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ANTI-DIABETIC AND NEUROPROTECTIVE ACTIVITIES OF PHYTOCHEMICALS IN TRADITIONALLY USED BOREAL PLANTS

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ABSTRACT

This thesis investigated the phytochemistry and pharmacology of boreal plant species used traditionally as treatments for symptoms and complications of diabetes and was completed in collaboration with the Team in Aboriginal Anti-Diabetic Medicine and the Cree of Eeyou Istchee in northern Québec.

Using a novel metabolomic-based approach, we first developed methods of phytochemical analysis to characterize *Vaccinium angustifolium* extracts and then other medicinal Ericaceae, identifying and quantifying an abundance of phenolic compounds. These methods were then employed to profile the phenolics in anti-diabetic species identified by Cree Elders.

Pharmacological assays of extracts focused primarily on *in vitro* inhibition of mechanisms involved in the pathophysiology of diabetic neuropathy, associated diabetic complications and neurodegenerative conditions. Upon testing for cytoprotective effects in two models of diabetic neuropathy, few extracts prevented cell loss mediated by serum/glucose deprivation but 10 of 18 significantly reduced glucotoxic cell death. The activities of *Picea glauca* needles, which was cytoprotective in both models, and *Sarracenia purpurea* leaves were of particular interest as observed organ-specific effects correlated with medicinal uses identified by Cree Elders. Cytoprotection, however, did not correlate with extract phenolic content or anti-oxidant activity. Active principles were therefore sought through assay-guided fractionation of *S. purpurea* extract, identifying quercetin-3-*O*-galactoside, which inhibited glucotoxicity at low micromolar concentrations. Extracts were then tested for effects on formation of advanced glycation endproducts. Most displayed concentration-dependent inhibition with IC_{50} values ranging from 0.4-38.6 μg/ml. Anti-

glycation activity was significantly correlated with extract phenolic content and anti-oxidant capacity.

The bioactivities of individual phenolic metabolites were then evaluated at physiologically relevant concentrations using molecular tools to determine cell proliferation, survival and death. A wealth of anti-apoptotic and mitogenic effects were observed and structure-activity relationships were separately identified among benzoic acid derivatives and flavonoids. To explore the neuroprotective potential of these two phenolic classes, collections of each were tested for the ability to inhibit platelet-activating factor (PAF) neurotoxicity. Three novel PAF inhibitors were identified, orsellinic acid, hesperetin and quercetin, and all prevented PAF-mediated executioner caspase activation. Together, these results validate the traditional knowledge shared by our Cree collaborators and highlight the multidimensional therapeutic potential of boreal plants as culturally appropriate complementary anti-diabetic therapies.

RÉSUMÉ

Cette thèse examine la phytochimie et la pharmacologie des plantes boréales utilisées comme traitements traditionnels pour les symptômes et complications du diabète. Elle a été complétée en collaboration avec l'équipe IRSC sur les médecines autochtones antidiabétiques et les Cris Eeyou Istchee au nord du Québec.

En utilisant une nouvelle approche métabolomique, nous avons premièrement développé des méthodes d'analyse phytochimique pour caractériser des extraits de *Vaccinium angustifolium* et, par la suite, des extraits d'autres éricacées médicinales, afin d'identifier et de quantifier l'abondance des composés phénoliques. Ces méthodes ont été utiles pour etablir un profil des composés phénoliques retrouvés dans autres espèces antidiabétiques reconnues par les Aînés Cris.

Les épreuves pharmacologiques portaient sur l'inhibition *in vitro* des mécanismes impliqués dans la neuropathie diabétique pathophysiologique, les complications diabétiques associées et les conditions neurodégénératives. Lorsque les extraits étaient analysés pour leurs effets cytoprotectrices chez deux modèles de neuropathie diabétique, peu d'entre elles ont prévenu la perte de cellules induit par la carence du glucose. Cependant, 10 des 18 extraits ont protégés contre la toxicité du glucose. Il est intéressant à noter que, dans les cas de *Picea glauca* et *Sarracenia purpurea*, l'organe de la plante où se retrouvait l'activité cytoprotectrice correspondait avec l'information fournie par les Aînés Cris. Cependant, la cytoprotection n'était pas expliquée par le contenu total des composés phénoliques présent dans les extraits ni par leurs activités antioxidantes. Les principes actifs de *S. purpurea* ont été recherchés par la méthide dite de fractionnement guidé par les bioessaie. La quercétine-3-*O*-glycoside a ainsi été isolée; cette molécule prévenant la toxicité du glucose à des

concentrations micromolaires. Les extraits ont ensuite été évalués pour identifier des effets sur la formation de produits de glycation avancée. La plupart des plantes ont exprimé une activité inhibitrice dépendante de la concentration avec des valeurs de IC_{50} se situant entre 0.4 et 38.6 μg/ml. Les activités d'antiglycation montraient une corrélation significative avec les composés phénoliques totaux et la capacité antioxydantes des extraits.

La bioactivité des métabolites phénoliques individuels a été évaluée à des concentrations physiologiquement pertinentes en utilisant des outils moléculaires pour déterminer la prolifération, la survie et la mort des cellules. Plusieurs effets mitogéniques, cytoprotecteurs et anti-apoptiques ont été observés et des relations structure-activité uniques ont été découvertes pour les acides benzoïques et les flavonoïdes, respectivement. Afin d'explorer le potentiel neuroprotecteur de ces deux classes de composés phénoliques, une sélection de membres de chacune d'entre elles a été évaluée pour identifier leur capacité à prévenir la neurotoxicité du facteur d'activation des plaquettes (platelet-activating factor (PAF)). Trois nouveaux inhibiteurs de PAF ont été identifiés; l'acide orsellinique, l'herperétine et la quercétine. Chacun prévenait l'activation des caspases exécutrices, caspase-3/7. Pris dans leur ensemble, ces résultats valident le savoir traditionnel des Cris et soulignent le potentiel multidimensionnel des plantes boréales comme thérapies complémentaires pour le diabète, particulièrement dans les communautés autochtones du Canada.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

Aβ	amyloid beta
AD	Alzheimer disease
AGE	advanced glycation endproduct
AMPK	adenosine 5' monophosphate-activated protein kinase
APCI	atmospheric pressure chemical ionization
BA	benzoic acid
BDE	bond dissociation enthalpy
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
C_{16}-PAF	1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine
CA	cinnamic acid
CD	conjugated diene
CEI	Cree of Eeyou Istchee
CGN	cerebellar granule neuron
CIHR	Canadian Institutes of Health Research
CML	Nε-(carboxymethyl)lysine
CNS	central nervous system
CV	coefficient of variation
DAG	diacylglycerol
DCCT	The Diabetes Control and Complications Trial
DMSO	dimethylsulfoxide
DPPH	diphenylpicrylhydrazyl
EGFP	enhanced green fluorescent protein
EMEM	Eagle's minimum essential medium
ERK	extracellular signal related kinase
ET	ethidium homodimer
FBS	fetal bovine serum
FNC	First Nations Centre
GSIS	glucose-stimulated insulin secretion
HPLC	high pressure liquid chromatography
HS	horse serum
IC_{50}	median inhibitory concentration, concentration at which 50% of maximum effect is observed
IDF	International diabetes federation
LD_{50}	median lethal dose, concentration at which treatment elicits death of 50% of cells
LDL	low-density lipoprotein
L-NAME	N-nitro-L-arginine methyl ester
LOD	limit of detection
log P	log of the partition co-efficient
LOQ	limit of quantitation
lyso-PAF	alkyl*lyso*glycerophosphocholine
MR	molecular refractivity
MS	mass spectrometer

m/z	mass over charge ratio
NCS	newborn calf serum
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NO	nitric oxide
NOS	nitric oxide synthase
PAA	phenylacetic acid
PAD	Photodiode array detector
PAF	platelet activating factor
PAF-AH	PAF acetylhydrolase
PAFR	platelet activating factor receptor
PARP	poly ADP-ribose polymerase
PBS	phosphate buffered saline
PKC	protein kinase C
PNS	peripheral nervous system
PPA	phenylpropanoic acid
PPAR	peroxisome proliferator-activated receptors
PTFE	polytetrafluoroethylene
PTK	protein tyrosine kinases
QSAR	quantitative structure-activity relationship
RAGE	receptor for advanced glycation endproducts
RCAP	Royal Commission on Aboriginal People
ROS	reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute medium
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SIV	Syndromic importance value
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAAM	Team in Aboriginal Anti-Diabetic Medicine
TBARS	thiobarbituric acid-reactive substances
TFA	trifluoroacetic acid
TIC	total ion current
TPH	total phenolic content
TUNEL	terminal deoxytransferase dUTP nick-end labeling
TZDs	thiazolidinediones
UKPDS	the United Kingdom Prospective Diabetes Study
UV	ultraviolet
WHO	World Health Organisation
WT	wild-type

CHAPTER 1 – GENERAL INTRODUCTION AND LITERATURE REVIEW

"For every sickness on this Earth, there is a medicine under your feet"

- Traditional Mi'kmac belief (Cook 2005)

1.1 INTRODUCTION

Plant phenolics are present in most plant-derived foods, beverages and medicines, are the most abundant anti-oxidants in the human diet and are believed to protect against chronic, degenerative conditions such as cardiovascular diseases, neurodenerative diseases, metabolic disorders and cancers (Mariappan et al. 2006; Middleton et al. 2000; Milne et al. 2007; Youdim and Joseph 2001). Despite these potential health benefits, the diets of many people around the world are becoming increasingly deficient in plant-derived anti-oxidants as processed and prepared foods of the 'western' diet have flooded the global marketplace. While the use of fresh produce, herbal medicines and dietary supplements has recently grown in parts of the developed world, westernized diets and healing practices have become more widespread, particularly in developing nations and among indigenous populations whose traditional diets were rich in phytochemicals. Many of these same populations now suffer from higher rates of diseases like diabetes. Although the causes of such health disparities are multifactorial and extend far beyond changes in diet, a return to traditional sources of natural anti-oxidants could nonetheless prove to be an effective tool for achieving improved health outcomes. Moreover, dietary or therapeutic intake of plant-based materials containing phenolic anti-oxidants and other secondary metabolites potentially represents an accessible, non-pharmaceutical means of preventing and managing degenerative diseases such as diabetes, a growing health concern in developed and developing nations alike. This

thesis explores the potential benefit of phenolics in the specific context of plant foods and medicines from the boreal forest and their traditional use by Canadian First Nations as anti-diabetic remedies.

1.2 LITERATURE REVIEW

1.21 – A Modern History of Diabetes Mellitus

Although the symptoms of diabetes mellitus were recognized by ancient Egyptian, Greek, Chinese and Ayurvedic physicians, the modern history of diabetes began in the late 19th century when von Mering and Minkowski observed diabetes-like symptoms in dogs with their pancreas' removed (Luft 1989). At that time, most diabetics suffered from Type 1 diabetes mellitus (T1DM), a disease that left patients with little hope for treatment or survival. T1DM, also referred to as early-onset or insulin-dependent diabetes, is caused by the destruction of pancreatic cells responsible for producing and secreting insulin, β-cells of the islets of Langerhans, often through autoimmune attack, viral infection, or exposure to exogenous toxins (LeRoith 2004)). Acting through the transmembrane insulin receptor expressed by certain cell types most prominently in muscle, adipose and liver, insulin mediates systemic energy metabolism by regulating the uptake, metabolism and storage of carbohydrates (predominantly glucose), lipids and proteins. With a deficiency or total lack of insulin, as seen in T1DM, insulin-dependent uptake of circulating glucose, fatty acids and amino acids does not occur and, in response to perceived starvation, gluconeogenesis and glycogenolytic, lipolytis and proteolytic breakdown of energy stores are initiated. Together, these effects underlie the various symptoms and complications of T1DM, including the catabolic cascade leading to ketoacidosis, coma and death (LeRoith 2004).

While the pathophysiology of T1DM was unknown at the time (and remains unclear today), the discovery of insulin in the 1920s by Banting and Best was hailed as the elusive cure for diabetes, the once fatal disease now treatable with regular injections of insulin. Indeed, insulin therapy with regular blood glucose monitoring and control has remained the standard of T1DM treatment to this day, providing patients with long-term management and a higher quality of life. Insulin, however, does not prevent all the metabolic defects, the ensuing complications or the reduced life expectancy of T1DM patients (Laing et al. 1999a; Laing et al. 1999b). The efficacy of insulin in T1DM, nonetheless one of the greatest successes of modern medicine, unfortunately does not apply to all types of diabetes.

Today, diabetes mellitus is considered a group of disorders that are etiologically and clinically distinct but share a common set of symptoms that includes excessive thirst, hunger, and urination with elevated levels of glucose in the blood and urine. In the decades following World War II, Type 2 diabetes mellitus (T2DM), otherwise known as age-onset or insulin-independent diabetes, was differentiated from T1DM. While both T1DM and T2DM are characterized by hyperglycemia and insufficient insulin production and/or action, their etiology and clinical manifestations are distinct. T1DM generally develops during childhood and requires exogenous insulin therapy while the onset of T2DM typically occurs in adulthood and treatment relies on lifestyle changes and hypoglycemic management through pharmaceutical intervention other than insulin. In contrast to T1DM, T2DM is commonly associated with obesity and adipocity (Chan et al. 1994; Shai et al. 2006).

T2DM is a functional disorder characterized by impaired glucose tolerance and progressive insulin resistance that result in compensatory over-secretion of insulin from β-cells and exhaustion of their production capacity. As such, insulin treatment alone is of

limited value as most patients are insulin-resistant but, when used in combination with other therapies, can be effective in cases of advanced T2DM with severely impaired β-cell function. Though dietary restriction had been used for treating diabetes since the turn of the century, the contributing role of diet to T2DM etiology and management was not fully recognized until the 1990's when strict glycemic control through lifestyle (diet and physical activity) and, if necessary, pharmacological intervention was identified as the best strategy for reducing diabetes related complications, morbidity and mortality (DCCT 1993; UKPDS 1998a; UKPDS 1998b). Prior to these studies, the need for effective pharmacotherapy to treat the rising population of Type 2 diabetics was first met by the sulphonylureas, a class of insulin secretagogue drugs targeting pancreatic β-cells. Although some success was observed, considerable side effects accompanied treatment, a problem that was largely rectified with the development of second generation sulphonylureas, which are still widely used today. The biguanides, namely metformin, were introduced in the 1990's and act by sensitizing peripheral tissues to insulin thereby reducing insulin resistance. With a relatively good safety profile, metformin remains the first-line drug of choice for most patients with T2DM today (Goodarzi and Bryer-Ash 2005; Inzucchi 2002).

Despite these advances in disease management, the prevalence of T2DM has been steadily climbing over the last 50 years, now accounting for approximately 90% of all cases of diabetes. With 246 million afflicted adults as of 2007, a number that has nearly doubled since 2003 (I.D.F. 2007) and increased 8-fold since 1985, T2DM has emerged as a global health concern reaching epidemic proportions. As prevalence climbs and age-of-onset drops, complications of T2DM, the main cause of morbidity and mortality among diabetics, are also now a matter of international health (Meetoo et al. 2007). With the increased allocation of

resources toward T2DM research and management, our biochemical, molecular, and clinical understanding of the disease continues to evolve and new anti-diabetic interventions continue to be developed. However, the personal, social and economic costs of T2DM nonetheless continue to rise in both developed and developing nations, particularly among the world's Aboriginal populations (Engelgau et al. 2003; Shai et al. 2006; Young et al. 2000; Zhao et al. 2008).

1.22 – T2DM Among Indigenous Peoples Worldwide

While T1DM was known to occur in many traditional societies, T2DM was virtually nonexistent prior to World War II. As T2DM became more prevalent in the years following the war, aboriginal people at first appeared to be less susceptible than non-native nationals but, as portended by the Pima of Arizona during the early 1980's (Knowler et al. 1981), different aboriginal groups from Australia to North America are now recognized as some of the world's highest at-risk populations (Daniel et al. 2002; Pollex et al. 2006; Shai et al. 2006). In effect, today's aboriginal populations suffer rates of T2DM that are 20-30% higher than dominant national cultures (for a review see Yu and Zinman (2007b)). In Canada, each of the three major indigenous groups, First Nations, Inuit and Métis, suffers above average incidence of T2DM compared to the non-indigenous population (Bruce 2003).

