

Ethnobotany, Pharmacology, and  
Metabolomics of  
Antidiabetic Plants used by the  
Eeyou Istchee Cree, Lukomir Highlanders,  
and Q'eqchi' Maya

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*The contemplative life is the best life.*

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## Abbreviations

Advanced glycation endproduct	AGE
Angiosperm Phylogeny Group 3	APG 3
Association of Universities and Colleges of Canada, PTP2	AUCC
Bosnia and Herzegovina	B&H
Bosnia and Herzegovina Mine Action Centre	BH MAC
Bobovica	B
Bovine serum albumen	BSA
Čuhovici	Č
Cross-cultural Health Initiative	CHI
Canadian International Development Agency	CIDA
Canadian Institute of Health Research Team for Aboriginal Antidiabetic	CIHR TAAM
Diode array detector	DAD
<i>Donji Lukomir</i>	DL
Informant consensus factor	F <sub>ic</sub>
<i>Gornji Lukomir</i>	GL
Garden outgroup	GO
High performance liquid chromatography	HPLC
Ultra performance liquid chromatography	UPLC
University of Ottawa Herbarium	OTT
Half maximal inhibitory concentration	IC <sub>50</sub>
Institute of Economic Botany	NYBG
Linear Angiosperm Phylogeny Group 3	LAPG 3
LOD	LOD
LOQ	LOQ
<i>Vodenica mlins</i>	M
Mass spectrometry	MS
The New York Botanical Garden Herbarium	NY
Hydrogen Nuclear magnetic resonance imaging	NMR
New York Botanical Garden	NYBG
1D NOESY	1D NOESY
University of Ottawa Herbarium	OTT
Principal Component analysis	PCA
Polytetrafluoroethylene	PTFE
Partnerships for Tomorrow Program Phase 2	PTP2
Syndromic importance value	SIV
Type 2 diabetes	T2D
TCI CryoProbe	TCI CryoProbe
Total phenolic estimation	TPE
Mass spectrometry-quadrupole time of flight	MS-QTOF
Wild outgroup	WO

## Abstract

A study was undertaken of plants used for treatment of diabetic symptoms by traditional healers of the Eeyou Istchee Cree (Canada), Lukomir Highlanders (Bosnia & Herzegovina), and Q'eqchi' Maya (Belize). All antidiabetic plants were ranked by syndromic importance value (SIV) based on 15 symptoms, all of which were recognized by the Cree and Maya and 8 by the Highlanders. The Cree used only 18 species, the Highlanders 41, and the Maya 150, numbers which reflect the diversity of flora in their region. *Vaccinium* (Ericaceae) was one of the few genera in all three regions and the only consensus genus between the Cree and Highlander study sites. The Q'eqchi' Maya ethnobotany did not present any cross-cultural consensus genera with Cree or Highlander medicinal plants, perhaps due to major biogeographic differences. In ethnopharmacological studies, *Vaccinium* species and Q'eqchi' antidiabetic plants were tested in an assay relevant to diabetes, the advanced glycation endproduct (AGE) inhibition assay. Boreal and tropical *Vaccinium* species were potent inhibitors of AGEs and demonstrated concentration dependent inhibition, with a half maximal inhibitory concentration (IC<sub>50</sub>) range of 5.93–100 µg/mL. Phenolic content ranged from 80.3 to 201 µg/mL in boreal samples and from 1470 to 2170 µg/mL in tropical samples. Tropical species have a greater phenolic content and AGE inhibition. Seven Q'eqchi' antidiabetic plant species were tested and all plant extracts showed AGE-inhibition. The IC<sub>50</sub>s ranged from 40.8 to 733 µg/mL, and the most active was *Tynanthus guatemalensis* Donn.. *Tynanthus guatemalensis* IC<sub>50</sub> was about five times greater (less active) than the mean ± SE IC<sub>50</sub> reported for six tropical *Vaccinium* species of *Vaccinium* (8.77 ± 0.79 µg/mL).

The highest consensus and most active Maya antidiabetic plant, *Tynanthus guatemalensis* Donn. Sm. was discovered to be an important plant recorded in archeological artifacts from the Late Classic Maya period (~750 CE). Ancient Maya used a cross shaped sign (*k'an* glyph) as a decorative element on Late Classic polychrome vessels and murals. The sign was believed to be the xylem template for a plant used as a flavouring in cacao drinks. However, the plant was incorrectly identified in the literature as *Pimenta dioica* (L.) Merr. (common name: Allspice) based on a common name and aromatic plant quality – not from a botanical voucher specimen. *Pimenta dioica* wood does not have a cross shape visible in the xylem but a unique character visible after a cross section of *T. guatemalensis*, is the xylem's cross shape organization. Wood of *T. guatemalensis* also has an "allspice" aroma. *Tynanthus guatemalensis* is most likely the true botanical template behind the ancient Maya *k'an* glyph and this finding would show the continuity of use of this medicinal plant from ancient to modern times.

*Vaccinium* was selected for an in depth phytochemical analysis using modern metabolomic methods. Nuclear magnetic resonance (<sup>1</sup>H NMR) was used to evaluate leaf extract spectra to provide information on (1) the taxonomic identity and (2) quantities of bioactive metabolites across multiple sites. Spectra clearly differentiated leaf samples of *V. angustifolium*, *V. boreale*, *V. corymbosum*, *V. macrocarpon*, *V. myrtilloides*, *V. myrtillus*, *V. ovalifolium*, and *V. uliginosum* according to generic, subgeneric, specific, phenotypic circumscriptions. Quantification of chlorogenic acid and hyperoside were replicated with a method that is highly reproducible across multiple sites with different NMR equipment. This methodology provides an important new approach to taxonomy and quality control for plants and natural health products.

## Résumé

Une étude a été réalisée sur l'utilisation des plantes par les guérisseurs traditionnels chez les Cris d'Eeyou Istchee (Canada), les Montagnards de Lukomir (Bosnie-Herzégovine) et les Mayas Q'eqchi' (Belize) pour le traitement des symptômes du diabète. Toutes les plantes antidiabétiques ont été classées selon leur valeur d'importance syndromique (SIV), calculée à partir de 15 symptômes, dont chacun a été reconnu par les Cris et Mayas et 8 par les Montagnards. Les 18, 41 et 150 espèces utilisées par les Cris, les Montagnards, et les Mayas, respectivement, représentent des chiffres reflétant la diversité floristique de chaque région. *Vaccinium* (Ericaceae) était l'un des quelques genres présent dans chacune de ces trois régions et le seul communément mentionné par les Cris et les Montagnards. Cependant, aucun consensus interculturel n'a été détecté entre l'ethnobotanique maya Q'eqchi' et les plantes médicinales cries ou montagnardes, peut-être en raison de grandes différences biogéographiques. Lors d'études ethnopharmacologiques, des espèces de *Vaccinium* et des plantes antidiabétiques q'eqchi' ont été évaluées à l'aide d'un bio-essai pertinent au diabète, soit l'inhibition des produits finaux de glycation avancée (AGE). Les espèces de *Vaccinium* boréales et tropicales étaient de puissants inhibiteurs d'AGE et démontraient une inhibition proportionnelle à la concentration avec une concentration inhibitrice médiane (CI<sub>50</sub>) variant de 5,93 à 100 µg/mL. Leur contenu phénolique variait de 80,3 à 201 µg/mL chez les espèces boréales et de 1470-2170 µg/mL chez les espèces tropicales. Ces dernières possédaient une teneur en composés phénoliques et une activité inhibitrice d'AGE plus élevées. Sept espèces de plantes antidiabétiques q'eqchi' ont aussi été testées et ont tous montré une activité inhibitrice d'AGE. Les CI<sub>50</sub> variaient de 40,8 à 733 µg/mL et le plus actif était *Tynanthus guatemalensis* Donn., avec une valeur d'environ cinq fois plus élevée (moins actifs) que la moyenne ( $\pm$  erreur type) rapportée pour les 6 espèces tropicales de *Vaccinium* ( $8,77 \pm 0,79$  µg/mL).

Il a été découvert que l'espèce antidiabétique maya la plus active, et possédant le consensus le plus élevé, *Tynanthus guatemalensis*, est une plante importante rapportée parmi les artefacts archéologiques de l'époque Maya du classique tardif (~ 750 CE). Les Mayas ont historiquement utilisés un signe en forme de croix (glyphe *k'an*) comme élément décoratif sur les vaisseaux et peintures murales polychromes du classique tardif. Le signe était considéré comme étant le modèle du xylème d'une plante utilisée comme aromatisant dans les boissons à base de cacao. Cependant, cette plante a été incorrectement identifiée dans la littérature comme étant *Pimenta dioica* (L.) Merr. (nom commun: piment de la Jamaïque) suite à l'un de ses noms communs et ses qualités aromatiques, au lieu d'un spécimen d'herbier. Quoique le bois de *P. dioica* n'a pas une forme de croix visible dans le xylème, cette-dernière est une caractéristique unique visible sur les coupes transversales de *T. guatemalensis*. Le bois de *T. guatemalensis* possède également un arôme ressemblant à celui du piment de la Jamaïque. *Tynanthus guatemalensis* est probablement le vrai modèle botanique derrière l'ancien glyphe maya *k'an*, une trouvaille démontrant l'utilisation continue de cette plante médicinale depuis l'Antiquité.

Le genre *Vaccinium* a été sélectionné pour une analyse phytochimique approfondie en utilisant les plus récentes méthodes métabolomiques. La résonance magnétique nucléaire (RMN 1H) a été utilisée pour analyser le spectre des extraits foliaires afin de fournir des informations sur (1) leur identité taxinomique et (2) la quantité des composantes bioactives entre plusieurs sites. Ces spectres permettent de clairement différencier les échantillons de *V. angustifolium*, *V. boreale*, *V. corymbosum*, *V. macrocarpon*, *V. myrtilloides*, *V. myrtillus*, *V. ovalifolium*, et *V. uliginosum* selon leur classification taxinomique et délimitation phénotypique. La quantification de l'acide chlorogénique et de l'hypéroside a été répliquée à l'aide d'une méthode hautement reproductible sur plusieurs sites et divers équipements RMN. Cette méthodologie fournit une nouvelle approche importante dans l'identification et le contrôle de la qualité des produits de santé naturels.

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- Fig 4.S2.** UPLC-MS-QToF analysis of *Tynanthus guatemalensis* crude extract. (A) Katchimoside analogues (564.1404) (B) 4-hydroxybenzoate (138.0235) (C) Salicylic acid (138.0097) (D) Crude extract trace. Exact mass and molecular formula search could not detect eugenol, leucosceptoside and isoverbascoside reported in Plaza et al. 2005.
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- Figure 5.3.** *Vaccinium myrtillus* and *V. poasanum* inhibition of advanced glycation endproducts. (A) The wild *V. myrtillus* (BBE 42) exhibits 5.44 IC<sub>50</sub> (R<sup>2</sup> = 0.903) and (B) the common garden *V. poasanum* (VPO11) exhibits a 7.62 IC<sub>50</sub> (R<sup>2</sup> = 0.953). The positive control, quercetin, gave 90% inhibition.
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- Figure 6.4.** Identification of species in section *Cyanococcus*. Principal Component analysis of wild *Vaccinium* samples from section *Cyanococcus* including: *Vaccinium angustifolium* (black x's, n = 88), *V. corymbosum* (blue dots, n = 21), *V. boreale* (green triangles, n = 9) leaf samples.
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- Figure 7.1.** NMR 1H spectral assignments (17) and molecular structure (inset) of chlorogenic acid. Signals at ca. 3.3 ppm and 2.5 ppm were assigned to the residual water and DMSO, respectively.
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- Figure 7.3.** Top and bottom overlay of spectra from the aromatic region of blueberry leaf extracts (blue) and pure hyperoside (red) and chlorogenic acid (green). Quantifiable hyperoside signal is shown at 7.7 ppm (inset).
- Figure 7.4.** Recovery chart displaying the amount of chlorogenic acid measured by NMR from doped blueberry leaf extract samples with known amounts of chlorogenic acid. Percent recovery represents the amount of calculated chlorogenic acid content versus the amount of added chlorogenic acid (minus the amount of chlorogenic acid in the sample).

**Figure 7.5.** One dimensional representation of the steps of the method tested during Youden's trial of ruggedness.

**Figure 8.1.** Average spectrum for each species – *Vaccinium angustifolium* (black line), *V. ovalifolium* (blue line), and *V. macrocarpon* (green line) (A) full spectrum (10.5 to -0.25 ppm) (B) expansion in the region containing the characteristic signals for chlorogenic acid (7.90 to 6.05 ppm).

**Figure 8.2.** Scores plot for all three species, *Vaccinium angustifolium* (black diamonds), *V. ovalifolium* (blue squares), and *V. macrocarpon* (green triangles). Hotelling ellipses, showing the region of the 95% confidence limit, are shown for each group by dashed lines of the matching color. Note that spectra for each species occupy a distinct region, with no overlap.

**Figure 8.3.** Representative calibration curve (from the David H. Murdock Research Institute's 600 MHz spectrometer). The plot shows integrated peak area, scaled by the 90-degree pulse length, versus the chlorogenic acid concentration. The data were fit to a line with a y-intercept of zero. The equation for the line and the square of the correlation coefficient ( $R^2$ ) are shown.

**Figure 8.4.** Scores plots for representative spectra for each species, with the spectra acquired in different laboratories included. In all three panels, spectra of the identical *Vaccinium angustifolium* sample sent to different laboratories are represented by red circles. Hotelling ellipses, showing the region of the 95% confidence limit, are shown for each group by dashed lines of the matching color. (A) Scores plot for *V. angustifolium* with the spectra acquired in different laboratories included. Spectra of the *V. angustifolium* samples collected at Bruker are represented by black diamonds. Note that the ellipse for the spectra acquired in different laboratories is almost completely within the ellipse for the diverse *V. angustifolium* sample set. (B) Scores plot for *V. ovalifolium* with the spectra acquired in different laboratories included. Spectra of the *V. ovalifolium* samples collected at Bruker are represented by blue squares. Note that the ellipse for the spectra acquired in different laboratories is well-separated from the ellipse for the *V. ovalifolium* samples. (C) Scores plot for *V. macrocarpon*, with the spectra acquired in different laboratories included. Spectra of the *V. macrocarpon* samples collected at Bruker are represented by green triangles. Note that the ellipse for the spectra acquired in different laboratories is well-separated from the ellipse for the *V. macrocarpon* samples.

**Figure 9.1.** Canadian Institutes of Health Research Team for Aboriginal Antidiabetic Medicine (CIHR TAAM) longhouse meeting in Misstissini, Eeyou Istchee Territory, Quebec, Canada.

## Chapter 1

# Introduction

Today, type 2 diabetes is a major health concern in societies. Type 2 diabetes (T2D) (formerly called non-insulin-dependent or adult-onset) results from the body's ineffective use of insulin and is characterized by hyperglycaemia. T2D incidence is three times higher in indigenous populations than that of the general population. These are some of the fastest growing yet most vulnerable populations in the world that often lack culturally appropriate primary healthcare (Alberti et al. 2004; Ahmed 2005; Helin 2008). T2D is responsible for 90% of diabetes cases (Alberti and Zimmet 1998) and is linked to rapid social change, genetics, dietary acculturation, excess carbohydrate consumption, physical inactivity, excess body weight, and cultural resistance to modern pharmaceuticals (Young 1994; Hegele et al. 1999; Young et al. 1992; Young et al. 2000; Ritenbaugh & Goodby 1989; WHO 2013a; CIHR TAAM 2006). In this thesis, partnerships with three indigenous communities who remain concerned about diabetes and the preservation of traditional medical knowledge and culture, led to a scientific investigation of traditional treatments.

### **Background and Literature Review**

#### ***The type 2 diabetes context in three indigenous communities***

The existing indigenous partners for this study were the Eeyou Istchee Cree of Canada; the Lukomir Highlanders of Bosnia and Herzegovina; and the Q'eqchi' Maya of Belize. All partners wish to alleviate the devastating effects of type 2 diabetes and identify relevant effective antidiabetic plants. The Eeyou Istchee Cree in Northern Quebec (James Bay) are a member of the Algonquian language family with nine communities located between 49° N and 55° N. The Eeyou Istchee Cree are a subpopulation of the greater Cree Nation, representing 14,131 of 77,970 Cree people in the 2006 census (Statistics Canada 2008). The prevalence of diabetes in the Eeyou Istchee territory was fast approaching 20% (CIHR TAAM 2006) in 2005, when the CIHR Team in Aboriginal Antidiabetic Medicines (CIHR TAAM) was formed as a partnership of two Cree communities and six Canadian

laboratories to study the safety and efficacy of traditional medicines (<http://www.taam-emaad.umontreal.ca>). The first step was an ethnobotanical survey of culturally relevant Cree antidiabetic plants using a new quantitative approach to ethnobotany.

The Lukomir Highlanders of Bosnia and Herzegovina (B&H) speak *Bosanski* and are one of Europe's last indigenous communities living a semi-nomadic lifestyle in the Bjelašnica region of the Dinaric Alps. There are approximately 60 families in residence in the highest settlement of B&H. The Lukomir Highlanders are historically Bogomil and their ancestry is marked by ancient *stećci* (gravestones) which are still present in their territory (Nikolić and Šarančić 2011). The Highlanders have a historic connection to semi nomadic peoples from the Podveležje Plateau near Mostar, B&H who travelled to Bjelašnica with their livestock in search of water during the summer months (Tourism Association of FB&H 2005). In my introductory meetings with families and traditional healers in Lukomir, the community wished to investigate matters of health and environment to help determine items which could assist in their health care. Following this request, we consulted with the one volunteer physician who holds a clinic each summer in Lukomir. We learned that Lukomir's priority health concerns are heart disease and diabetes. The World Health Organization (WHO) estimated that B&H had 111,000 people with diabetes in 2000 and was expected to increase to 180,000 by 2030 (WHO 2013b).

The Q'eqchi' Maya are one of 31 sub-tribes of the greater Maya (Coe 2011). The Q'eqchi' speak their own distinct native language Q'eqchi' and inhabit areas of Guatemala, Belize, and Mexico, with a strong representation between 15° N and 17° N in the vicinity of the Maya Mountains. Our laboratories' longstanding collaborations with the *Q'eqchi'* has produced a number of ethnobotanical surveys that were based out of Toldeo District, Belize (Awad 2009, Balick 2013, 2001; Bourbanais-Spear 2005; De Gezelle 2013; Treyvaud Amiguet 2005); however, there have been no Q'eqchi' ethnobotanical studies that focused specifically on diabetes. Between 2005 and 2006, studies by the Central American Diabetes Initiative (CAMDI 2009) demonstrated that the overall prevalence of T2D in Belize was 13.1%, while prevalence of T2D among Belize's native population was 8.3%. According to

the WHO and World Health Rankings (2011) diabetes is the leading cause of death in Belize.

### ***Quantitative ethnobotanical methods***

Given the cross-cultural risk of diabetes among the Cree, Highlander, and Q'eqchi' communities and their use of traditional medicines, quantitative cross-cultural ethnobotanical methods were required to numerically rank taxa used as medicine. The informant consensus ( $F_{ic}$ ) function, created by Trotter and Logan (1986) is often used as a quantitative method (Heinrich 2000; Amiguet 2005) in general ethnobotanical surveys. Because healers may use the same plant in different symptom categories the  $F_{ic}$  function incorporates two variables, the number use reports ( $n_{ur}$ ) from a number of taxa ( $n_{taxa}$ ) per usage category<sup>1</sup>. The data are quantified by adding up the individual reports on the uses of each plant for each category. A taxon may be used in several usage categories, but, each plant may only be considered once per healer per category. This means that if one informant used a plant to treat more than one disease in the same category, one would consider that, one use-report. Satisfying the  $F_{ic}$  function indicates how homogenous the ethnobotanical information is and supplies a numerical factor for categories of phytochemical and pharmacological interest.

In disease specific ethnobotanical surveys, the syndromic importance value (SIV) function can be utilized to prioritize taxa for pharmacological and phytochemical analyses. The SIV function was created by Leduc et al. (2006) with the Eeyou Istchee Cree and the CIHR TAAM antidiabetic project. SIV values are derived by accounting for: (1) the number of different symptoms for which a plant is cited, (2) the frequency of plant citation by individual informants, and (3) the association rank of symptoms for which a plant is cited to treat. The SIV function accounts for  $w$ , the weight of the symptom (absent from published literature),  $s$ , the symptom treated by the species,  $f$ , the frequency of citation for the species,  $S$ , the total number of symptoms used for the survey (used in Leduc et al. 2006, but was not

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<sup>1</sup> Categories are most often classified according to Cook's Economic Botany Data Collection Standard (1995).

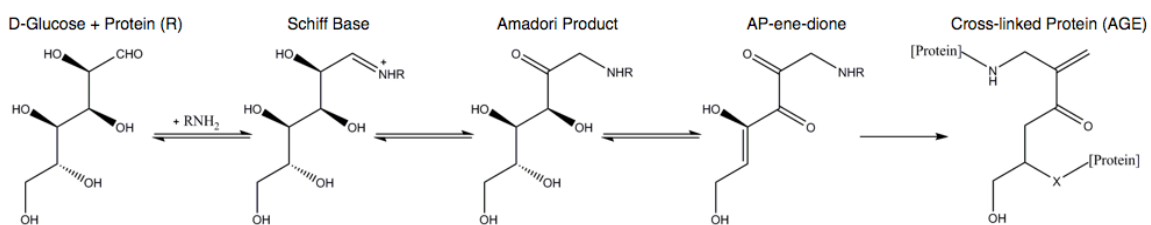
indicated in the denominator on the bottom right), and F the total number of interviews in the survey. Since the symptoms of diabetes would be a central component of an ethnobotanical questionnaire, and there are no published association weights ( $w$ ) to date for the SIV function, we were required to develop the association weights ( $w$ ) for the symptoms of diabetes.

By considering the variables of the SIV function, families and genera with large SIV values could potentially deliver new medicines for diabetes. Indeed, in cross-cultural studies like this, many families, genera, and species will present in a high ranked SIV group. How should one proceed with so many options in a limited amount of time? Studies have indicated that targeting closely related taxa is a sufficient strategy for bioactivity screening (Cox and Balick 1994; Rønsted et al. 2008; Saslis-Lagoudakis et al. 2011). However, taxa that have strong phylogenetic signal (SIV values for instance) plus cross-cultural consensus, have been demonstrated to contain more bioactive plants than random samples (Saslis-Lagoudakis et al. 2012).

### ***Diabetes and advanced glycation endproducts (AGEs)***

Studies have indicated that hyperglycaemia is the primary cause of diabetic complications (Brownlee et al. 1988). Evidence suggests that hyperglycemia is responsible for diabetes related tissue damage by (1) repeated acute changes in cellular glucose metabolism and (2) accumulation of glycated biomolecules and advanced glycation end products (AGEs) (Negre-Salvayre et al. 2009). Formation of AGEs is initiated when glucose and extracellular matrix proteins form chemically reversible adducts known as Schiff-bases. The Schiff-base, an early glycation product formed at a rate proportional to glucose concentration, rearranges to form an Amadori-type product in a matter of hours to weeks. The Amadori product is still chemically reversible (Brownlee et al. 1984). This is an important therapeutic window. Next, the early glycation product advances to a fluorescing cross-linked protein, trapping various molecules, and contributes to the development of retinopathy, cataracts, atherosclerosis, neuropathy, nephropathy, diabetic embryopathy and impaired wound healing in both diabetes and the aging process (Vik 2001; Ahmed 2005).

Recent studies suggest that the interaction of AGEs with the receptor for AGEs (RAGE) alter intracellular signalling, gene expression, release of pro-inflammatory molecules and free radicals that contribute towards the pathology of diabetic complications (Ahmed 2005). These diabetic insults impair glucose uptake of organ cells that do not require insulin for glucose uptake (the nervous system, heart, kidneys, and small blood vessels) which consequently leads to high concentrations of intracellular glucose especially after prolonged as well as and during hyperglycaemic periods. This results in impaired cell function and cell death (Ahmed, 2005). Since these T2D complications are mediated by the formation of AGEs, AGEs are an important therapeutic target with phytotherapies.



**Figure 1.1.** The chemistry of advanced glycation endproduct (AGE) formation (Rendered by Jonathan Ferrier © 2014) (Vik 2001). Cross-linking of proteins and the trapping of various molecules by AGEs and intermediates are associated with diabetes and the aging process, and contribute to the development of diseases such as atherosclerosis, kidney, vascular, and neurological diseases. Glycation is linked to multifactorial diseases such as Alzheimer's disease.

### *Floristics of the three ethnobotanical study sites*

The Eeyou Istchee Cree inhabit an area of northeastern Canada on the east coast of the world's largest inland sea, the Hudson Bay. The Eeyou Istchee territory is primarily taiga and boreal forest, bordered by temperate deciduous forest on the south and arctic tundra on the north. Common boreal families include the Pinaceae, Ericaceae, and the Betulaceae which are often surrounded by what the Cree call *maskek* (muskeg). Flora of North America is our primary taxonomic reference for the area (2008), while the Flora of Canada (Scoggan 1978) and Shrubs of Ontario (Soper & Heimburger 1982) were found to be useful for specific and sub-specific taxa and distributions. Herbaria at Acadia University (ACAD), University of Ottawa (OTT), and Montreal Botanical Gardens (MT) offer suitable references for the Eeyou Istchee territory.

The Lukomir Highlanders' territory is within the Dinaric Alps of the Bjelašnica Mountain area along the Rakitnica Canyon in Bosnia and Herzegovina (B&H). This area is classified as an alpine biogeographic region that is closely bordered by Mediterranean and continental biogeographic regions (European Environment Agency 2012a). The Lamiaceae thrive on the limestone south face of the Rakitnica Canyon. The forests of Lukomir are dominated by Betulaceae which are bordered by the trees and shrubs of the Rosaceae. Domac's Mala Flora Hrvatske i Susjednih Područja (1984) was our reference flora with voucher reference material from the University of Sarajevo. Land mines are still an issue in B&H and were avoided by consulting with Bosnia and Herzegovina Mine Action Center (BH MAC) (<http://www.bhmac.org>). The total mine contaminated area of B&H currently covers around 127420 km<sup>2</sup> or 2.5% of total country size.

The Q'eqchi' Maya source medical taxa from numerous vegetation sites including swamps and mangroves, semi-arid scrub and savanna, as well as coastal and montane pine and rain forests. There are 3,300 endemic species to Central America with 41 native to Belize (CPD 2013, Balick et al. 2001). Important medicinal families of the area include the Piperaceae, Rubiaceae, and Asteraceae which are often sourced from the limestone rich Maya Mountains. The Flora of Guatemala was our primary taxonomic reference for determining (Standley & Steyermark 1946), while the New York Botanical Gardens and additional vouchers from Costa Rica Herbaria were available. All plants in this dissertation follow tropicos.org.

When one examines the floristics within the ethnobotanical literature of our ethnobotanical partners' regions, the Ericaceae stand out with strong a record of traditional use in North America and Europe, especially the genus *Vaccinium* L.. Leduc's records show that the Eeyou Istchee Cree made use of numerous Ericaceae taxa, especially *Gaultheria hispidula* (L.) Muhl., *Kalmia angustifolia* L., *Rhododendron groenlandicum* (Oeder) Kron & Judd, *V. angustifolium* Aiton, and *V. vitis-idaea* L. (2006). While the Lukomir Highlanders do not have a published ethnobotanical survey (hence our investigations), Redžić determined that the Ericaceae represented 1.77% of all medicinal family use in B&H and

included *Arbutus unedo* L., *Calluna vulgaris* (L.) Hull., *V. myrtillus* L., and *V. vitis-idaea* L. (Redžić 2007, 2010).

The Ericaceae have a wide distribution in the northern hemisphere and can be found in most biogeographical regions except for deserts. Ericaceae and species of *Vaccinium* are often found on open mountain slopes in the tropics (Pedraza 2009; Camp 1942, 1943, 1945). *Vaccinium* use in Lukomir was highly probable due to the montane location; however, to uncover ethnomedical use of Ericaceae in Belize, one would have to target forests with oaks and pines such as the areas before the Maya Mountains at the Bladen Nature Reserve, Toldeo District, Belize, or in the mountain pine ridge forests near Caracol, at the Guatemala frontier. Interestingly, *Vaccinium* spp. are known to exist in central America with new species recently discovered in Mexico, in habitats similar to the Belize montane pine forests. *Vaccinium* species can be found in all countries bordering Belize, but to date remain absent from the Belize floristic record.

The genus *Vaccinium* belongs to the Asteridae order Ericales which is composed of 25 families containing 353 genera with more than 11,000 species (Stevens 2003). The Ericales' family Ericaceae contains 145 genera (Stevens 2003) grouped into eight subfamilies: Enkianthoideae, Pyroloideae, Monotropeoideae, Arbutoideae, Cassiopoideae, Ericoideae, Harrimanelloideae, Styphelioideae, and Vaccinioideae (Kron 2002). The Vaccinioideae's tribes include Oxydendreae, Lyonieae, Gaultherieae, Andromedeae, Vaccinieae. Vaccinieae's genus *Vaccinium* is comprised of 214 accepted, 265 synonyms, and 515 unresolved species. (The Plant List 2012).

### ***Delimiting the Ericaceae***

Kron et al. (2002) produced a new classification of Ericaceae based on phylogenetic analyses of nuclear and chloroplast DNA sequence data, morphology, anatomy, and embryology. The authors' aim was to delimit the Ericaceae beyond what traditional evolutionary taxonomic approaches were capable of with characters thought to be significant based on general notions of evolution. Ernisse & Kluge (1993) outlined that taxonomic

congruence and total evidence methods were paradigms in phylogenetic analyses. Kron et al. would follow the latter.

Morphological and character data was listed at the First International Ericaceae Conference where 91 character states were compiled. Genomic data was collected from sequenced nuclear ribosomal (18s) and chloroplast genes (*matK* and *rbcL*) from appropriate subset taxa of Ericaceae to construct phylogenetic trees and determine: (1) the sister groups to the Ericaceae, (2) the relationships of Empetraceae and Epacridaceae to Ericaceae, and (3) the tribal relationships within Ericaceae. Using the total evidence approach (Emissé & Kluge 1993) a combined analysis of morphology, *matK*, and *rbcL* was performed for 59 taxa of Ericaceae using PAUP\* 4.0 (Phylogenetic Analysis Using Parsimony) (Swofford 1999).

Kron et al. were able to conclude that all previous non-phylogenetic phylogenies of the Ericaceae were not perfect since many conclusions were based on character states that are extremely homoplasious – similarities not due to homology or common ancestry, but the result of independent evolutionary change, i.e. bracteole position, indumentum of the corolla and filaments, anther texture, presence and positioning of staminal appendages, fruit dehiscence, and texture (Kron et al. 2002). Future delimiting approaches should continue with a detailed phylogenetic analysis using several genes and a detailed survey of phenotypic characters.

The Ericaceae with eight subfamilies and 20 tribes are characterized by benzoquinones and naphthoquinones, route I secoiridoids +, ellagic acid 0; vessel elements with simple perforation plates; lamina vernation involute, margins entire to toothed, teeth associated with multicellular hairs; inflorescence terminal; tapetal cells uni-, bi- or multinucleate [*Empetrum*]; pollen >26µm, surface ± rugulate; stigma expanded; testa with outer wall unthickened; chloroplast *infA* gene defunct. Worldwide, but rare in lowland tropics. (Stevens 2003).

#### Vaccinieae Rchb.

In the same year Kron (2002b) addressed the phylogenetic relationships among the blueberries: Vaccinieae. The Ericaceae's Vaccinieae tribe are particularly diverse in the

neotropical montane forests producing brightly coloured flowers, fleshy berries, with many seeds, an inferior ovary, a valvate corolla and calyx, stamens equalling the corolla, coriaceous leaves, paracytic stomata, a white embryo and others (for more characters see Stevens et al. 2003). Twenty-eight of 33 Vaccinieae genera are native to the Neotropics, with approximately four to five genera found in the Old World tropics, and *Vaccinium* being the largest genus of ~450 species of both temperate and tropical distribution (Pedraza-Peñalosa 2011; Kron 2002b). To identify the major clades of Vaccinieae, Kron approached the tribe with a global treatment of appropriate species following the sections of Vaccinieae genera.

With sequence data from the nuclear internal transcribed spacer region (nrITS) and the matK gene Kron determined that the large genus *Vaccinium* is not monophyletic. *Vaccinium* has been described as an evolutionary grade group from which several more specialized clades have evolved. However, the representation of *Vaccinium* in phylogenetic studies is still too limited, with few treatments of the Old World species. To draw taxonomic conclusions many (Almeda & Breedlove 1992; Pedraza-Peñalosa 2011) still follow the traditional circumscriptions of *V. spp.*, provided their specimens fit well within the current limits (Stevens et al. 2004; Luteyn & Pedraza-Peñalosa 2006).

### ***Vaccinium L.***

*Vaccinium uliginosum* L. is the generitype of *Vaccinium* Linnaeus which was designated by Vander Kloet (1981). The genus *Vaccinium* remains associated to this type during any taxonomic revision. Species of *Vaccinium* are often found on open mountain slopes in the tropics (Camp 1942, 1943, 1945) with 40% of species native to Southeast Asia, 25% to North America, 10% to South and Central America, and the remaining 25% scattered globally (Luby et al. 1991; Song & Hancock 2011). Although many species are supported with >75% bootstrap values, many species still have poor resolution with <75% or no bootstrap support (Vander Kloet & Dickinson 2009). This matter combined with the fact that nuclear and chloroplast DNA data are not always congruent at the species level, while hybridization remains an issue to be resolved [i.e. *V. cereum* Forst.f. and the low acceptance within The Plant List (2012)], have lead Vander Kloet to continue with phenetic

classification to test the validity of *Vaccinium* sections using multiple numerical taxonomic approaches.

Vander Kloet & Dickinson used 65 descriptors with the distance form of Gower's general coefficient for mixed data (Gower 1971), and principal coordinates analysis (PCoA) (Gower 1966) of the distance matrix to provide a summary of variation in the sample [evaluated using the broken-stick criterion (Frontier 1976; Legendre and Legendre 1998)], and to depict clustering, and ordination. Vander Kloet & Dickinson show that sections follow geographic distributions that relate to their endemism. Section *Bracteata*, has a strictly south eastern Asian distribution, similar to Steven's description of *Eococcus sensu Stevens* (1969). The isolated taxonomic position of the SE Asian *Vaccinium fragile* Franch is corroborated by Kron et al. (2002) whose DNA sequence data from the chloroplast matK gene and the nrITS region gave *V. fragile* little or no support in their Vaccinieae consensus trees which kept it excluded from Kron et al's Bracteata-Orianthe clade. Also, of the 92 species of *Vaccinium* found in China, only *V. fragile* indicates acid soils. Finally, Vander Kloet & Dickinson's phenetic analyses suggested that sections *Cinctosandra* (Klotzsch) Hook.f. and *Pyxothamnus* Sleumer have a strong transatlantic affinity and connections to southern India. Vander Kloet & Dickinson's results show that section *Pyxothamnus* not only contains the Western North American *V. ovatum* Pursh, the Central American *V. consanguineum* Klotzsch, the Caribbean *V. meridionale* Sw., but also the South African *V. exul* Bolus. Similarly section *Cinctosandra* includes *V. wilburii* Luteyn from Central America, *V. emirnense* Hook. from Madagascar and *V. leschenaultii* Wight from Ceylon (Sri Lanka). These *Vaccinium* patterns are in congruence with the descriptions of Camp (1947) and Croizat (1958) who link the origins of the ericads to Antarctica while the continent was still attached to Africa, South America, India and Madagascar.

Vander Kloet & Dickinson note that their cladistic analyses of multistate and meristic descriptors demonstrated a high degree of homoplasy in their data which Kron (2002a) cited as being the source of imperfect delineations within the Ericaceae. Of course, cladistic analyses are very useful when applied to molecular data, but in Vander Kloet & Dickinson's case, the authors made use of the species and their OTUs to delineate their

samples for which only a fraction of molecular data was available. What Vander Kloet & Dickenson provided was a key to the sections that will help in a future total evidence approach in delimiting *Vaccinium* species.

***Metabolomics in taxonomy, identification, and quality control of traditional natural health products***

*Vaccinium* species have many current and potential applications in complementary medicine and therapeutic research in both aboriginal and non-aboriginal populations. *Vaccinium* spp. leaves are also widely used among traditional medicine practitioners in Quebec, Canada (Haddad 2003). Small and Catling (1999) describe the commercial and medical potential of Canadian sources including *V. macrocarpon* Aiton and *V. myrtillus* L. However, with many sympatric species, and *Vaccinium*'s notorious potential for hybridization (personal communication with S.P. Vander Kloet), the possibility of misidentification in taxonomy and natural health product (NHP) production is high especially for sterile samples, bulk collections, or powdered samples. There are now examples where substituted plant species have created toxic NHPs for sale on the marketplace (Awang 1991, Jiang et al. 2006). These circumstances demonstrate the need for recognizing the metabolomic identity of NHPs quickly and reliably on a lot to lot basis - preferably, with a taxonomic ruggedness. From a taxonomic perspective, chemometric data on this important and diverse genus is desirable for systematic total evidence approaches.

Recently Brown et al. (2012a,b) produced two articles discussing *Vaccinium* metabolomics. Brown et al. analyzed fruit extracts of *V. macrocarpon*, *V. oxycoccos* L., and *V. vitis-idaea* L. Using UPLC-MS-QToF they detected 10 038, 8035, 9285 compounds (respectively), “the five” major anthocyanins, as well as serotonin, melatonin, and ascorbic acid. Without fully identifying the compounds, Brown demonstrated that there were two unique compounds in *V. macrocarpon*, three in *V. oxycoccos*, and five in *V. vitis-idaea* (2012a). They also determined the number of unique compounds for five cultivars of *V. macrocarpon*: 136 (Ben Lear), 84 (Bergman), 91 (GH1), 128 (Pilgrim), and 165 (Stevens). These results indicate that discrimination is possible by metabolites and should necessarily be part of total evidence approaches.

## Rationale

Since diabetes is having a devastating impact on indigenous communities and is predicted to become a greater problem, culturally appropriate interventions are needed. Our partners, the Eeyou Istchee Cree, the Lukomir Highlanders, and the Q'eqchi' Maya, have a long history of use of traditional medicines, and an antidiabetic ethnobotanical survey was appropriate. The traditional ethnobotanical approach was also considered since our indigenous communities have less access to primary health, yet now require increased access to primary healthcare due to sociocultural, economic, geographic reasons, or war and post-war development. Dietary acculturation and cultural resistance to modern pharmaceuticals is also a phenomenon among indigenous societies, so an ethnobotanical survey of traditional medicines provides increased access to culturally appropriate medicine, food, and lifestyles to assist in the treatment and prevention of hyperglycaemia and diabetic insults.

For the ethnopharmacology study, ethnobotanical taxa with strong phylogenetic signal (SIV values) and cross-cultural consensus were given priority since they were demonstrated to contain more bioactive plants and because the process would allow our work groups to transfer antidiabetic technology more efficiently. The AGE assay was selected for evaluation of these taxa since the experiment indicates effectiveness in preventing diabetic related AGE formation which contribute to the development of retinopathy, cataracts, atherosclerosis, neuropathy, nephropathy, diabetic embryopathy and impaired wound healing.

Since our consensus taxa were likely to be of value as locally available treatments or possibly commercial natural health products, their characterization was considered. Phytochemical analyses were undertaken to account for the presence of known antidiabetic compounds for product quality control. Due to the use of taxa and their samples from these remote areas, plant identity is also an issue. Therefore plant systematics and chemometric analysis methods using NMR and LC-MS were used for identity assurance. These identity methods were developed for one large group of antidiabetic plants in the genus *Vaccinium*.

## Objectives and Hypotheses

### *Section 1: Ethnobotany*

My first objective addressed in section I (ethnobotany and ethnopharmacology) was to complete the ethnobotanical study in the three indigenous communities, where required. Since no ethnobotanical work had been completed with the Lukomir Highlanders both a general ethnobotany (chapter 2) and a diabetes ethnobotany (chapter 3) were undertaken. A general ethnobotany of the Q'eqchi' was already completed, so only a diabetes ethnobotany was undertaken (chapter 4). The Cree antidiabetic ethnobotany was already completed by Leduc et al. (2006) and Rapinski (2013) so no new ethnobotanical work is reported here. In order to rank taxa with the SIV function, our first step was to work with clinicians at the University of Ottawa Hospital to produce association weight values ( $w$ ) for the symptoms highly associated with diabetes. For the ethnopharmacology studies, the genus *Vaccinium* was investigated due to high phylogenetic signal and cross-cultural consensus between the Cree and Lukomir healers (Chapter 3). This study was also extended to a world collection of *Vaccinium* (chapter 5). For the Maya ethnobotany, the top ranked antidiabetic plants were selected for study in the ethnopharmacology evaluation.

### Section 1, hypotheses

1. Since Lukomir's priority health concerns are heart disease and diabetes (as indicated by Lukomir's visiting physician) we hypothesized that ethnobotanical heart disease, diabetes and panacea treatments and categories would be high frequency use categories.
2. The overarching ethnopharmacological hypothesis is that taxa with strong phylogenetic signal and cross-cultural consensus will be more effective in preventing the formation of advanced glycation endproducts than other ethnobotanical taxa or outgroups.

### *Section 2: Metabolomics and systematics of antidiabetic Vaccinium*

The objectives of this section were to develop an NMR metabolomics method for identity of *Vaccinium* species and a natural health product quality control method for key metabolites in the genus. The research was completed first in a single lab study, then

validated in a multi-lab study. Since NMR can provide quality assurance that can be easily replicated on any equipment, a quantitative NMR study of 2 key metabolites was undertaken.

### Section 2, hypothesis

Since there exists evolutionary distance to common ancestors among sampled taxa, from a subgeneric level to more specific levels, metabolomic analyses of leaves will discriminate taxa, based on these evolutionary distances in the metabolome.

## Section 1

# Ethnobotany

### Preface

Section 1 addresses our longstanding partnerships with Eeyou Istchee Cree, the Lukomir Highlanders, and the Q'eqchi' Maya communities who remain concerned about diabetes and the preservation of traditional medical knowledge. To date, a general ethnobotany of Q'eqchi' Maya and the Eeyou Istchee Cree are complete. A general ethnobotanical study of the Lukomir Highlanders would allow development of background for the diabetes context and support a comparison of specialized antidiabetic ethnobotany and ethnopharmacology. The antidiabetic ethnobotany of the Cree was published by Leduc et al. (2006) and the antidiabetic ethnobotany of Lukomir Highlanders and Q'eqchi' Maya is presented here in Section 1, Chapter 4. In this multilateral partnership, our focus on plant treatment commonalities (derived using quantitative ethnobotanical methodologies) has supported the elucidation of safe and effective antidiabetic plants for ethnopharmacological and phytochemical analysis in Section two.

## Chapter 2

# An Ethnobotany of the Lukomir Highlanders of Bosnia & Herzegovina<sup>2</sup>

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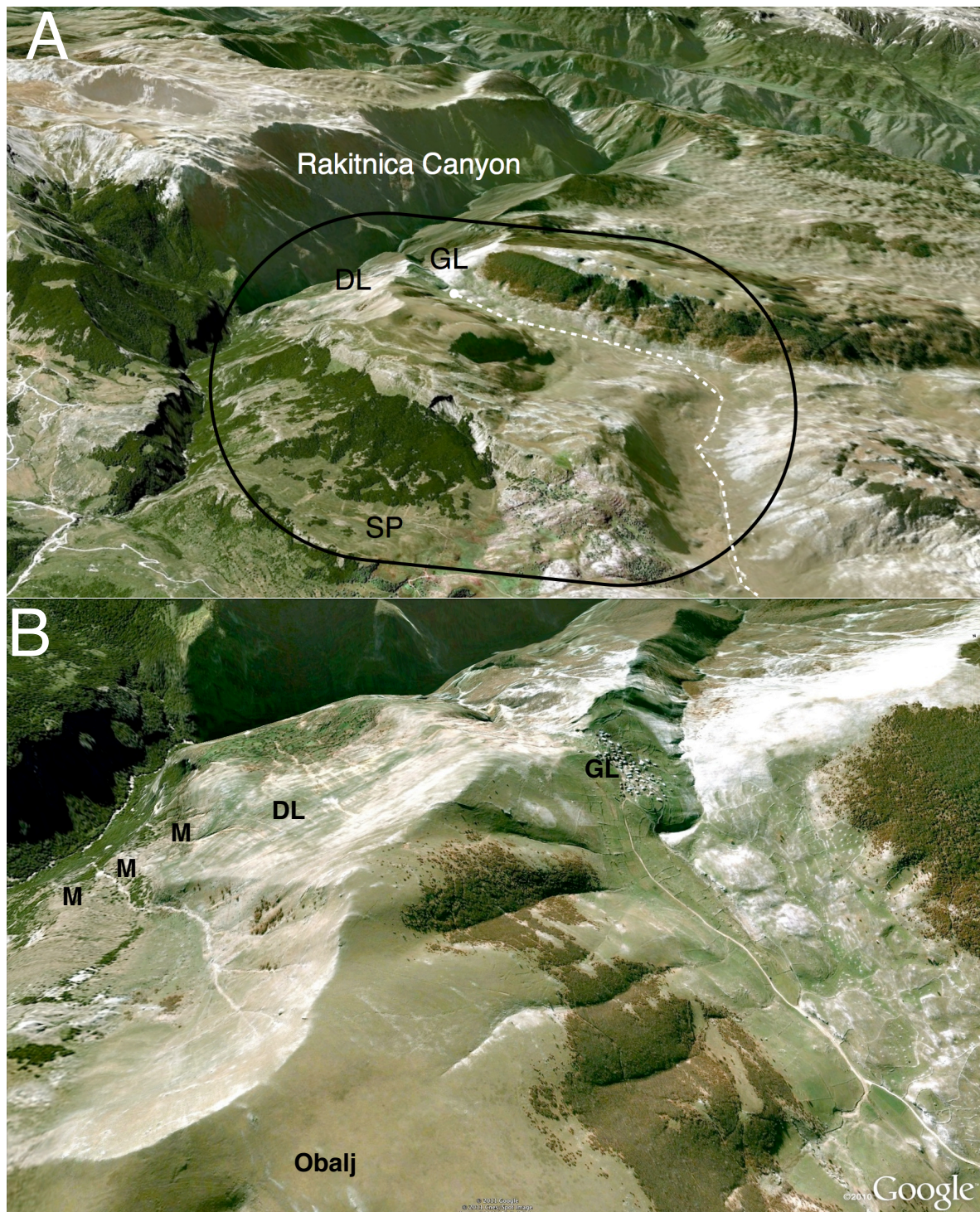
<sup>2</sup> Dedicated to the memory of Sulejman Redžić (1954-2013).

## **An Ethnobotany of the Lukomir Highlanders of Bosnia & Herzegovina**

This is the first ethnobotanical survey of Lukomir, Bosnia and Herzegovina (B&H). The Lukomir Highlanders speak *Bosanski*, and are one of Europe's last indigenous communities occasionally living a traditional semi-nomadic lifestyle. The Highlanders have inhabited the Lukomir territory (Figure 2.1) within the Dinaric Alps for centuries. The Lukomir Highlanders are historically Bogomil and their ancestry is marked by ancient *stećci* (gravestones), still present in their territory (Nikolić and Šarančić 2011). Autosomal STR loci studies link Lukomir to the isolated Adriatic islands of Brač, Hvar, and Korčula in Croatia (Marjanović et al. 2004). The Highlanders also have a historic connection to semi nomadic peoples from the Podveležje Plateau near Mostar, B&H who travelled to Bjelašnica with their livestock in search of water during the summer months (Tourism Association of FB&H 2005). Before the Highlanders adopted automobiles to transport livestock, shepherds with hundreds in their flocks (previously included goats but now exclusively sheep and cattle) set out on grazing journeys throughout the Lukomir territory and to neighbouring regions, especially Konjic, B&H. In the wild, shepherds slept with their sheep flocks while Lukomir family members culled the village pastures and gardens for enough food and fodder to last the long winter. Today, according to local conversations, there are fewer people staying all winter, with fewer families returning in the summer. In the winter of 2007, three families stayed in Lukomir for the winter. In 2008 and 2009, only two families remained all winter. The winter of 2011-2012 marked the first winter without inhabitants. This marks a transitional point in Lukomir's cultural history. This situation is due in part to 15 meter snow drifts and an impassable road. The residence of people in the village is talked about over the long winters.

From 1950 onward, the development of the only road prompted the Highlanders to gradually relocate from *Donji Lukomir* to their current location at *Gornji Lukomir*, which is now generally referred to as Lukomir (see DL and GL in Figure 2.1). Before *the* road, *Donji* Lukomir provided the most efficient access to health and trade services in neighbouring regions via the Rakitnica Canyon trails. Today, Lukomir still functions without a primary healthcare facility, but was noted to uphold the practice of traditional medicine by our late

co-author, and the ethnobotanical authority in B&H, Professor Sulejman Redžić (1947-2013)(Redžić 2006, 2007, 2008, 2010a,b, Redžić, Barudanović, & Radević 2008; Redžić & Ferrier 2011).



**Figure 2.1.** Maps of the Lukomir Highlander ethnobotanical study site. Donji Lukomir (DL), Gornji Lukomir (GL), *vodenica mlins* (M), and road (dotted white line).

The partnership with professor Redžić and Canadian researchers was developed based on a post war development grant from the Canadian International Development Agency (CIDA). The project was designed to address criteria of European Union Stabilization and Association agreement (SAA) for B&H including: 1) the awareness of native communities (Tourism Association of FB&H 2005); 2) environmental protection of endemic botanical regions (European Environment Agency 2012a,b; Redžić, Barudanović, & Radević 2009); and 3) female roles in traditional healthcare (Redžić 2010).

In introductory meetings with families and traditional healers in Lukomir, the community wished to investigate matters of health and environment to help determine items which could assist in their self sustaining development. Following this request, we consulted with the one volunteer physician who holds a clinic each summer in Lukomir. We learned that Lukomir's priority health concerns are heart disease and diabetes and therefore hypothesized that heart disease and diabetes treatments would be our high frequency use categories. Because Lukomir is an area of exceptional diversity in Europe, we also predicted that ethnobotanical reports would be associated with Bjelašnica's rare and endemic flora. We predicted that our ethnographic data would present health and environment themes with related information on the effects of the recent war in Bosnia and Herzegovina.

## **Methods**

### ***Partnership, Permits, and Prior Informed Consent***

This study was initiated with the community of Lukomir, B&H and J.F. & L.Š.'s organization, Cross-Cultural Health Initiative (CHI). International collaboration was initiated by the Association of Universities and Colleges Canada Partnerships for Tomorrow Program, Phase II funded by the Canadian International Development Agency (CIDA). Canadian partners were hosted by Professor Sulejman Redžić at the University of Sarajevo, Faculty of Science, Department of Biology, and by Emira Alikadić, at Foundation GEA+. Research permits were issued by the Municipality of Konjic with prior informed consent from Lukomir's leaders and informants.

***Study site: Lukomir, Municipality of Konjic, Bosnia & Herzegovina, Europe***

A consensus ethnobotany was conducted in the highlands of Bjelašnica with the Lukomir Highlanders of Bosnia and Herzegovina (B&H) (Figure 2.1). This area is classified as an alpine biogeographic region that is closely bordered by Mediterranean and continental biogeographic regions (European Environment Agency 2012a). Many community members are descendants of a Bogomil lineage who first settled in *Donji Lukomir* (Lower Lukomir) (43.632 lat, 18.194 lon, 1200 m a.s.l.) and eventually moved to *Gornji Lukomir* (Upper Lukomir, commonly referred to as Lukomir) (43.637 lat, 18.182 lon, 1460 m a.s.l.). Lukomir's informants included spiritual leaders, elders, and younger women and men. Informants described plants on field trips, garden tours, while shepherding, or in comfortable settings of their choice. Interview time was earned with Lukomir's healers by volunteering our time to shepherd, harvest food, and stack hay. Notes and photos were taken when participants chose to display preparation methods of plant and natural product remedies. Plant vouchers, the authors' working field guide, and an iPad (Apple, Cupertino, USA) were used to display collections and photos to elders who could not venture over the mountainside, or for informant review purposes (Thomas, Vandebroek, & Van Damme 2007).

Land mines were avoided by consulting with Bosnia and Herzegovina Mine Action Center (BH MAC) (<http://www.bhmac.org>). The total mine contaminated area of B&H currently covers around 1.274,20 km<sup>2</sup> or 2,5% of total country size. All plants were collected on trail sides or in areas that were constantly travelled by sheep herds, since only parts of the Lukomir territory were surveyed by BH MAC (Figure 2.2).



**Figure 2.2.** Bjelašnica mine map. Legend provides information on mine contaminated areas. Red dashes are areas hiked by Bosnia and Herzegovina Mine Action Center (<http://www.bhmac.org>).

Field work followed consensus methodology with individual semistructured interviews during which L.Š., J.F., and S.R. collected the following data: (1) specimen voucher number, (2) photo number, (3) common name, (4) scientific name, (5) family, (6) GPS coordinates, (7) altitude (m a.s.l.), (8) habitat, (9) sub-habitat, (10) flowering time and description, (11) medically active collection time, (12) use, (13) use category, (14) plant part used, (15) amount used, (16) medicine preparation method, (17) medicine administration method, (18) medicine dosing regimen, (19) ethnographic details, (20) informant name and number, and (20) date. Duplicate vouchers were collected (when sustainable) for deposition at University of Sarajevo Herbarium and OTT (Ferrier 351-458).

While this work was focused on medical plant use, a non exhaustive record of food and material uses was made in association with the medicinal plants. Due to the expense and time associated with ethnobotanical projects we felt that including the food and material mentions, rather than excluding them, would be of greater value to the Lukomir community and would serve as a preliminary account of medicinal food and material use in Lukomir.

## *Ethnobotanical analysis and statistics*

### Human selection of plants

The  $F_{ic}$  function, created by Trotter and Logan (1986), is completed by the number use reports ( $n_{ur}$ ) from the number taxa ( $n_{taxa}$ ) per usage category.

$$F_{ic} = \frac{n_{ur} - n_{taxa}}{n_{ur} - 1}$$

Satisfying the  $F_{ic}$  function supplies a numerical ranking factor for categories of phytochemical and pharmacological interest.

The Moerman regression analysis (1991) compares the medicinal flora to the total regional flora at the level of plant families and genera, leading to the identification of high use and low use families.

$$Y = 0.01434 * X + 0.04937$$

### Statistics and phylogenetic tree

Statistical analysis was conducted using Prism 6 software. A phylogenetic tree was constructed using TreeGraph 2.0.47-206 beta, FigTree v1.4.0, taxon with positive SIV values, and topology presented in Angiosperm Phylogeny Group (2009).

## **Results and Discussion**

### ***Interviews***

By volunteering our help to the Highlanders while shepherding, collecting food, and stacking hay, we had more time for interviews and recruited 25 informants who described plants on mountain and canyon field trips. During these interviews, the Highlanders identified 59 species from 35 families which were cited in 307 medicinal, 40 food, and seven material use reports (Table 2.1). The average consensus on use of medicinal plants was 3.5, and a maximum consensus of 15 was obtained on medicinal plants *Mentha longifolia* (L.) L. and *Salvia officinalis* L.. Average food plant consensus was three. The

maximum consensus on food plants was eight on *Urtica dioica* L.. Finally, there was a maximum consensus of two on material plants *Pinus nigra* Arnold and *Cornus mas* L..

**Table 2.1.** Medicinal, food, and material taxa used by the Lukomir Highlanders of Bosnia & Herzegovina (B&H). European endemic taxa are indicated by a dagger (†).

