A Multivariate Approach to Integration of Ethnobotanical, Pharmacological, and Phytochemical Analyses of Cree and Squamish Traditional Herbal Medicines for Anti-Diabetes Use

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ABSTRACT

This thesis investigated the integration of pharmacological and phytochemical data of medicinal plants from the Cree of Eeyou Istchee in Northern Quebec. Data from these 17 plant extracts were assessed for patterns of biological activity and chemical signals that could be explained by taxonomic or plant organ groupings. The Squamish medicinal plant *Oplopanax horridus* (Sm.) Miq. was also assessed for enzyme inhibition activity across multiple extracts and for bioactive compounds using an untargeted metabolomics approach.

A comprehensive data set was assembled documenting the relative activities on the 17 plant extracts in 69 cell-free and cell-based bioassays covering activity on glucohomeostasis, effects of hyperglycemia, and capacity for enzyme inhibition. Multivariate analysis suggests that the leaf part extracts are particularly associated with antioxidant and antiglycation activities, while another discrete group of extracts associate strongly with other sets of glucohomeostasis assays. The activity of extracts on enzyme inhibition appears to be the factor most strongly driving the majority of activity patterns, likely because extracts that interact strongly with more metabolic enzymes will have more effects on other targets in the body.

The phytochemical profiles of the Cree medicinal plants were assessed in two ways. First, spectroscopic and chromatographic data for the plant extracts was compared to a database of phytochemical standards using a proprietary Waters software, UNIFI, to match known signals of chemical standards to unidentified peaks in the plant extracts. Second, similarly collected spectroscopic data for the Cree plant extracts was processed using the software MZMine for multivariate analysis in R, revealing the chemical diversity of the bark extracts in relation to the fruit and leaf extracts. Additionally, marker signals were determined for major sample groupings, and the capacity for this analytical approach to be used to tentatively identify unique compounds was demonstrated.

Through bioassay guided fractionation of the *O. horridus* inner bark extract using the CYP 3A4 inhibition assay, the DCM subfraction midway through the non-polar elution on open column chromatography was determined to be the most potent. This fraction contained 10 major peaks on HPLC-DAD analysis. The hot water extract was found to have negligible activity on CYP 3A4 inhibition.

Together, this research provides the first integrated look at the pharmacological and phytochemical data from across the Cree anti-diabetic medicinal plants in a statistical way, as well as providing a first look at *O. horridus* for inclusion in the anti-diabetes project.

RÉSUMÉ

Cette thèse a examiné l'intégration des données pharmacologiques et phytochimiques de plantes médicinales des Cris d'Eeyou Istchee dans le Nord du Québec. Les données de ces 17 extraits de plantes ont été évaluées pour des modèles biologiques et des signaux chimiques qui peuvent être expliqués par des groupements de taxanomes ou d'organes de plantes. La plante médicinale du Squamish, *Oplopanax horridus* (Sm.) Miq., a été également évaluée pour l'activité d'inhibition enzymatique sur des extraits et pour les composés bioactifs en utilisant une approche métabolomique non ciblée.

Un ensemble de données a été rassembleré pour documenter les activités relatives des 17 extraits de plantes dans 69 essais biologiques sans cellules et aussi à base de cellules. Ces essais biologiques sont composes de trois categories: l'activité sur la glucohoméostase, la médiation des effets de l'hyperglycémie, et la capacité d'inhibition enzymatique. Une analyse multivariée suggère que la collection d'extraits des parties de feuille est particulièrement associée à des activités antioxydantes et antiglycantes, alors qu'une autres groupe de quatres d'extraits de plantes s'associe plus fortement à d'autres ensembles de dosages de la glucohoméostase. L'activité des extraits sur l'inhibition des enzymes semble d'être le facteur le plus déterminant dans la majorité des schémas d'activité. C'est plus que probable que les extraits qui interagissent fortement avec des enzymes plus métaboliques auront davantage d'effets sur d'autres cibles de l'organisme.

Les profils phytochimiques des plantes médicinales Cries ont été évalués de deux manières. Tout d'abord, les données spectrographiques et chromatographiques des extraits de plantes ont été comparées à une base de données d'étalons phytochimiques en utilisant un logiciel propriétaire de Waters, UNIFI, pour faire correspondre les signaux connus d'étalons chimiques aux métabolites non identifiés dans les extraits des plantes. Deuxièmement, les données spectrographiques qui ont été collectées d'une manière similaire pour les extraits de plantes Cries ont été traitées à l'aide du logiciel

MZMine pour l'analyse multivariée en R, révélant la diversité chimique des extraits d'écorce par rapport aux extraits de fruits et de feuilles. En addition, les signaux de marqueur ont été déterminés pour les groups principaux, qui a démontré la capacité de cette approche pour temporairement identifier des composés uniques.

Sur le fractionnement guidé par l'essai biologique de l'extrait d'écorce interne d'O*. horridus* à l'aide du dosage d'inhibition du CYP 3A4, la sous-fraction de DCM à mi-parcours d'élution non polaire par chromatographie sur colonne ouverte a été déterminée la plus puissante. Cette fraction contenait 10 métabolites majeurs sur l'analyse HPLC-DAD. L'extrait à l'eau chaude s'est révélé d'avoir une activité négligeable sur l'inhibition du CYP 3A4.

Ensemble, ces recherches fournissent le premier aperçu intégré des données pharmacologiques et phytochimiques provenant de plantes médicinales antidiabétiques Cries, ainsi qu'un premier apercu à l'inclusion d'O. *horridus* dans le projet anti-diabète.

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AGE	Antiglycation endproduct
CEI	Cree of Eeyou Istchee
CYP	Cytochrome P450
EET	Epoxyeicosatrienoic acid
G6P	Glucose 6-phosphate
GS	Glycogen synthase
HPLC	High performance liquid chromatography
OPLS-DA	Orthagonal partial least squares discriminant analysis
PCA	Principle component analysis
PRV	Pharmacological Rank Value
SIV	Syndromic Importance Value
T2D	Type 2 Diabetes Mellitus
TAAM	Team in Aboriginal Anti-diabetic Medicine
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UPLC	Ultra performance liquid chromatography

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 DIABETES MELLITUS

Diabetes mellitus is a chronic disease that results in the body's inability to process glucose effectively, and affects more than 400 million people in the world today (World Health Organization 2016). In Canada, more than 8% of the adult population has been diagnosed with diabetes. However, in First Nations communities, the value is disproportionately high, averaging over 17% in adult populations (Government of Canada 2011).

Insulin, produced by the pancreas beta cells, is an essential hormone in regulating glucose homeostasis, with the primary function of initiating the pathway for stimulating the uptake of glucose into cells. Type 1 diabetes is characterized by the inability to produce sufficient functional insulin and requires patients to take daily doses of insulin. Type 2 diabetes mellitus (T2D) is instead characterized by the body's inability to effectively use the insulin it produces, and is generally thought of as the desensitization of the body to insulin (World Health Organization 1999). This desensitization is usually followed by an overcompensation of the production of insulin, leading to exhaustion of the cells and a breakdown of blood glucose regulation in the body (Gavin et al. 2000). The long-term result is that less glucose is able to make it into cells for energy production, and instead continues to build up in the blood, leading to a state of hyperglycemia. This thesis will focus specifically on research directed toward management of T2D.

Poor regulation of blood glucose on its own can have serious metabolic and physiological consequences, as hyperglycemia can lead to side effects such as ketoacidosis, slow wound healing, and infections, while hypoglycemia leads to confusion and loss of consciousness. However, there are also many other complications and chronic conditions associated with the development of diabetes. These complications tend to arise from damage to the vascular system after long term exposure to extreme or

highly fluctuating blood glucose levels, leading to organ damage (Booth et al. 2006; Lok et al. 2003; Shamoon et al. 1993).

A large range of antidiabetic drugs with distinct pharmacological targets is currently available on the market (P. S. Haddad et al. 2012). The blood glucose lowering agent metformin is one of the most prescribed anti-diabetes medications worldwide, and is derived from the French lilac, *Galega officianalis* (Bailey 2017), setting a major precedence for the place of herbal medicine in the treatment of diabetes.

1.2 TEAM IN ABORIGINAL ANTI-DIABETIC MEDICINE

The Team in Aboriginal Anti-diabetic Medicine (TAAM) was established in 2003 by Dr. Pierre Haddad of the University of Montreal under CIHR funding to address the issue of rising rates of diabetes in Canadian First Nations communities. Working with the Cree of Eeyou Istchee (CEI), the team included researchers from three Canadian universities and community stakeholders representing both Elders and Public Health. The goal was to target traditional medicine as an accessible and culturally relevant method of prevention and management. Through multi-institutional collaborative work, the TAAM evaluated the ethnobotany, pharmacology, and phytochemistry of traditional Northern Cree medicinal plants in the context of diabetes. Based on the Cree ethnobotany, 17 plants were established as those with the greatest anti-diabetic potential. Dozens of publications (P. Haddad 2018) have since been produced looking at the bioactivity of these plants in more than 60 diabetes related bioassays.

1.3 ETHNOBOTANICAL APPROACHES

The traditional knowledge of Indigenous groups has been a long-standing interest of the scientific community around the world. Classical ethnobotanies were untargeted in nature, seeking mainly to document whatever traditional plants and uses Indigenous groups were willing to share with researchers at the time, and often gave little depth of focus to the medicinal uses (Core 1967; Turner

and Bell 1971) or focused only on specific plants (Chandler, Hooper, and Harvey 1982; Rymer 1976). However, as a result of an increase in plant-derived conventional medicines and a better understanding of the consequences of loss of Indigenous culture through world development, the medicinal focus of Indigenous plant knowledge became a more recent focus of the scientific community (Fabricant and Farnsworth 2001; Schultes 1994).

As the field of ethnobotany grew, studies began shifting from purely descriptive and qualitative approaches toward quantitative methods aiming to sift through the vast Indigenous plant knowledge using statistics (Prance 1991). Since then, numerous methods have been developed to help direct researchers towards the plants that had the greatest potential for use in targeted clinical settings (Höft, Barik, and Lykke 1999). To properly target traditional medicines in a specifically anti-diabetic context, Leduc et al. (2006) developed a novel data collection method that aimed to identify plants used for ailments considered major symptoms of diabetes by engaging with Cree elders in a more holistic view of plant use. Statistical methods were used to evaluate consensus at the same time as relatedness to diabetes, and this method resulted in the identification of the 17 Cree plants that became the basis for a decade of pre-clinical and phytochemical research by the TAAM.

1.4 THE TAAM'S APPROACH TO THE CREE ETHNOPHARMACOLOGY

When it comes to the use of natural products to combat the progression of diabetes, there are three main areas of research on which the TAAM has focused. Primarily, there is the investigation into direct antidiabetic effects. These studies usually focus on the cell types either most susceptible to the damages done by hyperglycemia or those most capable of reestablishing control over glucose levels in the blood. These have included assays involving liver, muscle, fat, and intestinal cells, among others. Multiple cell pathways govern the ways in which sugars are managed by cells, and a large focus of the project has been to identify plants that may target these pathways in ways comparable to contemporary

anti-diabetic medications (**Figure 1.1**). Biguanides, like metformin, target AMP kinase in muscle and liver cells in insulin-independent pathways to improve insulin sensitivity and glucose homeostasis (Bailey and Turner 1996). Glitazones act primarily on the PPARγ receptor of fat tissue to modulate adipocyte differentiation and lipid homeostasis with downstream effects of decreasing insulin resistance through adipokine ratio regulation (Henry 1997). In this thesis, bioassays that target mechanisms of lowering blood glucose are collectively referred to as "glucohomeostasis" assays. Other major pharmaceuticals include insulin secretagogues, alpha-glucosidase inhibitors, and incretins, but these pathways were not included in major ways in the project so will not be discussed.

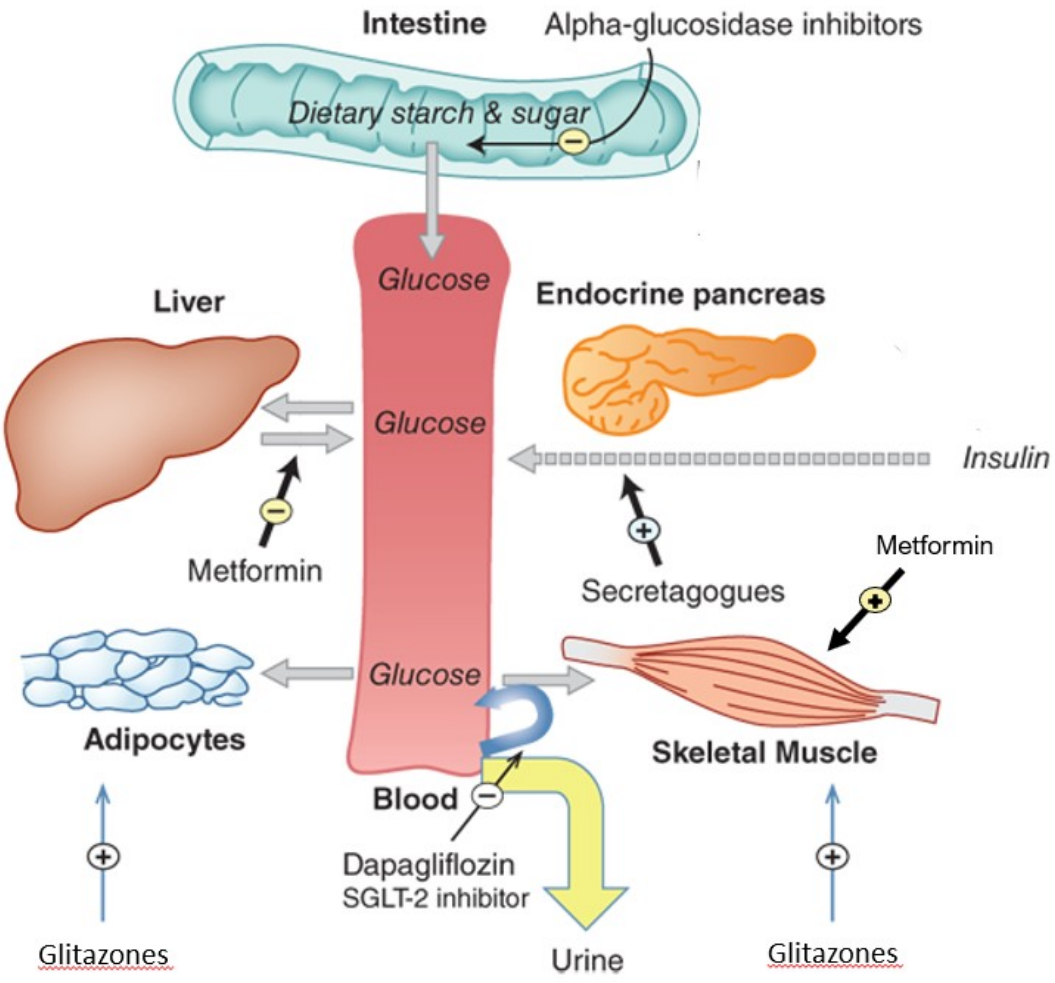


Figure 1.1. Endogenous and exogenous substances affecting fate of glucose in the body. Adapted from Katzung, Kruidering-Hall & Trevor, 2015.

Secondly, diabetic hyperglycemia leads to many other problems throughout the body, and one of the major effects is the reduction in body's ability to scavenge reactive oxygen species from the blood, leading to further tissue damage (Ceriello 2006). Identifying substances with potent antioxidant effects is key in helping to reestablish this balance and minimizing the effects of glycation in the body (Chetyrkin et al. 2008). Another negative result of hyperglycemia is the activation of inflammation pathways (Pickup 2004). Inflammation is linked with numerous other conditions diabetics are plagued with, such as arthritis; thus, identifying substances with anti-inflammatory capacity is essential. Further, the common diabetes symptom of neuropathy results from nerve damage due to multiple factors resulting from chronic hyperglycemia (Kelkar 2005), and evaluating plants' abilities to protect nerve cells from this type of damage can be used to measure the practical antioxidant and antiglycation effects of these plants. In this thesis, bioassays that target mechanisms of mitigating the physiological consequences of high blood glucose are collectively referred to as "effects of hyperglycemia" assays.

Finally, natural products run the risk of interacting with the cytochrome P450 (CYP) enzymes, the body's natural defenses against xenobiotics (Bushra, Aslam, and Khan 2011). These are also the main Phase 1 drug metabolizers, responsible primarily for first pass metabolism, and so must be considered seriously for how their activities change in the presence of natural health products. In this thesis, bioassays that target drug metabolizing enzymes are collectively referred to as "safety" assays.

These three foci of study are essential in developing our understanding of the capabilities of the traditional Cree pharmacopoeia, and so have been the focus of most of the TAAM's research goals. There has been some variation in the methods used by the TAAM to investigate these plant activities, but, in general, plant extracts were made with hot water (one of the most common preparations of traditional medicine in Indigenous communities (Uprety et al. 2012)) and/or organic solvents, normally hydroethanolic, representative of common laboratory practice as well as herbal tincture preparations. These extracts were then evaluated in various cell-free, cell-based, and *in vivo* bioassays to evaluate

biological activity and potential value in preventing or managing T2D. The major strength of this data set is that pharmacological data have been collected on the same set of plant extracts across a diverse yet overall related set of pharmacological bioassays. However, most of the time, each investigation focused on a relatively small and specific subset of anti-diabetic activity. This is unsurprising, given the exceptional amount of time and money required to evaluate all 17 Cree medicinal plants on a single assay. This practice ultimately (and unfortunately) results in a somewhat heterogeneous and fragmented view of the potential of these plants, and the multifaceted view of the effect of these plants within the framework of such a complex disease is easily missed. Nonetheless, the scope of the pre-clinical results, collected using standardized and validated methods, is unparalleled in the study of medicinal plants and represents a unique opportunity to consider their anti-diabetic potential.

The progression of bioactivity analysis of these plants started with a focus on both cell-free and cell-based assays to evaluate potential use. These can range from evaluating the effects of these plants on different enzyme activities (cell-free) to evaluating what regulatory pathways the plants may be influencing on various cell types (cell-based). Offering simplified models that may not reflect *in vivo* effects, these assays have the benefit of being efficient to replicate and run on such a large set of samples, and still provide an excellent first glimpse at what clinical potential the plants may have. Once observation of significant activity illuminated the potential mechanisms of specific plants, *in vivo* work (primarily on mouse models) provided a better view of the practical effects these plants can have on key diabetes measures such as weight gain, blood glucose/insulin levels, and tissue specific pathway activation.

In recent years, the TAAM's focus has shifted out of the lab and back into the First Nations communities once again. Current goals are to evaluate and implement community practices regarding culturally appropriate health care, community engagement on diabetes education, and implementation

of local intervention strategies to incorporate traditional practices into the regimen for diabetes prevention, management, and maintenance.

1.5 THE TAAM'S APPROACH TO PHYTOCHEMICAL ANALYSIS

At the outset of the project, the TAAM's first goal in terms of phytochemistry was to evaluate the complexity of Cree medicinal plant extracts and to identify marker compounds for each species. These first steps facilitated future chemical characterization and served as a minimum requirement for publication but do not necessarily provide insight on bioactive molecules.

A significant goal in the investigation of traditional plant medicines is the identification of active compounds contributing to the biological effect of interest. This goal is often achieved through the common practice of bioassay guided fractionation and has been a staple method in the TAAM's research of plant extracts once promising bioactivity had been established. Briefly, bioassay guided fractionation uses methods in chromatography to separate the chemical components of a bioactive plant extract by chemical properties (e.g. size, charge, polarity) into smaller, less complex fractions. These fractions are then tested again on the given bioassay to determine the one(s) with the greatest activity; active fractions can be separated again, with the process cycling until a single or small number of isolated active compounds can be determined using structure elucidation techniques, or spectral comparison with commercial chemical standards.

This process is considered a targeted phytochemical analysis and, while effective, it does have some limitations. First, the process is time consuming, as there are often hundreds or thousands of compounds to sift through, and separation through chromatography likely will take multiple steps to isolate pure compounds. Secondarily, considering the diversity of phytochemicals in a given plant extract, the targeted method does not always properly evaluate the potential of synergistic or additive effects of an extract. This is especially important given that the traditional consumption of herbal

medicine is not broken down into individual compounds, but is consumed as the full, complex extract. Finally, this method is only practical for evaluating a very small number of plants in a single investigation due to the lengthy process.

Recently, some untargeted methods have been used to evaluate the active phytochemistry among Cree medicinal plants, to some effect. Work by Shang et al. (2015) tested extracts for the effects on glucose uptake in muscle cells and classified samples using a bioactivity active-inactive binary system. Evaluation of the mass spectral data of all 17 plants, grouped as either active or inactive, permitted discriminant analyses that identified specific phytochemicals most associated with the more active plants. This method was successfully able to identify the active compounds quercetin-3-O-galactoside and quercetin-3-O-α-L-arabinopyranoside.

1.6 THE 3 OBJECTIVES OF THIS THESIS

The common theme of the TAAM's research has been its somewhat incremental nature. This approach has, for the most part, been out of necessity due to the large scope and novelty of the data. While this is often taken for granted as common scientific practice, it overlooks the holistic and interconnected views often associated with Indigenous methods. However, after years of this individualistic data collection, this has resulted in a truly unique opportunity to look at these plants anew from an integrated perspective.

Using the combined data of the ethnobotanical, pharmacological, and phytochemical studies conducted by the TAAM, I have attempted here to provide a new perspective of these plants that takes into account, through meta-analysis, all previous methods and analyses. Specifically, my three objectives are as follows:

1. To evaluate the collective pharmacology data of the TAAM for previously undetected patterns of activity.

2. To evaluate the collective metabolomics data of the TAAM for previously undetected patterns of phytochemistry.

3. To provide a first look at some of the metabolomics and safety activity of the medicinal plant *Opplopanax horridus*.

This thesis has two main purposes for evaluating the TAAM data in these ways.

First, the use of multivariate methods provides a unique opportunity to look at underlying patterns in the data, whether it be deciding if certain plant parts or families are producing similar activities or phytochemicals, or if patterns in activities could be used to establish hypotheses for mechanisms of action and targeting of specific secondary metabolites.

Second, this is the first time that data of this nature (ethnobotanical, pharmacological, and phytochemical) have been used in such an integrated way. As such, this thesis will provide a valuable comparison of the types of information that can be gained through such an analysis, and whether or not it is worth approaching similar data sets in this way in the future. Can the perspectives of 'Big Data' be adopted for such projects? Do such approaches even provide information that cannot otherwise be gained from traditional ethnopharmacological analysis? Can the work presented here potentially provide perspective on ways to collect such data in the future to maximize the informative power of the ethnobotanical method? These are the questions this thesis seeks to answer.

CHAPTER 2: REVIEW OF THE PHARMACLOGICAL ACTIVITY OF THE ANTI-DIABETIC MEDICINAL PLANTS OF THE CREE OF EEYOU ISTCHEE

2.1 INTRODUCTION

To address the rising rates of Type 2 Diabetes mellitus (T2D) in First Nations communities of the Cree of Eeyou Istchee (CEI) (Ekoe et al. 1990), the Team in Aboriginal Antidiabetic Medicine (TAAM) elected to use an ethnobotanical approach that specifically targeted traditional Cree herbal medicines used to treat diabetes symptoms. Classic ethnobotanies have tended to evaluate plant uses more generically, or to focus on a single plant at a time. By directing interview questions towards plants used for symptoms directly related to diabetes, the TAAM aimed to identify medicinally relevant plants that could be incorporated into culturally appropriate local intervention strategies for prevention and management of T2D (Leduc et al. 2006). To do so, the team developed a list of 15 major symptoms associated with the diabetes pathology, then conducted interviews with more than 100 Cree knowledge holders in the community of Mistissini in Northern Quebec on how they would care for people exhibiting any of these symptoms, using herbal medicine. This approach led to the identification of more than 40 local plants. After the success of this methodology, the process was repeated with the CEI community Whapmagoostui (Harbilas et al. 2009), and the data integration resulted in the 17 species used in this study (**Table 2.1**).

Table 2.1. Traditional medicinal plants of interest identified through interviews with elders of the Cree of Eeyou Istchee of Mistissini and Whapmagoostui. Syndromic Importance Value (SIV) indicates the degree of association with uses related to treating diabetes symptoms where values closer to 1 indicate stronger association.

Plant	Family	Part	SIV
***Abies balsamea* (L) Mill.**	Pinaceae	Bark	0.02446
***Alnus incana subsp. Rugose* (Du Roi) R. T.**	Betulaceae	Bark	0.02396
***Gaultheria hispidula* (L) Muhl.**	Ericaceae	Leaves	0.01005
***Juniperus communis* L.**	Cupressaceae	Berries	0.02030
***Kalmia angustifolia* L.**	Ericaceae	Leaves	0.03210
***Larix laricina* Du Roi (K. Koch)**	Pinaceae	Bark	0.03695
***Lycopodium clavatum* L.**	Lycopodaceae	Whole	0.00866
***Picea glaucaa* (Moench.) Voss**	Pinaceae	Leaves	0.03474
***Picea mariana* (P. Mill) BSP**	Pinaceae	Cones	0.03522
***Pinus banksiana* Lamb.**	Pinaceae	Cones	0.01798
***Populus balsamifera* L.**	Salicaceae	Bark	0.01170
***Rhododendron groenlandicum* (Oeder) Kron & Judd**	Ericaceae	Leaves	0.03773
***Rhododendron tomentsum* ssp. *subarcticum* (Harjama) G. Wallace**	Ericaceae	Leaves	0.03590
***Salix planifolia* Pursh**	Salicaceae	Bark	0.01782
***Sarracenia purpurea* L.**	Sarracenaceae	Whole*	0.01321
***Sorbus decora* (Sarg.) C. K. Schneid.**	Rosaceae	Bark	0.03122
***Vaccinium vitis-idaea* L.**	Ericaceae	Berries	0.01647

*Above ground material only

Part of the evaluation of each plant's potential as a tool for T2D management was the calculation of its Syndromic Importance Value (SIV). This value was calculated using data collected for 3 parameters: (1) how many symptoms was each plant used to treat; (2) the frequency of citations made by different elders for the same plant treating the same symptom; (3) the relative level of importance each of the symptoms had as a more unique and specific symptom of T2D.

Since their identification, these Cree medicinal plants were evaluated for anti-diabetic activity using an extensive suite of pre-clinical assays. This work has fallen into the 3 major categories of testing for i) effects on glucohomeostasis, ii) ability to mediate the effects of hyperglycemia, and iii) general

safety. Overall, more than 50 endpoints were evaluated across 5 cell types and a host of cell-free assays. Significant activity was identified by at least one plant in almost all cases, and all plants exhibited significant activity in at least one assay, but no two species shared identical activity profiles.

The investigation into the ability of Cree medicinal plants to modulate glucohomeostasis focused on two major factors. First, bioassays were run to test the possibility that these plants could prevent or reduce the production of glucose in liver cells (Nachar et al. 2013). Several plants exhibited inhibitory activity towards the enzyme glucose-6-phosphotase (G6Pase), a key enzyme in glucose production, but *P. glauca* and *A. balsamea* had levels of effect comparable to that of insulin. Also in hepatocytes, the team looked at the ability to increase sequestering of glucose into glycogen through stimulation of glycogen synthase (GS), finding significant activity in *A. balsamea* and *L. laricina*. Mechanistic work was also completed to evaluate activation of specific signaling pathways (Akt, AMPK, GSK). Second, bioassays evaluated the ability of these plants to increase the uptake of glucose from media into muscle and fat cells (Spoor et al. 2006). Many plants exhibited an insulinomimetic activity, whereby they increased basal glucose uptake in the absence of insulin, in both the differentiated muscle and fat cell lines. While most of the observed activity was significant relative to vehicle controls, no plant consistently stood out as a *most* active glucose uptake stimulator.

In association with the glucose uptake assays, the capacity for these plants to decrease the uptake of glucose into intestinal cells was also evaluated (Nistor Baldea et al. 2010). While nearly all plants exhibited an immediate inhibition of glucose uptake (relative to control), longer incubation led to a display of greatest activity in *P. mariana*.

The last glucohomeostasis activity that was evaluated was the adipogenic potential of the plant extracts (Spoor et al., 2006). It has been argued that both pro- and anti-adipogenic activity have potential value in treating diabetes, where reducing triglyceride content of differentiating adipocytes

suggests an anti-obesity outcome (Harp 2004), and stimulating triglyceride accumulation is an indicator of increased insulin sensitivity (Tontonoz, Hu, and Spiegelman 1995). Several plants displayed strong effects in increasing triglyceride content, primarily *L. laricina* and *R. groenlandicum*, while *P. balsamifera* was found to decrease triglyceride content well below the vehicle control, though non-significantly.

A major aspect of T2D is its association with a state of oxidative stress in the body (Ceriello 2006), which has been linked in some studies to chronic hyperglycemia (Yan 2014). This state manifests in numerous related ways, from the level of damaging modifications to proteins and DNA (Birben et al. 2012) to reduction in reproductive health (Agarwal et al. 2012), and so there are various methods for evaluating the ability of natural products to mitigate these effects.

Primarily, the antioxidant potential of Cree medicinal plants was evaluated using a set of complimentary biochemical assays (Fraser et al. 2007). All plants exhibited antioxidant activity to some extent, but no single or set of plants were found to be consistently the most active. *L. laricina* was determined to have the greatest average antioxidant effect, though. Also, strong positive correlations were observed between the antioxidant activity and ethnobotanical ranking by SIV.

Secondly, the potential of the Cree plant extracts to mitigate the biochemical and cytological effects directly related to the elevated sugar presence in the blood was tested. Antiglycation activity associated with hyperglycemic states was evaluated on two main metrics (C. Harris et al. 2011), and in both cases, *L. laricina*, followed closely by *R. tomentosum*, demonstrated a strong capacity for minimizing the formation of advanced glycation endproducts (AGEs). Next, the protective ability for these extracts in states of glucose toxicity or deprivation in undifferentiated PC12 cells was evaluated (Spoor et al., 2006), where *S. purpurea* held the greatest capacity to protect cells in toxic levels of glucose. Finally, the enzyme aldose reductase, responsible for the biochemical reaction leading from glucose to sorbitol, has been found in important tissues throughout the body and its activity has been

linked to multiple diabetes comorbidities in *in vivo* models, specifically processes leading to cardiovascular disease and retinopathy (Ramasamy and Goldberg 2010; Tarr et al. 2013). Experiments by Nguyen (Nguyen 2011) demonstrated the inhibitory action of some Cree plants on aldose reductase, a target for preventing cataract formation, led by *R. groenlandicum*.

The last major category of secondary effects of diabetes investigated by the TAAM was that of immunomodulation. Many natural product secondary metabolites have been associated with immunomodulation, but both pro- and anti-inflammatory activity has been observed in the Cree medicinal plants. With evidence that the state of inflammation in the body may have an effect on the development of T2D (Dandona and Aljada 2002), the immunomodulatory effects of Cree plants were evaluated through measuring their effect on levels of TNFα secretion in monocytes with and without immunostimulation by lipopolysaccharides (Walshe-Roussel 2014). Many plants exhibited anti-inflammatory activity in their ethanolic extractions, led by *P. mariana*. Although pro-inflammatory activity of these same extracts was minimal, the water extracts did exhibit some significant pro-inflammatory activity, particularly in *S. purpurea*, as well as *P. mariana* again.

Finally, an essential category of study for the activity of natural products is their safety. Natural plant compounds have been found to interact with metabolic enzymes (Foster, Arnason, and Briggs 2005), and therefore may interfere with the processing of foreign chemicals such as other medications. The enzymes primarily responsible for xenobiotic metabolism activity are the cytochrome P450 (CYP) isoforms, of which 57 have been identified in humans and 16 specifically been associated with drug metabolism (Guengerich 2008). Although CYP 3A4 is responsible for more drug metabolism in the body than other isoforms, diabetes medications are significantly metabolized by the isoforms 2C9 and 2C8, in addition to 3A4 (Triplitt et al. 2006). Beyond these 3 isoforms, Tam et al. (2009) completed extensive work evaluating the modulatory activity of the Cree plants on many other CYP enzymes. The variation in substrate specificity across CYPs provides the opportunity to consider a broader range of possible

bioactivities of the Cree plants in addition to herb-drug interactions relating to diabetes alone. The plants *L. clavatum* and *S. decora* were strong inhibitors of 2C8/9. Several mechanisms for 3A4 inhibition were evaluated, with members of the Pinaceae family often showing strongest inhibitory effects. On average, *R. groenlandicum* had the greatest mean inhibitory effect, whereas *V. vitis-idaea* had the least.

Together, these *in vitro* pharmacological and toxicological results – derived from 17 medicinal plant extracts tested systematically in validated assays – represents a unique opportunity to investigate relationships among and between plant species and their observed bioactivities in the context of diabetes using a meta-analysis framework.

Collectively, the activity of the tested plants is diverse and complex. While each bioassay individually produces useful data about the activity of plants in specific experimental paradigms related to diabetes, it is much less clear where the therapeutic potential of each plant fits in the full context of T2D. This chapter aims to evaluate the bioactivity of Cree traditional medicines using multivariate methods for an integrated view of their anti-diabetic potential, possible mechanisms of action, or phytochemical patterns.

2.2 METHODS

2.2.1 MATRIX DEVELOPMENT

Data for the meta-analysis was collected exclusively from research papers conducted within the TAAM group. Published research under the topic of *Cree* and under the authorship of Haddad, P* was collected, as well as unpublished data pertinent to the three categories of pharmacological activity of the Cree plants found in graduate studies theses of students under the TAAM. From these sources, the following data were collected: plant species evaluated, plant part evaluated, the bioassay used, whether the plant was found active or non-active relative to controls (depending on the design of the individual study), the position in rank of each plant's activity relative to controls, and significant notes from the discussion. In total, 69 individual bioassay results were collected for the 17 plants.

2.2.2 ANALYSIS

Initial analysis of the matrix explored basic distributions of activity as outlined by the sources, where plants were marked 'Active' if they achieved statistically significant activity relative to controls. As individual plant extract-bioactivity combinations were largely unreplicated, statistical comparisons between bioactivity studies was not applicable, and so only data trends were explored here. Species were evaluated for the total number of assays they were found active on; in addition, the ratio of total "Active" results to the actual number of assays each species was tested on was evaluated.