Various hypotheses have been forwarded to explain this increased incidence. The underlying causes, however, vary according to the population in question and are multi-factorial. "Acculturation and adoption of a 'Westernized' lifestyle" (Yu and Zinman 2007b), including diet, activities and traditions, and the corresponding loss of connectivity to indigenous land, community and culture have been repeatedly identified by researchers and aboriginals alike as contributing factors (Ferreira 2006; Rock 2003; Satterfield 2007;

Thompson and Gifford 2000). Moreover, although the 'thrifty gene' hypothesis, which proposes that genes that have evolved to maximize energy metabolism and storage within certain ethnic groups lead to diabetes in times of abundance, is generally not accepted in isolation, genetic predispositions whose effects are revealed upon changes in lifestyle likely contribute to rates of T2DM among aboriginal peoples (Hegele 2001; Joffe and Zimmet 1998). The impacts of socioeconomic status and geographical isolation, which relate to access to education, medical care, nutritious foods and other resources, no doubt vary between communities but cannot be overlooked. Similarly, the legacy of colonialism experienced by many indigenous groups has had devastating social, cultural and economic consequences that influence the health of individuals and the community at large (Ferreira 2006).

The T2DM crisis among indigenous people is further compounded by poor access to medical services and low compliance with modern management strategies, be it regular diagnostic screening, glucose monitoring, dietary intervention or pharmacotherapy (Mak et al. 2004; Roy 2005; Young et al. 2000). Together, these limitations often result in uncontrolled hyperglycemia and increased incidence and severity of diabetic complications and associated morbidity (Hanley et al. 2005; Young et al. 2000; Yu and Zinman 2007a). Whereas access to medical services can be largely attributed to the national standard of healthcare as well as the community's geographical isolation, issues of compliance are likely influenced by factors similar to those contributing to disparities in T2DM as described above. Poor compliance may also reflect the cultural inadequacies of most T2DM treatment strategies, which are designed by professionals unfamiliar with the needs of the local community and without consideration of cultural norms and accepted practices (Boston et al. 1997).

Around the world, traditional healing and medicine are increasingly viewed as an important component of aboriginal health and as a promising avenue for improved diabetes management and prevention. In Canada, First Nations believe "Traditional healing practices are a part of the integral fabric of the First Nations way of life and, as such, are tied to First Nations culture and health," (Assembly of First Nations, 2006) and that a return to these practices would improve health outcomes including diabetes (F.N.C. 2003). Similarly, the Royal Commission on Aboriginal People (R.C.A.P. 1996) reported that traditional healing practices promote mental, physical and spiritual well-being through cures using herbal medicines, ceremony and the wisdom of the Elders and called for an extended role for traditional medicine and healing practices to address the various aspects of health.

1.23 – Traditional Medicine, Ethnopharmacology and the Treatment of Diabetes

Traditional medicine refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (World Health Organization, WHO). Traditional medicine systems are found throughout the world, some dating back thousands of years while others evolved more recently. In all these systems, the use of medicinal plants figures prominently into healing practices with more than 60% of the global population currently believed to use herbal medicines (Farnsworth 1990). While much of this use is focused in the developing world where most people depend on traditional medicine for their primary healthcare needs, it also includes indigenous populations and a growing number of non-natives from developed nations (Moerman 1998; Tindle et al. 2005). Ethnopharmacology is the observation and experimental investigation of biological activities elicited by natural substances utilized in

traditional medicine of past and present cultures (Etkin and Elisabetsky 2005). The science of pharmacy, which originated largely from traditional plant medicines and still relies heavily on drugs developed from plant-derived compounds, provides testimony to the pharmacological value of traditional medicines and their chemical constituents.

Medicinal plants have similarly played a central role in the treatment of diabetes. Indeed, thousands of plant species have been used ethnopharmacologically or experimentally for the treatment of diabetic symptoms and complications. Moreover, despite the rapid emergence of T2DM over the last 50 years that has left Traditional Healers with little time to explore the disease and treatments, a remarkably high percentage of these plants demonstrate anti-diabetic properties when evaluated in a laboratory environment. For instance, in a comprehensive review of anti-diabetic plants including more than 1200 species assessed for hypoglycemic effects, over 80% of those traditionally used for anti-diabetic purposes were active (Marles 1995). A study of indigenous herbal medicines used for diabetes in India likewise reported that 80% of examined extracts effectively lowered blood glucose levels in rats with alloxan-induced diabetes (Kar et al. 2003). As evidenced by the increasing number of mechanistic studies stemming from growth in mainstream use of herbal remedies in diabetes management, this abundance of activity reflects, in part, a great variety of active constituents and mechanisms of action of plant compounds (Dunning 2006; Shane-McWhorter 2007). Guanidine, the active principle of *Galega officinalis* (French lilac) from which metformin was developed, provides an obvious example of the pharmaceutical potential of anti-diabetic medicinal plants (Witters 2001).

In aboriginal societies, the role of plants in physical, cultural, and spiritual health extends far beyond their pharmacological activities. Central to most traditional Aboriginal world views is the concept of cosmovision, the wholistic notion of Earth as an interconnected

web of life functioning in a complex ecosystem of relationships (Cohen 2001). As food, shelter, symbol, spirit, or feature of the local environment, plants are a prominent part of this web and provide connectedness to indigenous land and culture, elements repeatedly identified by Aboriginal people as central to personal and community health (Ferreira 2006; Roy 2005). The identification of traditionally used medicinal plants with pharmacologically validated anti-diabetic potential thus represents a promising approach to developing affordable complementary therapeutic options that are community-specific, locally accessible and, perhaps key to the treatment of this pandemic, culturally acceptable.

1.24 – Canada's boreal forest as a source of anti-diabetic plants

Stretching over 5000 km from the Yukon southeast to Newfoundland and Labrador, the Canadian boreal forest (Figure 1.1) occupies 35% of the country's total land area and supports about two thirds of its 140,000 species of plants, animals and other organisms (Government-of-Saskatchewan 2007). While several distinct ecozones provide the various habitats supporting this biodiversity, the boreal region is generally characterized by dense coniferous forest interspersed with literally more than a million lakes and river systems. Scattered innumerably over the landscape, wetlands (bogs, marshes and fens) cover 30% of Canada's boreal forest and are some of its most diverse and biologically productive ecosystems. Although the plant diversity of the boreal forest is generally considered to be low, plant life is abundant and, in general, highly adapted to frequent forest fires, acidic soils and a climate of long, dry, freezing winters opposite short, cool and moist summers.

The canopy of the boreal forest consists of approximately 20 tree species, which are predominantly coniferous species of spruce, pine, fir and tamarack with broad-leaf deciduous

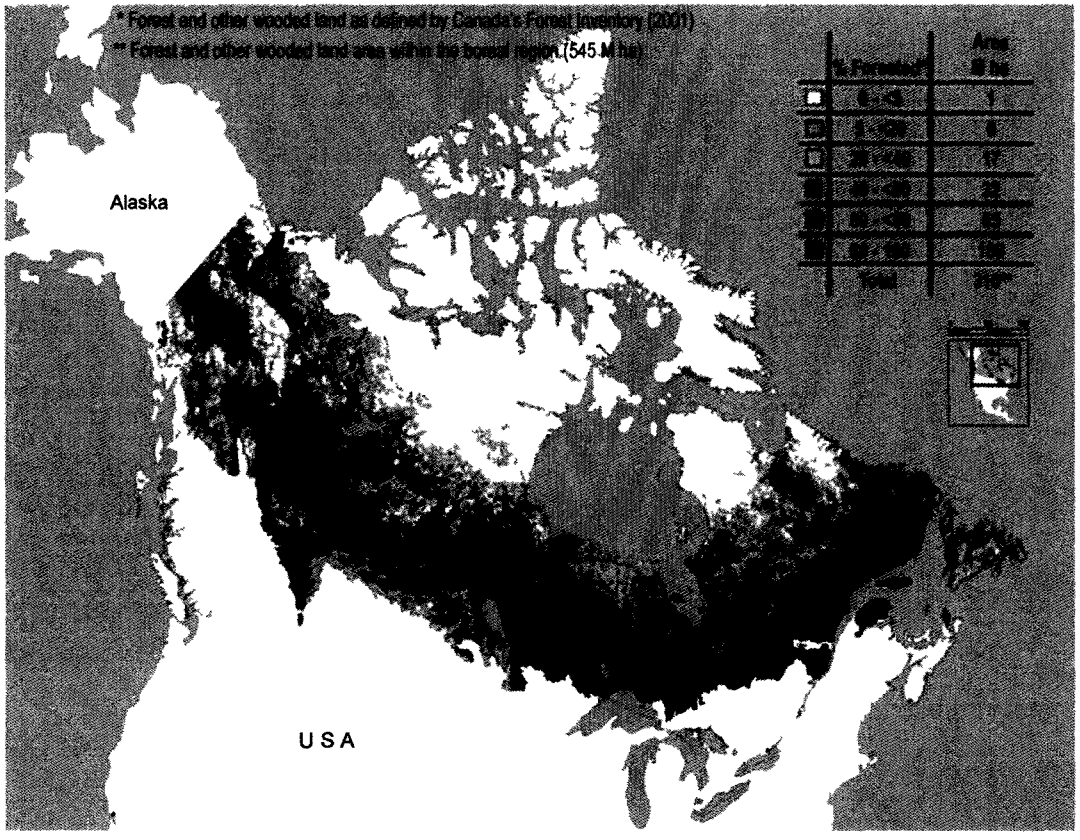

Figure 1.1 – The Canadian boreal forest. Representing 77% of Canada's forested and other wooded land, the boreal forest is one of the country's largest natural resources. Image modified from original production by Natural Resources Canada (www.hww.ca/hww2.asp?id=354).

species of poplar, birch and ash occurring less abundantly, particularly in the north. Beneath the trees, a variety of shrubs, herbs, vines, grasses, non-vascular plants and lichens thrive. Many species, such as *Picea mariana* (black spruce) and *Rhododendron groenlandicum* (labrador tea), are distributed across the entire region while others, such as *Pinus contorta* ssp. *latifolia* (lodgepole pine) and *Rhododendron tomentosum* (northern Labrador tea), are restricted to western and northern regions respectively (Hitchcock 1996; Marie-Victorin 1995). Together, the plants of boreal forest provide food and shelter for its many inhabitants, from microbes and insects to birds and humans.

Aboriginal people of Canada's boreal forest have sustained themselves for centuries on the regions natural resources. Though more widespread prior to colonialisation, thousands of Aboriginals, most notably of the First Nations, still reside within about 500 communities across the boreal forest. As reviewed by Arnason and Johns (1981), the First Nations of eastern Canada utilize hundreds of plant species for food, beverage and medicine (Table 1.1). With generations of accumulated ecological and botanical knowledge, these societies have distinguished the nutritive and healing properties of different plants, developing locally distinct, yet culturally and geographically linked, pharmacopoeias. Between the various aboriginal groups, hundreds of boreal plant species have been used to treat all types of human illness (Arnason 1981; Marles 2000; Moerman 1998). While few boreal remedies for T2DM *per se* have been identified due to the disease's recent emergence in the population, plants used to treat symptoms and complications of diabetes are plentiful and represent a valuable source of ethnobotanically selected bioactive compounds (McCune and Johns 2002; McCune and Johns 2003). Like the food, beverage and medicine plants listed in Table 1.1, species of Pinaceae, Ericaceae, Asteraceae, Rosaceae and Salicaceae are

Table 1.1 – Plant families most commonly used for food, beverage and medicine among First Nations of Eastern Canada. Summarized from Arnason (1981).

Food uses		Beverage uses		Medicinal uses	
Family	No. of species	Family	No. of species	Family	No. of species
Rosaceae	30	Rosaceae	9	Asteraceae	43
Ericaceae	13	Ericaceae	8	Rosaceae	33
Fagaceae	8	Pinaceae	7	Liliaceae	18
Saxifragaceae	8	Aceraceae	3	Ranunculaceae	16
Brassicaceae	6	Lamiaceae	3	Caprifoliaceae	13
Caprifoliaceae	6	Anacardiaceae	2	Ericaceae	13
Liliaceae	6	Betulaceae	2	Salicaceae	13
Aceraceae	5	Cuppressaceae	2	Apiaceae	12
Fabaceae	5	Lauraceae	2	Betulaceae	12
Salicaceae	5	Salicaceae	2	Lamiaceae	10
Asteraceae	4			Polygonaceae	10
Betulaceae	4			Polypodiaceae	10
Pinaceae	4			Pinaceae	9

Table 1.2 – Boreal plant species with recorded medicinal uses relevant to the treatment of diabetic symptoms and complications. Adapted from McCune and Johns (2002).

Plant family	Plant species	Common name	First Nations group(s)*
Anacardiaceae	*Rhus hirta* (L) Sudworth	Staghorn sumac	Alg , Del , Iroq , Mal
Apiaceae	*Heracleum lanatum* Michx	Cow parsnip	Cree, Micmac, Ojibwa
Araceae	*Acorus calamus* (L)	Sweet flag	Alg , Cree, Iroq , Mal , Ojibwa
	Arisaema triphyllum (L) Schott	Indian turnip	Mal , Ojibwa
Araliaceae	*Aralia nudicaulis* L	Wild sarsaparilla	Abe , Alg , Mont , Ojibwa
	Aralia racemosa L	Spikenard	Mal , Micmac, Ojibwa
Aristolochiaceae	*Asarum canadense* Ashe	Wild ginger	Mont , Ojibwa
Asteraceae	*Achillea millefolium* L	Yarrow	Alg , Cree, Ojibwa
	Solidago canadensis L	Goldenrod	Ojibwa
	Taraxecum officinale Weber	Dandelion	Alg , Cree, Iroq , Ojibwa
Betulaceae	*Corylus cornuta* Marsh	Beaked hazelnut	Abe , Alg , Cree
Celastraceae	*Celastrus scandens* L	Climbing bitter-sweet	Iroq , Ojibwa
Cornaceae	*Cornus stolonifera Michx*	Red-osier dogwood	Abe , Alg , Mal , Ojibwa
Cuppressaceae	*Thuja occidentalis* L	White cedar	Abe , Alg , Iroq , Mal , Micmac, Mont , Ojibwa
	Juniperus communis L	Juniper	Cree, Del , Mal , Micmac
	Juniperus virginiana L	Red cedar	Cree, Ojibwa
Ericaceae	*Gaultheria procumbens* L	Wintergreen	Alg , Cree, Iroq , Ojibwa
	Kalmia angustifolia L	Sheep laurel	Cree, Mal , Micmac, Mont
	Rhododendron groenladicum Oeder	Labrador tea	Alg , Cree, Mal , Micmac, Mont , Ojibwa
Fagaceae	*Quercus alba* L	White oak	Del , Mal , Micmac, Ojibwa
	Quercus rubra L	Red oak	Mal , Ojibwa
Lauraceae	*Sassafras albidum* (Nutt) Nees	Sassafras	Iroq , Ojibwa
Liliaceae	*Smalacina racemosa* (L) Desf	False spikenard	Abe , Alg , Ojibwa
Nymphaeaceae	*Nuphar variegatum* Durand	Yellow water-lily	Abe , Alg , Iroq , Micmac
Pinaceae	*Abies balsamea* (L) Mill	Balsam fir	Alg , Micmac, Mont , Ojibwa
	Picea glauca (Moench) Voss	White spruce	Abe , Alg , Cree, Micmac, Mont , Ojibwa
	Picea mariana (Mill) BSP	Black spruce	Alg , Cree, Ojibwa
	Tsuga canadensis (L) Carr	Hemlock	Abe , Alg , Mal , Micmac, Ojibwa
Rosaceae	*Prunus serotina* Ehrh	Rum cherry	Del , Iroq , Mal , Ojibwa
	Sorbus americana Marsh	Mountain ash	Alg , Cree, Mal , Mont , Ojibwa
Salicaceae	*Populus balsamifera* L	Balsam poplar	Alg , Cree, Mal , Micmac, Ojibwa
	Populus tremuloides Michx	Quaking aspen	Abi , Cree, Ojibwa
Scrophulariaceae	*Verbascum thapsus* L	Mullein	Iroq , Mal , Ojibwa
Taxaceae	*Taxus canadensis* Marsh	Yew	Abe , Alg , Mal , Micmac, Mont , Ojibwa
Thymylaaeceae	*Dirca palustris* L	Moosewood	Iroq , Ojibwa

Abe - Abenaki, Abi - Abitibi, Alg - Algonquin, Del - Delaware, Iroq - Iroquois, Mal - Malecite, Mont - Montagnais

* First Nations groups within the boreal region that used the species for ≥ 3 symptoms of T2DM (Arnason 1981)

some of the most widely used (Table 1.2). While their pharmacological activities have not yet been determined, certain medicinal species of the boreal forest, such as *Vaccinium angustifolium*, are already popular as alternative therapies for T2DM (Haddad et al. 2001; Haddad 2003). Others, particularly members of the Pinaceae and Ericaceae families, are closely related to established anti-diabetic species (Blumenthal 1998; Ju et al. 2008; Liu et al. 2004) and produce phenolic metabolites including flavonoids, phenolic acids and stilbenes, which possess known anti-diabetic activities (McCarty 2005; Pinent et al. 2004; Shane-McWhorter 2007; Su et al. 2006).