Family	Genus species	†	Common name	Habitat	Syntaxa* f	VN
Adoxaceae	<i>Sambucus nigra</i> L.		Zova, Zobovina, Elder	Village & shepherd trails	F	11 354
Adoxaceae	<i>Sambucus wightiana</i> Wall. ex Wight & Arn.		Haptovina	Mountainside slope	Ea, Art	1 376
Adoxaceae	<i>Sambucus wightiana</i> Wall. ex Wight & Arn.		Haptovina	Mountainside slope	Ea, Art	3 376
Amaranthaceae	<i>Chenopodium bonus-henricus</i> L.		Šćir, Good King Henry	Village & shepherd trails	Ch, O	5 370
Apiaceae	<i>Smyrniium perfoliatum</i> L.		Ljaljica, Perfoliate alexanders	Grassland	O, Arr	2 380
Apiaceae	<i>Smyrniium perfoliatum</i> L.		Ljaljica, Perfoliate alexanders	Grassland	O, Arr	2 380
Apiaceae	<i>Smyrniium perfoliatum</i> L.		Ljaljica, Perfoliate alexanders	Grassland	O, Arr	2 380
Aristolochiaceae	<i>Asarum europaeum</i> L.		Kopitnik/Kopitnjak, European wild ginger	Deciduous forest	F, Qp, O-co	1 382
Aristolochiaceae	<i>Asarum europaeum</i> L.		Kopitnik/Kopitnjak, European wild ginger	Deciduous forest	F, Qp, O-co	2 382
Aspleniaceae	<i>Phyllitis scolopendrium</i> (L.) Newman		Podrebica (male and female) indicated by sori	Riparian	F, Amph	10 357
Asteraceae	<i>Tussilago farfara</i> L.		Podbijel (♂ or ♀), Coltsfoot	Riparian	O, Pm	4 371
Asteraceae	<i>Matricaria matricarioides</i> (Less.) Porter ex Britton		Kamilica, Stomaklija, Pineappleweed	Village & shepherd trails	Pm	1 351
Asteraceae	<i>Matricaria matricarioides</i> (Less.) Porter ex Britton		Kamilica, Stomaklija, Pineappleweed	Village & shepherd trails	Pm	1 397
Asteraceae	<i>Matricaria matricarioides</i> (Less.) Porter ex Britton		Kamilica, Stomaklija, Pineappleweed	Village & shepherd trails	Pm	12 351
Asteraceae	<i>Matricaria matricarioides</i> (Less.) Porter ex Britton		Kamilica, Stomaklija, Pineappleweed	Village & shepherd trails	Pm	1 351
Asteraceae	<i>Artemisia absinthium</i> L.		Pelin, Absinthe wormwood	Mountainside slope	Art, O	1 396
Asteraceae	<i>Achillea millefolium</i> L.		Kunica, Yarrow	Village & shepherd trails	Arr, Pm, Ch	9 358
Asteraceae	<i>Achillea millefolium</i> L.		Kunica, Yarrow	Village & shepherd trails	Arr, Pm, Ch	1 358
Asteraceae	<i>Taraxacum officinale</i> F.H. Wigg.		Maslačak, Dandelion	Village & shepherd trails	Arr, O, Ag, Pm	1 412
Asteraceae	<i>Cichorium intybus</i> L.		Konjanik, Chicory	Village & shepherd trails	Art, O, Ag	1 411
Boraginaceae	<i>Symphytum officinale</i> L.		Gavez, Common comfrey, Boneset	Riparian	Ag, Bid, Art	2 383
Brassicaceae	<i>Capsella bursa-pastoris</i> (L.) Medik.		Rustemača, Shepherd's purse	Village & shepherd trails	Ch, O, Art	2 398
Caryophyllaceae	<i>Silene uniflora</i> Roth ssp. <i>prostrata</i> (Gaudin) Chater & Walters	†	Puca, Sea champion	Grassland	Arr, Be, O-Co	8 350
Caryophyllaceae	<i>Silene uniflora</i> Roth ssp. <i>prostrata</i> (Gaudin) Chater & Walters	†	Puca, Sea champion	Grassland	Arr, Be, O-Co	2 350
Caryophyllaceae	<i>Silene uniflora</i> Roth ssp. <i>prostrata</i> (Gaudin) Chater & Walters	†	Puca, Sea champion	Grassland	Arr, Be, O-Co	7 350
Caryophyllaceae	<i>Silene uniflora</i> Roth ssp. <i>prostrata</i> (Gaudin) Chater & Walters	†	Puca, Sea champion	Grassland	Arr, Be, O-Co	1 350

Family	Genus species	† Common name	Habitat	Syntaxa* f	VN
Caryophyllaceae	<i>Silene uniflora</i> Roth ssp. <i>glareosa</i> (Jord.) Chater & Walters	† Puca, Sea campion	Grassland	Arr, Be, O-Co	2 353
Caryophyllaceae	<i>Silene uniflora</i> Roth ssp. <i>glareosa</i> (Jord.) Chater & Walters	† Puca, Sea campion	Grassland	Arr, Be, O-Co	7 353
Caryophyllaceae	<i>Silene uniflora</i> Roth ssp. <i>glareosa</i> (Jord.) Chater & Walters	† Puca, Sea campion	Grassland	Arr, Be, O-Co	1 353
Caryophyllaceae	<i>Silene uniflora</i> Roth ssp. <i>glareosa</i> (Jord.) Chater & Walters	† Puca, Sea campion	Grassland	Arr, Be, O-Co	8 353
Cornaceae	<i>Cornus mas</i> L.	Drijen, European dogwood	Deciduous forest	O.Co, F,Qp,Ps	2 384
Cornaceae	<i>Cornus mas</i> L.	Drijen, European dogwood	Deciduous forest	O.Co, F,Qp,Ps	2 384
Cornaceae	<i>Cornus mas</i> L.	Drijen, European dogwood	Deciduous forest	O.Co, F,Qp,Ps	2 384
Cornaceae	<i>Cornus mas</i> L.	Drijen, European dogwood	Deciduous forest	O.Co, F,Qp,Ps	2 384
Cornaceae	<i>Cornus mas</i> L.	Drijen, European dogwood	Deciduous forest	O.Co, F,Qp,Ps	2 384
Cornaceae	<i>Cornus mas</i> L.	Drijen, European dogwood	Deciduous forest	O.Co, F,Qp,Ps	2 384
Crassulaceae	<i>Sedum sexangulare</i> L.	Zednjak, Tasteless stonecrop	Rockland		1 416
Crassulaceae	<i>Jovibarba hirta</i> (L.) Opiz	† Čuvarkuća, Beard of Jupiter, Hen and Eggs	Rockland	Amph	1 379
Crassulaceae	<i>Jovibarba hirta</i> (L.) Opiz	† Čuvarkuća, Beard of Jupiter, Hen and Eggs	Rockland	Amph	1 379
Cupressaceae	<i>Juniperus communis</i> L.	Smreka, Common juniper	Mountainside slope	Jun, O.Co	1 399
Equisetaceae	<i>Equisetum arvense</i> L.	Preslica, Field horsetail	Riparian	Pm, Ch	3 367
Equisetaceae	<i>Equisetum arvense</i> L.	Preslica, Field horsetail	Riparian	Pm, Ch	2 367
Ericaceae	<i>Vaccinium vitis-idaea</i> L.	Brusnica, Lingonberry	Mountainside slope	V	1 385
Ericaceae	<i>Vaccinium vitis-idaea</i> L.	Brusnica, Lingonberry	Mountainside slope	V	2 385
Ericaceae	<i>Vaccinium myrtillus</i> L.	Borovnica, European blueberry	Mountainside slope	V, V-P	1 368
Ericaceae	<i>Vaccinium myrtillus</i> L.	Borovnica, European blueberry	Mountainside slope	V, V-P	5 368
Euphorbiaceae	<i>Euphorbia myrsinites</i> L.	Mliječavac, Myrtle spurge	Rockland	Af, S-Ch, Be	1 400
Fabaceae	<i>Trifolium repens</i> L.	Bijela djetelina, White clover	Grassland	Ag, m, Arr	2 387
Fabaceae	<i>Trifolium pratense</i> L.	Crvena djetelina, Red clover	Grassland	Arr, Ag, Be	2 386
Fabaceae	<i>Ononis spinosa</i> L.	Gladišika, Spiny restharrow	Grassland	Be, S-Ch, Ps	3 377
Fabaceae	<i>Anthyllis vulneraria</i> L.	Ranjenik, Kidney vetch	Grassland	Be, St	4 372
Fabaceae	<i>Anthyllis vulneraria</i> L.	Ranjenik, Kidney vetch	Grassland	Be, St	4 372
Fagaceae	<i>Fagus sylvatica</i> L.	Bukva, European beech	Deciduous forest	F	1 410
Gentianaceae	<i>Gentiana lutea</i> L.	† Lincura, Great yellow gentian	Grassland	St, Pim	1 393
Gentianaceae	<i>Gentiana lutea</i> L.	† Lincura, Great yellow gentian	Grassland	St, Pim	1 393
Hypericaceae	<i>Hypericum perforatum</i> L.	Kantarion, Ženska trava, St. John's wort	Grassland	Orig, Ps, Be	2 355
Hypericaceae	<i>Hypericum perforatum</i> L.	Kantarion, Ženska trava, St. John's wort	Grassland	Orig, Ps, Be	11 355
Lamiaceae	<i>Thymus serpyllum</i> L.	Čubra (Crvena i Bijela), Majčina dušica, Breckland thyme	Rockland	Be, S-Ch	5 365

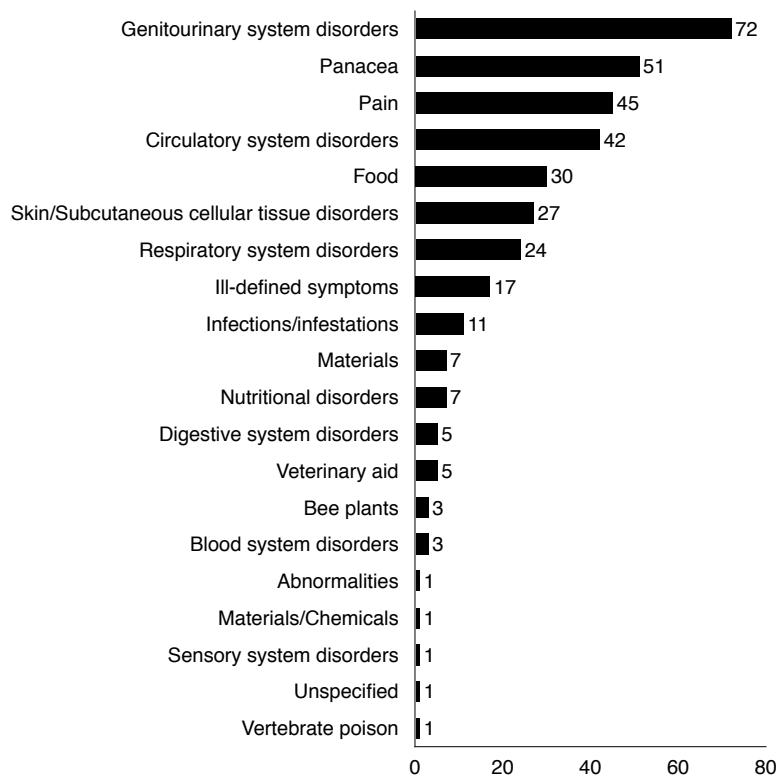
Family	Genus species	† Common name	Habitat	Syntaxa* f	VN
Lamiaceae	<i>Thymus serpyllum</i> L.	Čubra (Crvena i Bijela), Majčina dušica, Breckland thyme	Rockland	Be, S-Ch	7 365
Lamiaceae	<i>Teucrium montanum</i> L.	Iva, Mountain germander	Rockland	Be, S-Ch	12 352
Lamiaceae	<i>Satureja montana</i> L.	Vrijesak, Winter savory	Rockland	S-Ch, O- Co	6 366
Lamiaceae	<i>Salvia officinalis</i> L.	† Kadulja, Sage	Mountainside slope	S-Ch, O- Co	15 348
Lamiaceae	<i>Nepeta cataria</i> L.	Macina trava, Catnip	Rockland	O, Be	5 369
Lamiaceae	<i>Nepeta cataria</i> L.	Macina trava, Catnip	Rockland	O, Be	5 369
Lamiaceae	<i>Nepeta cataria</i> L.	Macina trava, Catnip	Rockland	O, Be	5 369
Lamiaceae	<i>Mentha pulegium</i> L.	Verem, European pennyroyal	Riparian	Bid, Mol, M-C	1 402
Lamiaceae	<i>Mentha longifolia</i> (L.) L.	Metvica, Nana, Horse mint	Riparian	Bid, Mol, M-C	1 413
Lamiaceae	<i>Mentha longifolia</i> (L.) L.	Metvica, Nana, Horse mint	Riparian	Bid, Mol, M-C	1 401
Lamiaceae	<i>Mentha longifolia</i> (L.) L.	Metvica, Nana, Horse mint	Riparian	Bid, Mol, M-C	15 349
Lamiaceae	<i>Satureja montana</i> L.	† Vrijesak, Winter savory	Rockland	S-Ch, O- Co	1 366
Lamiaceae	<i>Salvia officinalis</i> L.	† Kadulja, Sage	Mountainside slope	S-Ch, O- Co	1 348
Liliaceae	<i>Lilium bosniacum</i> (Beck) Fritsch	† Bosanski ljiljan, Zlatan, Bosnian lily	Grassland	Studen potuk	1 417
Liliaceae	<i>Lilium bosniacum</i> (Beck) Fritsch	† Bosanski ljiljan, Zlatan, Bosnian lily	Grassland	Studen potuk	1 417
Liliaceae	<i>Convallaria majalis</i> L.	Dudurika, Lily of the vally	Deciduous forest	Qp, F, O- Co	3 378
Malvaceae	<i>Tilia platyphyllos</i> Scop.	Lipa, Large-leaved linden	Grassland	F, Qp	4 374
Malvaceae	<i>Tilia platyphyllos</i> Scop.	Lipa, Large-leaved linden	Grassland	F, Qp	1 374
Melanthiaceae	<i>Veratrum album</i> L.	Čemerika, European white hellebore	Grassland	Ad, Arr	2 388
Parmeliaceae	<i>Cetraria islandica</i> (L.) Ach	Islandski lišaj, Mašina, Iceland moss	Mountainside slope	St, Be	1 403
Pinaceae	<i>Pinus nigra</i> Arnold	Bor, Black pine	Mountainside slope		1 418
Plantaginaceae	<i>Plantago major</i> L.	Bokvica, Broadleaf plantain	Village & shepherd trails	Pm, Ch	1 360
Plantaginaceae	<i>Plantago major</i> L.	Bokvica, Broadleaf plantain	Village & shepherd trails	Pm, Ch	9 360
Plantaginaceae	<i>Plantago lanceolata</i> L.	Bokvica ♀, Ribwort plantain	Grassland	Arr, Ag, Be	1 359
Plantaginaceae	<i>Plantago lanceolata</i> L.	Bokvica ♀, Ribwort plantain	Grassland	Arr, Ag, Be	9 359
Poaceae	<i>Elymus repens</i> (L.) Gould	Pirika, Couch grass	Grassland	Pm, Ag, Arr	1 389
Poaceae	<i>Elymus repens</i> (L.) Gould	Pirika, Couch grass	Grassland	Pm, Ag, Arr	1 389
Polygonaceae	<i>Polygonum bistorta</i> L.	Srčanik, Bistort	Grassland	Ad, Arr	11 356
Primulaceae	<i>Primula veris</i> L.	Jagorčevina, Cowslip	Grassland	Arr, Ad, Ps	4 373
Ranunculaceae	<i>Helleborus odorus</i> Waldst. & Kit. ex Willd.	† Kukurijek, Hellebore	Grassland	Ps, Cor, F, Qp	1 394
Rosaceae	<i>Rubus saxatilis</i> L.	Kupina, Stone bramble	Grassland	Af, S-Ch	1 407
Rosaceae	<i>Rubus idaeus</i> L.	Malina, Raspberry	Deciduous forest	Ea, Atr	1 406
Rosaceae	<i>Rosa glauca</i> Pourr.	Šipak/Šipina	Mountainside slope	O-Co, Orig	8 364
Rosaceae	<i>Prunus spinosa</i> L.	Trn, Trnjina, Blackthorne Rakija, Brandy	Mountainside slope	Ps, F, Qp	1 405
Rosaceae	<i>Prunus spinosa</i> L.	Trn, Trnjina, Blackthorne Rakija, Brandy	Mountainside slope	Ps, F, Qp	1 405
Rosaceae	<i>Prunus spinosa</i> L.	Trn, Trnjina, Blackthorne Rakija, Brandy	Mountainside slope	Ps, F, Qp	1 405
Rosaceae	<i>Crataegus monogyna</i> Jacq.	Glog, Gloginje, Hawthorn	Deciduous forest	Ps, Cor, Qp	2 361
Rosaceae	<i>Crataegus monogyna</i> Jacq.	Glog, Gloginje, Hawthorn	Deciduous forest	Ps, Cor, Qp	8 361

Family	Genus species	† Common name	Habitat	Syntaxa* f	VN
Rosaceae	<i>Rosa glauca</i> Pourr.	Šipak/Šipina, Redleaf rose	Mountainside slope	O-Co, Orig 8	364
Scrophulariaceae	<i>Verbascum thapsus</i> L.	Divizbina, Divizma, Great mullein	Grassland	Art, O 2	390
Solanaceae	<i>Solanum tuberosum</i> L.	Krompir, Potato	Cultivated	Cult, Ch 1	408
Urticaceae	<i>Urtica dioica</i> L.	Žara, Kopriva, Stinging nettle	Village & shepherd trails	Art, O 1	362
Urticaceae	<i>Urtica dioica</i> L.	Žara, Kopriva, Stinging nettle	Village & shepherd trails	Art, O 1	362
Urticaceae	<i>Urtica dioica</i> L.	Žara, Kopriva, Stinging nettle	Village & shepherd trails	Art, O 5	362
Urticaceae	<i>Urtica dioica</i> L.	Žara, Kopriva, Stinging nettle	Village & shepherd trails	Art, O 7	362
Vitaceae	<i>Vitis vinifera</i> L.	Sirće, Vinegar	Cultivated	Cult 1	409

\*Ad (Adiantetalia); Amph (Amphoricarpetalia); Arr (Arrhenatheretalia); Be (Brometalia erecti); C-Br (Cymbopogo-Brachypodietalia); Ch. (Chenopodietalia) Dj (Drypeetalia jacquiniana); Jun (Juniperetalia); Mp (Moltkaetalia petraeae); O (Onopordetalia); O-Co (Ostryo-Carpinetalia orientalis); Pa (Populeetalia albae); Pal (Paliuretalia aculeatae); Ph (Phragmitetalia); Pm Plantaginetalia majoris; P.s. -Prunetalia spinosae; Qi (Quercetalia ilicis); Qp (Quercetalia pubescentis); S-Ch (Scorzonero-Chrysopogonetalia); Sis (Sisymbrietalia); Sp - (Salicetalia purpureae); T-H (Trifolio-Hordetalia Arabidetalia).

### ***Frequency of use reports per usage category***

The number of use reports per category (Figure 2.3) varied from 1 to 72. High frequency categories include: genitourinary system disorders, panacea, pain, circulatory system disorders. Medium frequency categories include: food, skin/subcutaneous cellular tissue disorders, respiratory system disorders, ill-defined symptoms with many low frequency results (Figure 2.3).

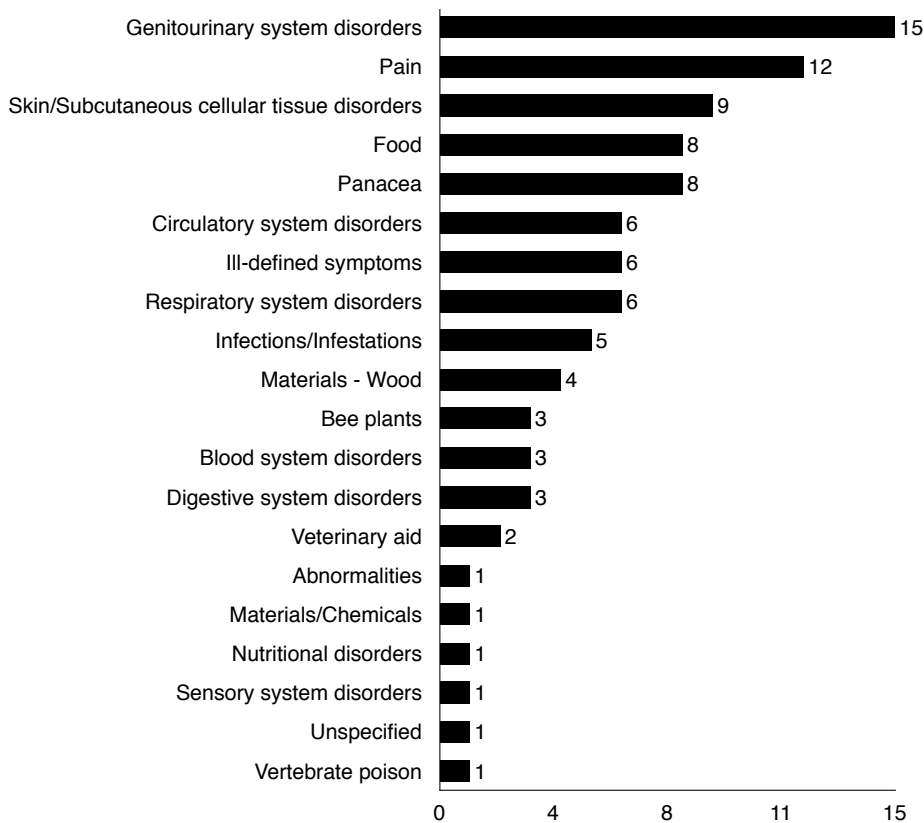


**Figure 2.3.** Distribution of medicinal, food, and material plants according to frequency of use reports.

The high use of plants for genitourinary system disorders (especially gynaecological uses) could be linked to researcher LŠ being a female, and the majority of interviewees being female; however, high female input was predicted based on our literature review (Redžić 2010). Panacea treatments were highly sought after since they were often cross listed as food plants, which also composed a number of economically important plants. High requests for panacea treatments is probably linked to diabetes, which the visiting physician cited as a top health concern. Diabetic insults affect many tissues and organs, leading to symptoms like frequent urination, neuropathy, slow healing sores, and panacea treatments are an important resource for Highlanders with these symptoms. Pain was a high frequency request likely due to hard labour work in wet and cold conditions. Heart disease was also described as a top concern by the visiting physician and again indicates that the community understands their health profile and are therefore requesting traditional circulatory system disorder medicines.

### *Frequency of taxa per usage category*

Frequency of taxa per usage category distributions revealed the type of usages that are of most concern (Figure 2.4). The medicinal categories employing the largest number of taxa include genitourinary system disorders, pain, skin/subcutaneous cellular tissue disorders and indicate that these categories are of high community concern. Medium categories included food, panacea, circulatory system disorders, ill-defined symptoms, respiratory system disorders, infections/infestations and may mark chronic or recurring health conditions that present more often than low frequency categories (four species or less).



**Figure 2.4.** Distribution of medicinal, food, and material plants according to frequency of taxa.

Table 2.2 breaks the number of taxa down to specific uses in their associated usage category. High diversity was found in uses including: cure alls, internal medicine, heart trouble, genitourinary system health, stomach pain, cuts and infections with five or more taxa each.

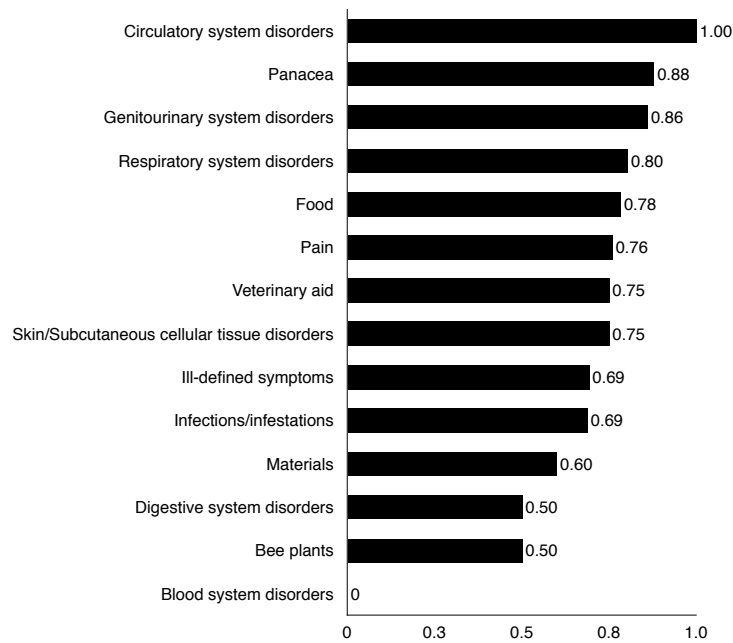
**Table 2.2.** The number of taxa for specific uses per usage category.

Ailment prophylaxis	1		
<b>Bee Plants</b>			
Honey	3		
<b>Blood system disorders</b>			
Blood clots	1	Circulation	2
<b>Circulatory system disorders</b>			
Heart trouble	5		
<b>Digestive system disorders</b>			
Diarrhoea	1	Liver aid	1
Hard stomach in children	1		
<b>Food</b>			
Vegetables	4	Preserve, jam	2
Beverage	1	Coffee substitute	1
Candy	1		
<b>Genitourinary system disorders</b>			
Female infertility	1	Heavy or irregular periods	2
Genitourinary system health	5	Infection	3
Kidney and bladder pain/stones	2	Urinary tract and kidney infection/ cleanser	1
Urinary tract and kidney health	4	Vaginal discharge	2
<b>III-defined symptoms</b>			
Internal medicine	6		
<b>Infections/infestations</b>			
Cold symptoms	1	Fever	3
Wounds	1		
<b>Materials</b>			
Flute and violin	1	Shingle	1
Furniture	1	Tools	1
Rake	1		
<b>Materials/Chemicals</b>			
Teeth cleaning	1		
<b>Nutritional disorders</b>			
Nutritional deficiency/low iron	1		
<b>Pain</b>			
Chest pain	2	Menstrual cramps and stomach pain	1
Ear pain (children)	1	Pain under ribs	1

Eye pain	1	Stomach pain	5
Headache	2		
<b>Panacea</b>			
Cure all	8		
<b>Respiratory system disorders</b>			
Asthma cough and chest pains	3	Cough/infection	4
<b>Skin/Subcutaneous cellular tissue disorders</b>			
Condylomata acuminata (warts)	2	Cuts and infections	5
Skin infection and irritations	1	Swollen skin and fat deposits under the skin	1
<b>Unspecified</b>			
Unspecified	1		
<b>Vertebrate poison</b>			
Chicken poison	1		
<b>Veterinary aid</b>			
Antivenin for sheep	1	Nit shampoo for sheep	1

### ***Informant consensus factor ( $F_{ic}$ ) per usage category***

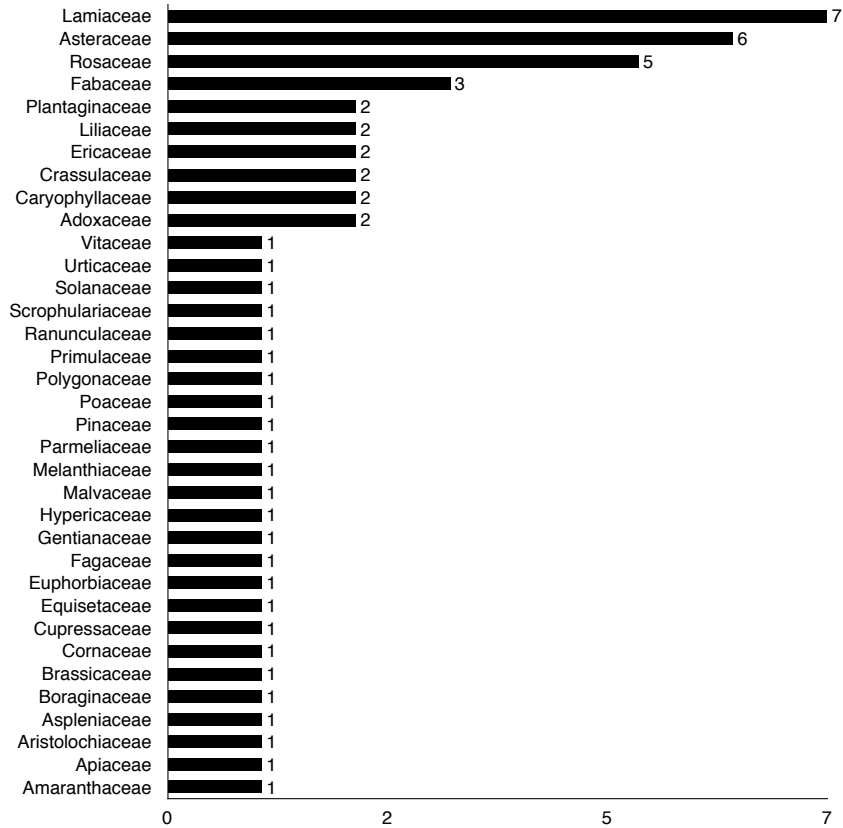
For a disease level analysis the informant consensus factors ( $F_{ic}$ ) were calculated (Figure 2.5). Here, 10 of the 15 categories have an  $F_{ic}$  value  $>$  or  $=$  0.69, which indicates a high degree of consensus among Lukomir's healers. The average  $F_{ic}$  was 0.64; but, in order to cross-culturally compare human medicinal plant use, non medicinal categories (bee plants, materials, veterinary aids, food) were removed from this Lukomir survey for an average  $F_{ic}$  of 0.76. Both Lukomir averages were greater than averages calculated from a study by Amiguet et al. (2005), with the *Q'eqchi'* Maya of Belize, (Avg.  $F_{ic}$  = 0.62) and the study with the Yucatec Maya in Mexico at 0.48. This difference in  $F_{ic}$  results may be explained by recognizing that the Maya informants were from isolated villages, while the Lukomir Highlanders, although semi nomadic, share a single village where they traditionally stay all winter, snowlocked, and where the interviews were conducted. These results indicate that these plants are well known in a well defined community based traditional knowledge system. When consensus is high, these categories are more likely to be active in condition-related pharmacological assays (Leaman et al. 1995).



**Figure 2.5.** Distribution of medicinal, food, and material plants according to frequency of Fic values.

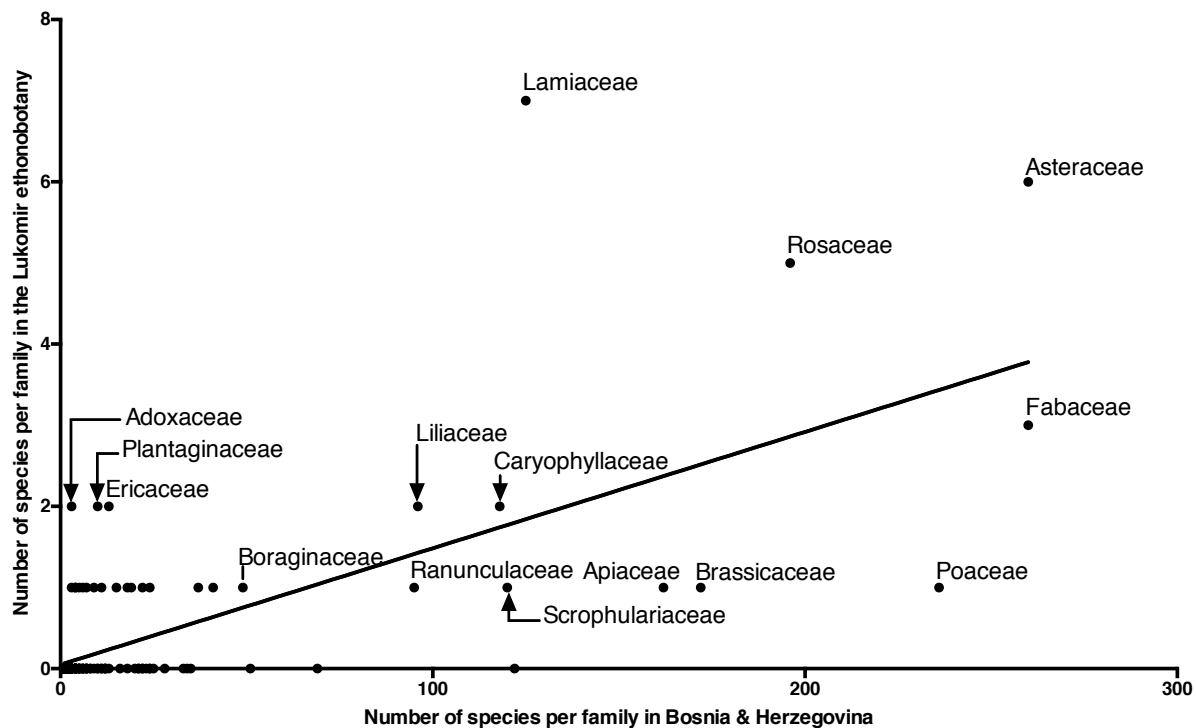
### *Analysis of medicinal plant families*

As a first step to identify key plant families, Figure 2.6 displays the number of medicinal, food, and material species by family. The three most diverse families were the Lamiaceae (7), Asteraceae (6), Rosaceae (5). Plant selection may merely be a function of family size and availability of species. However, a regression analysis accounts for the size of plant families and identifies high and low use families by plotting number of medicinal species versus total locally available species for the family (Figure 2.7). Several families have residual values greater than the standard deviation (Figure 2.8). The Highlander healers overuse 22 families and underuse six. The five families with the highest residuals (used more often than predicted) are the Lamiaceae, Asteraceae, Rosaceae, Adoxaceae, Plantaginaceae, and Ericaceae. Families with the lowest residuals (used less often than predicted) are Poaceae, Brassicaceae, Apiaceae, Scrophulariaceae, and Ranunculaceae.

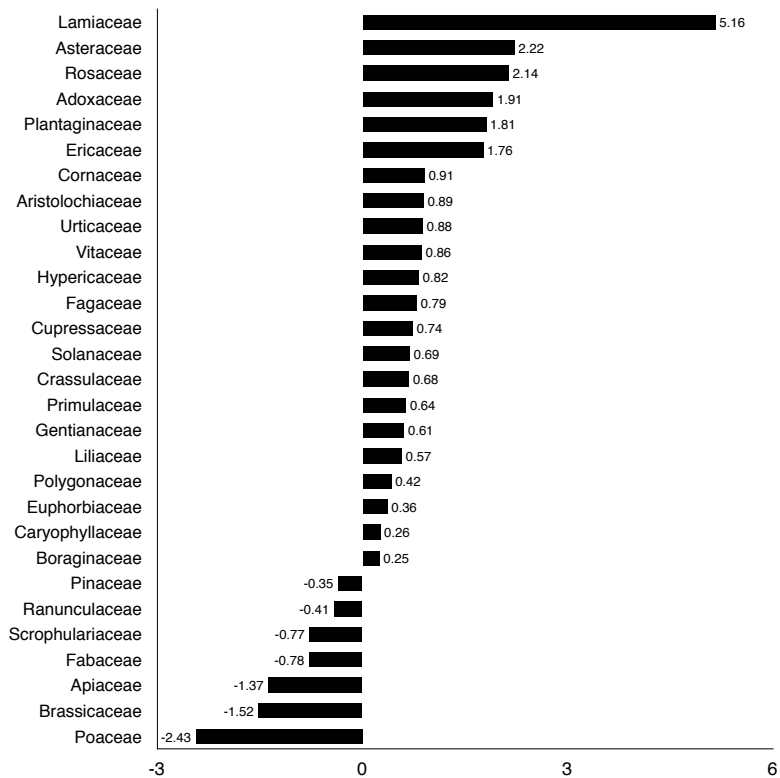


**Figure 2.6.** Frequency of species per family.

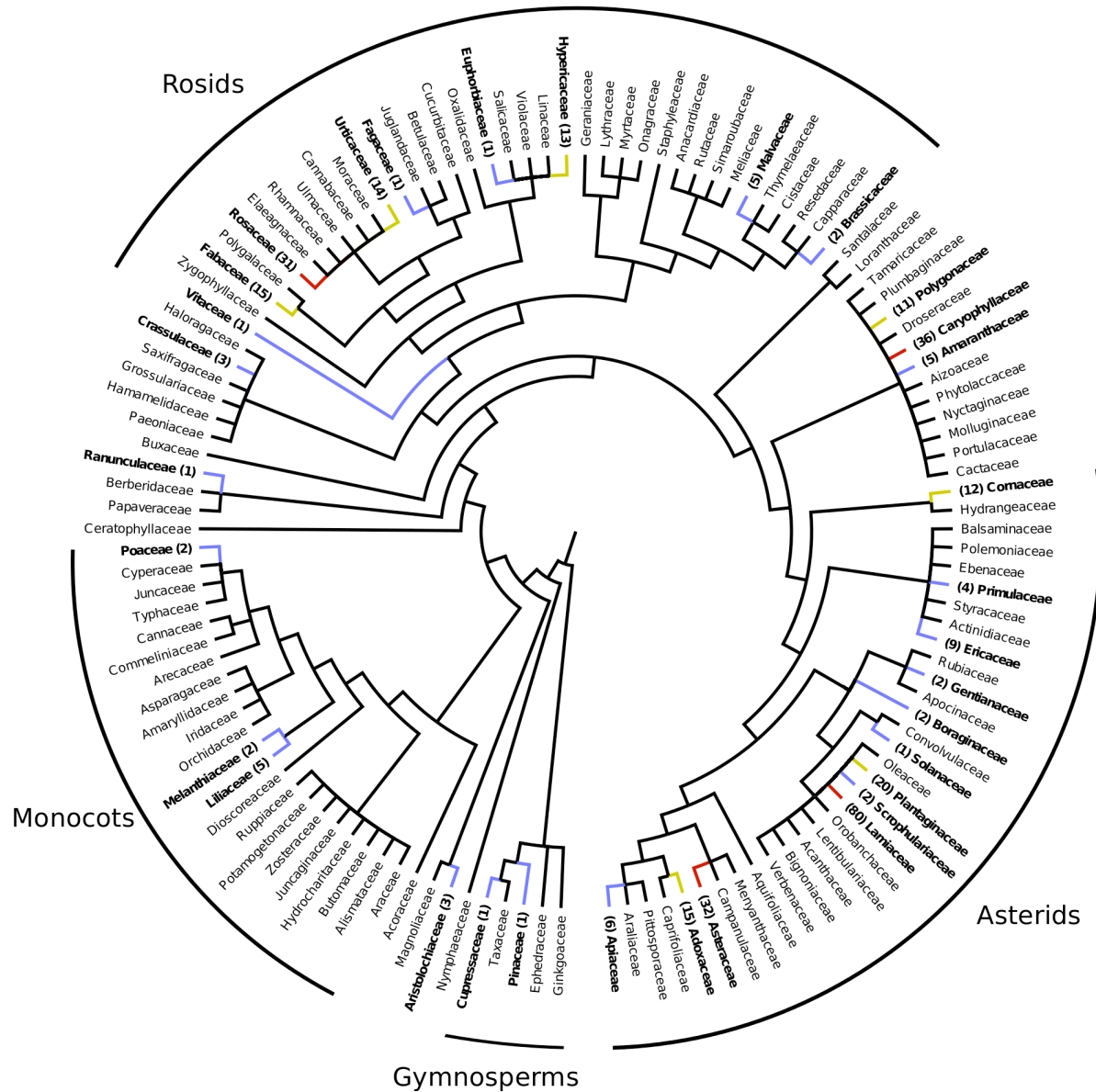
Moerman’s analysis reduces the impact of large families like Fabaceae which are otherwise ranked high (Figure 2.7). Moerman’s linear regression technique illustrates that the Highlanders’ plant selection is not a random event, but a cohesive traditional knowledge system specialized in recognizing and treating physician evaluated health concerns with species from small families.



**Figure 2.7.** Regression plot of ‘the number of plants per family used by the Lukomir Highlanders for medicines, foods, and materials and the ‘the number of taxa per family in the flora of Bosnia & Herzegovina ( $r^2 = 47$ ). The vertical distance between points and the regression line represent residual values.



**Figure 2.8.** Family residual values from the regression analysis in Figure 2.7.



**Figure 2.9.** A phylogenetic tree displaying plant families' use reports within the flora of B&H (Domac 1984).

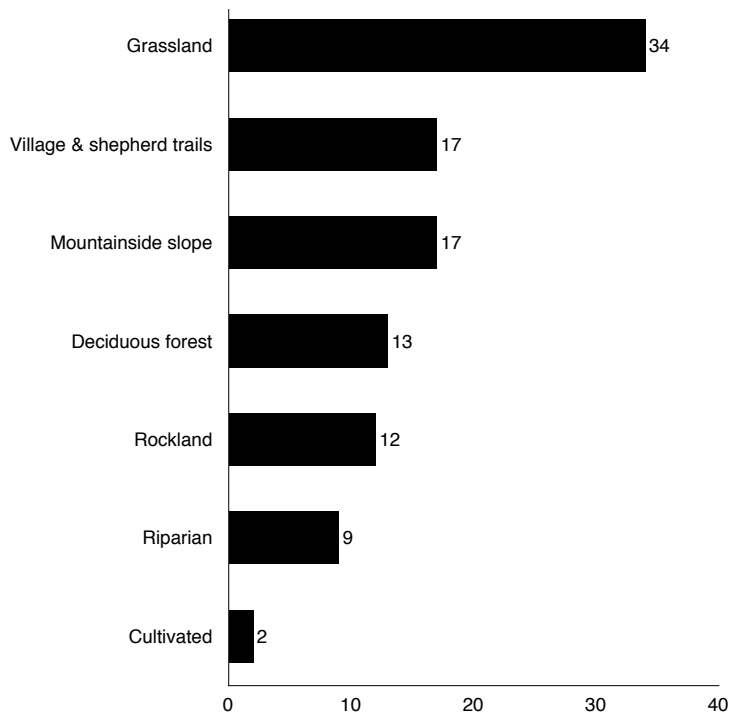
The phylogeny (Figure 2.9) links plant families to their use reports. The top three families included the Lamiaceae with 80 use reports. The Lamiaceae in Lukomir flourish in the Mediterranean environment on the south facing dolomite slopes of the Raktinica Canyon. The Lukomir cultural area is indeed a refuge of endemic Lamiaceae taxa as indicated by our ongoing floristic survey. The Caryophyllaceae were another family of high use and were often found fringing village trails and grasslands. Asteraceae presented with 32 use reports which was not surprising given the family's cosmopolitan distribution the family

is able to distribute throughout Lukomir's alpine, Mediterranean, and continental biogeographical regions.

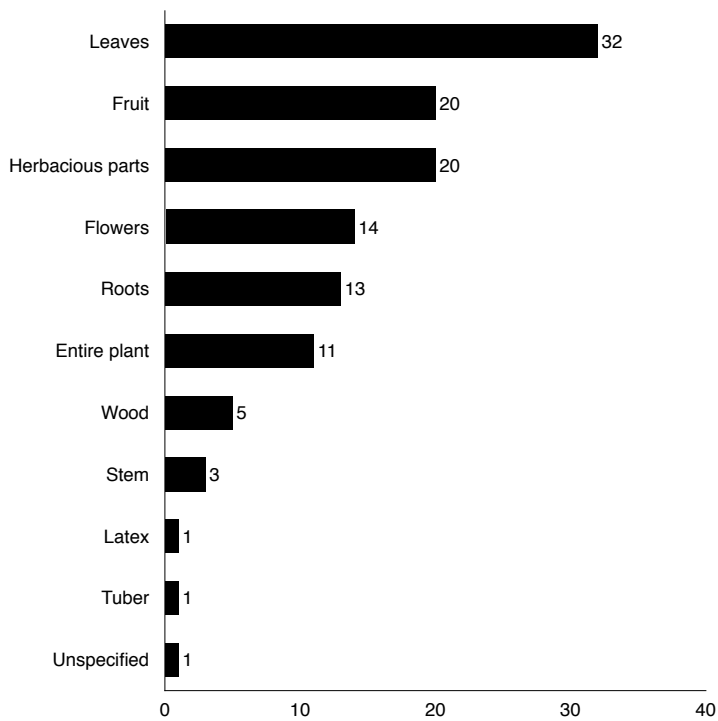
### ***Traditional use of rare and endemic plants***

Grassland habitats generated the most use reports of plants (Figure 2.10). In fact, collection of medical plants was greatest from mid July to early August as healers harvested hay from grasslands. From these taxa, the Highlanders make the highest use of leaves (Figure 2.11) and prepare most of their medicines as infusions (Figure 2.12). Interestingly, four medicinal taxa were cross-listed as foods. This shows that the Highlanders do not make a clear distinction between medicinal and food plants. These traditional foods are often cited for having disease preventing properties and the loss of traditional diets is often associated with the rise of chronic conditions such as heart disease and diabetes (Johns and Chapman 1995).

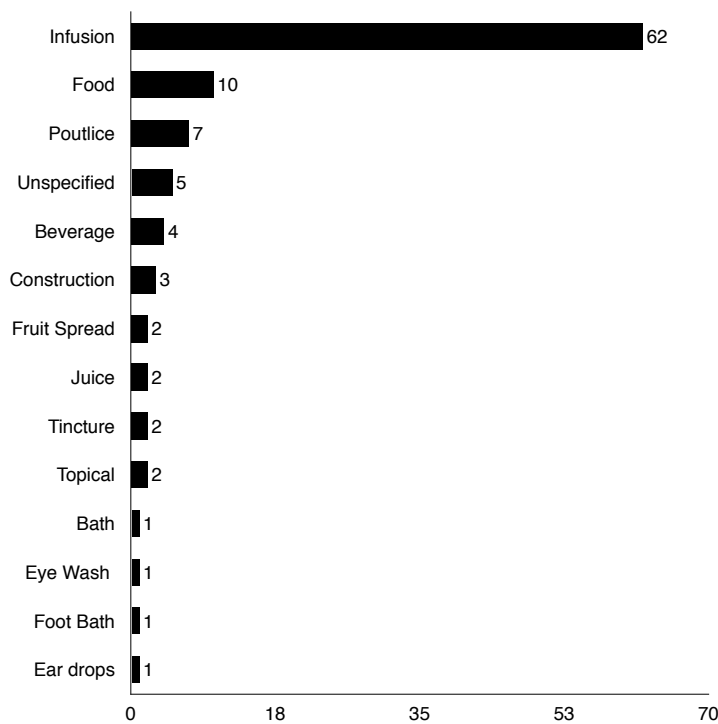
The Highlanders make use of eight endemic plant species: *Helleborus odoratus* Waldst. et Kit., *Gentiana lutea* L., *Lilium bosniacum* (Beck) Fritsch, *Silene uniflora* Roth ssp. *glareosa* (Jord.) Chater & Walters., *Silene uniflora* Roth ssp. *prostrata* (Gaudin) Chater & Walters, *Salvia officinalis* L., *Jovibarba hirta* (L.) Opiz, *Satureja montana* L.. Thirteen percent of all medicinal plant use is directly linked to endemic flora. This is not surprising since the Lukomir Highlanders are one of Europe's last indigenous communities, inhabiting one of Europe's most diverse biological hotspot (European Environmental Agency 2012). Endemic general and specific habitats included: 1. grasslands (St, Pim & Arr, Be, O-Co) 2. Rocklands and 3. Mountainside (Amphoricarpetalia (Amph.), Scorzonero-Chrysopogonetalia (S-Ch), and Ostryo-Carpinetalia orientalis (O-Co)). Endemic plant use exemplifies the community's native relationship the Highlanders have with their surrounding environment.



**Figure 2.10.** Habitats of plant species listed in Table 2.1.



**Figure 2.11.** Plant organ use of species listed in Table 2.1.



**Figure 2.12.** Plant preparation reports of plants listed in Table 2.1.

### ***Post war development, conservation, and the dietary shift in Lukomir***

Very recent changes have deteriorated the health and environment status in Lukomir. Prior to 1997, three *vodenica mlini* (hydro powered cereal mills) conducted the process of removing hard seed coats from Lukomir’s cereal crops (Figure 2.9). Once powered by the spring rains and snow melt, the mills are now in disorder on the skirts of Donji Lukomir (see M, Figure 2.1). From the peak of *Obalj*, through the canyons of the *Rakitnica*, runs the *Vodenica* waterway (Figure 2.1). The hardwood *Fagus sylvatica* L. (*Bukva*, Beech) was handcrafted into troughs to funnel the *vodenica* and hydropower the wooden turbines constructed of *F. sylvatica* vanes and axils. The turbine turned one of the two handmade grinding stones which produced the wholegrain cereal flour for centuries. Our informants indicated that the cereals routinely grown and milled in Lukomir were: *Avena sativa* L. (*Zob*, Oat), *Secale sereale* L. (*Raž*, Rye), *Hordeum vulgare* L. (*Ječam*, Barley), *Triticum aestivum* L. (*Pšenica*, Wheat), and *Zea mays* L. (*Kukuruz*, Corn). However, they are no longer cultivated and milled, and have been replaced by imported soft white wheat flour.

When we asked why the *mlini* are not used, informants explained that in 1997, one

year after the Bosnian war, water diversion began in Lukomir. Water pipelines were placed at the mouth of the Obalj mountain spring in order to share water with lower elevation communities within the Municipality of Konjic. Following diversion the waterways began to dry up by mid June, leaving the mills devoid of running water. There was no hydro power available for milling the June, July, and September cereal harvests. The consequence of this water work development was the collapse of the *vodenica mlini*, cereal landraces, and Lukomir's organic multigrain diet. Running the mills involved considerable physical effort and provided exercise.

The water work diversion lead to a community wide dietary shift. Many locals now depend on a food truck making deliveries of white flour once per week. Now, the time spent working to produce their own multi grain flour is gone, and the diet and lifestyle shift is contributing to Lukomir's greatest health concerns: heart disease and diabetes.

All rare and endemic flora and many other species listed in Table 2.1 can be found in the area from Obalj (1874 m) to the Rakitnica River (640 m). These areas are now more endangered because of the water work diversion and ongoing habitat loss. Although people from B&H should have equal and shared access to water, this particular development was placed in the centre one of the most biogeographic sensitive areas of the country, and the continent, without sufficient consultation and foresight. The collapse of this traditional food system comes at a time when tourism is increasing in the community and B&H is modeling itself as an international destination to experience European traditional culture. Endemic flora and organic multigrain products are popular attractions among hiking enthusiasts and health conscious visitors to the Dinaric Alps. Many women in the community depend on tourism as they open their homes to tourists, serve food, teas, and coffee, and sell their knitted merchandise.

Although post war development has contributed to the erosion of the self sustaining traditional lifestyle of the Lukomir Highlanders, they continue to have a strong traditional medicine and gathered food system. In planning for a durable, self sufficient future for the Lukomir Highlanders, emphasis should be placed on preservation of the *vodenica mlini* (hydro cereal mills), a unique cultural technology, as well as the tradition of plant use by one

of Europe's last indigenous communities in one of Europe's most biodiverse hotspots.



**Figure 2.13.** A *vodenica mlin* (hydro mill) on the dried up *Vodenica waterway* in July.

## Chapter 3

# Ways the Lukomir Highlanders of Bosnia & Herzegovina Treat Diabetes<sup>3\*</sup>

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\*Ferrier, J., Šaćiragić, L., Redžić, S., Trakić, S., Velić, S., Chen, E. C. H., Cuerrier, A., Balick, M. J., Arnason, J. T. (in press, 2014). Ways the Lukomir Highlanders of Bosnia & Herzegovina treat diabetes. In A. Pieroni & C. L. Quave (Eds.), *Ethnobotany and Biocultural Diversities in the Balkans Perspectives on Sustainable Rural Development and Reconciliation*. Section: The herbal reservoir of Europe: from folk medical uses to the modern medicinal plant trade. Springer.



Facing north-west on Obajl mountain, Lukomir, Bosnia and Herzegovina.

<sup>3</sup> Dedicated to the memory of Sulejman Redžić

## **Ways the Lukomir Highlanders of Bosnia & Herzegovina Treat Diabetes<sup>4</sup>**

Today, diabetes is a pandemic disease and a top concern in indigenous societies. The global burden of diabetes was estimated to affect 366 million people in the year 2011; by 2030, 552 million people are expected to have diabetes (IDF 2013). Type 2 diabetes (T2D) (formerly called non-insulin-dependent or adult-onset) results from the body's ineffective use of insulin and is characterized by hyperglycaemia. T2D is responsible for 90% of diabetes cases (Alberti and Zimmet 1998) and is linked to rapid social change, genetics, dietary acculturation, excess carbohydrate consumption, physical inactivity, and excess body weight (Young 1994; Hegele et al. 1999; Young et al. 92; Young et al. 2000; Ritenbaugh & Goodby 1989; WHO 2013a). The World Health Organization estimated that Bosnia and Herzegovina (B&H) had 111,000 people with diabetes in 2000 and the number is expected to increase to 180,000 by 2030 (WHO 2013b).

Long-term complications of T2D include vascular disease, heart disease, stroke, neuropathy, retinopathy, cataracts, atherosclerosis, nephropathy, and impaired wound healing. Impaired glucose uptake affects the cells of organs that do not require insulin for glucose uptake (the nervous system, heart, kidneys, and small blood vessels). As a consequence, these cells have high concentrations of intracellular glucose during elevated hyperglycaemic periods, resulting in impaired cell function and cell death (Ahmed, 2005). These T2D complications are mediated by the formation of advanced glycation endproducts (AGEs), which are a therapeutic target with phytotherapies (Figure 1.1).

T2D incidence is three times higher in indigenous populations. These are some of the fastest growing yet most vulnerable populations in the world that often lack culturally appropriate primary healthcare (Alberti et al. 2004; Ahmed 2005; Helin 2006). This is true for the Lukomir Highlanders of B&H, one of the last native communities in Europe, located in the Dinaric Alps, southwest of the capital city, Sarajevo (43.6° lat, 18.1° lon, 1460 m a.s.l.). Local health authorities have described diabetes and heart disease as the most prevalent diseases in Lukomir (Ferrier et al. 2013). One reason for the prevalence of

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<sup>4</sup> Dedicated to the memory of Sulejman Redžić

diabetes in Lukomir is due to a transition to modern diets and sedentary lifestyle. Municipal water diversion was a driver for this transition in Lukomir. This recent post war development project removed the source of water from Lukomir's cereal hydro mills, which in turn caused the collapse of the mills. This led to a nutrition shift from a traditional organic multigrain diet to a higher glycaemic diet based on soft white wheat flour (Ferrier et al. 2013). This development has eroded the Highlanders' traditional lifestyle, exercise, transformed habitats of medical flora, and increased the prevalence of T2D.

Since Lukomir has no primary health care facility, our objective was to identify the plants the Lukomir Highlanders use for the treatment of diabetes and highly associated symptoms. The plants were ranked using the syndromic importance value (SIV) function developed by Leduc et al. (2006) for a study of Cree traditional medicines. To assist future ethnobotanical studies, we present our physician-ranked symptom values required for completing Leduc's SIV function. As a pilot pharmacology and phytochemistry study, we investigated the bioactivity and phytochemistry of one genus that was prominent in the Lukomir pharmacopoeia and cross-culturally used by our Cree partners (Leduc et al. 2006).

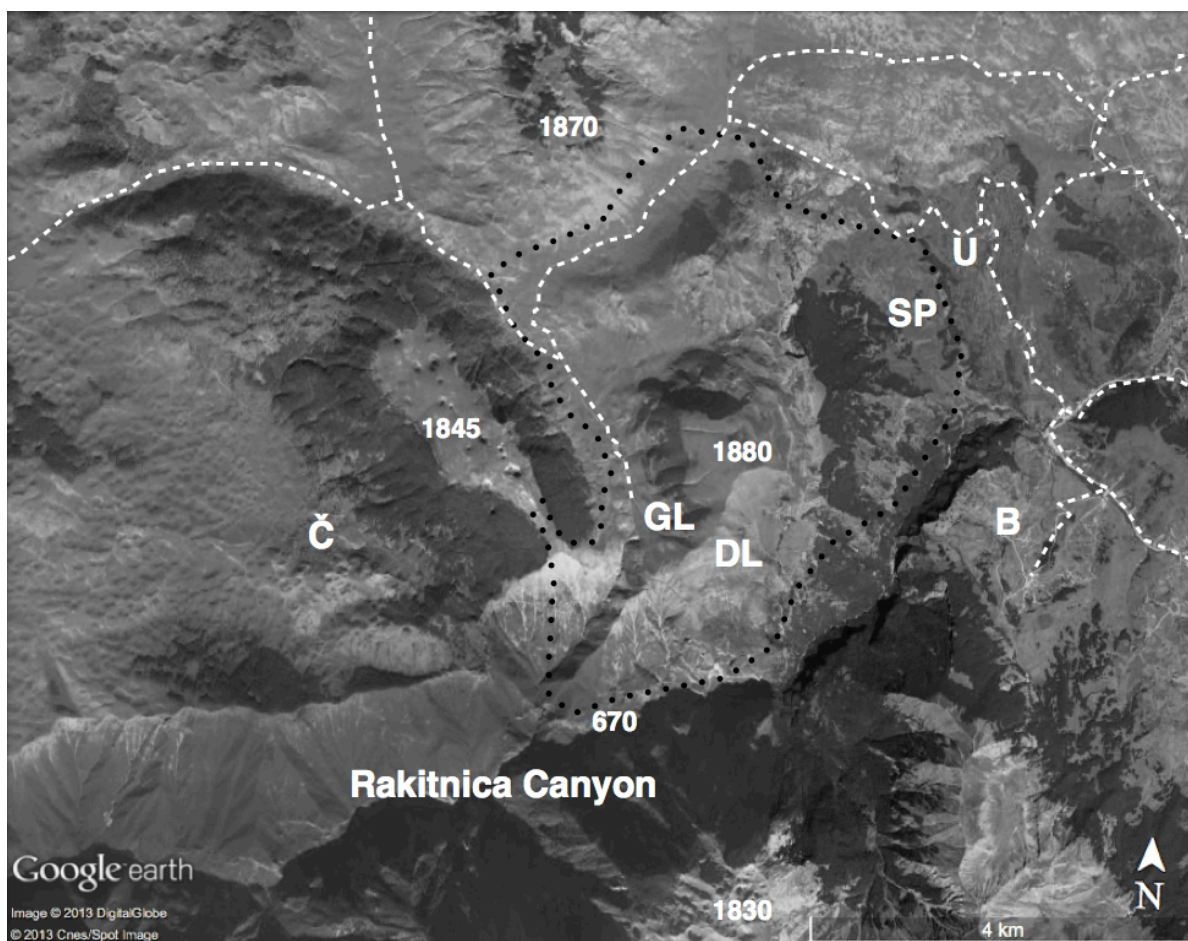
## **Methods**

### ***Partnership, permits, and prior informed consent***

This ethnobotanical study of plants was conducted between 2007-2013 with the Lukomir community in Bosnia and Herzegovina, and J.F. and L.Š.'s organization, Cross-cultural Health Initiative (CHI). An international collaboration was developed with the Partnerships for Tomorrow Program, Phase II (PTP) funded by the Canadian International Development Agency (CIDA). Members of CHI were hosted by the University of Sarajevo and Foundation GEA+. Research permits were issued by the Municipality of Konjic, University of Ottawa (H05-09-07), with prior informed consent from Lukomir's leaders and informants.