For multivariate analysis, the only data which were used were those of the 69 bioassay endpoints that were evaluated, the 17 Cree plants that were evaluated, and a matrix defining the rank each plant scored on each bioassay relative to the activity of the other plants. In such cases that one or more species did not have associated data for a given assay, the ranking was adjusted so that values equidistant from the highest and lowest ranks (1 and 17, respectively) were omitted. For example, if one species was missing from the analysis, the rank value '9' would be omitted, as it is the mid-value

between 1 and 17, and the rankings of the other plants on that assay were adjusted around it. Assays with more than 3 missing plants were omitted from the analysis completely. In such cases where two or more plants appeared to share an indistinguishable activity level and the raw data was not available for clarification, or that standard error values conflated the separation, the rank levels that these plants spanned was averaged and applied to those plants to share. From here, the data was divided into the three major distinctions between the bioassays: those evaluating effects on cell-based glucohomeostasis, those evaluating effects of hyperglycemia, and those evaluating interactions with common drug-metabolizing enzymes (Safety).

Once this new data set was organized for the multivariate analysis, matrices were subjected to principal component analysis (PCA) with groupings either by taxonomic family or the plant part used in the analysis, and separations of assays and plants were observed at multiple divisions of the data. Of the families, only Ericaceae and Pinaceae contained enough representatives for statistical visualization of their groupings. Of the plant parts, only Bark and Leaves contained enough representatives for statistical visualization of their groupings.

2.3 RESULTS

2.3.1 CUMULATIVE BIOACTIVITIES OF CREE MEDICINAL PLANTS ACROSS BIOASSAYS

The activity of the plant extracts across the full range of bioassays conducted by the research team was first evaluated for whether statistically significant activity was found on each assay. Overall activity was measured both as the total number and the percentage of assays where each species was active (**Table 2.1**). *L. laricina, R. groenlandicum*, and *S. purpurea* were found to have the greatest activity, both in terms of number and percentage of active assays. *P. balsamifera* and *V. vitis-idaea* had the lowest values on both metrics, despite being tested on more assays than other plants. Notably, significant activity was observed in more than 50% of tested assays for all 17 plants.

Table 2.2. Number and percentage of evaluated bioassays for which statistically significant activity was observed for Cree medicinal plant ethanol extracts.

Species	Number of Active Assays	Percentage of Active Assays (%)	Number of Assays Tested
A. balsamea	43	73	59
A. incana	43	67	64
G. hispidula	29	64	45
J. communis	30	64	47
K. angustifolia	37	70	53
L. laricina	48	79	61
L. clavatum	29	64	45
P. glauca	30	67	45
P. mariana	34	55	62
P. banksiana	34	55	62
P. balsamifera	27	50	54
R. groenlandicum	50	77	65
R. tomentosum	36	72	50
S. planifolia	29	62	47
S. purpurea	53	79	67
S. decora	44	72	61
V. vitis-idaea	27	50	54

Based on the total number of assays in which significant activity was observed in the previous studies (**Figure 2.1)**, *R. groenlandicum, S. purpurea, A. balsamea,* and *L. laricina* initially appear to be the most biologically active species. The separation of these four plants was seen through their combined activity across both sets of bioassays (Glucohomeostasis and Hyperglycemic Effects), although this distinction appears to be more driven by the results of the Glucohomeostasis bioassays specifically. Again, *P. balsamifera* appears to score last on all three metrics.

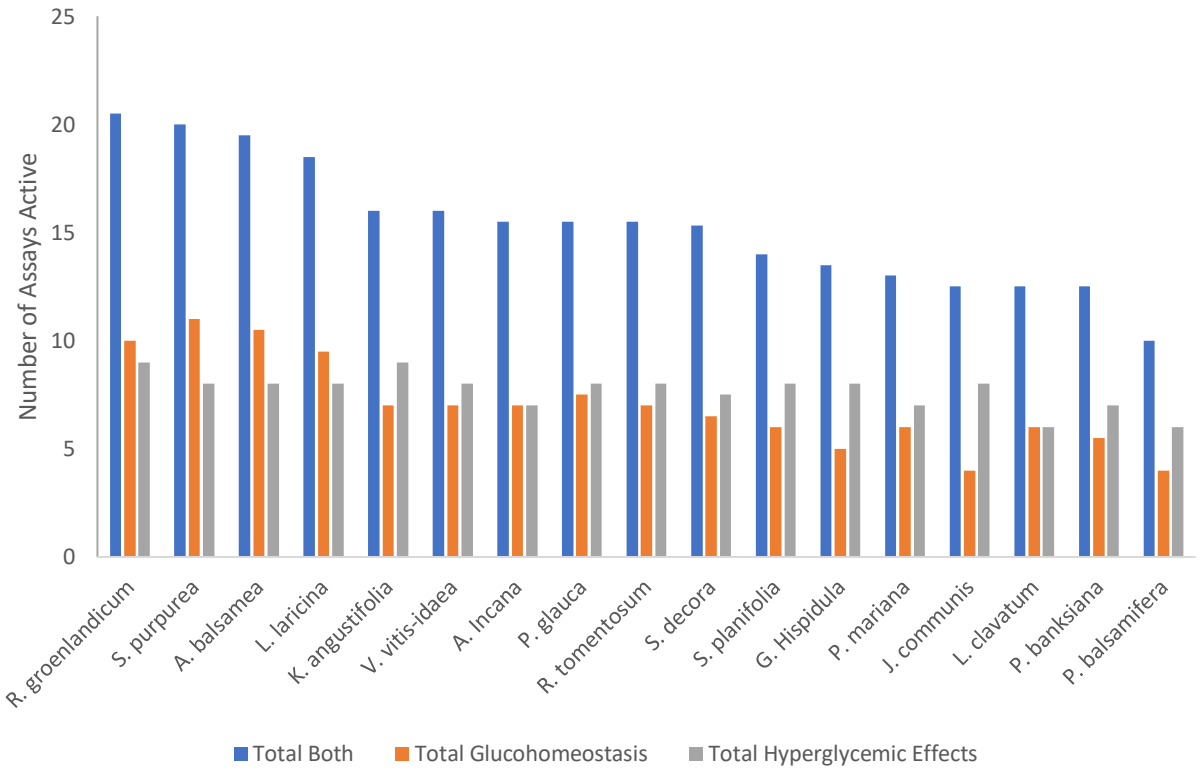


Figure 2.1. The total number of assays for which Cree medicinal plants were determined to have statistically significant activity on bioassays evaluating effects on glucohomeostasis and hyperglycemic effects. All species were tested on the same set of assays.

2.3.2 PRINCIPLE COMPONENT ANALYSIS OF BIOACTIVITY DATA BY SPECIES

When evaluating the full set of assays on all species (**Figure 2.2**), there was no obvious clustering of the data. When applying distinctions of the taxonomic families, although the regions occupied by the Ericaceae and the Pinaceae are distinct from each other, they still overlap extensively with the rest of the data. Moreover, there appears to be no pattern of distribution among the leaf and bark samples. However, although not containing enough representatives to form a statistical grouping, the two cone samples, *P. banksiana* and *P. mariana*, appear to be highly similar in their overall activity. Further, the highly localized distribution of the enzyme inhibition assays loading vectors suggests a dominance of both principle components, and that these assays should be evaluated on their own moving forward.

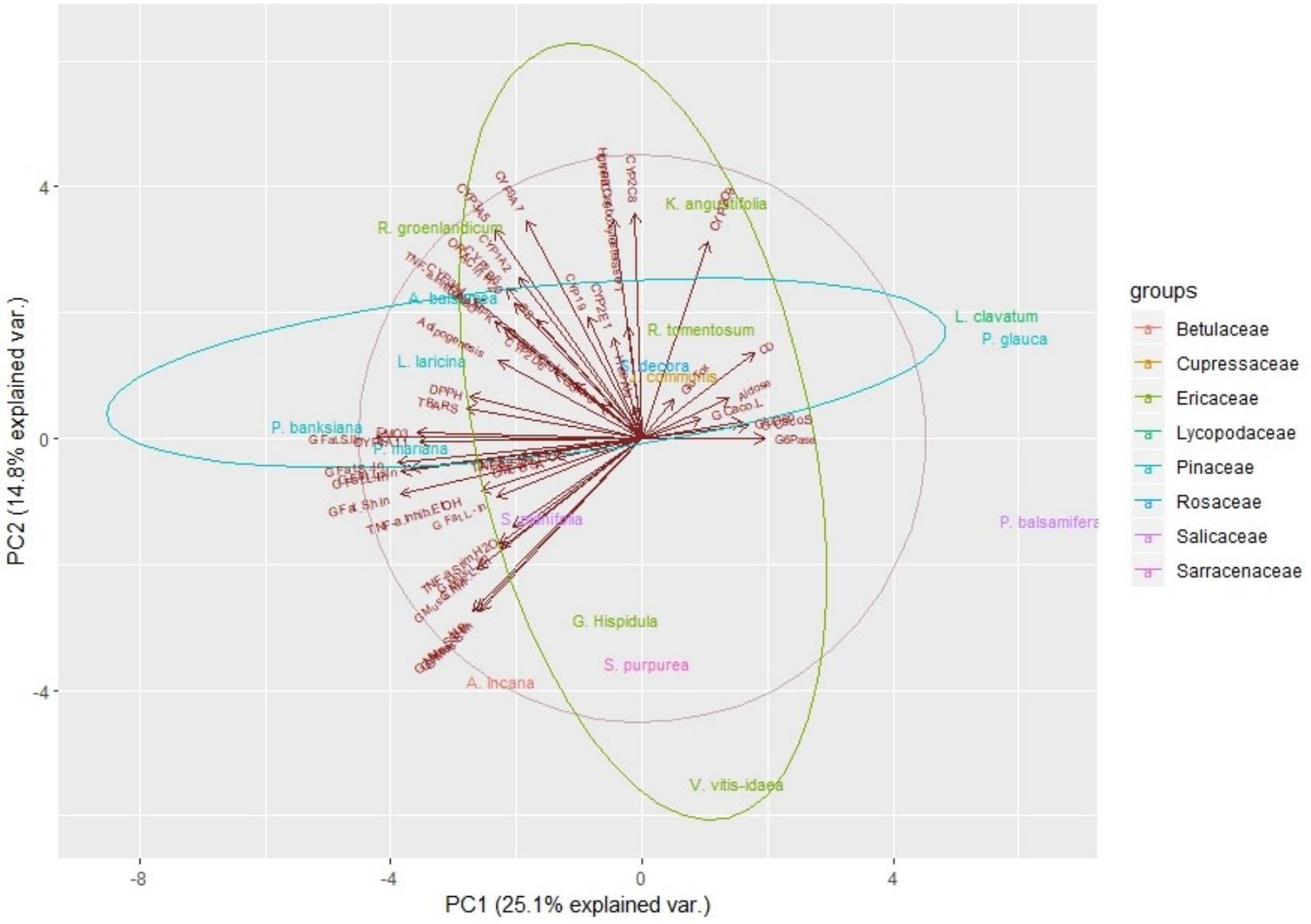
Looking at the enzyme inhibition assays alone (**Figure 2.3**), the unidirectional distribution of loadings along PC1 again indicate the strong bias in the data towards plants that interact with CYPs, and that this separation of the data is justified. There is some indication of members of the Pinaceae as particularly consistent inhibitors, although this is not universal. Again, the grouping of the two cone species is seen, and seems to be specifically associated with a subset of the enzymes: 1A2, 2E1, 4A11 and FMO3. The distribution of the major enzymes associated with diabetes drugs (2C8/9, 3A4/5) is less consistent.

Combining the assays for glucohomeostasis and hyperglycemic effects (**Figure 2.4**), the partial grouping of the two major families is seen again, but with only a single species from each extending the grouping to the other end of the associated principle component, *P. glauca* in the case of the Pinaceae and *V. vitis-idaea* in the case of the Ericaceae. In terms of the plant parts, the leaves appear to form a more consistent grouping associated with the main antioxidant and antiglycation assays. Also, the bark samples were widely distributed across both directions of the PCs, whereas the two cone species and the two berry species were grouped closely, suggesting that analyzing the leaves, cones, and berries separately from the other plant parts may be useful.

Reducing the data down to evaluating just these three plant parts (**Figure 2.5**), the separation of the three groups becomes more obvious, specifically when looking at the distribution of activity on the glucohomeostasis and hyperglycemia effects anti-diabetes assays without the enzyme inhibition activity. There does not appear to be any distinct associations between plant part groups and assay type distributions. However, there does appear to be a small trend associating the leaves with antioxidant activity, and the cones with glucose uptake stimulation in adipocytes.

Given that associations are more clearly discerned with plant parts than with plant families, subsequent analysis considered glucohomeostasis and hyperglycemic effect activities separately under the groupings of the plant parts (**Figure 2.6**). Looking at only the glucohomeostasis assays, the activity appears not to be associated with specific plant parts, except the cones, but more associated with specific plants, such as the top four species identified in **Figure 2.1**. The leaf samples group more strongly when looking at the hyperglycemia effects, as seen previously. This time however, their association to antioxidant and antiglycation assays is restricted, with barks of *A. incana* and *S. planifolia* associating with this assay group instead.

A


PC2 (14.8% explained var.)
PC1 (25.1% explained var.)
4
0
-4
-8
-4
0
4
K. angustifolia
R. groenlandicum
A. balsamea
L. clavatum
P. glauca
R. tomentosum
L. laricina
C. decora
communis
P. banksiana
P. mariana
P. balsamifera
S. purpurea
G. Hispidula
A. Incana
V. vitis-idaea
groups
Betulaceae
Cupressaceae
Ericaceae
Lycopodaceae
Pinaceae
Rosaceae
Salicaceae
Sarracenaceae

B

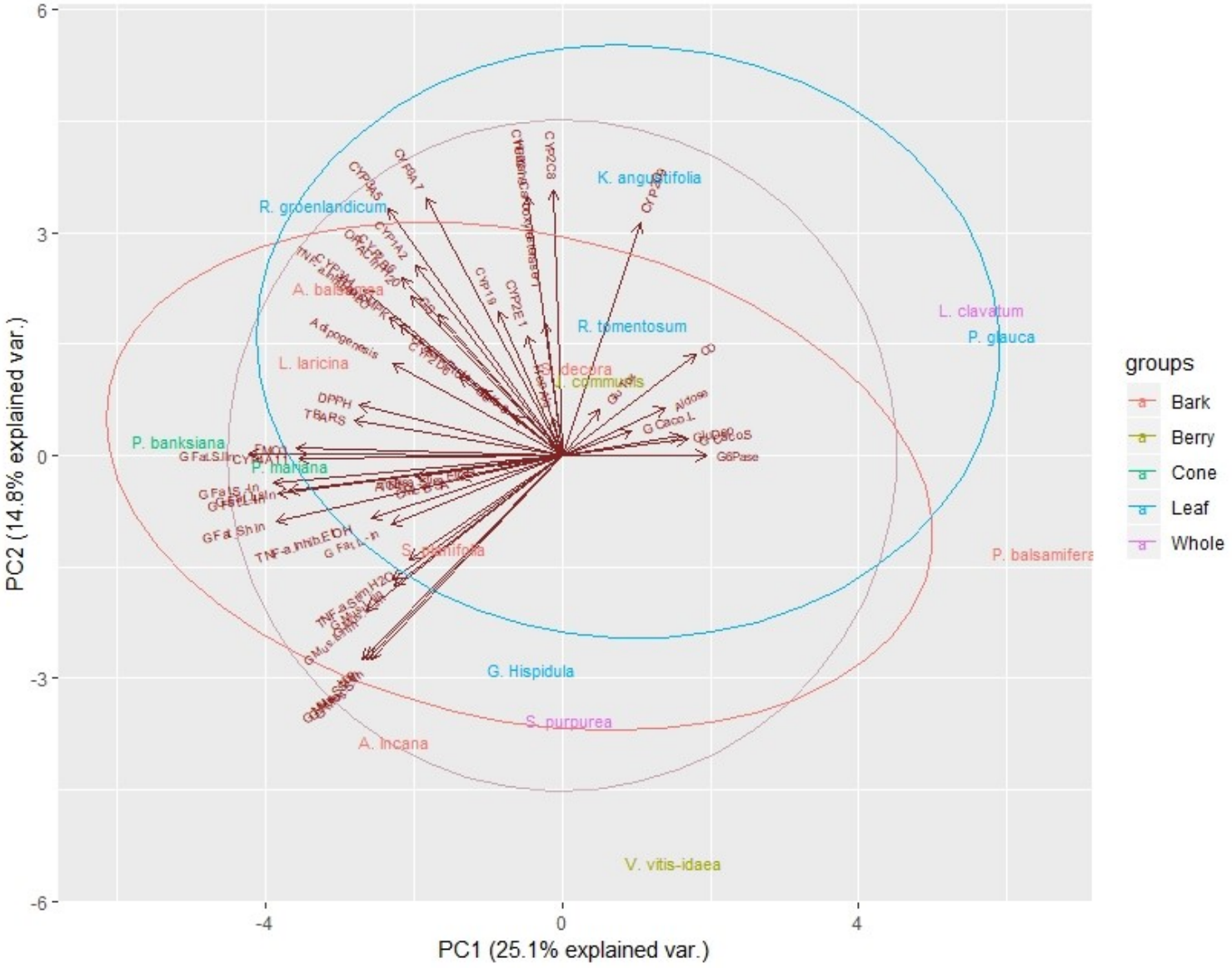
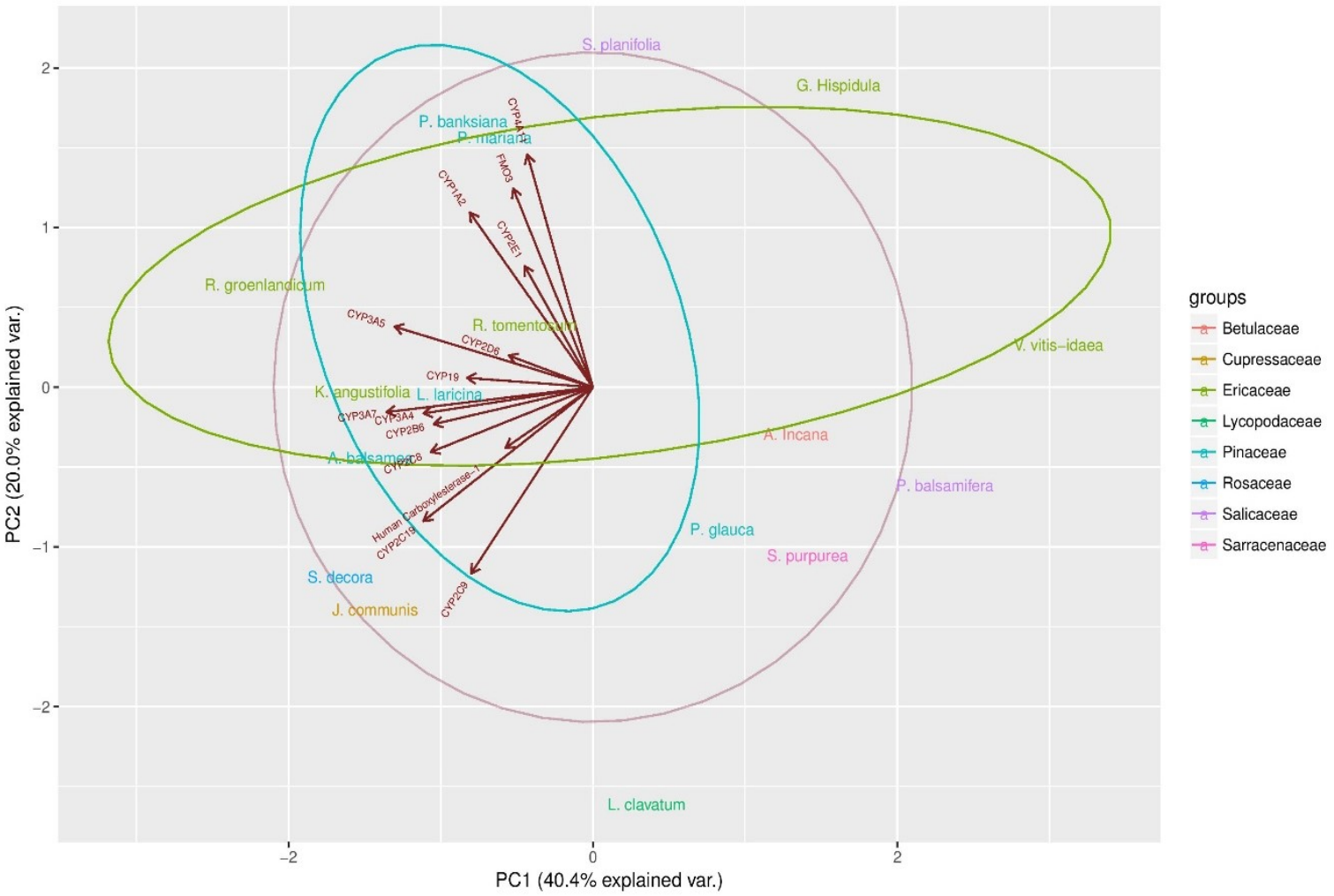


Figure 2.2. Principle component analysis of Cree medicinal plant biological activity. Groupings reflect (A) the taxonomic families of these plants or (B) the plant parts evaluated. Ellipses represent 95% confidence intervals on groupings. Central circle represents 95% confidence interval on full data set.

A


S. planifolia
G. Hispidula
P. banksiana
P. mariana
CYP4
FMO3
CYP1A2
CYP2E1
R. groenlandicum
CYP3A5
R. tomentosum
CYP2D6
V. vitis−idaea
CYP19
K. angustifolia
L. laricina
CYP3A7
CYP3A4
CYP2B6
A. incana
A. balsamea
CYP2C8
P. balsamifera
Human Carboxylesterase−1
CYP2C19
P. glauca
S. purpurea
S. decora
CYP2C9
J. communis
L. clavatum
PC2 (20.0% explained var.)
PC1 (40.4% explained var.)
groups
Betulaceae
Cupressaceae
Ericaceae
Lycopodaceae
Pinaceae
Rosaceae
Salicaceae
Sarracenaceae

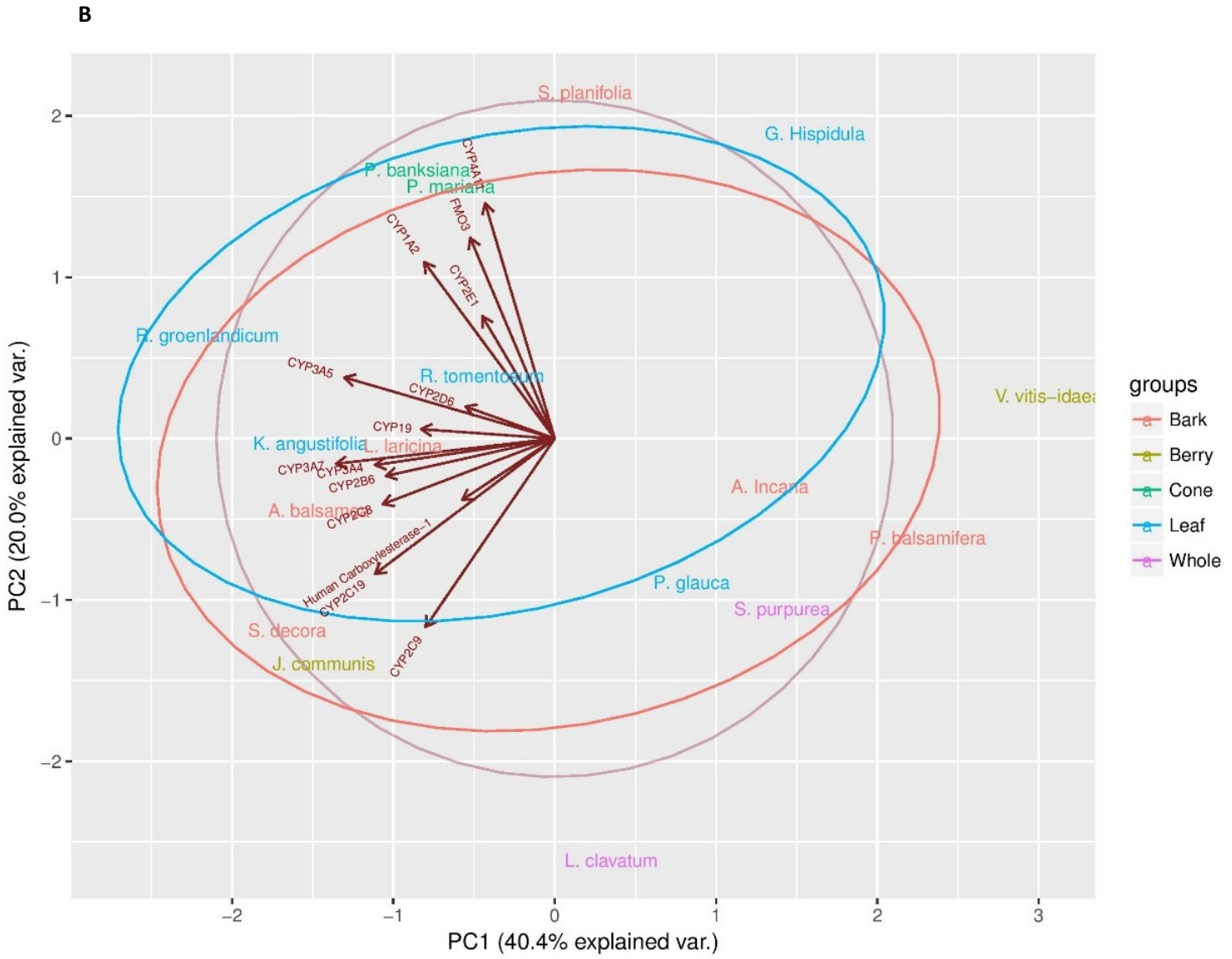
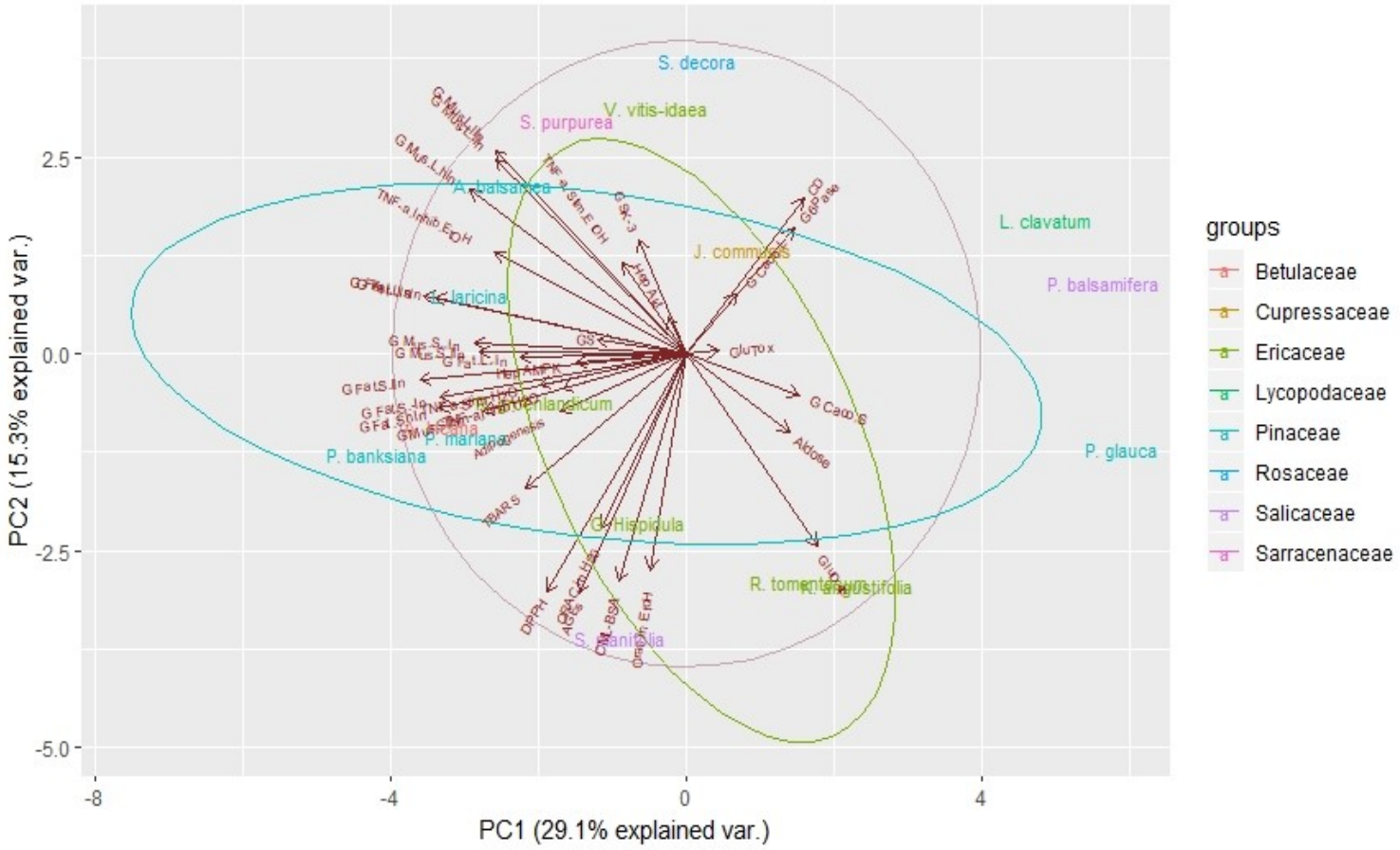


Figure 2.3. Principle component analysis of the Cree medicinal plant toxicological and safety biological activity. Groupings reflect (A) the taxonomic families of these plants or (B) the plant parts evaluated. Ellipses represent 95% confidence intervals on groupings. Central circle represents 95% confidence interval on full data set.

A


S. decora
V. vitis-idaea
S. purpurea
A. balsamea
L. clavatum
J. communis
P. balsamifera
P. laricina
P. glauca
P. banksiana
P. mariana
R. hispidula
R. tomentosum
R. groenlandicum
S. canadensis
groups
Betulaceae
Cupressaceae
Ericaceae
Lycopodaceae
Pinaceae
Rosaceae
Salicaceae
Sarracenaceae
PC2 (15.3% explained var.)
PC1 (29.1% explained var.)
2.5
0.0
-2.5
-5.0
-8
-4
0
4

B

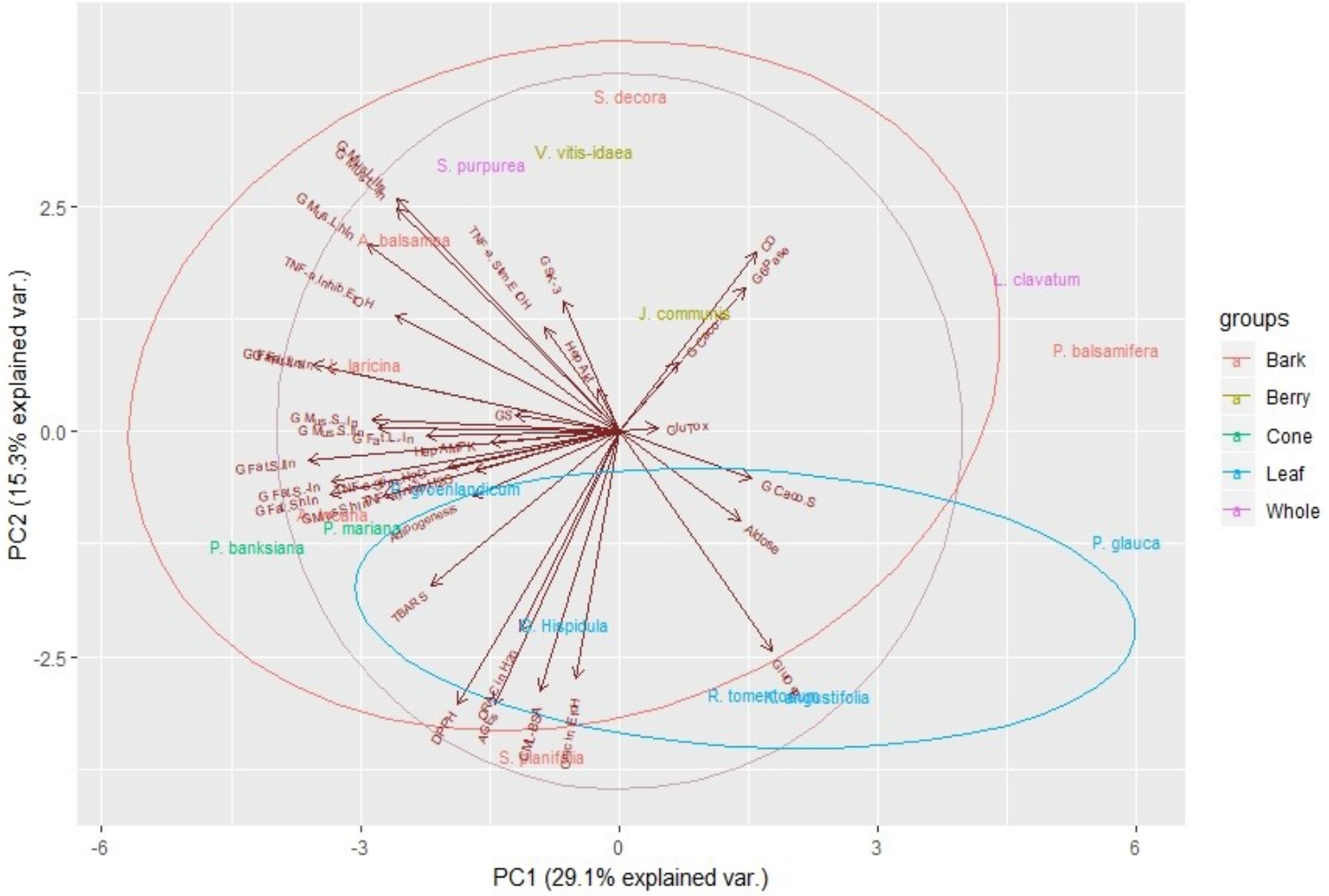
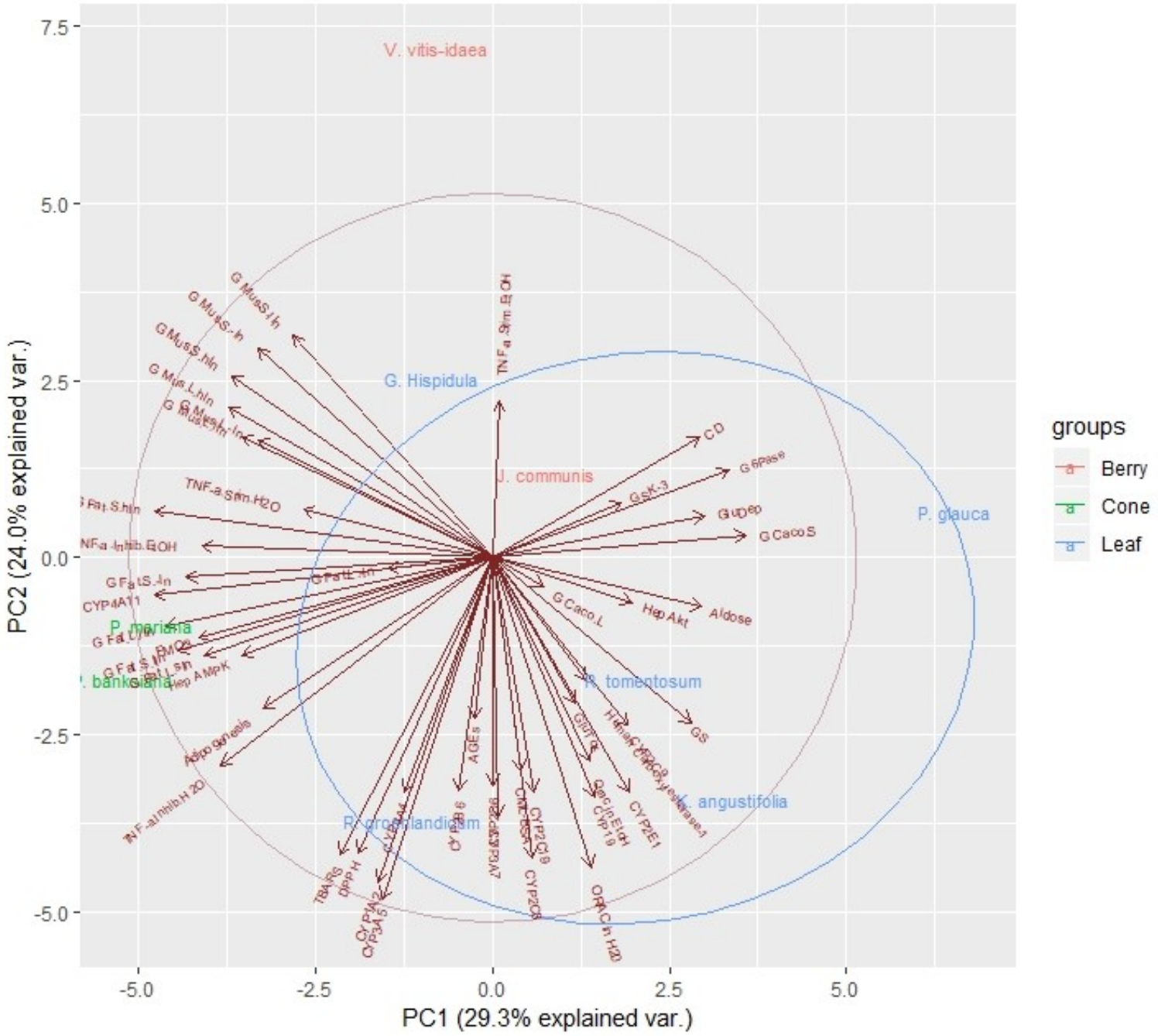


Figure 2.4. Principle component analysis of Cree medicinal plant glucohomeostasis and effects of hyperglycemia biological activity. Groupings reflect (A) the taxonomic families of these plants or (B) the plant parts evaluated. Ellipses represent 95% confidence intervals on groupings. Central circle represents 95% confidence interval on full data set.