Given the number of aboriginal communities and extent of their ethnopharmacological heritage, the boreal forest is clearly one of Canada's most promising sources of traditional medicine-based treatment options for managing T2DM.

1.3 RATIONALE

1.31 – The CIHR Team in Aboriginal Anti-diabetic Medicine

In 2003, The Canadian Institutes of Health Research (CIHR)-funded Team in Aboriginal Anti-diabetic Medicine (TAAM), led by Dr. P.S. Haddad at l'Université de Montréal, was initiated in response to the emerging diabetes crisis among Aboriginal Canadians and the lack of effective, culturally appropriate, and community-based therapeutic interventions. The primary objective of this transdisciplinary collaboration between university researchers, the Cree of Eeyou Istchee (CEI) (James Bay, Quebéc, Figure 1.2), and the Cree Board of Health and Social Services of James Bay, was to identify safe, locally-used, pharmacologically active traditional medicines of potential clinical value in the

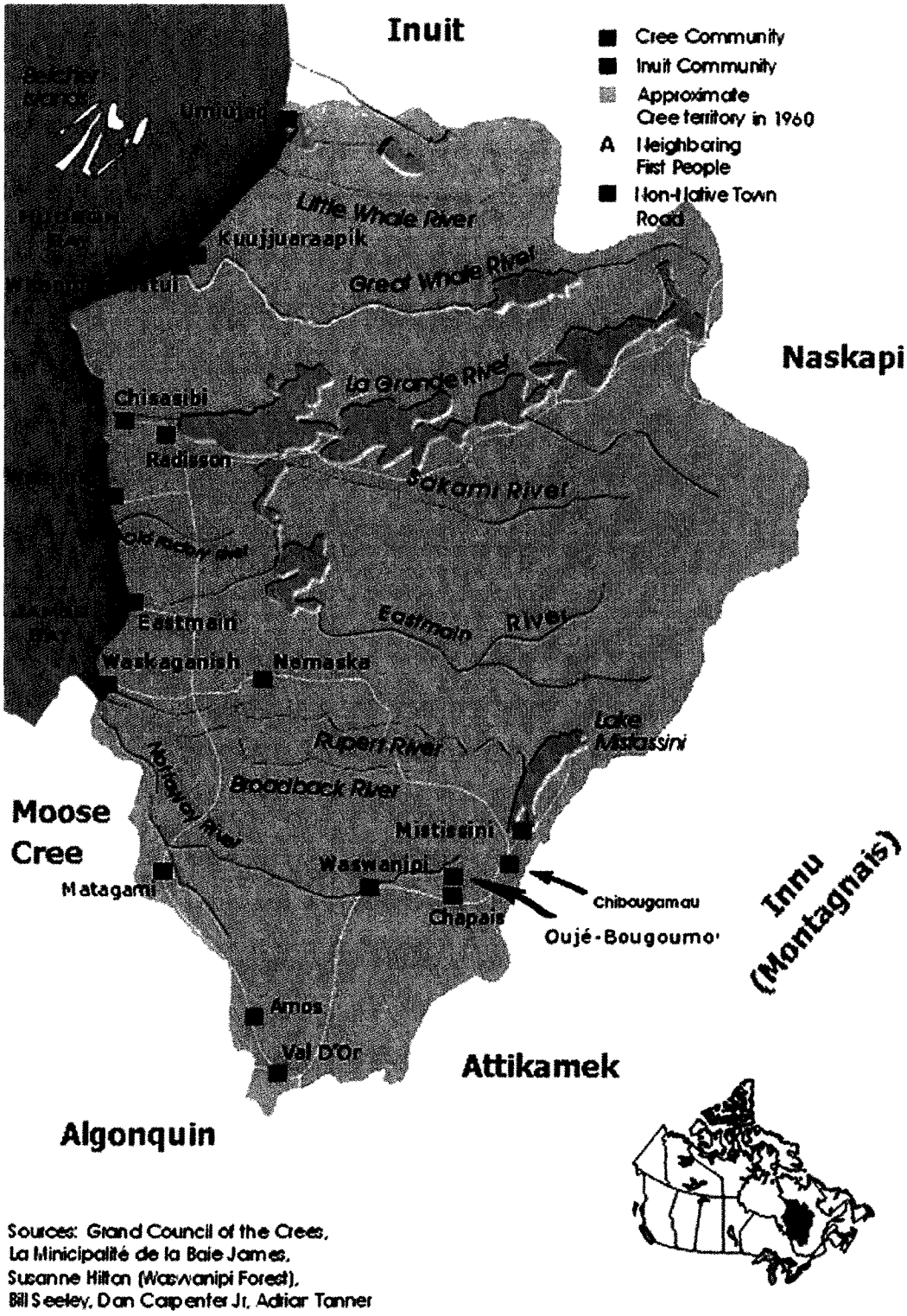

Figure 1.2 – Map of the Cree Nations of Eeyou Istchee, James Bay, Québec.
Adapted from www.ottertooth.com/Native_K/jbcree.htm.

management and prevention of T2DM. In addition, the TAAM aimed to establish safety and quality standards for Cree Traditional medicines recommended by the study, to provide scientific validation of Cree Traditional Knowledge and to promote an integrated approach to diabetes management in CEI communities. An overview of the project structure including collaborating laboratories and their respective contributions are presented in Figure 1.3 and Table 1.3.

For the first step in the project, the identification of anti-diabetic medicinal plants from the CEI pharmacopoeia for pharmacological validation, the TAAM employed an ethnobotanical approach consisting of semi-structured interviews. Because T2DM was practically non-existent in First Nations communities like the CEI until recently, the interviews only addressed diabetes *per se* in relation to participant perceptions of the disease and its causes. While participants were asked specifically about traditional remedies for diabetes, they were also asked about treatments for 15 symptoms of T2DM and related complications. The symptoms included 1) increased appetite, 2) increased thirst, 3) increased urination, 4) slow healing infections, 5) foot numbness and/or foot sores, 6) blurred vision 7) back and/or kidney pain, 8) heart and/or chest pain, 9) sore and/or swollen limbs, 10) arthritis/rheumatism, 11) swelling and/or inflammation, 12) frequent headaches, 13) abcesses and/or boils, 14) diarrhoea, and 15) general weakness. Following ethnobotanical data collection, identified plant species were assigned a syndromic importance value (SIV), a relative index of anti-diabetic importance calculated based on the plant's frequency of mention, number of symptoms treated, diabetic significance (weight) of symptoms and the total number of participants (Leduc et al. 2006). While additional communities of CEI have recently participated in TAAM research, SIV scores obtained through interviews with the inland community of Mistissini in southern CEI territory and the coastal community of

Whapmagoostui in the north (Figure 1.2), completed in 2003 and 2004 respectively, served as the basis for selecting plant species for laboratory research.

The ethnobotanically selected plants were harvested from the wild according to instructions provided by the Elders and, following species authentication by citing participants and TAAM botanist Dr. A. Cuerrier, were processed and extracted yielding dry extracts for distribution to all collaborating labs. The second stage of the project, which aimed to identify candidate plant species for *in vivo* assessment, consisted of two parallel arms of investigation, safety/quality control, and pharmacology. In natural health products such as herbal medicines, safety and quality control are inseparably linked and impact directly upon pharmacology and therapeutic viability (Vuksan and Sievenpiper 2005). For the purposes of this screening phase of the project, safety/quality control was divided into three components, phytochemistry, cytotoxicity and drug interactions. Phytochemical characterization served multiple functions vital to the development of safe and efficacious herbal products, most notably as a means of identifying marker compounds and active metabolites that can be used for extract standardization, authentication of processed plant materials and optimization of harvesting and processing protocols.

A common misconception about herbal products is a lack of toxicity or side effects (Bent 2008). While many boreal plants have a long history of human consumption as food and are generally considered as safe, some, such as sheep laurel (*Kalmia angustifolia*) and northern water hemlock (*Cicuta virosa*) are known to be toxic (Marie-Victorin 1995) and, as related by Traditional Healers, must be handled with caution (personal communication). Beyond direct toxicity, although a given treatment may appear to be safe when administered alone, dangerous side effects may appear when a second treatment is introduced. A well-documented mechanism through which plant medicines can cause adverse events is by

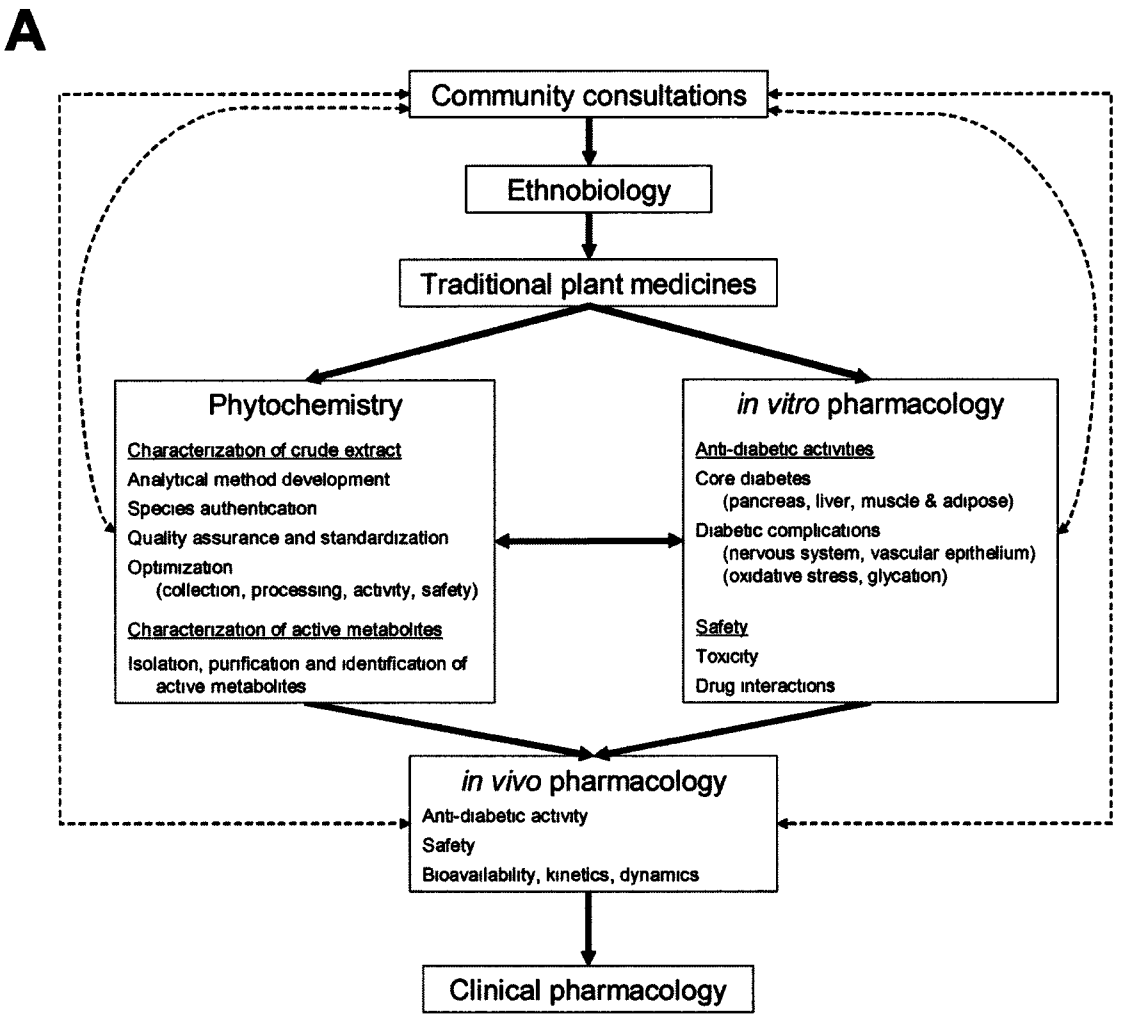

Figure 1.3 – Outline of proposed research for the CIHR Team in Aboriginal Anti-diabetic Medicine. Flow chart of basic research (A) beginning with community-based ethnobiological identification of anti-diabetic traditional medicines and progressing through *in vitro* and *in vivo* pharmacology to identify the plant medicines with the best safety and activity profiles for clinical evaluation (B).

Table 1.3 – Structure and contributing members of the CIHR Team in Aboriginal Anti-diabetic Medicine.

Supervisor (University)	Laboratory discipline	System / Experimental procedure	Anti-diabetic activity	Targeted pharmaceutical*
Dr. J.T. Arnason (University of Ottawa)	Phytochemistry (Safety)	Plant processing	n/a	n/a
		Phytochemical analyses	n/a	n/a
		Identification of active principles	n/a	n/a
	Pharmacology	Advanced glycation endproduct formation	Anti-glycation	In development
		Macrophage response	Anti-inflammatory	In development
Dr. S.A.L. Bennett (University of Ottawa)	Pharmacology	Neurons (PNS & CNS)	Neuroprotection Anti-apoptotic	In development
Dr. A. Cuerrier (L'Université de Montréal)	Ethnobiology	Traditional medicines	n/a	n/a
	Ethnoecology	Sustainable harvesting	n/a	n/a
Dr. D. Foster (University of Ottawa)	Pharmacology (Safety)	Drug interactions	n/a	n/a
		Cardiac toxicity	n/a	n/a
		Intestinal transcriptome profiling	n/a	n/a
Dr. P.S. Haddad Project leader (L'Université de Montréal)	Pharmacology	Peripheral insulin targets	↑ glucose uptake (insulin sensitizer)	Biguanide/TZDs
		(myocytes, adipocytes, hepatocytes)	Uncoupling of respiration	Biguanide/TZDs
			AMPK activation	Biguanides
		Adipocytes	Triglyceride accumulation	TZDs
			Anti-adipogenic	TZDs
		Pancreatic β-cells	Regeneration	In development
Dr. T. Johns (McGill University)	Ethnobiology	Traditional foods	n/a	n/a
	Pharmacology	Oxidative stress	Anti-oxidant	α-lipoic acid
		Epithelial cells	↑ NO synthase	In development
Dr. M. Prentki (L'Université de Montréal)	Pharmacology	Pancreatic β-cells	↑ insulin production / secretion	Sulphonylureas

n/a – not applicable; AMPK – adenosine 5' monophosphate-activated protein kinase; CNS – central nervous system; NO – nitric oxide; PNS – peripheral nervous system; TZDs – thiazolidinediones.

* Anti-diabetic pharmaceuticals that act through the given biological mechanism. 'In development' refers to drugs targeting the given mechanism in phase II or III clinical trials.

interacting with medications, such as grapefruit and pepper can influence bioavailability by altering the activity of enzymes involved in drug metabolism or of cellular pumps responsible for ejecting absorbed drugs or their active metabolites (Bhardwaj et al. 2002; de Castro et al. 2007; Foster et al. 2001). While a resulting drop in drug levels can reduce or abolish treatment efficacy, an increase can lead to overdose; both are potentially dangerous adverse effects. Alternatively, two co-administered medications may have additive or synergistic activities in a given tissue (for example, stimulatory effects on the heart) that can cause serious unforeseen harm, particularly in predisposed individuals. Therefore, in accordance with the precept *primum non nocere*, "First, do no harm", all extracts were evaluated for cytotoxic effects in both target (muscle, adipose, liver, pancreas, epithelial, nerve) and non-target (intestine, heart) cell types and for modulatory effects on enzymes and transporters regulating bioavailability.