### ***Study site: Lukomir, Municipality of Konjic, Bosnia & Herzegovina (B&H)***

A consensus ethnobotany was conducted in the Bjelašnica area of the Dinaric Alps, in Lukomir with the Lukomir Highlanders of Bosnia and Herzegovina (Figure 3.1).



**Figure 3.1.** Lukomir and nearby villages, Bosnia & Herzegovina. Taxa in Table 3.1 were collected within black dotted perimeter (~26km<sup>2</sup>). White dashes indicate roads leading to Lukomir. Numerals indicate meters above sea level (m a.s.l.). Bobovica (B = 1300 m a.s.l.), Čuhovici (Č = 1330 m a.s.l.), Donji Lukomir (DL = 1250 m a.s.l.), Gornji Lukomir (GL = 1460 m a.s.l.), Studeni Potok (SP = 1420 m a.s.l.).

This area is classified as an alpine biogeographic region that is closely bordered by Mediterranean and continental biogeographic regions (European Environment Agency 2012). Many community members are descendants of a Bogomil lineage who first settled in *Donji Lukomir* (Lower Lukomir) (43.632 lat, 18.194 lon, 1200 m a.s.l.) and eventually moved to *Gornji Lukomir* (Upper Lukomir, commonly referred to as Lukomir) (43.637 lat, 18.182 lon, 1460 m a.s.l.). Lukomir’s informants included spiritual leaders, elders, younger women and men. Informants described plants on field trips, garden tours, while shepherding, or in comfortable settings of their choice. We earned our interview time with Lukomir’s healers by volunteering our time to shepherd, harvest food, and stack hay. This allowed informants to spend more time on field collection trips and interviews. Notes and photos

were taken when participants choose to display preparation methods of plant and natural product remedies.

Land mines were avoided by consulting with Bosnia and Herzegovina Mine Action Center (BH MAC) (<http://www.bhmac.org>). All plants were collected on trail sides or in areas that were constantly travelled by sheep herds, since only parts of the Lukomir territory were surveyed by BH MAC.

Field work followed a quantitative consensus methodology with individual semistructured interviews during which L.Š., J.F., and S.R. collected the following data: (1) specimen voucher number, (2) photo number, (3) common name, (4) scientific name, (5) family, (6) GPS coordinates, (7) altitude (m a.s.l.), (8) habitat, (9) syntaxa, (10) flowering time and description, (11) medically active collection time, (12) use, (13) use category, (14) plant part used, (15) amount used, (16) preparation method, (17) administration method, (18) dosing regimen, (19) ethnographic details, (20) informant name, (21) date.

Determinations were made using a Domac's regional flora (1984), vouchers, and Tropicos.org (2013). Duplicate vouchers were collected (when sustainable) and are currently held at the University of Ottawa Herbarium (OTT) with voucher numbers in Table 3.1. Plant vouchers and an iPad (Apple, Cupertino, USA) were used to display collections to elders who could not venture over the mountainside, or for informant review purposes.

### ***Elucidation of plants for diabetes using syndromic importance values***

The syndromic importance value (SIV) function was adapted from (Leduc et al. 2006; Oubré et al. 1997; McCune and Johns 2002, 2003). SIV values allow ranking of plant species by accounting for: (1) the number of different symptoms for which a plant was cited, (2) the frequency of plant citation by individual informants, and (3) the association rank of symptoms for which a plant was cited to treat. The latter association rank of symptoms was determined by four physicians at The Ottawa Hospital, Ottawa, Canada, who diagnose and treat patients with diabetes. Symptoms were given a scale weight  $w$  from 1-4 for physician ranking, where 1 is a symptom highly associated with diabetes; 2, moderately associated with diabetes; 3, weakly associated with diabetes; and 4 is not at all associated with diabetes.

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

The SIV function accounts for  $w$ , the weight of the symptom;  $s$ , the symptom treated by the species;  $f$  the frequency of citation for the species;  $S$  the total number of symptoms used for the survey (used in Leduc et al. 2006, but was not indicated in the denominator on the right); and  $F$  the total number of interviews in the survey.

### ***Statistics and phylogenetic analysis***

Statistical analysis was conducted using Prism 6 software. A phylogenetic tree was constructed using TreeGraph 2.0.47-206 beta, taxon with positive SIV values, and based on topology presented by Angiosperm Phylogeny Group 3 (2009).

### ***Cross-cultural consensus***

In collaboration with the Eeyou Istchee Cree and their Canadian Institute of Health Research Team for Aboriginal Antidiabetic Medicines (CIHR TAAM) we compared the Lukomir ethnobotanical taxa with the Cree CIHR TAAM's taxa (Leduc et al. 2006) to find consensus specimens for cross-culturally relevant pharmacological and phytochemical investigations. Both communities contain alpine and continental type habitats and are isolated linguistically and geographically from each other.

### ***Extraction of consensus taxa and outgroup leaf material***

*Vaccinium* spp. and outgroup *Arctostaphylos uva-ursi* (L.) Spreng. leaves were collected and identified by S.T., S.R., and J.F., and deposited at the University of Ottawa Herbarium (OTT) (Table 3.2). Samples were dried at 35 °C and ground in a Thomas-Wiley Mill (1 mm mesh). Plant material was extracted twice in ethanol/water (95%:5%) at room temperature for 24 h per phase. The phases were centrifuged at 1000 xg, filtered, pooled, dried using a Speed Vac (Savant, Halbrook New York), and lyophilized (Edwards Super Modulyo Freeze Drier, Fisher Scientific, Ottawa, Canada).

### ***Inhibition of advanced glycation endproducts***

Inhibition of AGE formation was assessed as described by Farsi et al. (2008) with modifications. Bovine serum albumin (BSA) ( $1 \text{ mg}\cdot\text{mL}^{-1}$ ) was incubated with  $100 \text{ mmol}\cdot\text{L}^{-1}$  glucose /  $100 \text{ mmol}\cdot\text{L}^{-1}$  fructose in sodium phosphate monobasic monohydrate buffer (pH 7.4) with extract (experimental treatment), ethanol/water (4:1) (negative control), or quercetin, an antioxidant against glycation by way of phenolic hydroxyl groups in the flavonoid structure ( $24 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$  in assay) (Sengupta et al. 2006), which served as a positive control. To control for fluorescence of extracts, a treatment without BSA was included. To control for fluorescence of BSA, a treatment with BSA and vehicle was included. Stock solutions of extracts were serially diluted and tested at five concentrations that were optimized for dissolution and a linear concentration response ( $40$ ,  $20$ ,  $10$ ,  $5$ , and  $2.5 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$  in well concentration). Three replicates were tested in sterile opaque polystyrene 96-well clear bottom plates (Corning Inc., New York, N.Y., USA). Plates were covered, sealed with Parafilm, and incubated for 7 days at  $37 \text{ }^\circ\text{C}$  while shaking. Following incubation, fluorescence was measured using a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, Calif., USA) at excitation and emission wavelengths of 355 and 460 nm. Glucose/fructose and ethanol/water fluorescence was subtracted from all results, and percent inhibition and  $\text{IC}_{50}$  values were calculated as previously described (Farsi et al. 2008).

### ***HPLC-MS analysis of Vaccinium species***

Stock solutions of extracts were prepared to  $10 \text{ mg}\cdot\text{mL}^{-1}$  in ethanol/water (80%:20%) and filtered through a  $0.2 \text{ }\mu\text{m}$  PTFE non-sterile filter (Chromatographic Specialties Inc). HPLC analyses were performed using an Agilent 1100 chromatographic system (Agilent Technologies Inc.) consisting of an autosampler, quaternary pump, and diode array detector (DAD). Solvents were of HPLC grade (Fisher Scientific), and trifluoroacetic acid (TFA) was of analytical grade (J.T. Baker). A Synergi RP-Polar column ( $150 \text{ mm} \times 3 \text{ mm}$ ;  $4 \text{ }\mu\text{m}$  particle size) was kept at  $53 \text{ }^\circ\text{C}$  and a flow rate of  $0.5 \text{ mL}/\text{min}$  was maintained. The mobile phase consisted of aqueous TFA (0.05%) (solvent A), acetonitrile with TFA (0.05%) (solvent B), and methanol (solvent C). Initial conditions were set at 95%:5%:5% (A:B:C) and changed following a linear gradient of 5–9.2% B and 5–17.5% C in 25 minutes. The

column was then washed by increasing solvent B to 100% over 5 minutes and returned to initial conditions in the next 5 minutes. The column was allowed to re-equilibrate for 5 minutes, resulting in a total run time of 40 minutes. Ten  $\mu\text{L}$  of each  $10 \text{ mg}\cdot\text{mL}^{-1}$  leaf extract were injected for each run and the elution profiles were monitored at 350 nm, 280 nm, and 230 nm with band width kept at 4.

Phytochemical constituents were identified based on comparison to retention times and UV spectra of pure standards (95% purity) relative to a programmed library of known UV spectra, and further confirmed by mass spectrometry (MS) fragmentation patterns. Standard compounds used to monitor leaf extracts were: (+)-catechin, chlorogenic acid, quercetin-3-O-glucoside, (Extra-synthèse), rutin (quercetin-3-O-rutinoside), quercetin-3-O-rhamnoside (Sigma) para coumaric acid, taxifolin, quercetin-3-O-galactoside, and myricetin (Arnason lab inventory, University of Ottawa, ON).

## Results and Discussion

We were able to interview 25 informants who described plant uses on mountain and canyon field trips by volunteering to shepherd, collect food, and hay with the Highlanders between 2008 and 2010. Informants provided information on diabetic symptoms and did not initially provide any diabetes use reports, but during subsequent conversations on casual visits to Lukomir, many healers agreed that panacea remedies could be used to treat diabetes. Highlanders described eight panacea treatments which were subsequently treated as a diabetes SIV use report.

All eight species used as ‘diabetes’ treatments were cited as *čaj* (infusions). In one household’s case, we noticed that some of these taxa were present in their *čaj* collection, but were not cited as medicine: Asteraceae: *Matricaria matricarioides* (Less.) Porter ex Britton (*Kamilica*, *Stomaklija*, Pineappleweed) and *Salvia officinalis* L. (*Kadulja*, Sage); Lamiaceae: *Teucrium montanum* L. (*Iva*, Mountain germander); and one fern specimen never mentioned, Aspleniaceae: *Phyllitis scolopendrium* (L.) Newman (*Podrebница*<sup>5</sup>, Hart’s tongue fern)

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<sup>5</sup> Redžić (2008) informed us that Podrebница is translated “under the ribs”. The name refers to the sori which are aligned in such a way that they resemble a human rib cage, leading to a doctrine of signature use as a treatment for ailments under the ribs.

(Figure 3.2). When we noticed this *čaj* collection, we asked why they were not mentioned. The response: “We drink *čaj* for our health.” These plants were for their “medicine pot”, that was prepared with a prayer after meal consumption. This household also has one family member with T2D managed by prescription medicines. Perhaps these plants are best referred to as Pieroni and Quave (2006) describe them: “medicinal foods or food medicines”—prepared to obtain “medical action”, consumed in a “food context”, and in this case, not always cited as medicine.



**Figure 3.2.** Dairy (left) and plant based (right) *čaj* with a *čaj* plant collection (upper) including *Matricaria matricarioides*, *Phyllitis scolopendrium*, *Salvia officinalis*, and *Teucrium montanum*.

The Lukomir Highlanders used 41 species for the treatment of diabetes symptoms (Table 3.1). Antidiabetic reports (in parenthesis) were set in five general habitats: grassland (21), village and shepherd trails (12), mountain slopes (9) riparian zones (6), rocklands (6), deciduous forest (4), and cultivated (2). Antidiabetic preparation methods were infusion (46), poultice (7), food (3), beverage (2), ear drops (2), juice (1), foot bath (1), eye wash (1), and tincture (1) (Figure 3.S1).

**Table 3.1.** Taxa used by the Lukomir Highlanders to treat symptoms of diabetes. European endemic species are indicated by a dagger (†). Determinations followed legitimate names in Tropicos (2013).

Genus species	†	Common name (B,E)	Symptom	V#	SIV
<i>Achillea millefolium</i> L.	-	Kunica, Yarrow	Diabetes	358	0.00725
<i>Achillea millefolium</i> L.	-	Kunica, Yarrow	Swelling or inflammation	358	0.00725
<i>Anthyllis vulneraria</i> L.	-	Ranjenik, Kidney vetch	Slow healing infections	372	0.00095
<i>Asarum europaeum</i> L.	-	Kopitnik, kopitnjak, Wild ginger	Slow healing infections	382	0.00089
<i>Capsella bursa-pastoris</i> (L.) Medik.	-	Rustemača, Shepherd's purse	Swelling or inflammation	398	0.00289
<i>Cetraria islandica</i> (L.) Ach	-	Islandski lišaj, Mašina Iceland moss	Heart or chest pain	403	0.00192
<i>Cichorium intybus</i> L.	-	Konjanik, Chicory	General weakness	411	0.00235
<i>Cornus mas</i> L.	-	Drijen, European dogwood	Slow healing infections	384	0.00089
<i>Crataegus monogyna</i> Jacq.	-	Glog, gloginje, Hawthorn	Back or kidney pain	361	0.00553
<i>Crataegus monogyna</i> Jacq.	-	Glog, gloginje, Hawthorn	Diarrhoea	361	0.00553
<i>Elymus repens</i> (L.) Gould	-	Pirika, Couch grass	Heart or chest pain	389	0.00299
<i>Elymus repens</i> (L.) Gould	-	Pirika, Couch grass	Increased urination	389	0.00299
<i>Equisetum arvense</i> L.	-	Preslica, Field horsetail	Back or kidney pain	367	0.00588
<i>Equisetum arvense</i> L.	-	Preslica, Field horsetail	Swelling or inflammation	367	0.00588
<i>Gentiana lutea</i> L.	†	Lincura, Great yellow gentian	Sore or swollen limbs	393	0.00257
<i>Jovibarba hirta</i> (L.) Opiz	†	Čuvarkuća, Beard of Jupiter, Hen and Eggs	Slow healing infections	379	0.00363
<i>Jovibarba hirta</i> (L.) Opiz	†	Čuvarkuća, Beard of Jupiter, Hen and Eggs	Swelling or inflammation	379	0.00363
<i>Matricaria matricarioides</i> (Less.) Porter ex Britton	-	Kamilica, Stomaklija, Pineappleweed	Diabetes	351	0.00775
<i>Matricaria matricarioides</i> (Less.) Porter ex Britton	-	Kamilica, Stomaklija, Pineappleweed	Swelling or inflammation	351	0.00775
<i>Mentha longifolia</i> (L.) L.	-	Nana, Horse mint	Swelling or inflammation	349	0.00428
<i>Nepeta cataria</i> L.	-	Macina trava, Catnip	Swelling or inflammation	369	0.00321
<i>Ononis spinosa</i> L.	-	Gladišika, Spiny restharrow	Increased urination	377	0.00115
<i>Phyllitis scolopendrium</i> (L.) Newman	-	Podrebница (♂ or ♀), Hart's-tounge fern	Heart or chest pain	357	0.00259
<i>Plantago lanceolata</i> L.	-	Bokvica ♀, Ribwort plantain	Heart or chest pain	359	0.00304
<i>Plantago lanceolata</i> L.	-	Bokvica ♀, Ribwort plantain	Slow healing infections	359	0.00304
<i>Plantago major</i> L.	-	Bokvica ♀, Ribwort plantain	Heart or chest pain	360	0.00304
<i>Plantago major</i> L.	-	Bokvica ♀, Ribwort plantain	Slow healing infections	360	0.00304
<i>Polygonum bistorta</i> L.	-	Srčanik, Bistort	Heart or chest pain	356	0.00266
<i>Primula veris</i> L.	-	Jagorčevina, Cowslip	Diabetes	373	0.00382

<b>Genus species</b>	<b>† (B,E)</b>	<b>Common name</b>	<b>Symptom</b>	<b>V#</b>	<b>SIV</b>
<i>Prunus spinosa</i> L.	-	Trnjina, Blackthorn	Blurred vision	405	0.00428
<i>Prunus spinosa</i> L.	-	Rakija, Brandy	Swelling or inflammation	405	0.00428
<i>Rubus saxatilis</i> L.	-	Kupina, Stone bramble	Diabetes	407	0.00342
<i>Salvia officinalis</i> L.	†	Kadulja, Sage	Heart or chest pain	348	0.00296
<i>Sambucus wightiana</i> Wall. ex Wight & Arn.	-	Haptovina	Heart or chest pain	376	0.00192
<i>Sambucus nigra</i> L.	-	Zova, zobovina, Elder	Heart or chest pain	354	0.00266
<i>Satureja montana</i> L.	-	Vrijesak, Winter savory	Diabetes	366	0.00408
<i>Sedum sexangulare</i> L.	-	Zednjak, Tasteless stonecrop	Slow healing infections	416	0.00086
<i>Silene uniflora</i> Roth ssp. <i>glareosa</i> (Jord.) Chater & Walters	†	Puca, Sea champion	Heart or chest pain	353	0.00741
<i>Silene uniflora</i> Roth ssp. <i>glareosa</i> (Jord.) Chater & Walters	†	Puca, Sea champion	Increased urination	353	0.00741
<i>Silene uniflora</i> Roth ssp. <i>glareosa</i> (Jord.) Chater & Walters	†	Puca, Sea champion	Slow healing infections	353	0.00741
<i>Silene uniflora</i> Roth ssp. <i>glareosa</i> (Jord.) Chater & Walters	†	Puca, Sea champion	Swelling or inflammation	353	0.00741
<i>Silene uniflora</i> Roth ssp. <i>prostrata</i> (Gaudin) Chater & Walters	†	Puca, Sea champion	Heart or chest pain	350	0.00741
<i>Silene uniflora</i> Roth ssp. <i>prostrata</i> (Gaudin) Chater & Walters	†	Puca, Sea champion	Increased urination	350	0.00741
<i>Silene uniflora</i> Roth ssp. <i>prostrata</i> (Gaudin) Chater & Walters	†	Puca, Sea champion	Slow healing infections	350	0.00741
<i>Silene uniflora</i> Roth ssp. <i>prostrata</i> (Gaudin) Chater & Walters	†	Puca, Sea champion	Swelling or inflammation	350	0.00741
<i>Smyrniium perfoliatum</i> L.	-	Ljaljica, Perfoliate alexanders	Increased urination	380	0.00111
<i>Solanum tuberosum</i> L.	-	Krompir, Potato	Swelling or inflammation	408	0.00278
<i>Symphytum officinale</i> L.	-	Gavez, Common comfrey, Boneset	Slow healing infections	383	0.00089
<i>Teucrium montanum</i> L.	-	Iva, Mountain germander	Diabetes	352	0.00487
<i>Tilia platyphyllos</i> Scop.	-	Lipa, Large-leaved lime	Heart or chest pain	374	0.00215
<i>Tussilago farfara</i> L.	-	Podbijel (♂ or ♀), Coltsfoot	Heart or chest pain	371	0.00215
<i>Urtica dioica</i> L.	-	Žara, kopriva, Stinging nettle	Heart or chest pain	362	0.00222
<i>Urtica dioica</i> L.	-	Žara, kopriva, Stinging nettle	Slow healing infections	362	0.00222
<i>Vaccinium myrtillus</i> L.	-	Borovnica, European blueberry	Diabetes	368	0.00480
<i>Vaccinium vitis-idaea</i> L.	-	Brusnica, Lingonberry	Diabetes	385	0.00441
<i>Vaccinium vitis-idaea</i> L.	-	Brusnica, Lingonberry	Slow healing infections	385	0.00441
<i>Verbascum thapsus</i> L.	-	Divizbina, divizma, Great mullein	Heart or chest pain	390	0.00200
<i>Vitis vinifera</i> L.	-	Sirće, Common grape vine	Swelling or inflammation	409	0.00278

The frequency of taxa to treat diabetes and associated symptoms were heart and chest pain (15), swelling or inflammation (13), slow healing infections (12), panacea (8), increased urination (5), back or kidney pain (2), diarrhoea (1), blurred vision (1), general weakness (1), sore or swollen limbs (1) (Figure 3.S2).

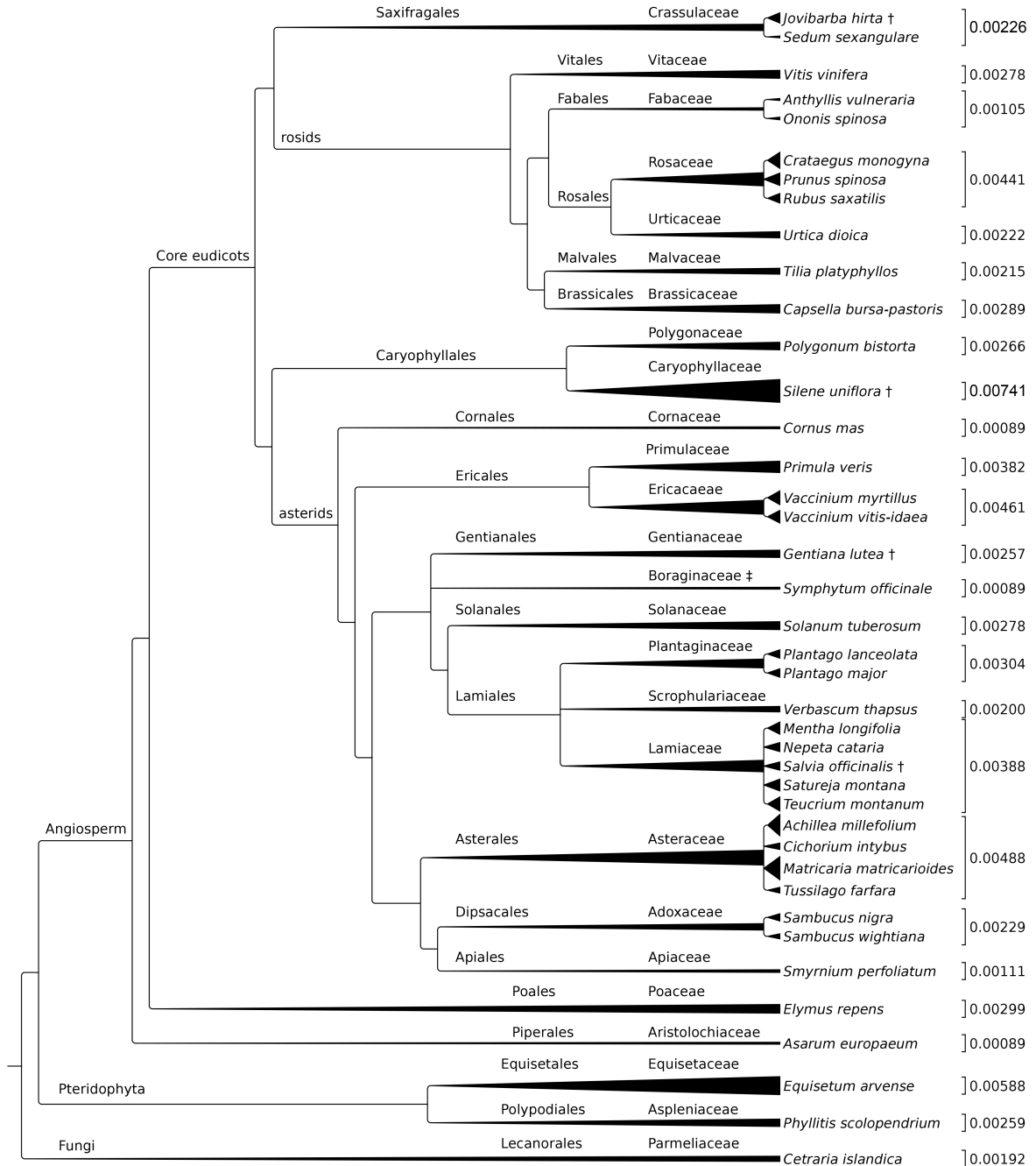
According to the Flora Europaea (Royal Botanic Garden Edinburgh 1998) The Lukomir Highlanders mentioned five European endemic taxa (†, Figure 3.3). Endemic taxa account for 20% of use reports: *Jovibarba hirta* (L.) Opiz (Crassulaceae), *Silene uniflora* ssp. *glareosa* (Jord.) Chater & Walters, *Silene uniflora* Roth ssp. *prostrata* (Gaudin) Chater & Walters (Caryophyllaceae), *Salvia officinalis* L. (Lamiaceae), and *Gentiana lutea* L. (Gentianaceae) was also listed as endangered (Figure 3.3).

### ***Ways the Lukomir Highlanders treat diabetes and associated symptoms***

In order to rank taxa from interviews with the SIV function, the weight ( $w$ ) of diabetes and 15 associated symptoms were given association to diabetes values by four physicians: increased thirst (1), slow healing infections (1), increased urination (1.25), foot numbness or foot sores (1.25), blurred vision (1.75), diarrhoea (2.25), heart or chest pain (2.25), abscesses or boils (2.25), frequent headaches (2.75), general weakness (2.75), increased appetite (2.75), sore or swollen limbs (3), arthritis or rheumatism (3.25), back or kidney pain (3.25), swelling and/or inflammation (3.25), diabetes (4).

SIV values of species were presented in Table 3.1. The top five SIV factors (in parentheses) belonged to *Matricaria matricarioides* (Less.) Porter ex Britton (0.0078), *Silene uniflora* ssp. *glareosa* (0.0074), *Silene uniflora* ssp. *prostrata* (0.0074), *Achillea millefolium* L. (0.0073), and *Equisetum arvense* L. (0.0059). Each species' SIV value was multiplied by 100,000 and presented as wedges to infer phylogenetic importance at various taxonomic levels (Figure 3.3). The top five families with the highest average SIVs were Caryophyllaceae (0.0074), Equisetaceae (0.0059), Asteraceae (0.0049), Ericaceae (0.0046), Rosaceae (0.0044). Considering the factors and framework of the SIV function, families and genera in Figure 3.3 with large SIV wedges have potential in delivering new medicines for diabetes. Furthermore, studies have indicated that targeting closely related taxa is an effective strategy for bioactivity screening (Cox and Balick 1994; Rønsted, Savolainen, and

Jäger 2008; Saslis-Lagoudakis et al. 2011). However, taxa that have strong phylogenetic signal (Figure 3.3) plus cross-cultural consensus, have been demonstrated to contain more bioactive plants than random samples (Saslis-Lagoudakis et al. 2012).



**Figure 3.3.** A phylogeny of taxa used to treat diabetes and highly associated symptoms of diabetes. Topology is based on Angiosperm Phylogeny Group 3 (2009). Average SIV values for each family are presented and daggers (†) indicate European endemic taxa.

### ***Cross-cultural consensus among Highlanders and Cree***

Two consensus families emerged when Leduc et al.'s study (2006) was compared with Table 3.1: Ericaceae and Rosaceae. *Vaccinium* of the Ericaceae was the only genus with cross-cultural consensus. There was one consensus species, *V. vitis-idaea* L., which was used by three Cree healers and two Lukomir healers for slow healing infections and urinary infections. *Vaccinium myrtillus* L. was used by four Highlander healers as a panacea. *V. angustifolium* Ait. was used by three Cree healers for frequent headaches. SIV factors from Lukomir ranked *V. myrtillus* as 8/41 plants and *V. vitis-idaea* as 9/41 plants (Table 3.1, Figure 3.S3). SIV factors from the Cree survey ranked *V. angustifolium* 11/15, and *V. vitis-idaea* 13/15. SIV values indicate that *Vaccinium* spp. are more important for the Highlanders than the Cree for treating diabetes.

### ***Inhibition of AGEs***

Both *Vaccinium myrtillus* and *V. vitis-idaea* showed linear concentration dependent inhibition of AGEs with activity higher or comparable to the positive control quercetin (Table 3.2). *V. myrtillus* (n = 3 at 50  $\mu\text{g}\cdot\text{mL}^{-1}$  initial concentration and n = 1 at 40  $\mu\text{g}\cdot\text{mL}^{-1}$  concentration) had an average  $\text{IC}_{50}$  of 12.4  $\mu\text{g}\cdot\text{mL}^{-1}$ , and *V. vitis-idaea* (n = 1) had an  $\text{IC}_{50}$  of 48.6  $\mu\text{g}\cdot\text{mL}^{-1}$ . The data for the most active species, *V. myrtillus*, is shown (Figure 3.4). *Vaccinium* spp. herein had approximately 61% greater activity than outgroup *Arctostaphylos uva-ursi* ( $\text{IC}_{50} = 100.2 \mu\text{g}\cdot\text{mL}^{-1}$ ). These results were comparable to  $\text{IC}_{50}$ 's from wild samples of *V. angustifolium* Aiton (McIntyre et al. 2009) ranging from 4.8 to 10.6  $\mu\text{g}\cdot\text{mL}^{-1}$  with a seasonal mean of 6.3  $\mu\text{g}\cdot\text{mL}^{-1}$ . *Vaccinium poasanum* Donn. Sm. was the most active taxon ( $\text{IC}_{50} = 4.2 \mu\text{g}\cdot\text{mL}^{-1}$ ) in an environmental and date controlled study consisting of numerous tropical specimens (Ferrier et al. 2012). Considering this comparison, the Highlanders' access to and use of *V. myrtillus* (*Borovnica*, European blueberry) is appropriate as a panacea remedy it may be an effective means of preventing AGE related insults and should be studied clinically.

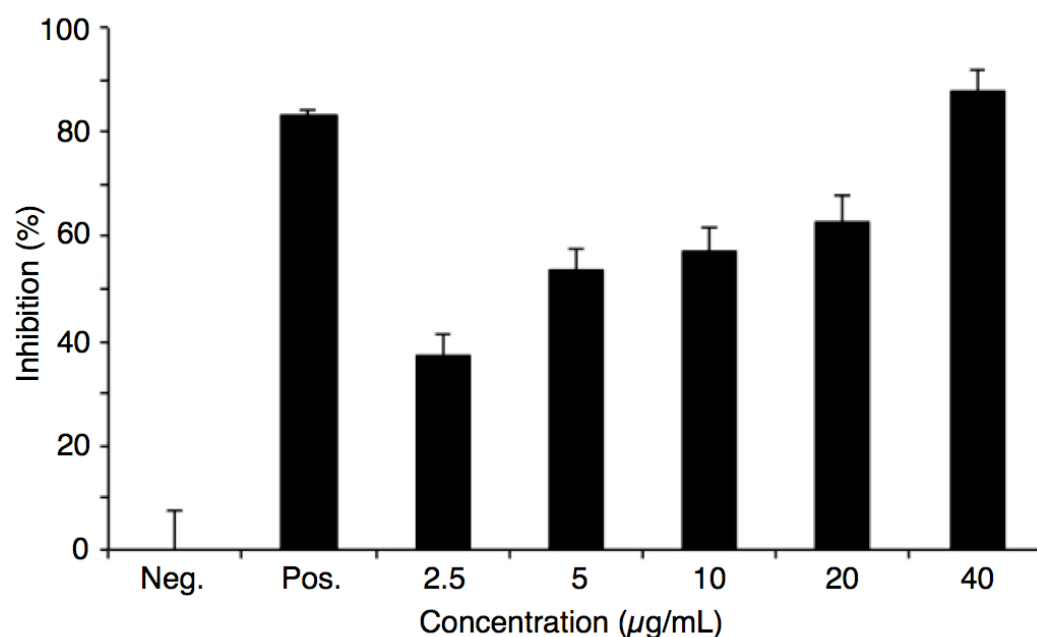
### ***HPLC-MS: Vaccinium myrtillus and V. vitis-idaea markers***

HPLC-MS analysis of *Vaccinium myrtillus* and *V. vitis-idaea* was compared with pure standards of (+)-catechin, chlorogenic acid, para coumaric acid, taxifolin, quercetin-3-O-

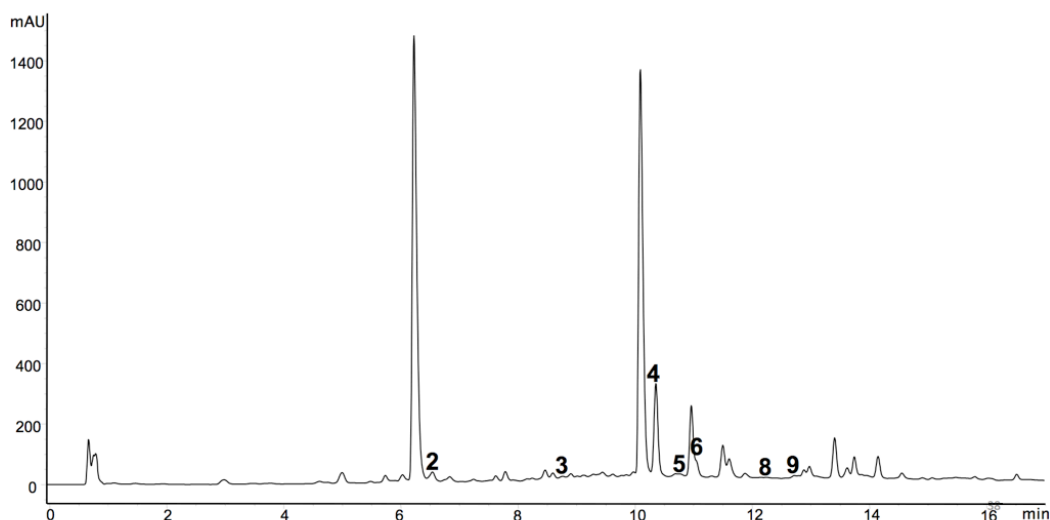
galactoside, quercetin-3-O-glucoside, rutin (quercetin-3-O-rutinoside), quercetin-3-O-rhamnoside, and myricetin. Rutin was absent in both species (Figure 3.5 and Figure 3.S4). *V. myrtillus* and *V. vitis-idaea* were separated based on the presence of (+)-catechin in *V. myrtillus* and quercetin 3-O-glucoside in *V. vitis-idaea*. All other metabolites identified were common to both species. Many of these compounds are good antioxidants and/or reported in other studies as active in antiglycation agents (McIntyre et al. 2009).

**Table 3.2.** *Vaccinium* spp. and outgroup *Arctostaphylos uva-ursi* leaf samples collected in Jahorina, B&H with IC<sub>50</sub> expressed in µg·mL<sup>-1</sup>. All sample vouchers were accessioned at OTT.

Genus species	IC <sub>50</sub>	Location	Alt. (m)	Date	Voucher	Voucher #
<i>V. myrtillus</i>	4.1	Igman, B&H	984	July, 1 2005	417	BBE 134
<i>V. myrtillus</i>	5.4	Jahorina, B&H	1730	July 24, 2005	418	BBE 42
<i>V. myrtillus</i>	17.35	Jahorina, B&H	1730	July, 1 2005	419	BBE 133
<i>V. myrtillus</i>	27.8	Jahorina, B&H	1730	July 5, 2005	420	BBE 125
<i>V. vitis-idaea</i>	48.6	Jahorina, B&H	1730	June 5, 2006	421	BBE 23
<i>A. uva-ursi</i>	100.2	Jahorina, B&H	1735	June 15, 06	422	ARC 1



**Figure 3.4.** Inhibition of advanced glycation endproducts by *Vaccinium myrtillus* L. (Ferrier 419) with a half maximal inhibitory concentration (IC<sub>50</sub>) of 17.35 µg·mL<sup>-1</sup>.



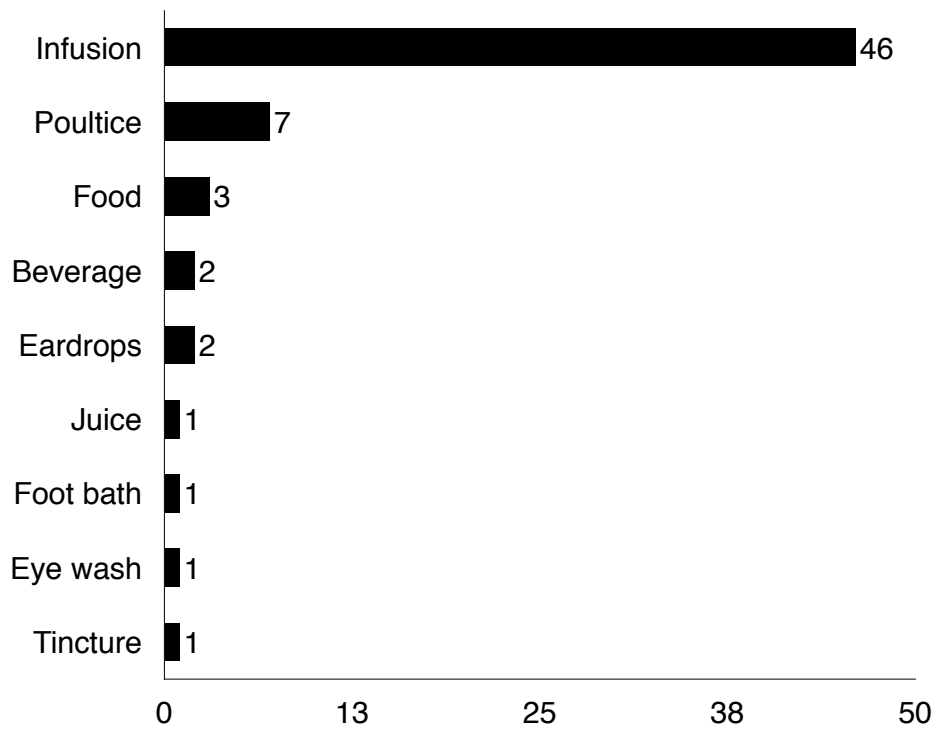
**Figure 3.5.** HPLC chromatogram with DAD at 280 nm of *Vaccinium myrtillus* leaf sample. Numbers indicate presence of chlorogenic acid (2), para coumaric acid (3), taxifolin (4), quercetin-3-O-galactoside (5), quercetin-3-O-glucoside (6), quercetin-3-O-rhamnoside (8), and myricetin (9). Rutin (quercetin-3-O-rutinoside) and (+)-catechin were absent.

This study has identified species of interest for study of complementary treatment of T2D, while our treatment of *Vaccinium* spp. from Lukomir demonstrates the high activity of at least this genus and identifies its major constituents. There are both medicinal and many more food species utilized by the Lukomir Highlanders that remain to be studied. For example, phenolics and saponins likely play an important role in radical scavenging and cholesterol-binding activity in the Lukomir diet as they do in pastoral Maasai communities (Lindhorst 1998, Chapman et al. 1997, Johns et al. 1999). Eventually clinical work is required to validate safe and effective use. Reinforcement or revival of traditional medicines and dietary plants with antidiabetic activity is especially important in these remote communities where modern health care is limited but traditional complementary medicines are abundant.

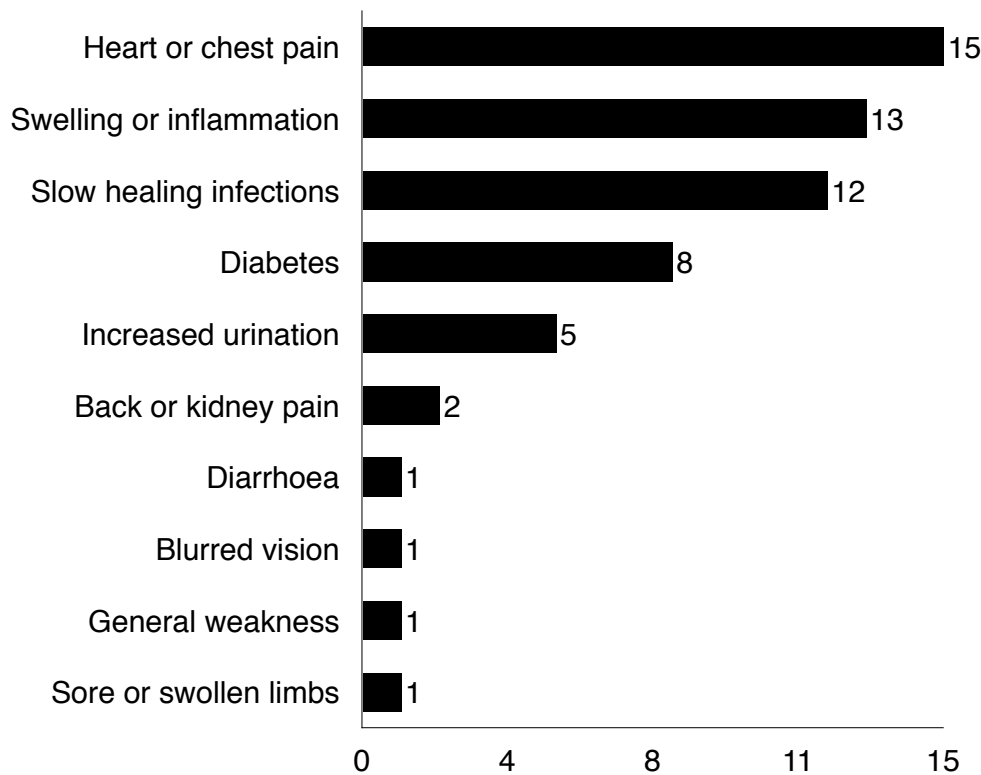
### Supplementary Tables and Figures

**Table 3.S1.** Metabolite standards and confirmation of their presence or absence in *Vaccinium myrtillus* and *V. vitis-idaea* (Rutin = quercetin-3-O-rutinoside).

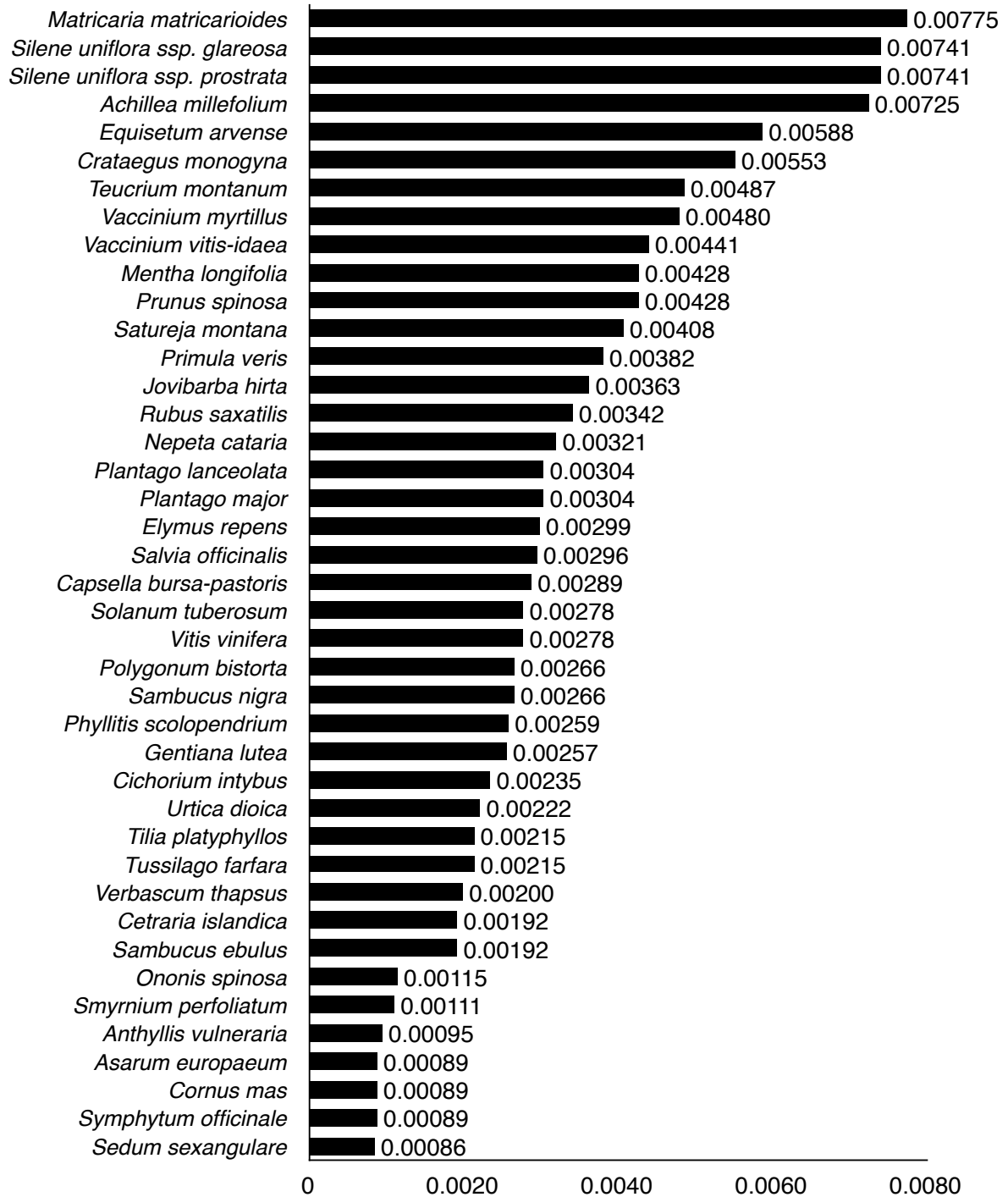
Sample	(+)-Catechin	Chlorogenic acid	Para Coumaric Acid	Taxifolin	Quercetin -3-O-galactoside	Quercetin -3-O-glucoside	Rutin	Quercetin-3-O-rhamnoside	Myricetin
<i>V. myrtillus</i>		X	X	X	X	X		X	X
<i>V. vitis-idaea</i>	X	X	X	X	X			X	X



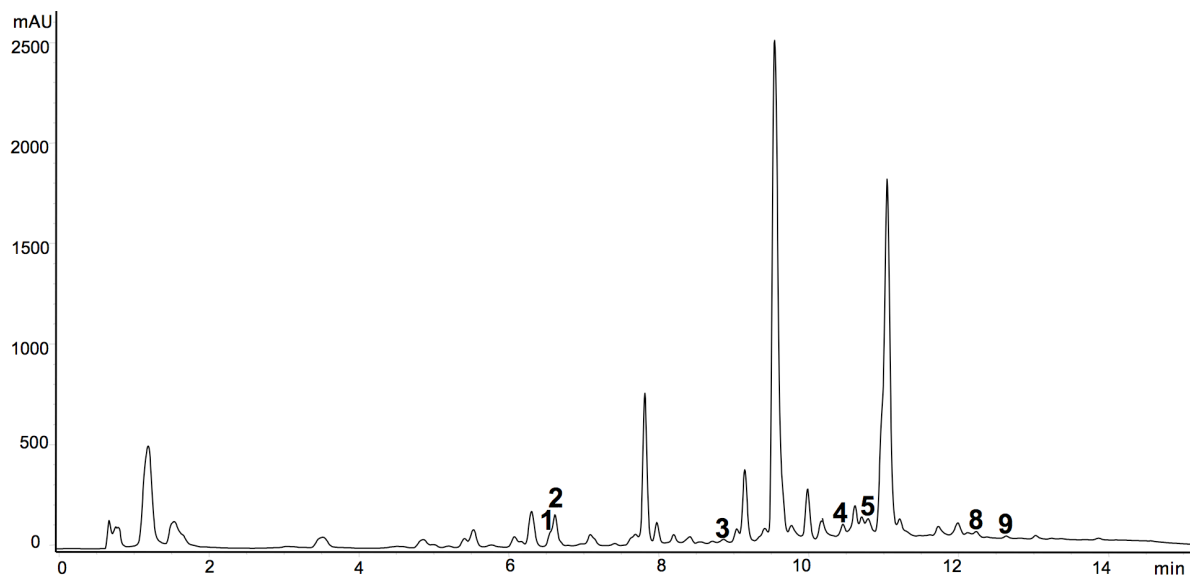
**Figure 3.S1.** Frequency of plant preparation reports from all species listed in Table 3.1.



**Figure 3.S2.** Frequency of taxa per diabetes and associated symptoms.



**Figure 3.S3.** Taxa and their syndromic importance values.



**Figure 3.S4.** HPLC chromatogram with DAD at 280nm of *Vaccinium vitis-idaea* leaf sample. Numbers indicate presence of chlorogenic acid (+)-catechin (1), chlorogenic acid (2), para coumaric acid (3), taxifolin (4), quercetin-3-O-galactoside (5), quercetin-3-O-rhamnoside (8), and myricetin (9). Rutin (quercetin-3-O-rutinoside) was absent.

## Chapter 4

# A Classic Maya Mystery of the Medicinal Plant & the *K'an* Glyph

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## A Classic Maya Mystery of the Medicinal Plant and the *K'an* Glyph

The Classic Maya *k'an* |K'AN| glyph is translated 'yellow' or 'precious' (Montgomery 2002a,b). In 2012, an article was published on the possible connection between the *k'an* glyph and the ancient ethnobotanical use of a plant (Weiss-Krejci 2012). This glyph is found in hieroglyphic texts and a variety of Classic Maya murals, pottery, bowls, and sacred vessels beverages made with cacao (*Theobroma cacao* L., *Kakaw*, Cacao tree). The glyph is a symmetric cross with equal arms but occasionally appears with tapered arms, like a Maltese cross. According to Friedel et al. (1993), the glyph is associated with the turtle shell from which the maize god sprouts in rebirth in the Maya book of creation, the *Popul Vuh*. As reported by Weiss-Krejci, *k'an* serves as an adjective for a specific cacao additive, hitherto read as "yellow, ripe", "cacao beverage" (2012). Furthermore, *k'an* is attested as a component of "emblem" glyphs (e.g., place names *k'anwitznal* |K'AN-na-WITZ-NAL| for Ucanal, or, *k'antumaak* |K'AN-tu-ma-ki| for Caracol), and the personal name *Itzamk'anahk* for Precious Itzam Turtle). Weiss-Krejci (2012), working with Maya in Belize, suggested that a plant with the common name "pimiento" was the *k'an* botanical template, based on a wood pattern that is similar to the glyph. We argue here that Weiss-Krejci was mistaken identifying the *k'an* template plant as "*Pimenta dioica*" (L.) Merr. (Allspice, Pimiento), a common tree of the Myrtaceae family in Mesoamerica and a prominent medicinal plant widely used as an aromatic flavouring agent and tea.

Ethnobotanical work on antidiabetic plants with Q'eqchi' Maya healers in Belize presented a different plant species, a woody liana with the *k'an* template - namely *Tynanthus guatemalensis* Donn. Sm. (*Chibayal*) of the Bignoniaceae family. After cross-sectioning the woody liana of *T. guatemalensis* we observed yellow tinted sap, a cross-shaped xylem organization, with an allspice-like aroma similar to *Pimenta dioica* (Figure 4.1). Q'eqchi' healers named *T. guatemalensis* their number one choice of treatments for diabetes. Subsequently, we tested the liana and seven phylogenetically related plants for antidiabetic activity in an inhibition of advanced glycation end products assay (AGEs). Advanced glycation endproducts are undesirable protein sugar adducts formed during hyperglycaemia

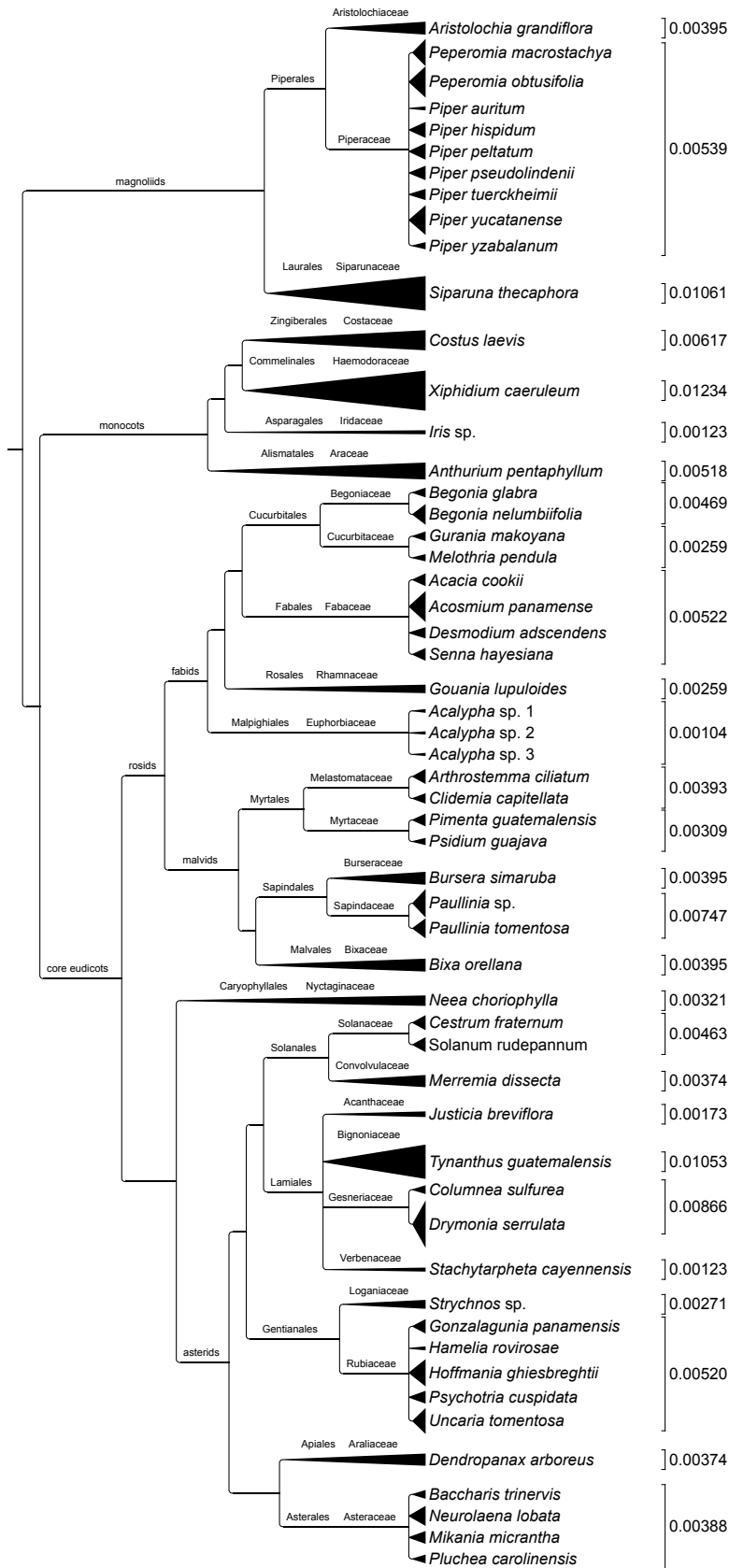
and responsible for the development of many diabetic symptoms. The Q'eqchi' Maya plant bearing the *k'an* symbol was the most active anti AGE treatment. Here, we provide evidence of the active principles responsible for this activity, and show a pharmacological basis for its' "precious" nature. Next, we determined the true botanical identity of the archaeological interpretation, of the Late Classic Maya *k'an* glyph, which is not symbolic of *Pimenta dioica*, but the potent contemporary Maya medicinal plant of our survey - *T. guatemalensis* (*Chibayal*).



