H4IIE hepatocytes (Benhaddou-Andaloussi et al., 2010).

Insulin sensitizers, including popular OHAs biguanides and TZDs and varieties of plants, could improve insulin sensitivity in the peripheral tissues, namely muscle, adipose tissue and liver. Metformin reduces blood glucose levels by suppressing hepatic production and by enhancing glucose uptake in skeletal muscle. Through PPARγ activation, TZDs improve insulin sensitivity partly by enhancing differentiation of pre-adipocyte to adipocyte to improve lipogenesis and avoid lipotoxicity in non-adipose tissue; by reducing lipolysis and hence FFAs in circulation; and regulating adipokines of adipose tissue. Plants belonging to this category includes *Mitragyna speciosa* Korth (Rubiaceae) (Purintrapiban et al., 2011), *Canna indica* root (Purintrapiban et al., 2006), *Artemisia princeps* (Yamamoto et al., 2010), *Glycyrrhiza uralensis* (Kuroda et al., 2003), *Cecropia obtusifolia* and *Cecropia peltata* (Andrade-Cetto and Vazquez, 2010) and *Malmea depressa* (Andrade-Cetto, 2011).

In our case, the selected CEI plants were reported to have no effect on glucose stimulated insulin secretion by pancreatic β cells in culture (Harbilas et al., 2009; Spoor et al., 2006). Interestingly, in general line with the previous study, the findings of the present thesis showed that ethanol and hot water extracts of several plant products from the pharmacopeia of the CEI have insulin sensitizing and/or insulin mimetic effects *in vitro*. For example, through both ethanol and hot water extractions, *R. groenlandicum* potentiated adipocyte differentiation and adipogenesis (glitazone-like activity) by activating PPARγ, SREBP-1 and C/EBPα; *S. purpurea* stimulated glucose transport in C2C12 cells by increasing GLUT4 protein content and activating phosphorylation of AMPK (for EE) or Akt (for HWE); *K. angustifolia* suppressed the hepatic G6Pase activity by activating phosphorylation of AMPK and Akt. Hence, CEI plant extracts, fractions and active principles altogether exert effects through both insulin-dependent and –independent biological activities.

5.3 Hot water extraction

As mentioned earlier, most of the initial work of the CIHR-TAAM adopted the classical phytochemical approach of using an organic solvent such as ethanol to optimally extract plant compounds (especially phenolics) in order to determine their biological activities. However, Aboriginal traditional preparations are based much more on hot water extraction. The second part of this thesis thus sought to evaluate and compare the biological activities of HWE and EE. In comparison to corresponding ethanol extracts, the HWE of CEI plants either maintained their antidiabetic activity, or exhibited partial or complete loss of the same. Ten of the 17 plants EE stimulated adipogenesis, hence indicating their antidiabetic potential; only 3 of the corresponding HWEs (those of *R. groenlandicum A. balsamea,* and *P. glauca*) still significantly stimulated adipogenesis, albeit at a lower level than their EE counterparts. Similarly, 3 species (both *Rhododendrons* and *S. purpurea*) either partially or fully maintained the capacity of their respective EE to stimulate muscle cell glucose transport when more traditional HWE preparations were used. In contrast, the inhibition of G6Pase could be observed for both the EE and HWE of 9 species. All inactive species remained inactive when HWE was used instead of EE.

Observing such differences in various plant species is not surprising because medicinal plants have complex chemical compositions, containing thousands of compounds. Hence, different extraction methods (methanol, ethanol, ethyl acetate and hot water extraction) might produce different combination of constituents (Khatun et al., 2012). Notably, ethanol is known to be a better solvent than water (Jeppesen et al., 2012; Khatun et al., 2012; Motawi et al., 2012) to extract several bioactive plant molecules (especially the large class of phenolic compounds).

However, this does not imply that preparation and usage by the traditional method is not efficient. This was discussed in Chapters 3 and 4 and is briefly summarized here. Firstly, EE and HWE extracts were tested at identical mg/mL concentrations of the crude extracts for purposes of proper pharmacological comparison. Future studies should use wide ranges of extract concentrations to better compare the pharmacological potential of EE and HWE plant preparations. Secondly, numerous parameters might affect the quality of extractions carried out using hot

water, such as extraction time, temperature, and water quality. Traditional medicinal plant preparations of Aboriginal healers might thus differ from the HWE conditions used in this study and could yield more concentrated solutions. Further studies should therefore compare crude plant extracts prepared by Cree healers with those prepared in the laboratory over a wider range of concentrations.

Many studies confirmed the biological effects of water extracts of plants for a variety of disease. For example, water extract of the *Bromelia plumieri* exhibited hypoglycemic activity in diabetic rats, with stronger effect at dose 350 mg/kg than that at 35 mg/kg (Andrade-Cetto and Medina-Hernandez, 2013). Hot water extract of *Euphorbia formosana* selectively inhibited the growth of human leukemic cancer cells (Hsieh et al., 2013). High glucose promotes mesangial cell proliferation, and water extract of *Poria cocos Wolf* inhibited this activity (Yoon et al., 2013). The water extract of *Argyreia speciosa Sweet* (Convolvulaceae) attenuated ethanol-induced gastric ulcer in rats, while the ethanol extract exhibited stronger therapeutic potency (Motawi et al., 2012).

5.4 Phytochemical analysis

The labor-intensive bioassay-guided fractionation is a classic approach to isolate the active constituents, but only the most active subtractions are generally selected for further fractionation steps and subsequent identification of key active principles from a given plant. Other active metabolites may thus exist in the active species, and several additional fractionation experiments would be necessary to make such determinations. The results of this thesis might provide a novel, additional and labor saving way to identify active principles that may be missed by the classic bioassay-guided fractionation approach; furthermore for more than one selected active plant species at a time.