In terms of pharmacology, anti-diabetic potential was first assessed by screening the Cree extracts through a series of cell-based and biochemical *in vitro* assays designed to detect a variety of activities pertinent to the treatment of T2DM. While many of the assays targeted core pathological mechanisms such as insulin action/secretion and glucose metabolism, others target mechanisms implicated in the development of diabetic complications. The selected assays not only modelled biological activities ascribed to currently available anti-diabetic drugs but to recently identified targets of drug development as well (Table 1.3). Although a comprehensive review of the molecular and biochemical processes contributing to the pathoetiology and progression of T2DM is beyond the scope of this thesis, additional information about the various assays and their relevance to diabetes management has been provided throughout Section 2. As the treatment of diabetic

neuropathy was central to the presented research, the mechanisms currently believed to be involved are summarized below.

To date, the TAAM has completed the first two stages of the project and, based on the safety and anti-diabetic screening results obtained during the *in vitro* investigations and on the feedback provided by participating Elders of CEI, selected plant extracts have begun evaluation *in vivo.* The current thesis presents my contributions to the TAAM, which focused primarily on phytochemical characterization of Cree plant extracts and evaluation of their *in vitro* activities pertaining to protection from diabetic neuropathy and other vascular complications of T2DM.

1.32 – Hyperglycemia, diabetic neuropathy and vascular damage

The pathophysiology of microvascular complications such as diabetic neuropathy, nephropathy, and retinopathy share several common mechanisms. Moreover, while T1DM and T2DM differ in terms of pathogenesis, the factors contributing to the different microvascular complications appear to be similar in both conditions. Several types of neuropathy may develop as a complication of diabetes and, though they may differ in pathology and clinical manifestations, common hyperglycemia-related neurotoxic effects exist. As such, the presented research is likely relevant to many types of diabetic neuropathy and to other microvascular complications as well. For the purpose of this thesis, however, the term "diabetic neuropathy" refers to distal symmetric sensorimotor polyneuropathy, which occurs to some degree in approximately 50% of all T2DM patients and is characterized by progressive loss of peripheral sensation, pain, and often leads to reduced mobility, unhealing wounds and infections that may ultimately result in amputation of the toes and feet (Dyck et al, 1993). As reviewed previously (Brownlee 2001; Hayden and

Tyagi 2004; Sheetz and King 2002), extensive research directed toward clarifying the pathogenic mechanisms of diabetic neuropathy has uncovered several interconnected factors but has not yet led to the development of effective pharmacotherapies. Hyperglycemia not only causes direct glucotoxic damage to peripheral neurons and support cells (Schmeichel et al. 2003) but also contributes to neuropathology indirectly by triggering a self-perpetuating cycle of oxidative stress, non-enzymatic formation of advanced glycation endproducts (AGEs), inflammation and vascular damage (Figure 1.4). To deal with excess glucose, cells increase both the rates of mitochondrial respiration, which results in superoxide production, and the flux through the polyol and hexosamine pathways, which respectively perturb cellular reductive capacity and protein function. Increased rates of glycolysis in turn lead to production of diacylglycerol (DAG) and subsequent activation of protein kinase C (PKC)-mediated gene expression and pro-inflammatory signaling (Aronson 2008; Brownlee 2005). While all of these factors have direct negative effects on neurons, they also cause vascular damage that impedes blood flow and the delivery of nutrients and trophic factors required for neuronal growth, function and survival (Sheetz and King 2002).

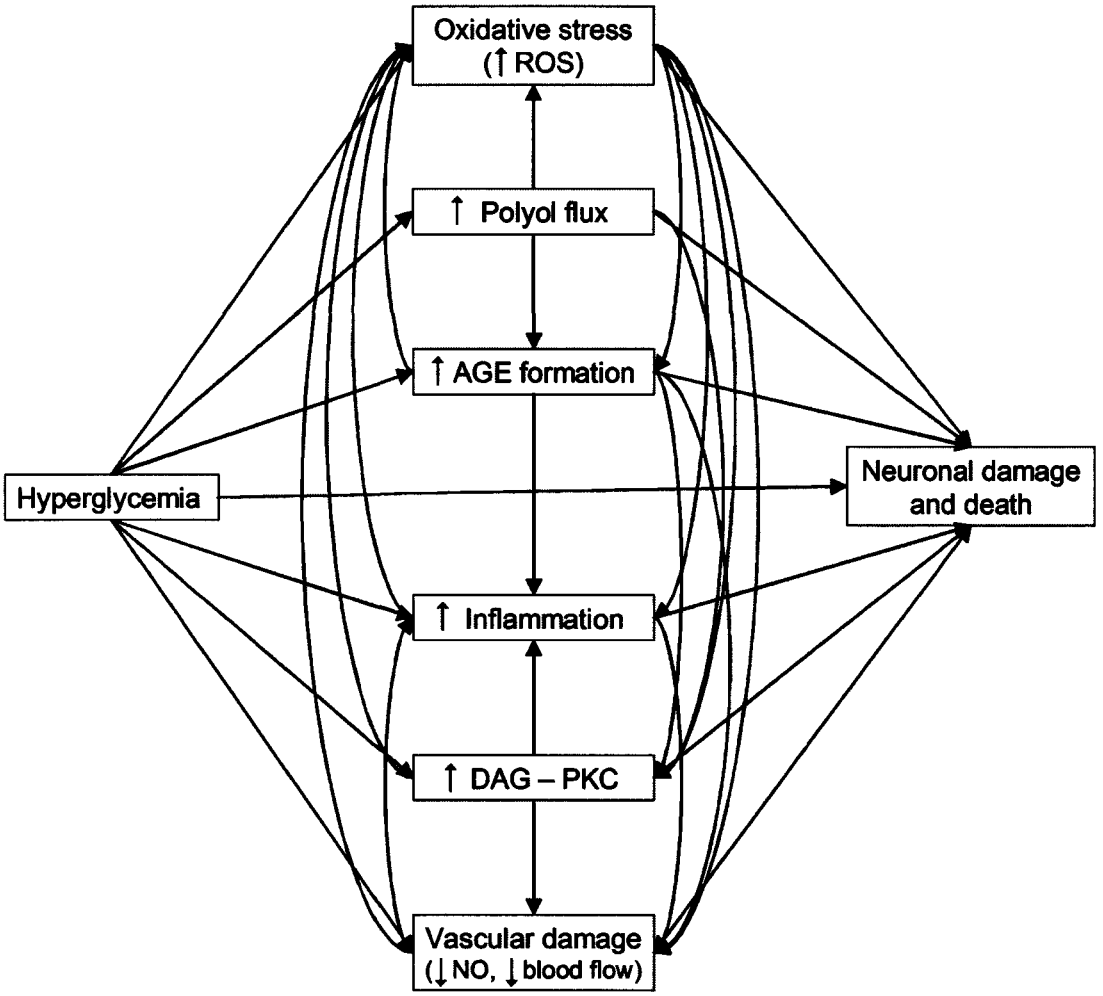

Figure 1.4 – Hyperglycemia-mediated factors contributing to the development of diabetic neuropathy. In addition to causing direct damage to neurons, hyperglycemia mediates increased production of reactive oxygen species (ROS), advanced glycation endproducts (AGEs), and diacyglycerol (DAG), which activate protein kinase C (PKC) leading to further oxidative stress, inflammation and vascular damage. Increased flux through the polyol pathway further propagates this cycle.

1.4 HYPOTHESES AND OBJECTIVES

The following three hypotheses and predictions guided my research:

Hypothesis 1: The selection of medicinal plants by Elders and Healers of CEI is not random but based, in part, on the plant's pharmacological properties.

Prediction: Extracts of plants or plant parts that are more frequently used by Cree Elders to treat symptoms of diabetes will elicit greater anti-diabetic effects than extracts of plants or plant parts that are seldom used.

Hypothesis 2: The anti-diabetic activity of Cree medicinal plants is mediated, in part, by their phenolic secondary metabolites.

Prediction: Plant extracts that are rich in phenolic metabolites will elicit greater anti-diabetic effects than extracts with low phenolic content.

Hypothesis 3: The anti-diabetic activity of Cree medicinal plants is mediated, in part, by their anti-oxidant properties.

Prediction: Plant extracts possessing strong anti-oxidant activity will elicit greater anti-diabetic effects than extracts with weak anti-oxidant activity.

In accordance with the CIHR TAAM, the overall objective of my research is to identify safe, locally-used, pharmacologically active plant medicines of potential clinical value in the management and prevention of diabetic neuropathy and related vascular

complications of T2DM. As a member of the two TAAM laboratories responsible for phytochemistry and neuropharmacology, respectively, the objectives of my contribution to the project are to provide phytochemical characterization of Cree medicinal plant extracts and to evaluate these extracts *in vitro* for protective activities relevant to diabetic neuropathy and related progressive neurodegenerative conditions. To meet these objectives and to test the hypotheses outlined above, the thesis has been divided into three sections, phytochemical characterization, anti-diabetic activities and neuroprotective activities, each with their own specific objectives.

Objective 1: To provide phytochemical characterization of Cree medicinal plant extracts.

Specific objective 1.1 – To develop and validate analytical methods for the characterization of representative plant extracts from the boreal forest by high pressure liquid chromatography (HPLC).

Specific objective 1.2 – To apply the methods developed in Specific objective 1.1 to identify and quantitate active metabolites and marker compounds in plant extracts and biological samples for further testing and the purposes of quality control.

Specific objective 1.3 – To measure the bulk phenolic content of extracts using the Folin-Ciocalteau method.

Objective 2: To evaluate Cree medicinal plant extracts *in vitro* for protective activities relevant to diabetic neuropathy and related vascular complications of T2DM.

Specific objective 2.1 – To evaluate extracts for cytoprotective activity in two *in vitro* models of diabetic neuropathy, glucose toxicity and glucose deprivation.

Specific objective 2.2 – To evaluate extracts for inhibitory activity on the *in vitro* formation of advanced glycation endproducts.

Specific objective 2.3 – To investigate possible relationships between the extracts' anti-diabetic activities and ethnobotanically determined diabetic SIV scores.

Specific objective 2.4 – To compare the anti-diabetic activities of extracts made from separated organs of active species in relation to their anti-diabetic use by the CEI.

Specific objective 2.5 – To investigate possible relationships between extracts' anti-diabetic activities and phytochemical profiles.

Specific objective 2.6 – To identify active metabolites of selected extracts through assay-guided fractionation (in cases when no relationship between activity and phytochemistry is revealed by Specific objective 2.5).

Specific objective 2.7 – To investigate possible relationships between identified anti-diabetic activities of extracts and anti-oxidant properties.

Objective 3: To evaluate phenolic metabolites characteristic of Cree medicinal plants *in vitro* for neuroprotective activities relevant to neurodegenerative conditions of the central nervous system.

Specific objective 3.1 – To characterize the cellular effects of physiologically relevant concentrations of structurally diverse phenolic compounds on cell viability in different stress paradigms.

Specific objective 3.2 – To investigate potential structure-activity relationships within structural classes of phenolics based on results obtained in Specific objective 3.1.

Specific objective 3.3 – To identify neuroprotective phenolic compounds in models of progressive neurodegenration.

Due to the collaborative nature of the CIHR TAAM project and its associated publications, the hypotheses and objectives outlined above are not always addressed sequentially but are instead addressed at relevant points throughout the thesis. Furthermore, while the majority of the research presented in the thesis was conducted by the author, additional data obtained by collaborating laboratories are included, when applicable, to address certain hypotheses or to provide greater insight into the context of the presented work and its contribution to the TAAM.

SECTION I

PHYTOCHEMICAL CHARACTERIZATION:

Method development and chemical analyses

CHAPTER 2 – CASE STUDY: DEVELOPMENT AND VALIDATION OF EXTRACTION AND ANALYTICAL METHODS FOR A KEY MEDICINAL PLANT SPECIES, *VACCINIUM ANGUSTIFOLIUM*

2.1 PREFACE

An essential step in any medicinal plant study is the development of reliable methods of quality control, which allow for species authentication, phytochemical standardization and identification of contaminants. Validation of selected methods of extraction and chemical analysis provides important insight into their efficiency, variability, and limitations. Because few methods for the analysis of medicinal plants from the boreal forest have been published, we first sought to develop a robust method for the characterization of different plant parts from a single species. A test plant, recommended by Quebec traditional practitioners and Cree Elders of Eeyou Istchee for the treatment of diabetes, was chosen to validate our protocol for identifying individual phenolic compounds. The overall objective was to provide proof of principle for a metabolomics methodology that would facilitate the characterization of other medicinal plants of the boreal forest.

2.2 STATEMENT OF AUTHOR CONTRIBUTIONS

CSH, AJB and JTA conceived and designed the experiments. CSH and AJB developed the analytical methods that were subsequently validated by CSH and AS. CSH wrote the manuscript with JTA and SALB. PML conducted initial extractions and chemical investigations. As the CIHR-TAAM project leader and project co-ordinator, respectively,

PSH and LCM have managed and directed the pharmacological assessment of Cree extracts and contributed to manuscript preparation.

2.3 A SINGLE HPLC-PAD-APCI/MS METHOD FOR THE QUANTITATIVE COMPARISON OF PHENOLIC COMPOUNDS FOUND IN LEAF, STEM, ROOT AND FRUIT EXTRACTS OF VACCINIUM ANGUSTIFOLIUM

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Publication:

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ABSTRACT

A method was developed for the analysis of *Vaccinium angustifolium* Ait. (Lowbush blueberry), which is a widely used natural health product, particularly for the treatment of diabetic symptoms. While the anthocyanin content of the fruit has been well characterized, the chemistry of the vegetative parts used in supportive therapy for diabetes has been largely ignored. Using a metabolomics-based approach for compound identification with an emphasis on phenolic metabolites, a single HPLC-PAD-APCI/MS method was developed for the separation and quantitation of the major metabolites found in the 95% ethanol extracts of leaf, stem, root and fruit. The leaf extract contained high concentrations of chlorogenic acid (~100 μg/mg extract) and a variety of quercetin glycosides that were also detected in the fruit and stem extracts. Flavan-3-ol monomers (+)-catechin and (-)-epicatechin were found in all plant parts but their procyanidin dimers were exclusively identified in the stem and root. The accuracy and precision of the presented method was corroborated by low intra- and inter-day variations in quantitative results in all plant part extracts. Further validation of the extraction and analytical protocols focused on identified compounds with reputed anti-diabetic activity, revealing recoveries greater than 80% and detection limits of 0.12 - 2.73 μg / ml.

INTRODUCTION

Various members of the genus *Vaccinium* have been used as traditional medicines for the treatment of diabetic symptoms (Jellin 2005; Leduc et al. 2006) and possess putative anti-diabetic activity (Blumenthal 1998; Chambers and Camire 2003; Cignarella et al. 1996). Although *V. myrtillus* L (European blueberry or bilberry) and *V. macrocarpon* Ait. (American cranberry) are recognized as sources of anti-diabetic phytochemicals, recent surveys have identified *V. angustofolium* Ait. (Canadian lowbush blueberry) as highly recommended by Quebec traditional practitioners and Cree Elders of Eeyou Istchee for treatment of diabetic symptoms and complications (Haddad 2003; Leduc et al. 2006).

Lowbush blueberry fruits are collected and consumed throughout regions of north-eastern North America and possess well-documented anti-oxidant properties (Kalt et al. 1999; Kay and Holub 2002; Rimando et al. 2004; Wang and Jiao 2000). By virtue of its popular consumption as a food, the phytochemistry of *V. angustifolium* fruits is relatively well described, particularly with regard to anthocyanin content (Kalt et al. 2001; Prior et al. 2001; Wu and Prior 2005). The traditional medicinal uses of the plant, however, include the leaves, stems and roots in addition to the fruits to treat different diabetic symptoms. Moreover, in a recent *in vitro* study, extracts of *V. angustifolium* leaf, stem, root and berry each elicited a different spectrum of anti-diabetic activities (Martineau et al. 2006). Few, if any, studies have investigated or compared the chemistry of these sources.

The present study describes the development and validation of an HPLC-PAD-APCI/MS method for analyzing the phytochemical content of *V. angustifolium* with an emphasis on phenolic compounds in different plant parts. Phenolic compounds were emphasized because of their abundance in closely related *Vaccinium* species (Witzell 2003) and because many different phenolics have demonstrated *in vivo* anti-diabetic activities

(Coskun et al. 2005; Kim et al. 2003). Using a metabolomics-based approach for preliminary compound identification and reference standards for confirmation, chemical profiles for each organ were determined and quantitative comparisons conducted.