**Figure 4.1.** *Tynanthus guatemalensis* and *Pimenta dioica* cross-sections. Left, *T. guatemalensis* liana cross-section showing *k'an* template. Right, *P. dioica* stem cross-section showing concentric growth rings.

## Results and Discussion

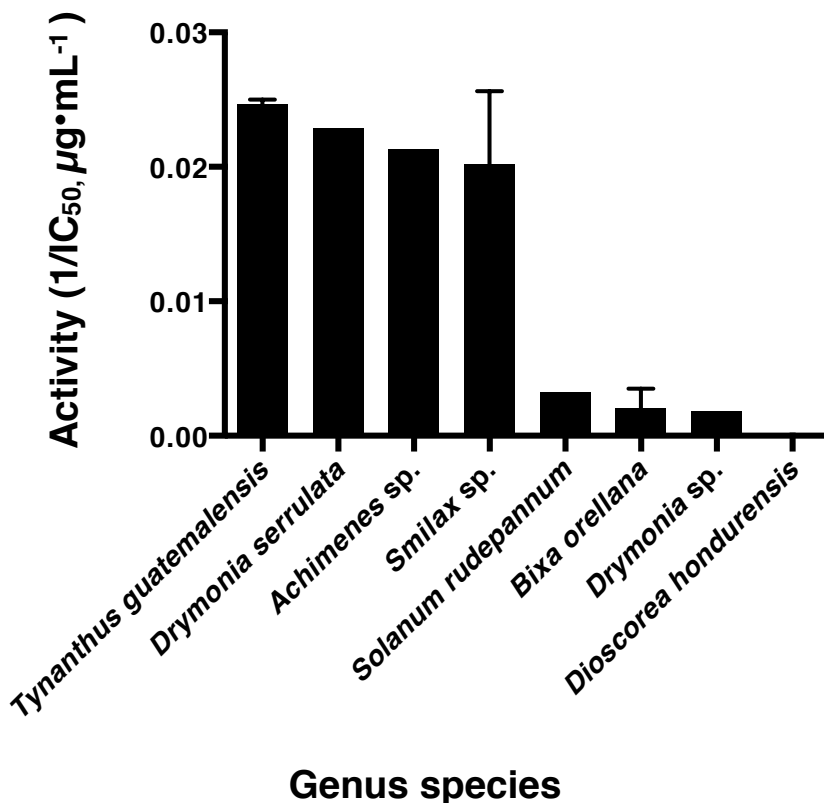
Detailed ethnobotanical interviews with five Q'eqchi' healers yielded data on 150 plants that were traditionally used to treat diabetes, and these were formally ranked by the syndromic importance value (SIV) developed with Cree healers in Canada (Leduc et al. 2006). Syndromic importance values ranged from 0.000987 to 0.0146 (Table 4.S1, Figure 4.2.). Plants at the lower end of the SIV scale were used for only one or two symptoms, or by a low number of healers, while plants at the high end of the scale were used for multiple symptoms by many healers. In a second set of interviews, healers ranked taxa within symptom categories (Table 4.S2), which were evaluated in a phylogenetic context.



**Figure 4.2.** A phylogeny of taxa used to treat diabetes and highly associated symptoms of diabetes. Topology is based on Angiosperm Phylogeny Group 3 (2009). Average SIV values for each family are presented.

Extracts were prepared and tested for AGE inhibition and half maximal inhibitory concentrations ( $IC_{50}$ ) were determined for each taxon (Figure 4.3). AGE inhibition ranged from the most effective, *Tynanthus guatemalensis* ( $IC_{50} = 40.8 \mu\text{g}\cdot\text{mL}^{-1}$ ), to, *Bixa orellana* L. ( $IC_{50} = 733.3 \mu\text{g}\cdot\text{mL}^{-1}$ ) of the Bixaceae, while the outgroup, a traditional birth control medicine, was the least effective: *Dioscorea hondurensis* R. Knuth ( $IC_{50} = >10,000 \mu\text{g}\cdot\text{mL}^{-1}$ ) of the Dioscoreaceae (Figure 4.3). The activity for *T. guatemalensis* was comparable to that reported for six tropical species of *Vaccinium* spp. (the Blueberry, Cranberry, Huckleberry genus) (Ferrier et al. 2012), but more than 200 times more active than reported for North American ginseng extract,  $>10 \text{ mg}\cdot\text{mL}^{-1}$  (McIntyre et al. 2011).

Out of 150 taxa, *Tynanthus guatemalensis* was the most recommended treatment for patients with diabetes (Table 4.S1). The SIV system placed *T. guatemalensis* 4th of 150 taxa (Table 4.S2).



**Figure 4.3.** AGE-inhibition activity by seven plants ( $n = 3$ , except for *Smilax* sp.  $n = 2$ ) used by the Q'eqchi' Maya for diabetes and one outgroup, *Dioscorea hondurensis* ( $n = 1$ ). Data are presented on the more intuitive activity basis ( $1/IC_{50} \text{ mg}\cdot\text{mL}^{-1}$ ) with four replicates.

### *Ethnobotanical notes*

In our research with Q'eqchi' Maya healers, *Tynanthus guatemalensis* was elucidated as a treatment for diabetes, heart and chest pain, increased thirst, and increased urination. The treatment was prepared by crushing 12 eight cm sections of the liana (plus the root for diabetes, and heart and chest pain) and boiling in 4 litres of water for 30 min. Healers administered the preparation as an infusion to be consumed or topically applied three to four times a day. On rainforest treks, Q'eqchi' healers used *T. guatemalensis* as a refreshing sterile water source, slicing the liana and collecting the sudden rush of sap. Sucking the liana in sections produced more water from the large vessel elements (Figure 4.4). In the field, the liana was also used as a coffee substitute (tea), and using the triangular sections outside the xylem as a cord, the Maya fastened tree poles for our forest shelter.



**Figure 4.4.** *Tynanthus guatemalensis*, a refreshing rainforest drink. (Left) Cutting and reversing the *T. guatemalensis* liana over the mouth to drip and consume the water. (Right) *T. guatemalensis* isopropyl sap is coloured a light golden yellow to brick red.

The flavour and aroma of *Tynanthus guatemalensis* is pleasant, and strikingly similar to the infusion prepared with *Pimenta dioica* leaves; since their common names are similar taxonomic misidentification is possible. Our GC-MS reads on *T. guatemalensis* isopropyl liquor (Figure 4.S1a) and a eugenol standard confirmed the presence of eugenol in the liquor

(Figure 4.S1b). Although not uncommon, *T. guatemalensis* is found in primary and secondary forests, often away from villages, and effort is required to find and collect the liana. For this reason, Maya medicinal plant specialists and botanists are aware of the two species and rarely confuse them. Lack of botanical expertise has led previously to errors in interpretation of ancient Maya botanical use (Lambert and Arnason 1982).

### ***Active compounds in Tynanthus guatemalensis***

Extractions of milled *Tynanthus guatemalensis* wood were made with solvents of increasing polarity (hexane, ethyl acetate, and methanol). The extracts were assayed, and activity was found mainly in the ethyl acetate fraction. Analysis of this active fraction by advanced analytical methods revealed the identity of several classes of compounds (Figure 4.S2), including benzoic acid derivatives, C-glycosyl flavonoids, and phenylpropenes. The presence of verbascoside and katchimoside and their antioxidant activity has been previously reported in the related species *T. panamensis* by Plaza et al. (2005). Available compounds from each group were tested in the AGE assay (Table 4.1) and although all have some activity, the phenyl propene verbascoside is clearly the most active, with an activity similar to the positive control quercetin (Figure 4.S5).

**Table 4.1.** Identification of compounds from *Tynanthus guatemalensis* and their advanced glycation endproduct (AGE) half maximal inhibitory concentration (IC<sub>50</sub>).

<b>Isolate</b>	<b>Biosynthetic class</b>	<b>Identified by</b>	<b>IC<sub>50</sub> (µg·mL<sup>-1</sup>)</b>
Salicylic acid	Benzoic acid derivative	UPLC-MS-QToF	Fluorescence interference
4 methoxy benzoic acid	Benzoic acid derivative	UPLC-MS-QToF	Fluorescence interference
Verbascoside	Phenylpropene glycoside	HPLC-MS-QTRAP	5.1
Katchimoside	C-glycosyl flavonoid	UPLC-MS-QToF	ND
Eugenol	Phenylpropene	GC-MS	Fluorescence interference
Quercetin	Flavonol	Not present (AGE + control)	4.6

### ***Taxonomic identification of the k'an template***

Common names can cause confusion when the name applies to more than one taxon. In Belize “pimiento” can allude to both *Pimenta dioica*, which we argue was incorrectly associated with the Maya *k'an* glyph, but also to “pimiento bejuco”, *Tynanthus guatemalensis* (Bignoniaceae) the Q’eqchi’ diabetes treatment. The Bignoniaceae, a mostly tropical family of trees, shrubs, and lianas is comprised of approximately 800 species and is not closely related to the Myrtaceae. The similar common name arises as both species are

rich in eugenol or its derivatives which gives both the *P. dioica* leaf and the *T. guatemalensis* liana a distinctive and pleasant “allspice” aroma.



**Figure 4.5.** Ways the Maya employ the mysterious *Tynanthus guatemalensis*' (Chibayal) *k'an* template. (A) Drawing of a *k'an* glyph (bottom left) from photograph K625 by Justin Kerr (drawing by Marc Zender). (B) The *k'an* cross on Late Classic Maya ceramic cacao vessels (photograph K8804© Justin Kerr) (C) The *k'an* cross on a Late Classic Maya ceramic bowl (D) Scaffold accession scene at the northern end of San Bartolo Pinturas Sub-1A West Wall implying the rope utility of the *T. guatemalensis* liana (rendering by Heather Hurst © 2008).

To solve the mystery of which species was the correct species represented on the Classic Maya glyph (Figure 4.5A), we collected both tree and liana stems at Itzamma Garden, Indian Creek, Belize. Vouchers of *Pimenta dioica* and *Tynanthus guatemalensis*

were collected and determined by Arnason and Ferrier in Toledo District Belize (Table 4.S1) and compared with *T. guatemalensis* type voucher (Smith 1488, NY 328979) and other Central American vouchers of *P. dioica* and *T. guatemalensis* at the New York Botanical Garden (NY). Information on and photos of *P. dioica* can be found at <http://www.tropicos.org/Name/22101787> and for *T. guatemalensis* at <http://www.tropicos.org/Name/3701480>. Cross sections of wood for comparison were prepared in the field for both species using a sharp machete. The *T. guatemalensis* cross section (Figure 4.1) showed clearly the *k'an* glyph template (Figure 4.5a), while *P. dioica* cross section showed concentric growth rings but no *k'an* cross (Figure 4.1). The *T. guatemalensis* pattern also clearly shows the tapered arms found on the cacao drinking vessel, bowl, and mural (Figure 4.5bc). Unusual xylem patterns of various types are common in tropical lianas but rare in trees. In subtropical areas with a distinct dry season, the growth pattern in trees normally shows annual rings similar to temperate trees whose growth is interrupted by winter. Although *T. guatemalensis* is a forest liana, and, unlike *P. dioica*, not easily grown in cultivated sites the appearance of the *k'an* cross and its association with the Popul Vuh, made *chibayal* a ritualistic component in Classic Maya art and hieroglyphic text.

Much has been written about Classic Maya civilization and the splendours of their language, astronomy, calendar, architecture, politics, economics, diet, and their medicine. Despite conquest, genocide, and forest destruction, the endurance of the Maya civilization is evident in contemporary cultural celebrations at Classic Maya city centres. As we have demonstrated, Classic Maya medical heritage was as important then as it is today. Numerous studies have testified to the ancient Maya's detailed utilization of hundreds of plant species, and their use in contemporary Maya culture, and the contributions to Maya medicine (Arvigo and Balick, 1993, Heinrich, 2000, Meckes et al. 1995, Trevaud Amiguet 2005, Arnason et al. 1980 among others). Like Ayurvedic medicine, Traditional Chinese medicine, and Persian Traditional Medicine, we should treat Maya Medicine (MM) too as one of the great medical traditions of the world.

## Supplementary Materials and Methods

### *Ethnobotany with the Q'eqchi' Maya Healers Association, Toledo District, Belize*

Research and collection permits were acquired from the Forest Department, Ministry of Natural Resources and the Environment, Belize, and ethics certificates were acquired from the University of Ottawa [H05-09-07] for an antidiabetic plant inquiry initiated by the Q'eqchi' Healers Association. Prior informed consent was received from all healers and the Belize Indigenous Training Institute. Fieldwork followed quantitative consensus methodology with individual semistructured interviews to collect the following: (1) symptom treated, (2) specimen treatment voucher and photo number, (3) common name, (4) scientific name, (5) family, (6) GPS coordinates, (7) altitude (m a.s.l.), (8) habitat, (9) syntaxa, (10) flowering time and description, (11) medically active collection time, (12) use (13) use category, (14) plant part used, (15) amount used, (16) preparation method, (17) administration method, (18) dosing regimen, (19) ethnographic details, (20) informant name, (19) informant #, (20) total # of informants per species, and (21) date. Triplicates of medicinal plant vouchers were made when sustainable and permitted and are currently held at the University of Ottawa Herbarium (OTT) with voucher numbers in Table 4.S1.

### *Elucidation of plants for diabetes using syndromic importance values*

The syndromic importance value (SIV) function was adapted from (Leduc et al. 2006 and Ferrier et al. 2013). SIV values allow ranking of plant species by accounting for: (1) the number of different symptoms for which a plant was cited, (2) the frequency of plant citation by individual informants, and (3) the association rank of symptoms for which a plant was cited to treat. The latter association rank of symptoms was determined by four physicians at the Ottawa Hospital, who diagnose and treat patients with diabetes. Symptoms were given a scale weight  $w$  from 1-4 for physician ranking, where 1 is a symptom highly associated with diabetes; 2, moderately associated with diabetes; 3, weakly associated with diabetes; and 4 is not at all associated with diabetes.

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

The SIV function accounts for  $w$ , the weight of the symptom;  $s$ , the symptom treated by the species;  $f$ , the frequency of citation for the species;  $S$ , the total number of symptoms used for the survey (used in Leduc et al. 2006, but was not indicated in the denominator on the right); and  $F$  the total number of interviews in the survey.

### ***Sample extraction and fractionation***

All plant samples were collected in the field and stored in isopropyl alcohol. Samples were blended in a food processor. Bark samples were dried and ground using a Wiley Mill (Fischer Scientific, Ottawa) and passed through a one mm mesh. Plant material was then extracted twice, by shaking for 24 h in 4:1 ethanol:water (10mL per g of material) at room temperature. The two extracted volumes were pooled, evaporated under continuous air flow (24-72 h) and lyophilized and stored at -20 °C for chemical analysis and bioassays.

### ***Advanced glycation endproduct (AGE) fluorescence inhibition assay***

AGE formation inhibition activity was assessed as described by Farsi et al. (2008) with modifications. Stock solutions of extracts were prepared with 4:1 ethanol:water. Samples were centrifuged (~15,000 x g) and filtered through a 0.2 µm polytetrafluoroethylene (PTFE) non-sterile filter (Chromatographic Specialties Inc., Brockville, ON, CAN). Bovine serum albumin (BSA) (1 mg·mL<sup>-1</sup>) was incubated with 100 mmol·L<sup>-1</sup> glucose / 100 mmol·L<sup>-1</sup> fructose in sodium phosphate monobasic monohydrate buffer (pH 7.4) with extract (experimental treatment), ethanol/water (4:1) (negative control), or quercetin, an antioxidant against glycation by way of phenolic hydroxyl groups in the flavonoid structure (24 mg·mL<sup>-1</sup> in assay) (Sengupta et al. 2006), which served as a positive control. A treatment without BSA was included in the bioassay analysis as a positive control for fluorescence measurements. Stock solutions of the extracts were serially diluted and tested at five concentrations that were optimized for dissolution and a linear concentration response (40, 20, 10, 5, and 2.5 mg·mL<sup>-1</sup> in assay). Three replicates were tested in sterile

opaque polystyrene 96-well clear bottom plates (Corning Inc., New York, N.Y., USA). Plates were covered, sealed, with Parafilm, and incubated for seven days at 37 °C while shaking. Following incubation, fluorescence was measured using a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, Calif., USA) at excitation and emission wavelengths of 355 and 460 nm. Glucose/fructose and ethanol/ water fluorescence was subtracted from all results, and IC<sub>50</sub> and percent inhibition (1/IC<sub>50</sub>) were calculated as previously described.

Verbascoside was tested for AGE-inhibitory activity. Verbascoside was dissolved in ethanol:water (4:1) and tested at final well plate concentrations of 15, 10, 5, 2.5 and 1.25 µg·mL<sup>-1</sup> in-well concentrations. Precautions were taken to reduce light exposure during sample preparation and assaying. Salicylic acid, 4-Hydroxy Benzoic Acid (4-HBA), and eugenol were also tested for AGE-inhibitory activity. Salicylic Acid was dissolved in 80% ethanol and was tested at 46.875, 23.4375, 11.71875, 5.8594, and 2.9297 µg·mL<sup>-1</sup> in-well concentrations. 4-HBA was dissolved in 80% ethanol and was tested at 4.6875, 2.3438, 1.1719, 0.5859, and 0.2930 µg·mL<sup>-1</sup> in-well concentrations. Eugenol was dissolved in 80% ethanol and was tested at 64.406, 32.2031, 16.1016, 8.0508, and 4.0254 µg·mL<sup>-1</sup> in-well concentrations.

### ***High performance liquid chromatography–mass spectrometry (HPLC-MS) analysis***

A verbascoside standard was obtained from Extrasynthese (Genay, France) and a stock solution (0.88mg·mL<sup>-1</sup>) was prepared at an initial concentration of 0.88mg µg·mL<sup>-1</sup> in 80:20 water/acetonitrile and then diluted 10, 100 and 1000-fold. Precautions were taken to avoid light exposure. Detection of verbascoside in *Tynanthus guatemalensis* was accomplished multiple reaction monitoring by 3200 Q-TRAP (AB Sciex, Concord, ON, Canada). Separations were performed on a Luna PFP column (150 mm × 3mm, particle size 3.5µm) at 45 °C and a flow rate of 0.5mL·min<sup>-1</sup>. Gradient elution was performed with water/ formic acid 0.1% (A) and acetonitrile/ formic acid 0.1% (B). A linear gradient was applied, starting with 5% B (and 95% A) and increasing to 95% B (and 5% A) in 5 min.

### ***Essential oil extraction***

The essential oil from *Tynanthus guatemalensis* was obtained by solvent extraction of the fresh liana. The liana, two cm in diameter, was cut into five cm long segments and placed in isopropanol. Sixteen mL of the solvent was filtered through a 13mm nylon 45 nm syringe filter (Canadian Life Science) to remove particulate matter. The remaining solution was extracted with 4 mL of hexanes and centrifuged at 2000 rpm for 20 min. The organic phase was collected, washed, dried over sodium sulphate and concentrated under reduced pressure. The method yielded 0.2 g of the oil. Two hundred mg of extracted oil was dissolved in 1 mL of hexanes for analysis via gas chromatography hyphenated mass spectrometry.

### ***Gas chromatography–mass spectrometry (GC-MS)***

The GC-MS analysis of the essential oil fraction was carried out on an Hewlett Packard 6890 series GC-MSD with 5913 inert Mass Selective Detector . Injector temperature was set to 250 °C and a 50:1 split ratio was employed using helium gas at a rate of 48 mL·min<sup>-1</sup>. Separations were performed on a DB-5 (dimethyl polysiloxane containing 95% methyl groups, and 5% phenyl groups) column (HP 19091A-102, 30m × 250µm × 0.25µm film thickness) at a helium carrier gas pressure of 14.63 psi and a flow rate of 0.9 mL·min<sup>-1</sup>. The temperature program was started at 40°C and raised to 300° C over a 26 min period at a steady rate of 10°C·min<sup>-1</sup>. The electron impact ionization mass spectra were obtained. Eugenol (cat# E51791-5G) was obtained from Sigma Aldrich and used as an external standard.

### ***Identification of the “allspice” essential oil component***

Eugenol was identified from essential oil by matching the mass spectra of pure compound with the peak eluting at same retention time under similar chromatographic and spectrometric conditions. Identity was further confirmed by spectral comparison with the Wiley275 database and NIST Chemistry Web Book entry (<http://webbook.nist.gov/chemistry/>). The calculated Kovats Retention Indices' were arrived using the linear retention

times of an n-alkane external standard according to the accepted method (Kováts 1958) and compared to the literature values (Choi 2003).

***Ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-qTOF-MS/MS) analysis of Tynanthus guatemalensis***

Identification was accomplished by comparison of retention time, monoisotopic mass, molecular formula and isotopic ratio using low energy spectra. The identifications were confirmed by comparison of high energy fragmentation spectra of target compounds and authentic standards analyzed under same chromatographic and spectrometric conditions.

Separations were performed on a UPLC Acquity BEH C18 2.1 × 100mm 1.7µm particle size column at flow rate of 0.5·min<sup>-1</sup> and column thermostat at 50°C . Mobile phases were A, water with 0.1% formic acid, and B, acetonitrile with 0.1% formic acid (Fisher Optima LC-MS). Flow rate 0.5 mL/min. Optimal mobile phase composition was 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). Autosampler needle was washed with 200 µL 90% acetonitrile+10% water followed by 600 µL wash with 10% acetonitrile:+90% water after 1µL sample injection.

MassLynx software, MSe ESI+ mode, lock mass Leucine Enkephalin 12C 556.2615, source temperature 120 °C, desolvation temperature 400 °C, Cone gas (N2) flow 50 L·hr<sup>-1</sup>, desolvation gas (N2) flow 1195 L·hr<sup>-1</sup>. MSe conditions, mass range 100-1500 Da, F1 CE, 6V, F2 CER 10-30V, Cone voltage 20V, Scan time 1 sec. Instrument was calibrated with 50-1000 Da sodium formate.

## Supplementary Tables and Figures

**Table 4.S1.** The syndromic importance value (SIV) of 150 antidiabetic plants identified at NY and OTT (Standley & Steyermark 1946; Balick et al. 2001). Asterisk (\*) indicate common names on vouchers without a scientific determination. *Pimenta dioica* (L.) Merr. (OTT 19950, 19951) was not recommended, but *P. guatemalensis* (Lundell) Lundell (*Pejns*, Allspice) had an SIV value of 0.0039.

Taxon	Syndromic importance value	JF collection number
<i>Drymonia serrulata</i> (Jacq.) Mart.	0.0146	1938, 1961, 1970, 1982, 2007, 2015, 2051, 2063
<i>Xiphidium caeruleum</i> Aubl.	0.0123	1940, 1954, 1978
<i>Siparuna thecaphora</i> (Poepp. & Endl.) A. DC.	0.0106	1997, 2032, 1933, 1952
<i>Tynanthus guatemalensis</i> Donn. Sm.	0.0105	1920, 1922, 1925, 1931, 1953, 2002, 2003, 2046
<i>Peperomia obtusifolia</i> (L.) A. Dietr.	0.0096	1972, 2001, 2034, 2061
<i>Acosmium panamense</i> (Benth.) Yakovlev	0.0095	1930, 1974, 2026, 2065
<i>Piper yucatanense</i> C. DC.	0.0091	1948, 2012, 2042
<i>Paullinia</i> sp.	0.0088	1945, 1946, 2039, 2050
<i>Hoffmannia ghiesbreghtii</i> (Lem.) Hemsl.	0.0084	1942, 1981, 2064, 2067
<i>Peperomia macrostachya</i> (Vahl) A. Dietr.	0.0084	1971, 2035, 2053, 2062
*Rax i jurich' aj pak'	0.0081	2006, 2013, 2036
<i>Uncaria tomentosa</i> (Willd. ex Schult.) DC.	0.0080	1921, 1975, 2010, 2049
<i>Smilax</i> sp.	0.0067	1976, 2009
<i>Begonia nelumbifolia</i> Schltld. & Cham.	0.0064	1937, 1941, 1973
<i>Costus laevis</i> Ruiz. & Pav.	0.0062	1977, 2017
<i>Neurolaena lobata</i> (L.) R.Br. ex Cass.	0.0062	1927, 1967
<i>Paullinia tomentosa</i> Jacq.	0.0062	2025, 2044
*Jol jol	0.0062	1929, 1958, 2073
<i>Lygodium venustum</i> Sw.	0.0059	1951, 2027
<i>Anthurium pentaphyllum</i> (Aubl.) G. Don.	0.0052	1979, 1984, 1962,
<i>Piper peltatum</i> L.	0.0051	1993, 1994, 2022, 2057, 2066
<i>Adiantum wilsonii</i> Hook.	0.0049	1944, 2011
<i>Piper hispidum</i> Sw.	0.0049	2033
<i>Arthrostemma ciliatum</i> Pav. Ex D. Don	0.0046	1923, 2030, 2058
<i>Cestrum fraternum</i> Morton, J. Arnold Arbor.	0.0046	1960, 1989, 2041
<i>Solanum rudepannum</i> Dunal	0.0046	1966, 2043
<i>Gonzalagunia panamensis</i> (Cav.) K.Schum.	0.0044	1939, 2071
<i>Piper pseudolindenii</i> C. DC.	0.0044	2028
<i>Acacia cookii</i> Safford	0.0039	1956
<i>Aristolochia grandiflora</i> Sw, see B's Kum pim	0.0039	1969
<i>Bixa orellana</i> L.	0.0039	2045
<i>Bursera simaruba</i> (L.) Sarg.	0.0039	1957
<i>Mandevilla subsagittata</i> (Ruiz & Pav.) Woodson	0.0039	2048
<i>Mikania micrantha</i> H.B.K.	0.0039	1968
<i>Pimenta guatemalensis</i> (Lundell) Lundell	0.0039	1955
<i>Psychotria cuspidata</i> Bredem. ex Schult.	0.0039	2047
<i>Senna hayesiana</i> (Britton & Rose) H.S. Irwin & Barneby	0.0039	2072
<i>Dendropanax arboreus</i> (L.) Decne. & Planch.	0.0037	1949
<i>Merremia dissecta</i> (Jacq.) H. Hallier F.	0.0037	1926, 2040
<i>Desmodium adscendens</i> (Sw.) DC.	0.0035	1996, 2019
<i>Desmodium axillare</i> var. <i>acutifolium</i> (Kuntze) Urb.	0.0035	1995, 2020
<i>Piper tuerckheimii</i> C. DC. ex Donn. Sm.	0.0035	1992, 2018
<i>Clidemia capitellata</i> (Bonpl.) D. Don	0.0032	1963
<i>Epiphyllum phyllanthus</i> (L.) Haw.	0.0032	2068
<i>Neea choriophylla</i> Standl.	0.0032	2016
<i>Anthurium willdenowii</i> Kunth.	0.0030	1980, 1983, 1985
<i>Begonia glabra</i> Aubl.	0.0030	2052
<i>Gurania makoyana</i> (Lem.) Cogn.	0.0030	2054
<i>Piper</i> sp. (Tzik' jwal pim)	0.0030	1932
<i>Baccharis trinervis</i> (Lam.) Pers.	0.0027	2031
<i>Bolbitis pergamentacea</i> (Maxon) Ching	0.0027	2070
<i>Columnnea sulfurea</i> Donn. Sm.	0.0027	2037
<i>Justicia</i> aff. <i>fimbriata</i> (Nees) V.A.W. Graham	0.0027	2038

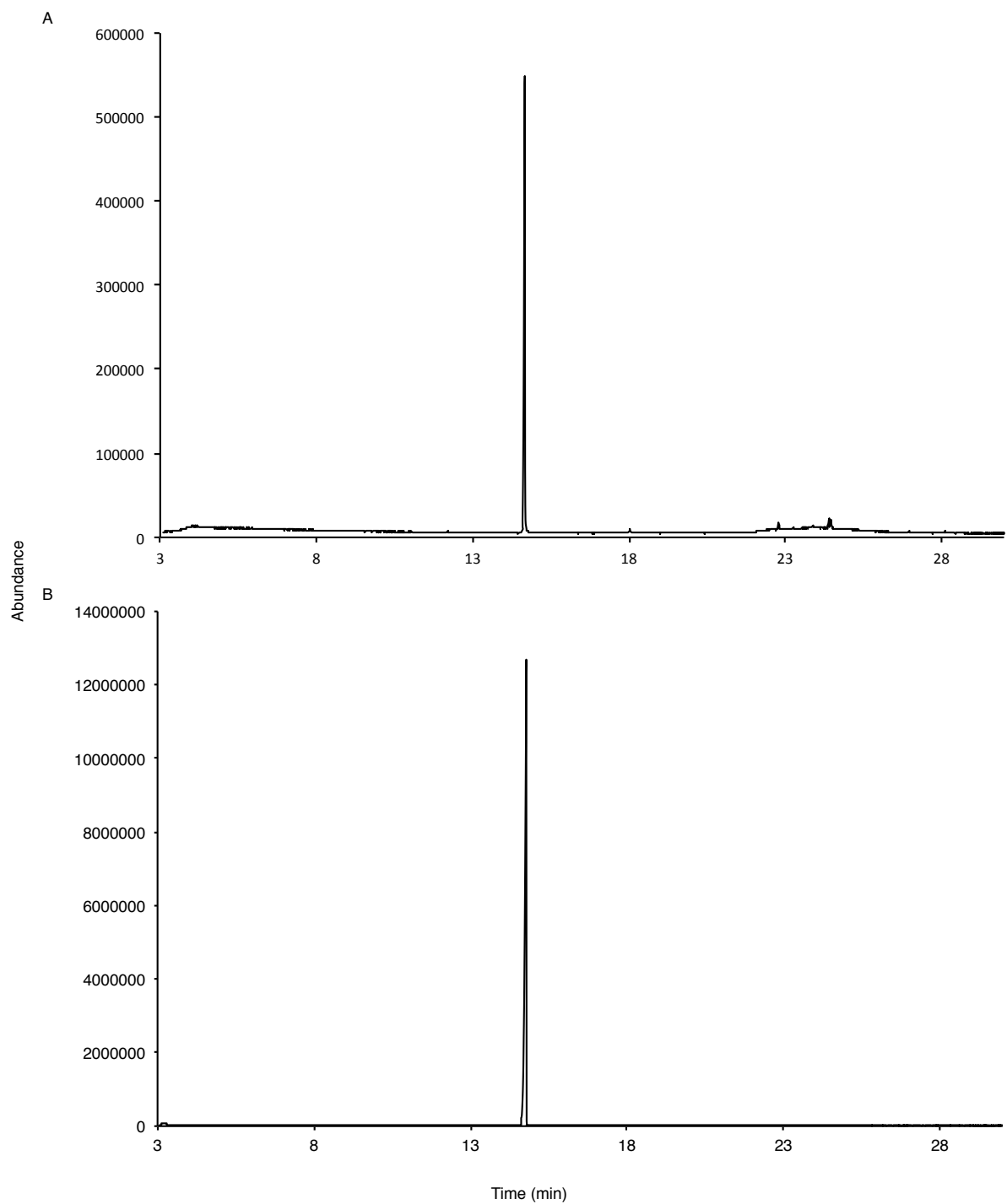
Taxon	Syndromic importance value	JF collection number
<i>Lygodium heterodoxum</i> Kunze	0.0027	1950
<i>Pluchea carolinensis</i> (Jacq.) G. Don	0.0027	1928
<i>Strychnos</i> sp.	0.0027	2008
<i>Gouania lupuloides</i> (L.) Urb.	0.0026	1947, 2024
<i>Justicia</i> sp.	0.0022	1943
<i>Melothria pendula</i> L.	0.0022	2005
<i>Piper yzabalanum</i> C. DC. ex Donn. Smith	0.0022	2000
<i>Psidium guajava</i> L.	0.0022	2023
<i>Drymonia</i> sp.	0.0017	2080
<i>Justicia breviflora</i> (Nees) Rusby	0.0017	2060
<i>Pityrogramma calomelanos</i> (L.) Link	0.0017	1998
<i>Begonia heracleifolia</i> Schltld. & Cham.	0.0012	2059
<i>Cestrum</i> sp.	0.0012	2056
<i>Hamelia rovirosae</i> Wernham	0.0012	1935
<i>Iris</i> sp.	0.0012	1924
<i>Piper auritum</i> Kunth.	0.0012	1934
<i>Piper</i> sp. (Che' puchuch)	0.0012	1990
<i>Stachytarpheta cayennensis</i> (L. Rich.) Vahl	0.0012	2079, 1936
<i>Acalypha</i> sp. 1	0.0012	1987
<i>Acalypha</i> sp. 2	0.0010	2055
<i>Acalypha</i> sp. 3	0.0010	1988
<i>Philodendron</i> sp.	0.0010	1959

**Table 4.S2.** Q'eqchi' healer rankings of plants for diabetes and highly associated symptoms.

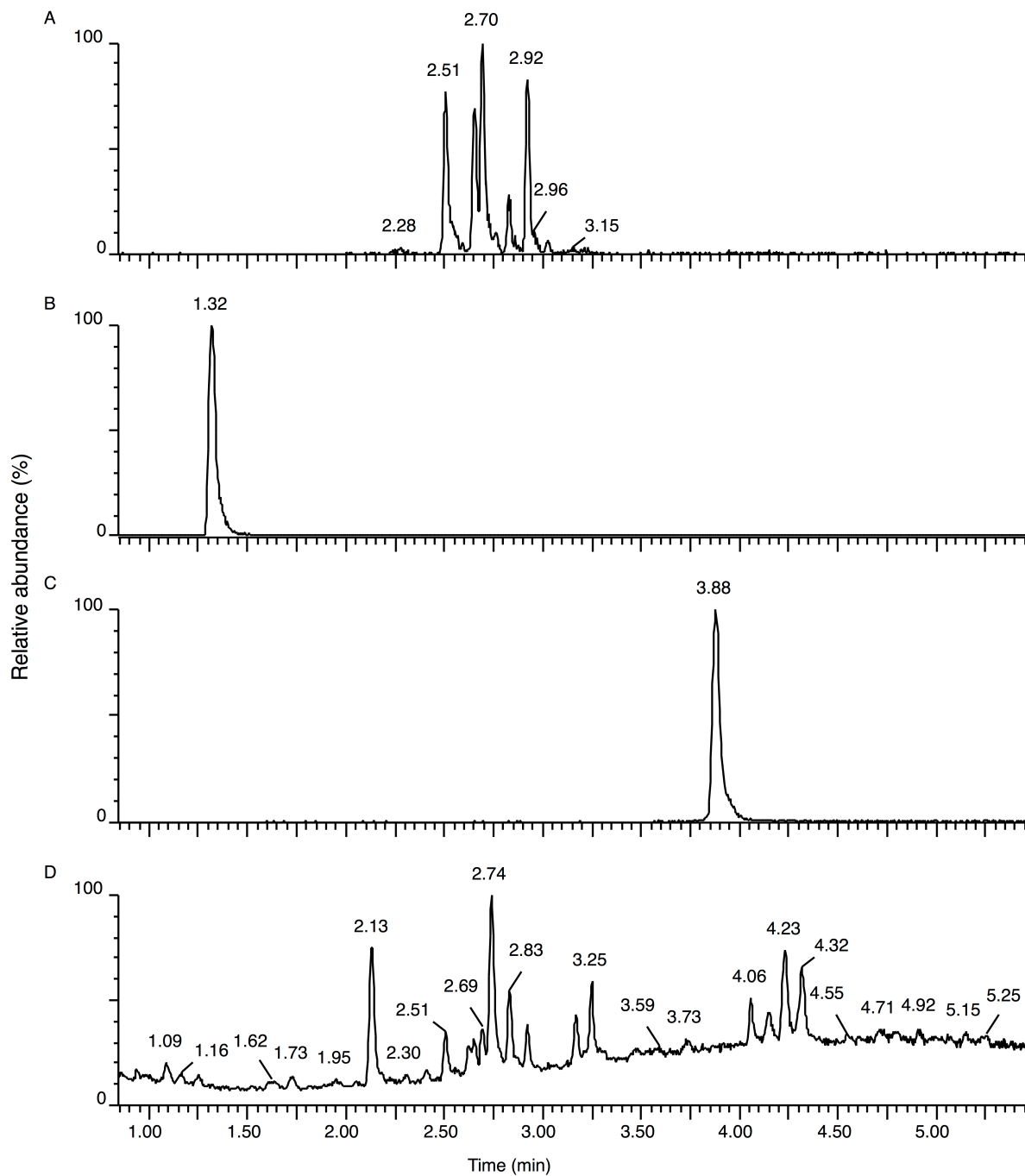
Genus species Authority	Healer rank	Symptom	Symptom rank
<i>Senna hayesiana</i> (Britton & Rose) H.S. Irwin & Barneby	NA	Diabetes	16
<i>Tynanthus guatemalensis</i> Donn. Sm.	1	Diabetes	16
<i>Tynanthus guatemalensis</i> Donn. Sm.	1	Diabetes	16
<i>Uncaria tomentosa</i> (Willd. ex Schult.) DC.	2	Diabetes	16
<i>Xiphidium caeruleum</i> Aubl.	2	Diabetes	16
<i>Pimenta guatemalensis</i> (Lundell) Lundell	3	Diabetes	16
<i>Acacia cookii</i> Safford	4	Diabetes	16
<i>Bursera simaruba</i> (L.) Sarg.	5	Diabetes	16
<i>Acosmium panamense</i> (Benth.) Yakovlev	NA	Diabetes	16
<i>Acosmium panamense</i> (Benth.) Yakovlev	NA	Diabetes	16
<i>Aristolochia grandiflora</i> Sw, see B's Kum pim	NA	Diabetes	16
<i>Bixa orellana</i> L.	NA	Diabetes	16
<i>Mandevilla subsagittata</i> (Ruiz & Pav.) Woodson	NA	Diabetes	16
<i>Mikania micrantha</i> H.B.K.	NA	Diabetes	16
<i>Neurolaena lobata</i> (L.) R.Br. ex Cass.	NA	Diabetes	16
<i>Paullinia tomentosa</i> Jacq.	NA	Diabetes	16
<i>Psychotria cuspidata</i> Bredem. ex Schult.	NA	Diabetes	16
<i>Smilax</i> sp.	NA	Diabetes	16
<i>Solanum rudepannum</i> Dunal	NA	Diabetes	16
<i>Solanum rudepannum</i> Dunal	NA	Diabetes	16
<i>Tynanthus guatemalensis</i> Donn. Sm.	NA	Diabetes	16
<i>Tynanthus guatemalensis</i> Donn. Sm.	NA	Diabetes	16
<i>Uncaria tomentosa</i> (Willd. ex Schult.) DC.	NA	Diabetes	16
<i>Uncaria tomentosa</i> (Willd. ex Schult.) DC.	NA	Diabetes	16
<i>Anthurium willdenowii</i> Kunth.	2	Sore/swollen limbs	16
<i>Drymonia serrulata</i> (Jacq.) Mart.	NA	Sore/swollen limbs	16
<i>Paullinia</i> sp.	NA	Sore/swollen limbs	16
<i>Drymonia serrulata</i> (Jacq.) Mart.	1	Sore/swollen limbs	15
<i>Anthurium pentaphyllum</i> (Aubl.) G. Don.	2	Sore/swollen limbs	15
<i>Anthurium willdenowii</i> Kunth.	2	Sore/swollen limbs	15
<i>Philodendron belizense</i> or <i>inaequalatum</i> (sp)	2	Sore/swollen limbs	15
<i>Anthurium pentaphyllum</i> (Aubl.) G. Don.	NA	Sore/swollen limbs	15

<i>Anthurium willdenowii</i> Kunth.	NA	Sore/swollen limbs	15
<i>Begonia glabra</i> Aubl.	NA	Sore/swollen limbs	15
<i>Begonia nelumbiifolia</i> Schltl. & Cham.	NA	Sore/swollen limbs	15
<i>Costus laevis</i> Ruiz. & Pav.	NA	Sore/swollen limbs	15
<i>Drymonia serrulata</i> (Jacq.) Mart.	NA	Sore/swollen limbs	15
<i>Gurania makoyana</i> (Lem.) Cogn.	NA	Sore/swollen limbs	15
<i>Hoffmania ghiesbreghtii</i> (Lem.) Hemsl.	NA	Sore/swollen limbs	15
<i>Peperomia macrostachya</i> (Vahl) A.Dietr.	NA	Sore/swollen limbs	15
<i>Peperomia macrostachya</i> (Vahl) A.Dietr.	NA	Sore/swollen limbs	15
<i>Peperomia obtusifolia</i> (L.) A. Dietr.	NA	Sore/swollen limbs	15
<i>Piper</i> sp.	NA	Sore/swollen limbs	15
<i>Siparuna thecaphora</i> (Poepp. & Endl.) A. DC.	NA	Sore/swollen limbs	15
<i>Xiphidium caeruleum</i> Aubl.	NA	Sore/swollen limbs	15
<i>Acalypha</i> sp. 1	NA	Slow healing infections	14
<i>Acalypha</i> sp. 1	NA	Slow healing infections	14
<i>Acalypha</i> sp. 2	NA	Slow healing infections	14
<i>Acalypha</i> sp. 3	NA	Slow healing infections	14
<i>Philodendron</i> aff. <i>inaequalifolium</i> (Ruiz & Pav.) Vahl	NA	Slow healing infections	14
<i>Piper auritum</i> Kunth.	NA	Foot numbness	13
<i>Cestrum fraternum</i> Morton, J. Arnold Arbor.	NA	Foot numbness and sores	13
<i>Cestrum</i> sp.	NA	Foot numbness and sores	13
<i>Piper peltatum</i> L.	NA	Foot numbness and sores	13
<i>Piper schiedeianum</i> Steud.	NA	Foot numbness and sores	13
<i>Piper</i> sp.	NA	Foot numbness and sores	13
<i>Hamelia rovirosae</i> Wernham	NA	Foot sores (itchiness)	13
<i>Begonia nelumbiifolia</i> Schltl. & Cham.	NA	Foot sores (pain)	13
<i>Cestrum fraternum</i> Morton, J. Arnold Arbor.	NA	Foot sores (pain)	13
<i>Drymonia serrulata</i> (Jacq.) Mart.	NA	Foot sores (pain)	13
<i>Stachytarpheta cayennensis</i> (L. Rich.) Vahl,	NA	Foot sores (pain)	13
<i>Arthrostemma ciliatum</i> Pav. Ex D. Don [Syn. <i>A. fragile</i> Lindl.]	NA	Increased urination	12
<i>Arthrostemma ciliatum</i> Pav. Ex D. Don [Syn. <i>A. fragile</i> Lindl.]	NA	Increased urination	12
<i>Begonia heracleifolia</i> Cham. & Schltl.?	NA	Increased urination	12
<i>Desmodium adscendens</i> (Sw.) DC.	NA	Increased urination	12
<i>Desmodium axillare</i> var. <i>acutifolium</i> (Kuntze) Urb.	NA	Increased urination	12
<i>Iris</i> sp.	NA	Increased urination	12
<i>Piper peltatum</i> L.	NA	Increased urination	12
<i>Piper peltatum</i> L.	NA	Increased urination	12
<i>Piper tuerckheimii</i> C. DC. ex Donn. Sm.	NA	Increased urination	12
<i>Tynanthus guatemalensis</i> Donn. Sm.	NA	Increased urination	12
<i>Pityrogramma calomelanos</i> L. var. <i>cal</i>	1	Blurred Vision	11
<i>Drymonia serrulata</i> (Jacq.) Mart.	2	Blurred vision	11
<i>Drymonia</i> sp.	NA	Blurred Vision	11
<i>Gonzalagunia panamensis</i> (Cav.) K.Schum.	NA	Blurred Vision	11
<i>Justicia breviflora</i> (Nees) Rusby	NA	Blurred Vision	11
<i>Peperomia obtusifolia</i> (L.) A. Dietr.	NA	Blurred Vision	11
<i>Siparuna thecaphora</i> (Poepp. & Endl.) A. DC.	NA	Blurred Vision	11
<i>Anthurium pentaphyllum</i> (Aubl.) G. Don.	1	Abscesses & boils	10
<i>Begonia nelumbiifolia</i> Schltl. & Cham.	NA	Abscesses & boils	10
<i>Drymonia serrulata</i> (Jacq.) Mart.	NA	Abscesses & boils	10
<i>Hoffmania ghiesbreghtii</i> (Lem.) Hemsl.	NA	Abscesses & boils	10
<i>Peperomia macrostachya</i> (Vahl) A.Dietr.	NA	Abscesses & boils	10
<i>Peperomia obtusifolia</i> (L.) A. Dietr.	NA	Abscesses & boils	10
<i>Piper yzabalanum</i> C. DC. ex Donn. Smith	NA	Abscesses & boils	10
<i>Xiphidium caeruleum</i> Aubl.	NA	Abscesses & boils	10
<i>Tynanthus guatemalensis</i> Donn. Sm.	NA	Increased thirst	9
<i>Tynanthus guatemalensis</i> Donn. Sm.	NA	Increased thirst	9
<i>Adiantum wilsonii</i> Hook.	NA	Heart/chest pain	8

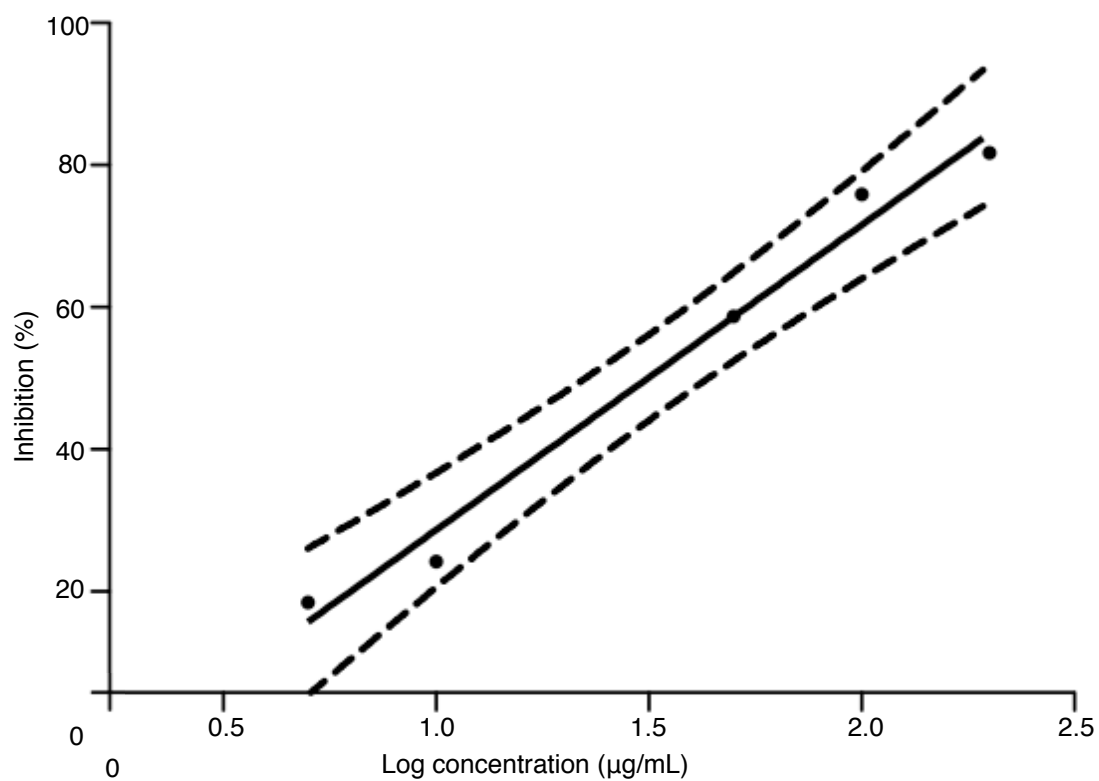
<i>Justicia</i> sp.	NA	Heart/chest pain	8
<i>Melothria pendula</i> L.	NA	Heart/chest pain	8
<i>Tynanthus guatemalensis</i> Donn. Sm.	NA	Heart/chest pain	8
<i>Drymonia serrulata</i> (Jacq.) Mart.	1	Increased appetite	7
<i>Strychnos</i> sp.	2	Increased appetite	7
<i>Acosmium panamense</i> (Benth.) Yakovlev	NA	Increased appetite	7
<i>Adiantum wilsonii</i> Hook.	NA	Weakness	6
<i>Piper yucatanense</i> C. DC.	NA	Weakness	6
<i>Smilax</i> sp.	NA	Weakness	6
<i>Uncaria tomentosa</i> (Willd. ex Schult.) DC.	NA	Weakness	6
<i>Clidemia capitellata</i> (Bonpl.) D. Don	NA	Swelling & Inflammation	5
<i>Costus laevis</i> Ruiz. & Pav.	NA	Swelling & inflammation	5
<i>Drymonia serrulata</i> (Jacq.) Mart.	NA	Swelling & inflammation	5
<i>Neea choriophylla</i> Standl.	NA	Swelling & inflammation	5
<i>Xiphidium caeruleum</i> Aubl.	NA	Swelling & inflammation	5
<i>Paullinia</i> sp.	1	Diarrhoea	4
<i>Paullinia</i> sp.	2	Diarrhoea	4
<i>Gouania lupuloides</i> (L.) Urb.	3	Diarrhoea	4
<i>Acosmium panamense</i> (Benth.) Yakovlev	NA	Diarrhoea	4
<i>Desmodium adscendens</i> (Sw.) DC.	NA	Diarrhoea	4
<i>Desmodium axillare</i> var. <i>acutifolium</i> (Kuntze) Urb.	NA	Diarrhoea	4
<i>Gouania lupuloides</i> (L.) Urb.	NA	Diarrhoea	4
<i>Neurolaena lobata</i> (L.) R.Br. ex Cass.	NA	Diarrhoea	4
<i>Paullinia tomentosa</i> Jacq.	NA	Diarrhoea	4
<i>Piper hispidum</i> Sw	NA	Diarrhoea	4
<i>Piper peltatum</i> L.	NA	Diarrhoea	4
<i>Piper peltatum</i> L.	NA	Diarrhoea	4
<i>Piper peltatum</i> L.	NA	Diarrhoea	4
<i>Piper tuerckheimii</i> C. DC. ex Donn. Sm.	NA	Diarrhoea	4
<i>Psidium guajava</i> L.	NA	Diarrhoea	4
<i>Piper yucatanense</i> C. DC.	1	Back & kidney pain	3
<i>Dendropanax arboreus</i> (L.) Decne.& Planch.	3	Back & kidney pain	3
<i>Arthrostemma ciliatum</i> Pav. Ex D. Don	NA	Back & kidney pain	3
<i>Dendropanax arboreus</i> (L.) Decne.& Planch.	NA	Back & kidney pain	3
<i>Epiphyllum phyllanthus</i> (L.) Haw.	NA	Back & kidney pain	3
<i>Hoffmania ghiesbreghtii</i> (Lem.) Hemsl.	NA	Back & kidney pain	3
<i>Lygodium venustum</i> Sw.	NA	Back & kidney pain	3
<i>Piper pseudolindenii</i> C. DC.	NA	Back & kidney pain	3
<i>Baccharis trinervis</i> (Lam.) Pers.	NA	Frequent headaches	2
<i>Bolbitis pergamentacea</i> (Maxon) Ching	NA	Frequent headaches	2
<i>Columnea sulfurea</i> Donn. Sm.	NA	Frequent headaches	2
<i>Gonzalagunia panamensis</i> (Cav.) K.Schum.	NA	Frequent headaches	2
<i>Justicia</i> aff. <i>fimbriata</i> (Nees) V.A.W. Graham	NA	Frequent headaches	2
<i>Lygodium heterodoxum</i> Kunze	NA	Frequent headaches	2
<i>Lygodium venustum</i> Sw.	NA	Frequent headaches	2
<i>Peperomia macrostachya</i> (Vahl) A.Dietr.	NA	Frequent headaches	2
<i>Peperomia obtusifolia</i> (L.) A. Dietr.	NA	Frequent headaches	2
<i>Piper hispidum</i> Sw.	NA	Frequent headaches	2
<i>Pluchea carolinensis</i> (Jacq.) G. Don	NA	Frequent headaches	2
<i>Siparuna thecaphora</i> (Poepp. & Endl.) A. DC.	NA	Frequent headaches	2
<i>Cestrum fraternum</i> Morton, J. Arnold Arbor.	NA	Arthritis/rheumatism	1
<i>Merremia dissecta</i> (Jacq.) H. Hallier F.	NA	Arthritis/rheumatism	1
<i>Merremia dissecta</i> (Jacq.) H. Hallier F.	NA	Arthritis/rheumatism	1
<i>Paullinia</i> sp.	NA	Arthritis/rheumatism	1
<i>Philodendron inaequilaterum</i> Liebm.	NA	Arthritis/rheumatism	1
<i>Piper yucatanense</i> C. DC.	NA	Arthritis/rheumatism	1
<i>Siparuna thecaphora</i> (Poepp. & Endl.) A. DC.	NA	Arthritis/rheumatism	1



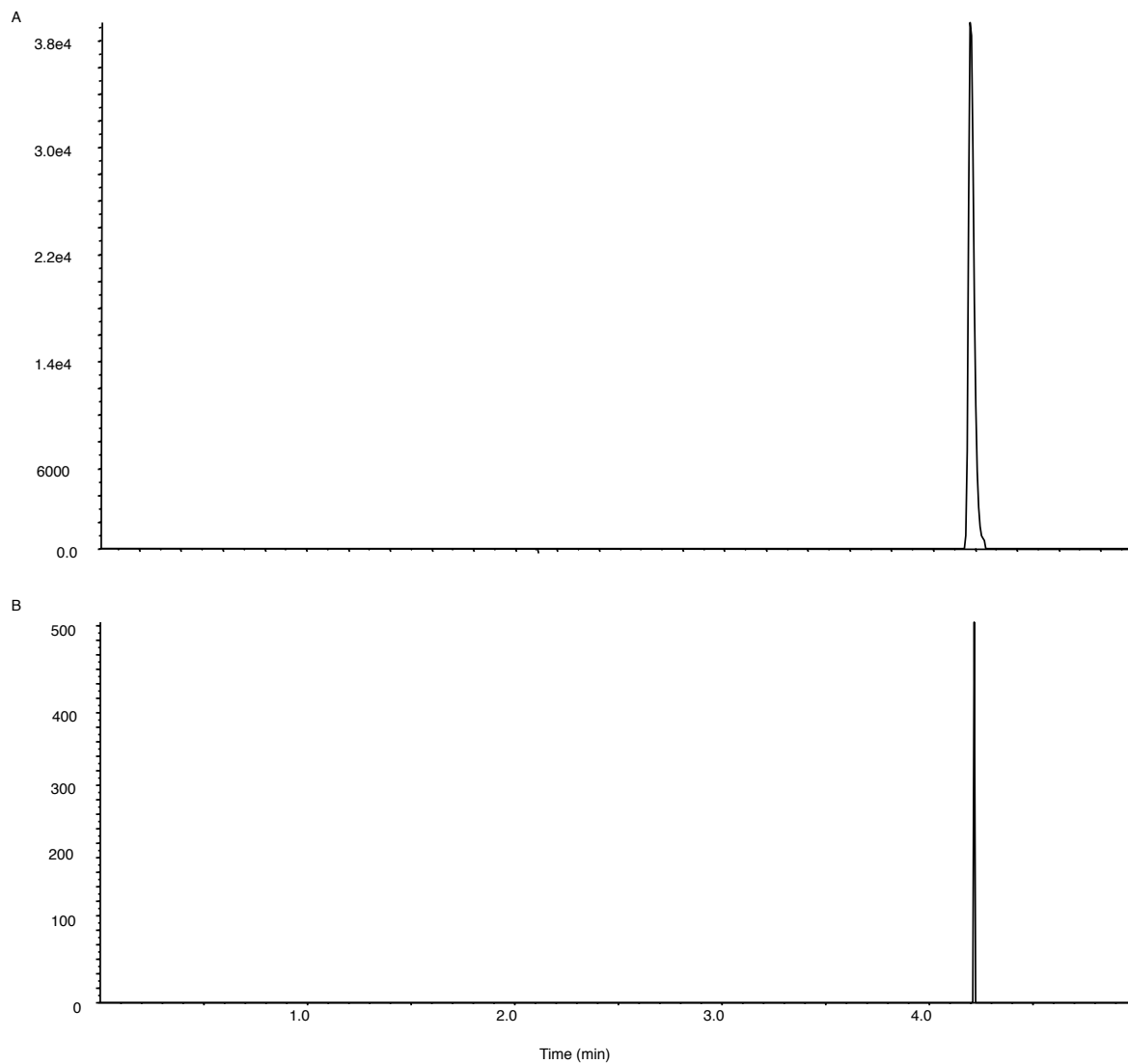
**Figure 4.S1.** Comparison of retention times (RT) and Kovát's Index (KI) of (A) *T. guatemalensis* isopropyl liquor (RT = 14.666 min, KI = 1338) and (B) eugenol standard (Rt 14.781, KI, 1346).