A


V. vitis-idaea
G. Hispidula
J. communis
P. glauca
P. mariana
P. banksiana
R. tomentosum
R. groenlandicum
V. angustifolia
TNF-a.Stim.EtOH
TNF-a.Stim.H2O
CD
G6Pase
GSK-3
GUDep
GCacoS
GCaco.L
HepAkt
Aldose
GS
AGEs
CYP2E1
CYP2C19
CYP2C8
ORACin H2O
Adipogenesis
PC1 (29.3% explained var.)
PC2 (24.0% explained var.)
groups
Berry
Cone
Leaf

B

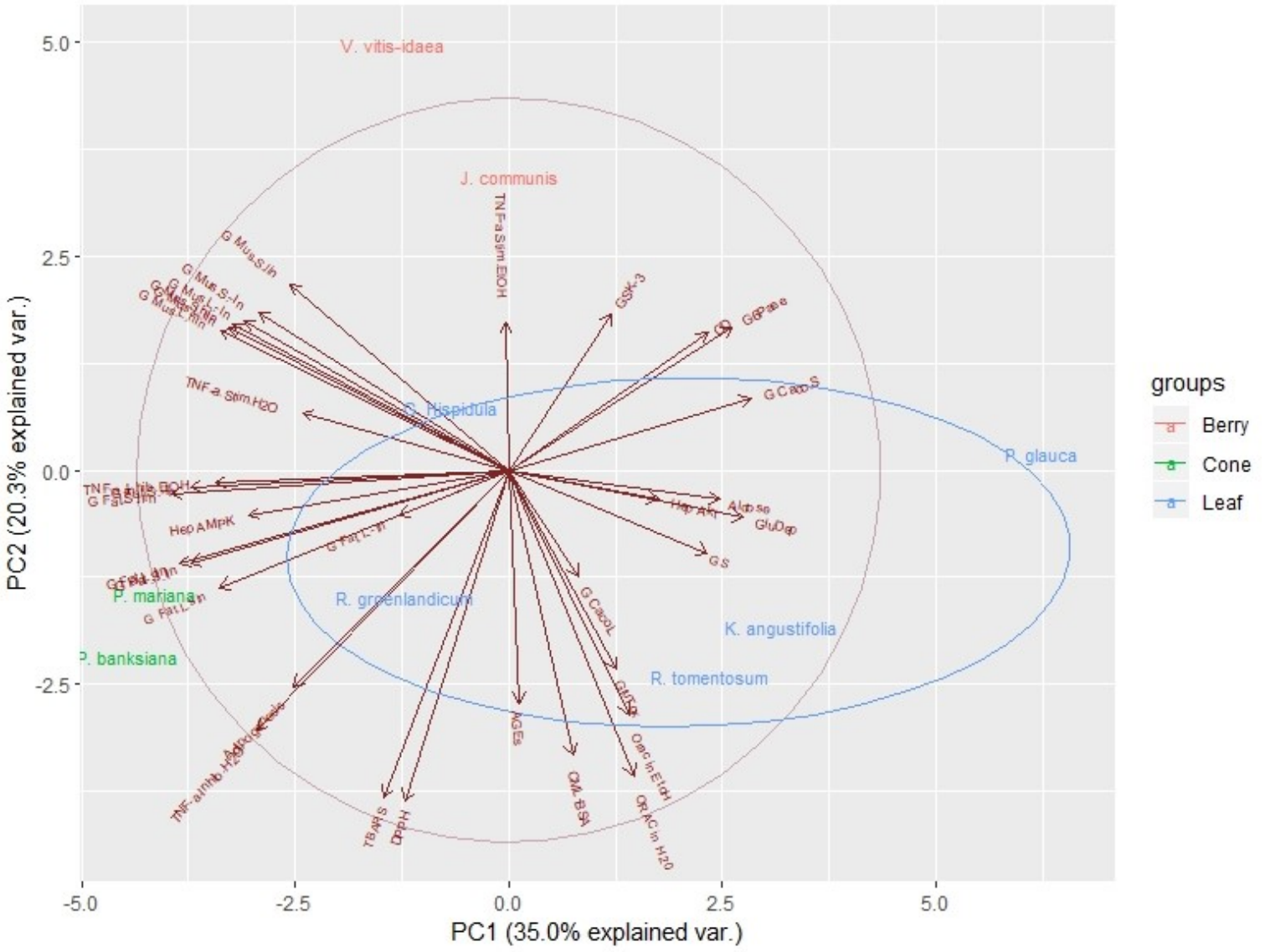
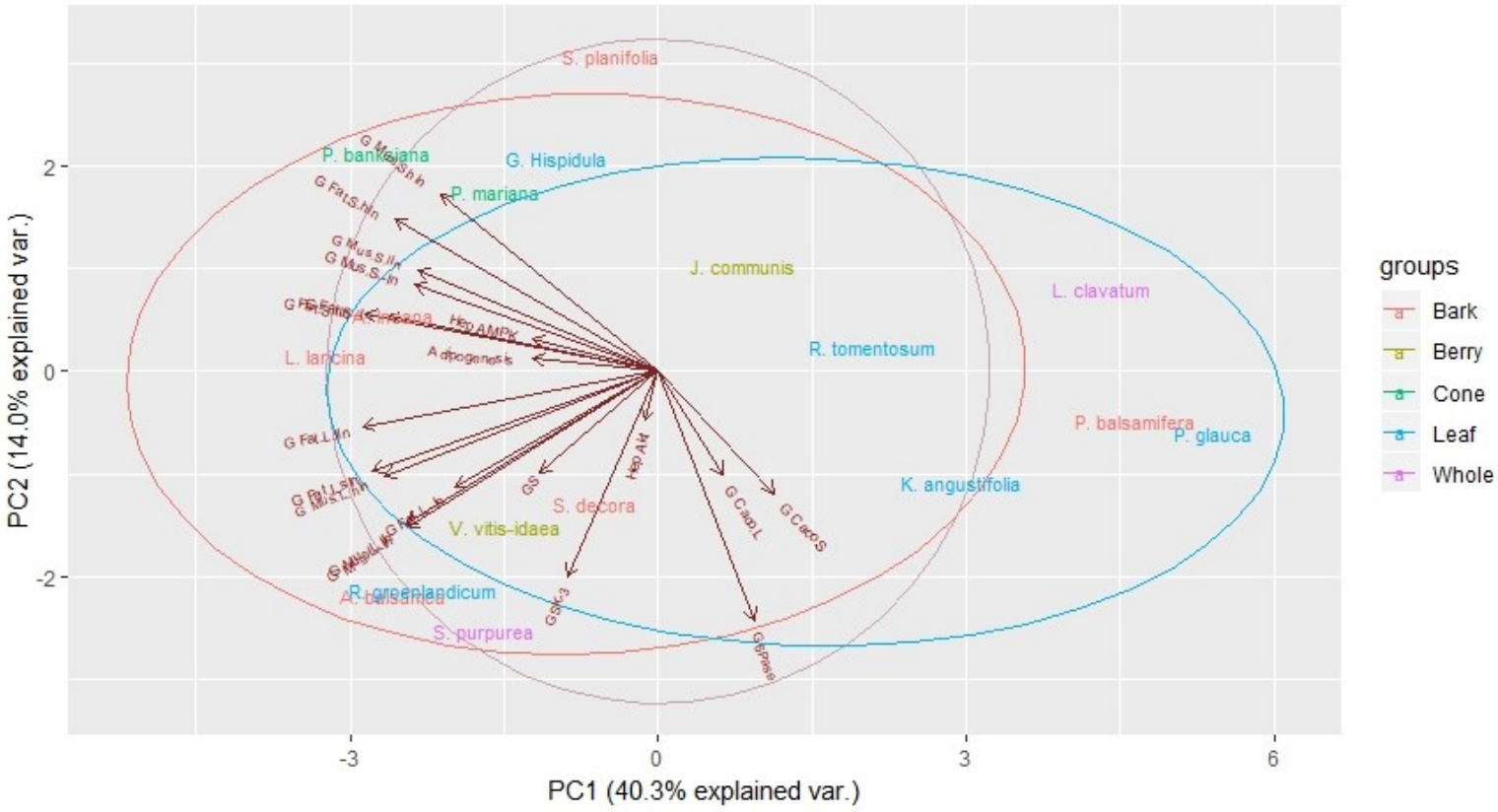


Figure 2.5. Principle component analysis of select Cree medicinal plant biological activity. (A) Analysis with all pharmacological activity evaluated on plants where Berry, Cone, or Leaf were tested. (B) Analysis without toxicological and safety activities evaluated on plants where Berry, Cone, or Leaf were tested. Ellipses represent 95% confidence intervals on groupings. Central circle represents 95% confidence interval on full data set.

A


S. planifolia
P. banksiana
G. Hispidula
P. mariana
J. communis
L. clavatum
R. tomentosum
L. lancina
P. balsamifera
P. glauca
K. angustifolia
S. decora
V. vitis-idaea
R. groenlandicum
A. balsamea
S. purpurea
Hep.AMPK
Adipogenesis
Hep.AH
GS
GSK3
GCasp3
GCaspL
GCaspS
GSpase
groups
Bark
Berry
Cone
Leaf
Whole
PC2 (14.0% explained var.)
PC1 (40.3% explained var.)
2
0
-2
-3
0
3
6

B

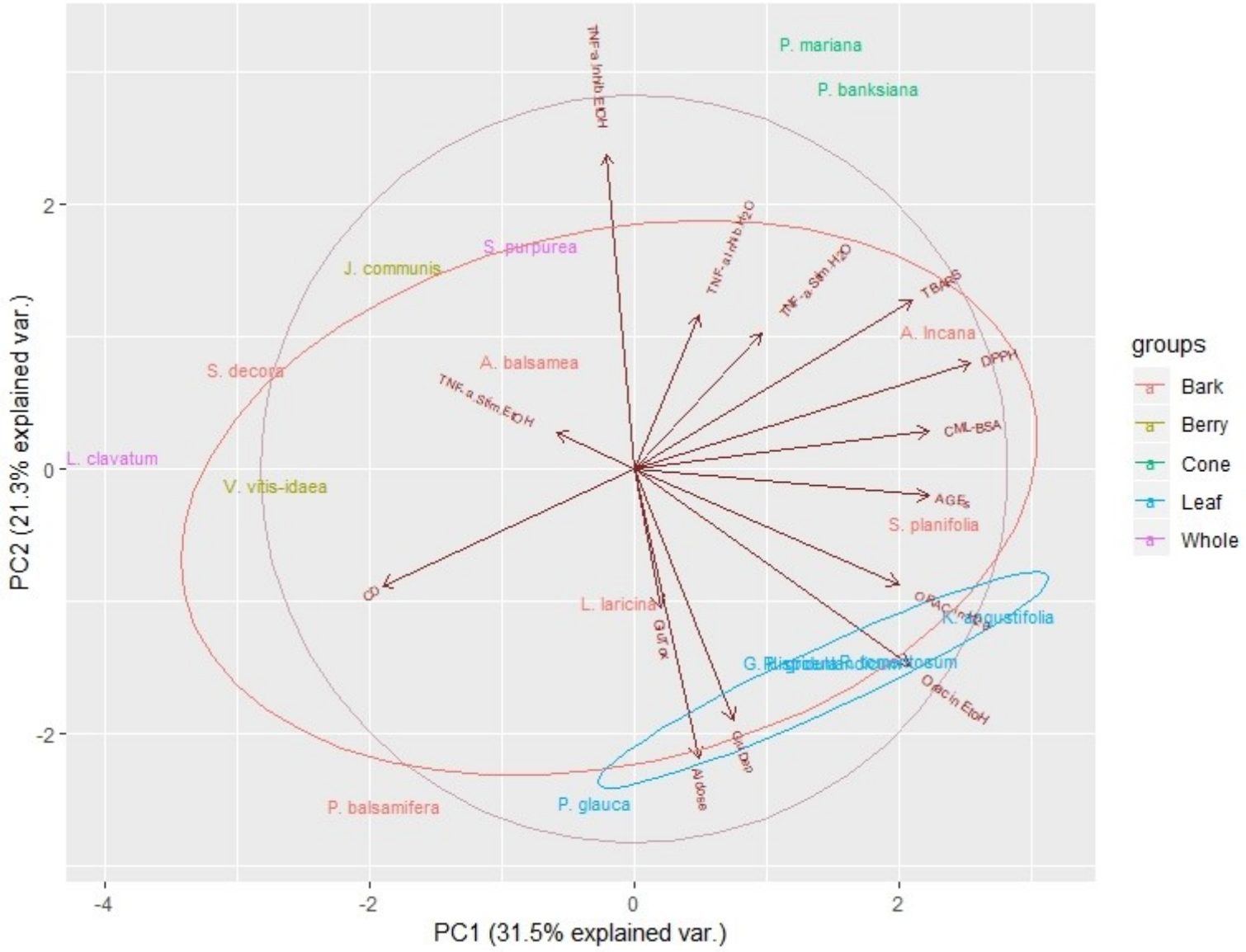
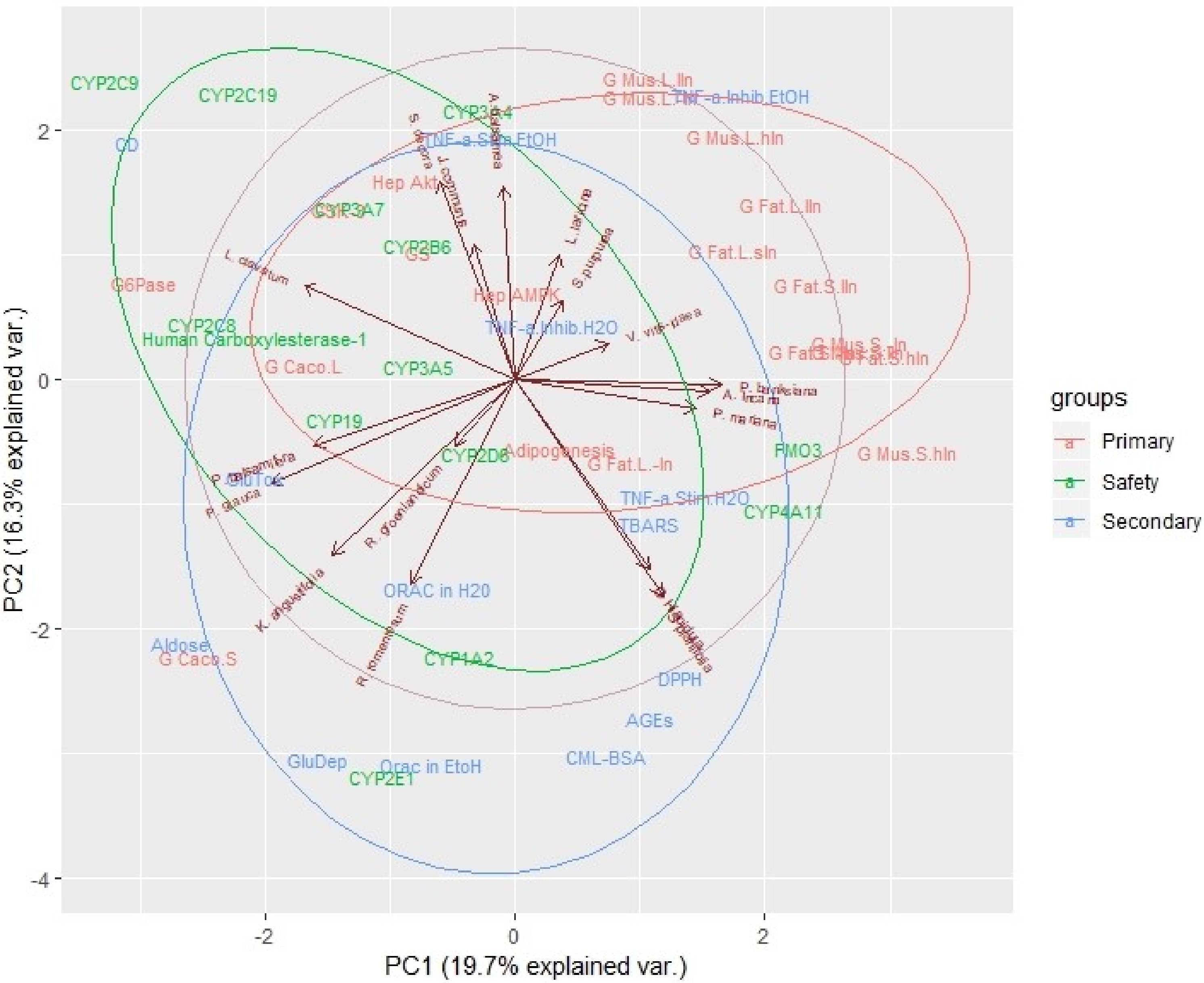


Figure 2.6. Principle component analysis of Cree medicinal plant biological activity. (A) Analysis with glucohomeostasis activity on all plants. (B) Analysis with diabetes complications biological activities on all plants. Ellipses represent 95% confidence intervals on plant part groupings. Central circle represents 95% confidence interval on full data set.

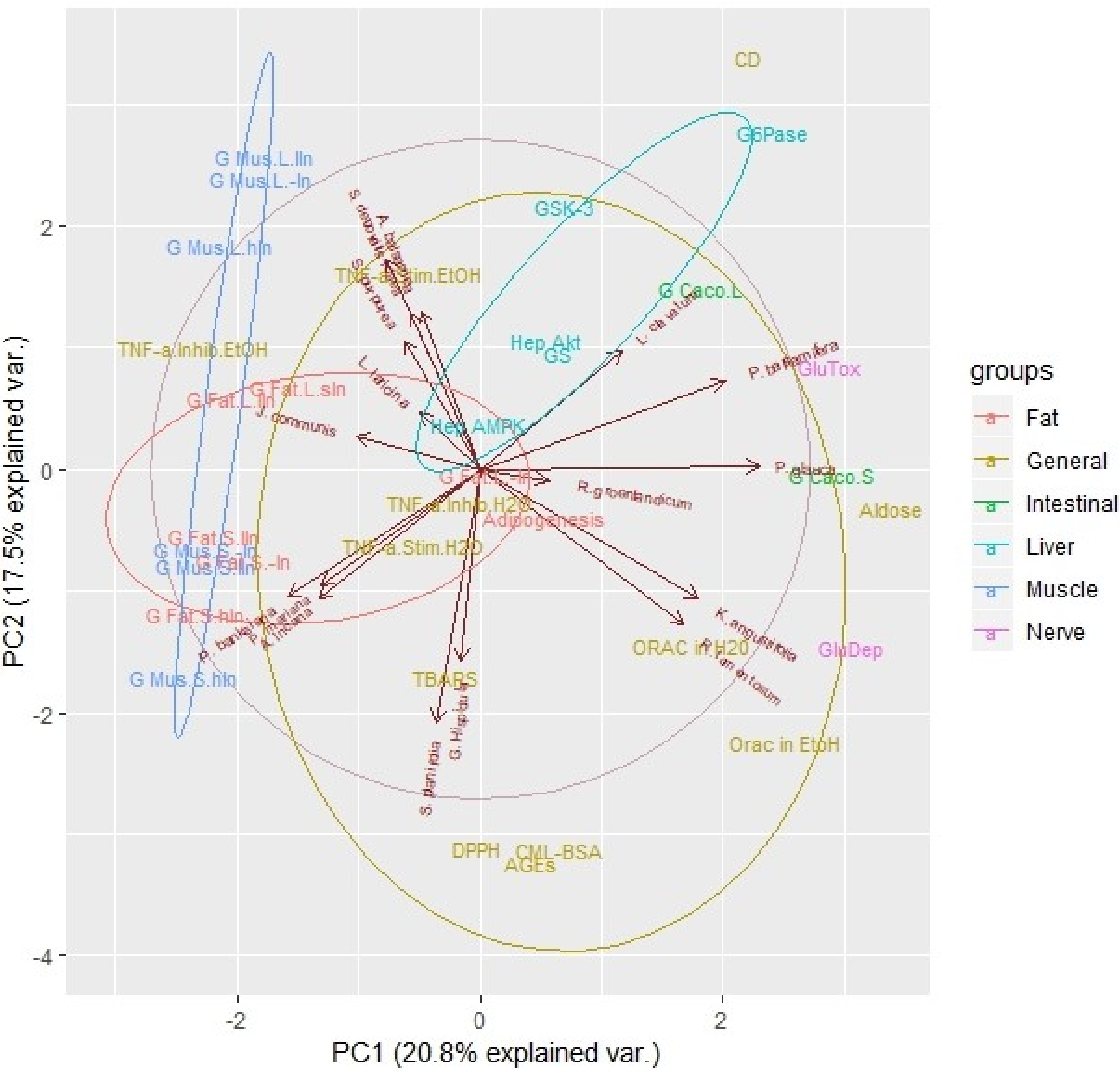
2.3.3 PRINCIPLE COMPONENT ANALYSIS OF BIOACTIVITY DATA BY ASSAY

Another way of looking at the data is by transposing the matrix to get scores for the distribution of bioassays, so that the relationships among different assay subtypes can be explored (**Figure 2.7**). Looking at all assays together, it is seen that there is a significant amount of overlap suggesting fairly common activities among most plants, but that each assay group has a smaller set of assays whose activity appears to be more unique to specific sets of plants (**Figure 2.7A**). For example, the leaf species are once again associated with the effects of hyperglycemia activity, specifically antioxidant and antiglycation assays; glucose uptake in muscle and fat associate with a diverse set of plants that include the cone plants; *L. clavatum* associated strongly with the CYP2Cs specifically. When eliminating the enzyme inhibition data from the analysis (**Figure 2.7B**), there again appears to be certain common groupings of assay activities, such as between glucose uptake and immunomodulation, between antioxidant and antiglycation activities, and with glucose management in the hepatocytes to be somewhat more unique. When switching the groupings to instead reflect the assay types more specifically (**Figure 2.7C**), the separation of assays relating to glucohomeostasis compared to those relating to hyperglycemic effects is again apparent.

A


CYP2C9
CYP2C19
CD
G Mus.L.lln
TNF-a.Inhib.EtOH
CYP3A4
TNF-a.Stim.EtOH
G Mus.L.hln
Hep Akt
CYP3A7
G Fat.L.lln
CYP2B6
G Fat.L.sln
L. clavatum
G6Pase
G Fat.S.lln
Hep AMPK
CYP2C8
TNF-a.Inhib.H2O
Human Carboxylesterase-1
V. vitis-idaea
G Caco.L
CYP3A5
P. mariana
CYP19
FMO3
G Mus.S.hln
Adipogenesis
CYP2D6
G Fat.L.-ln
P. glauca
TNF-a.Stim.H2O
CYP4A11
TBARS
R. groenlandicum
ORAC in H20
K. angustifolia
Aldose
G Caco.S
CYP1A2
DPPH
AGEs
CML-BSA
GluDep
Orac in EtoH
CYP2E1
PC1 (19.7% explained var.)
PC2 (16.3% explained var.)
-2
0
2
-4
groups
Primary
Safety
Secondary

B


CD
G6Pase
G Mus.L.lln
G Mus.L.-ln
GSK-3
G Mus.L.hln
TNF-a.Stim.EtOH
G Caco.L
Hep.Akt
GS
TNF-a.Inhib.EtOH
P. balsamifera
GluTox
G Fat.L.sln
G Fat.L.lln
J. communis
Hep.AMPK
P. glauca
G Caco.S
R. groenlandicum
TNF-a.Inhib.H2O
Aldose
Adipogenesis
G Fat.S.lln
TNF-a.Stim.H2O
G Mus.S.-ln
G Fat.S.-ln
G Mus.S.lln
G Fat.S.hln
K. angustifolia
ORAC in H2O
GluDep
G Mus.S.hln
TBARS
Orac in EtOH
DPPH
CML-BSA
AGEs
PC1 (20.8% explained var.)
PC2 (17.5% explained var.)
groups
Fat
General
Intestinal
Liver
Muscle
Nerve
-4
-2
0
2

C

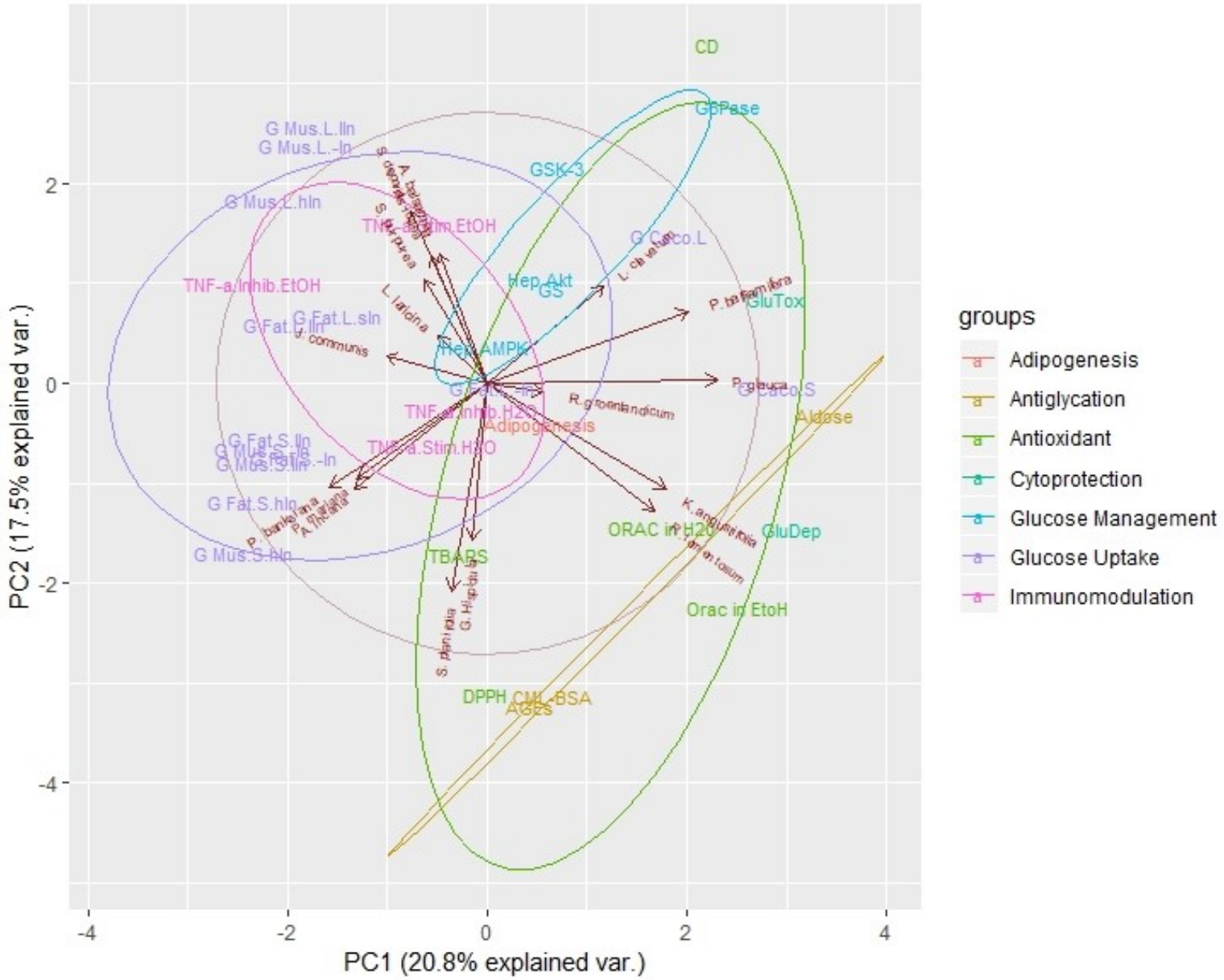


Figure 2.7. Principle component analysis of Cree medicinal plant biological activity with assays as loadings. (A) Analysis with all assays, grouped by major assay type, where Primary refers to glucohomeostasis and Secondary refers to effects of hyperglycemia. (B) Analysis with glucohomeostasis and effects of hyperglycemia, grouped by bioassay cell type. (C) Analysis with glucohomeostasis and effects of hyperglycemia assays, grouped by assay type subgroupings. Ellipses represent 95% confidence intervals on groupings. Central circle represents 95% confidence interval on full data set.

2.4 DISCUSSION

Both metrics of evaluating the active-inactive binary of the plant extracts on each assay have benefits and drawbacks (**Table 2**). The percent of active assays was determined by looking at the number of assays where a plant extract had significant activity, but only relative to the total number of assays on which that species was tested. Although looking at the percent of active assays more clearly represents the diversity and overall strength of a species' activity, it masks situations where species were ignored in multiple assays, perhaps due to poor prescreening results. For example, *G. hispidula* and *J. communis* had fewer assays they had activity in, but the proportion of the assays they were tested on and found to be active on was high, likely due to the fact that they were set aside after having low activity on certain assays and so not tested on related follow-up assays.

Key species to consider though are those that scored either high or low on both metrics. For example, *S. purpurea* had the highest values both for number of active assays and percent of active assays, suggesting that it has the potential for the widest and most diverse activities. Conversely, *V. vitis*-idaea, scored the lowest on both activity metrics. This can be explained by looking at the Safety data, where *V. vitis-idaea* was seen to be largely inactive in inhibiting almost all CYP isoforms, whereas all other species displayed varying inhibition in most of these assays (Tam et al. 2009).

Interesting to note is that, next to *V. vitis-idaea*, the species with the lowest scoring on both activity metrics is *P. balsamifera*, even though it has been tested on more assays than some of the other higher scoring species. This is noteworthy as, in Leduc's original anti-diabetic Cree ethnobotany (Leduc et al. 2006), *P. balsamifera* was not one of the species originally identified by the Cree elders in Mistissini as having a significant correlation of use with diabetic symptoms until later interviews were conducted with elders of other nearby Cree communities. While differences in accessibility to this species may contribute to this variation of use between communities, it appears that *P. balsamifera* was not as useful to them as other plants, and that is possibly being reflected in its lower activity here.

Looking further at rankings of plant use from the interviews, the top plants of interest can also be compared. Namely, the integrated consensus ratings and calculation of syndromic importance identified *R. groenlandicum*, *L. laricina*, and *A. balsamea* as the most important plants to the Cree, based on the symptoms of diabetes. Next to *S. purpurea*, these three species also scored highest on both number and percent of bioassays in which they were found active. Even though *S. purpurea* ranked much lower in Leduc's analysis, the consistency of these other three species again displays the significance of the knowledge of the Cree, and how important it is to listen to the wisdom of Indigenous leaders. Further, when evaluating the assays for which there was full species representation (**Figure 1),** the difference between the four most active species and the rest becomes much more distinct, especially when focusing on the assays for glucohomeostasis effects.