EXPERIMENTAL

Materials

HPLC solvents and analytical grade trifluoroacetic acid (TFA) were purchased from EMD (Darmstadt, Germany) and J.T. Baker (Phillipsburg, New Jersey, USA), respectively. Standards of (+)-catechin and quercetin-3-*O*-glycoside were obtained from Extrasynthase (Lyon, France), quercetin, chlorogenic acid and caffeic acid from Sigma (Oakville, Canada), and (-)-epicatechin from Fluka (Buchs, Switzerland). Procyanidins were graciously provided by Dr. D. Ferreira (University of Mississippi, USA) and the remaining standards (quercetin glycosides and compounds comprising the phenolic library) were isolated and purified 'in house' (>95% purity as determined by HPLC) (Lozoya et al. 1994).

Sample preparation and extraction

Fresh samples of wild *Vaccinium angustifolium* Ait. were collected near La Vérendrye Wildlife Reserve, Quebec, Canada on Aug 27 2005. Voucher specimens were stored at the University of Ottawa (UO#19190). After harvesting, the plant material was washed and manually separated into organs: leaves, stems, roots and fruits.

Fresh leaves were preserved in 95% ethanol then filtered, crushed and re-extracted three times with fresh ethanol (95%) at room temperature for 12 h. The four ethanolic phases were recovered, pooled, dried at 40°C on a rotary evaporator and lyophilised. Roots and stems were air-dried and stored at room temperature in the dark prior to extraction. The

fruits were frozen at -80°C. A Wiley mill with mesh size 40 was used for grinding each source and the resulting powder extracted three times in ethanol at room temperature for 12 h. The ethanolic phase was then dried and lyophilised as above. All extracts were stored at 4°C.

HPLC-PAD-APCI/MS analyses

HPLC-MS analyses were conducted on leaf, stem, root and fruit extracts. Since the anthocyanin content of fruit has been extensively characterized (Kalt et al. 2001; Prior et al. 2001; Rimando et al. 2004; Wu and Prior 2005), this family of compounds was not examined in detail. Analyses were performed on an Agilent (Palo Alto, CA, USA) 1100 LC MSD VL APCI system consisting of an autosampler, quaternary pump, photodiode array detector (PAD), and an online APCI/MS with mass range of 50-15000 amu. A Waters (Mississauga, Canada) YMC ODS-AM column (100 × 2 mm i.d..; 3 μm particle size), maintained at 50°C, was employed at a flow rate of 0.3 mL/min. The elution conditions were optimized with a mobile phase of aqueous TFA (0.05%), pH 3.4 (solvent A) and methanol (solvent B) as follows: initial conditions 92:8 (A:B) maintained 0-5 min, followed by four linear gradients of 8 – 13% B in 2 min, 13 – 30% B in 14 min, 30 – 60% B in 3 min, and 60 – 100% B in 2 min. The column was then washed with 100% B for 2 min, returned to the initial conditions (92:8) in 2 min, and re-equilibrated for 6 min, resulting in a total run time of 36 min. An aliquot (1 μl) of each extract was injected through the autosampler for each run and the subsequent elution profiles were monitored on-line at 325 nm and 280 nm (PAD) and by total-ion current (TIC). A monitoring wavelength of 520 nm was also used for qualitative analysis of anthocyanins in the fruit extract.

The mass spectrometer was tuned in dual polarity mode at the outset of all experiments. MS detection was performed in both positive and negative ionization modes. For positive ionization mode, the optimized spray chamber conditions were: drying gas flow rate of 6.0 L/min, nebulizer pressure of 40 psig, drying gas temperature of 300 °C, vaporizer temperature of 400 °C, capillary voltage of 3000 V, and corona current of 3.0 μA. For negative ionization mode, the conditions were: drying gas flow rate of 6.0 L/min, nebulizer pressure of 60 psig, drying gas temperature of 350 °C, vaporizer temperature of 400 °C, capillary voltage of –3000 V, and corona current of 15.0 μA. APCI was conducted at 300°C with the vaporizer at 400°C; nebulizer pressure, 40 psig; nitrogen (drying gas) flow rate, 6.0 L/min; fragmentation voltage, 20 V; capillary voltage, 3000 V; corona current, 3.0 μA. The MS was operated in scan mode within 100 to 800 amu with fragmentation voltages of 20 and –160 V for positive and negative ionization respectively.

Metabolomics-based compound identification

Over 120 purified phenolic compounds were injected in the HPLC system where the UV absorption spectrum was scanned and saved into a searchable library of reference standards. These spectra were used for comparison with the absorption spectra of the unknown compounds in the test extracts. A preliminary match to a library entry was defined by at least 95% similarity between the spectrum of an unknown peak and the library entry, as determined by the Chemstation software. Matches were further corroborated by visual inspection of the spectral match and by the presence of a major ion in the MS of the unknown peak's mass spectrum corresponding to the entry molecule or its major ion fragment (e.g. an ion peak corresponding to a monomeric procyanidin, along with a second peak corresponding to a dimer, would support a match to a procyanidin library entry).

Co-chromatography of standards of the identified compounds spiked into plant extracts was then performed to confirm matches, with retention time serving to distinguish between isomers (eg. quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside).

Compound quantification

Identified metabolites were quantified on the basis of area under the peak of HPLC-PAD chromatograms using calibration curves produced using pure standards analyzed on the same day. Phenolic acids and flavonols were quantified at 325 nm and catechins and procyanidins were quantified at 280 nm. Mean quantities were calculated from freshly dissolved triplicates of each extract quantified on three separate days (minimum n =9).

Method validation

Validation was performed for HPLC-PAD-APCI/MS in the positive ionization mode using the organic phase gradient program described above. Recovery experiments were undertaken by injecting aliquots of epicatechin and quercetin-3-*O*-glucoside standards (0.5, 1.0, 1.5 and 2.0 mg/mL) to stem material and aliquots of chlorogenic acid standard (2.5, 5.0, 7.5, and 10 mg/mL) to leaf material. Spiked and unspiked samples were extracted as described above. Experiments were carried out in triplicate and coefficients of variation determined accordingly.

The limit of detection (LOD) and limit of quantification (LOQ), respectively defined as a 3:1 and 10:1 peak to noise ratio, were determined for selected compounds by analysis of serially diluted leaf, stem, root and fruit extracts.

Statistical analysis

All statistical analyses were performed with Systat software version 10.0 (SPSS, Chicago, IL, USA); ANOVA analyses and subsequent Tukey *post-hoc* tests were used to compare plant-part specific metabolite levels. Recovery data were analyzed by simple linear regression.

RESULTS AND DISCUSSION

Chromatographic separations of extracts and compound identification

The ethanolic extraction of *V. angustifolium* leaves, stems, roots and fruits produced yields of 30, 9, 5 and 50% extract, respectively. Based on our previous work with phenolic-rich extracts (Bily et al. 2004), it was established that an aqueous mobile phase containing 0.05% TFA produced high quality peak shape and separation. Linear gradients of methanol and acetonitrile were individually optimized as the organic phase. Although compounds eluted more quickly from all extracts in acetonitrile, the resolution of early and late eluting peaks suffered and lower signal-to-noise ratios were observed during analysis of stems and roots, presumably due to high procyanidin/tannin content. While this noise was still apparent using methanol as the organic phase, subsequent optimization (as described in Experimental) with a 28-min methanol gradient programme yielded major peaks from all plant parts with sufficient separation for quantitation. Attempts to improve the separation of late eluting peaks by decreasing flow rate or by isocratic elution resulted in broader peaks. The improvement provided by extended gradient programs was marginal. MS results were also improved using methanol but only when the column was maintained at high temperature (50 °C). Note that the described methods were not optimized for separation of anthocyanins.

Metabolomics-based compound identification yielded several preliminary matches

that corresponded to caffeic acid derivatives, quercetin derivatives, flavan-3-ols and procyanidins. The identities of 10 compounds were confirmed within the leaf extract, 12 within the stem extract, and seven within the root extract. Excluding anthocyanin peaks, eight compounds were identified in the fruit extract. Additional tentative identifications were noted but not definitively confirmed and amounts were not quantitated (Table 2.1). While conclusive identifications could not be made for all major peaks, the metabolomics approach proved successful as a guide. Owing to the presence of many closely related compounds, similar UV absorption spectra and/or ion fragmentation patterns highlighted the need for tri-corroboration of these measures and retention time with those of standards. In cases where all criteria were not met adequately, confirmation was made (or rejected) by spiking the extract with a suitable amount of standard.

Distinct root and leaf compounds were identified while the stem extract appeared to be intermediate between leaf and root. Chlorogenic acid was identified as the most abundant phenolic in leaves consistent with previous analyses of leaves from *V. myrtillus* (Witzell and Shevtsova 2004). Beyond this early peak, most peaks in the leaf chromatograph corresponded to quercetin glycosides (Figure 2.2A). The identity of four of these glycosides was confirmed (quercetin-3-*O*-galactoside, Q-3-gal; quercetin-3-*O*-glucoside, Q-3-glu; quercetin-3-*O*-arabinoside, Q-3-ara; quercetin-3-*O*-rhamnoside, Q-3-rham) and two additional quercetin glycosides were identified, one containing a hexose and the other a pentose, according to their MS fragmentation pattern and absorption spectra (Table 2.1, Figure 2.2C). The remaining identified peaks corresponded to an isomer of chlorogenic acid (peak 1), (+)-catechin (catechin, peak 3), caffeic acid (peak 5), and (-)-epicatechin (epicatechin, peak 8). Small peaks eluting at retention times of 14.4 and 26.8 min, tentatively identified as a *p*-coumaroyl quinic acid and kaempferol-3-*O*-rutinoside

Table 2.1 – List of the compounds identified in *Vaccinium angustifolium* leaf, stem, root and fruit extracts by metabolomics-based HPLC-PAD-APCI/MS analysis.

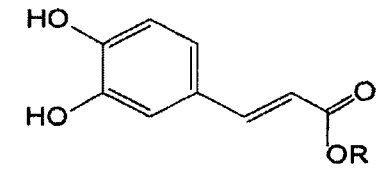
Peak	Rt[a] (min.)	$[M]^+$ & fragments (*m/z*)	Compound	Content (mg / g dry matter) ± SEM[b]			
				LEAF	STEM	ROOT	FRUIT
1	4.0	355/195/163	chlorogenic acid isomer*	✓	✓		
2	5.3	579/291	Procyanidin B1		0.81 ± 0.02	0.04 ± 0.007	
3	6.6	291/139	Catechin	6.16 ± 0.52	1.25 ± 0.05	0.05 ± 0.009	Trace
4	8.3	169	Vanillic acid		Trace	$0.01 \pm 4e^{-4}$	
5	9.2	195/179/163	Caffeic acid	0.36 ± 0.02	0.03 ± 0.007		
6	10.5	355/195/163	Chlorogenic acid	31.19 ± 0.55	0.09 ± 0.008	0.03 ± 0.007	1.54 ± 0.01
7	11.2	579/291	Procyanidin B2		0.98 ± 0.03	0.21 ± 0.01	
8	13.7	291/139	Epicatechin	7.25 ± 0.35	2.90 ± 0.10	0.70 ± 0.04	Trace
9	13.9	577/291	Procyanidin*		✓	✓	
10	14.9	579/291	Procyanidin*		✓	✓	
11	15.4	579/291	Procyanidin*		✓	✓	
12	24.2	465/303	Quercetin-3-*O*-galactoside	1.96 ± 0.05	0.45 ± 0.01		0.31 ± 0.02
13	24.8	465/303	Quercetin-3-*O*-glucoside	3.49 ± 0.08	0.13 ± 0.01		0.41 ± 0.02
14	25.2	465/303	Quercetin-hexoside*	✓	✓		✓
15	25.8	435/303	Quercetin-3-*O*-arabinoside	2.74 ± 0.06	0.67 ± 0.02		Trace
16	26.3	435/303	Quercetin-pentoside*	✓	✓		✓
17	26.6	449/303	Quercetin-3-*O*-rhamnoside	1.46 ± 0.05	$0.01 \pm 7e^{-4}$		Trace
18	26.8	595/449/287	Kaempferol-3-*O*-rutinoside*	✓			
19	27.6	303	Quercetin	1.24 ± 0.04	$0.01 \pm 6e^{-4}$		$0.03 \pm 2e^{-4}$

* indicates tentative identification without confirmation relative to a pure standard.

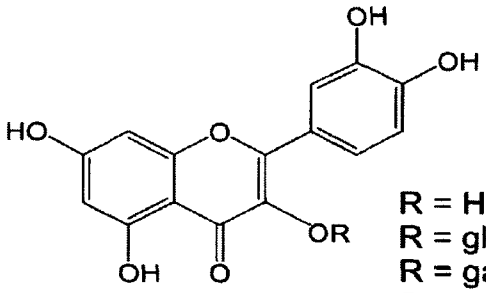
✓ indicates the detection but not quantification of a tentatively identified compound.

[a] Rt, retention time

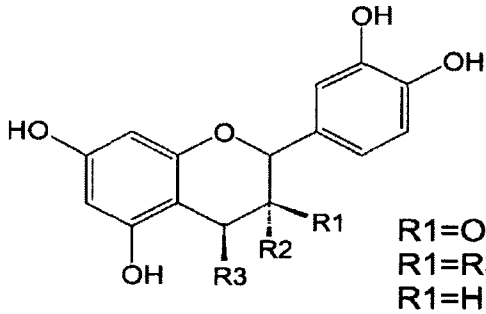
[b] SEM, standard error of the mean



R = H; caffeic acid
R = (5)-quinic acid; chlorogenic acid



R = H; quercetin
R = glucose; quercetin-3-*O*-glucoside
R = galactose; quercetin-3-*O*-galactoside
R = arabinose; quercetin-3-*O*-arabinoside
R = rhamnose; quercetin-3-*O*-rhamnoside



R1=OH, R2=R3=H; (+)-catechin
R1=R3=H, R2=OH; (-)-epicatechin
R1=H, R2=OH,
R3=(ß-8)-catechin; procyanidin B1
R1=H, R2=OH,
R3=(ß-8)-epicatechin; procyanidin B2

Figure 2.1 – Chemical structures of commonly identified phenolic metabolites of *Vaccinium angustifolium* extracts. Created using ChemDraw software version 8.0.

respectively, were seen only in the leaf chromatogram.

By contrast, stem extracts contained only low levels of chlorogenic acid. The same quercetin glycosides found in the leaf extract were identified in stems though in lower amounts and at different relative abundances (Figure 2.3A). In addition to the monomeric flavan-3-ols detected in leaf material (catechin and epicatechin), a variety of peaks corresponding to dimers (procyanidins) were eluted from the stem extract during the first 15 min of the gradient.

These procyanidin peaks were also seen in the root extract (Figure 2.4), revealing a chemical profile similar to that of the stem but unlike that of the leaf or fruit. The presence of procyanidins B1 and B2 was confirmed in both stem and root chromatographs. Peaks 9-11, each with catechin-matching absorption spectra, represent additional procyanidin species, as supported by MS data indicating two dimers (peaks 10 and 11) and a double-linked dimer (see Table 1, Figure 2C) (Nakamura 2003). Similarly, the compounds eluting from stem and root extracts between 19 and 21 min again produced catechin-like UV spectra and shared a common ion fragment ($m/z = 249$). To gain greater insight into the diversity of procyanidin oligomers occurring in the stem and root, an acetone extraction should be considered and a broader mass range employed during ion scanning. Finally, small amounts of vanillic acid were exclusively detected in the root extract (peak 4).