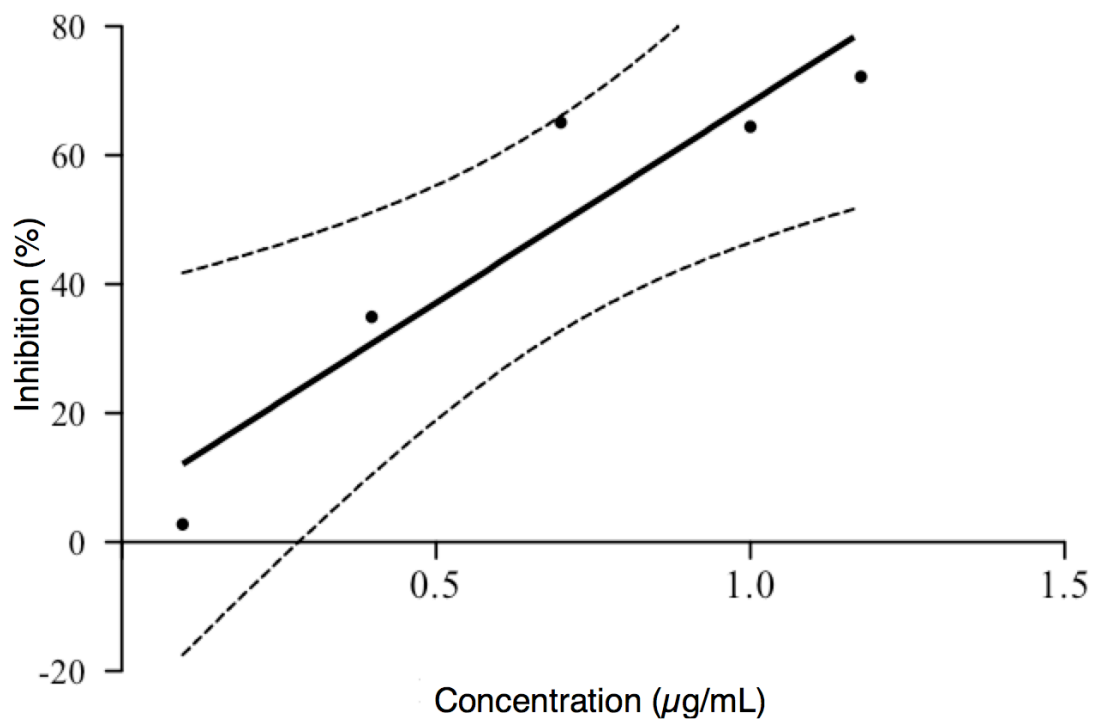
**Fig 4.S2.** UPLC-MS-QToF analysis of *Tynanthus guatemalensis* crude extract. (A) Katchimoside analogues (564.1404) (B) 4-hydroxybenzoate (138.0235) (C) Salicylic acid (138.0097) (D) Crude extract trace. Exact mass and molecular formula search could not detect eugenol, leucosceptoside and isoverbascoside reported in Plaza et al. 2005.



**Figure 4.S3.** Inhibition of AGEs by *Smilax* sp.. A typical result by *Smilax* sp. extract ( $IC_{50} = 42.8 \mu\text{g}\cdot\text{mL}^{-1}$ ) with 95% confidence interval of best fit-line ( $r^2 = 0.98$ ). The negative control exhibits no AGE-inhibition and the positive control, quercetin, exhibits an  $IC_{50} = 4.6 \mu\text{g}\cdot\text{mL}^{-1}$ .



**Figure 4.S4.** Comparison *Tynanthus guatemalensis* ethyl acetate fraction with verbascoside standard. (A) HPLC-MS-QTRAP chromatogram of verbascoside standard,  $8.8\mu\text{g}\cdot\text{mL}^{-1}$ , dissolved in 20% ACN and 80%  $\text{H}_2\text{O}$ . Negative multiple reaction monitoring, 623.2/160.8 amu. (B) HPLC-MS-QTRAP chromatogram of ethyl acetate fraction of *T. guatemalensis* showing verbascoside at retention time = 4.2 min.



**Figure 4.S5.** Dose-dependent inhibition of AGEs by verbascoside ( $IC_{50} = 5.1 \mu\text{g}\cdot\text{mL}^{-1}$ ) with 95% confidence intervals of best-fit line ( $r^2 = 0.87$ ). The negative control exhibits no AGE-inhibition and the positive control, quercetin, exhibits an  $IC_{50} = 4.6 \mu\text{g}\cdot\text{mL}^{-1}$ .

## Section 2

# Pharmacology and Metabolomics of the Antidiabetic Genus *Vaccinium*

## Preface

Consensus ethnobotany of antidiabetic plants with the Eeyou Istchee Cree and the Lukomir Highlanders, revealed a strong phylogenetic signal (syndromic importance value - SIV) in the genus *Vaccinium*. *Vaccinium* was not used by the lowland Q'eqchi' Maya, but it is present in the Central American highlands. Cross-cultural consensus taxa (*Vaccinium* spp.) were given priority since they were demonstrated to contain more bioactive plants than random samples (Saslis-Lagoudakis et al. 2012). This workflow would facilitate collaboration and efficiently transfer antidiabetic technology cross-culturally.

The advanced glycation endproduct (AGE) assay was selected for evaluation of *Vaccinium* species since the experiment indicates the plant's effectiveness in preventing AGE formation (linked to diabetes and contribute to the development of retinopathy, cataracts, atherosclerosis, neuropathy, nephropathy, diabetic embryopathy and impaired wound healing). Characterization and pharmacological assessment of *Vaccinium* spp. was conducted since consensus taxa are of value as internationally available medicines or as commercial natural health products. Metabolomic analyses were conducted to validate quality control measurements across single and multiple laboratories. Identity methods were developed were developed for a large group of antidiabetic *Vaccinium* species.

## Chapter 5

# Antiglycation Activity of *Vaccinium* spp. (Ericaceae) from Wild Sources and the Sam Vander Kloet Garden<sup>6\*</sup>

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\*Ferrier, J., Djeflal, S., Morgan, H.P., Vander Kloet, S.P., Redžić, S., Cuerrier, A., ... Arnason, J.T. (2012). Antiglycation activity of *Vaccinium* spp. (Ericaceae) from the Sam Vander Kloet collection for the treatment of type II diabetes. *Botany*, 406, 401–406. doi:10.1139/B2012-026



Ericaceae species *Vaccinium uliginosum*, *V. vitis-idaea*, and *Empetrum nigrum* growing together in Whapmagoostui, Nunavik.

<sup>6</sup> Dedicated to the memory of Sam Vander Kloet

## Antiglycation Activity of *Vaccinium* spp. (Ericaceae) from Wild Sources and the Sam Vander Kloet Garden

For millennia, Indigenous Peoples have used all plant parts from numerous species of *Vaccinium* L. (Ericaceae) in traditional medicine (Arnason et al. 1981; Lee et al. 2004; Redžić 2006, 2010; Zutter 2009). Leaves of northern *Vaccinium* spp. (blueberries, bilberries, huckleberries, and cranberries) have the following traditional uses: abortifacients, antiemetics, blood and ceremonial medicines, cold remedies, diaphoretics, dietary aids, eye medicines, febrifuges, gastrointestinal and gynecological aids, panacea, and pediatric aids (Moerman 2009; Ferrier et al. 2011). However, the use of leaves from tropical *Vaccinium* specimens is poorly documented or not accessible.

There are a few reports noting Indigenous Peoples use of *Vaccinium* leaves for treating diabetes specifically. Using *Vaccinium myrtillus* L. foliage for infusions was reported in Kelly's (1970) Colorado, USA, woody plants field guide. Ethnobotanical surveys in Lukomir, Bosnia and Herzegovina, report that 4 of 25 traditional healers use the leaves and (or) stems of *V. myrtillus* for a cure-all infusion (Ferrier et al. 2011). Antidiabetic pharmacological studies, guided by anecdotal traditional use reports of the European *V. myrtillus*, evaluated semi-pure and crude leaf extracts in animal and clinical trials with safe and positive antidiabetic effects on depancreatized dogs, insulin reduction and sensitization in diabetic patients, and statistically significant decreases in plasma glucose in streptozotocin-induced diabetic rats (Allen 1927; Cignarella et al. 1996).

The Eeyou Istchee Cree, Canada, in collaboration with the Canadian Institutes of Health Research Team in Aboriginal Antidiabetic Medicine undertook analyses of *Vaccinium angustifolium* Aiton. Cree healers recommended *V. angustifolium* and its leaves were shown to have its most potent advanced glycation endproduct (AGE) inhibition activity from leaf material collected in October (Leduc et al. 2006; McIntyre et al. 2009). Harris et al. (2007) demonstrated that *V. angustifolium* leaves contains high levels of the active phytochemicals chlorogenic acid ( $\sim 100 \mu\text{g}\cdot\text{mg extract}^{-1}$ ) combined with many quercetin glycosides at the end of August.

Prolonged hyperglycaemia in diabetes leads to impaired glucose uptake and damages the cells of organs that do not require insulin for glucose uptake (e.g., heart, kidneys, neurons, and small blood vessels). Consequently, these cells have high concentrations of intracellular glucose during elevated hyperglycaemic periods (Ahmed 2005). Cellular T2D complications begin with formation of “Schiff bases” between glucose and protein, which can progress into an irreversible AGE.

In this study we examined the AGE inhibition activity and phenolic content of *Vaccinium* species collected from wild populations in Canada and Bosnia and Herzegovina, as well as specimens that were available from Sam Vander Kloet’s common garden collection. Indigenous peoples are one of the fastest growing communities on the planet, and with rapidly increasing rates of type two diabetes (T2D) they are also some of the most susceptible populations (Helin 2008) that may benefit from diabetic therapies derived from traditional medicines. Because AGEs accumulate with sustained hyperglycaemia causing diabetic complications, AGEs are a therapeutic target. This study examines the relationship of AGE inhibition activity to phenolic content and the latitudinal geographic origin of *Vaccinium* species.

## **Methods**

### ***Vaccinium* sample collection**

Cuerrier, Ferrier, Redžić have collected and identified wild *Vaccinium* spp. with outgroup samples of *Gaylussacia baccata*. Voucher deposits were made at MT and OTT (Table 5.1). Determinations were made using ‘The Flora of Canada’ and ‘The Flora of North America’ for the QC, Canada region; ‘Mala Flora Hrvatske’ and ‘Flora Europaea’ for Bosnia and Herzegovina regions (Scoggan 1978; Vander-Kloet 2009; Domac 1984; Tutin et al. 1972).

Table 5.1. A list of samples collected from the wild with habitat information and test results.

Genus species	Section	North	West	Alt. (m)	Total phenolic:IC <sub>50</sub>	Pop. # - d.m.y	Habitat	OTT Voucher #
<i>V. boreale</i> (V. Hall & Alders)	Cyanococcus	48.61278	-70.79658	959	111.5	52.0 Pop. 1 - 8.7.7	Mont-Vain, QC, Canada: rocky peak with boreal surroundings	BBE 129
<i>V. boreale</i> (V. Hall & Alders)	Cyanococcus	48.61278	-70.79659	959	179.9	19.3 Pop. 2 - 8.7.7	Mont-Vain, QC, Canada: rocky peak with boreal surroundings	BBE 32
<i>V. corymbosum</i> L. f. <i>corymbosum</i>	Cyanococcus	45.46453	-70.79661	23	124.7	61.9 Pop. 1 - 5.6.7	Snake River, QC, Canada: 2m shrubs, river mouth, beach area below forest	BBE 80
<i>V. corymbosum</i> L. f. <i>corymbosum</i>	Cyanococcus	45.46453	-70.79661	23	185.3	9.8 Pop. 1 - 5.6.7	Snake River, QC, Canada: 2m shrubs, river mouth, beach area below forest	BBE 81
<i>V. corymbosum</i> f. <i>albiflorum</i> (Hook.) Fernald	Cyanococcus	45.67957	-74.6647	132	103.4	15.3 Pop. 2 - 6.6.7	Rivière Rouge, QC, Canada: rain, cloud forest of conifers, ferns, moss, gravel	BBE 84
<i>V. caespitosum</i> Michx.	Myrtillus	48.61278	-70.79658	984	110.0	16.9 Pop. 3 - 6.7.7	Mont-Vain, QC, Canada: passing shower, arid, windy, rocky summit	BBE 98
<i>V. caespitosum</i> Michx.	Myrtillus	49.0063	-66.01054	824	130.9	22.6 Pop. 5 - 17.7.7	Mine Madeleine, QC, Canada: pine forest with birch and some spruce	BBE 101
<i>V. caespitosum</i> Michx.	Myrtillus	48.9821	-65.9574	1157	114.7	17.4 Pop. 7 - 18.7.7	Mont Jacques Cartier, QC, Canada: stunted forest, stunted birch at 2 m, scattered snow, caribou	BBE 136
<i>V. caespitosum</i> Michx.	Myrtillus	49.00485	-65.99695	1053	192.9	23.4 Pop. 6 - 17.7.7	Mine Madeleine, QC, Canada: between two peaks, softwood forest stunted to 2.5 meters	BBE 99
<i>V. ovalifolium</i> Smith	Myrtillus	48.6187	-70.80218	877	133.4	41.1 Pop. 1 - 6.7.7	Mont-Vain: rain, along trail, near the refuge, 1 km from the summit with sand, pine, and rocks	BBE 77
<i>V. ovalifolium</i> Smith	Myrtillus	49.00503	-65.94468	1052	129.8	9.8 Pop. 8 - 19.7.7	Mont Jacques Cartier, QC, Canada: mixed forest, trailside	BBE 90
<i>V. ovalifolium</i> Smith	Myrtillus	48.966672	-66.02696	807	80.3	78.4 Pop. 10 - 20.7.7	Mont Richardson, QC, Canada: with conifers and alders	BBE 95
<i>V. macrocarpon</i> Aiton	Oxycoccus	45.99502	-73.98824	350	195.0	14.6 Pop. 1 - 23.5.7	Station de l'Université de Montréal, Canada: Lake 4, sunny, small peat island	BBE 100
<i>V. macrocarpon</i> Aiton	Oxycoccus	45.99502	-71.19058	307	200.9	13.2 Pop. 3 - 29.5.7	Lac à la Barbe, QC, Canada: sunny with wind, floating islands on lake	BBE 103
<i>V. macrocarpon</i> Aiton	Oxycoccus	45.99502	-71.18272	313	189.6	19.0 Pop. 4 - 31.5.7	Lac à la Barbe, QC, Canada: floating island near dock	BBE 104
<i>V. uliginosum</i> *	Vaccinium	49.00677	-66.01161	1054	145.0	3.6 Pop. 5 - 17.7.7	Mine Madeleine, QC, Canada: pre summit, scree corridor, with Lab tea and stunted birches	BBE 34
<i>V. uliginosum</i> *	Vaccinium	49.00677	-66.01161	1054	125.7	52.5 Pop. 5 - 17.7.7	Mine Madeleine, QC, Canada: pre summit, scree corridor, with Lab tea and stunted birches	BBE 45
<i>V. uliginosum</i> *	Vaccinium	49.00677	-66.01161	1054	132.4	42.7 Pop. 5 - 17.7.7	Mine Madeleine, QC, Canada: pre summit, scree corridor, with Lab tea and stunted birches	BBE 63
<i>V. uliginosum</i> *	Vaccinium	49.00677	-66.01161	1054	110.8	36.9 Pop. 5 - 17.7.7	Mine Madeleine, QC, Canada: pre summit, scree corridor, with Lab tea and stunted birches	BBE 66
<i>V. myrtillus</i> L.	Myrtillus	43.72839	+18.56319	1730	233.9	27.8 Pop. 1 - 6.15.7	Jahornia, Bosnia and Herzegovina: mountainside	BBE 125
<i>V. myrtillus</i> L.	Myrtillus	43.72839	+18.56320	1730	140.9	5.4 Pop. 1 - 6.15.7	Jahornia, Bosnia and Herzegovina: mountainside	BBE 42
<i>V. vitis-idaea</i> L.	Myrtillus	43.81028	+18.56321	984	248.6	4.1 Pop. 1 - 6.20.8	Igman, Bosnia & Herzegovina, mountainside	BBE 134
<i>V. vitis-idaea</i> L.	Myrtillus	43.72839	+18.56322	1730	243.5	48.6 Pop. 1 - 6.15.7	Jahornia, Bosnia and Herzegovina: mountainside	BBE 23
<i>G. baccata</i> (Wangenh.) K. Koch	NA	45.46893	-74.02575	14	112.2	71.0 Pop. 5 - 6.6.7	Parc d'Oka, QC, Canada: Beach side, forest	G 1
<i>G. baccata</i> (Wangenh.) K. Koch	NA	45.80153	-71.18353	338	144.2	25.1 Pop. 4 - 1.6.7	Parc de Frontenac, QC, Canada: pine forest, near cottage, near lac des îles	G 3
<i>G. baccata</i> (Wangenh.) K. Koch	NA	45.46893	-74.0258	14	109.2	58.2 Pop. 5 - 6.6.7	Parc d'Oka, QC, Canada: Beach side, forest	G 4

\*ssp. *pubescens* Wormsk. ex Hornem.

Table 5.2. A list of samples collected from a common garden with habitat information and test results.

Genus species	Section	North	West	Alt. (m)	Total phenolic:IC <sub>50</sub>	Pop. # - d.m.y	Habitat	ACAD voucher #
<i>V. barandatum</i> S. Vidal	Bracteata	16.583572	+120.883592	2455	624.0	16.2 Pop. 1 - 30.9.9	Mt. Pulog, Luzon, Philippines	17(X)92 (AUV.00122)
<i>V. barandatum</i> S. Vidal	Bracteata	16.583572	+120.883593	2455	427.3	6.5 Pop. 2 - 30.9.9	Mt. Pulog, Luzon, Philippines	17(X)92 (AUV.00123)
<i>V. barandatum</i> S. Vidal	Bracteata	16.583572	+120.883594	2455	557.2	11.6 Pop. 3 - 30.9.9	Mt. Pulog, Luzon, Philippines	17(X)92 (AUV.00124)
<i>V. consanguineum</i> Klotzsch	Calciocolus	9.979167	-83.8525	3304	412.6	10.2 Pop. 1 - 30.9.9	Irazu Volcan, Costa Rica: 3432 msm; volcanic ash surrounding the crater	714686 (AUV.00128)
<i>V. consanguineum</i> Klotzsch	Calciocolus	9.979167	-83.8525	3304	460.7	6.0 Pop. 2 - 30.9.9	Irazu Volcan, Costa Rica: 3432 msm; volcanic ash surrounding the crater	714686 (AUV.00128)
<i>V. consanguineum</i> Klotzsch	Calciocolus	9.979167	-83.8525	3304	257.7	6.1 Pop. 3 - 30.9.9	Irazu Volcan, Costa Rica: 3432 msm; volcanic ash surrounding the crater	714686 (AUV.00128)
<i>V. myrsinites</i> Lam.	Cyanococcus	31.775278	-83.8525	61	437.1	12.1 Pop. 1 - 30.9.9	Pine Grove, Appling Co., Georgia: pine flat woods with Palmetto	422574 (AUV.00295)
<i>V. gauthierifolium</i> *	Galeopetalum	22.223	+103.7373	622	508.4	11.2 Pop. 1 - 30.9.9	Phan Xi Páng, Vietnam: base of mountain	1231197 (AUV.00204)
<i>V. gauthierifolium</i> *	Galeopetalum	22.223	+103.7374	622	585.7	8.9 Pop. 4 - 30.9.9	Phan Xi Páng, Vietnam: base of mountain	1231197 (AUV.00204)
<i>V. gauthierifolium</i> *	Galeopetalum	22.223	+103.7375	622	415.1	8.6 Pop. 5 - 30.9.9	Phan Xi Páng, Vietnam: base of mountain	1231197 (AUV.00204)
<i>V. poasanum</i> Donn.Sm.	Oreades	10.2	-84.2	2200	431.0	7.6 Pop. 1 - 30.9.9	Mt. Poas, Costa Rica: collected by M. Zinck, July 20, 1990	20790B (AUV.00139)
<i>V. poasanum</i> Donn.Sm.	Oreades	9.509425	-83.711556	2500	625.5	4.2 Pop. 3 - 30.9.9	Cerro de la Muerte, Costa Rica: on hummock in bog large plant; red berries	113686 (AUV.00124)
<i>V. tonkinense</i> Dop	NA	21.516667	+105.55	483	564.4	6.6 Pop. 3 - 30.9.9	Tam Dao, Vietnam: common epiphyte in Rhododendron shrubs	127 1197 p. 331 (AUV.00142)
<i>V. tonkinense</i> Dop	NA	21.516667	+105.55	483	485.2	8.8 Pop. 4 - 30.9.9	Tam Dao, Vietnam: common epiphyte in Rhododendron shrubs	127 1197 p. 332 (AUV.00142)
<i>V. tonkinense</i> Dop	NA	21.516667	+105.55	483	581.3	7.0 Pop. 5 - 30.9.9	Tam Dao, Vietnam: common epiphyte in Rhododendron shrubs	127 1197 p. 334 (AUV.00142)
<i>D. rimbacchi</i> (A.C.Sm.) Luteyn	NA	NA	NA	1995	489.8	13.6 Pop. 1 - 30.9.9	Ecuador: montane moist forest	Outgroup: 8011

(Griff.) Hook. f. ex C.B. Clarke.

We were very fortunate to have access to the late Sam Vander Kloet's global collection of tropical *Vaccinium* species growing at the Harriet Irving Botanical Gardens at Acadia University, Nova Scotia, Canada (ACAD). All common garden leaf samples were collected by J.F. and identified by Sam Vander Kloet (Table 5.2). *Disterigma rimbachii* (A.C. Sm.) Luteyn was available at ACAD and selected as a common garden Ericaceae outgroup to determine if *Vaccinium* spp. treated herein were more effective than the referenced outgroup family member.

Sam Vander Kloet's specimens were propagated from their wild seeds or cuttings, which grow together under common environmental conditions at the Harriet Irving Botanical Gardens (latitude 45.08686, longitude -64.3681). All common garden specimens grew outdoors during the spring, summer, and autumn; in late autumn plants were moved into a climate controlled greenhouse for the winter period.

All collections were made from healthy aboveground foliage from plants, older than 10 years. Leaves were collected in paper bags and tobacco offerings were made according to Algonquian custom. Each sample was dried overnight at 37 °C and stored at room temperature prior to a standardized extraction procedure.

For comparison purposes, only six temperate *Vaccinium* spp. (*V. boreale* I.V. Hall & Aalders, *V. corymbosum* L., *V. caespitosum* Michx., *V. ovalifolium* Sm., *V. macrocarpon* Aiton, and *V. uliginosum* L.) were collected in northeastern Canada, processed, and assayed in a similar way. Full details of collection data and activity of individual species will be reported later.

### ***Sample preparation and extraction***

All leaves were prepared and extracted using the following standardized method. Dried leaf samples were ground using a Wiley Mill and passed through a 1 mm mesh. Plant material was extracted twice in 95% ethanol (1 g·10 mL<sup>-1</sup>) at room temperature for 24 h per extraction. The recovered extractions were pooled and dried using a rotary evaporator (Savant Speed-Vac) at room temperature for 24 h to remove EtOH. Extracts were then placed in a freezer for 2 h at -20 °C before lyophilization at -40 °C for 24 h to remove water

from the extract. Lyophilization yielded between 0.10 and 0.21 g crude extract·g dried leaf<sup>-1</sup>. Extracts were stored at -20 °C. Stock solutions were prepared in EtOH and distilled H<sub>2</sub>O (4:1) for both total phenolic quantification and AGE inhibition assays. All samples were filtered through a 0.2 mm polytetrafluoro-ethylene (PTFE) nonsterile filter (Chromatographic Specialties Inc., Brockville, Ont., Canada) prior to analysis.

#### ***Total phenolics estimation assay***

Total phenolic content of *Vaccinium* and other extracts was estimated colourimetrically with a Folin–Ciocalteu (F–C) reagent based assay (Singleton and Rossi 1965) and modified to reduce volumes as previously described by Farsi et al. (2008), Spoor et al. (2006), and Harbilas et al. (2009). A stock solution of 7.5% NaHCO<sub>3</sub> in distilled H<sub>2</sub>O was prepared. All extracts were diluted using a 10 mg·mL<sup>-1</sup> stock solution to optimal assay concentrations of 2 mg·mL<sup>-1</sup>, 1 mg·mL<sup>-1</sup>, and 0.5 mg·mL<sup>-1</sup>. Quantification was based on the standard curve generated at 725 nm of solutions of 0.4, 0.2, 0.1, 0.05, and 0.025 mg·mL<sup>-1</sup> of quercetin prepared in ethanol/water (4:1). Eighty microlitres of extract or standard solution was added to 400 mL of F–C. The mixture was then vortexed briefly and left to stand at room temperature for 5 min. Two-hundred-seventy microlitres of the NaHCO<sub>3</sub> solution was added to the mixture and gently stirred. Samples were transferred to a clear bottom non sterile 96-well plate (Nalge Nunc International, Rochester, N.Y., USA) in three replicates of 200 mL per well. The plate was covered and incubated at room temperature in the dark for 2 h. Absorbance was read at 725 nm and total phenolic content was calculated in terms of micrograms quercetin equivalents per milligrams of extract.

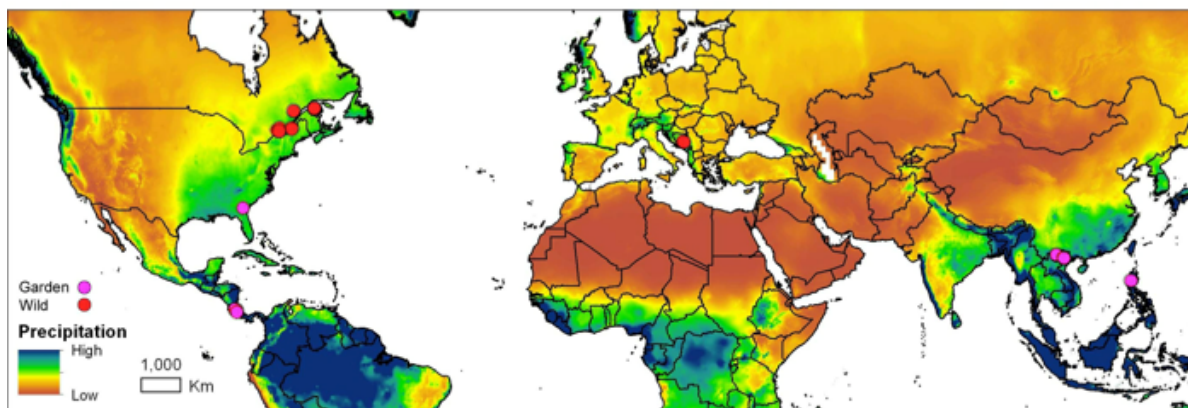
#### ***AGE formation inhibition assay***

Advanced glycation endproduct formation inhibition activity was assessed as described by Farsi et al. (2008) with modifications. Bovine serum albumin (BSA) (1 mg·mL<sup>-1</sup>) was incubated with 100 mmol·L<sup>-1</sup> glucose / 100 mmol·L<sup>-1</sup> fructose in sodium phosphate monobasic monohydrate buffer (pH 7.4) with extract (experimental treatment), ethanol/water (4:1) (negative control), or quercetin, an antioxidant against glycation by way

of phenolic hydroxyl groups in the flavonoid structure (24 mg/mL in assay) (Sengupta et al. 2006), which served as a positive control. To control for fluorescence of extracts, a treatment without BSA was included; to control for fluorescence of BSA, a treatment with BSA and vehicle was included. Stock solutions of extracts were serially diluted and tested at five concentrations that were optimized for dissolution and a linear concentration response (40, 20, 10, 5, and 2.5 mg·mL<sup>-1</sup> in assay). Three replicates were tested in sterile opaque polystyrene 96-well clear bottom plates (Corning Inc., New York, N.Y., USA). Plates were covered, sealed with Parafilm, and incubated for 7 days at 37 °C while being shaken. Following incubation, fluorescence was measured using a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, Calif., USA) at excitation and emission wavelengths of 355 and 460 nm. Glucose/fructose and ethanol/water fluorescence was subtracted from all results, and percent inhibition and IC<sub>50</sub> values were calculated as previously described (Farsi et al. 2008).

### ***Statistics and geographic information system analysis***

Statistical analysis was conducted using Prism 5.0d trial software. All specimen localities were imported into ArcGIS version 9.3 and projected to an Albers projection with a World Geodetic System 1984 datum. Utilizing the altitude variable from the Worldclim dataset at 30 arc seconds resolution (Hijmans et al. 2005), values were extracted for each unique specimen locality using the “Extract Values to Points” tool in the Spatial Analyst extension of ArcGIS version 9.3. These altitude values were compared between the latitudinal geographic origins of wild and garden groups using the non-parametric Kolmogorov–Smirnov z test, because assumptions of data normality and homogeneity of the variance were not met (data not shown).



**Figure 5.1.** Genetic origins of all examined species and annual precipitation.

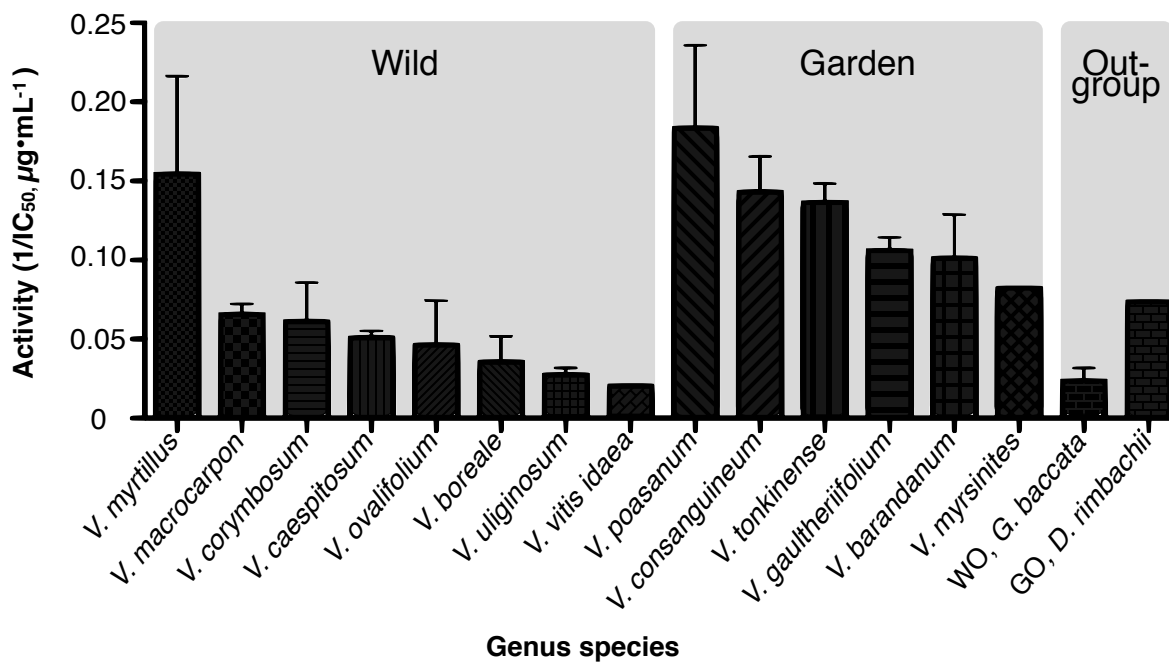
## Results

### *Inhibition of AGE formation by wild and garden Vaccinium spp.*

All *Vaccinium* species extracts tested in the AGE inhibition assay displayed highly linear concentration dependent inhibition of AGE formation (Figure 1.1). While leaf extracts of all *Vaccinium* species showed inhibition of AGE formation (Table 5.1 and 5.2), the mean  $IC_{50}$  for the northern species (wild group) was 29.8, while the tropical and subtropical (common garden group) had a substantially lower mean  $IC_{50}$  of 8.99. This result suggests that the tropical *Vaccinium* spp. were, on average, 3x more inhibitory. The range of  $IC_{50}$ s was from 4.1 to 79  $\mu\text{g/mL}$  for the wild group (~20x), and from 4.2 to 16  $\mu\text{g/mL}$  (~4x) in the common garden group. This probably reflects the reduced environmental effects in the common garden even though the species are different.

To illustrate the results on an activity ( $1/IC_{50}$ ) basis,  $1/IC_{50}$  is plotted in Figure 5.2. The highest mean activities ( $1/IC_{50}$ ) from the wild group were 0.155 from *Vaccinium myrtillus* (Bosnia and Herzegovina), and 0.0657 from *V. macrocarpon* (Canada). The highest mean activity ( $1/IC_{50}$ ) from the common garden group was 0.184 from *V. poasanum* (Costa Rica) (Figure 5.3). The activities of *Vaccinium* spp. were comparable to or greater than the respective out-group plants (*Gaylussacia baccata* for northern species and *Distigmea rimbacchii* for tropicals).

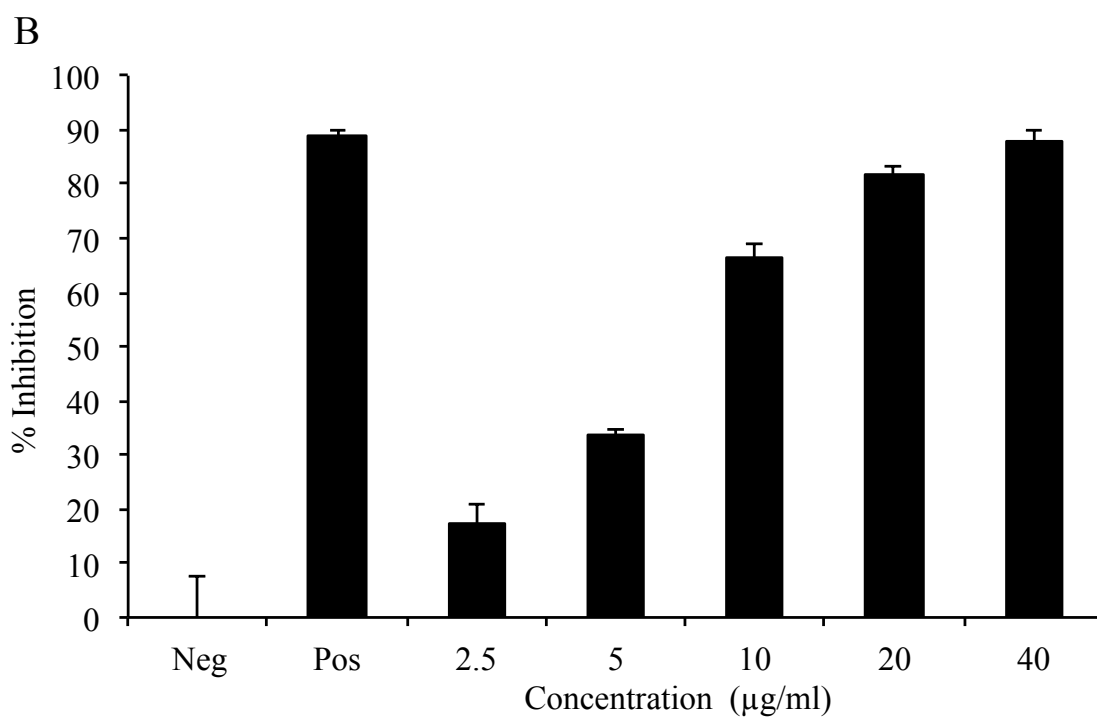
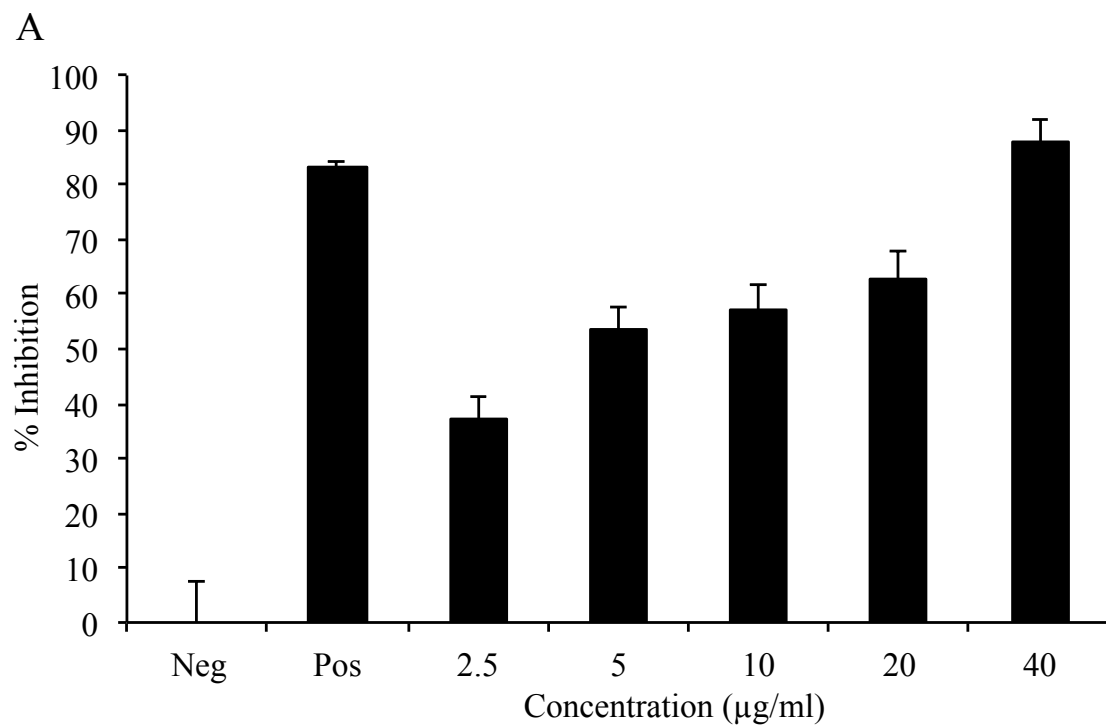
A linear regression showed that there was a relationship between IC<sub>50</sub> values for garden species and latitudinal genetic origin (R<sup>2</sup>= 0.314 and P< 0.037) (Figure 5.4). This relationship found with garden species was not present with the wild group.



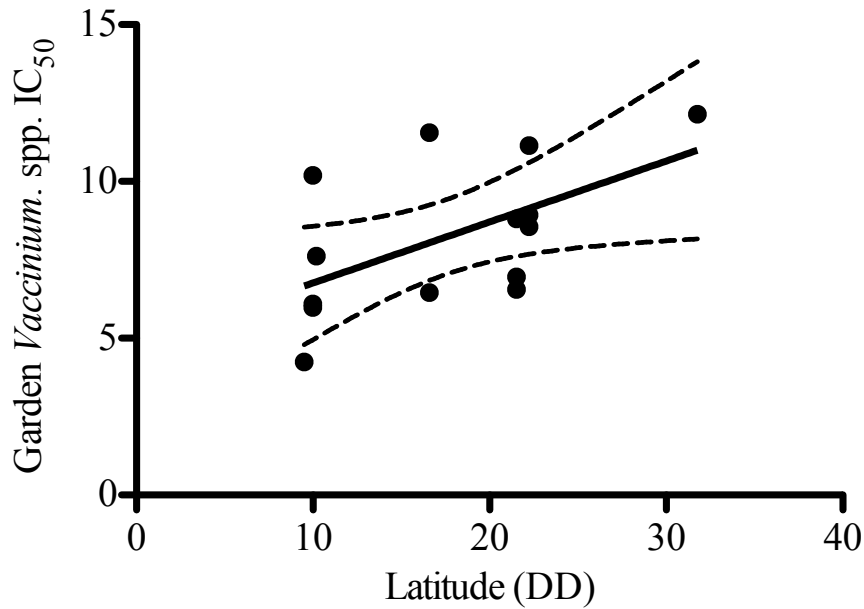
**Figure 5.2.** *Vaccinium* and out-group mean AGE inhibition activity (µg·mL<sup>-1</sup>) from wild and common garden populations (Wild out-group = WO, Common garden out-group = GO).

***Phenolic content of wild and common garden Vaccinium spp.***

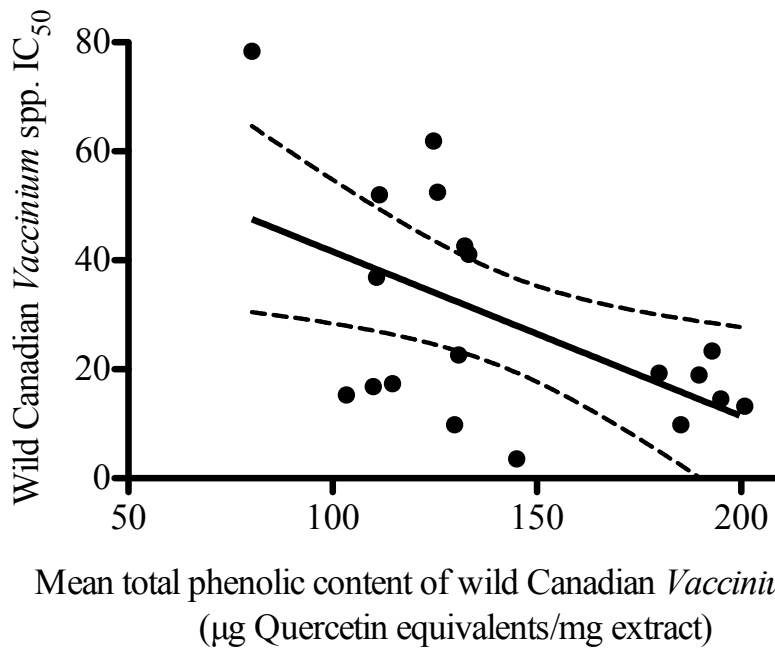
Mean phenolic concentration was higher (492 µg quercetin equivalents/mg extract) in the tropical *Vaccinium* species compared to the northern *Vaccinium* species (155 µg Quercetin equivalents/mg extract), but the range of phenolics was larger in the northern species (80–248 µg quercetin equivalents/mg extract) or 3.1x compared to the range (271-675) in the tropics (2.5x). Only the wild Canadian collection of *Vaccinium* spp. (with Bosnia and Herzegovina’s species excluded) displayed a relationship between IC<sub>50</sub> value and total phenolic concentration values (R<sup>2</sup>= 0.294; P = <0.017) (see Figure 5.5).



**Figure 5.3.** *Vaccinium myrtillus* and *V. poasanum* inhibition of advanced glycation endproducts. (A) The wild *V. myrtillus* (BBE 42) exhibits 5.44  $IC_{50}$  ( $R^2 = 0.903$ ) and (B) the common garden *V. poasanum* (VPO11) exhibits a 7.62  $IC_{50}$  ( $R^2 = 0.953$ ). The positive control, quercetin, gave 90% inhibition.



**Figure 5.4.** A linear regression (with 95% confidence intervals) testing the relationship between latitudinal genetic origins (Latitude) and  $IC_{50}$  of garden *Vaccinium* spp. (minus one *V. barandanum* outlier) ( $R^2 = 0.314$ ;  $P < 0.0371$ ).



**Figure 5.5.** A linear regression with 95% confidence intervals of wild *Vaccinium* samples from Canada, showing the relationship between mean total phenolics and  $IC_{50}$  ( $R^2$  square = 0.294;  $P = 0.0165$ ).

### ***Geographical information system***

The values extracted for each bioclim variable at each of the specimen localities demonstrate important bioclimatic differences between the wild and the garden *Vaccinium* spp. groups. For example, the original localities of garden species were found to have a higher annual mean temperature (Bio1) and higher annual precipitation (Bio12) when compared with wild species. Conversely, the localities of wild species experienced lower minimum temperatures in the coldest month (Bio6) and higher annual and diurnal temperature ranges (Bio2 and Bio7) when compared with the garden species, which are located at latitudes closer to the equator.

The Kolmogorov-Smirnov Z test found significant differences in climatic parameters for the locations of wild and garden species for all bioclim variables except precipitation of the coldest quarter.

**Table 5.3.** Average values for wild and garden specimen localities for each of the 19 bioclim variables. Temperature = (°C \* 10), precipitation = mm. Z= results of Kolmogorov-Smirnov Test comparing wild and garden specimens.

<b>Variable</b>	<b>Wild</b>	<b>Garden</b>	<b>Z</b>	<b>P</b>
BIO1 = Annual Mean Temperature	-13	163	2.851	***
BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp))	92	84	1.719	**
BIO3 = Isothermality (BIO2/BIO7) (* 100)	24	59	2.986	***
BIO4 = Temperature Seasonality (standard deviation *100)	9990	2438	2.787	***
BIO5 = Max Temperature of Warmest Month	205	235	1.394	*
BIO6 = Min Temperature of Coldest Month	-180	80	2.986	***
BIO7 = Temperature Annual Range (BIO5-BIO6)	385	155	2.787	***
BIO8 = Mean Temperature of Wettest Quarter	123	186	1.575	*
BIO9 = Mean Temperature of Driest Quarter	-74	139	2.986	***
BIO10 = Mean Temperature of Warmest Quarter	139	190	1.439	*
BIO11 = Mean Temperature of Coldest Quarter	-120	129	2.986	***
BIO12 = Annual Precipitation	1158	2239	2.787	***
BIO13 = Precipitation of Wettest Month	118	383	2.986	***
BIO14 = Precipitation of Driest Month	71	33	2.588	***
BIO15 = Precipitation Seasonality (Coefficient of Variation)	15	65	2.986	***
BIO16 = Precipitation of Wettest Quarter	34	1021	2.986	***
BIO17 = Precipitation of Driest Quarter	232	136	2.045	***
BIO18 = Precipitation of Warmest Quarter	331	799	2.986	***
BIO19 = Precipitation of Coldest Quarter	257	197	1.195	ns

\*\*\* p<0.001, \*\* P<0.01, \* P<0.05, NS not significant.

## Discussion

The inhibition of AGE formation is comparable to that previously reported for *Vaccinium angustifolium*, and considerably higher than for *Panax quinquefolius* (Ginseng), plants with reported anti-diabetic activity (McIntyre et al. 2009). Our results confirm that all species tested in the genus *Vaccinium* are a significant source of anti-glycation agents.

### ***Common garden Vaccinium samples***

All tropical/subtropical *Vaccinium* species grown in the Harriet Irving Botanical Garden showed higher activity than northern species except for the wild *V. myrtillus* from Bosnia and Herzegovina, which had the second highest activity overall. Tropical species had greater mean phenolic content in their leaves than wild species and the antioxidant properties of phenolics are known to inhibit the formation of AGEs (Farsi et al 2008). If one is to make definitive conclusions about the activity of the two groups, the northern and southern species should be compared using plants which all come from a common garden. Unfortunately this was not possible, due to the limited time available for the present research. However, compared to the northern wild collections the common garden reduced environmental variability and the tropical plants grown there had a lower range of variation of IC<sub>50</sub> and phenolic content.

Tropical *Vaccinium* species often grow at high altitudes with high UV irradiances. As a genetic adaptation to these environments, these species appear to have high phenolic production, possibly to avoid photo-oxidant damage by the antioxidant properties of phenolics. For example, in other Ericaceae, Rapinski et al. (2011) found a relationship between phenolic concentrations and mean July solar radiation. The high AGE inhibition of the *Vaccinium* species is also related to their high phenolic content. The garden group displayed significant relationships between both phenolic content or activity and their latitude of genetic origin. Latitude is not a parameter affecting plant physiology or genetic adaptation directly, but latitudinal variation in climatic parameters affecting plant growth are likely primarily responsible for this correlation.

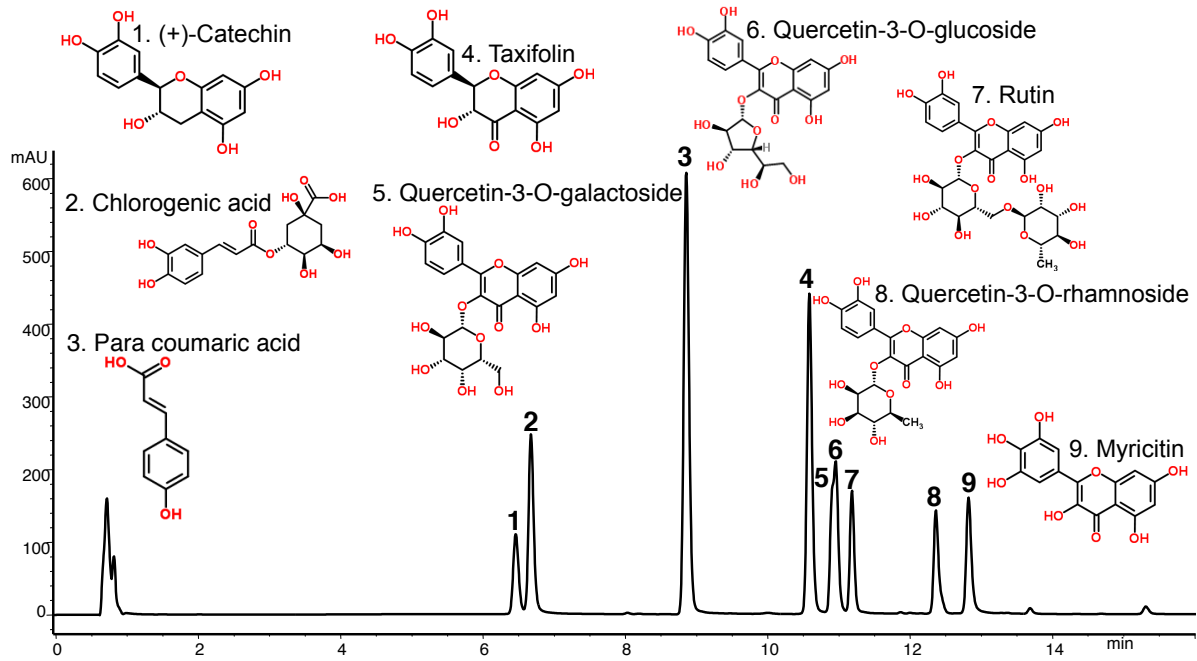
### ***Wild Vaccinium samples***

*Vaccinium myrtillus*, the most active wild species in terms of AGE formation inhibition, grows across Eurasia to the west coast of North America, had the highest phenolic content from the wild group. The wild group of *Vaccinium* had greater variability than the common garden group. The wild group also displayed a significant relationship of IC<sub>50</sub> with phenolic content across species not seen in the common garden group.

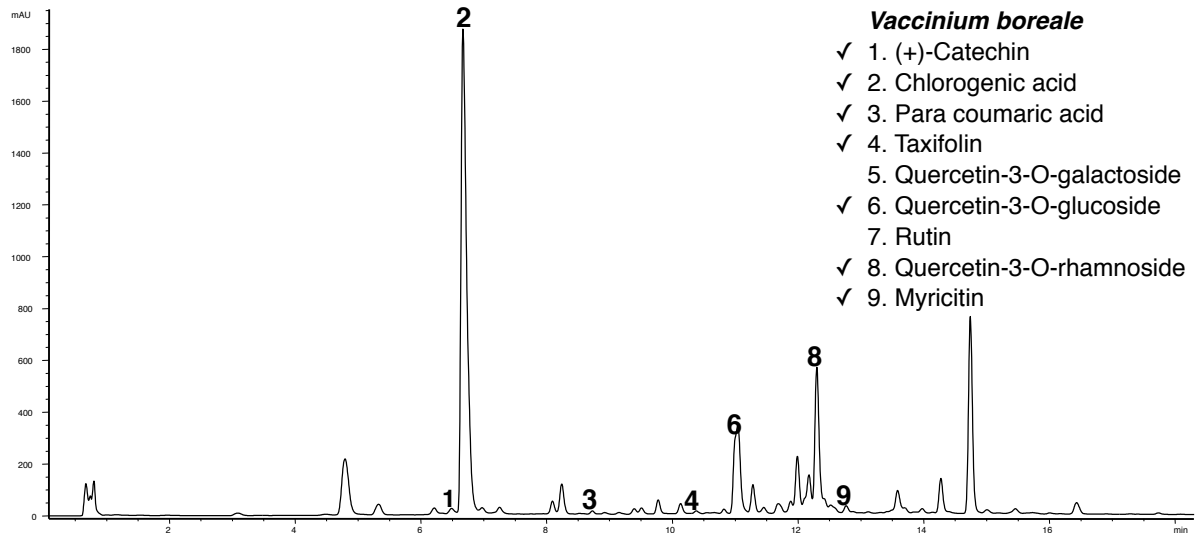
*Vaccinium species* have a long history of use among First Peoples (Moerman 2009). Traditional use suggests they are also very safe for consumption. This report shows that most species have the potential to reduce complication in type two diabetes. The genus clearly merits further pharmacological and phytochemical study.