Exploring these four species within the PCA analyses, consistent themes emerge. In the context of all the assays, *A. balsamea*, *L. laricina*, and *R. groenlandicum* all cluster closely together in terms of similar activities (**Figure 2**), but this is seen to be driven by the enzyme inhibition activities (**Figure 3**), where the same grouping occurs. All four species grouped closely when evaluating the combined glucohomeostasis and hyperglycemia effects assays with specific association to the glucose uptake assays (**Figure 4**), although *R. groenlandicum* also falls under the association of the other leaf samples and their activities in the antioxidant/antiglycation assays. Similar trends are seen when comparing the glucohomeostasis assays to those of the hyperglycemia effects assays. In the glucohomeostasis assays specifically (**Figure 6**), *R. groenlandicum* closely groups with *A. balsamea* and *S. purpurea* while associating with activity on several hepatocyte glucose management assays and the long-term effect on glucose uptake in fat and muscle cells. *L. laricina* associates more closely with short term effect on glucose uptake. However, in the context of hyperglycemia effects activities, *R. groenlandicum* only groups with the other leaves in their association with antioxidant and antiglycation activity. What all these observations suggest is that there is a distinct similarity in phytochemical composition within

these four plants driving the glucose uptake activity, while *R. groenlandicum* also contains a phytochemical and activity profile similar to other leaves.

Previous studies have identified strong phenolic content in leaf parts of bioactive plants, and also that a higher phenolic content is strongly correlated with a plant's antioxidant (Fraser et al. 2007) and antiglycation (C. Harris et al. 2011) potential. Comparisons with extracts from other market produce has found that many traditional Boreal plant medicines, including many evaluated here, have significantly higher radical scavenging abilities, and have antioxidant effects comparable to green tea, Vitamin C, and Vitamin E (McCune and Johns 2002).

As for the cone species, *P. banksiana* and *P. mariana* consistently associated with specific groups throughout the analysis. Primarily, their association of activity on stimulating glucose uptake, specifically in fat cells but also muscle, was seen in each analysis. Further, their activity was strongly associated with both adipogenesis and immunomodulatory assays. In addition, the two cones had strong activity associated with inhibition of CYPs 1A2, 2E1, and 4A11, a group of enzymes known for their metabolism of endogenous fatty acids into important signaling molecules (Westphal, Konkel, and Schunck 2011). Specifically, the CYP mediated metabolite of arachidonic acid, epoxyeicosatrienoic acid (EET), has been shown to have cytoprotective effects for maintaining Akt and AMPK signaling in numerous cell lines and preventing insulin resistance in high fat diets of *in vivo* models (He et al. 2016; Xu et al. 2013). Indeed, it may prove useful to focus bioactive phytochemical compound identification of these two species' cones to either EET-like fatty acids or other fatty acids that may be metabolized by this set of enzymes.

Several issues with this data set must be identified. First, different metrics for significance were used to specify activity. Some studies determined significance relative to positive controls, whereas others used negative controls. Further, some publications quantified these differences with p-values

while others did not, and even then there were cases where different post-hoc tests of significance and different sample sizes yielded different clarifications of the active-non-active binary in different publications (C. S. Harris 2008; Harbilas et al. 2009). This combination of problems is one of the main reasons why it was decided to evaluate the data both as a binary (Active vs Inactive, according to statistical significance) and as a rank (highest to lowest activity, according to raw data). Second, not every plant was evaluated on every assay, leading to holes in the matrix. Third, collection times and ranges were not considered, but may have significant effects on rates of phytochemical biosynthesis due to seasonal and latitudinal variations in light intensity, climate, and soil composition (Jaakola and Hohtola 2010). As a result, it must be acknowledged that the results discussed here may not be properly representative of the diversity of activity that may be experienced by members of different communities throughout the Cree of Eeyou Istchee territory.

2.5 CONCLUSION

Taken together, what these results suggest is that there is a distinct separation in the modes of activities of the traditional medicines investigated here. First, there are those plants that appear most useful in promoting general health through their antioxidant and antiglycation activity, such as the set of Ericaceae leaves, and this activity is possibly associated with the diverse phenolic content of these plants. Second, there are those plants with more unique phytochemicals that may interact with specific cell signaling pathways for anti-diabetic effects. These may include fatty acids in the Pinaceae cones. Also, given the similarities in activity between the four major plants (*A. balsamea*, *L. laricina*, *R. groenlandicum*, and *S. purpurea*), it would be important to evaluate whether these activities are associated with a specific set of related compounds, given the diversity of these species, or whether their methods of activity are unique. Either way, this evaluation should provide guidance for traditional plant use in informed community health practices in diabetes prevention and management.

CHAPTER 3: REVIEW OF THE METABOLOMIC PROFILES OF THE ANTI-DIABETIC MEDICINAL PLANTS OF THE CREE OF EEYOU ISTCHEE

3.1 INTRODUCTION

When investigating the bioactivity of plants, a primary objective is often to identify which phytochemical components elicit the observed activity. Although much of Western medicine focuses on the use of single entity medications, it is also understood that plant extracts can contain numerous phytochemicals associated with a variety of activities, and often with additive or synergistic effects (Nasri et al. 2014; Rajasekaran, Sivagnanam, and Xavier 2008). In the context of using research to provide recommendations for use of natural medicine, it becomes essential to gain a better understanding of what phytochemicals are present, and in what quantities they can be expected. This understanding is especially important if those compounds have the potential to interfere with drug metabolism, as any changes to this process poses the risk of such drugs either falling below the therapeutic concentration or accumulating to toxic dosages (Boullata 2005).

The Team in Aboriginal Anti-diabetic Medicine (TAAM) has produced a wealth of insight into the uses and potential of Cree traditional medicine in the prevention and management of Type 2 Diabetes (T2D). In navigating the complexities of this data, however, it is essential to take a step back. Often, medicinal plant research begins with screening numerous plants to identify the most active plants for a given targeted activity, then to isolate individual phytochemicals associated with that activity. What is less commonly done is to compare the full metabolomic profile of all plants of interest at the same time.

With more than 40 publications on the phytochemistry and pharmacology of the traditional medicine of the Cree, many individual aspects of these plants have been investigated (e.g. identifying species biomarkers (Spoor et al. 2006; Harbilas et al. 2009), quantifying phenolic metabolites (C. Harris et al. 2011; Saleem et al. 2010). However, an evaluation of the similarities and differences in phytochemical makeup in these plants has yet to be done. While several of the plants have had specific

active components identified (**Table 3.1**), the diversity and variation of secondary metabolites across plant species, parts, and life stages makes the notion of identifying all actives a daunting one (Moore et al. 2014; Hartmann 1996). Developing a better understanding of the level of chemical diversity among these species, however, will help direct focus to the chemical signals that distinguish active samples. Further, in the context of the Cree traditional pharmacopeia, different communities may not always have the same level of access to all plant species, and understanding phytochemical diversity and patterns of similar chemical signals may also be important in providing evidence for the use of different plants for equal or similar purposes, based on availability.

In working towards the goal of a more holistic understanding of the Cree traditional medicine phytochemical profile, this project applied two complementary metabolomic approaches to evaluate the Cree anti-diabetic plants. First, a database was created using the chemical standards collection of Dr. John T. Arnason, which included many standards previously associated with bioactivity in the Cree medicinal plant extracts. This library was built by integrating chromatographic (e.g. retention time) with spectral (e.g. mass-charge ratio, fragmentation) data of each of the phytochemicals. Using proprietary Waters software, previously unidentified components in the Cree plants were compared to evaluated standards in the database. Second, multivariate analysis of all components detected by Quadrupole Time-of-Flight Ultra Performance Liquid Chromatography and Mass Spectrometry (QTOF HPLC-MS) was used to evaluate relationships between species at the metabolomic level. By taking on an approach that evaluates the larger context of the metabolomic profiles of Cree plant extracts, multivariate analyses can provide unique perspectives on where the similarities and differences between these plants lie.

3.2 METHODS

3.2.1 PLANT MATERIAL AND EXTRACTION

The 17 medicinal Cree plants had been collected previously under the guidance of Cree elders over the course of collection periods associated with 2 projects, both in the area of Mistissini and Whapmagoostui of Northern Quebec, Canada (Spoor et al. 2006; Harbilas et al. 2009). Plant material was ground with a Wiley Mill (Arthur H. Thomas, Swedesboro, USA) with a 2-millimetre filter, and extracted in 80% ethanol (10 mL/g dry material) twice for 24 h on a mechanical shaker. Extracts were then filtered, combined, lyophilized, and stored at 4^{o}C.

3.2.2 DEVELOPMENT OF UNIFI

The UNIFI database was built with 828 previously identified plant metabolites, and contains compound name, CAS ID, ChemSpider ID, elemental composition, neutral and adduct masses, chemical classification, examples of plants containing the compound, a brief description of its activity, and chemical structure. The structures within this database represent all major classes of plant metabolites, with a focus on secondary metabolites, in particular phenolics and terpenes.

Where good quality commercial chemical standards were available in the collection of Dr. John T. Arnason, retention times and mass fragments of these compounds were evaluated using chromatography and mass spectrometry for inclusion in the database. Standards were dissolved in DMSO at a concentration of 1mg/mL, then diluted to 10ppm in 1:1 methanol:water. The analysis was conducted using an ultra-performance liquid chromatography system connected to a quadrupole time-of-flight mass spectrometer (UPLC-QTOF; Waters Acquity Xevo G2 QTOF, Waters Corp). The column used to separate samples was a VanGuard HSS T3 pre-column, Acquity HSS T3, C18 (1.8µm 2.1mm x 100 mm), with Fisher Optima LC-MS grade mobile phase (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile) at a flow rate of 0.8 mL/min, with a linear gradient from 5% to 95% of

solvent B over 4 minutes. Column temperature was maintained at 65°C. 1µL of standard was injected, followed by a 200 µL 50% acetonitrile 50% water post injection needle wash, then by 600 µL 10% acetonitrile 90% water.

QTOF analysis was conducted in MS^e mode with positive electrospray ionization, with rapid alternate collision energies on each peak above the noise level, to capture both precursor ions and product ions. The instrument was mass calibrated with sodium formate prior to analysis. The conditions for MS analysis were as follows: source temperature, 500°C; cone voltage, 35V; scan time, 0.08 seconds; cone gas flow (N2), 50 L/hr; desolvation gas flow (N2), 1200 L/hour; *m/z* range, 100 to 1500 amu. Low energy setting 6V, high energy setting 10V-50V. Data was acquired with MassLynx v4.1 (Waters). The mass calibration was achieved by Leucine Lock mass correction applied post analysis after the import of the raw data from MassLynx to UNIFI.

Mass fragments for standards were also validated, where possible, in the Metlin database (https://metlin.scripps.edu/) to ensure that observed fragments were accurate.

Plant extracts were dissolved in 4:4:2 Acetonitrile:Methanol:Water at a concentration of 1 mg/mL, sonicated for 5 minutes, then filtered through a 0.2 micron PTFE syringe filter. Chromatography and mass spectrometry data was collected using the same methods described previously for the phytochemical standards.

3.2.3 UNIFI APPLICATION

The chromatographic and spectral data for Cree plant extracts was compared to the standards database using Water's UNIFI informatics platform (1.8.2.169). The retention time tolerance was set at 0.03 minutes, and mass tolerance was set at 10 mDa for the adduct mass, and 5 mDa for fragments. +H, +K, +Na, and -e were all searched as positive adducts. For all library compounds, UNIFI predicted *in*

silico fragments which contributed to their identification. If multiple adduct matches were detected for a compound that did not have experimental retention time assignment, all detected adducts along with corresponding matching theoretical fragments were reported at each observed retention times. Matches were assigned in two main categories: a good match is based on a combination of accurate parent mass and at least 1 fragment mass and retention time, and a tentative match is based only on masses (both parent and fragments).

3.2.4 DATA COLLECTION FOR MULTIVARIATE ANALAYSIS

The data used for the multivariate analysis was obtained from Dr. Ammar Saleem, which was collected for Shang et al. (2015). Cree plant extracts were reconstituted in DMSO at 1 mg/mL, sonicated, and filtered through a 0.2 micron PTFE syringe filter. 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 UPLCTM 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 74 wash 200 µL (90% acetonitrile+10% water) and weak wash 600 µL (10% acetonitrile+90% water).

Mass spectrometric analysis was performed using a Waters QTOF UPLC-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. The retention times and the protonated masses were generated at a noise threshold of 500 counts and no smoothing was applied.

Using ProteoWizard (Chambers et al., 2012), the fragmentation signal data was removed from the raw data file. The main portion of data processing occurred in MZMine (Pluskal et al. 2010), where the normalization data was filtered out and thresholds were set to minimize the incorporation of signal noise. Major peaks within a retention time threshold of 0.01 minutes were then identified and lined up across the samples for comparison, and data was extracted for peak retention times, mass-charge ratio, peak height and peak area. The exported data was evaluated to remove signals associated with methanol blanks that were also injected.

3.2.5 STATISTICAL ANALYSIS

Peak area data was analyzed using R software (R Core Team, 2013), including the specialized metabolomics R package *muma* (Gaude et al. 2012). MZMine processed signal data underwent linear normalization relative to average peak intensity for each sample, as well as pareto scaling to the square root of the standard deviation for each signal. Data was evaluated using hierarchal cluster analysis, principal component analysis, and supervised orthagonal partial least squares discriminant analysis.

Correlation analysis was used to evaluate the degree of association between the (+)-catechin presence in all species, as determined by UNIFI, and antiglycation activity data extracted from Harris et al. (2011).

When evaluating comparisons of different sample groupings, most groupings did not contain enough representatives for statistical analysis, and so most conclusions are observational. The groups of barks, leaves, Pinaceae, and Ericaceae contained enough representatives for statistical analysis.

3.3 RESULTS AND DISCUSSION

3.3.1 UNIFI EVALUATION OF CREE PLANTS

Initially, the Cree related literature of the TAAM was searched for instances where specific compounds were identified through bioassay guided isolation for anti-diabetes related activity, summarized in **Table 3.1.** This list was used as a guide to focus evaluation of the UNIFI database on known activity. Of the 13 compounds in **Table 3.1**, three were present in the UNIFI database: (+)-catechin, (-)-epicatechin, and hyperoside (**Table 3.2**).

Table 3.1. Active compounds previously identified by the TAAM through bioassay guided isolation of Cree medicinal plants and validation through commercial standards.

Compound	Formula	Theoretical m/z	Associated Activity	Associated Plant	Reference
(+)-Catechin	$C_{15}H_{14}O_6$	291.0869	AGE	*V. vitis-idaea*	Beaulieu et al., 2010
			Adipogenesis Activation	*R. gorenlandicum*	Eid et al., 2010
(-)-Epicatechin	$C_{15}H_{14}O_6$	291.0869	Adipogenesis Activation	*R. groenlandicum*	Eid et al., 2016
Quercetin-3-O-galactoside (Hyperoside)	$C_{21}H_{20}O_{12}$	465.1033	AGE	*V. vitis-idaea*	Beaulieu et al., 2010
			Cytoprotection; AGE; Antioxidant	*S. purpurea*	Muhammed et al., 2012; Harris et al., 2012; Harris et al., 2011
Cyanidin-3-O-glucoside	$C_{21}H_{20}O_{11}$	448.1006	AGE	*V. vitis-idaea*	Beaulieu et al., 2010
Morroniside	$C_{17}H_{26}O_{11}$	407.1553	Cytoprotection; AGE; Antioxidant	*S. purpurea*	Muhammed et al., 2012; Harris et al., 2012; Harris et al., 2011
Quercetin	$C_{15}H_{10}O_7$	303.0505	AMPK activation	*V. vitis-idaea*	Eid et al., 2010
			Adipogenesis Activation	*R. groenlandicum*	Eid et al., 2010
23,28-dihydroxylupan-20(29)-ene-3β-caffeate	$C_{39}H_{56}O_6$	621.4155	C2C12 Glucose uptake	*S. decora*	Guerrero-Analco et al., 2012
Salicortin	$C_{20}H_{24}O_{10}$	425.1448	Glucose and lipid regulation;	*P. balsamifera*	Harbilas et al., 2013
			Anti-adipogenesis	*P. balsamifera*	Martineau et al., 2010
Oregonin	$C_{24}H_{30}O_{10}$	479.1917	Anti-adipogenesis	*A. incana*	Martineau et al., 2010
6-O-caffeoylgoodyeroside	$C_{19}H_{22}O_{11}$	427.1240	Inhibit G6Pase	*S. purpurea*	Muhammad et al., 2012
Gooderyoside	$C_{10}H_{16}O_8$	265.0923	Inhibit G6Pase	*S. purpurea*	Muhammad et al., 2012
23-oxo-3α-hydroxycycloart-24-en-26-oic acid	$C_{30}H_{46}O_4$	471.3474	Adipogenesis Activation	*L. laricina*	Shang et al., 2012
13-epitorulosol	$C_{20}H_{34}O_2$	307.2637	Adipogenesis Activation	*L. laricina*	Shang et al., 2012

Table 3.1. cont.

Compound	Formula	Theoretical m/z	Associated Activity	Associated Plant	Reference
Abietic acid	$C_{20}H_{30}O_2$	303.2319	Inhibit G6Pase; Stimulate GS	*A. balsamea*	Nachar et al., 2015
Dehydroabietic acid	$C_{20}H_{28}O_2$	301.2162	Inhibit G6Pase; Stimulate GS	*A. balsamea*	Nachar et al., 2015
Squalene	$C_{30}H_{50}$	411.3985	Inhibit G6Pase; Stimulate GS	*A. balsamea*	Nachar et al., 2015

Table 3.2. Confirmed matches of anti-diabetic phytochemicals to Cree medicinal plants using UNIFI analysis software.

Compound	Number of Confirmed Matches in Cree Plant Extracts	Number of Confirmed Matches Previously Unreported
(+)-Catechin	13	9
(-)-Epicatechin	12	8
Hyperoside	8	3

Although only three of the active compounds could be explored, analysis through UNIFI identified matches in numerous Cree medicinal plants, including many matches that had previously been unreported in the greater literature (**Appendix A**). Apart from the identification capacity of this approach, relative abundances of individual compounds can be evaluated in the form of number of detector hits. In the original TAAM publication that evaluated the antiglycation capacity of the Cree medicinal plant extracts (Harris et al., 2011), the antiglycation endproduct (AGE) activity of extracts was positively correlated with total phenolic content across the extracts. The UNIFI analysis of detector hits per extract on specific active compounds also results in a mild correlation, but only with the (+)-catechin compound specifically (**Figure 3.1**). This example demonstrates the potential for the UNIFI system to be used in similar ways in the future.

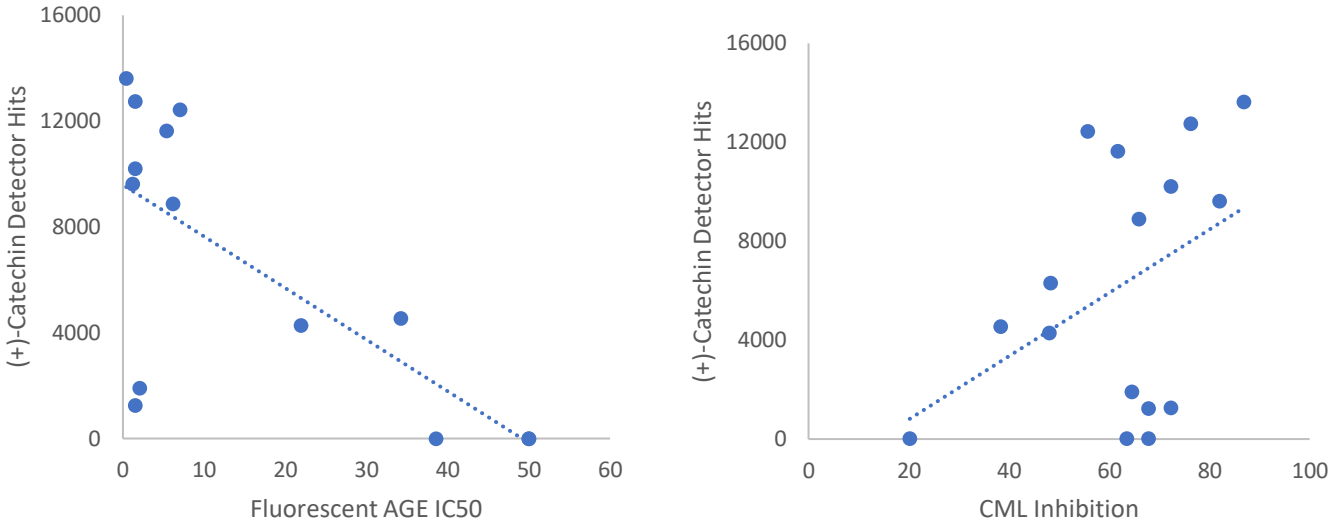


Figure 3.1. Pearson correlation of Cree plant extract inhibition of (A) AGE formation, R^2=0.498, and (B) inhibition of Nε-(carboxymethyl)lysine (CML) adducts, R^2=0.179, with (+)-catechin content as determined by analysis through UNIFI.

3.3.2 MULTIVARIATE ANALYSIS OF CREE METABOLOMICS

Hierarchal cluster analysis of the Cree metabolomics data with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) linkage (**Figure 3.2**) presented several distinct groupings. The groupings of the branches can be seen to be more aligned with the plant parts that were tested than with the associated plant families. Distinct clusters appeared with the leaves of the Ericaceae, and with the category of "fruiting bodies", specifically berries and cones. There was a distinct lack of grouping among the bark samples.

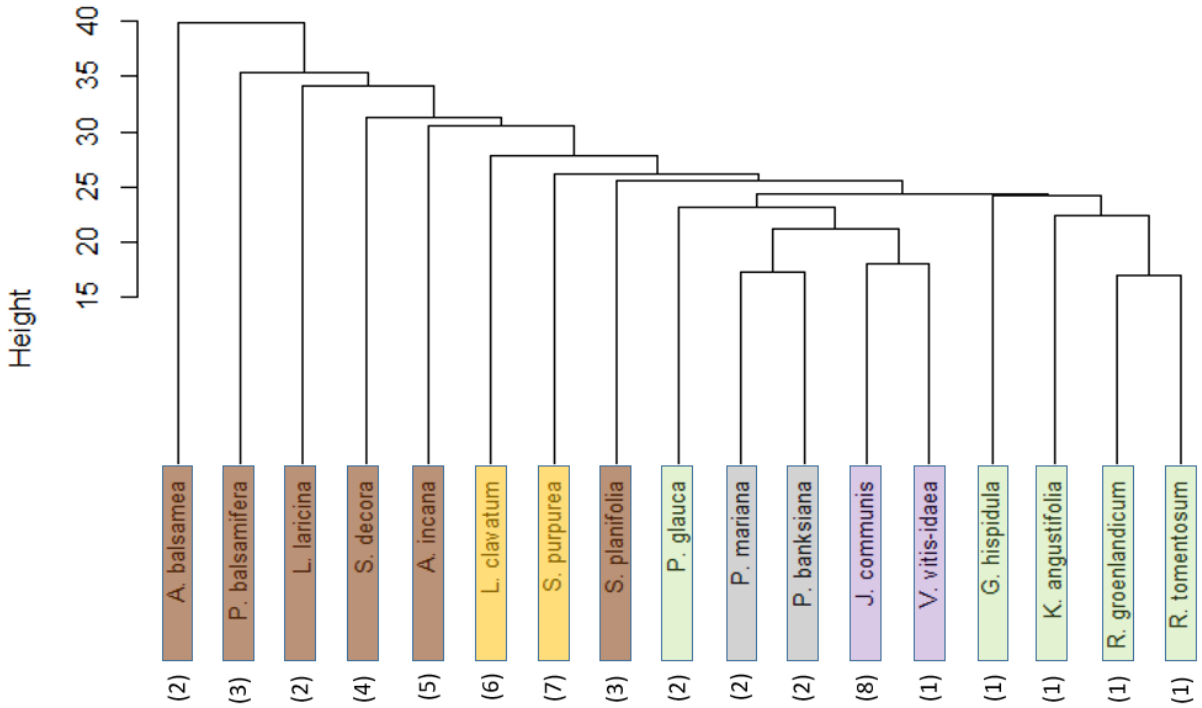


Figure 3.2. Dendrogram resulting from Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis of Cree medicinal plant metabolomes. Species colouring indicates the plant part investigated: Brown (Bark), Yellow (Whole), Green (Leaf), Blue (Cone), Purple (Berry). Numbers indicate species family: 1 (Ericaceae), 2 (Pinaceae), 3 (Salicaceae), 4 (Rosaceae), 5 (Betulaceae), 6 (Lycopodaceae), 7 (Sarracenaceae), 8 (Cupressaceae).

One way to explain the high variability of the bark chemistry compared to the other plant parts is by looking at the natural diversity of compound classes often associated with different plant parts. Namely, terpenes have been thoroughly analyzed for their role as a major mechanism of defense of conifer tree species (Keeling and Bohlmann 2006), and although they are also present in leaves it has been seen to in much lesser quantity and diversity (Courtois et al. 2012). The dissimilarity of the barks may also be related more simply to the fact that 4 plant families were evaluated across the barks, whereas only 2 were evaluated across the leaves.

PCA was used to evaluate the distribution of the metabolite signals across the plants (**Figure 3.3**). The evaluation of all plants suggests 3 semi-distinct branches of distribution of metabolomic signals. The greatest concentration of signals is associated with the left branch (consisting of members of the Pinaceae family) and the upper branch, consisting of a collection of varied plant families and parts. The lower right branch consists of the plants of the Ericaceae family whose leaves were tested, forming a distinct group to be evaluated.

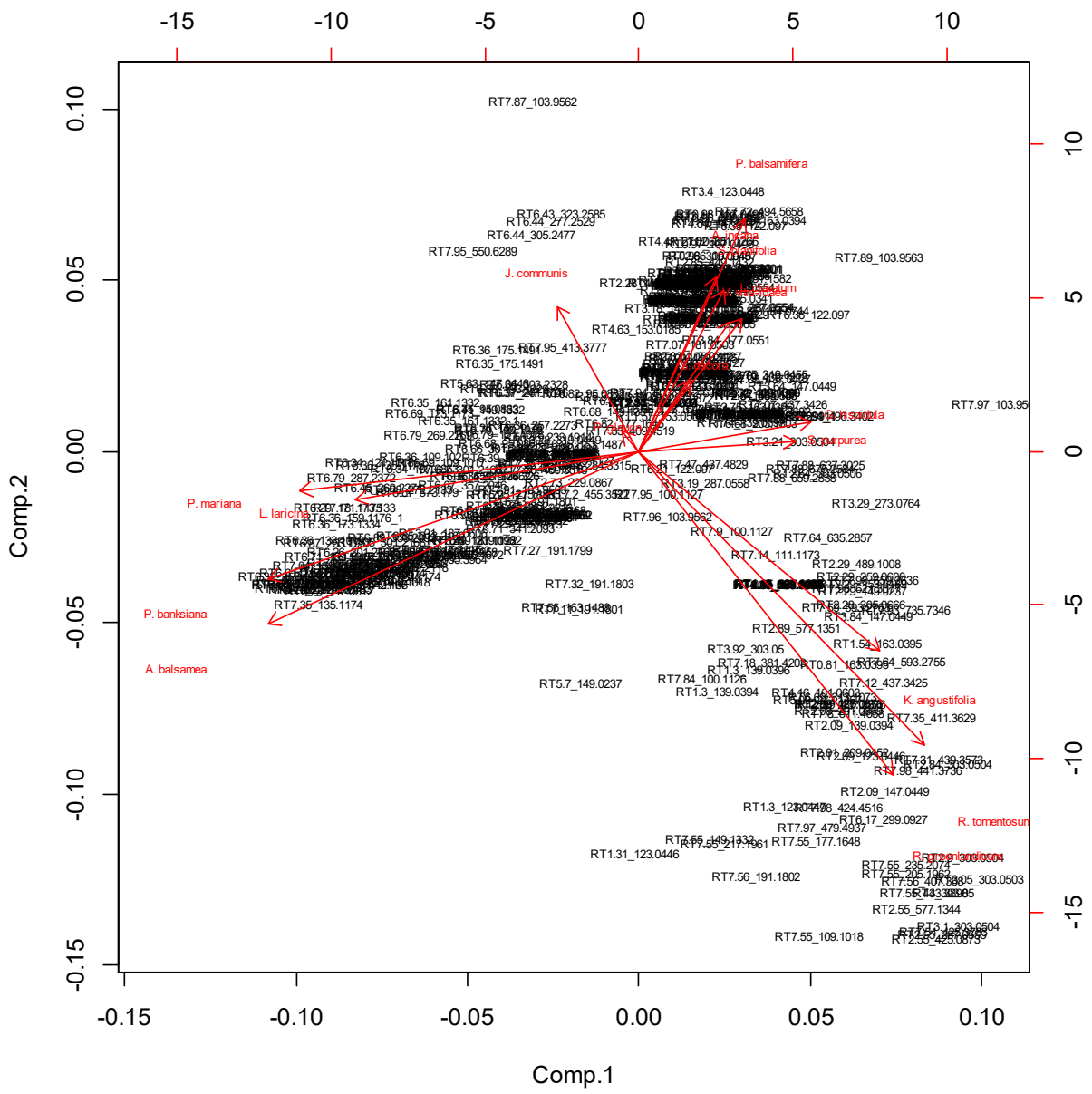


Figure 3.3. Principal component analysis of Cree medicinal plants metabolomics. Loadings indicate the compound signal retention time followed by the mass-charge ratio as detected by HPLC analysis. PC1 = 16.3% variance explained, PC2 = 9.5% variance explained.

Evaluation of principle component analysis loadings distributions (**Figure 3.4**) in combination with the discriminant analysis (**Figure 3.5**) is the primary method used here for identifying chemical signals of interest to the two plant families, and the most notable signals from each group are presented for comparison with the other groupings (**Table 3.2**).

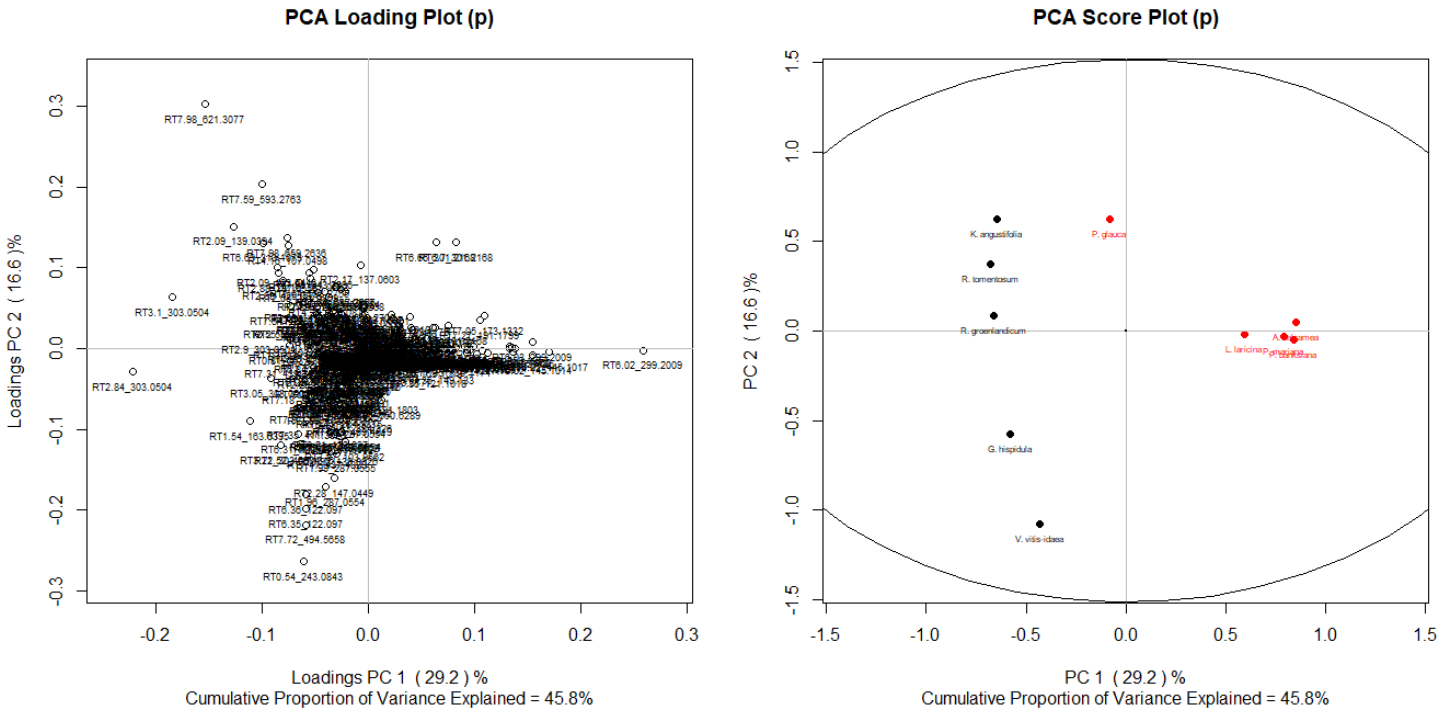


Figure 3.4. PCA of Cree medicinal plants metabolomics, grouped by plant families Ericaceae (black) and Pinaceae (red). (A) PCs 1 and 2 for metabolomics signal loadings. (B) PCs 1 and 2 for plant scores.

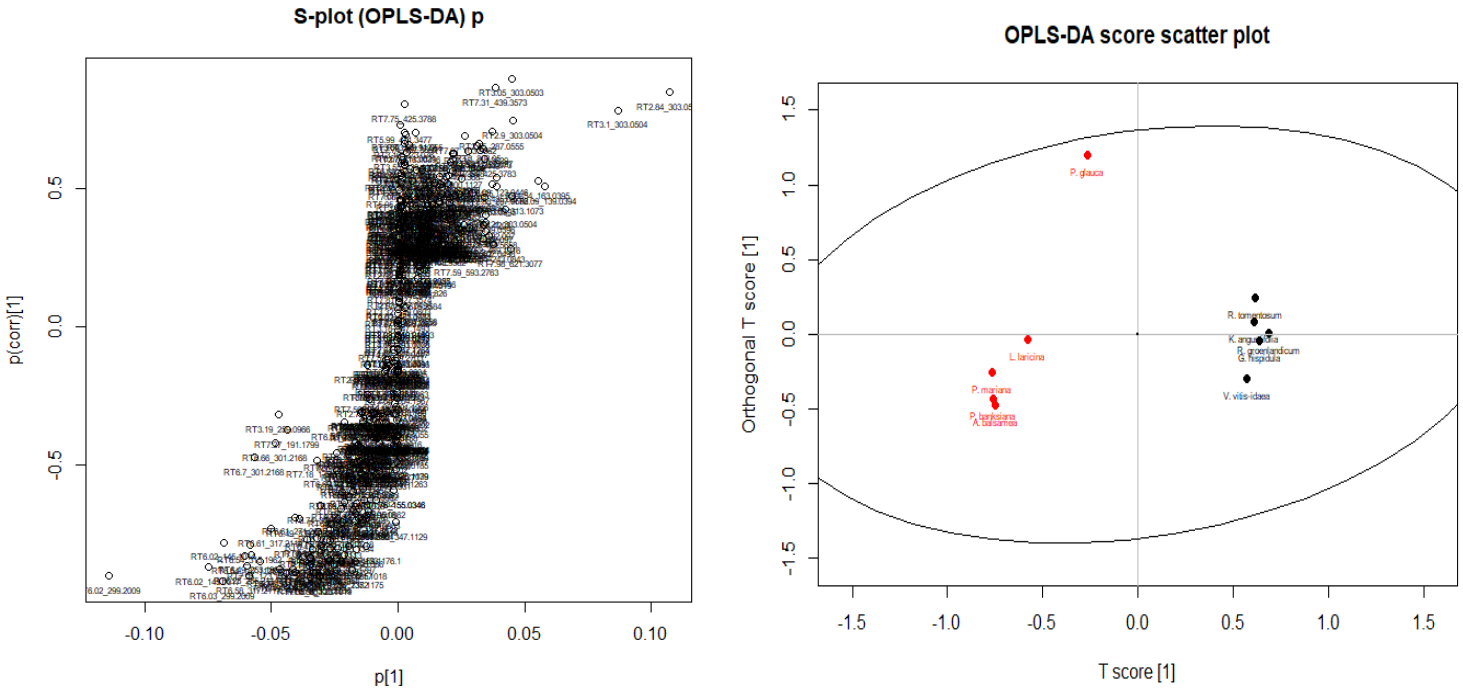


Figure 3.5. Orthoganol Partial Least Squares Discriminant Analysis (OPLS-DA) of Cree medicinal plants metabolomics, grouped by plant families Ericaceae (black) and Pinaceae (red). (A) Distribution of metabolomics signal loadings. (B) Distribution of plant scores.