In this study, the fruit chromatograph strongly resembled the leaf chromatograph; excluding anthocyanin peaks, the peaks in the fruit chromatograph were representative of chlorogenic acid, quercetin glycosides and catechins (Figure 2.5). These findings are consistent with the compounds previously identified from lowbrush blueberries (Taruscio et al. 2004). PAD and MS data for the quercetin hexosides indicated that these compounds eluted independently of anthocyanins. In the case of Q-3-ara and Q-3-rham, co-elution with

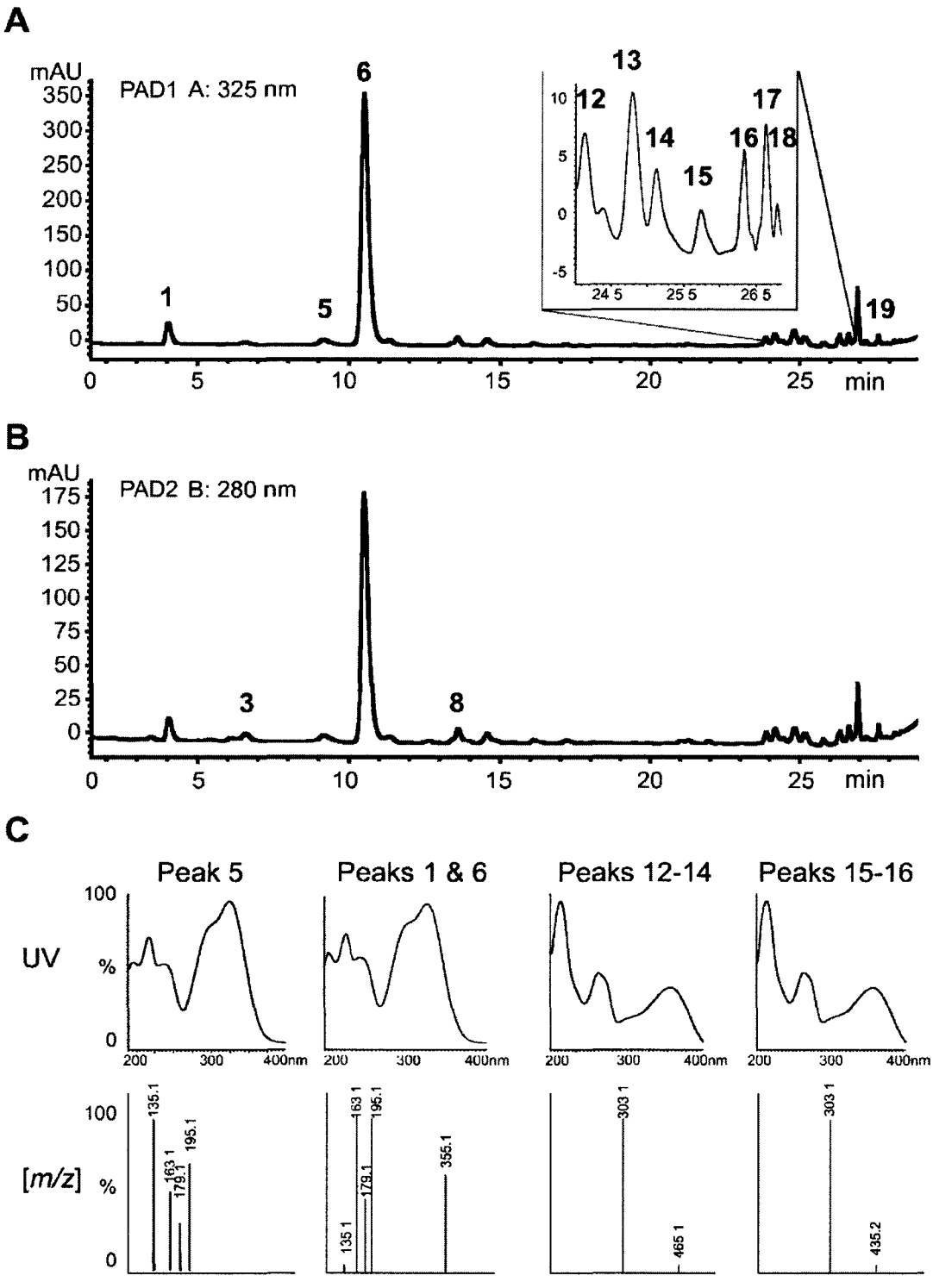


Figure 2.2 – HPLC chromatograms of a leaf extract of *Vaccinium angustifolium* with photodiode array detection (PAD) at 325 nm (A) and 280 nm (B). Numbered peaks represent identified compounds as reported in Table 1. The UV absorption and mass spectral data obtained from selected peaks identified multiple caffeic acid and quercetin derivatives (C, [m/z] data reported for ions with relative abundances >15%).

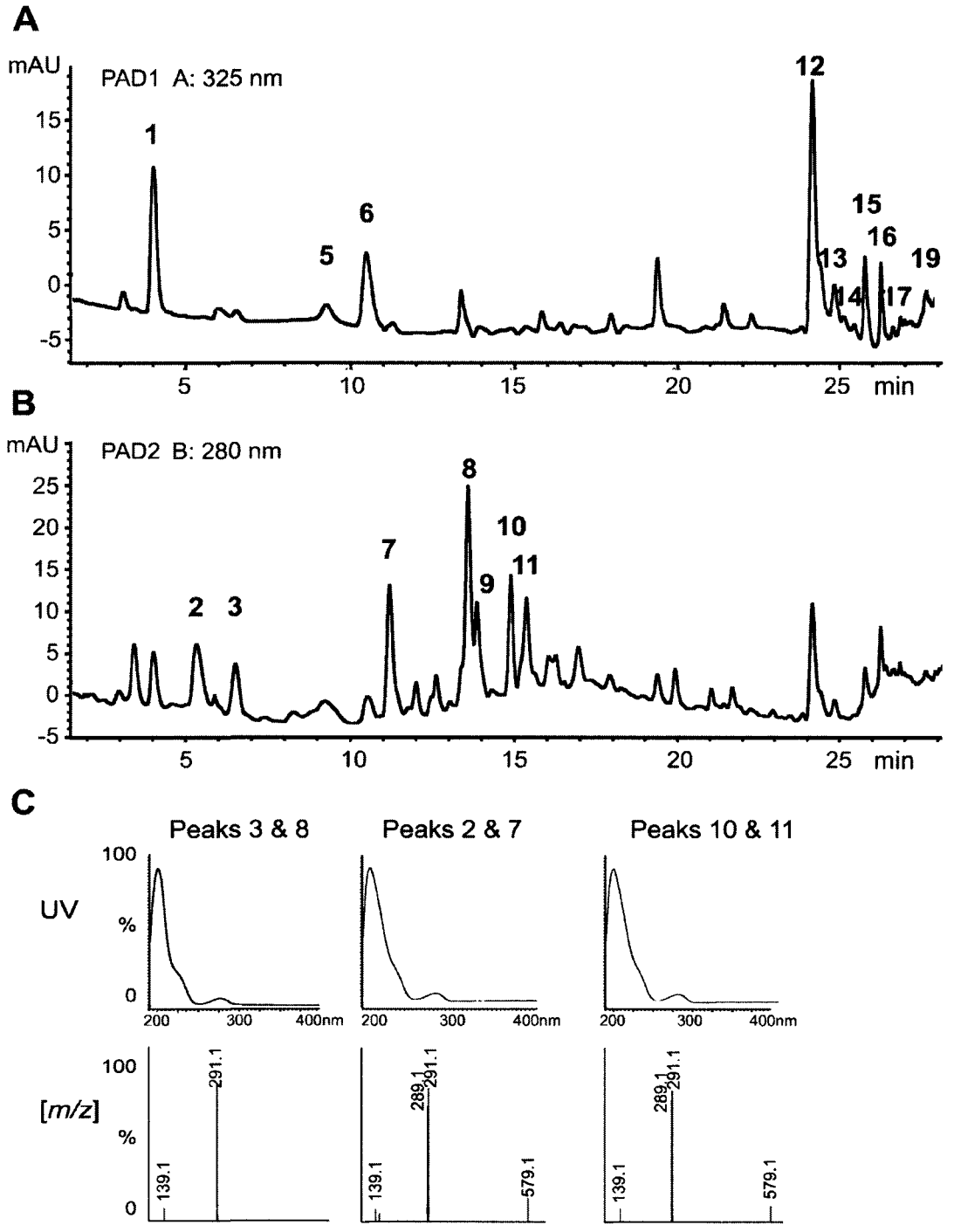


Figure 2.3 – HPLC chromatograms of a stem extract of *Vaccinium angustifolium* with photodiode array detection (PAD) at 325 nm (A) and 280 nm (B). Numbered peaks represent identified compounds as reported in Table 1. The UV absorption and mass spectral data obtained from selected peaks identified several closely related compounds representing procyanidin species (C, [m/z] data reported for ions with relative abundances >15%).

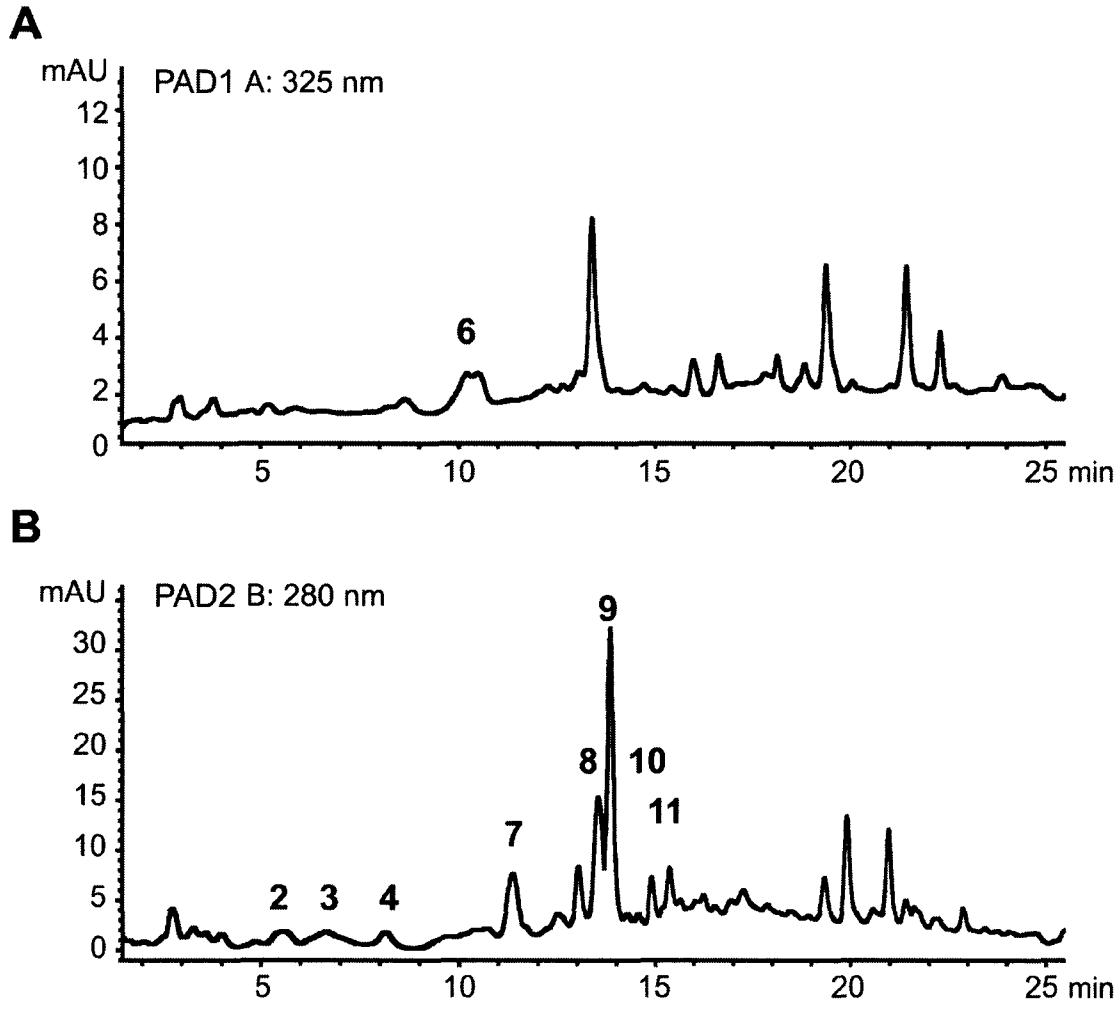


Figure 2.4 – HPLC chromatograms of a root extract of *Vaccinium angustifolium* with photodiode array detection (PAD) at 325 nm (A) and 280 nm (B). Numbered peaks represent identified compounds as reported in Table 1.

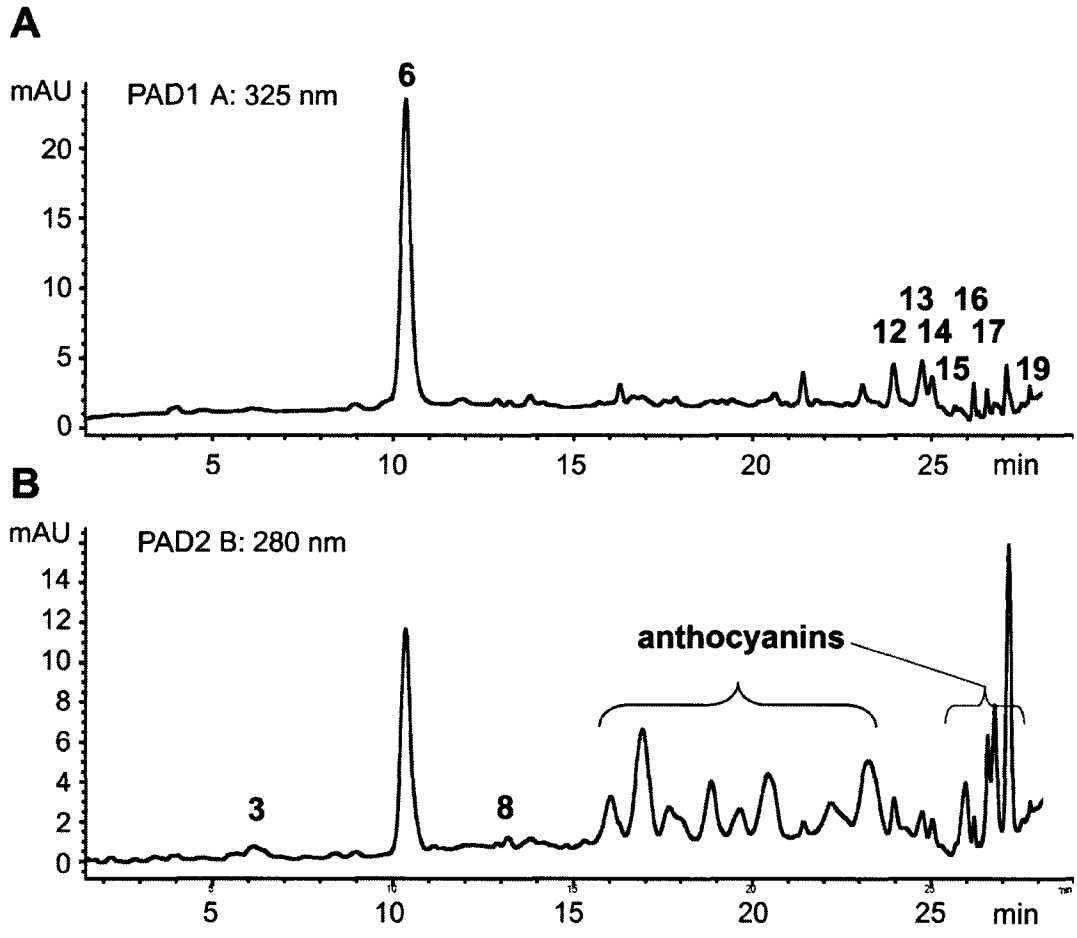


Figure 2.5 – HPLC chromatograms of a fruit extract of *Vaccinium angustifolium* with photodiode array detection (PAD) at 325 nm (A) and 280 nm (B). Numbered peaks represent identified compounds as reported in Table 1.

anthocyanins prevented accurate quantitation and confirmation by MS data (Fig. 2.5B). Interfering anthocyanins (eluting between 26-27 min) were tentatively identified as malvidin glycosides, producing ion fragments of 331 m/z (data not shown). Separation of anthocyanins improved drastically, resolving 20 peaks, with a 5% formate aqueous phase during chromatography, as previously reported (Prior et al. 2001).

Although low levels of procyanidins (3 μg/g dry wt) have been reported in lowbush blueberry fruits (Prior et al. 2001), only trace amounts of monomeric catechin and epicatechin were detected using the presented methods. Higher procyanidin yields can be achieved with an acetone extraction but phenolic content of each plant part is also subject to both temporal and environmental variation (Witzell and Shevtsova 2004; Witzell 2003), which may contribute to variation between reports. Development of a validated method for constituent identification and quantification is therefore a necessary step if comparisons are to be made.