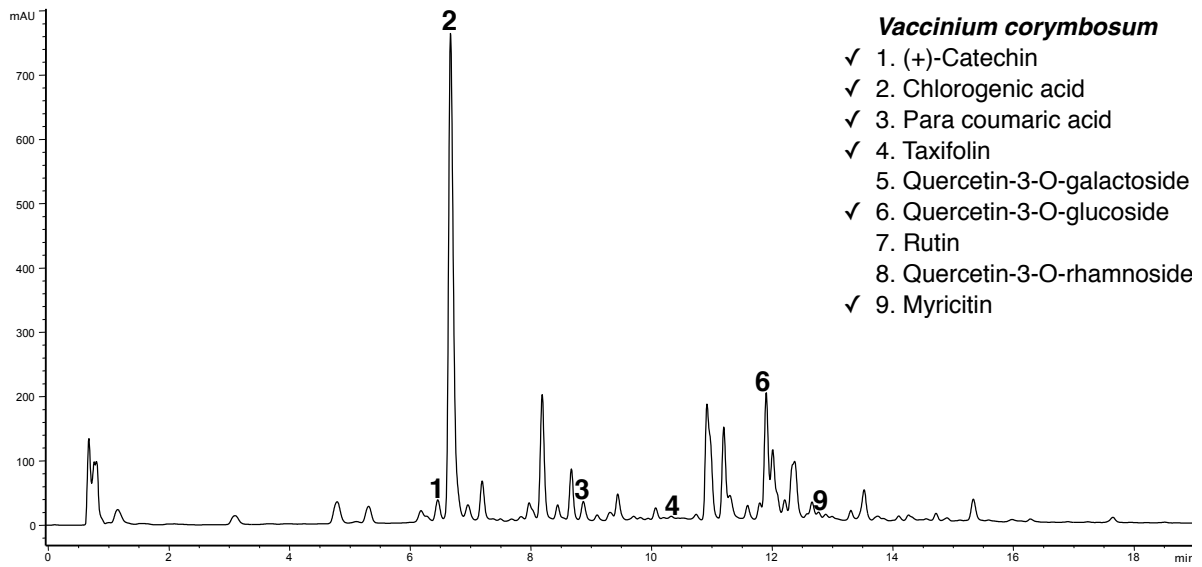
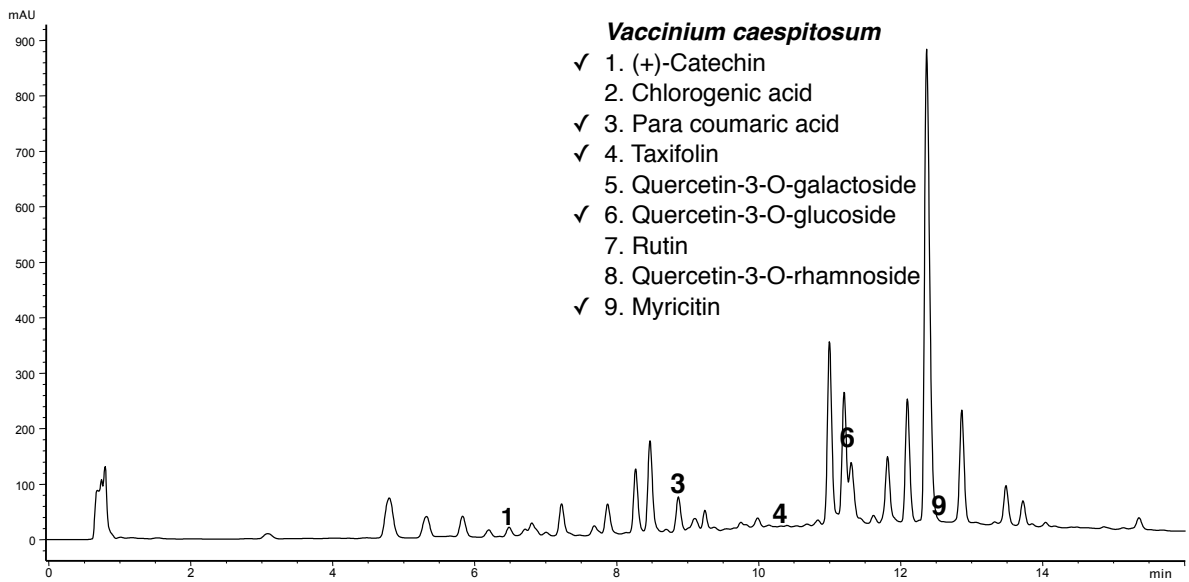
## Supplementary Figures

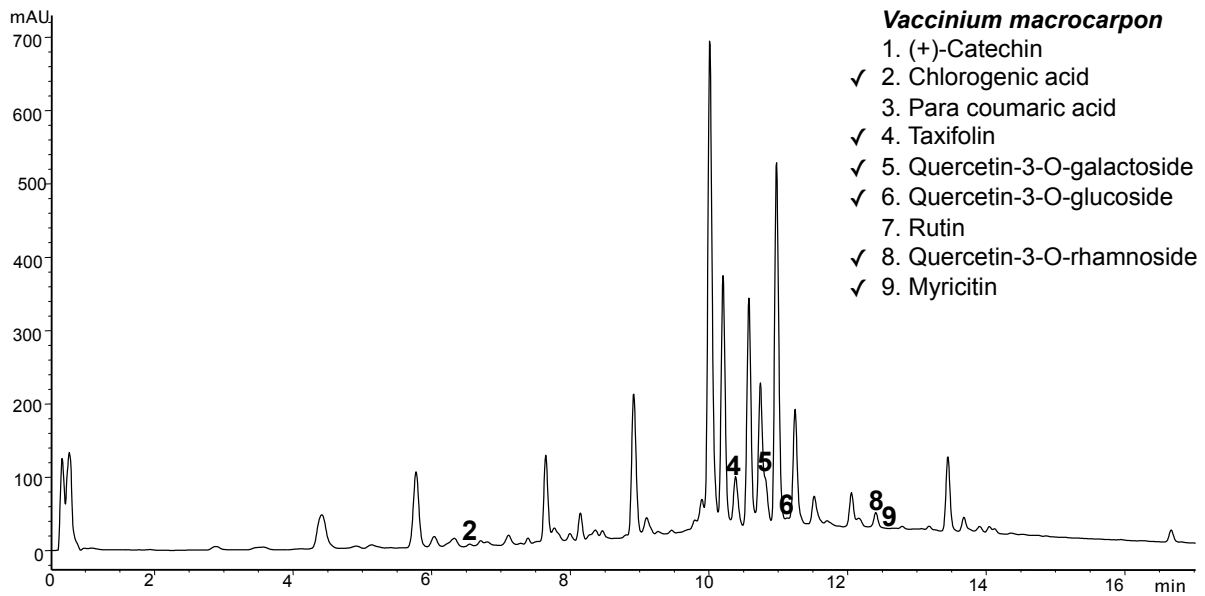
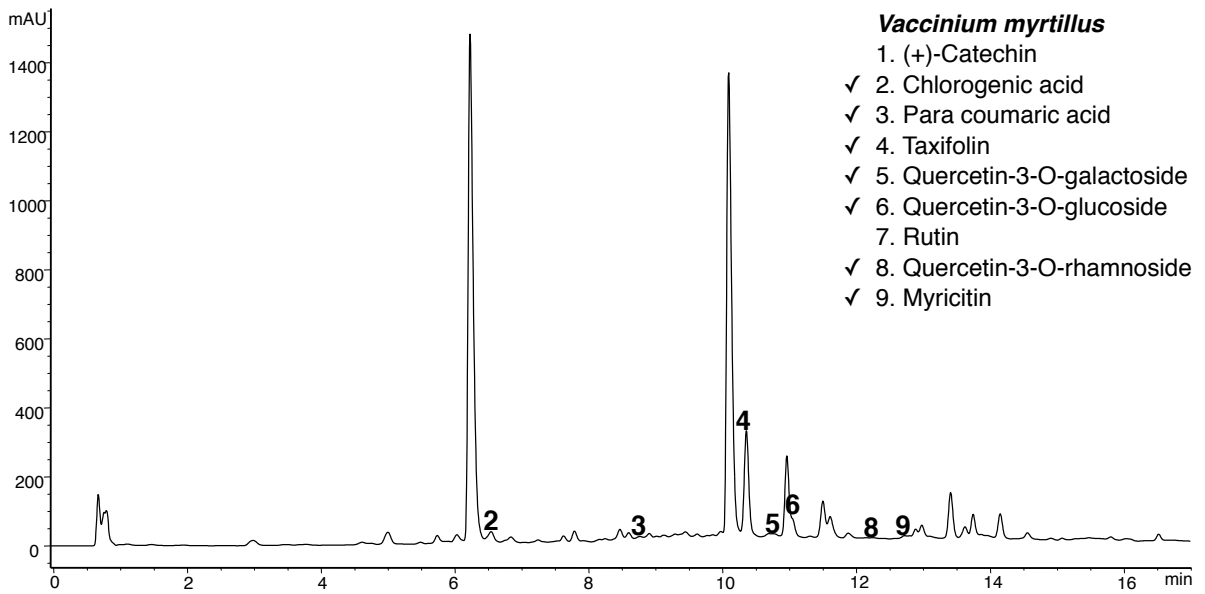
**A**

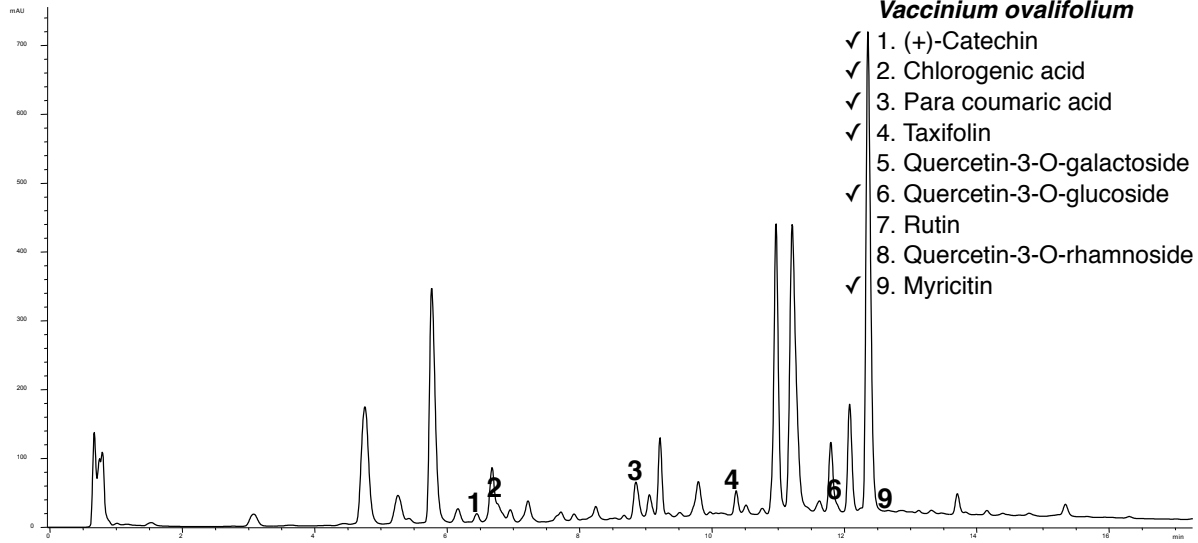
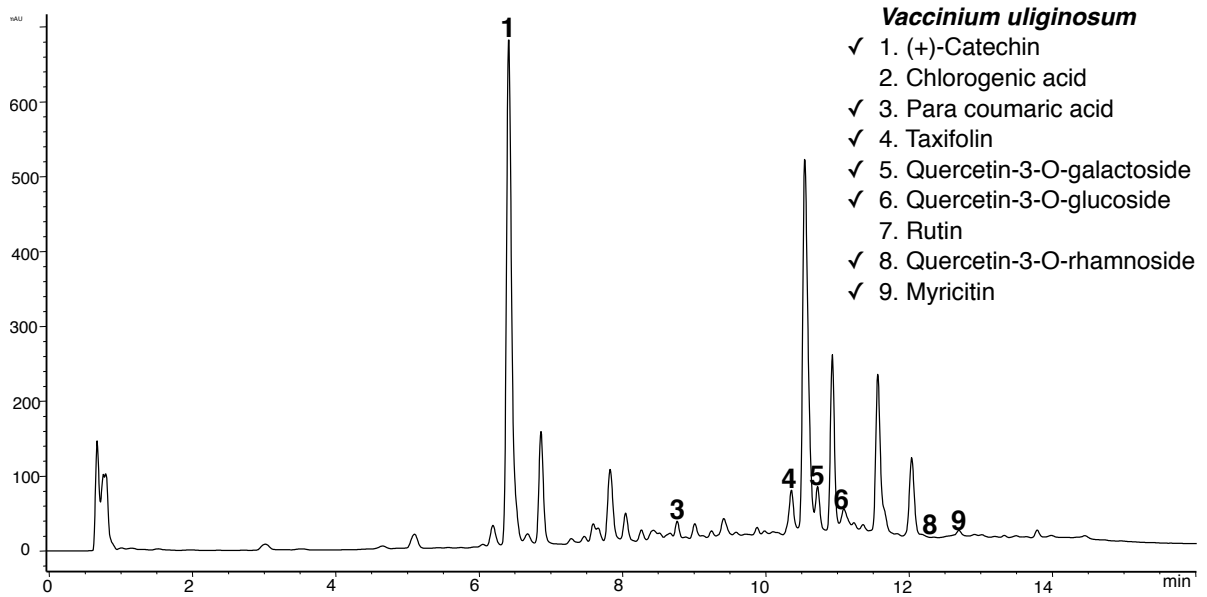


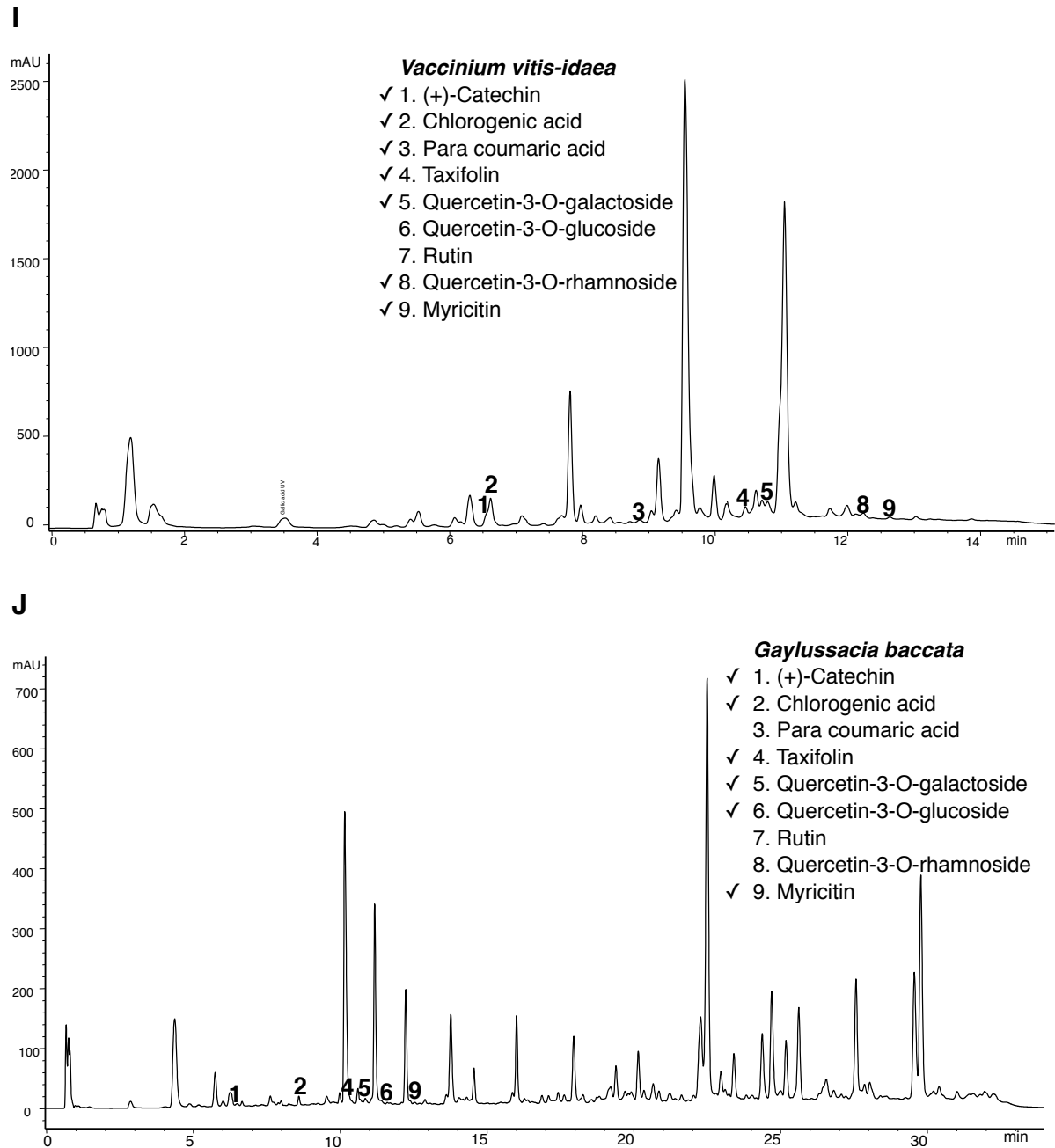
**B**



**C****D**

**E****F**

**G****H**



**Figure 5.S1.** HPLC-MS chromatograms of *Vaccinium* species and outgroup *Gaylussacia baccata* marker compounds.

**Table 5.3.** Presence of metabolite standards in *Vaccinium* species and outgroup *Gaylussacia baccata*

<b>Genus species</b>	<b>(+)-Catechin</b>	<b>Chlorogenic acid</b>	<b>P coumeric acid</b>	<b>Taxifolin</b>	<b>Quercetin</b>	<b>Quercetin</b>	<b>Rutin</b>	<b>Quercetin-3-O-rhamnoside</b>	<b>Myricetin</b>
<i>V. boreale</i>	x	x	x	x		x		x	x
<i>V. corymbosum</i>	x	x	x	x		x			x
<i>V. caespitosum</i>	x		x	x		x			x
<i>V. macrocarpon</i>		x		x	x	x		x	x
<i>V. myrtilus</i>		x		x	x	x		x	x
<i>V. ovalifolium</i>	x	x		x		x			x
<i>V. uliginosum</i>	x		x	x		x		x	x
<i>V. vitis-idaea</i>	x	x	x	x	x	x		x	x
<i>G. baccata</i>	x			x	x				x

## Chapter 6

# Taxonomic Nuclear Magnetic Resonance (NMR) Metabolomics: “Leafprints”<sup>7</sup> for Systematics and Medicinal Quality Control of *Vaccinium* L.

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5. Montreal Botanical Garden, Montreal, Canada.

**Field identification:** David Garbary, John Peter, Neville Crabbe, Nick Harris, Chieu Anh Tah, Alex McCalvay, Al von Finster

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<sup>7</sup> First time in print, to my knowledge

## **Taxonomic Nuclear Magnetic Resonance (NMR) Metabolomics: “Leafprints” for Systematics and Medicinal Quality Control of *Vaccinium* L.**

The genus *Vaccinium* L. (Ericaceae) includes a number of important and taxonomically interesting species of bilberries, blueberries, cranberries, deerberries, huckleberries, and whortleberries. Ericaceae origins have been linked to Laurasia (Kron and Luteyn 2005) with a high density of *Vaccinium* species in China (92 species, 51 endemic) (Stevens 2005). Vander Kloet (2009) estimated that there are approximately 500 species of *Vaccinium*, with 40% of species native to Southeast Asia, 25% to North America, 10% to South and Central America, and the remaining 25% scattered globally (Luby et al. 1991; Song & Hancock 2011). The Plant List (2013) records 171 accepted species, 185 synonyms, and 570 unresolved species. The boreal region of Canada and adjacent U.S.A. host 17 species of *Vaccinium* including *V. boreale* I.V. Hall & Alders and *V. uliginosum* L. which are becoming increasingly rare in NS, Canada due to climate warming (Scoggan 1978; Ferrier et al. 2010).

*Vaccinium* species' leaves are used in many North American and Eurasian aboriginal medical applications as diaphoretics, ineberratives, gastrointestinal aids, gynaecological aids, panacea, and as additives in ceremonial smudges and smoking mixtures known as *kinikinik* (combined with *Arctostaphylos uva-ursi* (L.) Spreng.) (Arnason et al. 1981; Moerman 1998; Pahlow 1993; Redžić 2007; Ferrier et al. 2012). *Vaccinium* species have many current and potential applications in complementary medicine and therapeutic research in both aboriginal and non-aboriginal populations. *Vaccinium* spp. leaves are also widely used among traditional medicine practitioners in Quebec, Canada (Haddad 2003). However, with many sympatric species, and *Vaccinium*'s notorious potential for hybridization, the possibility of misidentification in taxonomy and natural health product (NHP) production is high, especially for sterile samples or bulk collections and powdered samples.

Substituted plant species have created toxic natural health products (NHPs) for sale on the marketplace (Awang 1991) with recent examples occurring with an adulteration of Black cohosh (*Cimicifuga racemosa* (L.) Nutt., syn. *Actaea racemosa* L., Ranunculaceae) with an Asian species of *Actaea* (Jiang et al. 2006). These circumstances demonstrate the

need for recognizing the metabolomic “leafprint” of NHPs, quickly and reliably, on a lot to lot basis. From a taxonomic perspective, chemometric data on this diverse genus is desirable for total evidence approaches. Data from an increased number of operational taxonomic units (OTUs) from new approaches in ‘metric’ and ‘omic’ analyses is necessary in incontrovertibly describing the evolutionary history and relationships of taxa.

The following  $^1\text{H}$  NMR approach is a high resolution, non-destructive, structurally definitive technique that can provide rapid, accurate quantitative and qualitative assessment of a mixture’s phytochemical profile. By analyzing the fine structure of  $^1\text{H}$  NMR *Vaccinium* spp. spectra, we predicted that these spectra could be utilized to create a standard metabolomic leafprint region for comparison and determination purposes. Our objective in this study was to develop a rapid leafprint methodology to discriminate between subgeneric sections, species and their source habitats.

## Methods

### *Wild Vaccinium samples*

Samples of wild *Vaccinium* leaf material was harvested between May and October of 2006-2012 (Table 6.1) by J.F., A.C. S.T., D.G., A.M., C.T., A.B., and N.C.. North American specimens were obtained between latitudes N42 & N52 and can be found from Greenland to California (Vander Kloet 2009). European samples *Vaccinium myrtillus*, *V. vitis-idaea*, and *Arctostaphylos uva-ursi* samples were collected from Bosnia and Herzegovina, Croatia, and Montenegro between latitudes N42 & N52, and grow from Iceland to Turkey, reaching China and Western Canada (Scoggan 1978, Tutin et al. 1972, Stevens 1972) (Table 6.1). Many wild *Vaccinium* samples herein have a circumboreal distribution and are found throughout acidic bogs and heathlands, boreal and coastal forests, exposed mountain slopes, extending north into arctic tundra regions, and south into mixed deciduous and conifer forest regions. Leaves were collected in paper bags, and tobacco offerings were made according to Algonquin custom. Each sample was dried overnight at 37°C and stored at room temperature prior to a standardized extraction procedure.

### ***Garden Vaccinium samples***

#### Harriet Irving Botanical Gardens at Acadia University, Wolfville, NS, Canada (ACAD)

We were very fortunate to have access to the late Sam Vander Kloet's global collection of *Vaccinium* specimens (Garbary 2012). Leaf samples were collected by J.F. and identified by Sam Vander Kloet on Sept. 30, 2009 (Table 6.1). *Disterigma rimbachii* (A.C. Sm.) Luteyn was available at ACAD and selected as a common garden Ericaceae outgroup. *D. rimbachii* was used as a putative metabolic outlier when compared to other *Vaccinium* spp. in a principal component analysis.

Sam Vander Kloet's specimens were propagated from collections of wild seeds found in their geographic origins (Table 6.1). All ACAD specimens grow together under common environmental conditions at the Harriet Irving Botanical Gardens (45.08686, -64.3681). All common garden specimens grew outdoors during the spring, summer, and autumn, and winter, except for those samples indicated by a dagger † in Table 6.1 which were moved into a climate controlled greenhouse for the winter. All collections were made from healthy aboveground foliage from plants that were older than 10 years. Leaves were collected in paper bags and tobacco offerings were made according to Algonquian custom. Each sample was dried overnight at 37 ° C and stored at room temperature prior to a standardized extraction procedure.

#### The New York Botanical Garden, Bronx, New York, U.S.A. (NY)

We were also very fortunate to have access to New York Botanical Garden global collection of *Vaccinium* specimens. *Vaccinium* spp. leaf samples were collected by J.F. and J.P. on May 30, 2012 (Table 6.1). NYBG's specimens were propagated from collections of wild seeds found in their geographic origins (Table 6.1). All NYBG's specimens grow together under common environmental conditions (40.8636 N 73.8783 W). All NYBG specimens grew outdoors during the spring, summer, autumn, and winter, except for those indicated with a double dagger (‡) in Table 6.1, which grew in a climate controlled greenhouse year round. All collections were made from healthy aboveground foliage from plants that were 10 years of age or older. Leaves were collected in paper bags, and tobacco

offerings were made according to First Nations' custom. Each sample was dried overnight at 37 °C and stored at room temperature prior to a standardized extraction procedure.

### ***Sample preparation and extraction***

All leaves were prepared and extracted using the following standardized method. Dried leaf samples (except for asterisks \* in Table 1 which were extracted fresh) were ground using a Wiley Mill (Thomas Scientific, Swedesboro, NJ, U.S.A.) and passed through a 1 mm mesh. Plant material was extracted twice in 95% EtOH, 5% H<sub>2</sub>O (1 g · 10 mL<sup>-1</sup>) at room temperature for 24 h per extraction. The recovered extractions were pooled and dried using a rotary evaporator (Savant Speed-Vac) at room temperature for 24 h to remove EtOH. Extracts were then placed in a freezer for 2 h at -20°C before lyophilization at -40 °C for 24 h to remove water from the extract. Lyophilization yielded between 0.10 and 0.21 g crude extract·g dried leaf<sup>-1</sup>. Extracts were stored at -20 °C.

### ***<sup>1</sup>H NMR***

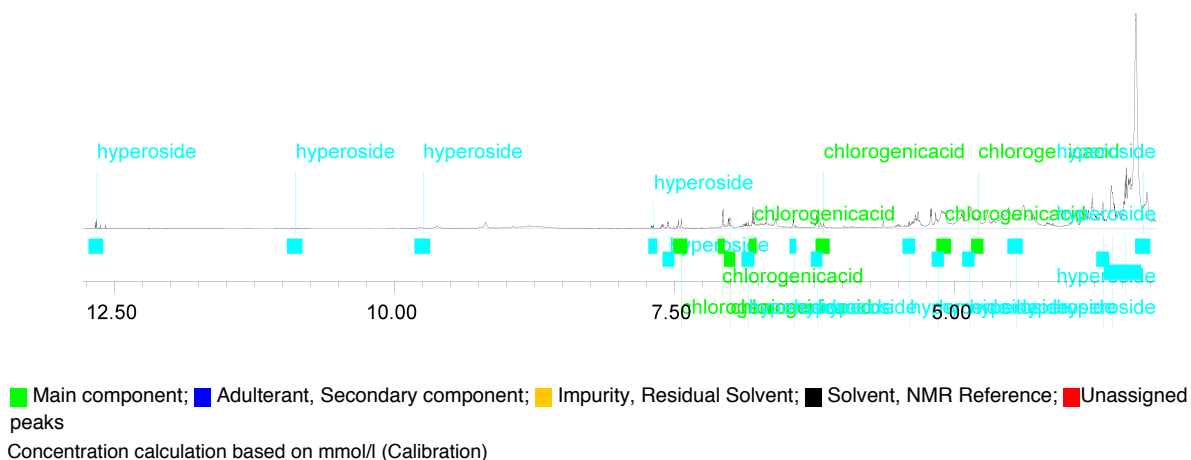
Twenty-five mg of each crude extract was dissolved in 1.0 mL of DMSO-d<sub>6</sub>. Six hundred µL of supernatant was added to a 5 mm <sup>1</sup>H NMR tube with 1 microliter of 300 mM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid). <sup>1</sup>H NMR spectra were recorded using a 600 MHz Bruker AVANCE III Spectrometer equipped with a 5 mm TCI CryoProbe. <sup>1</sup>H NMR spectra were referenced to DSS at 0.0 ppm to calibrate the chemical shift scale. This signal was also used as an internal quantitative reference standard. Standard 1D NOESY and 1D CPMG spectra were acquired at 298 K for each sample. Pulse width variation between samples was accounted for by performing an automated pulse calibration prior to acquisition on each sample. <sup>1</sup>H NMR data were processed and baseline corrected using TopSpin 3.1 (Bruker BioSpin, Billerica, MA, U.S.A.). Statistical analysis was performed with AMIX (Bruker BioSpin, Billerica, MA, U.S.A.). Variation occurring in our method and instrument was reported in a recent single laboratory validation publication (Hicks et al. 2012). Any results obtained here should be reproducible in any laboratory with different equipment and operators as demonstrated in our multilab publication (Chapter 8), as compared to HPLC-MS-MS analysis which is more difficult to reproduce.

## Principal component analysis of leaf spectra

Principal Component analysis (PCA) of leaf spectra was conducted using the AMIX™ software, version 3.9.14 (Bruker BioSpin, Billerica, MA) with rectangular bucketing and Pareto scaling. Spectra were aligned by the DSS signal before bucketing and baselines were corrected. The spectra were divided into buckets ( $n = 800$ ) in the chemical shift region from 8.40 to 0.40 ppm with a bucket width of 0.01 ppm. Exclusions were made in the regions of peaks for DMSO (2.66-2.40 ppm), water (3.42-3.23 ppm), ethanol (1.23-0.93, 3.52-3.42, and 4.47-4.31 ppm), methanol (3.23-3.17 ppm), and DSS (0.125 to -0.125 ppm).

## Results and Discussion

This  $^1\text{H}$  NMR methodology for sample identity analysis was found to be extremely time and cost efficient with minimal sample handling. The entire  $^1\text{H}$  NMR acquisition time took approximately 7 minutes 40 seconds per sample. Representative spectra from *Vaccinium* samples are shown in Figure 6.1. Since  $^1\text{H}$  NMR does not destroy the sample, the sample is available for bioactivity and pharmacology studies after chemometric analysis.



Compound	Category	Mean	Std.Dev.	Status
chlorogenicacid	Main component	5.04 mmol/l	0.00	quantified
hyperoside	Secondary component	1.22 mmol/l	0.00	quantified

**Figure 6.1.** Representative 600MHz  $^1\text{H}$  1D NOESY NMR spectrum of *Vaccinium angustifolium* (Extract 591) with Assure-raw material quantification of the main component chlorogenic acid and a secondary component hyperoside.

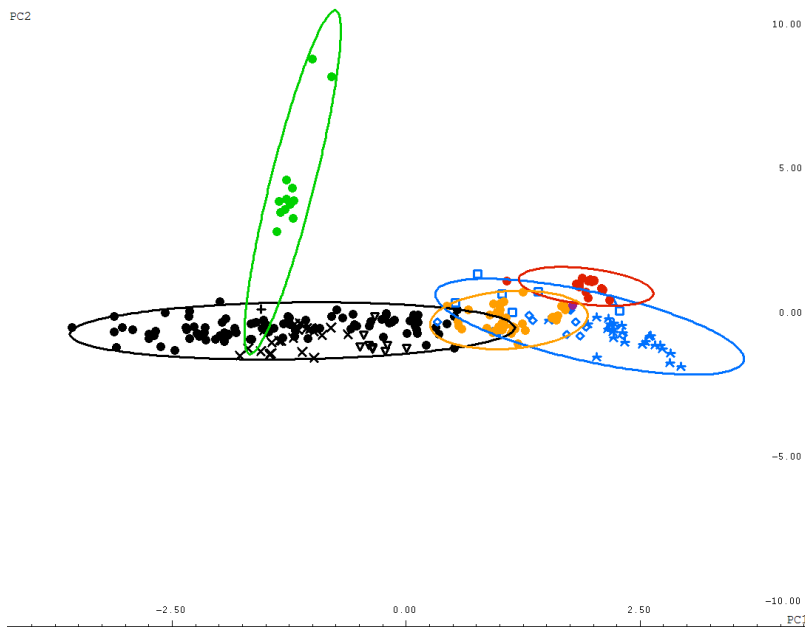
### ***Generic and subgeneric identification***

Our first principal component analysis tested whether the method could separate genera (Figure 6.2). Here, the outgroup *Gaylussacia baccata* (green) clearly separates from all other samples. This PCA also tested if the method could group the *Vaccinium* samples into their subgeneric sections and separate the sections as described by Odell & Vander Kloet (1989), Kron et al. (2002), Vander Kloet (2004), Vander Kloet & Dickinson (2009), Ehlenfeldt & Ballington (2012). This analysis used wild *Vaccinium* leaf samples from different sections: *Cyanococcus* (*V. angustifolium* n = 88; *V. corymbosum* n = 23); *Myrtillus* (*V. caespitosum* n = 12; *V. myrtillus* n = 7; *V. ovalifolium* n = 24); *Oxycoccus* (*V. macrocarpon* n = 15, *V. oxycoccus* n = 1); *Vaccinium* (*V. uliginosum* n = 38), *Vitis-idaea* (*V. vitis-idaea* n = 1). The two dimensional PCA scores plot of these <sup>1</sup>H NMR spectra (Figure 6.2) indicate that sections *Cyanococcus*, *Oxycoccus*, and *Vitis-idaea* have no overlapping species.

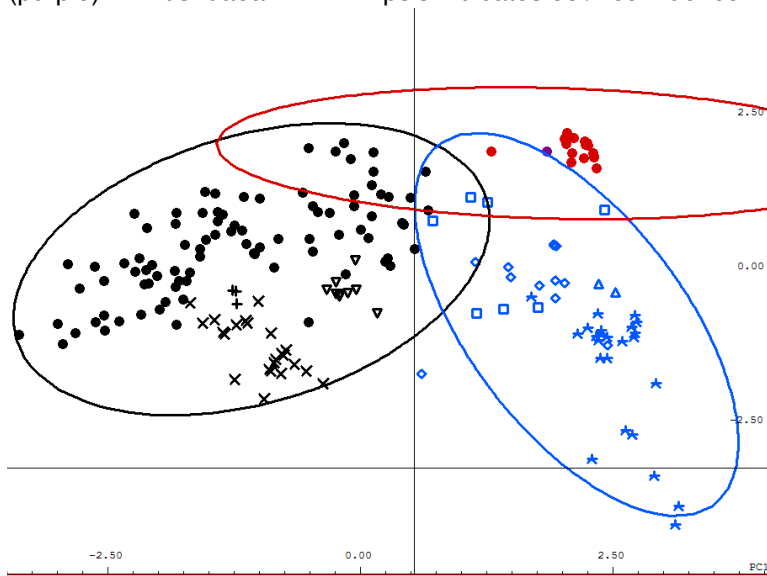
In Figure 6.2, sections *Vaccinium* (orange) and *Myrtillus* (blue), located between sections *Oxycoccus* (red) and *Cyanococcus* (black), had species samples in close proximity to neighbouring section species. This was especially true for *Vaccinium uliginosum* sect. *Vaccinium* which had samples overlapping with sect. *Cyanococcus*, *Myrtillus*, and *Vitis-idaea*. Section *Vaccinium* and *Myrtillus* 95% confidence ellipsis overlapped with all the sections except with the outgroup *Gaylussacia baccata*. The *V. uliginosum* metabolomic fingerprint was very similar to *V. angustifolium* sect. *Cyanococcus*, as well *V. caespitosum* and *V. myrtillus* sect. *Myrtillus*.

Section *Oxycoccus* (red) fringed upon sect. *Myrtillus* (blue) with one sample of *Vaccinium macrocarpon* sect. *Oxycoccus* beginning to eclipse *V. corymbosum*. One sample of *V. oxycoccus* appeared outside the confidence interval of sect. *Oxycoccus*. This was due to *V. oxycoccus* n = 1 and was appropriately represented as an outlier to the *V. macrocarpon* dominated section in the PCA. The confidence ellipsis of sect. *Oxycoccus* overlapped less with sect. *Vaccinium* and more with sect. *Myrtillus*.

These results discriminate according to the subgeneric circumscriptions of species within *Cyanococcus*, *Myrtillus*, *Oxycoccus*, and *Vitis-idaea* (albeit n = 1). However, the results indicate that a section versus section comparison is more suitable as in Figure 6.3.



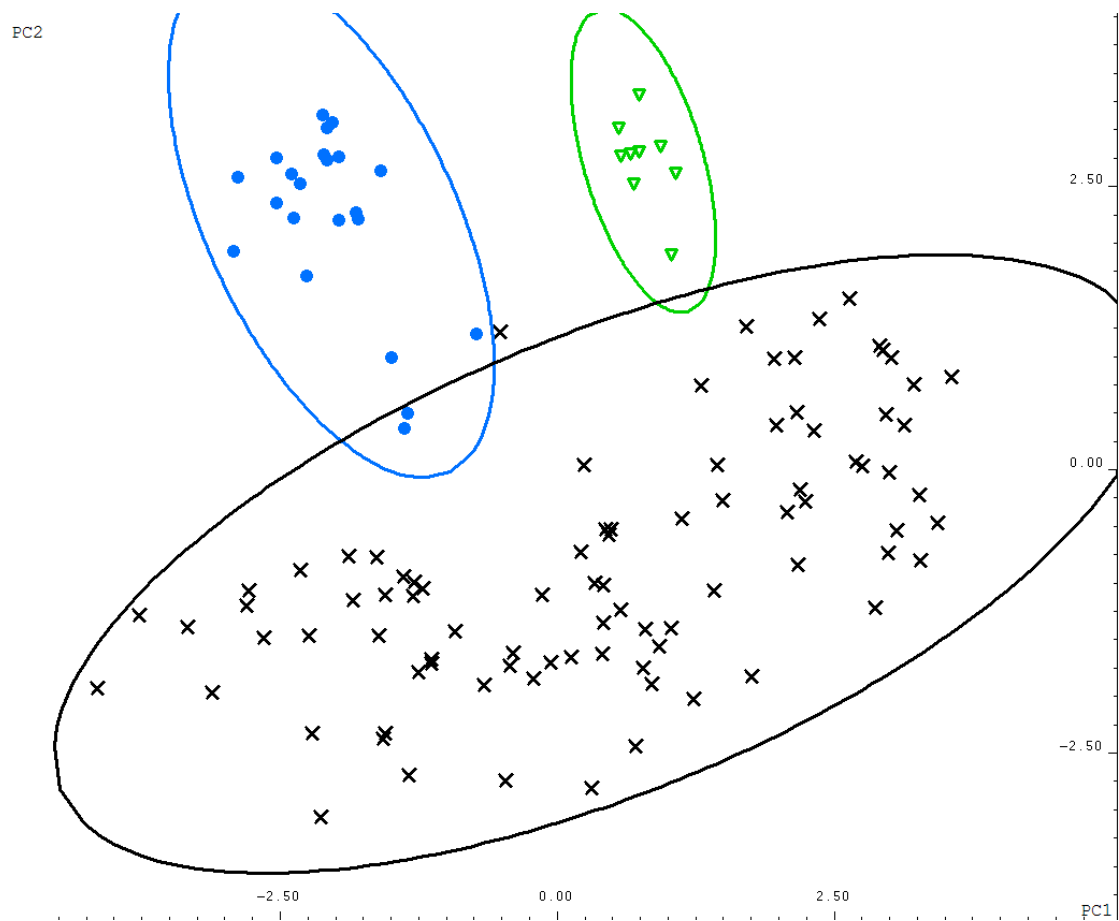
**Figure 6.2.** Identification of genera and subgenera by principal component analysis. Principal component analysis of outgroup *Gaylussacia baccata* (n = 13) and wild *Vaccinium* spp. leaf samples from sections *Cyanococcus* (black): *V. angustifolium* n = 88, *V. corymbosum* n = 23, *V. boreale* n = 8; *Myrtillus* (blue): *V. caespitosum* n = 12, *V. myrtillus* n = 7, *V. ovalifolium* n = 24; *Oxycoccus* (red): *V. macrocarpon* n = 15, *V. oxycoccus* n = 1; *Vaccinium* (orange): *V. uliginosum* n = 38; *Vitis-idaea* (purple): *V. vitis-idaea* n = 1. Ellipsis indicates 95% confidence interval.



**Figure 6.3.** Resolution of sections *Cyanococcus* (black), *Myrtillus* (blue), and *Oxycoccus* (red). Sample *Vaccinium vitis-idaea* (purple, n = 1) of section *Vitis-idaea* appears in closer proximity to *V. macrocarpon* than *V. oxycoccus* (n = 1).

### *Specific identification*

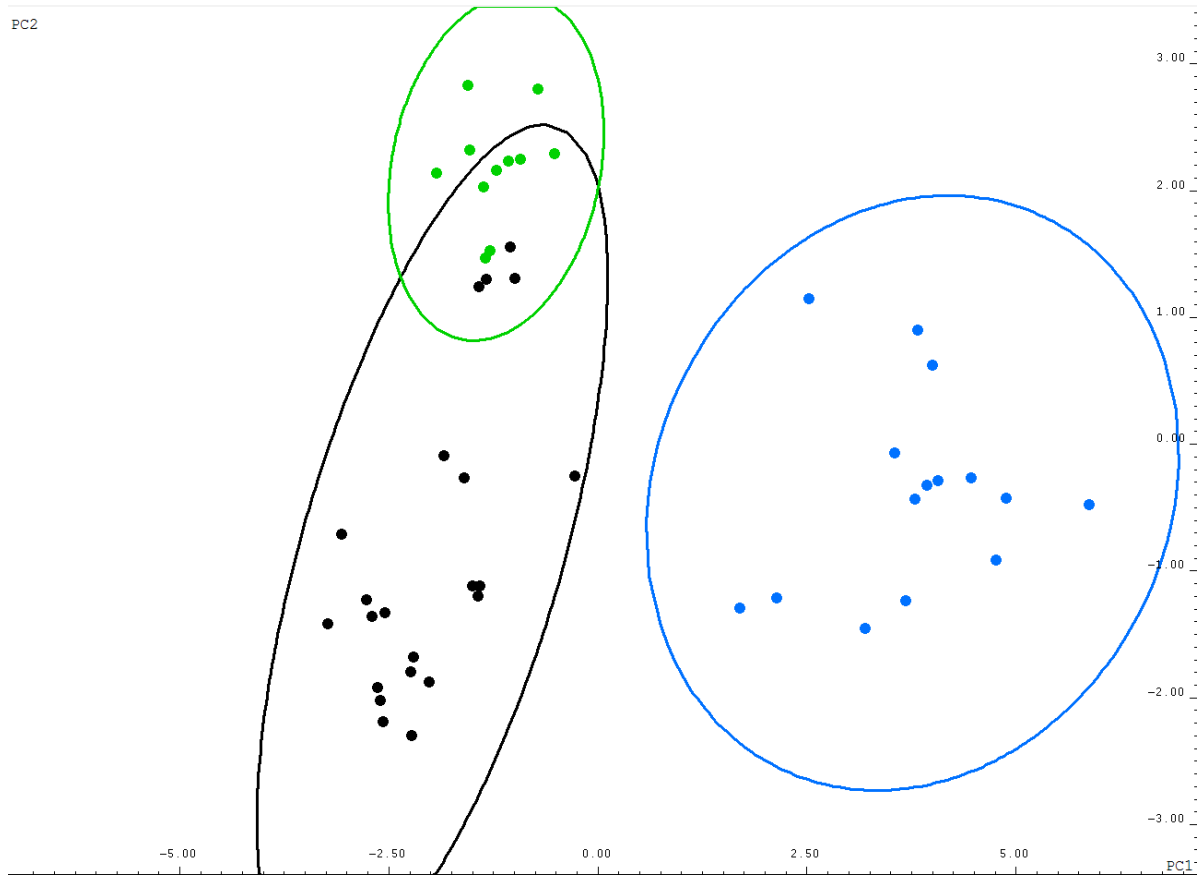
Next, a specific analysis of wild leaf samples from the sect. *Cyanococcus* to determine if highly related species from the same section could be separated (Figure 6.4). A clear separation was observed in the PCA scores plot of *Vaccinium angustifolium* (n = 88), *V. corymbosum* (n = 21), and *V. boreale* (n = 9) leaf samples. Hotelling ellipses at the 95% confidence level indicate the degree of overlap between the groups. Figure 6.4 indicates that this leafprint method is able to discriminate between highly related *Vaccinium* species of the same section. Identification of highly related taxa is important for taxonomists, agriculturalists, and the NHP industry since original formula specimens and adulterants often share a common name or are related species with the adulterants having undesirable or toxic effects (Awang 1991; Jiang et al 2006).



**Figure 6.4.** Identification of species in section *Cyanococcus*. Principal component analysis of wild *Vaccinium* samples from section *Cyanococcus* including: *Vaccinium angustifolium* (black x's, n = 88), *V. corymbosum* (blue dots, n = 21), *V. boreale* (green triangles, n = 9) leaf samples.

### *Phenotypic identification*

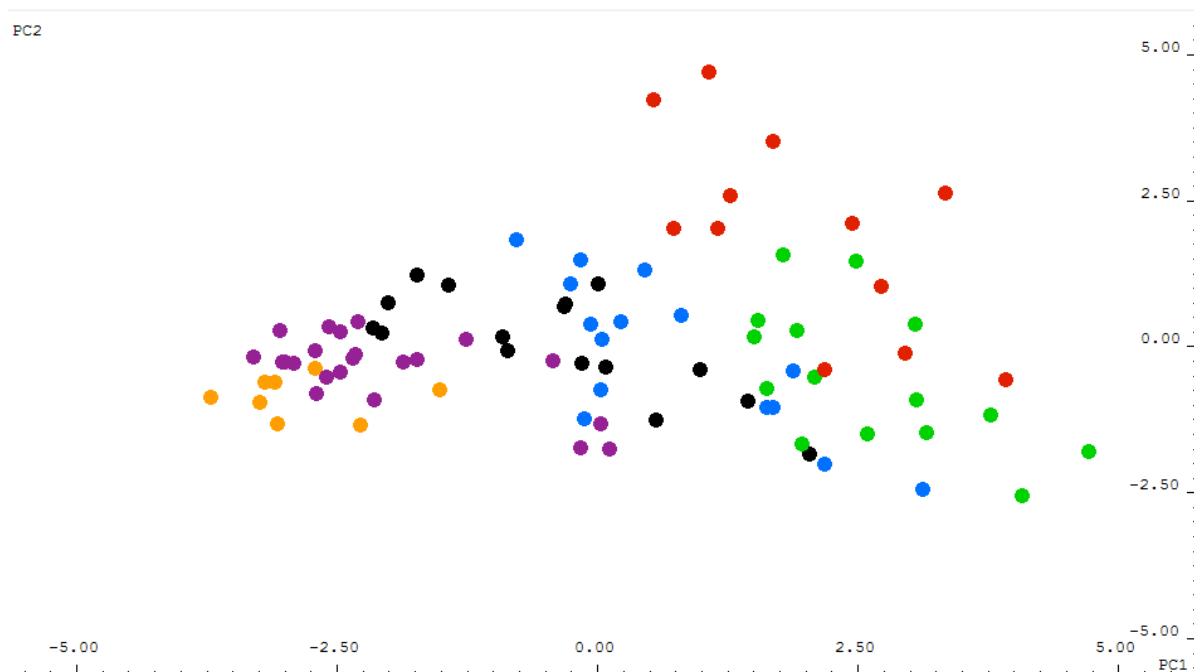
We then reduced the level of taxonomic analysis to a single species and tested whether the method could discriminate between phenotypes of *Vaccinium corymbosum*. Figure 6.5 shows a PCA scores plot from wild and garden *V. corymbosum* samples. Wild samples (n = 22), and garden samples from NYBG (n = 11) and ACAD (n = 15) separate clearly. Figure 6.4 shows that NHPs from various locations must be carefully considered since the phytochemistry of a species changes with location and therefore affects NHP constitution and thus quality. Yuk et al. (2013ab) were also able to show separation of the metabolome when studying highly related landraces of *Panax quinquefolius* L. (American ginseng).



**Figure 6.5.** Principal component analysis of wild and garden *Vaccinium corymbosum* samples. Wild *V. corymbosum* samples are black (n = 22), and garden *V. corymbosum* samples from NY are green (n = 11) and ACAD blue (n = 15).

### ***Seasonal variation of Vaccinium angustifolium***

Finally, to test our method's ability to display a trend in the seasonal variation of leaf metabolomes, we conducted a PCA of *Vaccinium angustifolium* samples collected in May (n = 12), June (n = 15), July (n = 15), August (n = 16), September (n = 20), and October (n = 8) as shown in Figure 6.6. Collections gradually shift and overlap from month to month moving from left (May) to right (October) as the growing season begins and ends. Our results are similar to those of McIntyre et al. (2009) who reported a seasonal decline in chlorogenic acid, rutin (quercetin-3-O-rutinoside), and quercetin 3-arabinoside in leaves, and, a significantly greater advanced glycation endproduct inhibition activity at the end of the growing season.



**Figure 6.6.** Principal component analysis of *Vaccinium angustifolium* samples collected in May (red, n = 12), June (green, n = 15), July (blue, n = 15), August (black, n = 16), September (purple n = 20), and October (orange n = 8).

Although we were successful in differentiating major sections relevant to North America our attempts to simultaneously differentiate 14 subgeneric sections were less promising. These findings are important to learn which sections are closely related to develop future methods for identifying these taxa. Discriminant analysis to identify section biomarkers may allow discrimination using this <sup>1</sup>H NMR profiling technique in the future.

Again, collection times should be considered in <sup>1</sup>H NMR taxonomic applications and by NHP manufacturers when producing NHPs for human consumption.

Our objective was to develop a rapid subgeneric plant identification method based on <sup>1</sup>H NMR metabolomic leafprints. We were able to distinguish between metabolomic leafprints for identification of subgeneric sections, species, and phenotypes for numerous *Vaccinium* taxa. This allows the analytical chemist to have a taxonomic and phenotypic level of discrimination with raw, extracted, powdered, or other morphologically destroyed samples and does not require field visitation. This is also a rapid and useful tool for distinguishing between specimens which, at the time of collection, are without reproductive parts or other operational taxonomic units necessary for morphological taxonomy, or sample groups which are not well delineated or exceedingly difficult to identify, or easily confused (for example Piperaceae spp.).

**Table 6.1.** *Vaccinium* spp. and outgroups.

Section	Genus	Species	Date	Type	Site	Prov/ State	Country	Extract #
-	<i>Arctostaphylos</i>	<i>uva-ursi</i> (L.) Spreng.	June 15, 06	Wild	Jahorina	-	Bosnia & Herzegovina	ARC 1
-	<i>Arctous</i>	<i>alpina</i> (L.) Nied.	Sept. 18, 09	Wild	Iqaluit	NU	Canada	ARC 2
-	<i>Arctous</i>	<i>alpina</i> (L.) Nied.	Sept. 18, 09	Wild	Iqaluit	NU	Canada	ARC 3
-	<i>Cavendishia</i>	sp.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	CAV 1
-	<i>Disterigma</i>	<i>rimbachii</i> (A.C.Sm.) Luteyn	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	629
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 1, 07	Wild	Parc de Frontenac	QC	Canada	GAY 13
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 1, 07	Wild	Parc de Frontenac	QC	Canada	GAY 3
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 1, 07	Wild	Parc de Frontenac	QC	Canada	GAY 7
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 6, 07	Wild	Parc d'Oka	QC	Canada	GAY 1
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 6, 07	Wild	Parc d'Oka	QC	Canada	GAY 10
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 6, 07	Wild	Parc d'Oka	QC	Canada	GAY 11
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 6, 07	Wild	Parc d'Oka	QC	Canada	GAY 12
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 6, 07	Wild	Parc d'Oka	QC	Canada	GAY 2
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 6, 07	Wild	Parc d'Oka	QC	Canada	GAY 4
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 6, 07	Wild	Parc d'Oka	QC	Canada	GAY 5
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 6, 07	Wild	Parc d'Oka	QC	Canada	GAY 6
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 6, 07	Wild	Parc d'Oka	QC	Canada	GAY 8
-	<i>Gaylussacia</i>	<i>baccata</i> (Wangenh.) K. Koch	June 6, 07	Wild	Parc d'Oka	QC	Canada	GAY 9
-	<i>Vaccinium</i>	[Polaris]	May 30, 12	Garden NY	Hardy	NY	USA	746
-	<i>Vaccinium</i>	[Polaris]	May 30, 12	Garden NY	Hardy	NY	USA	747
-	<i>Vaccinium</i>	[Rubel]	May 30, 12	Garden NY	Hardy	NY	USA	748

Section	Genus	Species	Date	Type	Site	Prov/ State	Country	Extract #
-	<i>Vaccinium</i>	[Rubel]	May 30, 12	Garden NY	Hardy	NY	USA	749
-	<i>Vaccinium</i>	sp.	May 30, 12	Garden NY	Hardy	NY	USA	757
-	<i>Vaccinium</i>	sp. [Northsky]	May 30, 12	Garden NY	Hardy	NY	USA	762
<i>Bracteata</i> Nakai	<i>Vaccinium</i>	<i>barandanum</i> S. Vidal	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	625
<i>Bracteata</i> Nakai	<i>Vaccinium</i>	<i>barandanum</i> S. Vidal	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	626
<i>Bracteata</i> Nakai	<i>Vaccinium</i>	<i>barandanum</i> S. Vidal	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	627
<i>Calcicolus</i> (Vander Kloet 2009)	<i>Vaccinium</i>	<i>gaultheriifolium</i> (Griff.) Hook. f. ex C.B. Clarke	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	723
<i>Calcicolus</i> (Vander Kloet 2009)	<i>Vaccinium</i>	<i>gaultheriifolium</i> (Griff.) Hook. f. ex C.B. Clarke	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	612
<i>Calcicolus</i> (Vander Kloet 2009)	<i>Vaccinium</i>	<i>gaultheriifolium</i> (Griff.) Hook. f. ex C.B. Clarke	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	613
<i>Calcicolus</i> (Vander Kloet 2009)	<i>Vaccinium</i>	<i>gaultheriifolium</i> (Griff.) Hook. f. ex C.B. Clarke	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	614
<i>Calcicolus</i> (Vander Kloet 2009)	<i>Vaccinium</i>	<i>gaultheriifolium</i> (Griff.) Hook. f. ex C.B. Clarke	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	615
<i>Conchophyllum</i> Sleumer	<i>Vaccinium</i>	<i>tonkinense</i> Dop	Sept 30, 07	Garden ACAD	ACAD	NS	Canada	621
<i>Conchophyllum</i> Sleumer	<i>Vaccinium</i>	<i>tonkinense</i> Dop	Sept 30, 07	Garden ACAD	ACAD	NS	Canada	622
<i>Conchophyllum</i> Sleumer	<i>Vaccinium</i>	<i>tonkinense</i> Dop	Sept 30, 07	Garden ACAD	ACAD	NS	Canada	623
<i>Conchophyllum</i> Sleumer	<i>Vaccinium</i>	<i>tonkinense</i> Dop	Sept 30, 07	Garden ACAD	ACAD	NS	Canada	624
<i>Conchophyllum</i> Sleumer	<i>Vaccinium</i>	<i>tonkinense</i> Dop	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	711
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 16, 06	Wild	Ramsay Lake	ON	Canada	567
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 16, 06	Wild	Ramsay Lake	ON	Canada	568
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 16, 06	Wild	Ramsay Lake	ON	Canada	571
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 16, 06	Wild	Ramsay Lake	ON	Canada	572
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 16, 06	Wild	Sand Pit	ON	Canada	565
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 16, 06	Wild	Sand Pit	ON	Canada	566
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 16, 06	Wild	Sand Pit	ON	Canada	569
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 16, 06	Wild	Sand Pit	ON	Canada	570
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 3, 06	Wild	Ramsay Lake	ON	Canada	559
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 3, 06	Wild	Ramsay Lake	ON	Canada	560
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 3, 06	Wild	Ramsay Lake	ON	Canada	563
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 3, 06	Wild	Ramsay Lake	ON	Canada	564
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 3, 06	Wild	Sand Pit	ON	Canada	557
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 3, 06	Wild	Sand Pit	ON	Canada	558
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 3, 06	Wild	Sand Pit	ON	Canada	561
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Aug. 3, 06	Wild	Sand Pit	ON	Canada	562
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 19, 06	Wild	Ramsay Lake	ON	Canada	547
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 19, 06	Wild	Ramsay Lake	ON	Canada	548
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 19, 06	Wild	Ramsay Lake	ON	Canada	555
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 19, 06	Wild	Ramsay Lake	ON	Canada	556
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 19, 06	Wild	Sand Pit	ON	Canada	545
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 19, 06	Wild	Sand Pit	ON	Canada	546
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 19, 06	Wild	Sand Pit	ON	Canada	553
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 19, 06	Wild	Sand Pit	ON	Canada	554
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 6, 06	Wild	Ramsay Lake	ON	Canada	543
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 6, 06	Wild	Ramsay Lake	ON	Canada	544
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 6, 06	Wild	Ramsay Lake	ON	Canada	551
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 6, 06	Wild	Ramsay Lake	ON	Canada	552
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 6, 06	Wild	Sand Pit	ON	Canada	541
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 6, 06	Wild	Sand Pit	ON	Canada	542
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 6, 06	Wild	Sand Pit	ON	Canada	549
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	July 6, 06	Wild	Sand Pit	ON	Canada	550
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 21, 06	Wild	Ramsay Lake	ON	Canada	519
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 21, 06	Wild	Ramsay Lake	ON	Canada	520
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 21, 06	Wild	Ramsay Lake	ON	Canada	539
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 21, 06	Wild	Ramsay Lake	ON	Canada	540
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 21, 06	Wild	Sand Pit	ON	Canada	517
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 21, 06	Wild	Sand Pit	ON	Canada	518
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 21, 06	Wild	Sand Pit	ON	Canada	537
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 21, 06	Wild	Sand Pit	ON	Canada	538
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 7, 06	Wild	Ramsay Lake	ON	Canada	510
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 7, 06	Wild	Ramsay Lake	ON	Canada	511
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 7, 06	Wild	Ramsay Lake	ON	Canada	530
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	June 7, 06	Wild	Ramsay Lake	ON	Canada	531