Muma also makes use of a univariate analysis function for this data. However, due to the lack of effective replication, the reliance on p-values for selecting signals of interest was forgone in favour of just the visual analysis. P-values and fold changes were recorded where possible.

Although less variance is explained in the PCAs comparing the signals in the plants where bark or leaves were tested (**Figures 3.6-7**), the signals related to the leaves do show separation from those of the barks, enough so to be isolated for comparison (**Table 3.2**). The smaller variance explained can perhaps be attributed to the apparent greater variation in the chemistry of the barks, as indicated by the greater separation of bark scores and supported by the lack of groupings from the cluster analysis (**Figure 3.2**).

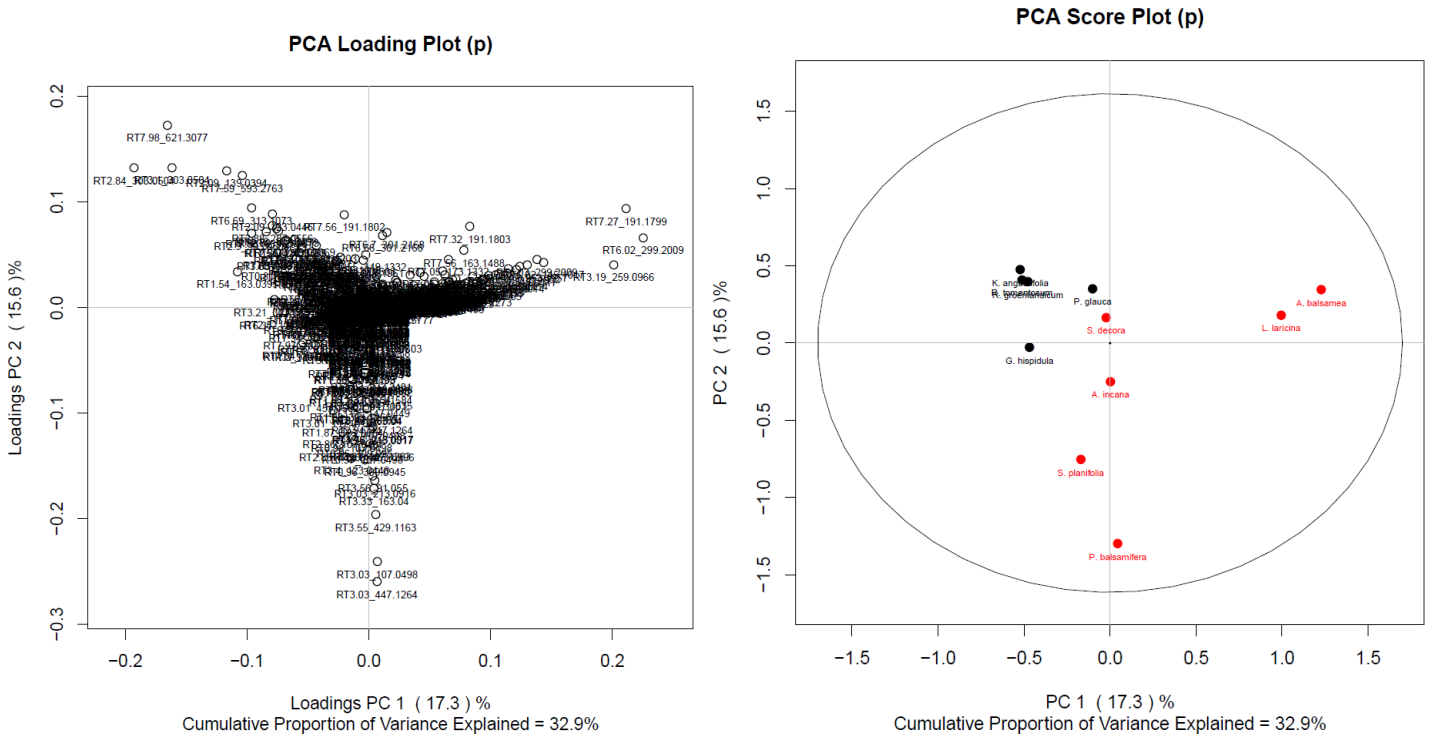


Figure 3.6. PCA of Cree medicinal plants metabolomics, grouped by plant parts leaves (black) and barks (red). (A) PCs 1 and 2 for the metabolomics signal loadings. (B) PCs 1 and 2 for plant scores.

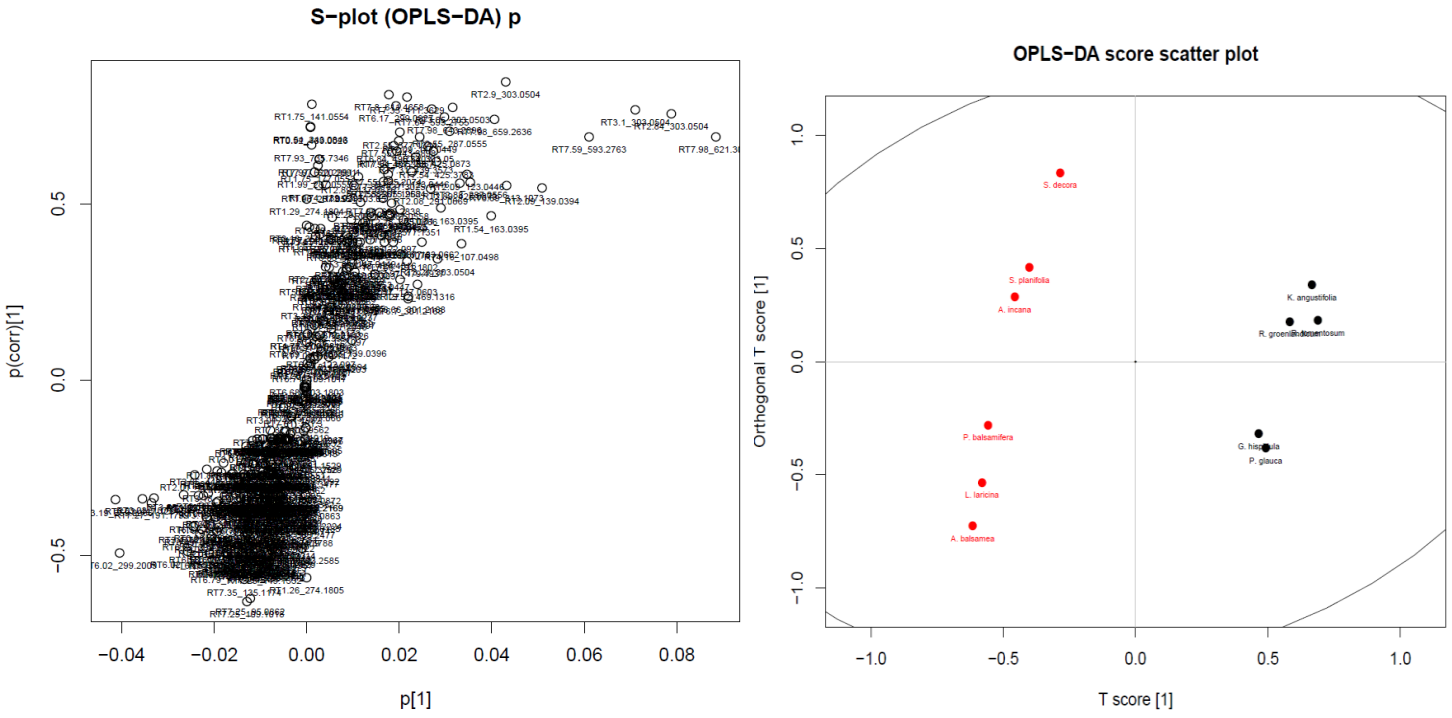


Figure 3.7. OPLS-DA of Cree medicinal plants metabolomics, grouped by plant parts leaves (black) and barks (red). (A) Distribution of metabolomics signal loadings. (B) Distribution of plant scores.

As indicated by the groupings suggested by the cluster analysis (**Figure 3.2**) and the distinct variation of bark phytochemical signals (**Figure 3.6**), the comparison of the signal profiles of the leaves, cones, and berries was also completed (**Figure 3.8**). The extent of the variation inherent in this data set across the Cree medicinal plant chemical profiles rendered the statistical comparison of signals across groups invalid, while the visual representation of signal separation is still accurate, if not quite as precise. Also due to the multiple groups, the discriminant analysis S-plot was not applicable here. However, there does appear to be distinct separation of signals associated with these 3 plant parts.

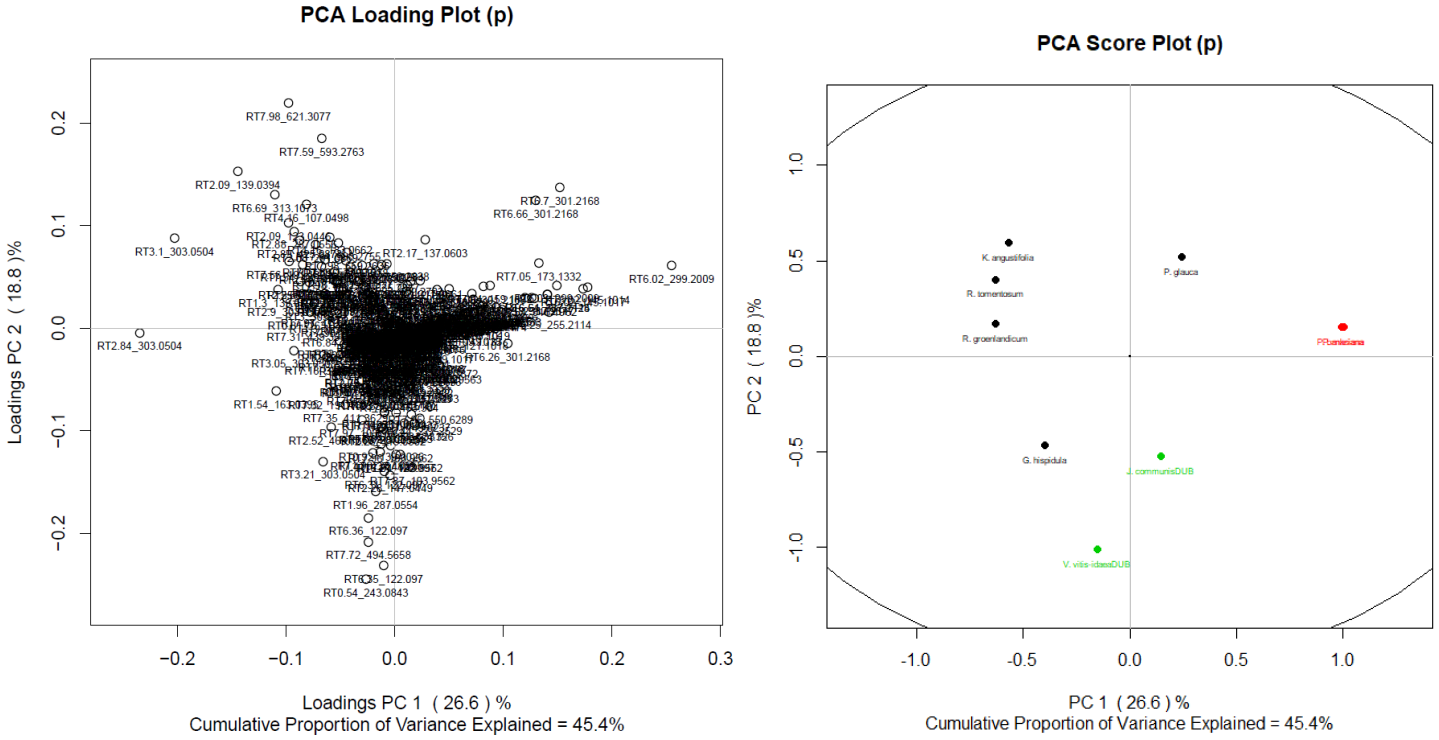


Figure 3.8. PCA of Cree medicinal plants, grouped by plant parts leaves (black), berries (green), and cones (red). (A) PCs 1 and 2 for the metabolomics signal loadings. (B) PCs 1 and 2 for plant scores.

Important relationships should be noted based off of the orientation of some of the plant scores. First, the upper right branch of loadings signals appears to be associated with the cones of *P. mariana* and *P. banksiana*, both species of the family Pinaceae. The point for the leaf of *P. glauca* is located halfway between the cones and the other leaves, all species of the Ericaceae; however, the fact that the leaf of *P. glauca* is actually a needle, also of the Pinaceae, indicates that the conserved nature of the other leaves may be attributable to how distinctly different these families are. Second, the lower branch of loadings signals appears to be associated with the berries of *V. vitis-idaea* and *J. communis*, but with apparent similarities with the leaves of *G. hispidula*. Throughout the TAAM project, the leaves of *G. hispidula* were primarily collected and tested; however, the berries of this plant were also collected and tested in other capacities. The fact that the signals of this sample are so closely related to the other berries that were tested may indicate a misrepresentation of what plant part was actually used in the chemical analysis and should be kept in mind moving forward.

As suggested by the collection of previous analyses, the grouping of the Ericaceae leaves is one of the most distinct. Indeed, the PCA of this grouping (**Figure 3.9**) very clearly shows the separation of samples and the most abundant signals associated with them. Keeping to one plant family and one plant part has also significantly reduced the inherent variation in the data, allowing the PCA to explain a much greater amount of the variance with only 2 principle components. However, the problem of having enough replicates for univariate analysis persists.

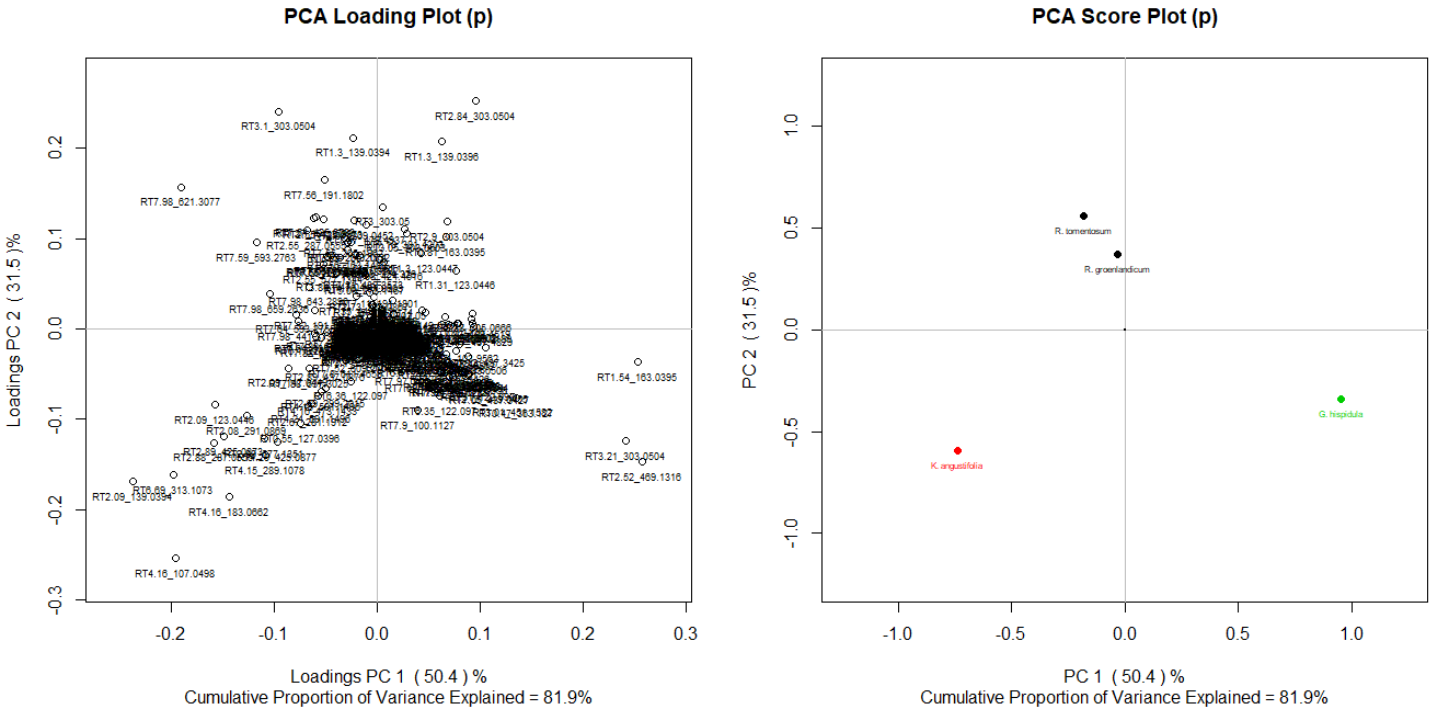


Figure 3.9. PCA of Cree medicinal Ericaceae leaves metabolomics, grouped by genera *Rhododendron* (black), *Kalmia* (red), and *Gaultheria* (green). (A) PCs 1 and 2 for metabolomics signal loadings. (B) PCs 1 and 2 for plant scores.

Table 3.3. Key signals identified through evaluation of multivariate analysis loadings figures. P-values and fold changes extracted through univariate pairwise group comparisons using R package muma where possible. Tentative IDs based off of evaluation of signal masses in Metlin.scripps online database.

Signal	Association	p-value	Fold Change	Tentative ID
Ericaceae and Pinaceae				
RT2.84_303.0504	Ericaceae	0.092	630.527	Quercetin derivative
RT7.31_439.3573	Ericaceae	0.092	13.861	Triterpene
RT6.02_299.2009	Pinaceae	0.156	0.000642	Diterpene
Leaves and Barks				
RT7.98_621.3077	Leaf	0.695	7.544	Protein
RT2.84_303.0504	Leaf	0.447	16.241	Quercetin derivative
RT3.10_303.0504	Leaf	0.447	137.751	Quercetin derivative
RT7.59_593.2763	Leaf	0.646	15.196	Chlorophyll derivative Pheophorbide A
RT6.02_299.2009	Bark	0.945	0.0604	Diterpene
Leaves, Cones and Berries				
RT7.98_621.3077	Leaf	-	-	Protein
RT7.59_593.2763	Leaf	-	-	Chlorophyll derivative Pheophorbide A
RT2.09_139.0394	Leaf	-	-	Phenolic acid
RT3.10_303.0504	Leaf	-	-	Quercetin derivative
RT0.54_243.0843	Berry	-	-	Sulfur compound
RT6.35_122.097 NIX	Berry	-	-	Amino acid derivative
RT7.72_494.5658	Berry	-	-	Lipid
RT6.36_122.097 NIX	Berry	-	-	Amino acid derivative
RT6.02_299.2009	Cone	-	-	Diterpene
Ericaceae Leaves				
RT4.16_107.0498	K. angustifolia	-	-	Unknown
RT4.16_183.0662	K. angustifolia	-	-	Unknown
RT2.09_139.0394	K. angustifolia	-	-	Phenolic acid
RT6.69_313.1073	K. angustifolia	-	-	Flavone derivative
RT1.54_163.0395	G. hispidula	-	-	Coumarin/carbaldehyde derivative
RT3.21_303.0504	G. hispidula	-	-	Quercetin derivative
RT2.52_469.1316	G. hispidula	-	-	Glycoside
RT3.10_303.0504	Rhododendron sp.	-	-	Quercetin derivative
RT1.30_139.0394	Rhododendron sp.	-	-	Phenolic acid

The identification of key signals of interest came through using comparisons of different groupings of the plants data to evaluate significant signals from those groups. As mentioned, the high degree of variation between these samples lead to several ways to place the plants into different groups. This poses both as useful, in the way that it provides many ways to evaluate the data, and as a difficulty, as it distinctly complicates the goal of determining which grouping best explains an associated chemical signal. A tool provided by the muma package to help simplify this process is the univariate analysis which provides the p-values and fold changes for each signal between the groupings. Unfortunately, these tools are not as useful with the Cree data set due to the lack of replication, so that not all groups can be compared effectively, and due to the high degree of inherent variability, which leads to insignificant p-values. For these reasons, the signals of interest have been chosen based off of their orientations on the multivariate loadings plots instead of those with the most significant p-values or fold changes, although these values have been included where possible (**Table 3.3**).

The first comparison came between the plants from the families Ericaceae (n=5) and Pinaceae (n=5) (**Figures 3.4-5**). With these groupings, the majority of signals most strongly associated with the Ericaceae family had a m/z ratio of 303.0504, a mass shared by the polyphenol quercetin and its numerous isomers and derivatives. The other major signal associated with the Ericaceae was RT7.31_439.3573, a mass indicating a triterpene. Ursolic acid has been identified as one of the few triterpenes in these Ericaceae species before (Rogachev et al. 2007; Szakiel and Mroczek 2007), and has been associated with cellular protection, anti-inflammatory, and antihyperlipidemic activities (J. Liu 1995). Additionally, ursolic acid has been detected as the active constituent in 9 plant species used therapeutically to treat diabetes mellitus specifically (Marles and Farnsworth 1995). A comparison of possible molecular weights indicate that this signal could represent ursolic acid without a water molecule. The most distinct signal associated with the grouping of Pinaceae samples is likely a diterpene, commonly found in conifer barks and resins (Hall et al. 2013). More specific identification

without comparison to commercial standards or compound isolation and structure elucidation is unlikely, as the chemical diversity of Pinaceae barks is so great, not only in terms of terpenoid content but even down to the level of diversity in terpene synthases across Pinaceae species (Bohlmann, Gershenzon, and Aubourg 2000).

The second comparison was between the samples where the leaves (n=5) or the barks (n=6) were tested (**Figures 3.6-7**). Most of the major distinctive signals were similar to the previous comparison, with quercetin derivatives showing commonly for the leaves, and the same diterpene signal showing for the barks. This was to be expected, as most of the leaves were from the Ericaceae, and several of the barks were from the Pinaceae. The p-values were less significant in this comparison, possibly due to the greater degree of phytochemical variation in the barks, as indicated by the cluster analysis (**Figure 3.2**). Two new significant signals were identified through this comparison, though. The signal RT7.98_621.3077, when searched for in Metlin, indicates that this compound may be a small protein. The other new signal, RT7.59_593.2763, has a mass consistent with the chlorophyll derivative pheophorbide A, which makes sense to be found in the leaf sample.

While these two comparisons do indicate important signals in some of the major groups, many of the plants that were used in this comparison have some of the most variable metabolic profiles, as indicated by the cluster analysis. *A. balsamea* had the greatest dissimilarity in the cluster analysis, but was part of both previous comparisons as a Pinaceae species and as a bark that was tested. Unfortunately, this likely means that major signals identified for either of these groups are more associated with *A. balsamea* specifically than with the larger grouping. This may be why the major identifying compound for these two groups was the same (RT6.02_299.2009). In fact, looking at the original signal data, this signal is present in *A. balsamea* in a significantly greater amount than in any other plants in its own grouping, and so should not be considered a key signal for any grouping other than its own sample. Given this new understanding of the association of this signal with *A. balsamea*, it

is likely that this signal indicates the diterpene dehydroabietic acid, a compound isolated from *A. balsamea* specifically for its activity in inhibiting G6Pase and stimulating glycogen synthase in hepatocytes (Nachar et al. 2015), a major model for anti-diabetic activity. This is important given that the most recent review of *Abies* phytochemistry does not contain representation from *A. balsamea* (Yang et al. 2008), nor does it contain the compound squalene, a bioactive compound that was isolated with dehydroabietic acid in Nachar's study.

By focusing on the groupings where inherent variation appears to be smaller (berries, cones, and leaves), perhaps signals more representative of the groupings can be identified. Although there are not enough replicates for statistical significance, performing PCA is still possible and can provide useful information (**Figure 3.8**). Again, there were several signals from previous comparisons that were also major signals in this comparison. While the major *A. balsamea* signal RT6.02_299.2009 was seen here associated with the cones (2 species which have been identified for dehydroabietic presence (Schuh and Benjamin 1984; Micales et al. 1994)), the intensity was much more equal between these two samples. The same protein, quercetin and chlorophyll derivative signals were still considered major leaf signals compared to the other two plant parts, but the new major signal RT2.09_139.0394 appears to be associated with many possible simple phenolics in Metlin. The major signals for the berries seemed to be the most unique so far, for the first time indicating potential fatty acids and amino acids as key signals, and one that appeared to be a sulfur containing compound (RT0.54_243.0843). Compounds containing sulfur, common in fruit and often contribute to the fruit's aromatic quality, have been identified in other *Vaccinium* species (Zhu et al. 2016), though none match the molecular weight indicated by this signal.

As seen by the lack of variance explained by major principal components, the great amount of inherent signal variation between samples makes identifying important signals for individual plants difficult. By focusing on a smaller group which shares major features, more focus can be placed on the

distribution of signals. In the case of the Ericaceae leaves, not only is variation reduced by evaluating only a single plant part and taxanomic family, but it has also been suggested by the cluster analysis that this group has the greatest similarity of signal distribution, and therefore provides a better opportunity to identify important signals for individual plants. There was strong separation of the 3 genera, and signals were associated for each that had previously not been identified by the other comparisons (**Figure 3.9**). For example, the signal RT2.52_469.1316 was identified for its strong association with G. hispidula. Top results for this signal mass in Metlin included the glycosides lucuminic acid and bungeiside D, both of which have the chemical formula C19H26O12, shared by the compound gaultherin isolated from Chinese species of *Gaultheria* (W.-R. Liu et al. 2013), but as of yet unconfirmed in *G. hispidula* and not present in the Metlin database. These results indicate the benefit of focusing on smaller associated sample groups with this type of analysis.

3.4 CONCLUSION

With the great leaps being made forward in the field of chemical analysis, the ease with which precise and accurate phytochemical data is being made accessible is positioning metabolomic analysis as a key contributor to understanding plant bioactivities. UNIFI has allowed the simple and speedy evaluation of specific compounds in plant extracts with high accuracy due to incorporation of fragmentation and phytochemical standards' chromatographic/spectrometric data, and the usefulness of the software will continue to grow as the database expands and is validated. Alternative analysis of the same data through the MZMine program allows for a simple entry into the multivariate analysis of large metabolomics datasets. Together, the breadth and depth of understanding of plant activity and chemical data has never been better.

These two tools allowed the exploration of the Cree plant phytochemistry in a novel way that resulted in generating signals of interest for many of the species that were evaluated, and provided suggestions on some of the possible compounds or chemical classes indicated by the signals. These methods have also allowed a better understanding the variability of the bark biochemistries relative to the conserved nature of the other plant parts.

CHAPTER 4: EVALUATION OF *OPLOPANAX HORRIDUS* (SM.) MIQ. PHYTOCHEMISTRY AND POTENTIAL HERB-DRUG INTERACTIONS

4.1 INTRODUCTION

The Squamish Nation of Canada consists of 24 main settlements, with more than 4000 registered members (Statistics Canada, 2018). The people of this Nation are descended from the Coast Salish peoples, and are now mainly located in the Vancouver area, Gibson's Landing, and the Squamish River watershed (Squamish Nation, 2013). The mild, rainforest-like climate of British Columbia provides excellent conditions for the significant plant diversity and subsequently rich ethnobotany of the Coast Salish peoples (Turner and Bell 1971), including qwáʔtʌp, the Squamish word for Devil's Club.

The genus Oplopanax, in the Family Araliaceae, contains only 3 closely related species. *Oplopanax elatus* (Nakai) Nakai, *Oplopanax japonicus* Nakai, and Devil's Club, *Oplopanax horridus* (Sm.) Miq., are all shrubs characterized by their abundance of small spines covering the plant, large, lobed leaves, greenish flowers in panicles, and bright red drupe-like fruit (**Figure 4.1**) (Pojar & MacKinnon, 1994). *O. horridus* is of particular interest due to its widespread occurrence across North America and related history of traditional use by numerous Indigenous groups across its range (**Figure 4.2**). Turner (1982) summarized more than a dozen medicinal uses of *O. horridus* across more than two dozen Indigenous groups in North America. While the major uses of the plant were for the treatment of arthritis and rheumatism, specific treatment of diabetes is included in its known uses, with this use being tested on mammals as far back as 1938 (Large and Brocklesby 1938), resulting in a hypoglycemic effect. Since then, only one other study looked at the potential anti-diabetic effect (Thommasen, Wilson, and Mcilwain 1990) without finding evidence for the hypoglycemic effect. All studies were small scale, however, and did not all have consistent use of dosages, plant parts, or preparation methods, leaving much room for further study.

Figure 4.1. *Oplopanax horridus* (Alaska Beachcomber, 2013).

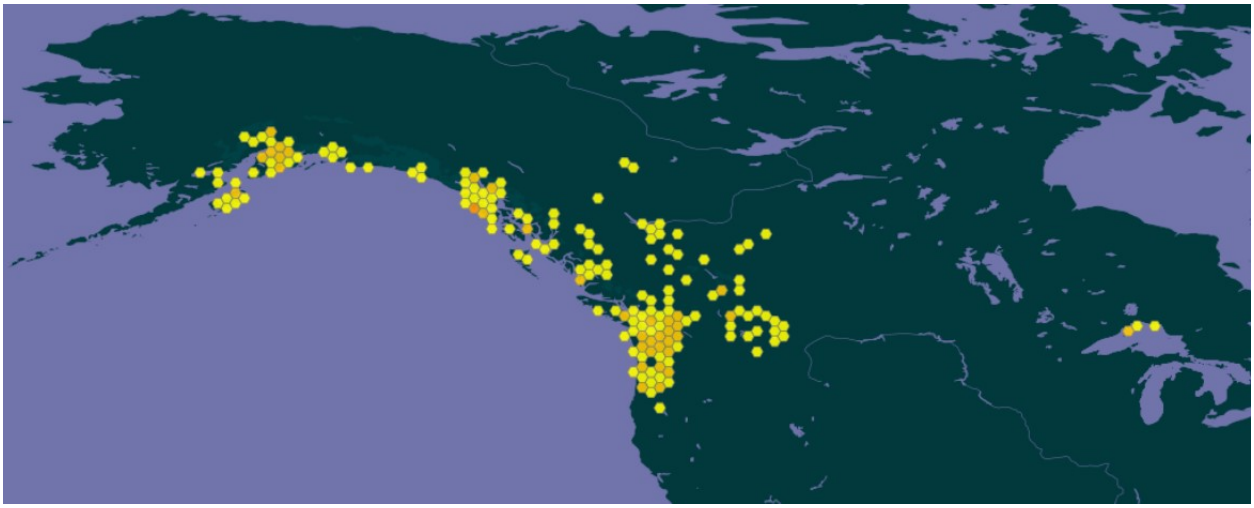


Figure 4.2. Distribution of *O. horridus* in North America. Darkness of hexagons refers a relatively more dense region of identified collections. Adapted from GBIF.org, 2018, https://www.gbif.org/species/3036356.

Extensive phytochemical analysis has been completed on the three Oplopanax species, characterizing triterpenes and other glycosides, polyynes, polyenes, and lignans as major constituents (Calway et al. 2012). These include several phytochemicals unique to this genus, specifically a collection of oplopantriols, oploxynes, and oplopanphesides (W.-H. Huang et al. 2011). However, phytochemical analysis of *O. horridus* has focused primarily on the roots and has left the inner bark of the stem largely untouched. The lack of study on the inner bark is strange, given that among 167 entries for *O. horridus* in the Native American Ethnobotany Database (Moerman, 2003), over 50% of the documented uses included the bark of this plant (**Appendix B**).

While specific estimates of diabetes prevalence in British Columbia and its First Nations are unavailable, the most recent overall predictions for diabetes risk in Canadian First Nations (17% incidence in adult populations, (Government of Canada 2011)) are still of great import. Although the First Nations of British Columbia have initiated multiple programs to aid in the management and prevention of the further spread of diabetes, including multiple events through the Squamish Nation promoting traditional knowledge regarding medicinal plant collection, preparation and use (Joseph 2018), concerns have been raised for their effectiveness in the face of new policy changes where the First Nations Health Authority joined with the BC Pharmacare health plan which no longer covers multiple anti-diabetes medications (Dawson 2018). In light of these concerns, it is now more important than ever for members of First Nations communities to have access to more local and accessible methods to prevent and manage their diabetes.

With concerns of consistent and culturally appropriate health care assistance, the incorporation of traditional medicines into people's regular care plans is essential to address. At the very least, the potential risk for plant medicines to interfere with enzymatic function is a first step, as any herb-drug interactions introduced to a care program that is already compromised may exacerbate the existing problem of medication availability. The cytochrome P450s (CYPs) are a class of enzyme associated with

the metabolism of both endogenous compounds and xenobiotics, foreign chemical compounds that enter the body and have potential to interact with normal body function, a major category of these xenobiotics being pharmaceutical drugs (Lewis 2004). However, many cases of natural substances interfering with the function of CYPs have been documented (Williams and Feely 2002; Foster, Arnason, and Briggs 2005), and the result often is a loss of accurate dose management of such pharmaceuticals, with the potential for either toxic or ineffective levels.