Whereas certain compounds (catechin, chlorogenic acid and epicatechin) were common to all plant organs, none was ubiquitously abundant and most were restricted in distribution. In this study, chlorogenic acid, the predominant phenolic acid derivative detected, was 30 times more concentrated in leaf extract than in fruit and >100 times more concentrated than in stem or root extracts (Figure 2.6A). The distribution of identified quercetin derivatives likewise differed significantly between plant parts. While Q-3-glu and Q-3-rham accounted, respectively, for 36 and 18% of the quantified quercetin glycosides in leaf extract, they represented just 10 and 1% in stem extract. In the fruit extract, where glycoside species were present in similar ratios to those seen in the leaf, the content was approximately ten fold less (Figure 2.6B). With regard to flavan-3-ols, leaf and stem extracts had similar quantities but the concentration in roots was significantly lower (Figure 2.6C).

The detected ratio of epicatechin to catechin was more variable, roughly 1:1 in the leaf extracts, 2:1 in the stem and 14:1 in the root. Although the stem extract contained higher levels of procyanidins B1 and B2, the area of peak 9 (Figure 2.3 and 2.4), corresponding to an unknown procyanidin dimer, was substantially greater in the root.

Considering that, in a separate pharmacological study, each extract (leaf, stem, root and fruit) elicited a range of in vitro anti-diabetic activities (Martineau et al. 2006), it is interesting that all the identified classes of phenolic compounds, chlorogenic acid, quercetin derivatives, procyanidins and anthocyanins, have demonstrated anti-diabetic potential in vitro and in vivo (Ajay et al. 2006; Coskun et al. 2005; Dias et al. 2005; Hemmerle et al. 1997; Jayaprakasam et al. 2006; Kim et al. 2003; Pinent et al. 2004; Rodriguez de Sotillo and Hadley 2002; Tsuda et al. 2003). Validation of the extraction and analytical protocols therefore focused on those compounds with reputed anti-diabetic activity. However, since *V. angustifolium* contains additional secondary metabolites that may possess biological activity, further chemical analyses and assay-guided fractionation are required to identify the active constituents.

Method validation of marker compounds

Dual PAD monitoring of UV absorption at wavelengths of 325 and 280 nm provided optimal detection of all identified metabolites except anthocyanins. Moreover, detection of anthocyanins in the fruit extract was severely dampened at 325 nm, simplifying the detection of quercetin derivatives (Figure 2.5). Should anthocyanin characterization be required, a wavelength of 520 nm would identify these compounds (Figure 2.5B).

The production of reference calibration curves during compound quantification revealed linear response profiles ($R^2 > 0.999$) for all quantified compounds at minimum

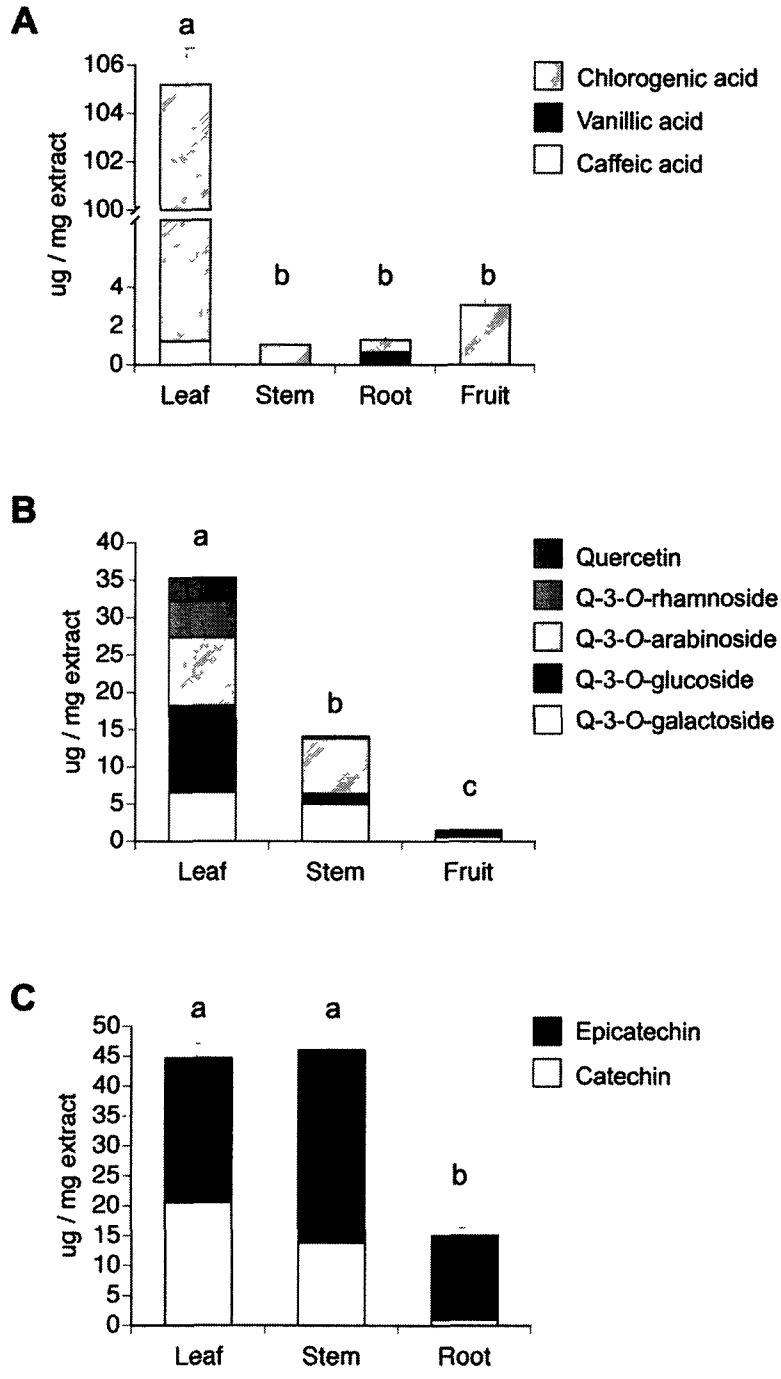


Figure 2.6 – Quantitative comparisons of commonly identified metabolites in leaf, stem, root and/or fruit extracts. Chlorogenic acid was the most abundant phenolic acid in all extracts (A). The total quantified quercetin derivatives were highest in the leaf extract (B). Total monomeric procyanidin content was similar in leaf and stem extracts but was significantly lower in the root (C). Significant differences ($P<0.05$), as determined by one-way analysis of variance with Tukey post hoc test, are designated by letters (a, b, c).

ranges of 6.6-330 μg/mL (Q-3-ara) to 7.7-1440 μg/mL (chlorogenic acid) (data not shown).

To assess the accuracy and precision of the presented method, variation in the results for each quantified compound were calculated within and between days. Expressed as the co-efficient of variation (CV, standard deviation / mean x 100%), variation between successive trials (intraday) was minimal on days 1 and 2 (Table 2.2). The slightly more pronounced variability on the third day was likely due to column degradation as this third trial was conducted several months following the first two. Nevertheless, with the exception of a few compounds detected at levels near or below 1 μg/mg extract, interday variation was generally less than 10% (Table 2.2).

Analysis of serially diluted samples and subsequent peak to noise ratio calculation generated LOD and LOQ values for a subset of identified compounds (Table 2.3). LOD and LOQ values are reported as the lowest detectable and quantifiable amounts of a compound present in a 1mL sample (i.e., total content of the sample, regardless of extract concentration) and did not differ significantly between plant parts. The LOD values of chlorogenic acid, Q-3-gal, Q-3-glu and quercetin were 329, 415, 437, and 116 ng, respectively, while the catechins and procyanidins were not detectable until concentrations exceeding 1.5 μg/mg extract. LOQ values were consistently lower for compounds quantified at 325 nm than those quantified at 280 nm; to increase sensitivity to flavan-3-ols, a lower monitoring wavelength can be employed (230 nm) but baseline drift and/or noise will also increase. The recommended extract concentrations for quantifiable detection of major metabolites is 10 mg/mL for leaf extract and 25 mg/mL for stem, root and fruit extracts.

Table 2.2 – Intraday and interday variation in quantitative results.

	Intraday Variation (SD/ mean amount x 100%)			Interday variation
	Day 1	Day 2	Day 3	Days 1-3
Leaf				
Catechin	0.63	1.56	6.45	5.27
Caffeic acid	14.32	7.26	6.40	21.71
Chlorogenic acid	0.14	0.94	1.39	3.23
Epicatechin	0.12	8.61	12.74	7.71
Quercetin-3-*O*-galactoside	0.36	0.54	2.74	3.85
Quercetin-3-*O*-glucoside	0.23	0.64	3.65	3.49
Quercetin-3-*O*-arabinoside	0.23	1.40	8.25	9.71
Quercetin-3-*O*-rhamnoside	0.19	2.16	0.91	0.79
Quercetin	0.77	2.84	6.40	6.38
Stem				
Procyanidin B1	0.78	0.20	13.40	6.43
Catechin	1.00	0.77	4.26	7.84
Chlorogenic acid	2.71	1.23	5.20	9.83
Procyanidin B2	0.85	2.29	8.76	7.41
Epicatechin	4.00	4.98	10.94	9.58
Quercetin-3-*O*-galactoside	1.29	1.23	0.69	2.10
Quercetin-3-*O*-glucoside	1.33	0.32	10.32	12.82
Quercetin-3-*O*-arabinoside	0.63	0.41	9.77	6.51
Quercetin-3-*O*-rhamnoside	1.50	0.70	14.07	5.09
Quercetin	1.16	2.72	9.82	22.30
Root				
Procyanidin B1	5.25	1.00	5.79	7.05
Catechin	0.91	2.46	7.89	5.49
Vanillic acid	4.32	2.04	17.66	11.1
Chlorogenic acid	9.15	0.29	13.02	8.04
Procyanidin B2	9.92	0.53	7.90	8.72
Epicatechin	5.61	5.20	4.25	4.54
Fruit				
Chlorogenic acid	1.61	0.61	4.68	2.07
Quercetin-3-*O*-galactoside	7.12	24.32	4.47	2.86
Quercetin-3-*O*-glucoside	4.73	0.43	3.25	2.08
Quercetin	1.18	2.11	3.57	0.88

SD = standard deviation

Table 2.3 – Methodological limits of detection and quantification and their corresponding minimum concentrations for detection in the leaf, stem, root and fruit extracts of *V. angustifolium*.

Compound	Limit of Detection (μg / mL ± SEM)	Limit of Quantification (μg / mL ± SEM)
Procyanidin B1	1.63 ± 0.30	5.42 ± 0.99
Catechin	2.73 ± 0.40	9.11 ± 1.12
Chlorogenic acid	0.33 ± 0.04	1.10 ± 0.11
Procyanidin B2	2.03 ± 0.19	6.77 ± 0.49
Epicatechin	1.77 ± 0.15	5.91 ± 0.44
Quercetin-3-*O*-galactoside	0.42 ± 0.06	1.38 ± 0.19
Quercetin-3-*O*-glucoside	0.44 ± 0.06	1.46 ± 0.18
Quercetin	0.12 ± 0.02	0.39 ± 0.09

SEM = standard error of the mean

Table 2.4 – Recovery of selected compounds from leaves and stems of *V. angustifolium*.

	CV[(a)] of yield (%)	Recovery (% recovered) Level 1	Level 2	Level 3	Level 4	Mean*	R^{2} [(b)]
Leaf		(+2.5 mg)	(+5.0 mg)	(+7.5 mg)	(+10 mg)		
Chlorogenic acid	*6.1*	*88.0*	*84.0*	*100.4*	*107.7*	*103.8*	*0.985*
Stem		*(+0.5 mg)*	*(+1.0 mg)*	*(+1.5 mg)*	*(+2.0 mg)*		
Epicatechin	*4.8*	*100.3*	*89.6*	*87.8*	*92.3*	*91.0*	*0.962*
Quercetin-3-O-glucoside	*4.8*	*77.8*	*83.3*	*78.1*	*101.4*	*80.1*	*0.970*

* As determined from slope of regression analysis of injected and recovered quantities

[a] CV, coefficient of variation; standard deviation / mean × 100%

[b] R^2, R-squared value of linear regression

Recovery analyses were restricted to three representative compounds spiked into either leaf or stem material prior to extraction. Plotting recovered amounts versus spiked amounts, the corresponding regression line provides the mean recovery across concentrations (the slope), the linearity of recovery over the range of spiking (R^2) and the approximate content of unspiked samples (y-intercept). Despite spiking high amounts of chlorogenic acid into leaves, all were recovered during the extraction procedure (Table 2.4). Considering that separate extractions conducted on separate samples produced a yield with a CV of 6.1%, a mean recovery of 104% is reasonable. While epicatechin and Q-3-glu were recovered well with mean recoveries of 91 and 80%, respectively, and R^2 values greater than 0.96 (Table 2.4), more complex procyanidins were likely recovered less efficiently. In general, the best recoveries were observed from extracted leaves, which were preserved fresh. We therefore recommend using 80% ethanol for extraction of dried samples.

Following these results, the validated method is currently being used to evaluate the phytochemical content and corresponding medicinal properties of the plant from population to population and from season to season. The wide distribution and range of habitats occupied by the species suggests that, as *V. angustifolium* becomes more popular as a medicinal plant and commercial products are made available, the value of a method for rapid analysis of marker compounds increases.

ACKNOWLEDGEMENTS

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CHAPTER 3 - PHENOLIC FINGERPRINTS OF 14 SPECIES FROM AN IMPORTANT BOREAL MEDICINAL PLANT FAMILY, THE ERICACEAE

3.1 PREFACE

Having developed a validated method for the extraction and characterization of compounds in a single species, *Vaccinium angustifolium*, I expanded these applications to the analysis of other Ericaceae family members since it is a major boreal plant family. Here, I evaluated 5 of the 17 most highly rated anti-diabetic species identified in collaboration with the members of our CIHR-TAAM. My objective was to identify marker compounds in leaf extracts of boreal Ericaceae species and develop a rapid fingerprinting method that could serve purposes of both quality control and qualitative/quantitative comparisons between species. While a methodical analysis of all 17 Cree plant extracts is beyond the scope of this chapter and thesis, further phytochemical characterization is provided throughout Section 2 and a complete set of chromatograms (with methods) is presented in Appendix 1.

3.2 STATEMENT OF AUTHOR CONTRIBUTIONS

CSH, AS and JTA conceived and designed the experiments. AS and CSH equally contributed to the phytochemical analyses and wrote the manuscript with JTA. AC collected and summarized the ethnobotanical data presented in Table 3.2 thanks in part to knowledge shared by the Elders of CEI. JC assisted with the collection of plant materials. As the CIHR-TAAM project leader and project co-ordinator, respectively, PSH and LCM have managed and directed the pharmacological assessment of Cree extracts and contributed to manuscript preparation.

3.3 *METABOLOMIC METHODS FOR THE IDENTITY AND QUALITY ASSURANCE OF NORTHERN MEDICINAL ERICACEAE*

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ABSTRACT

Because of the importance given to Ericaceous medicinal plants by the Eeyou Istchee Cree and other northern peoples of North America, a reliable species authentication method was developed for reversed phase-high performance liquid chromatography (RP-HPLC). Twenty-three marker compounds were identified in the leaf extracts of 14 Ericaceae species using a metabolomics approach focusing on phenolic constituents. This approach offered the assignment of unique fingerprints of known marker compounds for each species under study. The performance of chromatographic methodology was evaluated by determining the retention factors of marker compounds, which ranged from 3.9 – 18.0 min, while the peak asymmetry varied from 0.84 for quercetin-3-*O*-glucoside to 1.22 for myricetin. The limits of detection and limits of quantification were 0.30 ± 0.03 and 1.01 ± 0.11 μg/ml for (+)-catechin and plate numbers for selected markers were in the range of 23900 –46100. The extraction yields were notably high, ranging from 30.9% (*Rhododendron groenlandicum*) to 50.1% (*Arctostaphylos uva-ursi*) while the recovery of quecetin-3-*O*-rhamnoside, one of the most ubiquitous markers among species, was 86 %. The present study describes a rapid method for the characterization and quantitation of marker compounds that can be used for the authentication of medicinal species and quality assurance of herbal products used for local or commercial purposes. As illustrated by the inclusion of a species from Costa Rica, the outlined methods could be further applied to other members of the Ericaceae family.