Section	Genus	Species	Date	Type	Site	Prov/ State	Country	Extract #	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Sept. 27, 09	Wild	Mt Thom/ Kemptville Commercial #3	NS	Canada	663	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>angustifolium</i> Aiton	Sept. 27, 13	Wild	Mt Thom/ Kemptville Commercial #1	NS	Canada	665	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	2007	Wild		QC	Canada	666	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	2007	Wild		QC	Canada		
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	2007	Wild		QC	Canada	102	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	July 7, 06	Wild	Mt. Valin	QC	Canada	27	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	July 7, 06	Wild	Mt. Valin	QC	Canada	32	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	July 7, 06	Wild	Mt. Valin	QC	Canada	41	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	July 7, 06	Wild	Mt. Valin	QC	Canada	129	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	July 7, 06	Wild	Mt. Valin	QC	Canada	130	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	July 7, 06	Wild	Mt. Valin	QC	Canada	131	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	July 7, 06	Wild	Mt. Valin	QC	Canada	132	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	Sept. 30, 09	Wild	Garden ACAD	ACAD	NS	Canada	682
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> I.V.Hall & Aalders	Sept. 30, 09	Wild	Garden ACAD	ACAD	NS	Canada	694
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> x <i>myrtilloides</i>	Sept. 30, 09	Wild	Garden ACAD	ACAD	NS	Canada	648
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>boreale</i> x <i>myrtilloides</i>	Sept. 30, 09	Wild	Garden ACAD	ACAD	NS	Canada	653
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> [Berkeley]	May 30, 12	Wild	Garden NY	Hardy	NY	USA	736
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> [Berkeley]	May 30, 12	Wild	Garden NY	Hardy	NY	USA	741
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> [Bluegold]	May 30, 12	Wild	Garden NY	Hardy	NY	USA	744
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> [Bluegold]	May 30, 12	Wild	Garden NY	Hardy	NY	USA	745
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> [Duke]	May 30, 12	Wild	Garden NY	Hardy	NY	USA	742
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> [Duke]	May 30, 12	Wild	Garden NY	Hardy	NY	USA	743
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> [Herbert]	May 30, 12	Wild	Garden NY	Hardy	NY	USA	737
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> [Herbert]	May 30, 12	Wild	Garden NY	Hardy	NY	USA	740
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> [Jersey]	May 30, 12	Wild	Garden NY	Hardy	NY	USA	738
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> [Jersey]	May 30, 12	Wild	Garden NY	Hardy	NY	USA	739
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 6, 07	Wild	Rivière Rouge	QC	Canada	10	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 6, 07	Wild	Rivière Rouge	QC	Canada	12	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 6, 07	Wild	Rivière Rouge	QC	Canada	44	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 6, 07	Wild	Rivière Rouge	QC	Canada	47	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 6, 07	Wild	Rivière Rouge	QC	Canada	48	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 6, 07	Wild	Rivière Rouge	QC	Canada	52	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 6, 07	Wild	Rivière Rouge	QC	Canada	71	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 6, 07	Wild	Rivière Rouge	QC	Canada	73	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 6, 07	Wild	Rivière Rouge	QC	Canada	83	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 6, 07	Wild	Rivière Rouge	QC	Canada	84	
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 6, 07	Wild	Rivière Rouge	QC	Canada	85	

Section	Genus	Species	Date	Type	Site	Prov/ State	Country	Extract #
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 7, 07	Wild	Rivière Rouge	QC	Canada	29
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 7, 07	Wild	Rivière Rouge	QC	Canada	49
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> fo. <i>albiflorum</i> (Hook.) Camp	June 8, 07	Wild	Tourbière Blanville	QC	Canada	74
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> fo. <i>corymbosum</i>	2007	Wild	Snake River	QC	Canada	72
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> fo. <i>corymbosum</i>	May 6, 07	Wild	Snake River	QC	Canada	80
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> fo. <i>corymbosum</i>	May 6, 07	Wild	Snake River	QC	Canada	81
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> fo. <i>corymbosum</i>	May 6, 07	Wild	Snake River	QC	Canada	82
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	-	Wild	Mer Bleue	ON	Canada	512
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	-	Wild	Mer Bleue	ON	Canada	513
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	-	Wild	Mer Bleue	ON	Canada	532
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	-	Wild	Mer Bleue	ON	Canada	533
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	-	Wild		QC	Canada	124
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	2007	Wild		QC	Canada	122
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	May 30, 12	Garden NY	Hardy	NY	USA	754
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	640
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	644
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	646
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	647
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	652
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	677
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	678
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	679
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	680
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	684
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	685
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	686
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	689
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	691
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	697
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	700
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	701
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	702
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	703
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	704
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	715
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	720
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	721 empty bag
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	empty bag
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	QC	Canada	707
Cyanococcus A. Gray	Vaccinium	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	QC	Canada	710

Section	Genus	Species	Date	Type	Site	Prov/ State	Country	Extract #
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	QC	Canada	725
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>corymbosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	QC	Canada	Missing
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>darrowii</i> Camp	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	638
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>hirsutum</i> Buckley	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	633
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>hirsutum</i> Buckley	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	634
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>hirsutum</i> Buckley	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	657
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>hirsutum</i> Buckley	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	675
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>hirsutum</i> Buckley	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	726
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrsinites</i> Lam.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	628
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	June 15, 06	Wild	Mer Bleue	ON	Canada	514
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	June 15, 06	Wild	Mer Bleue	ON	Canada	534
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	June 18, 06	Wild	Val-Morin	QC	Canada	515
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	June 18, 06	Wild	Val-Morin	QC	Canada	516
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	June 18, 06	Wild	Val-Morin	QC	Canada	535
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	June 18, 06	Wild	Val-Morin	QC	Canada	536
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	Sept. 26, 06	Wild	Grand Lake	NB	Canada	604
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	Sept. 27, 09	Wild	Beaver Mountain	NS	Canada	637
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	Sept. 27, 09	Wild	Beaver Mountain	NS	Canada	649
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	Sept. 27, 09	Wild	Beaver Mountain	NS	Canada	655
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	635
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	687
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	693
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	<i>myrtilloides</i> Michx.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	699
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	sp. [tophat]	May 30, 12	Garden NY	Hardy	NY	USA	764
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	sp. [tophat]	May 30, 12	Garden NY	Hardy	NY	USA	765
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	sp. [tophat]	May 30, 12	Garden NY	Hardy	NY	USA	766
<i>Cyanococcus</i> A. Gray	<i>Vaccinium</i>	sp. [tophat]	May 30, 12	Garden NY	Hardy	NY	USA	767
<i>Euepigynium</i> Schltr.	<i>Vaccinium</i>	<i>horizontale</i> Sleumer	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	639
<i>Euepigynium</i> Schltr.	<i>Vaccinium</i>	<i>myrtoides</i> Miq.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	705
<i>Euepigynium</i> Schltr.	<i>Vaccinium</i>	<i>myrtoides</i> Miq. x <i>horizontale</i> Sleumer	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	658
<i>Euepigynium</i> Schltr.	<i>Vaccinium</i>	<i>myrtoides</i> Miq. x <i>horizontale</i> Sleumer	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	662
<i>Euepigynium</i> Schltr.	<i>Vaccinium</i>	<i>myrtoides</i> Miq. x <i>horizontale</i> Sleumer	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	729
<i>Euepigynium</i> Schltr.	<i>Vaccinium</i>	<i>myrtoides</i> Miq. x <i>horizontale</i> Sleumer	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	731
<i>Hemimyrtillus</i> Sleumer	<i>Vaccinium</i>	<i>arctostaphylos</i> L.	May 30, 12	Garden NY	Hardy	NY	USA	771
<i>Hemimyrtillus</i> Sleumer	<i>Vaccinium</i>	<i>arctostaphylos</i> L.	May 30, 12	Garden NY	Hardy	NY	USA	772
<i>Hemimyrtillus</i> Sleumer	<i>Vaccinium</i>	<i>arctostaphylos</i> L.	May 30, 12	Garden NY	Hardy	NY	USA	773
<i>Myrtilius</i> Dumortier	<i>Vaccinium</i>	<i>caespitosum</i> Michx.	2007	Wild	-	QC	Canada	98
<i>Myrtilius</i> Dumortier	<i>Vaccinium</i>	<i>caespitosum</i> Michx.	July 17, 07	Wild	Mine Madeleine	QC	Canada	99
<i>Myrtilius</i> Dumortier	<i>Vaccinium</i>	<i>caespitosum</i> Michx.	July 17, 07	Wild	Mine Madeleine	QC	Canada	101
<i>Myrtilius</i> Dumortier	<i>Vaccinium</i>	<i>caespitosum</i> Michx.	July 17, 07	Wild	Mine Madeleine	QC	Canada	117
<i>Myrtilius</i> Dumortier	<i>Vaccinium</i>	<i>caespitosum</i> Michx.	July 18, 07	Wild	Mont Jacques Cartier	QC	Canada	118
<i>Myrtilius</i> Dumortier	<i>Vaccinium</i>	<i>caespitosum</i> Michx.	July 18, 07	Wild	Mont Jacques Cartier	QC	Canada	136
<i>Myrtilius</i> Dumortier	<i>Vaccinium</i>	<i>caespitosum</i> Michx.	July 6, 07	Wild	Mt. Valin	QC	Canada	98

Section	Genus	Species	Date	Type	Site	Prov/ State	Country	Extract #
Myrtilius Dumortier	Vaccinium	caespitosum Michx.	July 6, 07	Wild	Mt. Valin	QC	Canada	114
Myrtilius Dumortier	Vaccinium	caespitosum Michx.	July 6, 07	Wild	Mt. Valin	QC	Canada	127
Myrtilius Dumortier	Vaccinium	caespitosum Michx.	July 6, 07	Wild	Rivière Rouge	QC	Canada	3
Myrtilius Dumortier	Vaccinium	caespitosum Michx.	July 6, 07	Wild	Rivière Rouge	QC	Canada	120
Myrtilius Dumortier	Vaccinium	caespitosum Michx.	July 8, 07	Wild	Bellevue Peak	QC	Canada	53
Myrtilius Dumortier	Vaccinium	caespitosum Michx.	July 8, 07	Wild	Bellevue Peak	QC	Canada	119
Myrtilius Dumortier	Vaccinium	caespitosum Michx.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	683
Myrtilius Dumortier	Vaccinium	caespitosum Michx.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	709
Myrtilius Dumortier	Vaccinium	caespitosum Michx.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	712
Myrtilius Dumortier	Vaccinium	caespitosum Michx.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	716
Myrtilius Dumortier	Vaccinium	caespitosum Michx.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	718
Myrtilius Dumortier	Vaccinium	myrtilus L.	-	Wild	Kotlovi	-	Bosnia & Herzegovina	2
Myrtilius Dumortier	Vaccinium	myrtilus L.	Aug, 5, 2007	Wild	Pivska Planina	-	Montenegro	128
Myrtilius Dumortier	Vaccinium	myrtilus L.	July 1, 07	Wild	Lotlovi Bjelasnica	-	Bosnia & Herzegovina	36
Myrtilius Dumortier	Vaccinium	myrtilus L.	July 2005	Wild	Igman	-	Bosnia & Herzegovina	134
Myrtilius Dumortier	Vaccinium	myrtilus L.	July 2005	Wild	Jahorina	-	Bosnia & Herzegovina	133
Myrtilius Dumortier	Vaccinium	myrtilus L.	July 24, 2005	Wild	Jahorina	-	Bosnia & Herzegovina	42
Myrtilius Dumortier	Vaccinium	myrtilus L.	July 24, 2005	Wild	Lukomir	-	Bosnia & Herzegovina	2
Myrtilius Dumortier	Vaccinium	myrtilus L.	July 5, 2005	Wild	Jahorina	-	Bosnia & Herzegovina	125
Myrtilius Dumortier	Vaccinium	myrtilus L.	July 6, 07	Wild	Lukomir	-	Bosnia & Herzegovina	126
Myrtilius Dumortier	Vaccinium	myrtilus L.	May 30, 12	Garden NY	Hardy	NY	USA	769
Myrtilius Dumortier	Vaccinium	myrtilus L.	May 30, 12	Garden NY	Hardy	NY	USA	770
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	2007	Wild	Lake 22	WA	USA	631
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 17, 07	Wild	Mine Madeleine	QC	Canada	89
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 17, 07	Wild	Madeleine	QC	Canada	94
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 17, 07	Wild	Mt. Richardson	QC	Canada	111
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 19, 07	Wild	Mont Jacques Cartier	QC	Canada	90
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 19, 07	Wild	Mont Jacques Cartier	QC	Canada	113
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 20, 07	Wild	Mt. Xalibu	QC	Canada	70
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 20, 07	Wild	Mt. Xalibu	QC	Canada	86
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 6, 07	Wild		QC	Canada	93
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 6, 07	Wild		QC	Canada	95
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 7, 07	Wild		QC	Canada	78
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 7, 07	Wild		QC	Canada	88
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 7, 07	Wild		QC	Canada	92
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 7, 07	Wild		QC	Canada	96
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 7, 07	Wild		QC	Canada	135
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 8, 07	Wild	Bellevue Peak	QC	Canada	123
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 8, 07	Wild	Bellevue Peak	QC	Canada	17
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 8, 07	Wild	Bellevue Peak	QC	Canada	79
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 8, 07	Wild	Bellevue Peak	QC	Canada	97
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	July 8, 07	Wild	Bellevue Peak	QC	Canada	112
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	June 7, 07	Wild	Mt. Valin	QC	Canada	16
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	June 7, 07	Wild	Mt. Valin	QC	Canada	76
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	June 7, 07	Wild	Mt. Valin	QC	Canada	77
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	June 7, 07	Wild	Mt. Valin	QC	Canada	91
Myrtilius Dumortier	Vaccinium	ovalifolium Sm.	May 24, 06	Wild		QC	Canada	115
Myrtilius Dumortier	Vaccinium	sp.	May 30, 12	Garden NY	Hardy	NY	USA	774
Myrtilius Dumortier	Vaccinium	sp.	May 30, 12	Garden NY	Hardy	NY	USA	775
Myrtilius Dumortier	Vaccinium	sp.	May 30, 12	Garden NY	Hardy	NY	USA	776
Nesococcus Copel.	Vaccinium	auriculifolium Sleumer	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	708

Section	Genus	Species	Date	Type	Site	Prov/ State	Country	Extract #
<i>Nesococcus</i> Copel. x <i>Euepigynium</i> Schltr.	<i>Vaccinium</i>	<i>acrobacteatum</i> K.Schum. x <i>horizontale</i> Sleumer	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	654
<i>Nesococcus</i> Copel. x <i>Euepigynium</i> Schltr.	<i>Vaccinium</i>	<i>acrobacteatum</i> K.Schum. x <i>horizontale</i> Sleumer	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	730
<i>Nesococcus</i> Copel. x <i>Euepigynium</i> Schltr.	<i>Vaccinium</i>	<i>acrobacteatum</i> K.Schum. x <i>horizontale</i> Sleumer	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	733
<i>Oreades</i> Sleumer	<i>Vaccinium</i>	<i>poasanum</i> Donn. Sm.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	620
<i>Oreades</i> Sleumer	<i>Vaccinium</i>	<i>poasanum</i> Donn. Sm.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	717
<i>Oreades</i> Sleumer	<i>Vaccinium</i>	<i>poasanum</i> Donn. Sm.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	724
<i>Oreades</i> Sleumer	<i>Vaccinium</i>	<i>poasanum</i> Donn. Sm.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	619
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>oxycoccus</i> L.	May 24, 06	Wild	Sand Pit	ON	Canada	121
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>oxycoccus</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	676
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>oxycoccus</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	728
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>oxycoccus</i> L. x <i>macrocarpon</i> Aiton	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	643
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>parvifolium</i> Sm.		Wild	Oyster Dome	WA	USA	630
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>parvifolium</i> Sm.		Wild	Rattlesnake Lake	WA	USA	632
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> [Stevens]	May 30, 12	Garden NY	Hardy	NY	USA	768
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	July 11, 07	Wild	Pointe-Taillon	QC	Canada	51
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 23, 07	Wild	Station de l'Université de Montréal, Lake 4	QC	Canada	7
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 23, 07	Wild	Station de l'Université de Montréal, Lake 4	QC	Canada	100
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 23, 07	Wild	Station de l'Université de Montréal, Lake 4	QC	Canada	109
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 24, 07	Wild	Lac Geai	QC	Canada	8
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 24, 07	Wild	Lac Geai	QC	Canada	9
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 24, 07	Wild	Lac Geai	QC	Canada	87
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 29, 07	Wild	Lac à la Barbue	QC	Canada	6
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 29, 07	Wild	Lac à la Barbue	QC	Canada	103
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 29, 07	Wild	Lac à la Barbue	QC	Canada	104
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 29, 07	Wild	Lac à la Barbue	QC	Canada	105
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 29, 07	Wild	Lac à la Barbue	QC	Canada	106
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 29, 07	Wild	Lac à la Barbue	QC	Canada	107
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 29, 07	Wild	Lac à la Barbue	QC	Canada	108
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	May 29, 07	Wild	Lac à la Barbue	QC	Canada	110
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	681
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	690
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	695
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> Aiton	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	735
<i>Oxycoccus</i> (Hill) W.D.J. Koch	<i>Vaccinium</i>	<i>macrocarpon</i> [Hamilton]	May 30, 12	Garden NY	Hardy	NY	USA	756
<i>Pyxothamnus</i> Sleumer	<i>Vaccinium</i>	<i>consanguineum</i> Klotzsch	May 30, 12	Garden NY	Tropical	NY	USA	750
<i>Pyxothamnus</i> Sleumer	<i>Vaccinium</i>	<i>consanguineum</i> Klotzsch	May 30, 12	Garden NY	Tropical	NY	USA	751
<i>Pyxothamnus</i> Sleumer	<i>Vaccinium</i>	<i>consanguineum</i> Klotzsch	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	608

Section	Genus	Species	Date	Type	Site	Prov/ State	Country	Extract #
<i>Pyxothamnus</i> Sleumer	<i>Vaccinium</i>	<i>consanguineum</i> Klotzsch	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	616
<i>Pyxothamnus</i> Sleumer	<i>Vaccinium</i>	<i>consanguineum</i> Klotzsch	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	617
<i>Pyxothamnus</i> Sleumer	<i>Vaccinium</i>	<i>consanguineum</i> Klotzsch	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	618
<i>Pyxothamnus</i> Sleumer	<i>Vaccinium</i>	<i>consanguineum</i> Klotzsch <i>x ovatum</i> Pursh	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	645
<i>Pyxothamnus</i> Sleumer	<i>Vaccinium</i>	<i>ovatum</i> [Austin Creek]	May 30, 12	Garden NY	Tropical	NY	USA	752
<i>Pyxothamnus</i> Sleumer	<i>Vaccinium</i>	<i>ovatum</i> Pursh	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	722
<i>Pyxothamnus</i> Sleumer	<i>Vaccinium</i>	<i>ovatum</i> Pursh	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	726
<i>Pyxothamnus</i> Sleumer x <i>Cinctosandra</i>	<i>Vaccinium</i>	<i>meridionale</i> Sw. x <i>exul</i> Bolus	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	651
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Aug. 7, 07	Wild	Bellevue Peak	QC	Canada	3
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Aug. 7, 07	Wild	Bellevue Peak	QC	Canada	38
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Aug. 7, 07	Wild	Bellevue Peak	QC	Canada	40
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Aug. 7, 07	Wild	Bellevue Peak	QC	Canada	50
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Aug. 7, 07	Wild	Bellevue Peak	QC	Canada	54
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Aug. 7, 07	Wild	Bellevue Peak	QC	Canada	58
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Aug. 7, 07	Wild	Bellevue Peak	QC	Canada	116
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Aug. 12, 09	Wild	Dempster Highway	Yuko n	Canada	650
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Aug. 12, 09	Wild	Dempster Highway	Yuko n	Canada	667
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Aug. 15, 09	Wild	Clinton Creek Road	Yuko n	Canada	671
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 14, 07	Wild	Montagne Blanche	QC	Canada	11
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 14, 07	Wild	Montagne Blanche	QC	Canada	35
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 14, 07	Wild	Montagne Blanche	QC	Canada	60
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 14, 07	Wild	Montagne Blanche	QC	Canada	64
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 17, 07	Wild	Mine Madeleine	QC	Canada	19
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 17, 07	Wild	Mine Madeleine	QC	Canada	62
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 17, 07	Wild	Mine Madeleine	QC	Canada	69
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 18, 07	Wild	Mont Jacques Cartier	QC	Canada	25
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 18, 07	Wild	Mont Jacques Cartier	QC	Canada	43
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 18, 07	Wild	Mont Jacques Cartier	QC	Canada	59
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 19, 07	Wild	Lac à la René	QC	Canada	4
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 19, 07	Wild	Lac à la René	QC	Canada	14
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 19, 07	Wild	Mont Jacques Cartier	QC	Canada	13
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 20, 07	Wild	Mt. Xalibu	QC	Canada	18
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 20, 07	Wild	Mt. Xalibu	QC	Canada	31
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 20, 07	Wild	Mt. Xalibu	QC	Canada	56
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 20, 07	Wild	Mt. Xalibu	QC	Canada	68
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 7, 07	Wild	Mt. Valin	QC	Canada	22
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 7, 07	Wild	Mt. Valin	QC	Canada	55
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 7, 07	Wild	Mt. Valin	QC	Canada	57
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 7, 07	Wild	Mt. Valin	QC	Canada	61
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	July 7, 07	Wild	Mt. Valin	QC	Canada	67
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	June 7, 07	Wild	Mt. Valin	QC	Canada	20
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Sept. 18, 09	Wild	Iqualuit	Nuna vut	Canada	672
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Sept. 18, 09	Wild	Iqualuit	Nuna vut	Canada	673
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	688
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	732
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> L.	Sept. 6th, 09	Wild	Iqualuit	Nuna vut	Canada	668
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> subsp. <i>pedris</i> (Harshb.) S.B. Young	July 17, 07	Wild	Mine Madeleine	QC	Canada	34
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> subsp. <i>pedris</i> (Harshb.) S.B. Young	July 17, 07	Wild	Mine Madeleine	QC	Canada	45

Section	Genus	Species	Date	Type	Site	Prov/ State	Country	Extract #
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> subsp. <i>pedris</i> (Harshb.) S.B. Young	July 17, 07	Wild	Mine Madeleine	QC	Canada	46
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> subsp. <i>pedris</i> (Harshb.) S.B. Young	July 17, 07	Wild	Mine Madeleine	QC	Canada	63
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> subsp. <i>pedris</i> (Harshb.) S.B. Young	July 17, 07	Wild	Mine Madeleine	QC	Canada	65
<i>Vaccinium</i> L.	<i>Vaccinium</i>	<i>uliginosum</i> subsp. <i>pedris</i> (Harshb.) S.B. Young	July 17, 07	Wild	Mine Madeleine	QC	Canada	66
<i>Vitis-idaea</i> Dumortier	<i>Vaccinium</i>	<i>vitis-idaea</i> L.	June 5, 06	Wild	Jahorina	-	Bosnia & Herzegovina	23
<i>Vitis-idaea</i> Dumortier	<i>Vaccinium</i>	<i>vitis-idaea</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	674
<i>Vitis-idaea</i> Dumortier	<i>Vaccinium</i>	<i>vitis-idaea</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	692
<i>Vitis-idaea</i> Dumortier	<i>Vaccinium</i>	<i>vitis-idaea</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	696
<i>Vitis-idaea</i> Dumortier	<i>Vaccinium</i>	<i>vitis-idaea</i> L.	Sept. 30, 09	Garden ACAD	ACAD	NS	Canada	706
<i>Vitis-idaea</i> Dumortier	<i>Vaccinium</i>	<i>vitis-idaea</i> var. <i>minus</i> Lodd., G. Lodd. & W. Lodd.	May 30, 12	Garden NY	Hardy	NY	USA	755

## Chapter 7

# Single-Lab Validation of a Method for the Quantification of Chlorogenic Acid and Hyperoside Directly from Crude Blueberry (*Vaccinium angustifolium* Aiton) Leaf Extract by Nuclear Magnetic Resonance (NMR) Analysis\*

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## **Single-Lab Validation of a Method for Quantification of Chlorogenic Acid and Hyperoside Directly from Crude Blueberry (*Vaccinium angustifolium* Aiton) Leaf Extract by Nuclear Magnetic Resonance (NMR) Analysis**

Chlorogenic acid is the most abundant antioxidant found in blueberry leaf extract Harris et al. (2007) comprising up to 10% of the total dried extract weight. Formation of chlorogenic acid results from the esterification of caffeic acid and (-)-quinic acid and is an important intermediate in the formation of cell wall lignin. Chlorogenic acid is known to possess antifungal properties and antidiabetic properties (Van Dam 2006). Hyperoside [quercetin-3-O-galactoside], also found in other medicinal plants such as *Crataegus* spp. (hawthorn), has multiple activities, such as cardioprotective, neuroprotective, antioxidant, and anti-inflammatory properties (Boots et al. 2008).

Currently, there are no known validated methods for screening these two key constituents by nuclear magnetic resonance (NMR). NMR is a highly reproducible, non-destructive analytical technique commonly used for identifying compounds and it is well accepted as a definitive structural confirmation technique. The ability to quantify, and qualitatively assess the presence or absence of constituents directly from the mixture spectrum provides a means of accelerating metabolite identification and quantification without laborious liquid chromatography (LC) steps. This report presents a method for determining the concentration and identification of chlorogenic acid and hyperoside directly from crude blueberry leaf extracts (BBE).

## Method

### *Principle*

Ethanol extracts of milled blueberry leaves are lyophilized and suspended into a solution of DMSO. The solvated extract is measured by NMR and the chemical shift signals associated with the compounds are used to identify and quantify the constituents.

### *Scope*

The method is suitable for the determination of chlorogenic acid and hyperoside. The method is applicable to the determination of 0.01 mM or greater of each compound in *Vaccinium angustifolium* Aiton leaf extracts.

### *Apparatus*

- (a) *NMR Spectrometers* (Bruker BioSpin) - 600 MHz (14 Tesla), AV-III, 5mm TCI CryoProbe, with temperature regulation and 400 MHz (9.3 Tesla), AV-III, 5mm TXI probe, with temperature regulation
- (b) *NMR Spectrometer tubes* – Wilmad PP-535 and Bruker Z107374
- (c) *Centrifuge tubes* – VWR: 87003-294 (1.5 ml)
- (d) *Pipettes* – Eppendorf Research
- (e) *Pipette tips* – VWR sterol/aerosol tips
- (f) *Centrifuge* – Capsule Tomy HF-120
- (g) *Centrifuge* – Eppendorf 5810 R
- (h) *Freeze drier* – Edwards Super Modulyo
- (i) *Analytical balance* – 4-digit, Mettler Toledo AG245 (Mettler-Toledo, Columbus, OH) and 5-digit, Mettler Toledo AG104 (Mettler-Toledo, Columbus, OH)
- (j) *HPLC system* - 1100 series HPLC-DAD system (Agilent, Palo Alto, CA, USA) consisting of an autosampler, a column thermostat, a quaternary pump, and a diode array detector DAD.

### ***Samples***

- (a) *Vaccinium angustifolium* var. *laevifolium* House was collected and identified from the “Sand Pit” location (+45.59974, -76.09649) at Ramsay Lake, ON, Canada, by John Thor Arnason and Jonathan Ferrier. The determination was made using Scoggan’s, ‘The Flora of Canada’ (Scoggan 1978), and a herbarium voucher remains at the University of Ottawa Herbarium (OTT).

### ***Reagents***

- (a) *Chlorogenic acid* – greater than 95% Chlorogenic acid (Sigma-Aldrich Chemical Co.) C3878, batch number 070M1331. COA of greater than 99% purity via HPLC, confirmed by NMR.
- (b) DMSO-d<sub>6</sub>: 99.9% Dimethyl Sulfoxide-d<sub>6</sub> (DLM-10-100 PSO#10K-221, Cambridge Isotopes)
- (c) DSS-d<sub>6</sub>: 4,4-dimethyl-4-silapentane-1-sulfonic acid – d<sub>6</sub> (613150 Lot TV0142, Isotec)
- (d) DMSO/DSS solvent solution: add 157 microliters 300 mM DSS-d<sub>6</sub> (in DMSO-d<sub>6</sub>) to 100 milliliters of DMSO-d<sub>6</sub>

### ***Calibration***

- (a) *Standard solutions* – Solutions of known chlorogenic acid were prepared by dissolving dried, solid powder in 600 microliters of DMSO-d<sub>6</sub>. Chlorogenic acid standards were created using a 5-digit scale [1.51, 3.29, 7.10, 8.89, 12.84, 22.49, and 88.20 mM] and using a 4-digit scale [2.8, 7.5, 9.9, 15.1, 24.0, 51.7, and 68.2 mM].
- (b) *Spectroscopy* – Integration of known chemical shift signals (I) was used to generate calibration curves. Scaled integrals (Is) were calculated based on the physical properties of the sample as reflected in the 90 degree pulse calibration in accordance with the PULCON principle (Wider and Dreier 2005) as follows:

### ***Preparation of Blueberry leaf extracts (BBE)***

- (a) Leaves (dried in a plant dehydrator over night) were ground in a Wiley Mill through a mesh size of 40. Ground material was weighed and extracted using 95% EtOH (10 mL/g leaf material) by shaking for 24 hrs. The supernatant was decanted (Phase 1) and the pellet was extracted again using 95% EtOH (5mL/g leaf material) and shaken for another 24 hrs. The supernatant was decanted (Phase 2) and phase 1 and 2 were pooled and centrifuged in an Eppendorf 5810 R for 5 minutes at 3000 rpm at room temperature. The supernatant was separated from the pellet and transferred into a round bottom flask wrapped with aluminium foil and all alcohol was removed in a Speed Vac at water bath temperature of 37 °C. To remove the water content the samples were transferred into pre-weighed 50 mL self standing tubes then lyophilized in a Supermudlyo freeze dryer overnight. All extracts were stored at 20 °C.

#### ***Preparation of NMR samples***

- (a) Samples of blueberry leaf extract (BBE) were prepared by dissolving a dried extract solid into DMSO-d<sub>6</sub> at a ratio of approximately 25 mg per milliliter. Specific sample preparation weights were measured to the digitization limits of the appropriate gravimetric balance. Samples were vortexed for 10 seconds and subsequently centrifuged for 10 seconds at 6,400 rpm. 600 microliters was drawn from the microfuge tube and placed directly into the NMR tube.

#### ***System suitability***

- (a) The system suitability tests used were the <sup>1</sup>H SINO using 0.1% ethylbenzene in CDCl<sub>3</sub> and the <sup>1</sup>H lineshape and resolution test using 1% CHCl<sub>3</sub> in acetone-d<sub>6</sub> (or 0.3% CHCl<sub>3</sub> in acetone-d<sub>6</sub> for CryoProbes). Additionally, the temperature was confirmed with the 99.8% MeOD temperature standard before instrument operation.

#### ***NMR analysis***

- (a) *Instrumentation* – All samples were run on Bruker NMR spectrometers temperature calibrated to 298 K.

- (b) *Experiment* – Acquisition parameters were set to acquire 64 K data points, relaxation delay of 10 seconds, 64 scans, 8 dummy scans using the NOESY1D pulse sequence with a d8 of 0.01 seconds for a total experiment time of 9:13 minutes. The same number of points, scans, and dummy scans were used for the ZGPR pulse sequence and a presaturation pulse of 25 Hz optimized on water (ca. 3.33 ppm) for a total experiment time of 7:40 minutes. A 90-degree pulse calibration was generated automatically using the nutation echo scheme (14). Spectra were processed using exponential broadening (LB = 0.3 Hz) and phased with 0-order phase corrections using TopSpin 3.0 (Bruker BioSpin). Spectra were referenced to DSS at 0.0 ppm.
- (c) *Chlorogenic Acid* – In all crude extracts, all the chlorogenic acid signals were found in the downfield region between 6.0 and 7.5 ppm (Figure 7.1) and can be used individually as quantifiable protons.
- (d) *Hyperoside* – Identification of hyperoside was found in the same NMR spectrum and the isolated chemical shifts at 7.7 ppm were used for quantification (Figure 7.1 inset).
- (e) *Analysis* – All spectra were analyzed using AMIX™ (Bruker BioSpin) software, 3.9.9.

### **Calculations**

- (a) *Quantification* – Determination of quantification by NMR was done by determining the integral of a signal representing a known number of protons at a known concentration. This integral of the known sample was directly correlated to the signals of an unknown concentration with a known number of protons. The calculations for quantification and additional considerations for different 90 degree pulse lengths were adopted from Wider et al. (Wider and Dreier 2005).
- (b) *LOD and LOQ* – To determine the LOD (defined as signal-to-noise at 3:1) and LOQ (defined as signal-to-noise at 10:1) for chlorogenic acid (or any constituent), noise levels were determined by finding a section of the spectrum

(minimum 512 points) where there are no real peaks and the region has the most Gaussian-like distribution of noise. Noise thresholds can be calculated as follows:

$$\text{Noise threshold} = 815.86 + (F * 472.54)$$

***Youden ruggedness testing***

- (a) Youden's trial (16) was run by altering seven items as outlined in Table 7.1. Items were chosen based on the known factors which would most likely result in changes in the quantification of chlorogenic acid including NMR field strength, mesh size during extraction, NMR tube glass quality, the beginning amount of extract powder, precision of balance in creating integration curves/interpreting concentration, NMR pulse sequence, and different acquisition days.

**Table 7.1.** Youden ruggedness trial design and calculations.

Factor	High	Low
NMR field strength	600 (A)	400 (a)
Extraction mesh size	40 (B)	20 (b)
NMR tube	PP-535 (C)	Z107374 (c)
Extract powder	25.52 mg (D)	9.18 mg (d)
Balance	5-digit (E)	4-digit (e)
Pulse sequence	NOESY (F)	ZGPR (f)
Different days	0 (G)	1 (g)

Experiment No.	Combination	Measurement
1	ABCDEFG	X1
2	ABcDefg	X2
3	AbCdEfg	X3
4	AbcdeFG	X4
5	aBCdeFg	X5
6	aBcdEfG	X6
7	abCDefG	X7
8	abcDEFg	X8

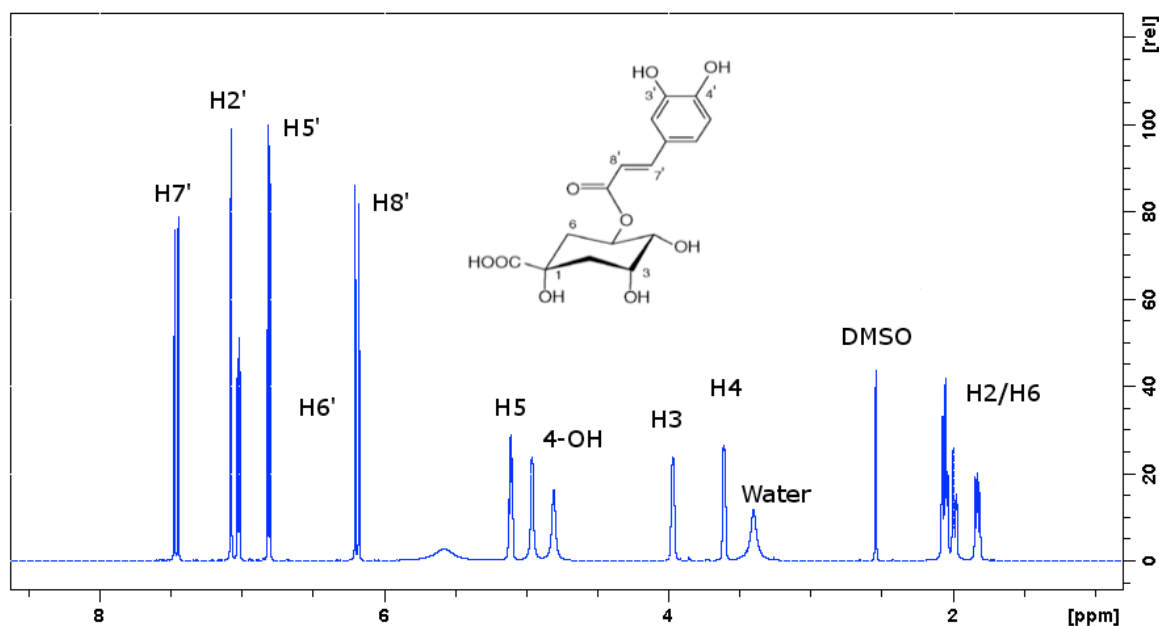
  

Effect	Equation
Effect of "A" and "a"	$= [(X1 + X2 + X3 + X4)/4] - [(X5 + X6 + X7 + X8)/4]$ $= -0.12$
Effect of "B" and "b"	$= [(X1 + X2 + X5 + X6)/4] - [(X3 + X4 + X7 + X8)/4]$ $= -0.01$
Effect of "C" and "c"	$= [(X1 + X3 + X5 + X7)/4] - [(X2 + X4 + X6 + X8)/4]$ $= 0.04$
Effect of "D" and "d"	$= [(X1 + X2 + X7 + X8)/4] - [(X3 + X4 + X5 + X6)/4]$ $= 0.03$
Effect of "E" and "e"	$= [(X1 + X3 + X6 + X8)/4] - [(X2 + X4 + X5 + X7)/4]$ $= 0.06$
Effect of "F" and "f"	$= [(X1 + X4 + X5 + X8)/4] - [(X2 + X3 + X6 + X7)/4]$ $= 0.03$
Effect of "G" and "g"	$= [(X1 + X4 + X6 + X7)/4] - [(X2 + X3 + X5 + X8)/4]$ $= 0.00$

## Results

### *Chlorogenic acid and hyperoside identification*

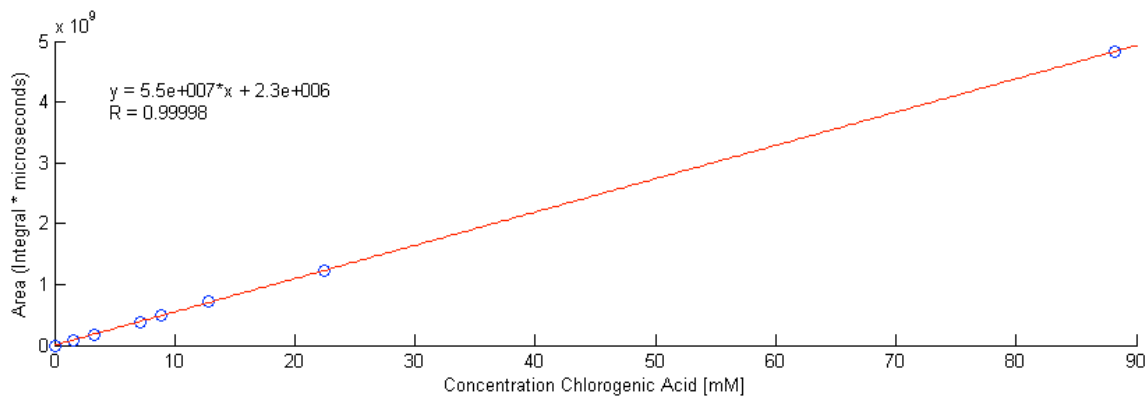
The proton and carbon NMR assignments of chlorogenic acid and hyperoside are well known (17, 18). Proton chemical shifts and coupling patterns of the stock chlorogenic acid solutions were first confirmed by NMR. Figure 7.1 shows the molecular structure view as well as the proton chemical structure profile with correlated assignments.



**Figure 7.1.** NMR  $^1\text{H}$  spectral assignments (17) and molecular structure (inset) of chlorogenic acid. Signals at ca. 3.3 ppm and 2.5 ppm were assigned to the residual water and DMSO, respectively.

### *Calibration linearity*

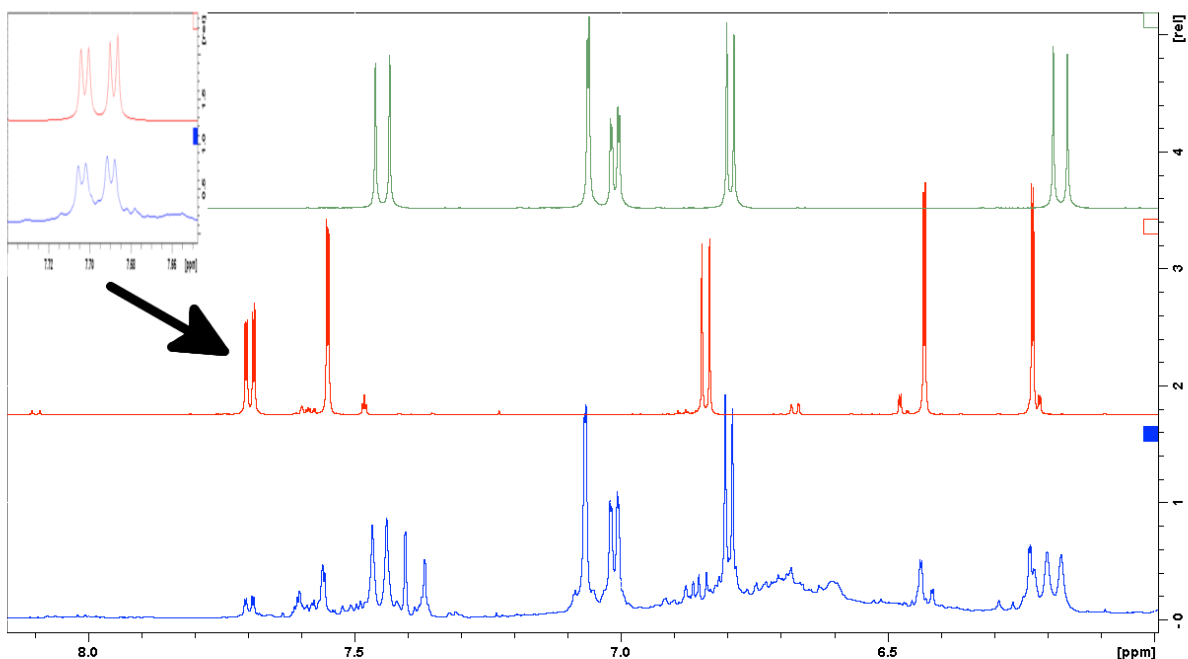
Standard calibration curves of chlorogenic acid were generated using seven concentration points of 1.51, 3.29, 7.10, 8.89, 12.84, 22.49, and 88.20 mM. Figure 7.2 shows the best linear fit using the integral of the peak at 6.8 ppm. The regression analysis showed the best fit line of  $y = 2.59294 \times 10^{-9} * x$  with residuals of 1.0000 ( $R=0.99998$ ) using a 5-digit scale. Calibration curves of chlorogenic acid (2.8, 7.5, 9.9, 15.1, 24.0, 51.7, 68.2 mM) using a 4-digit scale had a best fit line of  $y = 1.9321 \times 10^{-8} * x$  with residuals of 0.9982. Single point calibration is typically used in NMR, but multi-point calibration curves were generated for both the 4-digit and 5-digit scale for the Youden's trial.



**Figure 7.2.** Calibration curve of known chlorogenic acid standards measured on a 5-digit gravimetric scale, prepared in DMSO-d6 against the area determined from the NMR signal at 6.8 ppm.

### ***Quantification of chlorogenic acid and hyperoside in BBE***

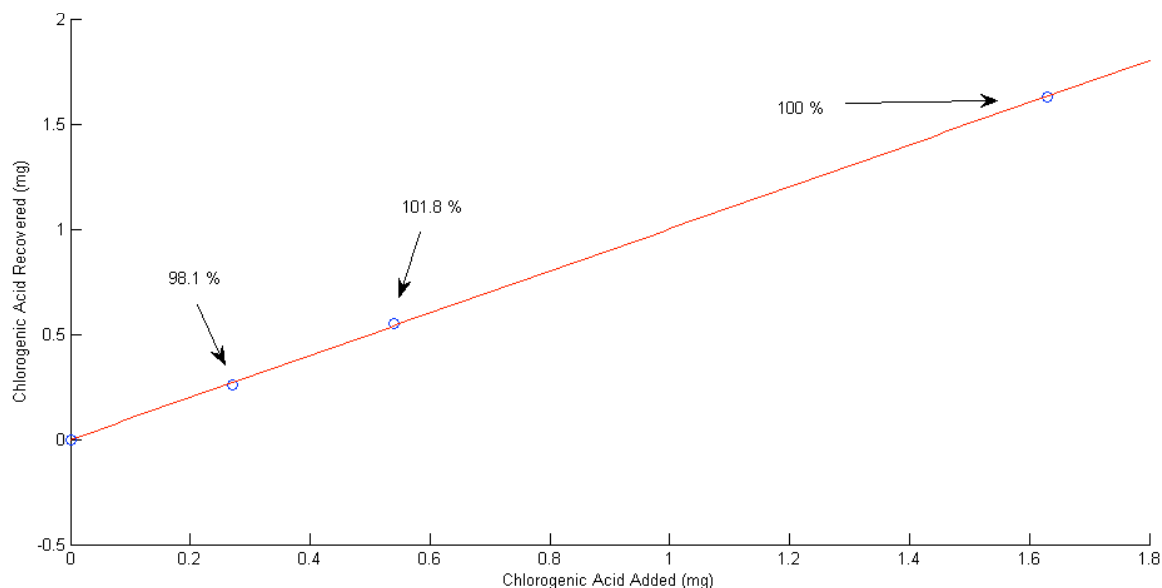
The identification of the signals corresponding to chlorogenic acid and hyperoside in the crude extract is exemplified by overlaying spectra (Figure 7.3). The quantification of the content of the two constituents was done by integrating only the peaks, and not the underlying broad signals that the peaks sit on. The concentration of chlorogenic acid in the crude extract was determined to be 7.53 (+/- 0.15) mM. Because NMR is a measurement of the proton content regardless of what structure it is bound to, the integral can be used to calculate the concentration of any integratable signal within the spectrum. Thus, the quantification of 0.77 (+/- 0.03) mM hyperoside was undertaken directly from the chlorogenic acid calibration curve. The comparison of HPLC-DAD (Saleem et al. 2010) quantification showed no noticeable difference from NMR quantification.



**Figure 7.3.** Top and bottom overlay of spectra from the aromatic region of blueberry leaf extracts (blue) and pure hyperoside (red) and chlorogenic acid (green). Quantifiable hyperoside signal is shown at 7.7 ppm (inset).

### ***Recovery of chlorogenic acid from doped crude extract***

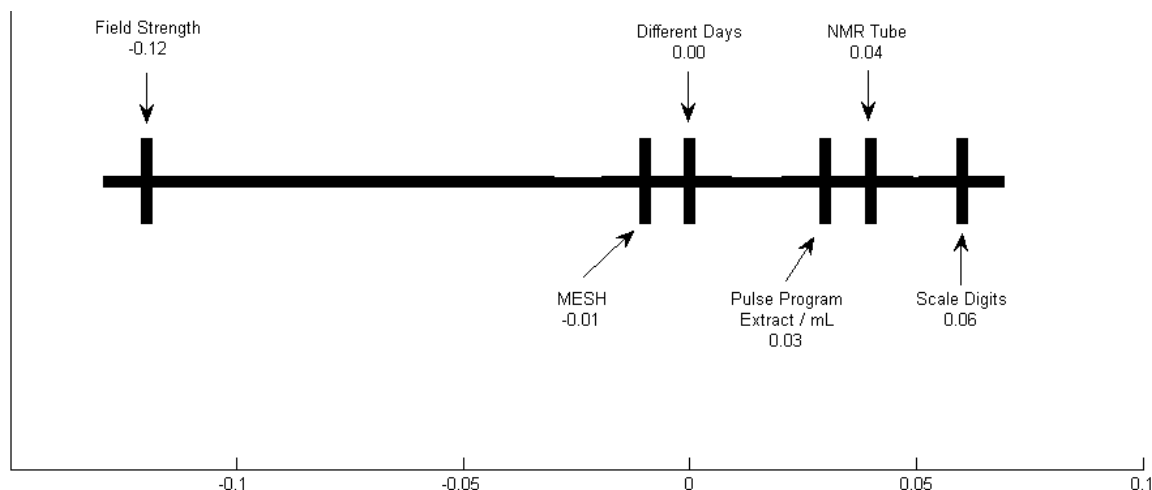
Three progressive doping recovery samples were made by adding different amounts of chlorogenic acid powder to dried blueberry leaf extract then solvated. Measurement of the chlorogenic acid content in the doped samples was done by NMR. Figure 7.4 summarizes the recovery of doping showing (after correcting the endogenous amounts in the BBE sample) the ability of the selected solvent to measurably recover and fully solubilize the chlorogenic acid from the lyophilized state. The experiment showed an average recovery of 99.9%.



**Figure 7.4.** Recovery chart displaying the amount of chlorogenic acid measured by NMR from doped blueberry leaf extract samples with known amounts of chlorogenic acid. Percent recovery represents the amount of calculated chlorogenic acid content versus the amount of added chlorogenic acid (minus the amount of chlorogenic acid in the sample).

### *Youden's ruggedness trial*

The results from Youden's trial presented in Figure 7.5 show the method sensitivity to different factors ("high" and "low", Table 7.1) on the quantification of chlorogenic acid in the mixture. The largest contributing factor is field strength ( $A/a = -0.12$ ) which correlates to a 2.1% (-0.15 mM) decrease in accuracy of measuring chlorogenic acid directly from the mixture in a 400MHz spectrometer as compared to a 600MHz spectrometer. The method sensitivity to scale precision ( $E/e$ ), NMR tube glassware ( $C/c$ ), pulse program ( $F/f$ ), and beginning dry-weight powder ( $D/d$ ) affected the results to a lesser extent. Mesh size ( $B/b$ ) and different days of acquisition ( $G/g$ ) were determined to be the least likely method variables to affect the ability to measure the concentration.



**Figure 7.5.** One dimensional representation of the steps of the method tested during Youden's trial of ruggedness.

### ***LOD and LOQ***

Using the formula for noise calculation in the method section, LOD and LOQ levels (which are applicable to any constituent in the mixture) were determined to within significant figures to be 0.01 mM and 0.01 mM (0.005 mM and 0.013 mM to non-significant figures), respectively.

## **Discussion**

The novel single lab validation study presented here shows a timely, simple, accurate, non-destructive method for monitoring chlorogenic acid content directly from crude blueberry leaf extracts. The extraction technique, minimal sample handling, and direct quantification from mixtures were chosen to minimize sample handling. The ability to simultaneously monitor multiple constituents has been presented, but not limited to the quantification of chlorogenic acid and hyperoside. The method was demonstrated to be highly robust in terms of precision, accuracy and ruggedness. NMR provides a means of directly calculating the concentration of constituent within the complex mixture and is scalable in experiment time to change LOD and LOQ thresholds. In this method, the NMR experimental time was 9:13 minutes. This method is considerably simpler and less time consuming than comparable HPLC methods (Harris et al. 2007).

Youden's trial for ruggedness indicates that none of the possible quantification-susceptible variables in the method contributed significantly to the error in determining the concentration of chlorogenic acid in the crude extract. The largest effect on accuracy was the measurement at 400 MHz versus 600 MHz which corresponded to a 2.1% error in quantification. Notably, the signal to noise is related to the field strength and by increasing the experiment time to achieve comparable signal to noise on the 400 MHz instrument it would be possible to account for this error. The next largest effect on the method was from the gravimetric scale when comparing a 4-digit scale to a 5-digit scale. The improvement in residuals correlates directly with the improvement in scale digitization and the high degree of linearity across the concentration range indicating that NMR is a more accurate with a wider dynamic range than gravimetric methods and justifies single point calibration.

Both NMR glassware and pulse program selection had similar effects on the method. Generally NMR glassware considerations are addressed by increasing the glass quality in parallel with an increase in NMR field strength. The decrease in performance of the ZGPR experiment versus the NOESY1D experiment can largely be attributed to less defined baselines and lineshapes which result from the ZGPR experiment. These spectral features lead to a reduced ability to accurately integrate the peak shape. Additionally, the theoretical benefits of the NOESY1D provide for a simpler data processing scheme which makes it an ideal experiment for automation. The smallest effect on the method was a result of varying the mesh size used in the milling of the leaf samples.

Importantly, changing the acquisition day had no effect on the quantification results. There are many outcomes of the ability to acquire accurate and precise data continuously eliminating the need for instrument 'conditioning' or revalidation and a single point calibration that applies to many constituents. As a result of the high reproducibility, a multilab trial has been initiated.

## Chapter 8

# Multi-Lab Validation for Distinguishing *Vaccinium* Species by Chemical Leafprinting using Proton Nuclear Magnetic Resonance ( $^1\text{H}$ NMR) Spectra

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## **Multi-Lab Validation for Distinguishing *Vaccinium* Species by Chemical Leafprinting using Proton Nuclear Magnetic Resonance (<sup>1</sup>H NMR) Spectra**

Species from the genus *Vaccinium* L. (heath family, *Ericaceae*) have been used traditionally by indigenous cultures of the circumboreal floristic region (the largest floristic region in the world which includes most of Canada, Europe, and Russia) for food and for treatment of a variety of disease conditions (Moerman 1998). The genus includes plants that bear edible berries such as blueberry, bilberry, huckleberry, and cranberry. There are over 40 species in North America and about 450 species worldwide. Examples of medicinal preparations include blueberry leaf extract, which is a traditional Cree medicine shown to have anti-diabetic properties (Leduc et al. 2006, Ferrier et al. 2012). The Lukomir Highlanders of Bosnia also use blueberry leaf extract for medicinal purposes (Ferrier et al. 2012). As interest increases in traditional herbal medicines, these medicines are the subject of modern clinical trials to validate effectiveness (Ly et al. 2013ab).

For use as an herbal product, it is important to verify the identity of the plant materials collected. Here we use nuclear magnetic resonance (NMR) fingerprinting in a multi-laboratory setting to distinguish the taxonomic identity of blueberry leaf extracts, demonstrating the usefulness of NMR spectroscopy as a tool to examine crude botanical extracts. The NMR methodology employed is part of a broader effort to establish validated conditions for highly reproducible qualitative and quantitative (qNMR) NMR spectroscopy, to be used for the analysis of complex samples including natural products. Non-chromatographic botanical fingerprinting has previously been applied to discriminate between *Panax* species, in particular *P. ginseng*, *P. quinquefolius*, and *P. notoginseng* (Chen et al. 2011). However, in that study, ultraviolet (UV) spectroscopy, near infrared spectroscopy and mass spectrometry were used, rather than NMR. The reproducibility of NMR across sites has recently been demonstrated in a study including seven labs (Viant et al. 2009), but the samples included test mixtures and animal tissue (flounder liver extracts), not plant materials. Thus the current report fills the gap of NMR applied to the analysis of plant materials and features a test across five laboratories to demonstrate the linearity and reproducibility of NMR across sites.

Blueberry leaves contain a variety of phytochemicals such as phenyl propanoids, flavonoids, and procyanidins, which are well-known antioxidants, that may explain some of their numerous reported biological activities. A method to identify and quantify marker compounds in blueberry leaves using high performance liquid chromatography (HPLC) and mass spectrometry has previously been reported (Harris et al. 2007). Subsequently it was shown that the chemical markers chlorogenic acid (COA) and hyperoside can be identified and quantitated by NMR in crude extract material without further separation (Hicks et al. 2012). Qualification of the crude extracts of blueberry by chemometric modeling for three *Vaccinium* species is reported here. A future manuscript will extend our statistical models beyond these three species.

In the current study, 123 samples from three different species (*Vaccinium angustifolium* Ait. – 82 samples, *V. ovalifolium* Sm. – 22 samples, *V. macrocarpon* L. – 19 samples) were analyzed by NMR. Principal component analysis (PCA) of the NMR spectra readily differentiated between these species. With the ability to differentiate species established, one sample of *V. angustifolium* was sent to five different laboratories for acquisition of NMR spectra. Spectra were acquired according to rigorously defined conditions on spectrometers whose performance had been validated. PCA shows that the spectra for this sample from different sites group with *V. angustifolium* and not the other species. Thus, the NMR fingerprinting confirms the species identification by comparison to reference spectra. In addition, it was shown that this identification is insensitive to details of the NMR spectroscopy including probe type, spectrometer hardware and software, field strength, operator, and laboratory.

## Experimental

### **Reagents**

- (a) *DMSO-d<sub>6</sub>* – 99.9% dimethyl sulfoxide-d<sub>6</sub> (DLM-10-100 PSO#10K-221, Cambridge Isotopes, Andover, MA)
- (b) *DSS-d<sub>6</sub>* – 4,4-dimethyl-4-silapentane-1-sulfonic acid-d<sub>6</sub> (613150 Lot TV0142, Isotec)

- (c) *DMSO/DSS solvent solution* – add 157  $\mu\text{L}$  of 300 mM DSS- $\text{d}_6$  (in DMSO- $\text{d}_6$ ) to 100 mL of DMSO- $\text{d}_6$
- (d) *Chlorogenic acid* – greater than 95% chlorogenic acid (Sigma-Aldrich Chemical Co.) C3878, batch number 070M1331. COA of greater than 99% purity via HPLC, confirmed by NMR.