Of particular interest is the Cytochrome P450 CYP 3A4. Not only is CYP3A4 the dominant enzyme in metabolism of many xenobiotics (Wilkinson 2005), but it is also the major enzyme for metabolism of most diabetes medications specifically (Scheen 2007; Kajosaari et al. 2006; Triplitt et al. 2006; Siest, Jeannesson, and Visvikis-Siest 2007).

Although *O. horridus* is already currently used extensively within First Nations of the North American west coast, most documented use of the inner bark has been for external applications, and enzyme inhibition has not previously been evaluated for this plant. With the revitalization of traditional medicinal plant use as a cultural approach to health, understanding more about the risks and rewards of using *O. horridus* is more important than ever. The primary objectives here were to determine the relative safety of use of *O. horridus* inner bark extracts by evaluating its inhibitory activity on CYP 3A4. With the confirmation of bioactivity, bioassay guided fractionation was used to determine what potential compounds may be responsible.

4.2 METHODS

4.2.1 PLANT MATERIAL PREPARATION

Collections of *O. horridus* were made and prepared by Shirley Lewis (Squamish Nation), Dr. Pierre Haddad and Dr. Alain Cuerrier in the summer of 2016 in the region of the Squamish River Watershed. Species identification was confirmed by taxonomist Dr. Alain Cuerrier. Plants were harvested under the guidance of Squamish knowledge holders and branches stripped to the inner bark under traditional practices. This inner bark was then transferred to the University of Ottawa for processing. Plant material was then milled using a Wiley Mill and mesh size 20 (1 mm).

4.2.2 CRUDE PLANT EXTRACTION

Plant material was initially extracted in two ways. In one batch, 20g of ground material was extracted in 1L boiling water for 75 minutes. Filtrate was collected through vacuum filtration, then freeze dried to obtain the dry extract.

In the second batch, 500mL of 80% ethanol was added 50g of ground material in a flask. Flasks were sealed with parafilm, covered with aluminum foil, and then placed on a shaker overnight (200 rpm). Filtrate was collected through vacuum filtration and stored at 4^{o}C. The plant material was then re-extracted and filtered by the same method in 250mL of 80% ethanol. Filtrates were combined, roto-evaporated, then lyophilized to produce a crude extract free of any remaining water.

4.2.3 SOLVENT SERIES EXTRACTION

Following identification of greater inhibitory activity in the non-polar extract, a serial extraction of *O. horridus* was performed. 50g of newly ground plant material was extracted in 500mL of hexanes in the dark on a shaker at 200rpm for 1 hour. Filtrate was collected through vaccuum filtration and stored

at 4°C, while the plant material was re-extracted by the same method, after which filtrates were combined. The plant material was subsequently re-extracted sequentially using dichloromethane, ethyl acetate, methanol, and mili Q water, each time again with two washes of 500mL of the solvent. Organic extracts were rotoevaporated, then lyophilized to eliminate any remaining water. The water extract was lyophilized only.

4.2.4 OPEN COLUMN SEPARATION

Once the DCM fraction had been selected for fractionation, due to patterns of anti-diabetic activity evaluated by Sanchez et al. (2017, unpublished), 1.3g of the dried DCM fraction were loaded onto a 50 x 5cm Silica-packed glass column and separated using a step-wise gradient (**Table 1**). Collected samples of approximately 50mL each were pooled into 9 fractions based on HPLC chromatographs, and those 9 fractions were subsequently tested for CYP 3A4 inhibition activity.

Table 4.1. Solvent gradients used for separation of the DCM fraction of *O. horridus*. Each elution step used 1L of the specified solvent combination.

Elution Step	% Hexanes	% Ethyl Acetate	% Methanol
1	100	0	0
2	80	20	0
3	50	50	0
4	20	80	0
5	0	100	0
6	0	50	50
7	0	0	100

4.2.5 UPLC-MS QTOF ANALYSIS AND CONDITIONS

Extracts were resuspended in DMSO at 1mg/mL, followed by a 5 minute sonication and filtering through 0.2 micron PTFE syringe filter.

Chromatographic separations were performed on a Waters Acquity Xevo G2 series Ultraperformance Liquid Chromatograph connected to a time of flight mass spectrometer UPLC-QTOF system (Waters Corp) equipped with an Acquity BEH C18 column (1.7um 2.1 x 50mm). The separation method started with isocratic 98% A (0.1% TFA in LCMS grade water) and 2% B (0.1% TFA in LCMS grade acetonitrile) over the first 2 minutes at 65^{o}C, followed by a linear gradient for the next 3 minutes ending at 95% B, followed by another 2 minutes at isocratic conditions, followed by a linear gradient back to starting conditions over the next 0.01 minute, ending with an isocratic condition for the last 3 minutes. A flow rate of 0.8 mL/min and injection volume of 5 µl, followed by a post injection needle wash of 200 µl 50% acetonitrile and 600 µl 10% acetonitrile, was used.

QTOF analysis was conducted in MSe mode with positive electrospray ionization, with rapid alternate collision energies on each peak above the noise level, to capture both precursor ions and product ions. The instrument was mass calibrated with sodium formate prior to analysis. The conditions for MS analysis were as follows: source temperature, 500°C; cone voltage, 35V; scan time, 0.08 seconds; cone gas flow (N2), 50 L/hr; desolvation gas flow (N2), 1200 L/hour; m/z range, 100 to 1500 amu. Low energy setting 6V, high energy setting 10V-50V. Data was acquired with MassLynx v4.1 (Waters). The mass calibration was achieved by Leucine Lock mass correction applied post analysis after the import of the raw data from MassLynx to UNIFI.

The literature for all phytochemical identifications associated with *Oplopanax* species was searched, and the review by Huang et al, (2014) provided the most complete evaluation of such research. In said review, 123 phytochemicals identified in various parts from all three *Oplopanax*

species were discussed. The accurate masses of these compounds were used as the search terms for directed signal identification of the HPLC chromatograms in MassLynx analysis software (Waters Corp.).

4.2.6 HPLC-DAD CONDITIONS

Extracts were resuspended in methanol at 1mg/mL, followed by 5 minutes of sonication and filtering through 0.2 micron PTFE syringe filter.

Chromatographic separations were also performed on an Agilent 1100 series analytical HPLC-DAD system (Agilent Technologies, Montreal, QC, Canada) equipped with a Phenomenex Luna® C-18 column (150 mm x 2 mm, 3 micron, Phenomenex, Torrance, CA, USA). To separate within phenolics and also within terpenes, methods were optimized by applying a linear gradient over the first 8 minutes starting at 100% A (0.1% TFA in water) and 0% B(0.1% TFA in acetonitrile), and ending at 40% B, followed by a linear gradient over the next 8 minutes starting at 40% B and ending at 90% B, followed by a linear gradient over the next 4 minutes starting at 90% B and ending at 100% B, followed by an isocratic flow at 100% B, with a flow rate of 4 ml/min and an injection volume of 5 µl. A five minute post-run equilibration was completed at 100% A. The samples were run at a temperature of 50^{o}C.

4.2.7 CYP 3A4 INHIBITION ACTIVITY

Inhibitory capacity for select *Oplopanax* extracts on CYP 3A4 was determined using a microtiter fluorometric assay procedure adapted from Tam et al. (2011). Assays were prepared in triplicate in white walled, clear bottomed 96-well plates under red light and repeated on three separate occasions (n=3). Fluoresence was measured using a Cytation-3 Image Reader. Percent inhibition was determined relative to activity of the methanol control. Dried extracts were solubilized in methanol and diluted 10-

fold in water before plating 10µL per well. Plate wells were organized by methanol vehicle control, ketoconazole positive control, and extract samples.

The final in-well concentration for the crude extracts and solvent series fractions was 50 µg/mL. In-well concentration of the DCM subfractions was 5µg/mL. Multiple concentrations were tested to determine the IC50s for the hot water, ethanol, and DCM fraction extracts.

Samples were then incubated with a combination of three solutions. Solution A contained 1.08mM NADPH, and substrate dibenzylfluorescein in 0.25 potassium phosphate buffer (pH 7.4). Solution B contained CYP 3A4 in 0.13M buffer, while solution C contained the inactive CYP 3A4 from insect microsomes, also in 0.13M buffer. 100µL of solution A was added to each well. Half the wells for each sample set then received 90µL of solution B while the other half received the same amount of solution C, for a total volume of 200µL per well. Wells with solution C were considered Blanks. The plate was then transferred under darkness to the plate reader where it was shaken for 3 seconds before initial fluorescence ($F_{initial}$) was recorded. After a 20 minute incubation at 37°C, final fluorescence (F_{final}) was measured. Percent inhibition was determined with the following formula:

$$\left[1-\left(\frac{(Sample_{Ffinal}-Sample_{Finitial})-(Sample\ Blank_{Ffinal}-Sample\ Blank_{Finitial})}{(Control_{Ffinal}-Control_{Finitial})-(Control\ Blank_{Ffinal}-Control\ Blank_{Finitial})}\right)\right]x100\%$$

4.3 RESULTS

4.3.1 UPLC-MS QTOF ANALYSIS

Of the 123 compounds identified by Huang's review, 31 were associated with *O. horridus* parts, but only four were associated with the inner bark of *O. horridus*: neroplomacrol, neroplofurol, oploxyne A, and 4',7-Epoxy-4,9,9'-trihydroxy-3,3'-dimethoxy-5',8-lignan 4,9-bis[O-b-d-glucopyranoside]. Using the accurate mass of these compounds for signal searching, 16 were found to have highly accurate matches against predicted values (<5 mDa), based off chemical formulas (**Table 4.2).** These compounds were chosen as the best candidates for signal annotation using commercial standards and MRM development on the QTRAP system. Reference standards for five of these compounds were commercially available, but MRMs were not developed due to lack of signal presence.

In addition, 5 signals strongly matching the predicted m/z of the sesquiterpene formula C15H24 were identified, but due to the 17 potential *Oplopanax* compound matches associated with this formula, these compounds were not pursued for identification.

Table 4.2. Top potential matches from UPLC-MS QTOF analysis of *O. horridus* ethanol (EE) and hot water (HWE) extracts in positive (+) and negative (-) ion mode. Matches based off of signals with < 5 mDa mass-charge (m/z) difference from calculated m/z and <3 compounds with the same chemical formula.

Compound	Formula	# Potential Matching Signals	Extract	Ion Mode
(R)-Curcumene (a-Curcumene)*	C15H22	7	EE	+
(+)-Spathulenol (espatulenol)*	C15H24O	1	EE	+
(3S,8S)-Falcarindiol*	C17H24O2	4	EE/HWE	±
(9Z,11S,16S)-11,16-Dihydroxyoctadeca-9,17-diene-12,14-diyn-1-yl acetate*	C20H28O4	1	EE	-
Oplopantriol B*	C18H28O3	1	EE	+
Falcarinol^	C17H24O	2	EE	+
Oploxyne B	C18H30O4	5	EE/HWE	-
(1R,2S)-3'-Methoxy-1-(3,4-dihydroxyphenyl) propane-1,2,3-triol 4'-O-b-d-glucopyranoside	C16H24O10	1	EE	-
(1S,2R)-3'-Methoxy-1-(3,4-dihydroxyphenyl) propane-1,2,3-triol 5'-O-b-d-glucopyranoside	C16H24O10	1	EE	-
3-O-Caffeoylquinic acid	C16H18O9	12	EE/HWE	±
1-O-Caffeoylquinic acid	C16H18O9	12	EE/HWE	±
Oplopanpheside A*	C21H28O13	2	EE/HWE	-
Oplopanpheside B*	C22H30O14	1	HWE	-
Oplopanpheside C*	C23H32O13	2	EE/HWE	-
5-Methoxylariciresinol 4-O-b-d-glucopyranoside	C27H36O12	2	EE/HWE	-
(+)-5'-Methoxyisolariciresinol 3-O-b-d-glucopyranoside	C27H36O12	2	EE/HWE	-

*Previously identified in *O. horridus*, other plant parts.

^Previously identified in *O. horridus* inner bark.

4.3.2 CYP INHIBITION IN EXTRACTS

Initial assays of the crude hot water and ethanol extracts revealed only weak activity for hot water whereas the ethanol extract completely inhibited CYP3A4 activity, comparable to the positive control ketoconazole (**Figure 4.3**).

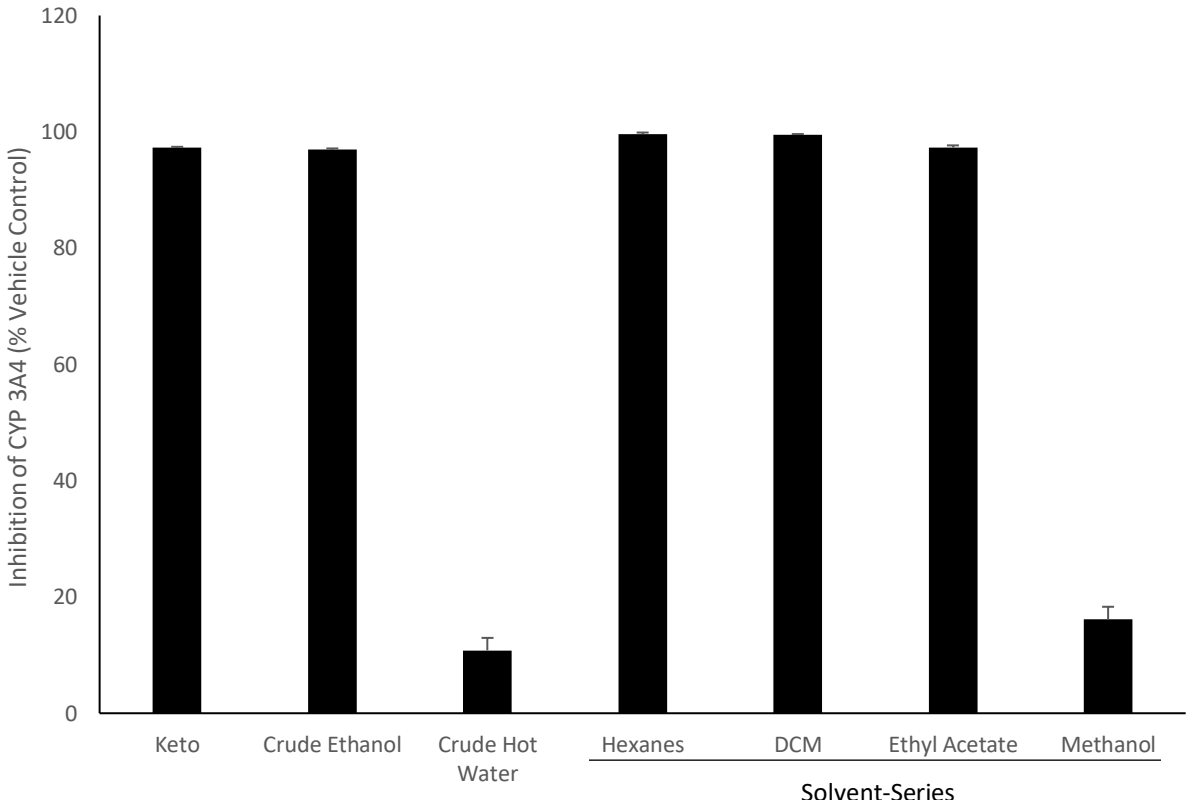


Figure 4.3. Percent inhibition of crude and solvent series extractions of *O. horridus* at 50 µg/mL. Standard error bars presented (n=3).

Following solvent-series extraction of the inner bark, the methanol fraction showed markedly less inhibitory activity of CYP3A4 than the other organic solvents. Although this extract concentration was too high to discern relative activity, the crude ethanol, hexanes, DCM and ethyl acetate extracts all elicited maximum inhibition, revealing that activity is mostly found in the non-polar fractions of the

solvent series. Further, preliminary work completed by Sanchez et al. (2018) on these extracts revealed the greatest anti-diabetic activity of the same non-polar fractions, with marginally greater activity seen in the DCM fraction (**Appendix B**), supporting the focus on the DCM fraction moving forward.

The 50% inhibitory concentration for the crude ethanol extract, crude hot water extract, and DCM fraction was determined (**Table 4.3**). Based on relative potency of inhibition, DCM was subfractionated by open column for further study.

Table 4.3. Mean IC50 values ± SEM calculated from individual samples (n=3) for CYP 3A4 inhibition.

Sample	**IC50 (µg/mL) ± SEM**
Crude Ethanol	88.57 ± 14.38
Crude Hot Water	>1000
DCM Fraction	26.16 ± 2.37

4.3.3 COMPOSITION OF DCM FRACTION

HPLC analysis comparing the peaks in the ethanol extract and DCM fraction to known phenolics (**Figure 4.4**) demonstrates that the DCM fraction contains a much greater enrichment of less polar compounds than the ethanolic extract. Even though the scales are slightly different, the major peaks in the DCM fraction approach a value of 200 mAU, whereas these peaks are barely visible in the ethanol extract near the 100 mAU level. Further, it can be seen that the DCM fraction is likely primarily composed of non-phenolic compounds, demonstrated by separation of the elution zone for the selected phenolic standards.

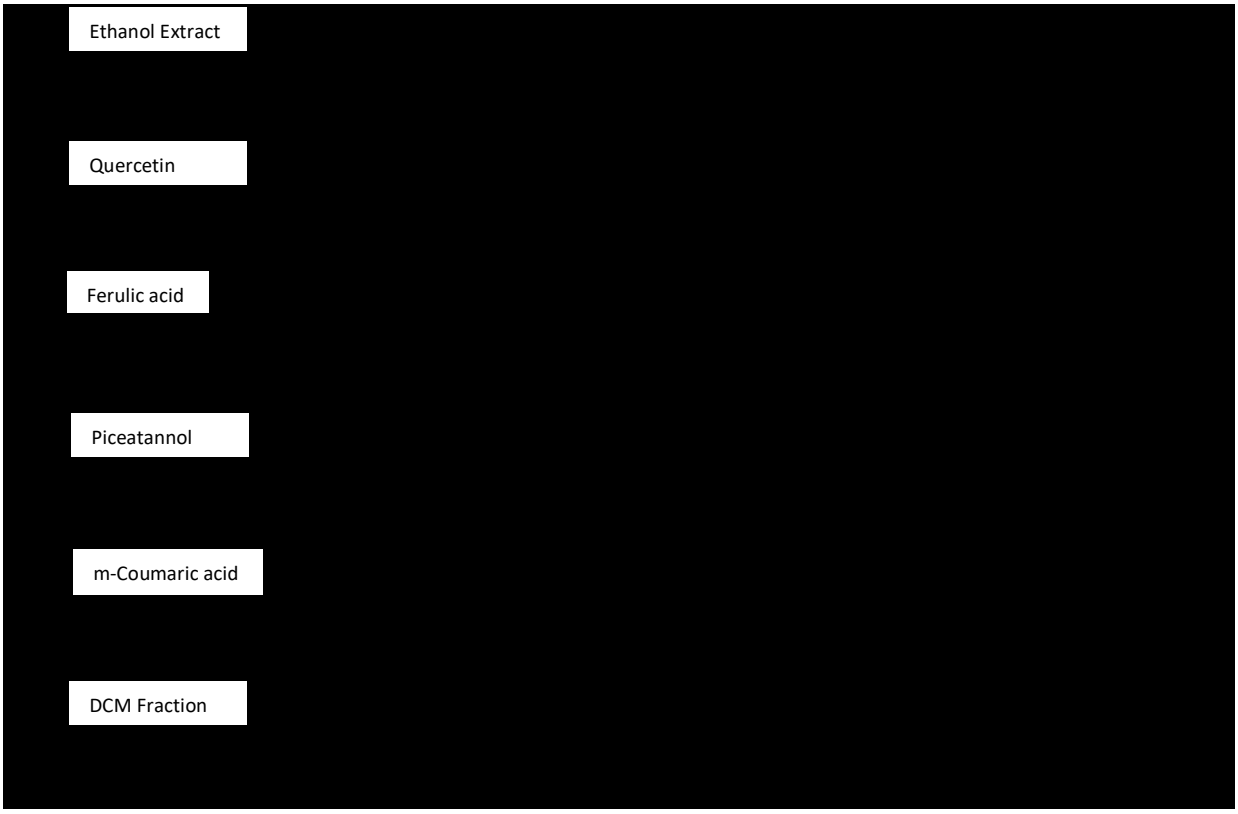


Figure 4.4. HPLC-DAD Chromatogram of *O. horridus* inner bark extracts and phenolic standards at 245nm.

4.3.4 CYP INHIBITION IN DCM SUBFRACTIONS

Column chromatography of the DCM fraction resulted in 51 subfraction collections. These subfractions were pooled into 9 fractions based on separation and signal evaluation of HPLC-DAD. Of these subfractions, 1 and 9 did not result in enough dry material to be used in the CYP 3A4 inhibition assay. Subfractions 2 through 8 were evaluated (**Figure 4.5**).

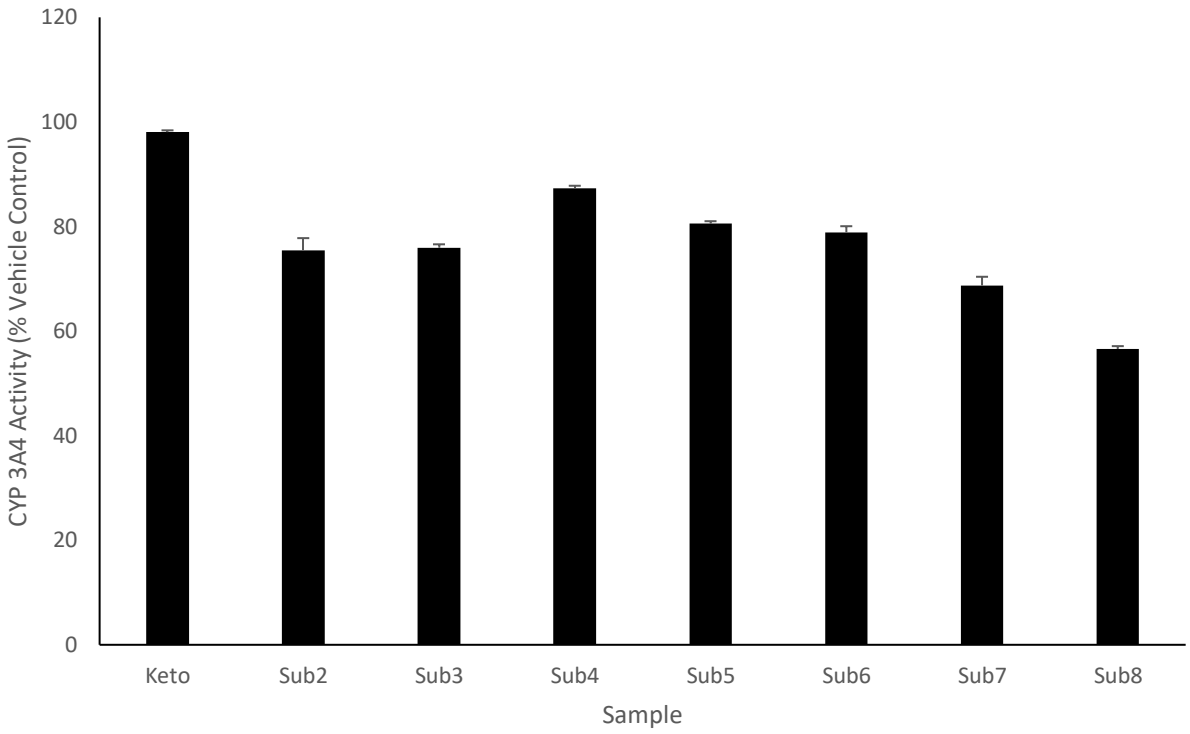


Figure 4.5. Percent inhibition of DCM open column separation subfractions of *O. horridus* at 5 µg/mL in well. Standard error bars presented (n=3).

The subfraction with the apparent greatest inhibitory activity was subfraction 4, with a trend of decreasing activity in the earlier and later subfractions. An evaluation of the neighbouring subfractions on HPLC-DAD (**Figure 4.6**) displays that there is an overlap of like compounds, but the abundances are highest in subfraction 4, suggesting that these may be the compounds most likely involved in the 3A4 inhibition activity.

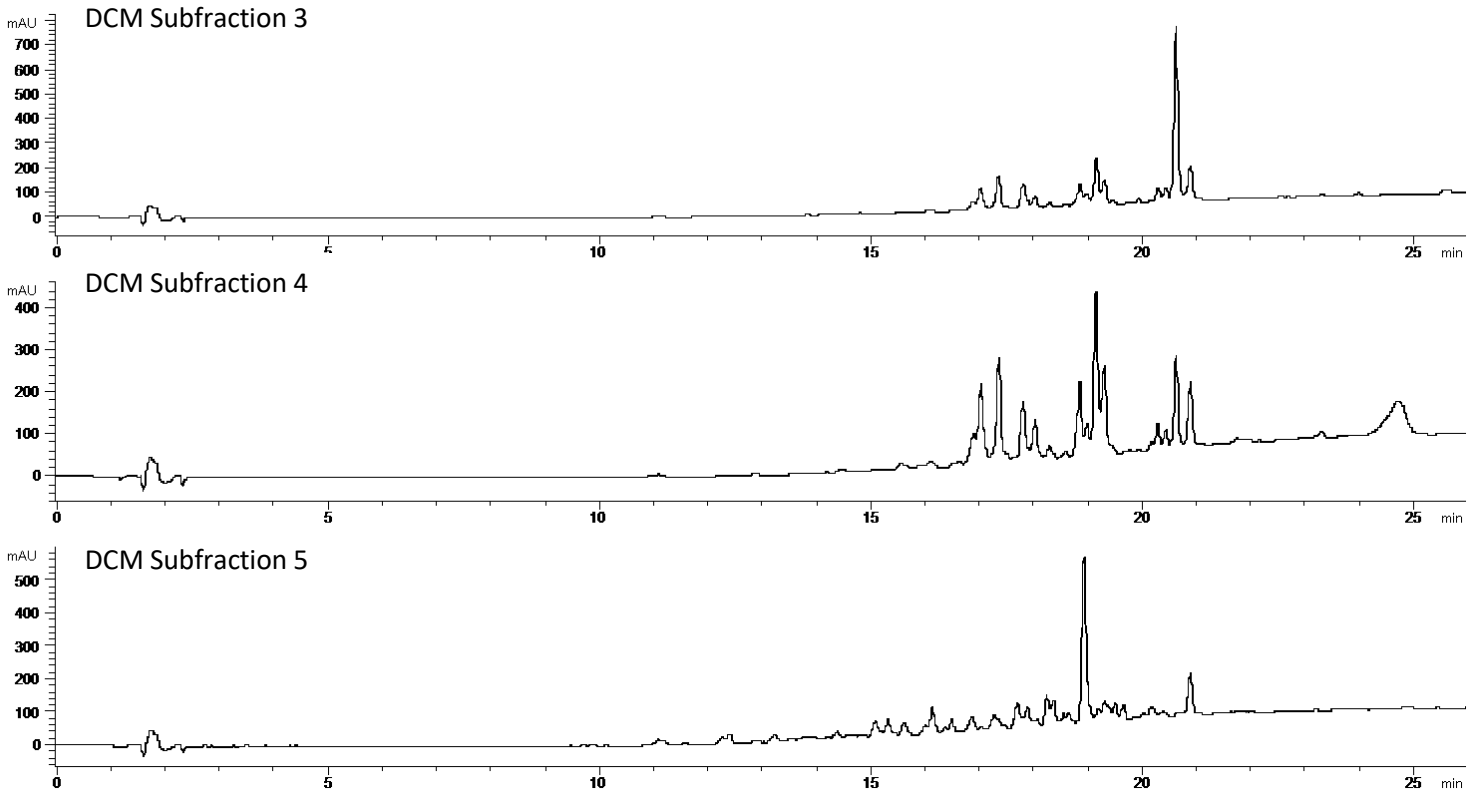


Figure 4.6. HPLC-DAD chromatogram of select DCM subfractions at 245 nm.

4.4 DISCUSSION AND LIMITATIONS

4.4.1 DISCUSSION

In evaluating the results of the CYP 3A4 inhibition assays, it is seen that the greatest activity occurs in the less polar extractions of the plant material (**Figure 4.3**). When specifically evaluating the DCM subfractions, strong inhibitory activity was seen across the majority of the samples. Although there was a lack of material from DCM subfractions 1 and 9 to be used on the CYP 3A4 inhibition assay, the trend of highest activity in subfraction 4 with decreasing activity on either side suggests that phytochemicals of interest will be present in the greatest amount in this central range from subfraction 3 to 5. With 13 major signals detected here associated with sesquiterpenoid structures and 3 possible matches with bioactive triterpenes gypsogenin, 3α-hydroxylup-20(29)-ene-23,28-dioic acid, and 3β-hydroxylup-20(29)-ene-23,28-dioic acid (Heller et al., 2014; Li et al., 2007; Kim et al., 2010), terpenes and related compounds may be a good compound class to begin a more targeted activity analysis.

Despite the focus here on the large terpenes, the chemical makeup of *O. horridus* is highly complex. The polyene compound nerolidol has been identified in large amounts in the roots and stem of *O. horridus* and is largely what contributes to the oily quality of these extracts (W. Huang et al. 2010). The non-polar extracts and fractions of the stem bark completed here were indeed highly oily, although nerolidol was not identified as a constituent on the QTOF UPLC-MS. This may be because the previous study that identified nerolidol used gas chromatography (GC), which is known for being much more effective at elucidating volatile terpenes like nerolidol (Huie 2002). Similarly, much of *Oplopanax*'s secondary metabolome is so unique that few commercial standards were available. The ones that were obtained were all volatile polyynes and sesquiterpenes, and none elucidated well enough on LC for comparison with the extracts here. Reanalysis with GC methods instead would help further our understanding of the bark's composition.

In terms of clinical relevance of the observed 3A4 inhibition, previous studies have identified extracts of St. John's Wort to be responsible for changes in pharmacokinetics of 3A4 metabolised medications when taken concomitantly (Gurley et al. 2005), and evaluations of the IC_{50} of St. John's Wort extracts have found it comparable to that of the DCM extract presented in this thesis (Hellum and Nilsen 2008). However, the use of non-polar solvents such as DCM outside of the laboratory setting is highly unlikely and should not be concerning to local use by the Squamish community. Even though the crude ethanol extract of *O. horridus* has a more moderate inhibition, it still presents a greater potency of inhibition on CYP 3A4 than other natural products evaluated by Hellum & Nilsen, and therefore users should be cautious. Also, the reduction in potency from the DCM fraction to the ethanol extract may correspond to the greater relative presence of the less polar compounds in the elution region of 15-20 minutes. That being said, future research on the potency of these extracts should consider that there may be multiple sets of different compounds responsible for this inhibitory activity, given that macimum inhibition was not reached with this fraction. Meanwhile, the hot water extract of *O. horridus* appears to pose no threat to clinically relevant CYP 3A4 inhibition, but further testing on other cytochrome types would be essential. Compared to even just the ethanol extract, the chromatographic peaks for the hot water extract are present in a much lower amount and occupy a more polar range, further suggesting that the compounds that are present do not possess the same inhibitory risk as those in the other extracts (**Appendix B**). However, it would be important to gain a better perspective on the inhibitory capacity of *O. horridus* hot water extract since, as mentioned previously, there are other CYPs that interact strongly with diabetes medications (2C8/9), and even beyond the context of diabetes, understanding this plant's effect on the other CYP isoforms is necessary if new uses start going beyond traditional practices. Although *O. horridus* is not known to be toxic to humans, one of its traditional uses is as an emetic (Calway et al. 2012), so a greater understanding of this pathway may also be important.

4.4.2 LIMITATIONS

In doing work with the analysis of traditional indigenous medicines, researchers must aim to address both the full pharmacological potential of a plant as well as be realistic in terms of its expected use. While many traditional preparations are referenced, the specific methods for these are often either not fully disclosed, or highly variable. However, it is known that both the time frame and the temperatures used in these methods affect the amount and types of compounds that can be extracted from the plant material (Tam et al. 2014). Therefore, it is important to recognize that the extraction methods used here are limited relative to traditional preparations, especially if part of the goal is to identify the true risk to potential users. Specifically, by only testing a single hot water extraction at 1 hour and one temperature, this must be identified as not truly being representative of the possibilities of the traditional preparation. Similarly, the use of organic solvents, other than alcohol, is also not a good representation of traditional practice or likely use by active users.

4.4.3 NEXT STEPS

This research provides a very early yet essential look at the phytochemical makeup and safety of the inner bark of *O. horridus*. Further research should be done to continue towards identifying active compounds in the DCM subfractions, particularly in subfraction 4. This step may include further column chromatography to work towards isolation of individual compounds. Keeping in mind practical application of this research in the Squamish community, further research should also be done on evaluating the safety of this plant material in a greater variety of preparations, including longer boiling times and at varying temperatures. Further, this study looked only at basic IC50 identification. To provide a more accurate prediction of potential health effects, mechanisms of inhibition of *O. horridus* should be evaluated. This can be achieved with evaluations of NADPH- and time-dependence. Additionally, given its previous association with hyperglycemia reduction as well as the recent data

matching the less polar fractions to anti-diabetic activities, completing bioactivity guided isolation for these assays is pertinent and attainable.

CHAPTER 5: DATA INTEGRATION AND GENERAL DISCUSSION

A major component of this thesis has been about moving away from the focus on small sets of activity that has been characteristic of this team so far, and instead attempt to glean some meaning from the data by looking at how each of the plant species are behaving within the whole context of the project.

In Chapter 2's evaluation of the collective pharmacology assay data, interpretation of the multivariate data led to suggestions of certain relationships among the plants. First, four plants displayed both diversity and consistency in their activities related to the glucohomeostasis anti-diabetes models: *A. balsamea*, *L. laricina*, *R. groenlandicum*, and *S. purpurea*. This grouping is surprising given how different they are taxonomically and by the parts of each plant that were tested, as well as intriguing in how three of the four were the top most selected species by Cree elders for the treatment of diabetes symptoms. Second, the activities of the two cones from *P. mariana* and *P. banksiana* on fatty acid metabolism CYPs as well as fat-related bioassays, such as glucose uptake in fat, adipogenesis, and immunomodulation, suggest that these activities may be associated with the fatty acid content of these plants. However, these observations also indicate that caution should be used with regards to the relationship with the particular CYP inhibition. Fatty acids are important signaling molecules, both in and beyond diabetes, and interference with their metabolism has the potential of negative impacts. Finally, there appeared a trend separating plants generally active on the hyperglycemia effect assays, namely high phenolic content leaves, from plants more active on specific sets of glucohomeostasis activities. This trend suggests potential use of some plants for general system health while others may be used to target more specific cell activities for minimizing states of hyperglycemia.