Using the optimal UPLC–QTOF/MS, representative fingerprints for all HWE and EE of the 17 plants were obtained. This generated a matrix of data comprising qualitative (relating to phytochemical compounds, notably retention time and accurate mass) and quantitative (signal intensities) components. By applying PCA and OPLS-DA on these data for the Cree plants, we evaluated their phytochemical

profiles in relation to their biologic activity. The results of the thesis uncovered significant differences in the chemical composition of the HWE of plants with glucose transport stimulating effects versus inactive plants. This also held true for the EE of active versus inactive plants inducing adipogenesis; especially when plant species were segregated by their botanical family. Notably, two compounds were identified as possessing strong potential to be responsible for the biological activity of three active HWE, due to their highest contribution to first separation of clustering. One of these, quercetin-3-O-galactoside, was previously identified by our team through the classical bioassay-guided fractionation approach and was confirmed to be one of the active principles capable of enhancing glucose transport in C2C12 through the activation of AMPK (Eid et al.; Muhammad et al., 2012). The second antidiabetic compound putatively identified as quercetin 3-O-α-L-arabinopyranoside, has not been isolated in prior bioassay-guided fractionation studies by our group or associated with this biological activity by others. Hence, other active metabolites may exist in the active species and quercetin 3-O-α-L-arabinopyranoside may thus contribute to the antidiabetic potential of some CEI plant species. Future studies will be needed to carefully assess the biological activity of this compound in the context of glucose metabolism and diabetes.

Chapter 6: Conclusion and perspective

The results of this thesis showed that active principles were successfully isolated form *L. laricina* by adipogenesis-guided fractionation and the antidiabetic activity, as well as cellular and molecular mechanism of ethanol and hot water extracts of 17 Cree plants were evaluated and compared.

For the fractionation of *L. laricina*, we isolated several known compounds and identified a new active cycloartane triterpene, which strongly enhanced adipogenesis in 3T3-L1 cells and was partly responsible for the adipogenic (potentially glitazone-like insulin sensitizing) activity of the ethanol extract of the bark of *L. laricina*.

As discussed in the first article, we hypothesize that a synergistic relationship might exist between the isolated compounds that could contribute to the activity of the crude extract and to the most active fraction LLE-5. Firstly, the maximal effect of this triterpenoid was still less than the most active fraction LLE-5. Secondly, the expected activity of the new triterpenoid at the concentration found in LLE-5 only accounts for about two thirds of its adipogenic activity. So future work will be necessary to search for the synergy effects of these isolated compounds from *L. laricina*.

Then, *in vivo* mice model could be used to evaluate the antidiabetic effect of the active compound or combination of the active compounds, particularly, reduction of glycemia or improvement of insulin resistance.

We also evaluated the antidiabetic activity and mechanism of the actions of ethanol and hot water extracts of 17 Cree plants on lipid and glucose homeostasis *in vitro*. For the adipocyte lipid metabolism, the results confirmed that several EE potentiated adipocyte differentiation and adipogenesis, whereas 2 inhibited the same. For the glucose homeostasis, results showed that several EE stimulated muscle glucose uptake and inhibited hepatic G6Pase activity. Generally, corresponding HWE maintained, or exhibited partial or complete loss of such antidiabetic activity in comparison to EE. Only one plant (*R.groenlandicum*) retained similar potential between EE and HWE in the glucose metabolism assays.

Targeting phosphorylation of AMPK and/or Akt or activation of PPAR γ appears to be involved in the mechanism of the active plant species. Indeed, by both ethanol and hot water extractions, *R. groenlandicum* potentiated adipogenesis by increasing PPARγ, SREBP-1 and C/EBPα; *S. purpurea* stimulated glucose uptake in C2C12 cells by increasing GLUT4 protein content and activating phosphorylation of AMPK (for EE) or Akt (for HWE); *K. angustifolia* suppressed the hepatic G6Pase activity by activating phosphorylation of AMPK and Akt.

As we discussed previously, to confirm potentially higher biological activities of HWE, future studies should use wide ranges of extract concentrations to obtain a clearer picture of their full pharmacological potential. Similarly, actual medicinal plant recipes prepared by Aboriginal healers according to their traditions should be studied in the laboratory and compared with those prepared in the laboratory.

Finally, by applying PCA and OPLS-DA, we compared the chemical composition of some plants. For lipid metabolism part, we uncovered clustering of active versus inactive species, notably when segregation by plant family was applied. This confirmed significant differences in the chemical profiles of these groups. This also suggests that certain compounds or groups thereof may be common to several Boreal plant species and underlie some of the potential antidiabetic effects reported herein. Indeed, the PCA and OPLS-DA approach enabled us to identify two such biomarkers in the HWE of species stimulating muscle cell glucose transport; notably, quercetin 3-O-α-L-arabinopyranoside for which our work represents a novel finding. Future discriminant analysis should similarly be applied to active and inactive EE plants in adipogenesis to identify other biomarkers. Our work supports the notion that PCA and OPLS-DA represent a novel and powerful tool to complement the classic bioassay-guided fractionation.

In conclusion, we isolated several known compounds and identified a new active triterpene through fractionation of *L. laricina*. This thesis further provides direct evidence for insulin-like or insulin-sensitizing actions of ethanol and/or hot water extracts of CEI plants extract at the level of muscle, liver and adipose tissue. Part of their action may be related to stimulation of insulin-dependent and -independent intracellular signaling pathways, as well as to PPARγ activation. Our

results indicate that plant species, target tissues or cells, as well as extraction methods are all significant determinants of the biological activity of Cree medicinal plants on glucose and lipid metabolism.

Chapter 7: Reference

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