### ***Samples***

- (a) *Preparation of leaf extracts* - Leaves from the *Vaccinium* spp. were harvested from reproductively isolated sites across Canada. Identification was made by Alain Cuerrier and Jonathan Ferrier using Scoggan's, 'The Flora of Canada' (Scoggan 1978), and herbarium vouchers were deposited at the University of Ottawa Herbarium (OTT) and the Marie-Victorin Herbarium (MT). Leaves were dried overnight in a dehydrator. Dried leaves were ground in a Wiley Mill through a size 40 mesh and extracted with 95% ethanol (10 mL per gram leaf material) with shaking at room temperature for 24 hours. After 24 hours, the solvent was decanted (Phase 1) and the ground material was extracted again using 95% EtOH (5 mL per gram of leaf material) and shaken for another 24 hours. Subsequently, the solvent was decanted (Phase 2) and phases 1 and 2 were pooled and centrifuged in an Eppendorf 5810 R at 1000 g for 5 minutes at room temperature. The solvent was decanted into a round bottom flask wrapped with aluminium foil, and all alcohol was removed in a Speed Vac (Thermo Scientific, Asheville, NC) at a water bath temperature of 37  $^{\circ}\text{C}$ . To remove water, the samples were transferred into pre-weighed 50 mL self-standing tubes and then lyophilized in a SuperModulyo freeze dryer overnight. All extracts were stored at -20  $^{\circ}\text{C}$ .
- (b) *Preparation of NMR samples* - 25 mg of crude extract were dissolved in 1.0 mL of DMSO/DSS solvent solution. Specific sample preparation weights were measured to the digitization limits of the appropriate balance. Samples were vortexed for 10 seconds and subsequently centrifuged for 10 seconds at 3,800 g. 600  $\mu\text{L}$  of the resulting supernatant was transferred to a 5 mm NMR tube for spectroscopy.

## *Apparatus*

- (a) *NMR Spectrometers*: (Bruker BioSpin, Billerica, MA) – Bruker: 600 MHz (14.1 Tesla), AV-III, 5mm TCI CryoProbe, with temperature regulation; DHMRI-1: 600 MHz (14.1 Tesla), AV-III, 5mm DCH CryoProbe, with temperature regulation; DHMRI-2: 700 MHz (16.4 Tesla), AV-III, 5mm QNP CryoProbe, with temperature regulation; NRC IMB: 700 MHz (16.4 Tesla), AV-III, 1.7 mm TCI CryoProbe, with temperature regulation; UIC: 600 MHz (14.1 Tesla), AV-I, 5mm TCI (room temperature) probe, with temperature regulation; AAFC: 600 MHz (14.1 Tesla), AV-III, 1.7 mm TXI (room temperature) probe, with temperature regulation. Most spectrometers were equipped with the TopSpin software for data acquisition and processing; the UIC instrument was equipped with an earlier software package, Xwin-nmr (Bruker BioSpin, Billerica, MA).
- (b) *NMR tubes* – Wilmad PP-535 and Bruker Z107374
- (c) *Centrifuge tubes* – VWR: 87003-294 (1.5 ml)
- (d) *Pipettes* – Eppendorf Research
- (e) *Pipette Tips* – VWR sterol/aerosol tips
- (f) *Centrifuge* – Capsule Tomy HF-120
- (g) *Centrifuge* – Eppendorf 5810 R (Eppendorf AG, Hamburg, Germany)
- (h) *Freeze drier* – Edwards Super Modulyo (Thermo Electron Corporation, Waltham, MA)
- (i) *Analytical balance* – Readability of 0.1mg (4 decimal places), Mettler Toledo AG245 (Mettler-Toledo, Columbus, OH) and readability of 0.01mg (5 decimal places), Mettler Toledo AG104 (Mettler-Toledo, Columbus, OH)
- (j) *HPLC system* – 1100 series HPLC-DAD system (Agilent, Palo Alto, CA, USA) consisting of an autosampler, a column thermostat, a quaternary pump, and a diode array detector DAD.

### ***System suitability***

System suitability tests were used to validate the performance of the NMR spectrometer prior to acquiring data on the test samples. The system suitability tests used were: (1) <sup>1</sup>H sensitivity (which measures the signal-to-noise ratio, SINO) using 0.1% ethylbenzene in CDCl<sub>3</sub>, (2) <sup>1</sup>H lineshape, using 1% CHCl<sub>3</sub> in acetone-d<sub>6</sub> (or 0.3% CHCl<sub>3</sub> in acetone-d<sub>6</sub> for CryoProbes), (3) The temperature was confirmed with a 99.8% MeOD temperature standard before instrument operation.

### ***Standard curves***

- (a) *Standard solutions* - Solutions of known chlorogenic acid concentration were prepared by dissolving known weights of dried, solid powder in 600 μL of DMSO-d<sub>6</sub>.
- (b) *Analysis of spectra* – For each spectrum, a signal associated with chlorogenic acid was integrated using AMIX 3.9.14 (Bruker BioSpin, Billerica, MA). The integrals were scaled by the calibrated 90-degree pulse length to account for the physical properties of the sample in accordance with the PULCON principle (Wider & Dreier 2006) and the Q correction method (Burton et al. 2005).
- (c) *Linear regression* – The scaled integrals as a function of chlorogenic acid concentration were plotted in Microsoft Excel 2010 (Microsoft, Redmond, WA) and fitted to a line.

### ***Data acquisition, processing, and analysis***

- (a) *Temperature regulation* - All samples were run on Bruker NMR spectrometers with the temperature calibrated to 298 K. Each spectrometer was equipped with a pre-cooling and temperature stabilization unit for conditioning of gas feeding the probe.
- (b) *Pulse calibration* - A 90-degree pulse calibration was generated automatically using the nutation echo scheme (Wu & Otting 2005) prior to acquiring the data, implemented as the “pulsecal” command in TopSpin.

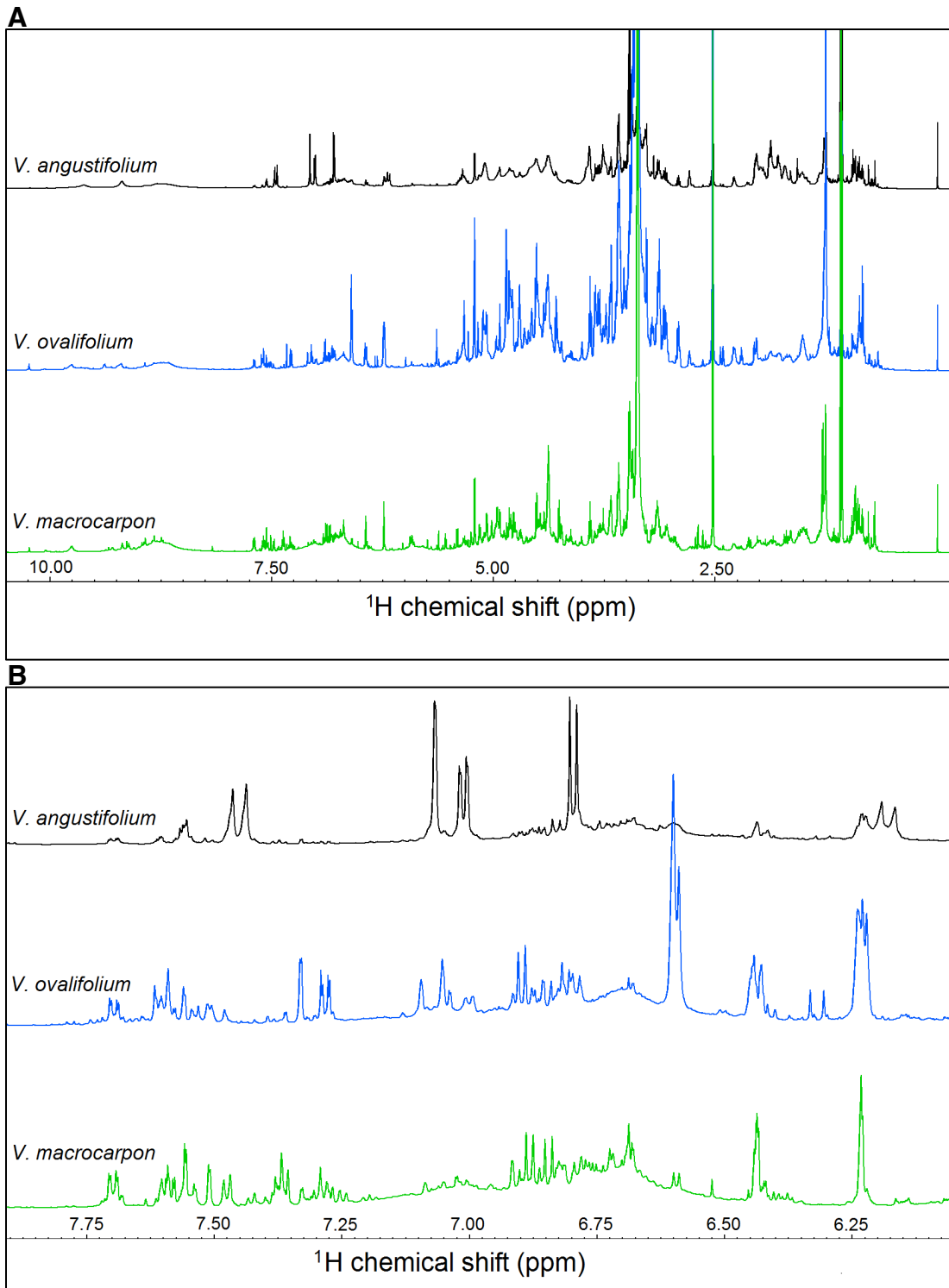
- (c) *Experiment* - Proton NMR spectra were acquired using the one-dimensional proton nuclear Overhauser effect spectroscopy pulse sequence (noesy 1d). For the standard procedure, 64 K data points were collected and the recycle delay was 4 seconds. A total of 64 scans were accumulated, with four dummy scans and a mixing time of 0.01 seconds, for an experiment time of 7:40 minutes.
- (d) *Data processing* - Spectra were processed using the TopSpin software, version 3.1. Free induction decays (fids) were multiplied with exponential line broadening (0.3 Hz) before Fourier transform. Spectra were phased to zero-order automatically using the apk0 command and referenced to DSS at 0.000 ppm.
- (e) *Data analysis* - All spectra were analyzed using AMIX™ software, version 3.9.14 (Bruker BioSpin, Billerica, MA). The spectra were divided into buckets in the chemical shift region from 8.40 to 0.40 ppm with a bucket width of 0.01 ppm. Exclusions were made in the regions of peaks for DMSO (2.66-2.40 ppm), water (3.42-3.23 ppm), ethanol (1.23-0.93, 3.52-3.42, and 4.47-4.31 ppm), methanol (3.23-3.17 ppm), and DSS (0.125 to -0.125 ppm). SIMCA models were generated using tools in Assure-RMS™ (Bruker BioSpin, Billerica, MA).
- (f) *Statistics* - One-way analysis of variance (ANOVA) followed by post hoc Tukey multiple-comparison tests of the data were performed on the PCA scores to indicate whether or not the species groups were significantly different from each other. All results were considered to be statistically significant if  $p < 0.05$ . ANOVA and Tukey multiple-comparison tests were performed using SPSS 19.0 (IBM, Somers, NY). For comparison of the spectra acquired in different laboratories to the reference set for a single species, the Student's t-test was used with two-tailed distribution and two-sample unequal variance (heteroscedastic), as implemented in Microsoft Excel (Microsoft, Redmond, WA).

## Results

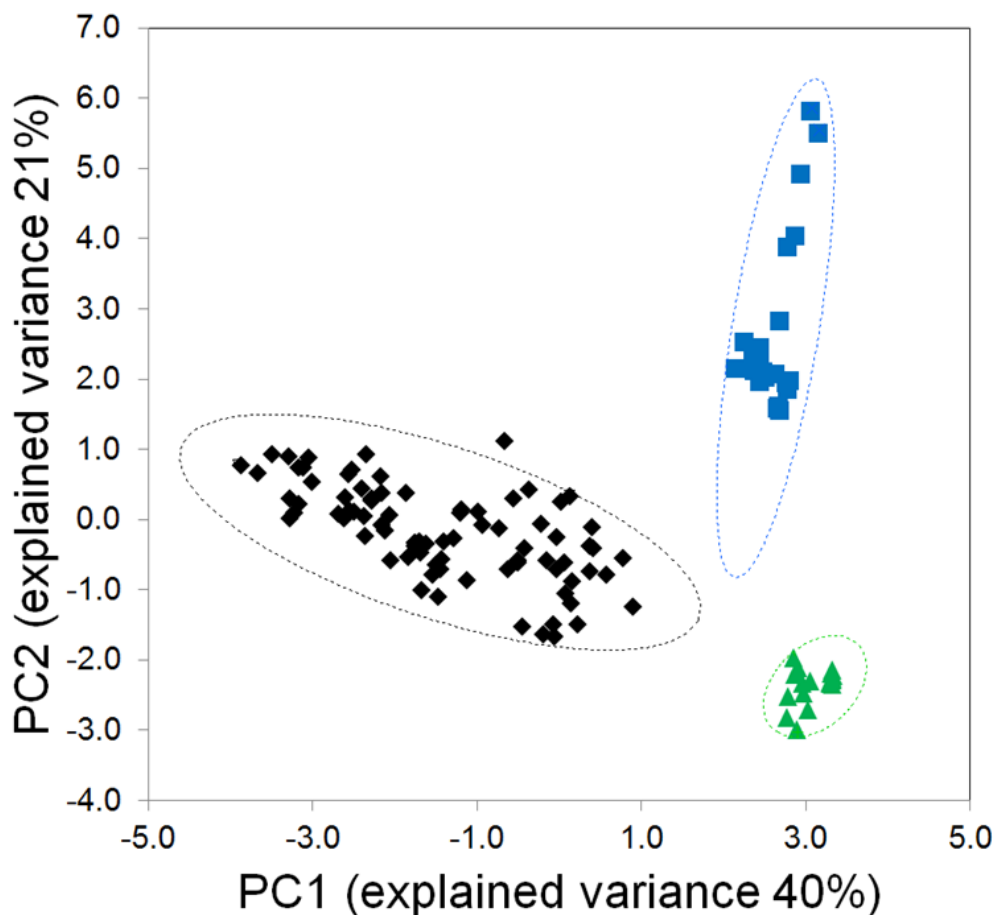
The 1D <sup>1</sup>H NMR spectra of the set of samples available for each species (*Vaccinium angustifolium* – 82 samples, *V. ovalifolium* – 22 samples, *V. macrocarpon* – 19 samples)

were collected at Bruker. The average spectrum for each species is shown in Figure 8.1. Although all the samples represent leaf extracts of plants from the same genus, visual inspection reveals noticeable variations between the species (as shown in Figure 8.1B).

The spectra of the leaf extracts are a complex superposition of many components, represented at high digital resolution to yield many potential variables. Taking advantage of the power of multivariate statistics to handle this type of data, principal component analysis (PCA) was selected to further explore the inter-species differences. The PCA plot comparing *Vaccinium angustifolium*, *V. ovalifolium*, and *V. macrocarpon* is shown in Figure 8.2. Spectra for the three species clearly cluster into three distinct groups, with no overlap between their Hotelling ellipses at the 95% confidence limit. One-way ANOVA, followed by Tukey multiple comparisons, shows that in the PC1 dimension (horizontal axis in Figure 8.2), *Vaccinium angustifolium* (black diamonds) is significantly different ( $p < 0.05$ ) from *V. ovalifolium* (blue squares) and *V. macrocarpon* (green triangles), while *V. ovalifolium* and *V. macrocarpon* are not significantly different from each other ( $p = 0.364$ ). In PC2 (vertical axis in Figure 8.2), all three species show statistically significant differences ( $p < 0.05$ ). Thus these three species can be distinguished by PCA of the NMR spectra using the first two PCs.



**Figure 8.1.** Average spectrum for each species – *Vaccinium angustifolium* (black line), *V. ovalifolium* (blue line), and *V. macrocarpon* (green line) (A) full spectrum (10.5 to -0.25 ppm) (B) expansion in the region containing the characteristic signals for chlorogenic acid (7.90 to 6.05 ppm).



**Figure 8.2.** Scores plot for all three species, *Vaccinium angustifolium* (black diamonds), *V. ovalifolium* (blue squares), and *V. macrocarpon* (green triangles). Hotelling ellipses, showing the region of the 95% confidence limit, are shown for each group by dashed lines of the matching color. Note that spectra for each species occupy a distinct region, with no overlap.

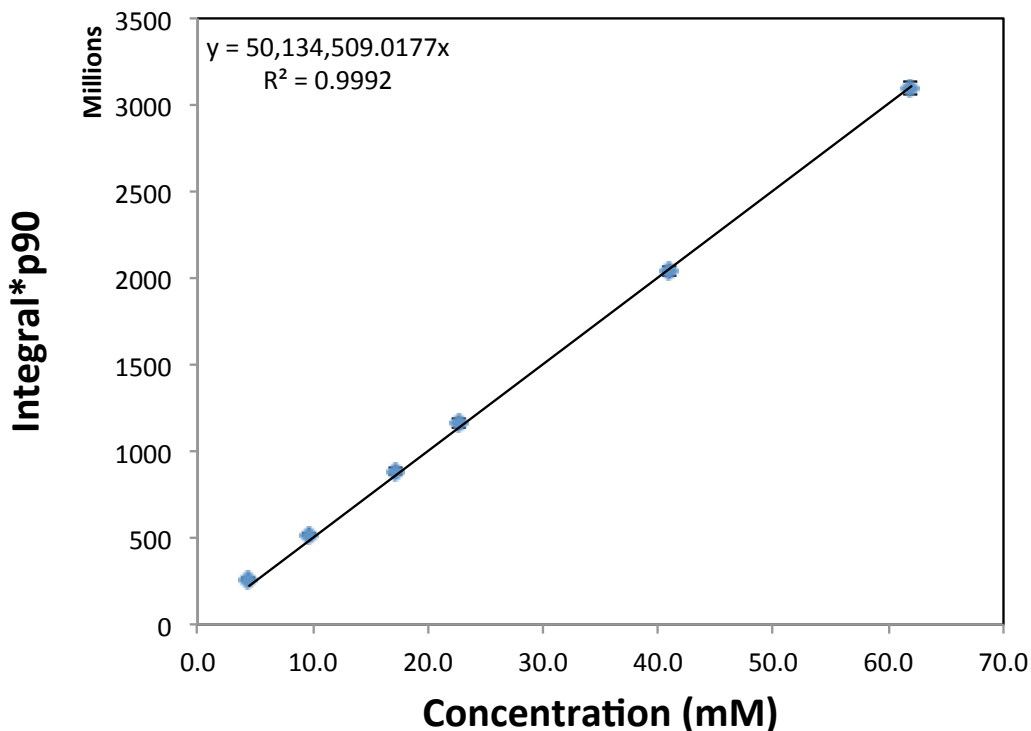
Building on the observation that the *Vaccinium* species can be distinguished based on NMR spectra collected in one laboratory (Bruker), we tested how sensitive the PCA was to differences in the details of the data acquisition. For this purpose, the identical sample of *V. angustifolium* extract was sent to several different laboratories for measurement of the NMR spectrum, which was then used to compare the sample against the set of reference spectra for the three species. Note that the original study design included ten laboratories. However, upon examining the spectra from all sites, we realized there were changes over the time it took to ship the samples and run the spectra, as seen in other plant extract studies (Liu & Murphy 2007). Preliminary assays exploring the activity of the blueberry extracts suggest the activity decreases with time dissolved in DMSO over a time frame similar to that

observed in this study (J.F.). In addition, it is well established that DMSO, despite its universal application, does not necessarily represent an inert solvent for (q)NMR applications (Pauli et al. 2012, 2005). Therefore, the present study considered only the spectra that were recorded within the first month of dissolving the sample in DMSO-d<sub>6</sub> to yield five participating laboratories. These laboratories demonstrated their experimental proficiency by measuring a standard curve for chlorogenic acid, one of the compounds identified in blueberry leaf extract (Figure 8.3). The integrated area of a chlorogenic acid peak in the NMR spectrum, scaled by the 90-degree pulse length according to the PULCON (Wider & Dreier 2005) and probe Q (Burton et al. 2005) principles, increases linearly with the concentration of chlorogenic acid. This linearity of signal strength with sample concentration is a basic property of NMR (Pauli 2005). In practice, study participants observed a very tight correlation, with R<sup>2</sup> coefficients of 0.9782 and higher (Table 8.1).

**Table 8.1.** Comparison of the experimental configuration at different laboratories in this study. Sites: Bruker: Bruker BioSpin Corporation, Billerica, MA, DHMRI: David H. Murdock Research Institute, Kannapolis, NC (there are two spectrometers at this site), NRC IMB: NRC Institute for Marine Biosciences, Halifax, Nova Scotia, UIC: University of Illinois at Chicago, Chicago, IL, AAFC: Agriculture and Agri-Foods Canada, Charlottetown, Prince Edward Island.

Feature	Bruker	DHMRI-1	DHMRI-2	NRC IMB	UIC	AAFC
R <sup>2</sup> for standard curve	0.9999	0.9782	0.9992	0.9987	0.9990	0.9998
Signal/noise COA peak*	3620.3	3696.6	3689.1	1464.2	673.5	492.0
Field strength (MHz)	600	700	600	700	600	600
Console	AVIII	AVIII	AVIII	AVIII	AVI	AVIII
Probe	TCI	QNP	DCH	TCI	TCI	TXI
CryoProbe	yes	yes	yes	yes	no	no
Sample diameter (mm)	5.0	5.0	5.0	1.7	5.0	1.7
Software	TopSpin 3.1	TopSpin 2.1	TopSpin 2.1	TopSpin 2.1	Xwin-nmr	TopSpin 2.1

\*doublet at 6.797 ppm in the same sample of *Vaccinium angustifolium*.

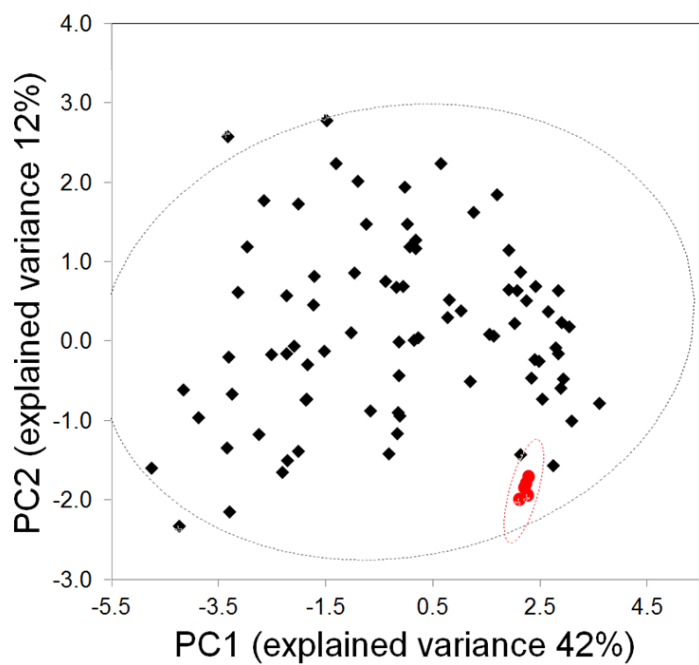


**Figure 8.3.** Representative calibration curve (from the David H. Murdock Research Institute's 600 MHz spectrometer). The plot shows integrated peak area, scaled by the 90-degree pulse length, versus the chlorogenic acid concentration. The data were fit to a line with a y-intercept of zero. The equation for the line and the square of the correlation coefficient ( $R^2$ ) are shown.

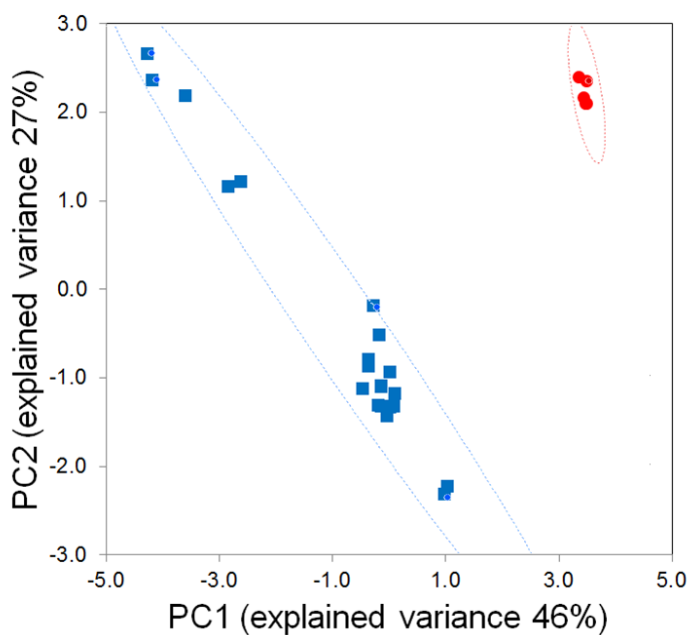
The *Vaccinium angustifolium* spectra acquired at different sites were compared against the *V. angustifolium* spectra acquired at Bruker (Billerica, MA), as shown in Figure 8.4A. The spectra collected at different sites (red circles) were well within the 95% confidence limits for the set of *V. angustifolium* spectra acquired at Bruker (black dotted line). The Student's t-test returned a low probability that the test spectra are from the same distribution as the *V. angustifolium* set, likely because the test spectra were acquired on one sample and cluster very tightly. Figure 8.4B shows the comparison of the test *V. angustifolium* spectra against the *V. ovalifolium* spectra collected at Bruker. The test spectra form a distinct cluster, separate from the *V. ovalifolium* spectra, as clearly visualized by the non-overlapping Hotelling ellipses for the data sets. Due to the distribution of values for *V. ovalifolium*, this separation is statistically significant ( $p < 0.05$ ) for both PC1 and PC2 in the Student's t-test. Figure 8.4C shows the comparison of *V. angustifolium* against *V.*

*macrocarpon*. Again, the scores plot of PC2 versus PC1 clearly suggests distinction between the test sample and the *V. macrocarpon* samples acquired at Bruker. For *V. macrocarpon*, the distinction based on PC1 is statistically significant in the Student's t-test ( $p < 0.05$ ), which corresponds to the observed separation in the horizontal axis in Figure 8.4C.

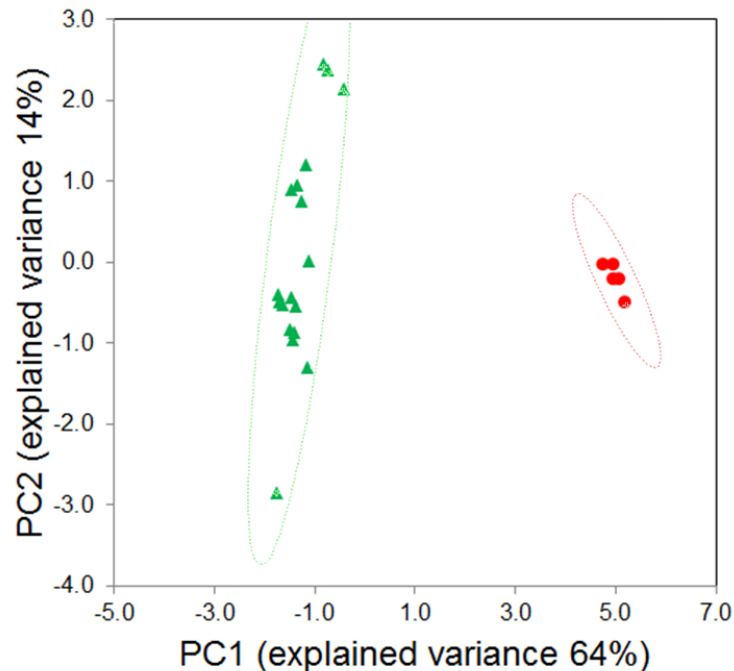
**A**



**B**



C



**Figure 8.4.** Scores plots for representative spectra for each species, with the spectra acquired in different laboratories included. In all three panels, spectra of the identical *Vaccinium angustifolium* sample sent to different laboratories are represented by red circles. Hotelling ellipses, showing the region of the 95% confidence limit, are shown for each group by dashed lines of the matching color. (A) Scores plot for *V. angustifolium* with the spectra acquired in different laboratories included. Spectra of the *V. angustifolium* samples collected at Bruker are represented by black diamonds. Note that the ellipse for the spectra acquired in different laboratories is almost completely within the ellipse for the diverse *V. angustifolium* sample set. (B) Scores plot for *V. ovalifolium* with the spectra acquired in different laboratories included. Spectra of the *V. ovalifolium* samples collected at Bruker are represented by blue squares. Note that the ellipse for the spectra acquired in different laboratories is well-separated from the ellipse for the *V. ovalifolium* samples. (C) Scores plot for *V. macrocarpon*, with the spectra acquired in different laboratories included. Spectra of the *V. macrocarpon* samples collected at Bruker are represented by green triangles. Note that the ellipse for the spectra acquired in different laboratories is well-separated from the ellipse for the *V. macrocarpon* samples.

The features of the spectra acquired in different laboratories, i.e. – tight clustering in the (PC1, PC2) scores plots, grouping within *Vaccinium angustifolium* but not *V. ovalifolium* or *V. macrocarpon* – are all the more striking when the differences in the instrumentation at each site are considered (Table 8.1). Spectra were acquired at two different fields strengths (600 versus 700 MHz), with the accompanying difference in sensitivity attributable to field strength (26% higher at 700 MHz), resolution (17% higher at 700 MHz), and observed couplings (their fixed values in hertz show different values in ppm after scaling by field

strength). The spectra were all acquired with NMR consoles of the Avance (AV) spectrometer series which feature digital electronics and advanced radio frequency (RF) signal generation, manufactured within a 10 year time frame. Most spectra were acquired with the AVIII generation which features an enhanced digital receiver and pulse programmer. The AVI and AVIII consoles differ in the baseline linearity that was compensated for by manual baseline correction of the spectra from the AVI console. The spectra were acquired with a variety of probes, some optimized for  $^1\text{H}$  sensitivity and lineshape (inverse triple resonance probes: TCI and TXI), some optimized for detection of multiple nuclei (observe dual and quad nucleus probes: DCH and QNP). Some of the probes were cryogenically cooled (CryoProbes at Bruker, DHMRI-1, DHMRI-2, and NRC IMB) to reduce noise and enhance sensitivity. Also, the probes accommodated two different diameter sample tubes, 5 mm and 1.7 mm. The larger probe diameter allows measurement on higher volume samples, but the smaller diameter probe benefits from higher sensitivity and better magnetic field homogeneity over a smaller sample. Even the spectrometer operating software varied, in one case using the Xwin-NMR package compared to the various versions of the TopSpin software (Bruker Biospin). This demonstrates that NMR produces the same robust result for the same sample across a variety of measuring conditions.

Since the species can be distinguished based on PCA, we built soft independent modeling of class analogies (SIMCA) models for each species. SIMCA has the advantage that it is based on the same spectra used in the PCA without further assumptions. SIMCA has no implicit assumption of non overlapping classes, so in principle a test sample can belong to multiple classes (Harris 1986). After building a SIMCA model for each species, the set of spectra for each species was tested against each model. The results are summarized in Table 8.2. For *Vaccinium ovalifolium* and *V. macrocarpon*, all the spectra used to build the model classify within the model (100%). For *V. angustifolium*, the model based on 82 spectra fits most of the spectra, with 77 (94%) classified as within the model. Note that there is no confusion among the species using these models (0% for the off-diagonal elements in Table 8.2).

**Table 8.2.** Classification of the *Vaccinium* species. A SIMCA model was generated for each species. Then, samples from each species were tested against that model. The percentage of samples (rows) that test within the model (columns) are reported.

Species	<i>V. angustifolium</i>	<i>V. ovalifolium</i>	<i>V. macrocarpon</i>
<i>V. angustifolium</i>	94%	0%	0%
<i>V. ovalifolium</i>	0%	100%	0%
<i>V. macrocarpon</i>	0%	0%	100%

### Conclusion

Using crude extracts of botanically-defined *Vaccinium* spp. as a test case, the current study shows that the NMR spectrum of a chemically complex sample is a characteristic feature of that sample, independent of the details of the measurement within the variation tested here. Spectra acquired at five different sites with differences in field strength, console, probe, sample diameter, and software clustered tightly together in the PCA. Comparison of these spectra against sets of reference spectra for three species acquired at yet another site allowed correct identification of the species of the test sample.

The study also shows that NMR is a valuable tool for identifying plant materials by spectroscopic fingerprinting. Specifically, it was possible to distinguish different *Vaccinium* species using PCA of the 1D <sup>1</sup>H spectra of samples of leaf extract. Currently there are a sufficient number of samples to serve as inclusivity panels (Harnly 2012) for *V. angustifolium* (lowbush blueberry), *V. ovalifolium* (oval leaf huckleberry) and *V. macrocarpon* (cranberry) and build models. Preliminary assessment of spectra from additional species of blueberry and bilberry (such as *V. corymbosum*, *V. boreale*, and *V. caespitosum*) suggest that additional species can also be distinguished by the same NMR method. Further results will be reported in due course.

## Chapter 9

# General Discussion

This dissertation presented three ethnobotanical studies in partnership with the Eeyou Istchee Cree, the Lukomir Highlanders, and the Q'eqchi' Maya, and found a strong cross-cultural phylogenetic signal with the antidiabetic genus *Vaccinium*. *Vaccinium*, outgroups, and other high ranked antidiabetic taxa collected from Eeyou Istchee Cree, Lukomir Highlander, and Q'eqchi' Maya floras were assessed for antidiabetic activity. *Vaccinium* species were the most active group in our diabetes target assay (advanced glycation endproduct (AGE) inhibition assay). *Vaccinium* and high ranked antidiabetic taxa presented as important natural health products, and traditional pharmacologic use and activity of antidiabetic taxa from the three communities (in current and historical contexts) demonstrated the need for phytochemical analyses. We determined the presence, absence, and quantities of metabolites from the numerous antidiabetic taxa to characterize active components of extracts and to solve ethnographic problems using <sup>1</sup>H NMR, UPLC MS-QTOF, and HPLC-MS. We then designed a taxonomic NMR metabolomics methodology for systematics and medical quality control of the *Vaccinium* genus and were able to discriminate between closely related genera, subgenera, species, phenotypes, and collections made across a growing season. Nuclear magnetic resonance spectra of three *Vaccinium* spp. test samples were acquired at multiple sites with known apparatus differences in field strength, console, probe, sample diameter, and software versions. Comparison of multi site test results demonstrated tight clustering in a principal component analysis, indicating a highly reproducible taxonomic quality control method.

This was the first presentation of a general ethnobotany and a diabetes focused ethnobotany with the Lukomir Highlanders of Bosnia and Herzegovina. This is also the first diabetes focused ethnobotany with the Q'eqchi' Maya of Belize. Our work with the Q'eqchi' was able to define the botanical typographic inspiration of the Maya word '*k'an*' and the implications of the word and the 'precious' plant has in Classic and contemporary Maya culture.

The cross-cultural genus here, *Vaccinium*, the vastly important economic taxon, served as a model for a different way of performing taxonomy. We now know that using our NMR metabolomics methods can work as a taxonomic tool and offers a rigorous systematic quality control technique to the natural health product industry.

### ***Section 1: Ethnobotany***

Section 1 addressed our longstanding partnerships with the Eeyou Istchee Cree, the Lukomir Highlanders, and the Q'eqchi' Maya communities, all of whom remain concerned about diabetes and the preservation of traditional medical knowledge. An antidiabetic ethnobotany of the Eeyou Istchee Cree was published by Leduc et al. (2006) and three comparable studies with the Lukomir Highlanders and Q'eqchi' Maya were presented in chapters two, three, and four. Ethnobotanical studies were completed in Lukomir, Bosnia and Herzegovina and Toledo District, Belize for cross-cultural analyses with the Eeyou Istchee Cree to understand the context of diabetes and to elucidate antidiabetic taxa with a strong phylogenetic signal and cross-cultural consensus.

### **Hypothesis one**

In chapter one, I hypothesized that traditional heart disease and panacea (recommended for diabetes) treatments would be high frequency use categories since Lukomir's visiting physician in chapter two listed the communities' priority health concerns as (1) heart disease and (2) diabetes. In chapter three, diabetes treatments were described as panacea use reports since diabetic insults affect many tissues and organs, leading to symptoms like frequent urination, neuropathy, and slow healing sores. Circulatory system disorders included use reports for taxa that treated heart trouble.

The frequency of use reports per category (Figure 2.3) marked panacea and circulatory system disorders as high frequency categories. Species in panacea treatments were highly sought after by the Highlanders and often cross listed as economically important food plants. High requests for panacea treatments by the Lukomir community are likely linked to both heart disease and diabetes, and are likely supplemented with medicinal foods to address Lukomir's physician's concerns. Circulatory system disorders were another high

frequency category. The Highlander healers' plant use for "heart trouble" aligns with the visiting physician's highest concern.

Frequency of taxa per usage categories (Figure 2.4) indicated that panacea, circulatory system disorders were medium frequency categories. Medium frequency categories according to Amiguet et al. (2005), mark chronic or recurring health conditions that present more often than low frequency categories.

Finally, informant consensus factors ( $F_{ic}$ ) per usage category (Figure 2.5) ranked circulatory system disorders first and panacea second which matched the physician's rank top health concerns (heart disease and diabetes). These  $F_{ic}$  results indicate that these plants are well known in a well-defined community-based traditional knowledge system. When consensus is high, these categories are more likely to be active in condition-related pharmacological assays (Leaman et al. 1995). The use of diabetes and heart disease plants was foreshadowed by the physician and likely exacerbated by post war development conditions illustrated in chapter two.

I accepted hypothesis one since these results indicated that Highlanders recognize chronic or recurring symptoms of diabetes and heart disease, as their physician has, and are selecting plants from a well defined and presumably effective traditional pharmacopoeia.

#### Cross-cultural ethnobotany and type 2 diabetes

In this multilateral partnership, our focus on plant treatment commonalities (derived using quantitative cross-cultural ethnobotanical analysis) supported elucidation of safe and effective antidiabetic plants for ethnopharmacological and phytochemical analysis in section two. The ethnobotanical syndromic importance value (SIV) function was completed by working with clinicians at the Ottawa Hospital to produce association weight values ( $w$ ) for symptoms highly associated with diabetes. Leduc et al. (2006) presented the symptoms without  $w$  values which were needed to calculate the SIV value. We then conducted a cross-cultural analysis of our partner Eeyou Istchee Cree, Lukomir Highlander, and Q'eqchi' Maya antidiabetic floras to elucidate taxa with strong phylogenetic signal (SIV values) and cross-cultural consensus.

Of the three biogeographic regions, in relation to each other, we described the Eeyou Istchee territory to be of low biodiversity with 18 SIV species; the Lukomir Highlander territory of medium diversity with 41 SIV species, and the Q'eqchi' Maya territory of high biodiversity with 150 SIV species (Table 9.1). This comparison demonstrates a two and three fold for each increase of diversity. One major difference between each study is the number of informants. The Q'eqchi' Maya with approximately seven times fewer interviewees, produced approximately eight times more SIV citations (Table 9.1). This is indeed a testament of biodiversity wealth of Belize, and the intense pharmacopeial knowledge of the Q'eqchi' Maya.

In a cross-cultural ethnographic analysis of the three study sites (Table 9.1), one can notice that the Eeyou Istchee Cree have a more “modern” occupation sense and a diabetes prevalence of approximately 18%. The Lukomir Highlanders and Q'eqchi' Maya are much more traditional in an occupational sense and while diabetes is a major health concern in these communities, the prevalence of diabetes is less than half that of the Eeyou Istchee Cree. These data seem to mark a case of diabetes being a “lifestyle disease” (Rapinski 2013) where a change in occupation may be a strong factor in influencing traditional activities, income, and dietary acculturation. As welfare and office jobs have likely exacerbated the diabetes prevalence among the Cree, this case may foreshadow the future of modernizing traditional societies (Kuhnlein & Turner 1991 and 2009), including the Lukomir Highlanders and the Q'eqchi' Maya communities.

Seven consensus families emerged across the three study sites. The Eeyou Istchee Cree antidiabetic flora (Leduc et al. 2006) and the Lukomir Highlanders antidiabetic flora (chapter three, Ferrier et al. 2013) shared two consensus families: Ericaceae and Rosaceae. The Lukomir Highlanders and the Q'eqchi' Maya (chapter four) shared five consensus families: Aristolochiaceae, Asteraceae, Euphorbiaceae, Fabaceae, and Solanaceae. There were zero consensus families recorded between the Eeyou Istchee Cree and the Q'eqchi' Maya. *Vaccinium* of the Ericaceae was the only cross-cultural consensus genus. There was one consensus species, *V. vitis-idaea* L., which was used by three Cree healers and two Lukomir Highlander healers for slow healing infections and urinary infections. *V.*

*angustifolium* Aiton was used by three Cree healers for frequent headaches and *V. myrtillus* L. was used by four healers as a panacea and gynaecological aid. *Vaccinium* SIV factors from Lukomir ranked *V. myrtillus* 8/41 and *V. vitis-idaea* 9/41 (Table 3.1, Figure 3.S5). *Vaccinium* SIV factors from the Cree survey ranked *V. angustifolium* 11/15 and *V. vitis-idaea* 13/15 (Leduc et al. 2006). *Vaccinium* were prioritized for section two metabolomic analyses due to the phylogenetic signal and cross-cultural generic specificity. Also, a pharmacological analysis of consensus Q’eqchi’ Maya healer and SIV ranked species were assayed.

**Table 9.1.** An ethnographic cross-cultural summary table.

Community	Floristics	Ethnographics	Consensus taxa
Eeyou Istchee Cree	SIV species: 18 Biogeography: alpine, Taiga Biodiversity: low	Informants: 34 Collection: August-September Diabetes prevalence: 17.7% Occupation: mining, government, hunting gathering	Families: Ericaceae, Rosaceae Genera: <i>Vaccinium</i> L. Species: <i>V. vitis-idaea</i> L.
Lukomir Highlanders	SIV species: 41 Biogeography: alpine, Mediterranean, continental Biodiversity: medium	Informants: 25 Collection: June-August Diabetes prevalence: 7.54% Occupation: pastoralists, agriculturalists	Families: Aristolochiaceae, Asteraceae, Ericaceae, Euphorbiaceae, Fabaceae, Rosaceae, and Solanaceae Genera: <i>Vaccinium</i> L. Species: <i>V. vitis-idaea</i> L.
Q’eqchi’ Maya	SIV species: 150 Biogeography: Tropical Biodiversity: high	Informants: 5 Collection: all year Diabetes prevalence: 8% (Natives) Occupation: pastoralists, agriculturalists	Families: Aristolochiaceae, Asteraceae, Euphorbiaceae, Fabaceae, and Solanaceae. Genera: NA Species: NA

### Inhibition of advanced glycation endproducts

Both *Vaccinium myrtillus* and *V. vitis-idaea* leaves showed linear concentration dependent inhibition of advanced glycation endproducts (AGEs) with activity higher or comparable to the positive control quercetin (Table 3.2). As reported in chapter three, *V. myrtillus* had an average  $IC_{50}$  of  $12.4 \mu\text{g}\cdot\text{mL}^{-1}$ , and *V. vitis-idaea* had an  $IC_{50}$  of  $48.6 \mu\text{g}\cdot\text{mL}^{-1}$ . *Vaccinium* spp. herein had approximately 61% greater activity than outgroup *Arctostaphylos uva-ursi* (L.) Spreng ( $IC_{50} = 100.2 \mu\text{g}\cdot\text{mL}^{-1}$ ). These results were comparable to  $IC_{50}$ 's from wild samples of *V. angustifolium* leaves (McIntyre et al. 2009) ranging from 4.8 to  $10.6 \mu\text{g}\cdot\text{mL}^{-1}$  with a seasonal mean of  $6.3 \mu\text{g}\cdot\text{mL}^{-1}$ . In chapter five, *Vaccinium posanum* Donn. Sm. was the most active taxon ( $IC_{50} = 4.2 \mu\text{g}\cdot\text{mL}^{-1}$ ) in an environmental and date controlled study consisting of numerous tropical specimens (chapter 5, Ferrier et al. 2012). Considering this comparison, the Highlanders' traditional use of *V. myrtillus* (*Borovnica*) is appropriately described as a panacea remedy, may be an effective traditional method of preventing AGE related insults, and should be studied clinically.

In chapter four, a phylogenetic selection of Q'eqchi' Maya SIV ranked plants were forwarded to the inhibition of advanced glycation endproducts (AGEs) assay. Out of 150 taxa, *Tynanthus guatemalensis* was the most recommended treatment for patients with diabetes (Table 4.S1). The SIV system placed *T. guatemalensis* 4th of 150 taxa (Table 4.S2). Inhibition of AGEs ranged from the most effective, *Tynanthus guatemalensis* ( $IC_{50} = 40.8 \mu\text{g}\cdot\text{mL}^{-1}$ ), to, *Bixa orellana* L. ( $IC_{50} = 733.3 \mu\text{g}\cdot\text{mL}^{-1}$ ), while the outgroup, a traditional birth control medicine, was the least effective: *Dioscorea hondurensis* R. Knuth ( $IC_{50} = >10,000 \mu\text{g}\cdot\text{mL}^{-1}$ ) (Figure 4.2). The activity for *T. guatemalensis* was comparable to that reported for six tropical species of *Vaccinium* spp. (Ferrier et al. 2012), but more than 200 times more active than reported for North American ginseng root extract,  $>10 \text{ mg}\cdot\text{mL}^{-1}$  (McIntyre et al. 2011).

### Hypothesis two

In chapter one, I hypothesized that taxa with strong phylogenetic signal and cross-cultural consensus would be more effective in preventing the formation of advanced

glycation endproducts (AGEs) than other ethnobotanical taxa or outgroups. In Table 9.2 one can notice that consensus plays an important role in elucidating anti-AGE taxa. *Tynanthus guatemalensis* was a top ranked healer plant and was the most active of all tested Q'eqchi' plants. *Vaccinium* had a high SIV phylogenetic signal and was the only cross-cultural genus in this study. Compared with a number of taxa within the genus, *V. poasanum* (from Costa Rica) was had the greatest activity at the lowest concentrations among all plants tested in this dissertation. Although this study was not an exhaustive study of all antidiabetic taxa from the three study sites, I still accept hypothesis three since *Vaccinium* spp. were the most active plants.

**Table 9.2.** Inhibition of advanced glycation endproducts by top ranked antidiabetic plants. Taxa presented here were included here by way of strong phylogenetic signal and, or, cross-cultural consensus.

Sample group	Consensus Antidiabetic Taxon	Lowest IC <sub>50</sub>
Eeyou Istchee Cree	<i>Vaccinium angustifolium</i>	4.8 µg·mL <sup>-1</sup>
Lukomir Highlanders	<i>Vaccinium myrtilus</i>	12.4 µg·mL <sup>-1</sup>
Q'eqchi' Maya	<i>Tynanthus guatemalensis</i>	40.8 µg·mL <sup>-1</sup>
Vander Kloet <i>Vaccinium</i> Garden	<i>Vaccinium poasanum</i>	4.2 µg·mL <sup>-1</sup>
Outgroups	<i>Arctostaphylos uva-ursi</i>	100.2 µg·mL <sup>-1</sup>
	<i>Disterigma rimbachii</i>	13.6 µg·mL <sup>-1</sup>
	<i>Dioscorea hondurensis</i>	>10000 µg·mL <sup>-1</sup>
	<i>Gaylussacia baccata</i>	51.4 µg·mL <sup>-1</sup>

*Vaccinium* were given priority in section two since they were demonstrated to contain more effective antidiabetic plants and because the process would allow a more efficient transfer of antidiabetic technology. The AGE assay was selected for evaluation of these taxa since the experiment indicates effectiveness in preventing diabetic related AGE formation which contributes to the development of retinopathy, cataracts, atherosclerosis, neuropathy, nephropathy, and diabetic embryopathy and impaired wound healing.

### **Section 2: NMR metabolomics**

The objective of this section was to use NMR to develop A) an NMR metabolomics method for taxonomic identification of *Vaccinium* taxa first in a single lab study, then

validated in a multi-lab study and B) a method for quantitative determination of marker phytochemicals in *Vaccinium*. Taken together, the NMR metabolomics technology provides new methods for identity and quality assurance of *Vaccinium* based natural health products.

### Hypothesis three

In chapter one, I hypothesized that NMR spectra of leaves will discriminate taxa according to current taxonomic circumscriptions since there exists evolutionary distance between common ancestors among sampled taxa. I accepted this hypothesis since I was able to discriminate between metabolomic leafprints of *Vaccinium* at the generic, subgeneric, specific, and phenotypic levels for numerous samples.

Taxonomic metabolomics is a new area of research, and all entries at this point are pioneering, solving problems in methodology, and moving the field forward. Recently, Brown et al. (2012a) conducted a pioneering metabolomics study of three *Vaccinium* species. Using UPLC-MS-QTOF the work focused on the analysis of *Vaccinium* fruit and developed a targeted chemometric analysis that supports recognition of cranberry taxa. What is intriguing in their analyses, using two or more principal components, is that their fruit samples of *Vaccinium macrocarpon* Aiton and *V. oxycoccus* L. (two similar plants with similar bog habit, vines, section *Oxycoccus*) seem to cluster in some cases, closer than *V. vitis-idaea* (rock and montane habitat, a rhizomatous shrub, section *Vitis-idaea*). A systematic grouping of the cranberries within subgenera would follow section *Oxycoccus*: *V. macrocarpon* and *V. oxycoccus*; and section *Vitis-idaea*: *V. vitis-idaea*. Inspecting the Brown (2012a) data and the Ph.D. thesis (Brown 2011), one can notice that when Brown combines members of the opposite sections, the number of unique compounds is always greater than those in *V. macrocarpon* and *V. oxycoccus*. With a systematic grouping, one may be able to discriminate the species according to sections and rationalize the presence of unique compounds according to the subgeneric delineations of sections *Oxycoccus* and *Vitis-idaea*.

Resolving taxa using metabolomics is not always clear. While metabolomics, using NMR and UPLC-MS-QTOF, is proving to be a useful tool for total evidence approaches in systematic methods, the standard alternative is DNA barcoding. Barcoding is a quick and

cost effective method of identifying species. Starr et al. (2009) investigated 34 species of the cosmopolitan *Carex* using proposed plant barcoding loci (*matK*, *rbcL*, *rpoC1*, *rpoB*, *trnH-psbA*), to find that a single or multi locus approach did not achieve greater than 60% resolution. This relatively low resolution seems to be the case for large international systematic inquiries in metabolomics and DNA barcoding. In most cases however, international levels of analysis are not usually required, except for cases of method development and proof on concept investigations. Most systematic research endeavours will deal with regional flora specimens where both metabolomics and DNA barcoding work very well.

Standard operating procedures are important for establishing reproducible results in metabolomics based taxonomy, or with metabolomics based quality control procedures in the food and drug industry. One should use an outgroup or regional control in order to account for the variability within the taxa in question, for instance, three taxa from one region would be preferable, not three taxa from three different locations since discrimination seems entirely plausible given the taxa were sampled from three regions and then compared to each other.

### Organ collection

Most genetic studies use leaves since they contain evolutionarily stable loci related to chloroplasts and ribosomes. Leaves are often the most available organ, and collection of this organ causes the least harm, is more sustainable, and would allow for uniform sampling of herbarium vouchers, than roots would, which are often missing from the voucher, and stems, which pose a problem with maturity and chemical variation and therefore not have the comparison ruggedness. Since mature stems may be woody, there is also a good chance that the stem sample would not be accessioned. Fruits may present difficulties given monoecious and dioecious character states of some taxa, but in turn may help resolve taxa when leaves are not sufficient. Leaves also contain a larger diversity of secondary metabolites than fruit (J.T. Arnason personal communication).

Collection dates are also important. Our seasonal studies show a “right to left” trend on the x axis across the growing season. There is also an “up and down” sample variation on the Y axis, which is wide in the beginning of the season and much narrower towards the end of season (see Figure 6.7). For this reason and others, botanical garden collections are very valuable since gardens provide numerous taxa growing in a common environment (controlling for environment) and samples are collectable on the same date.

Below, I provide a list of suggested standard operating procedures, that should be considered in the design of metabolomics based taxonomic studies.

Suggested metabolomics based taxonomy standard operating procedures:

1. Treatment of taxa should follow accepted and referenced circumscriptions ([www.tropicos.org](http://www.tropicos.org)) to the fullest extent possible.
2. As many taxa as possible should be sampled from the same region or closest proximity possible, with a highly related control outgroup taxa.
3. Sample populations should be reproductively isolated from comparison populations.
4. Leaf samples are suggested as the taxonomic standard in organ sampling. Other organs may be suitable in more specific studies or necessary to resolve some taxa.
5. The higher the number of taxon samples from a population the better. If sample size is a problem, perhaps making multiple samples from at least one specimen is a way forward.
6. Outgroup taxa should be selected with a close phylogenetic relationship, from similar habitat, and with similar habit.
7. Sample extraction should follow a method that allows for the widest spectrum of chemistry to be observed, until principal components have determined that targeted extractions are suitable.
8. Samples should be redissolved at random and be in solution for equal lengths of time prior to metabolomic analysis.

The single lab NMR validation study shows a timely, simple, accurate method for monitoring chlorogenic acid content directly from crude blueberry leaf extracts. These results are also highly reproducible across multiple sites with known differences in NMR field strength, console, probe, sample diameter, and software. Minimal sample handling, and direct quantification from mixtures were chosen to minimize sample handling. NMR provides a means of directly calculating the concentration of constituents and is considerably simpler and less time consuming than comparable HPLC methods (e.g. Harris et al. 2007). NMR quantitation does have limitations in that it is less sensitive and is reliable for quantification of fewer compounds than HPLC.

### **Ways forward**

Nuclear magnetic resonance spectra worked well for conducting *Vaccinium* systematic analysis and determination. The *Vaccinium* are a large genera of approximately 500 species and can be a problematic and difficult genus to identify or source due to their cohabitation. Nuclear magnetic resonance imaging is a rapid and useful tool for distinguishing between specimens which, at the time of collection, are sometimes without reproductive parts or other operational taxonomic units necessary for morphological taxonomy, or sample groups which are not well delineated, difficult to identify, or easily confused (e.g. *Vaccinium* and *Piper* species). This technique will become an important tool for identification and quality control of plants for scientific inquiry and quality control of natural health products. I will continue on this theme of inquiry for disease diagnosis and treatment.

Collaboration is an important way to make strides in research and discovery. Interdisciplinary collaboration worked very well in our metabolomics studies but also in our cross-cultural ethnobotany studies. Ethnobotanical collaboration allowed our team to realize and demonstrate ways in which *Vaccinium* are important in traditional systems of food and medicine, especially in the context of diabetes. After realizing the global importance of *Vaccinium*, we transferred species of the complex genus to our metabolomics models to deliver a strong case for the promise NMR metabolomics in food and drug quality control and for taxonomic inquiry. Here, cross-cultural collaboration linked three communities to a

project grounded in both the humanities and sciences; an interdisciplinary approach utilizing avenues of anthropology and languages, international development, biology, taxonomy, pharmacology and medicine, chemistry, physics, and industry. I believe that great things can and will happen at the intersection of the humanities and sciences.

While interdisciplinary collaboration enables great possibilities, the planning process can be highly complex, especially in precede-proceed research model development. There were a number of items I felt were important to stakeholders, that researchers, project and community leaders should consider in their preceding responsibilities. For instance, how should intellectual knowledge be handled when discoveries and data have economic value?

Take, for example a case of a communities' ethnobotanical resources published in ways that do not conceal intellectual knowledge. The ability for a community to patent and pursue their intellectual property (developed over millennia) and develop culturally relevant startup business and job opportunities is diminished. I believe that these opportunities should be considered, especially in a time when the need for self driven culturally relevant manufacturing, education, and health programs are in demand by native communities, Canada and abroad, especially ones that have adopted cash based economies.

Alain Cuerrier noted that one of the great accomplishments of the CIHR Team for Aboriginal Antidiabetic Medicines (CIHR TAAM), lead by the Eeyou Istchee Cree and Pierre Haddad, was that the interdisciplinary collaboration between Cree and CIHR TAAM participants elicited the need to start recalling pharmacological and other scientific terminology in the Cree language! Elders and healers were able to recall the past to embrace the future. How can natives or first nations embrace health and medicine, or as Kuhnlein and Turner point out, food security and sovereignty, if they are not masters of their own destiny and in touch with their linguistic heritage, as the Coady International Institute and Wade Davis tirelessly illustrate? Not one of Canada's native languages are official – the official languages are Germanic and Romance. An interesting way forward being considered and pursued by Canada's aboriginal leaders and communities is the right to practice self government. Could this be a way to embrace native heritage and inspire a self sustaining future? Time will tell.

“Don’t only practice your art, but force your way into its secrets, for it and knowledge can raise men (and women) to the divine.” - Ludwig van Beethoven

“Diabetes will end when there is sweetness in our souls.” - Cree elder



**Figure 9.1.** Canadian Institutes of Health Research Team for Aboriginal Antidiabetic Medicine Team for Aboriginal Antidiabetic Medicine (CIHR TAAM) longhouse meeting in Misstissini, Eeyou Istchee Territory, Quebec, Canada.

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