Chapter 3 explored the use of several methods to evaluate the metabolomic profiles of the Cree plants. Using the UNIFI data to target standards that have been isolated from active plants through bioassay guided fractionation, the presence of active compounds such as hyperoside, catechin and

epicatechin was identified in multiple other Cree plants for the first time. The use of this data in analysis using MZMine resulted in observations that the phytochemical profiles of the different bark species that were investigated were highly variable, especially compared to the much more conserved profiles observed in the berry, cone, and leaf parts. Further, discriminant analysis of these groups led to identification of potential marker signals.

Finally, the investigation of *O. horridus* in Chapter 4 found the hot water extract of the plant's inner bark to be largely inactive in the inhibition of CYP 3A4. Other extractions of the plant, particularly the dichloromethane fraction, exhibited much more potent inhibition of the 3A4 enzyme, with subfractionation indicating greatest activity in a group of compounds associated with terpenoid elution region. Further, mass analysis of compounds detected in other *Oplopanax* species and *O. horridus* plant parts identified 16 compounds with highly accurate masses previously unreported in *O. horridus* inner bark.

5.1 INTEGRATION OF INTERDISCIPLINARY DATA

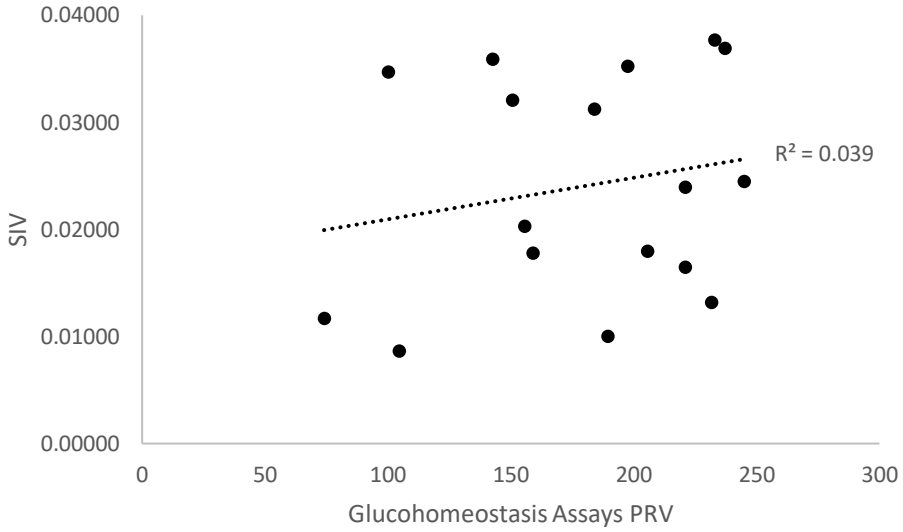
While the meta-analysis of pharmacology or phytochemical data within their own sets has proven useful, there is still further to go, as there are ways that these overarching investigations into the pharmacology, phytochemistry, and ethnobotany of the Cree plants can be combined and investigated with reference to each other as well.

For instance, much of the justification for this work has been about evaluating the place of traditional knowledge as a guide for local intervention strategies with regards to T2D. However, it becomes very easy to take the initial studies from which these plants were identified, and from there leave behind the human connection to the use of these plants. Incorporating the anti-diabetic potential of the plants derived from the interviews with Cree elders, the SIV analysis developed in Leduc's original work (Leduc et al., 2007), we can start by comparing how the plants ranked in their importance of traditional use to the number of assays in which they were then found to be active (**Figure 5.1**).

Earlier chapters have demonstrated the need to separate the pharmacology data into the three categories for effective evaluation, so the same has been done here. First, there appears to be very little correlation between the predicted usefulness of the plants (SIV) and the observed activity on the glucohomeostasis assays, despite a small trend towards plants with a higher SIV being more active. Instead, there appears to be a stronger correlation between their traditional use and the plant activity on the hyperglycemia effects bioassays, and a much stronger correlation between traditional use and inhibition of metabolic enzymes. The correlation with the hyperglycemia effect activity may be a result of phenolic content, as Harris et al. (2011) outlined a strong correlation between total phenolics and both antioxidant and antiglycation activity, and Fraser et al. (2007) found a similar correlation between antioxidant activity and the SIV rankings. However, correlation between total phenolics and SIV has not been evaluated.

When evaluating the same relationships on principle component analysis (**Figure 5.2**), it initially appears that there is a distinct association between plants of higher SIV value and overall performance on the pharmacological assays. However, by once again separating the enzyme inhibition data, it is seen that it is actually the enzyme inhibition which drives the groupings. This association matches the correlation from **Figure 5.1**, and strongly suggests the selection of these plants by the Cree elders based off very real metabolomic interactions, and not just through the general health benefits of high phenolic content associated with antioxidant activity. This is especially true given Tam et al.'s finding (Tam et al. 2009) that phenolic content was not generally correlated with enzyme inhibition.

A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0.03000
0.02000
0.01000
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SIV
R² = 0.039
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50
100
150
200
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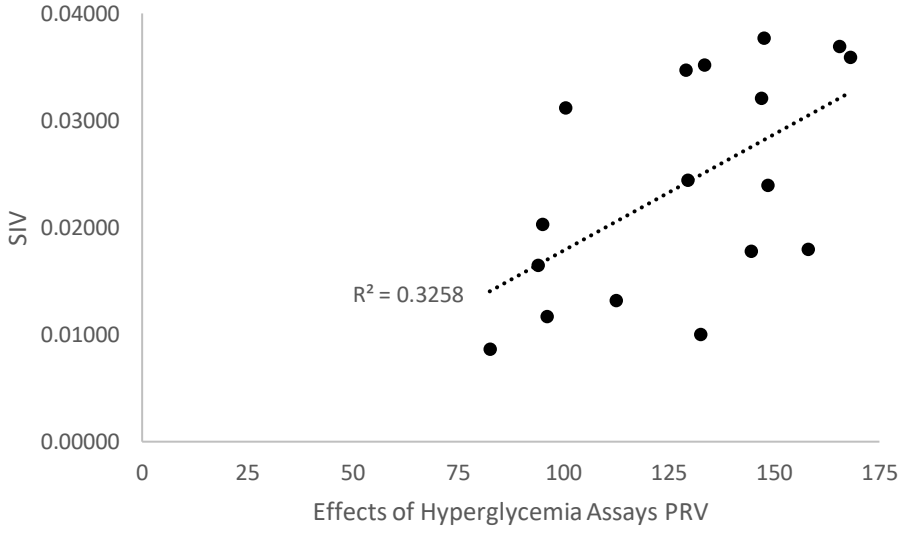
300
Glucohomeostasis Assays PRV

B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100
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150
175
Effects of Hyperglycemia Assays PRV

C

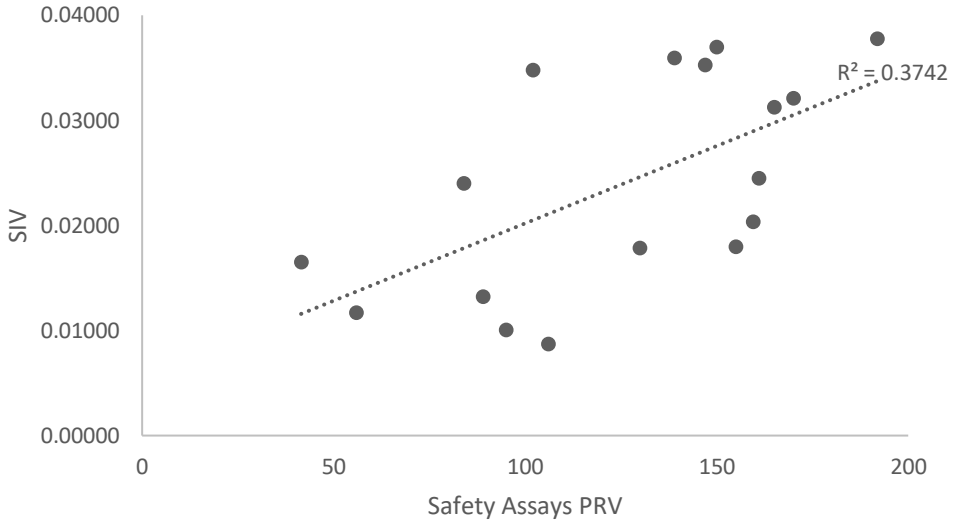
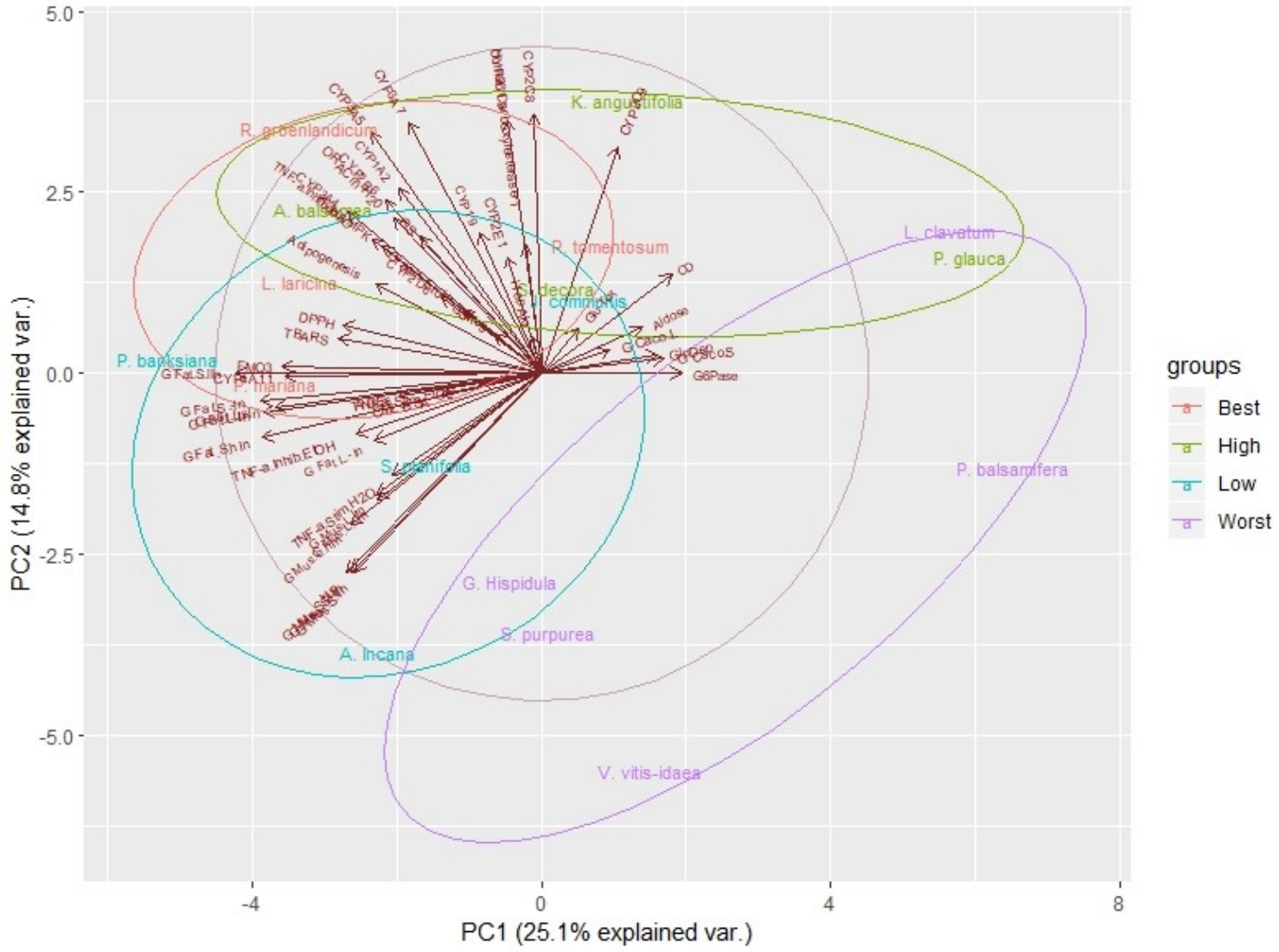
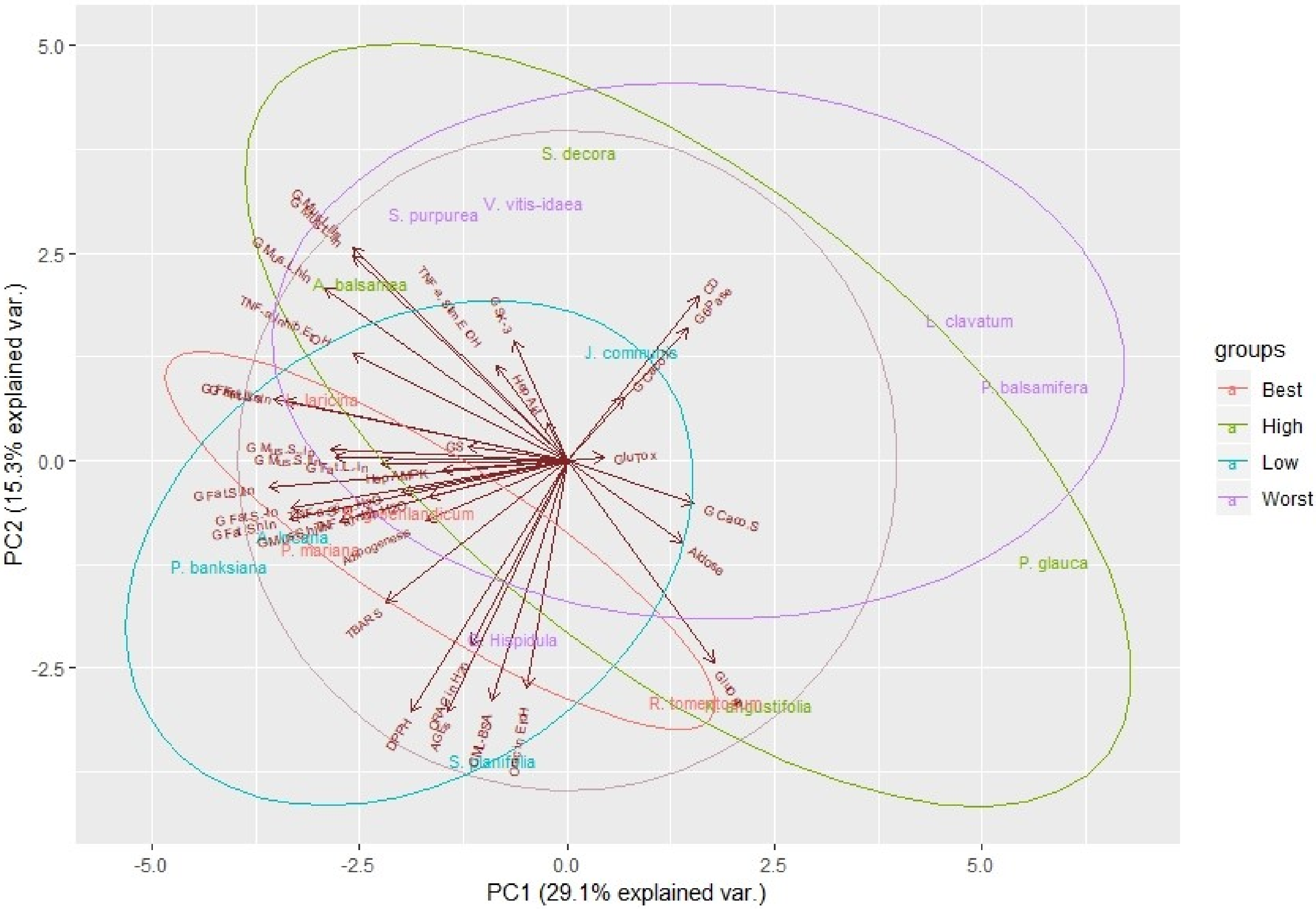


Figure 5.1 Pearson correlation plots of the SIV and PRV of Cree medicinal plants bioactivity in (A) Core Anti-diabetes Assays, *r* =0.1616, (B) Diabetes Complications Assays, *r* =0.6026 and (C) Safety Assays, *r* =0.6117. Greater SIV values indicate greater associated anti-diabetic potential based on ethnobotanical evidence. Lower PRV values indicate more significant relative activity in associated bioassays

A


5.0
2.5
0.0
-2.5
-5.0
PC2 (14.8% explained var.)
-4
0
4
8
PC1 (25.1% explained var.)
K. angustifolia
R. groenlandicum
A. balsamea
P. tomentosum
L. laricina
S. decora
commonis
L. clavatum
P. glauca
P. banksiana
P. mariana
S. planifolia
P. balsamifera
G. Hispidula
S. purpurea
A. Incana
V. vitis-idaea
DPPH
TBARS
Adipogenesis
Aldose
G6Pase
groups
Best
High
Low
Worst

B


5.0
2.5
0.0
-2.5
-5.0
-2.5
0.0
2.5
5.0
PC1 (29.1% explained var.)
PC2 (15.3% explained var.)
groups
Best
High
Low
Worst
S. decora
S. purpurea
V. vitis-idaea
A. balsamea
L. clavatum
J. communis
P. balsamifera
P. glauca
P. banksiana
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R. tomentosum
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S. planifolia
TBARS
DPPH
Aldose

C

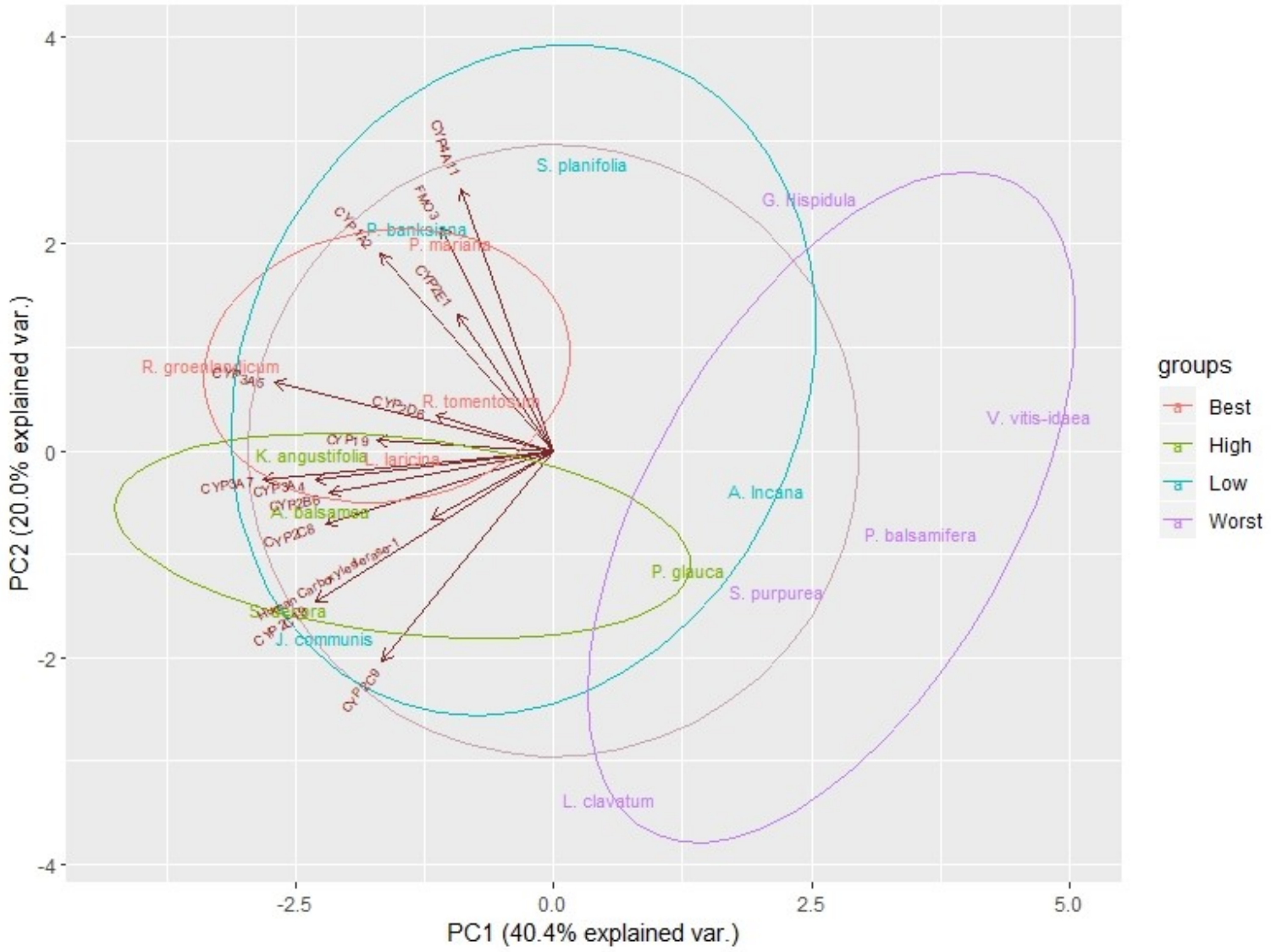

Figure 5.2. Principle component analysis of Cree medicinal plant bioactivity. Groupings reflect rankings based off Syndromic Importance Value assigned to each plant through interviews with Cree elders; goodness of the groups refer to the degree of use with relation to the diabetes symptoms. (A) Analysis with all pharmacological bioassays. (B) Analysis with core anti-diabetic and secondary diabetes complications bioassays. (C) Analysis with toxicological and safety bioassays. Ellipses indicate 95% confidence intervals on groupings.

Given this multivariate approach, the analysis also provides opportunities to look at the ways that the phytochemistry of these plants may relate to determining associations with pharmacological activity. Similar methods have been explored before within this team (Shang et al., 2015), where the dichotomy of plant activity on a given assay was used as the basis of grouping metabolomic signals in discriminant analysis. The metabolomics data for that investigation was processed manually, so re-evaluating the results from the program-based metabolomics data generation (Chapter 3) is highly useful (**Figure 5.3**). The signals associated with the most active species are summarized in Table 1.

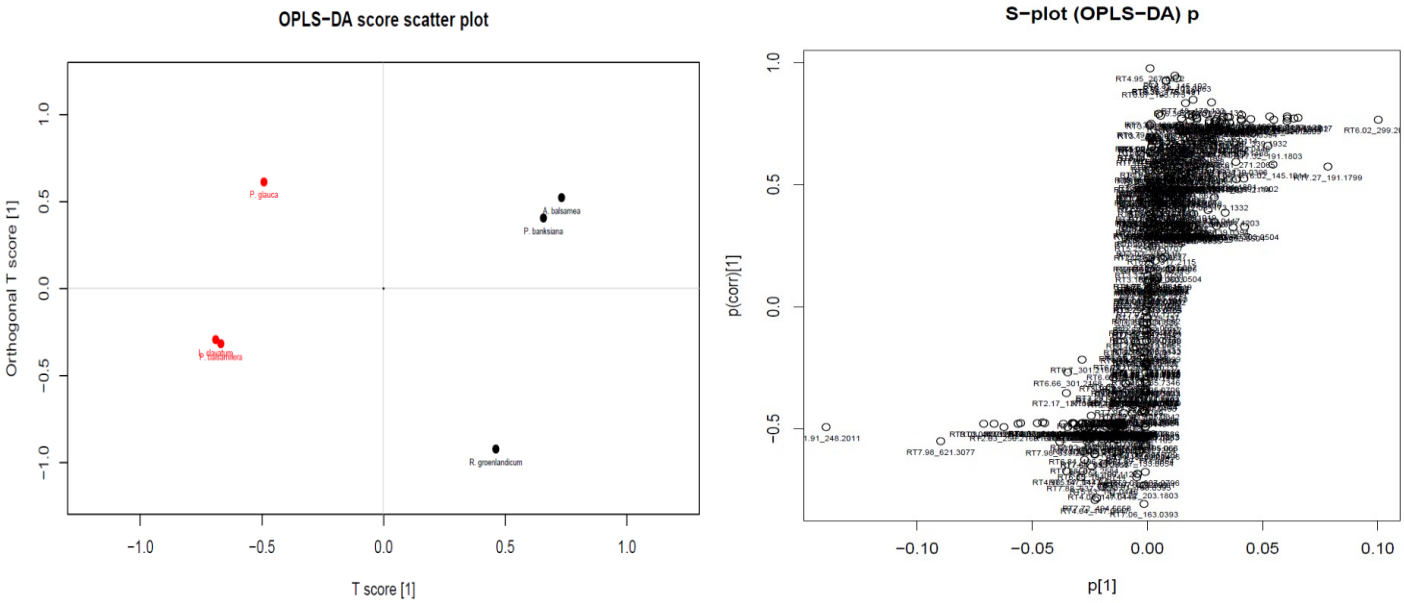

Figure 5.3. Orthagonal partial least squares discriminant analysis (OPLS-DA) of the most (black) and least (red) active species for stimulation of glucose uptake in fat cells.

Table 5.1. Discriminant metabolomic signals associated with the most active species on select anti-diabetes bioassays.

Assay	Top Species	Bottom Species	Key Signals	
			m/z	*Tentative ID*
Inhibition of glucose uptake into intestinal epithelium	*L. clavatum* *P. glauca* *R. tomentosum*	*J. communis* *G. hispidula* *P. banksiana*	248.2011 250.2169	Lycopodine Amide
Inhibition of G6Pase	*P. glauca* *A. balsamea* *V. vitis-idaea*	*P. banksiana* *G. hispidula* *S. planifolia*	301.2168 621.3077	Diterpene or HEPE Protein
Stimulation of glycogen synthase	*A. balsamea* *L. laricina* *R. groenlandicum*	*P. banksiana* *V. vitis-idaea* *P. balsamifera*	191.1799 259.0966	C14 Fatty acid Sesquiterpene C15H14O4
Stimulation of glucose uptake into adipocytes	*A. balsamea* *P. banksiana* *R. groenlandicum*	*P. glauca* *L. clavatum* *P. balsamifera*	299.2009 191.1799	Diterpene (dehydroabietic acid derivative) C14 Fatty acid
Stimulation of glucose uptake into muscle cells	*A. incana* *S. decora* *S. purpurea*	*P. glauca* *L. clavatum* *P. balsamifera*	381.4203 409.4519 411.1290	Unknown Unknown Unknown

Across these analyses, there were distinct signals of interest associated with the top three plants. However, when looking at these signals in the original data, most of these signals are more representative of individual species and not the top set of species. For example, the signal for 248.2011 m/z was highly distinct in the discriminant analysis, but only had notable presence in one species, *L. clavatum*. In fact, the second major signal associated with the inhibition activity of intestinal enterocytes was also only present in *L. clavatum*. These details make the interpretation of such data

more difficult, for although this provides strong reasoning to attempt the isolation and testing of these specific compounds, an explanation for the strong activity of *P. glauca* and *R. tomentosum* is still lacking. This problem is consistent across the rest of the assays that were evaluated here. While possible, it is unrealistic to expect the same active compound to be present across species and plant parts. More likely is that similar activities are resulting from similar types of compounds, although similar activities can arise from very different compounds too, seen also in diabetes specifically (Marles & Farnsworth, 1995).

One set of signals was of interest, however. While most signals could be tentatively identified to the level of chemical class using the Metlin Scripps database tool, those associated with the active plants on the glucose uptake into muscle cells assay defied such identification. These three signals, 381.4203, 409.4519, and 411.1290, either matched a much too diverse and complex set of possible chemical classes, or received no matches at all, even when considering major adducts. Further, of the major signals identified, these had a greater level of association between signal intensity and the three most active plants than the other assays that were evaluated in this way. Isolation and identification of these signals would not only be beneficial from a potential active compound standpoint, but also one of potentially unknown compound characterization.

5.2 LIMITATIONS AND FUTURE DIRECTIONS

One of the purposes of this thesis was to explore the power and potential for multivariate analysis of multidisciplinary data in the context of the ethnopharmacology. The ideal achievement would have been to put together predictive models where the general activities of a given plant could be projected based on its taxonomic family, the part used, or a quick screen of its phytochemical profile. Perhaps unsurprisingly, despite the few patterns that were discussed here, it became apparent that there was still quite a long way to go before a model of such power could be fully realized. My results, however, can inform the design of future studies with fewer limitations and greater statistical power for investigating potential relationships between ethnobotany, phytochemistry, and pharmacology.

First, changes would need to be made in the methods for collection of the pharmacological activity data, or a large amount of data manipulation would otherwise be required to standardize all the data. Currently, activity analysis has been completed by using the maximum concentration of each extract before it becomes cytotoxic, and there tends to be a large variation between each plant as to what this concentration is. If links are to be made between active plants and active compounds, concentrations must be comparable, if not by experimental design that uses consistent concentrations across extracts, then at least by standardization of the raw chemical data to correct for differences in assay-tested concentration. Further, it would be highly useful to have multiple diabetes drugs tested consistently across the assays to get a better idea of what patterns of activity are present between the plants and the known modes of action of these drugs.

The other major change would be to the extent of the data collection for the metabolomics. Currently, there is metabolomic data for only one sample of each plant and, even then, only for the plant part specified by the elders. To reach the level of accuracy needed for a predicative model, data for a multitude of other factors needs to be collected. Starting simply, data from multiple individual plants will be more telling and useful than just replicates of the same pool of collected plant material.

However, for this to be useful, replicated representatives would need to be collected across both space and time to give better resolution to the phytochemical fluctuations that occur over the course of the landscape and the seasons. Ideally, metabolomic data would also be collected for all plant parts of each species to gain the best understanding of the medicinal capacity across the full plant, but doing so would insinuate the same expansion of plant parts to the pharmacological analysis, which would be significantly more work.

It becomes clear that such a process may be excessive to put together. However, the presence of such a model would be a major boon to both the ethnopharmacological and medicinal fields, as new plants and their metabolomes could be added continuously to keep the model growing ever stronger. The ability to analyze a plant extract and, based on metabolome, receive a list of its most likely biological activities would revolutionize this kind of research. All over the world there are individual labs looking at individual parts of individual plants for small sets of activities at a time and connecting all that research in a larger collaboration could lead to better direction of use of natural health products, easier identification of active compounds, and could be used as a tool for reinvigorating motivation for maintenance of biological diversity.

On more of a practical note, there are things that can also be done immediately within the currently existing capacities of the TAAM that are more directly related to the project itself. A goal of this research since its inauguration has always been to find ways to incorporate traditional Cree medicines in a more directed way for local intervention strategies for prevention and management of diabetes. While the team has made great strides in identifying potential directed uses for these plants, it has also had to be very careful on what kind of recommendations it can actually make when it comes to the consumption of plants with possible safety concerns. That is one of the main reasons that a 'Plant Medicine Plan' has yet to be enacted in these communities, despite this research running for more than

a dozen years. The other likely reason is that the activities of these plants has been complex than originally expected and have not organized themselves in a way that is easy to interpret and use.

Through the multivariate analysis, this thesis identified specific groups of interest, of which there were usually a few species representatives. What should be done next is to take different combinations of those group representatives and test their extracts in combination for both their combined activity and safety on the previously evaluated assays. As a whole, additive and synergistic effects have so far not been evaluated on the Cree plants, and since it seems to look like certain groups of plants are associated with certain groups of activity, it makes sense to combine them to cover as large a range of anti-diabetic activity as possible. Combining all 17 plants is impractical, but by giving people just a few major categories to cover and which contain a couple different plant options to choose from, based on what is most closely available, this would give people the power to use their traditional medicines in this non-traditional diabetes context, but in a way that is directed and safe. A proposed separation of plants is presented in **Table 5.2**.

Table 5.2. Potential categories of Cree Plant Use

Glucose Management in Muscle Cells	**Glucose Management in Fat Cells**	**General Antioxidant/Antiglycation Effects**
A. balsamea bark	*P. banksian* cones	*G. hispidula* leaves
L. laricina bark	*P. mariana* cones	*K. angustifolia* leaves
R. groenlandicum leaves		*R. groenlandicum* leaves
S. purpurea		*R. tomentosum* leaves

The directions to be taken with the research on *O. horridus* is much simpler, as it essentially only has to follow the process constructed for evaluation of First Nations plants by TAAM over its history. More extensive work needs to be completed on this plant's capacity to interfere with other P450 enzymes, and even though work is currently being completed by Mayra Sanchez of the Haddad lab in Montreal on its effects on glucohomeostasis assays, there is still much work to complete related to its hyperglycemia effects activities.

REFERENCES

Agarwal, Ashok, Anamar Aponte-Mellado, Beena J Premkumar, Amani Shaman, and Sajal Gupta. 2012. "The Effects of Oxidative Stress on Female Reproduction: A Review." *Reproductive Biology and Endocrinology*. Vol. 10. https://doi.org/10.1186/1477-7827-10-49.

Bailey, Clifford J. 2017. "Metformin: Historical Overview." *Diabetologia* 60 (9): 1566–76. https://doi.org/10.1007/s00125-017-4318-z.

Bailey, Clifford J., and Robert C. Turner. 1996. "Metformin." Edited by Alastair J.J. Wood. *New England Journal of Medicine* 334 (9): 574–79. https://doi.org/10.1056/NEJM199602293340906.

Birben, Esra, Umit Murat Sahiner, Cansin Sackesen, Serpil Erzurum, and Omer Kalayci. 2012. "Oxidative Stress and Antioxidant Defense." *The World Allergy Organization Journal* 5 (1): 9–19. https://doi.org/10.1097/WOX.0b013e3182439613.

Bohlmann, Jörg, Jonathan Gershenzon, and Sébastien Aubourg. 2000. "Biochemical, Molecular Genetic and Evolutionary Aspects of Defense-Related Terpenoid Metabolism in Conifers." In , 109–50. https://doi.org/10.1016/S0079-9920(00)80006-4.

Booth, Gillian L, Moira K Kapral, Kinwah Fung, and Jack V Tu. 2006. "Relation between Age and Cardiovascular Disease in Men and Women with Diabetes Compared with Non-Diabetic People: A Population-Based Retrospective Cohort Study." *The Lancet* 368 (9529): 29–36. https://doi.org/10.1016/S0140-6736(06)68967-8.

Boullata, Joseph. 2005. "Natural Health Product Interactions with Medication." *Nutrition in Clinical Practice* 20 (1): 33–51. https://doi.org/10.1177/011542650502000133.

Bushra, Rabia, Nousheen Aslam, and Arshad Yar Khan. 2011. "Food-Drug Interactions." *Oman Medical Journal* 26 (2): 77–83. https://doi.org/10.5001/omj.2011.21.

Calway, Tyler, Guang-Jian Du, Chong-Zhi Wang, Wei-Hua Huang, Jing Zhao, Shao-Ping Li, and Chun-Su Yuan. 2012. "Chemical and Pharmacological Studies of Oplopanax Horridus, a North American Botanical." *Journal of Natural Medicines* 66 (2): 249–56. https://doi.org/10.1007/s11418-011-0602-2.

Ceriello, Professor Antonio. 2006. "OXIDATIVE STRESS AND DIABETES-ASSOCIATED COMPLICATIONS." *Endocrine Practice* 12 (Supplement 1): 60–62. https://doi.org/10.4158/EP.12.S1.60.

Chandler, R. F., S. N. Hooper, and M. J. Harvey. 1982. "Ethnobotany and Phytochemistry of Yarrow,Achillea Millefolium, Compositae." *Economic Botany* 36 (2): 203–23. https://doi.org/10.1007/BF02858720.

Chetyrkin, Sergei V., Missy E. Mathis, Amy-Joan L. Ham, David L. Hachey, Billy G. Hudson, and Paul A. Voziyan. 2008. "Propagation of Protein Glycation Damage Involves Modification of Tryptophan Residues via Reactive Oxygen Species: Inhibition by Pyridoxamine." *Free Radical Biology and Medicine* 44 (7): 1276–85. https://doi.org/10.1016/j.freeradbiomed.2007.09.016.

Core, Earl L. 1967. "Ethnobotany of the Southern Appalachian Aborigines." *Economic Botany* 21 (3): 199–214. https://doi.org/10.1007/BF02860370.

Courtois, Elodie A., Christopher Baraloto, C.E. Timothy Paine, Pascal Petronelli, Pierre-Alain Blandinieres, Didier Stien, Emeline Höuel, Jean-Marie Bessière, and Jérôme Chave. 2012. "Differences in Volatile

Terpene Composition between the Bark and Leaves of Tropical Tree Species." *Phytochemistry* 82 (October): 81–88. https://doi.org/10.1016/j.phytochem.2012.07.003.

Dandona, P, and A Aljada. 2002. "A Rational Approach to Pathogenesis and Treatment of Type 2 Diabetes Mellitus, Insulin Resistance, Inflammation, and Atherosclerosis." *American Journal of Cardiology* 90 (5A): 27–33.

Dawson, K. 2018. "Diabetes Care in First Nations Populations in British Columbia." *BRITISH COLUMBIA MEDICAL JOURNAL* 60 (9): 451–54.

Ekoe, Jean-Marie, Jean-Pierre Thouez, Claude Petitclerc, Peter Michael Foggin, and Parviz Ghadirian. 1990. "Epidemiology of Obesity in Relationship to Some Chronic Medical Conditions among Inuit and Cree Indian Populations in New Quebec, Canada." *Diabetes Research and Clinical Practice* 10 (January): S17–27. https://doi.org/10.1016/0168-8227(90)90135-G.

Fabricant, Daniel S., and Norman R. Farnsworth. 2001. "The Value of Plants Used in Traditional Medicine for Drug Discovery." *Environmental Health Perspectives* 109 (March): 69. https://doi.org/10.2307/3434847.

Foster, Brian C., J. Thor Arnason, and Colin J. Briggs. 2005. "NATURAL HEALTH PRODUCTS AND DRUG DISPOSITION." *Annual Review of Pharmacology and Toxicology* 45 (1): 203–26. https://doi.org/10.1146/annurev.pharmtox.45.120403.095950.

Fraser, Marie-Hélène, Alain Cuerrier, Pierre S. Haddad, John T. Arnason, Patrick L. Owen, and Timothy Johns. 2007. "Medicinal Plants of Cree Communities (Québec, Canada): Antioxidant Activity of Plants Used to Treat Type 2 Diabetes Symptoms." *Canadian Journal of Physiology and Pharmacology* 85 (11): 1200–1214. https://doi.org/10.1139/Y07-108.

Gaude, Eduardo, Francesca Chignola, Dimitrios Spiliotopulous, Silvia Mari, Andrea Spitaleri, and Michela Ghitti. 2012. "Muma: Metabolomics Univariate and Multivariate Analysis. R Package." https://cran.r-project.org/package=muma.

Gavin, JR, KGMM Alberti, MB Davidson, and et al. 2000. "Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus." *Diabetes Care* 23: S4–19. http://apps.webofknowledge.com/full_record.do?product=WOS&search_mode=GeneralSearch&qid=1&SID=3CVYqmVXp8ZNsG7JhjW&page=1&doc=1.

Government of Canada, Public Health Agency of Canada. 2011. "Chapter 2 – The Health Impact of Diabetes on Canadians - Diabetes in Canada: Facts and Figures from a Public Health Perspective - Public Health Agency of Canada."

Guengerich, F. Peter. 2008. "Cytochrome P450 and Chemical Toxicology." *Chemical Research in Toxicology* 21 (1): 70–83. https://doi.org/10.1021/tx700079z.

Gurley, Bill J, Stephanie F Gardner, Martha A Hubbard, D Keith Williams, W Brooks Gentry, Yanyan Cui, and Catharina Y W Ang. 2005. "Clinical Assessment of Effects of Botanical Supplementation on Cytochrome P450 Phenotypes in the Elderly." *Drugs & Aging* 22 (6): 525–39. https://doi.org/10.2165/00002512-200522060-00006.

Haddad, Pierre. 2018. "Publications." CIHR Team in Aboriginal Antidiabetic Medicine. 2018. http://www.taam-emaad.umontreal.ca/publications/publication_scientif.html.

Haddad, Pierre S., Lina Musallam, Louis C. Martineau, Cory Harris, Louis Lavoie, John T. Arnason, Brian

Foster, et al. 2012. “Comprehensive Evidence-Based Assessment and Prioritization of Potential Antidiabetic Medicinal Plants: A Case Study from Canadian Eastern James Bay Cree Traditional Medicine.” *Evidence-Based Complementary and Alternative Medicine*. https://doi.org/10.1155/2012/893426.

Hall, Dawn E, Philipp Zerbe, Sharon Jancsik, Alfonso Lara Quesada, Harpreet Dullat, Lina L Madilao, Macaire Yuen, and Jörg Bohlmann. 2013. “Evolution of Conifer Diterpene Synthases: Diterpene Resin Acid Biosynthesis in Lodgepole Pine and Jack Pine Involves Monofunctional and Bifunctional Diterpene Synthases.” *Plant Physiology* 161 (2): 600–616. https://doi.org/10.1104/pp.112.208546.

Harbilas, Despina, Louis C. Martineau, Cory S. Harris, Danielle C.A. Adeyiwola-Spoor, Ammar Saleem, Jennifer Lambert, Dayna Caves, et al. 2009. “Evaluation of the Antidiabetic Potential of Selected Medicinal Plant Extracts from the Canadian Boreal Forest Used to Treat Symptoms of Diabetes: Part II.” *Canadian Journal of Physiology and Pharmacology* 87 (6): 479–92. https://doi.org/10.1139/Y09-029.

Harp, Joyce B. 2004. “New Insights into Inhibitors of Adipogenesis.” *Current Opinion in Lipidology* 15 (3): 303–7. http://www.ncbi.nlm.nih.gov/pubmed/15166786.

Harris, Cory, Louis-Philippe Beaulieu, Marie-Hélène Fraser, Kristina McIntyre, Patrick Owen, Louis Martineau, Alain Cuerrier, et al. 2011. “Inhibition of Advanced Glycation End Product Formation by Medicinal Plant Extracts Correlates with Phenolic Metabolites and Antioxidant Activity.” *Planta Medica* 77 (02): 196–204. https://doi.org/10.1055/s-0030-1250161.

Harris, Cory S. 2008. “Anti-Diabetic and Neuroprotective Activities of Phytochemicals in Traditionally Used Boreal Plants.” *ProQuest Dissertations and Theses*. http://search.proquest.com.ezp-prod1.hul.harvard.edu/docview/911791953?accountid=11311%5Cnhttp://sfx.hul.harvard.edu/sfx_local?url_ver=Z39.88-2004&rft_val_fmt=info:ofi/fmt:kev:mtx:dissertation&genre=dissertations+&+theses&sid=ProQ:ProQuest+Dissertations+&+T.

Hartmann, Thomas. 1996. “Diversity and Variability of Plant Secondary Metabolism: A Mechanistic View.” *Entomologia Experimentalis et Applicata*. Vol. 80. Kluwer Academic Publishers. https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1570-7458.1996.tb00914.x.

He, Jinlong, Chunjiong Wang, Yi Zhu, and Ding Ai. 2016. “Soluble Epoxide Hydrolase: A Potential Target for Metabolic Diseases.” *Journal of Diabetes* 8 (3): 305–13. https://doi.org/10.1111/1753-0407.12358.

Hellum, Bent H, and Odd Georg Nilsen. 2008. “In Vitro Inhibition of CYP3A4 Metabolism and P-Glycoprotein-Mediated Transport by Trade Herbal Products.” https://doi.org/10.1111/j.1742-7843.2008.00227.x.

Henry, R R. 1997. “Thiazolidinediones.” *Endocrinology and Metabolism Clinics of North America* 26 (3): 553–73. https://doi.org/10.1016/S0889-8529(05)70267-X.

Höft, M, S K Barik, and A M Lykke. 1999. “Quantitative Ethnobotany Applications of Multivariate and Statistical Analyses in Ethnobotany.” http://pure.au.dk/portal/files/17477574/hoeftbariklykke1999.pdf.

Huang, Wei-Hua, Qing-Wen Zhang, Lan-Zhen Meng, Chun-Su Yuan, Chong-Zhi Wang, and Shao-Ping Li. 2011. “Oplopanphesides A—C, Three New Phenolic Glycosides from the Root Barks of Oplopanax

Horridus." *CHEMICAL & PHARMACEUTICAL BULLETIN* 59 (5): 676–79. https://doi.org/10.1248/cpb.59.676.

Huang, Weihua, Jing Yang, Jing Zhao, Chong-zhi Wang, Chun-su Yuan, and Shao-ping Li. 2010. "Quantitative Analysis of Six Polyynes and One Polyene in Oplopanax Horridus and Oplopanax Elatus by Pressurized Liquid Extraction and On-Line SPE–HPLC." *Journal of Pharmaceutical and Biomedical Analysis* 53 (4): 906–10. https://doi.org/10.1016/j.jpba.2010.06.021.

Huie, Carmen W. 2002. "A Review of Modern Sample-Preparation Techniques for the Extraction and Analysis of Medicinal Plants." *Analytical and Bioanalytical Chemistry* 373 (1–2): 23–30. https://doi.org/10.1007/s00216-002-1265-3.

Jaakola, Laura, and Anja Hohtola. 2010. "Effect of Latitude on Flavonoid Biosynthesis in Plants." *Plant, Cell and Environment* 33 (8): 1239–47. https://doi.org/10.1111/j.1365-3040.2010.02154.x.

Joseph, L. 2018. "The Yetwanay Project." 2018. https://www.leighjoseph.com/blog/.

Kajosaari, Lauri I., Tiina Jaakkola, Pertti J. Neuvonen, and Janne T. Backman. 2006. "Pioglitazone, an in Vitro Inhibitor of CYP2C8 and CYP3A4, Does Not Increase the Plasma Concentrations of the CYP2C8 and CYP3A4 Substrate Repaglinide." *European Journal of Clinical Pharmacology* 62 (3): 217–23. https://doi.org/10.1007/s00228-005-0093-8.

Keeling, C., and J. Bohlmann. 2006. "Genes, Enzymes and Chemicals of Terpenoid Diversity in the Constitutive and Induced Defence of Conifers against Insects and Pathogens." *New Phytologist*, no. 170: 657–75. https://journals-scholarsportal-info.proxy.bib.uottawa.ca/pdf/0028646x/v170i0004/657_geacotocaiap.xml.

Kelkar, Praful. 2005. "Diabetic Neuropathy." *Seminars in Neurology* 25 (02): 168–73. https://doi.org/10.1055/s-2005-871325.

Large, R G, and H N Brocklesby. 1938. "A HYPOGLYCAEMIC SUBSTANCE FROM THE ROOTS OF THE DEVIL'S CLUB (FATSIA HORRIDA)." *Canadian Medical Association Journal* 39 (1): 32–35. http://www.ncbi.nlm.nih.gov/pubmed/20321017.

Leduc, C., J. Coonishish, P. Haddad, and A. Cuerrier. 2006. "Plants Used by the Cree Nation of Eeyou Istchee (Quebec, Canada) for the Treatment of Diabetes: A Novel Approach in Quantitative Ethnobotany." *Journal of Ethnopharmacology* 105 (1–2): 55–63. https://doi.org/10.1016/j.jep.2005.09.038.

Lewis, David FV. 2004. "57 Varieties: The Human Cytochromes P450." *Pharmacogenomics* 5 (3): 305–18. https://doi.org/10.1517/phgs.5.3.305.29827.

Liu, Jie. 1995. "Pharmacology of Oleanolic Acid and Ursolic Acid." *Journal of Ethnopharmacology* 49 (2): 57–68. https://doi.org/10.1016/0378-8741(95)90032-2.

Liu, Wei-Rui, Wen-Lin Qiao, Zi-Zhen Liu, Xiao-Hong Wang, Rui Jiang, Shu-Yi Li, Ren-Bing Shi, and Gai-Mei She. 2013. "Gaultheria: Phytochemical and Pharmacological Characteristics." *Molecules*, no. 18: 12071–108.

Lok, CE, MJ Oliver, DM Rothwell, and JE Hux. 2003. "The Growing Volume of Diabetes-Related Dialysis: A Population Based Study." *NEPHROLOGY DIALYSIS TRANSPLANTATION* 19 (12): 3098–3103. http://apps.webofknowledge.com.proxy.bib.uottawa.ca/full_record.do?product=WOS&search_mode=GeneralSearch&qid=12&SID=4F1kHR4ZfmiaeE3WMiO&page=1&doc=1.

Marles, R.J., and N.R. Farnsworth. 1995. “Antidiabetic Plants and Their Active Constituents.” *Phytomedicine* 2 (2): 137–89. https://doi.org/10.1016/S0944-7113(11)80059-0.

McCune, Letitia M., and Timothy Johns. 2002. “Antioxidant Activity in Medicinal Plants Associated with the Symptoms of Diabetes Mellitus Used by the Indigenous Peoples of the North American Boreal Forest.” *Journal of Ethnopharmacology* 82 (2–3): 197–205. https://doi.org/10.1016/S0378-8741(02)00180-0.

Micales, J. A., J. S. Han, J. L. Davis, and R. A. Young. 1994. “Chemical Composition and Fungitoxic Activities of Pine Cone Extractives.” In *Mycotoxins, Wood Decay, Plant Stress, Biocorrosion, and General Biodeterioration*, 317–32. Boston, MA: Springer US. https://doi.org/10.1007/978-1-4757-9450-2_25.

Moore, Ben D., Rose L. Andrew, Carsten Külheim, and William J. Foley. 2014. “Explaining Intraspecific Diversity in Plant Secondary Metabolites in an Ecological Context.” *New Phytologist* 201 (3): 733–50. https://doi.org/10.1111/nph.12526.

Nachar, Abir, Ammar Saleem, John T. Arnason, and Pierre S. Haddad. 2015. “Regulation of Liver Cell Glucose Homeostasis by Dehydroabietic Acid, Abietic Acid and Squalene Isolated from Balsam Fir (Abies Balsamea (L.) Mill.) a Plant of the Eastern James Bay Cree Traditional Pharmacopeia.” *Phytochemistry* 117 (September): 373–79. https://doi.org/10.1016/j.phytochem.2015.07.001.

Nachar, Abir, Diane Vallerand, Lina Musallam, Louis Lavoie, Alaa Badawi, John Arnason, and Pierre S. Haddad. 2013. “The Action of Antidiabetic Plants of the Canadian James Bay Cree Traditional Pharmacopeia on Key Enzymes of Hepatic Glucose Homeostasis.” *Evidence-Based Complementary and Alternative Medicine* 2013: 1–9. https://doi.org/10.1155/2013/189819.

Nasri, Hamid, Azar Baradaran, Hedayatollah Shirzad, and Mahmoud Rafieian-Kopaei. 2014. “New Concepts in Nutraceuticals as Alternative for Pharmaceuticals.” *International Journal of Preventive Medicine* 5 (12): 1487–99. http://www.ncbi.nlm.nih.gov/pubmed/25709784.

Nguyen, San. 2011. “Inhibitory Properties of Funtional Food Plants on CYP Enzymes and Cree Traditional Medicines on Aldose Reductase.”

Nistor Baldea, Lidia A., Louis C. Martineau, Ali Benhaddou-Andaloussi, John T. Arnason, Émile Lévy, and Pierre S. Haddad. 2010. “Inhibition of Intestinal Glucose Absorption by Anti-Diabetic Medicinal Plants Derived from the James Bay Cree Traditional Pharmacopeia.” *Journal of Ethnopharmacology* 132 (2): 473–82. https://doi.org/10.1016/j.jep.2010.07.055.

Pickup, John C. 2004. “Inflammation and Activated Innate Immunity in the Pathogenesis of Type 2 Diabetes.” *Diabetes Care* 27 (3): 813–23. http://www.ncbi.nlm.nih.gov/pubmed/14988310.

Pluskal, L, S Castillo, A Villar-Briones, and M Oresic. 2010. “MZMine 2.0.” *BMC Bioinformatics* 11: 395.

Prance, Ghillean T. 1991. “What Is Ethnobotany Today?” *Journal of Ethnopharmacology* 32 (1–3): 209–16. https://doi.org/10.1016/0378-8741(91)90120-3.

Rajasekaran, Aiyalu, G Sivagnanam, and Rathinam Xavier. 2008. “Nutraceuticals as Therapeutic Agents: A Review PhD Scholar View Project Pharmaceutical Analysis View Project.” *Research Journal of Pharmacology and Technology* 1 (4): 328–40. https://www.researchgate.net/publication/268427982.

Ramasamy, Ravichandran, and Ira J. Goldberg. 2010. “Aldose Reductase and Cardiovascular Diseases,

Creating Human-Like Diabetic Complications in an Experimental Model." *Circulation Research* 106 (9): 1449–58. https://doi.org/10.1161/CIRCRESAHA.109.213447.

Rogachev, A D, N I Komarova, S V Morozov, V V Fomenko, and N F Salakhutdinov. 2007. "Phytochemical Studies of Rhododendron Adamsii Rehder. Quantitative Determination of Ursolic and Oleanolic Acids in Some Representatives of Ericaceae Family." *Chemistry for Sustainable Development*, no. 15: 561–64. http://www.sibran.ru/upload/iblock/22c/22cd660421573811af3037b4eb032cf5.pdf.

Rymer, L. 1976. "The History and Ethnobotany of Bracken." *Botanical Journal of the Linnean Society* 73 (1–3): 151–76. https://doi.org/10.1111/j.1095-8339.1976.tb02020.x.

Saleem, Ammar, Cory S. Harris, Muhammad Asim, Alain Cuerrier, Louis Martineau, Pierre S. Haddad, and John T. Arnason. 2010. "A RP-HPLC-DAD-APCI/MSD Method for the Characterisation of Medicinal Ericaceae Used by the Eeyou Istchee Cree First Nations." *Phytochemical Analysis* 21 (4): 328–39. https://doi.org/10.1002/pca.1203.

Scheen, A. 2007. "Drug-Drug and Food-Drug Pharmacokinetic Interactions with New Insulinotropic Agents Repaglinide and Nateglinide." *Clinical Pharmacokinetics* 46 (2): 93–108. https://doi.org/10.2165/00003088-200746020-00001.

Schuh, Beth A., and D. M. Benjamin. 1984. "The Chemical Feeding Ecology OfNeodiprion Dubiosus Schedl,N. Rugifrons Midd., AndN. Lecontei (Fitch) on Jack Pine (Pinus Banksiana Lamb.)." *Journal of Chemical Ecology* 10 (7): 1071–79. https://doi.org/10.1007/BF00987514.

Schultes, Richard Evans. 1994. "Amazonian Ethnobotany and the Search for New Drugs." https://onlinelibrary-wiley-com.proxy.bib.uottawa.ca/doi/pdf/10.1002/9780470514634.ch8.

Shamoon, H, H Duffy, N Fleischer, and et al. 1993. "THE EFFECT OF INTENSIVE TREATMENT OF DIABETES ON THE DEVELOPMENT AND PROGRESSION OF LONG-TERM COMPLICATIONS IN INSULIN-DEPENDENT DIABETES-MELLITUS." *NEW ENGLAND JOURNAL OF MEDICINE* 329 (14): 977–86. http://apps.webofknowledge.com.proxy.bib.uottawa.ca/full_record.do?product=WOS&search_mode=GeneralSearch&qid=16&SID=4F1kHR4ZfmiaeE3WMiO&page=1&doc=1.

Siest, Gérard, Elise Jeannesson, and Sophie Visvikis-Siest. 2007. "Enzymes and Pharmacogenetics of Cardiovascular Drugs." *Clinica Chimica Acta* 381 (1): 26–31. https://doi.org/10.1016/j.cca.2007.02.014.

Spoor, Danielle C.A., Louis C. Martineau, Charles Leduc, Ali Benhaddou-Andaloussi, Bouchra Meddah, Cory Harris, Andrew Burt, et al. 2006. "Selected Plant Species from the Cree Pharmacopoeia of Northern Quebec Possess Anti-Diabetic Potential." *Canadian Journal of Physiology and Pharmacology* 84 (8–9): 847–58. https://doi.org/10.1139/y06-018.

Szakiel, Anna, and Agnieszka Mroczek. 2007. "Distribution of Triterpene Acids and Their Derivatives in Organs of Cow-Berry (Vaccinium Vitis-Idaea L.) Plant *." www.actabp.pl.

Tam, Teresa W., Rui Liu, John T. Arnason, Anthony Krantis, William A. Staines, Pierre S. Haddad, and Brian C. Foster. 2009. "Actions of Ethnobotanically Selected Cree Anti-Diabetic Plants on Human Cytochrome P450 Isoforms and Flavin-Containing Monooxygenase 3." *Journal of Ethnopharmacology* 126 (1): 119–26. https://doi.org/10.1016/j.jep.2009.07.036.

Tam, Teresa W., Rui Liu, Ammar Saleem, John T. Arnason, Anthony Krantis, Pierre S. Haddad, and Brian C. Foster. 2014. "The Effect of Cree Traditional Medicinal Teas on the Activity of Human Cytochrome P450-Mediated Metabolism." *Journal of Ethnopharmacology* 155 (1): 841–46.

https://doi.org/10.1016/j.jep.2014.06.045.

Tarr, Joanna M, Kirti Kaul, Mohit Chopra, Eva M Kohner, and Rakesh Chibber. 2013. “Pathophysiology of Diabetic Retinopathy.” *ISRN Ophthalmology* 2013 (January): 343560. https://doi.org/10.1155/2013/343560.

Thommasen, H, R Wilson, and R Mcilwain. 1990. “EFFECT OF DEVILS CLUB TEA ON BLOOD-GLUCOSE LEVELS IN DIABETES-MELLITUS.” http://apps.webofknowledge.com.proxy.bib.uottawa.ca/full_record.do?product=WOS&search_mode=GeneralSearch&qid=12&SID=5BfBY83r4OYCW874hGk&page=1&doc=1.

Tontonoz, P, E Hu, and B M Spiegelman. 1995. “Regulation of Adipocyte Gene Expression and Differentiation by Peroxisome Proliferator Activated Receptor Gamma.” *Current Opinion in Genetics & Development* 5 (5): 571–76. http://www.ncbi.nlm.nih.gov/pubmed/8664544.

Triplitt, C., L. Glass, Y. Miyazaki, E. Wajcberg, A. Gastaldelli, E. De Filippis, E. Cersosimo, and R. A. DeFronzo. 2006. “Comparison of Glargine Insulin Versus Rosiglitazone Addition in Poorly Controlled Type 2 Diabetic Patients on Metformin Plus Sulfonylurea.” *Diabetes Care* 29 (11): 2371–77. https://doi.org/10.2337/dc06-0564.

Turner, Nancy Chapman, and Marcus A. M. Bell. 1971. “The Ethnobotany of the Coast Salish Indians of Vancouver Island.” *Economic Botany* 25 (1): 63–99. https://doi.org/10.1007/BF02894564.

Uprety, Yadav, Hugo Asselin, Archana Dhakal, and Nancy Julien. 2012. “Traditional Use of Medicinal Plants in the Boreal Forest of Canada: Review and Perspectives.” *Journal of Ethnobiology and Ethnomedicine* 8 (1): 7. https://doi.org/10.1186/1746-4269-8-7.

Walshe-Roussel, Brendan. 2014. “An Ethnobiological Investigation of Q’eqchi Maya and Cree of Eeyou Istchee Immunomodulatory Therapies.”

Westphal, Christina, Anne Konkel, and Wolf-Hagen Schunck. 2011. “CYP-Eicosanoids—A New Link between Omega-3 Fatty Acids and Cardiac Disease?” *Prostaglandins & Other Lipid Mediators* 96 (1–4): 99–108. https://doi.org/10.1016/j.prostaglandins.2011.09.001.

Wilkinson, Grant R. 2005. “Drug Metabolism and Variability among Patients in Drug Response.” *New England Journal of Medicine* 352 (21): 2211–21. https://doi.org/10.1056/NEJMra032424.

Williams, David, and John Feely. 2002. “Pharmacokinetic-Pharmacodynamic Drug Interactions with HMG-CoA Reductase Inhibitors.” *Clinical Pharmacokinetics* 41 (5): 343–70. https://doi.org/10.2165/00003088-200241050-00003.

World Health Organization. 1999. “Definition, Diagnosis and Classification of Diabetes Mellitus and Its Complications. Part 1: Diagnosis and Classification of Diabetes Mellitus.”

World Health Organization. 2016. “Global Report on Diabetes.” Geneva.

Xu, Xizhen, Ling Tu, Wenjing Feng, Ben Ma, Rui Li, Changlong Zheng, Geng Li, and Dao Wen Wang. 2013. “CYP2J3 Gene Delivery Up-Regulated Adiponectin Expression via Reduced Endoplasmic Reticulum Stress in Adipocytes.” *Endocrinology* 154 (5): 1743–53. https://doi.org/10.1210/en.2012-2012.

Yan, Liang-Jun. 2014. “Pathogenesis of Chronic Hyperglycemia: From Reductive Stress to Oxidative Stress.” *Journal of Diabetes Research* 2014: 1–11. https://doi.org/10.1155/2014/137919.

Yang, Xian-Wen, Su-Mei Li, Yun-Heng Shen, and Wei-Dong Zhang. 2008. “Phytochemical and Biological

Studies OfAbies Species." *Chemistry & Biodiversity* 5 (1): 56–81. https://doi.org/10.1002/cbdv.200890015.

Zhu, J, F Chen, L Wang, Y Niu, H Chen, H Wang, and Z Xiao. 2016. "Characterization of the Key Aroma Volatile Compounds in Cranberry (Vaccinium Macrocarpon Ait.) Using Gas Chromatography-Olfactometry (GC-O) and Odor Activity Value (OAV)." *Journal of Agricultural and Food Chemistry* 64 (24): 4990–99.

APPENDIX A: ADDITIONAL PHYTOCHEMICAL ANALYSIS OF CREE MEDICINAL PLANT EXTRACTS

Table A-1. Tentative and confirmed identifications for metabolites in Cree plant extracts through UNIFI (modified from Sunstrum et al., 2018).

Species	Plant Part	Tentative matches	Confirmed matches	Number of unreported confirmed matches*
Alnus incana (L.) Moench	Small branches	221	15	11
	Large branches	241	17	12
	Total		21	15
Arctostaphylos uva-ursi (L.) Spreng.		305	27	16
Gaultheria hispidula (L.) Muhl. ex Bigelow	Stems	267	30	21
	Shoot	237	28	18
	Total		41	27
Kalmia angustifolia L.	Leaf	246	24	14
Rhododendron tomentosum (Stokes) Harmaja ssp. subarcticum (Harmaja) G. Wallace*	Leaf	270	27	17
Rhododendron groenlandicum (Oeder) Kron & Judd	New leaf	223	22	13
	Old leaf	260	25	14
	Total		31	18
Lycopodium clavatum L.	Whole plant	163	11	5
Abies balsamea (L.) Mill.	Bark	195	7	5
Juniperus communis L.	Fruit	202	17	7
Larix laricina (Du Roi) K.Koch	Bark	221	15	11
Picea glauca (Moench) Voss	Bark	195	13	7
	Cone	208	14	8
	Leaf	238	23	16
	Total		33	22
Picea mariana (Mill.) Britton, Sterns & Poggenb.	Cone	155	13	6
Pinus banksiana Lamb.	Cone	213	18	13
Sorbus decora (Sarg.) C.K.Schneid.	Bark	287	19	14
Populus balsamifera L.	Bark	238	25	23
Salix planifolia Pursh	Bark	238	15	11
Sarracenia purpurea L.	Root	252	17	13
	Leaf	307	31	24
	Total		39	30

*Reporting status based on Kanaya database http://kanaya.naist.jp/KNApSAcK/.

APPENDIX B: ADDITIONAL ETHNOBOTANICAL, PHARMACOLOGICAL, AND PHYTOCHEMICAL EVALUATION OF *O. HORRIDUS*

Table B-1. Summary of *O. horridus* use entries found in the Native American Ethnobotany Database. (Moerman, 2003)

Part	**Use Category**						
	Cardiology	Dermatology	Gastrointestinal	Gynecological	Neurological	Orthopedic	Pulmonary
Leaves	0	0	0	0	0	1	0
Root	0	0	3	0	4	3	2
Stem/Bark	3	13	15	3	6	12	13

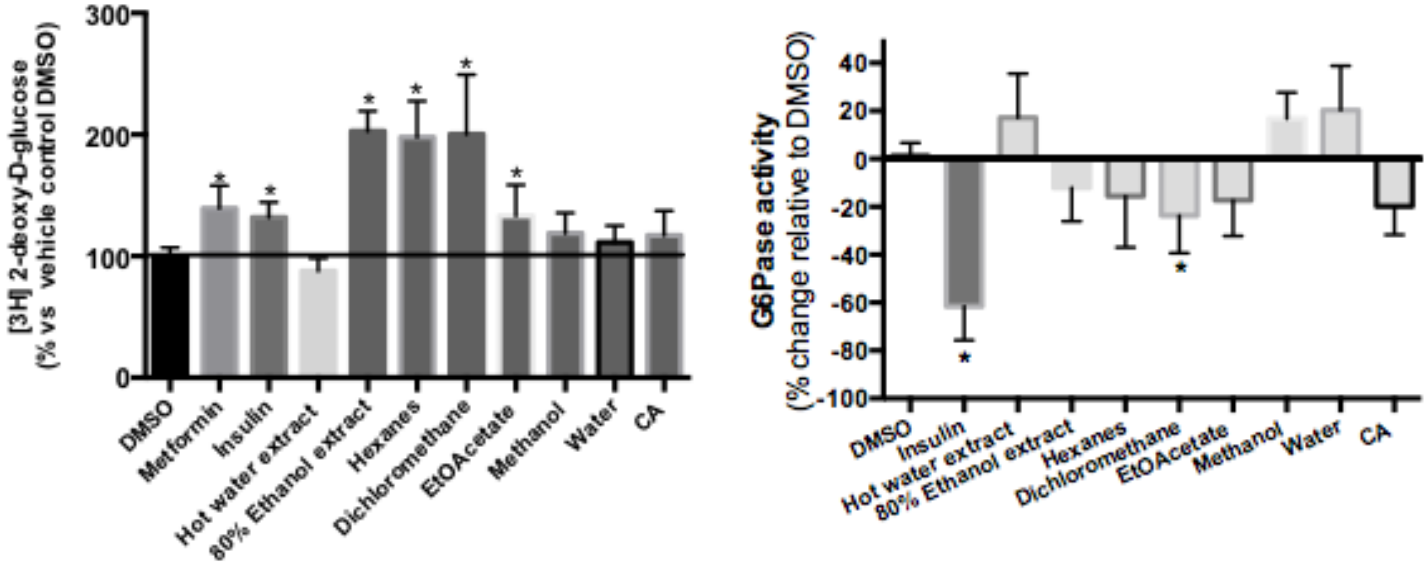

Figure B-1. Anti-diabetic activity of *O. horridus* extracts. (Sanchez et al., 2018).

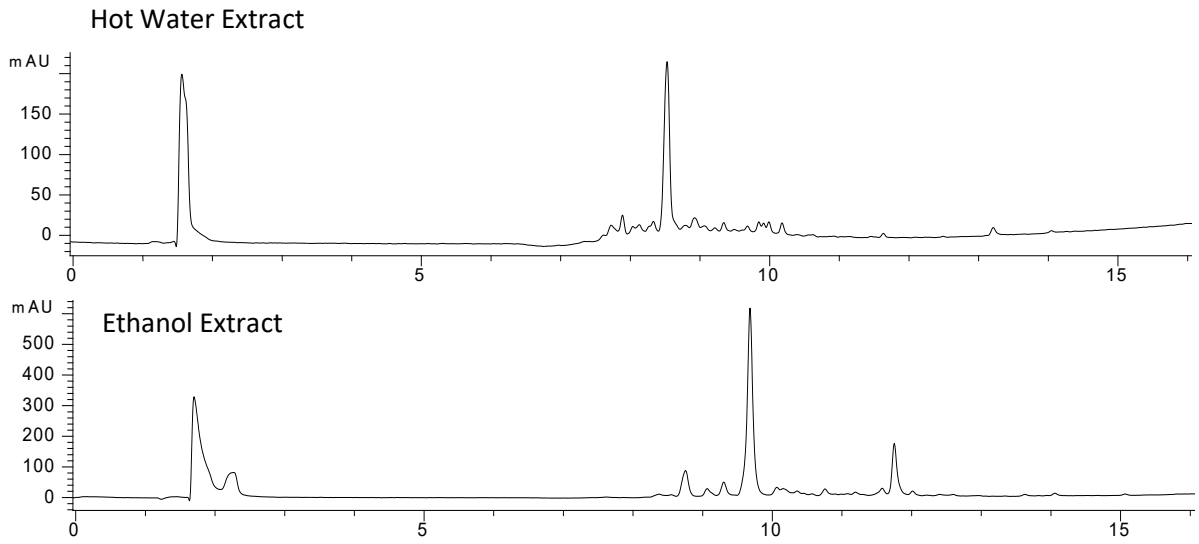

Figure B-2. HPLC-DAD Chromatogram of *O. horridus* inner bark extracts at 245nm.