INTRODUCTION

The Ericaceae (heath family) are mostly shrubs which are characteristic species of Northern and mountainous regions of North America, although there are many species in the tropics. The family comprises about 125 genera and 3500 species worldwide. In Canada there are 18 genera, representing some of the most important native medicinal and edible plants, including blueberry, bilberry, cranberry, Labrador tea, bearberry etc., (Scoggan 1973). In our review of the historic ethnobotanies of Eastern Canada, there were 80 medicinal usage records for the family by the 24 First Nations cultures represented (Arnason 1981). It is one of the top ten plant families in terms of usage mentions for medicine with only the Asteraceae, Rosaceae and Pinaceae families higher in ranking (Jones 2000). Using a Moerman analysis, Jones (2002) found that the use of Ericaceae species as medicinal plants is significantly higher than expected based on the number of species present in the flora. Some specific usage examples in the published literature include *Chimiphila umbellata* for kidney problems, *Rhododendron groenlandicum* for colds, *Kalmia angustifolia* for headache and *Arctous alpina* (Alpine Bearberry) for treating rheumatism (Moerman 1998). Many more medicinal Ericaceae are described in Moerman's (1998) comprehensive review of native American ethnobotany.

Recently, in collaboration with Cree elders of Eeyou Istchee First Nations, we have been undertaking an evaluation of the traditional medicines for type 2 diabetes mellitus (T2DM) and associated conditions. Using scores for 15 symptoms associated with T2DM, a quantitative ethnobotany analysis used to calculate the syndromic importance value for each species indicated that the most important species for treatment of diabetes was Labrador tea (Leduc *et al.* 2006). Four other Ericaeae were found to be important in this survey and others have been identified in ongoing ethnobotanical studies.

The pharmacological significance of the *Rhododendron* and *Vaccinium* treatments has been demonstrated by their stimulation of glucose uptake in skeletal muscle cells and insulin release by pancreatic cells (Martineau et al. 2006; Spoor et al. 2006). Moreover, many of the Ericaceae have been shown to possess anti-glycation and anti-oxidant activities that are relevant to treatment and prevention of diabetic complications (Fraser 2007; McCune and Johns 2002). We have also found these activities to be related to phenolic content (Fraser 2007, unpublished data).

One of the key requirements of a program evaluating medicinal plants is the development of methods to identify plant materials with confidence and to determine product quality by quantitative analysis. Building from a validated method for one Ericaceae medicinal species, the lowbush blueberry *Vaccinium angustifolium* (Harris et al. 2007a), we sought to develop methods for species identity and quality assurance of other medicinal Ericaceae. Using reversed phase-high performance liquid chromatography with diode-array detection and atmospheric pressure chemical ionization mass spectrometry (HPLC-PAD/APCI-MS), we applied a metabolomics method based on a phenolic library to identify marker phytochemicals in 14 species of Ericaceae and provide quantitative data for representative collections of plant materials.

EXPERIMENTAL

Ethnobotanical data collection

As part of the Canadian Institutes of Health Team in Aboriginal Anti-Diabetic Medicines, interviews were conducted with informed and consenting Elders of two Cree communities of northern Quebec, Mistissini (48°51'N and 72°12'W) and Whapmagoostui (55°16′N, 77°45′W). For complete descriptions of the field sites, participants and interview

process, refer to Leduc *et al.* (2006). Briefly, semistructured interviews were targeted toward identifying traditional medicines for the treatment of 15 defined symptoms of diabetes. There were no specific questions regarding use of the Ericaceae. Medicinal uses of Ericaceae by other Native American peoples were collected from various sources reviewed by Moerman (1998).

Materials

All solvents and materials were obtained from Fisher Scientific (Ottawa, Ontario, Canada), unless otherwise indicated. Analytical grade trifluoroacetic acid (TFA) was purchased from J.T. Baker (Phillipsburg, New Jersey, USA). Chlorogenic acid, (+)-cacechin, (+)-epicatechin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside and myricetin were purchased from Extrasynthase (Lyon, France) while arbutin, para-coumaric acid, taxifolin, myricitrin, rutin, quercetin-3-*O*-arabinoside and quercetin-3-*O*-rhamnoside were purchased from Sigma (Oakville, Ontario). Procyanidin B2 was generously provided by Dr. D. Ferreira (University of Mississippi, USA).

Sample preparation and extraction

Wild samples of Ericaceae leaves were collected during August and September of 2006. Collection sites, indicated in Table 3.1, varied according to both species distribution and regions of traditional use. Voucher specimens were deposited at the University of Ottawa herbarium and Montreal Botanical Garden. After harvesting, the leaves were dried at 40°C using an electric food dehydrator (Nesco/American Hervest WI, USA) and stored in darkness at room temperature (RT) until extraction. For each species, two grams of dried leaves were ground to powder using a Wiley Mill (mesh size 40) then extracted three times

with 25 ml 80% ethanol at RT with 6 hours of circular shaking at 200 RPM on an Orbit Shaker (Lab Line, Mumbai, India). The three ethanolic supernatants were recovered, pooled, centrifuged at 1000 x g for 15 min at room temperature and vacuum filtered through a Whattman filter paper #1 prior to drying by rotary evaporation and lyophilisation. All extracts were stored at 4 °C. A 1 ml sample of each extract (10 mg/ml) was prepared in HPLC grade methanol and filtered through a PTFE membrane (0.2 μm pore size, lot # 0681) (Chromatographic Specialties Inc. Brockville, Canada).

HPLC-PAD-APCI/MS analyses

Analyses were performed on an Agilent 1100 series HPLC-PAD-APCI/MS system (Agilent Technologies Inc.CA, USA). The system was equipped with an online degasser, a quaternary pump (maximum pressure limit 400 bars), an auto sampler with a 100 ml injection loop, a column thermostat compartment and an on line APCI-MS (version 1964C). In order to identify a suitable column for the separation of selected markers, an initial scouting gradient (0-100% acetonitrile in 60 min) was performed on three columns with different chromatographic selectivity: a Zorbax XDB C8, 150 × 3.0 mm I.D. particle size 4 micron (Agilent Technologies,Montreal, Canada), a Restek Ultra C18, 50 × 2.1 mm I.D. particle size 3 micron (Restek Bellefonte, PA , USA) and a Synergi Polar RP-C18 column, 150 × 3.0 mm I.D., particle size 4 micron, (Phenomenex, CA, USA). The method development was finalized on a newly purchased Synergi Polar RP-C18 column (product number 00F-4366-YO, serial number 354770-3, batch number 5343-36) dedicated to the project.

Table 3.1 – Collection sites and extraction yields for the 14 species of Ericaceae in this study.

Botanical Nomenclature	Common name	Collection site	Voucher number[a]	Extraction yield[b]
Andromeda polifolia ssp. *glaucophylla*	Bog rosemary	Whap, QC	UO1731	31.2
Arctostaphylos uva-ursi	Bearberry	Gat, QC	UO1732	44.3
Empetrum nigrum	Crowberry	Whap, QC	UO1733	44.4
Gaultheria hispidula	Creeping snowberry	Mist, QC	UO1734	37.3
Gaultheria procumbens	Wintergreen	Gat, QC	UO1735	35.3
Kalmia angustifolia	Sheep laurel	Mist, QC	UO1736	47.7
Rhododendron groenlandicum	Labrador tea	Mist, QC	UO1737	30.9
Rhododendron lapponicum	Lapland rosebay	Whap, QC	UO1738	41.8
Rhododendron tomentosum ssp. *subarcticum*	Northern Labrador tea	Whap, QC	UO1739	35.2
Vaccinium angustifolium	Lowbush blueberry	Gat, QC	UO1740	38.7
Vaccinium myrtilloides	Velvetleaf blueberry	Den, QC	UO1741	47.9
Vaccinium oxycoccus	Northern cranberry	Mist, QC	UO1742	34.4
Vaccinium poasanum	No common name	Cerro del Muerto[c]	UO1743	42.6
Vaccinium vitis-idaea	Bog cranberry	Whap, QC	UO1744	38.1

Gat = Gatineau, Mist = Mistissini, Whap = Whapmagoostui, Den = Denholm, QC = Québec

[a] Vouchers are deposited at the University of Ottawa Herbarium (UO)

[b] The extractions were performed on dried ground leaves. See Sample Preparation and Extraction section for extraction details, the results are averages, n = 3

[c] Cerro del Muerto, Costa Rica

Chromatographic method development

Based on preliminary identifications in the Ericaceae extracts, 13 standards were selected for method development [two phenolic acids (**2**, and **4**), three flavanols (**3**, **5** and **6**), a flavanone (**13**), a flavone (**15**) and five flavonols (**16**, **17**, **18**, **21**, **22** and **23**)]. To estimate the separation behaviour, methanolic solutions of pure standards at known concentrations were combined and injected (1 μL) into the HPLC system (results not shown). Standard arbutin (**1**), injected separately, showed an impurity peak which eluted earlier than true arbutin peak and its mass spectrum was obtained in negative polarity due to its quinone nature.

After acquiring the spectral data, the individual chromatograms were carefully inspected for coelutions, resolutions, retention factors, plate numbers, baseline drifts and peak asymmetry factors in order to optimize the integration. The optimal integration conditions were, slope sensitivity 0.99, peak width 0.27, area reject 4, and height reject 0.27 and shoulders off while the shoulders were set to tan for correctly detecting and quantifying markers which had closely eluting unknown peaks particularly for marker 3 in extracts EL4, EL7, EL9 and EL12.

Identification of markers by metabolomics library match

Following HPLC separation, PAD spectra of each marker compound were obtained with the following settings: 5 spectra per peak, up-slope, apex and down-slope, UV wavelength range 200 – 400 nm and threshold 5 mAU. The spectra were processed without smoothing factors. After confirming their purity using peak purity tests on both UV and MS data, the spectra of the 13 standards were re-added to the metabolomics library. The library consisted of UV spectra of 131 compounds, which were predominantly phenolics. Co-

chromatography with standards was then performed to distinguish between related isomers such as catechin and epicatechin.

The identification of marker compounds in the crude Ericaceae extracts was defined by at least 99% similarity between the spectra of unknown peaks with those of matching compounds stored in the library, as determined by the Chemstation software (Version B.03.01). The spectral matching conditions were set so that an appropriate threshold for each peak was based on its individual signal to noise ratio. When matching an unknown spectrum with a reference spectrum, the minimum threshold of purity pure was set at 990.

Quantification of markers

To determine the suitable UV wavelengths for PAD monitoring and quantitation, the area under the peak and peak height of 12 selected markers were compared. Special emphasis was given to the base line drift and peak asymmetry integration results (data not shown). Identified metabolites were quantified on the basis of area under the peak of HPLC-PAD chromatograms at the monitoring wavelengths of 280 nm relative to calibration curves produced using pure standards analyzed on the same day. Where possible, compounds with no corresponding standard were quantified as equivalents of the most appropriate standard available. Mean quantities were calculated from results obtained on three separate days (minimum n = 3).

Limit of detection and limit of quantification

The determination of LOD and LOQ of selected markers was undertaken by mixing the standard markers and preparing serial dilutions for analysis. The LOD (3:1 signal to noise ratio) and LOQ (10:1 signal to noise ratio) are average values of three runs calculated

from signal to noise ratios obtained from performance + noise data of Chemstation B.03.01. All markers were analyzed as one mixture at the concentrations of 58.8 μg / ml, 11.76 μg / ml, 5.88 μg / ml, and 0.588 μg / ml except **5** and **16**, which were analyzed at concentrations of 88.2 μg / ml, 16.44 μg / ml, 8.22 μg / ml, and 0.88 μg / ml. The retention factor k is calculated as $k = \frac{Rt - t0}{t0}$ where Rt = retention time of a marker and t_0 (column dead volume) = volume of mobile phase inside the column divided by the flow rate (0.5 ml/min).

Recovery

The recovery of one of the most ubiquitous markers, quercetin-3-O- rhamnoside (**23**), was performed by serially diluting a fresh stock solution of standard in ethanol and adding 1, 2 or 4 mg of standard into triplicate samples of dried and blended *Gaultheria hispidula* leaf tissue. Three additional tissue samples were injected with 95% ethanol only. Samples were stored in a fumehood for 48 hours to allow the ethanol to evaporate prior to performing extractions in triplicate as described above.

RESULTS

The 14 species species of Ericaceae included in this study were collected from boreal forest areas of North-Eastern Canada (Table 3.1). One Ericaceae from Costa Rica was provided as an outlier. Only leaves were studied due to their predominant use as traditional remedies. The extraction strategy required a small amount of material and provided remarkable extraction yields of 34.4 – 47.9 % (Table 3.1).

The traditional medicinal uses of the Ericaceae in northern North America (Table 3.2) show a wide range of applications including urinary problems, which is not surprising

Table 3.2 – Review of medicinal uses of Ericaceae species by the Cree Elders of Eeyou Istchee and other Native American peoples.

Latin name	Cree name	Part used	Diabetes symptoms [other uses][1]	Medicinal uses found in the literature[2]
Andromeda polifolia ssp. glaucophylla	Kakupukw	Whole	General weakness (1/W) [Fainting]	Stomach ache (Metis)
Arctostaphylos uva-ursi		Leaf		Rheumatism, blood medicine, panacea, headache (Ojibway); slow excessive menstrual bleeding, prevent miscarriage, recovery after childbirth, diarrhea (Cree); cough (Dene); kidney trouble (Metis)
Empetrum nigrum	Iischiiminh	Whole	Back/kidney pain (1/W)	Diarrhea, hypothermia, inflammation (Inuit); cold, stomachache (Gwich'in)
Empetrum nigrum	Iischiiminh	Fruit	Increased urination (1/W)	Cathartic (Bella Coola), diuritic (Cree); diarrhea, cold, sore eyes, kidney troubles (Tanana)
Gaultheria hispidula	Piyeumanaan	Fruit	Heart/chest pain (2/M)	Digestive (Algonquin); cough (Cree)
Gaultheria procumbens		Leaf		Headaches, cold (Algonquin); cold, flu, stomach ache (Atikamekw); rheumatism (Ojibway); cold, kidney problems, rheumatism, anti-helmintic, blood purifier (Iroquois); urinary problems, cold, upset stomach (Malecite); cold (Chippewa)
Kalmia angustifolia	Uischichipukw	Leaf	Inflammation/swelling (2/M; 3/W); Back/kidney pain (1/M; 8W); Increased thirst (1/W); Headaches (4/W); Diarrhea (2/W); Arthritis/rheumatism (3/W); Sore and/or swollen limbs (6/W); Heart/ chest pain (8/W); Foot numbness/ sores (4/W); Increased urination (2/W); Slow healing infections (3/W); Abscesses and/or boils (3/W) [Toothache, stomach ache]	Cold (Algonquin); cold, backache, rheumatism, stomach ache (Innu); diarrhea, stomachache, tonic (Cree); headaches , swelling, panacea (Mi'qmaq); cold, nasal inflammation (Abenaquis); headaches (Atikamekw); pain, swelling (Malecite)
Rhododendron groenlandicum	Kaachepukw	Leaf	Diabetes (1/W); Diarrhea (1/M;15/W); General weakness (2/M; 6/W); Heart/chest pain (3/M; 21/W); Slow healing infections (1/M; 7/W);	Cold, nasal inflammation (Abenaki); headaches, tonic, cold, childbirth (Algonquin); analgesic, stomach ache (Bella Coola); burns, diuretic, emetic, backache, rheumatism, skin problems, flu,

Latin name	Cree name	Part used	Diabetes symptoms [other uses][1]	Medicinal uses found in the literature[2]
R.groenlandicum cont.			Headaches (1/M; 13/W); Back/kidney pain (5/M; 18/W); Foot sores (1/M; 12/W); Inflammation/swelling (2/M; 8/W); Arthritis/rheumatism (1/M; 5/W); Sore/swollen limbs (2/M; 15/W); Increased thirst (8/W); Abscesses and/or boils (20/W); Blurred vision (3/W); Increased urination (3/W); Increased appetite (2/W) [Fainting, toothache, vomiting blood, cold, asthma, sore throat, sickness, stomach ache, cough]	diarrhea, sore hands, urinary infections, heart pain, chest pain, kidney pain, pneumonia, cough and whooping cough, arthritis, headaches, chills, bad breath, teething pain, system cleanser, hair loss, eye problems (dry, infection), infected wounds, umbilical scab, clean out stomach, cuts and wounds, general health, diabetes (Cree); kidney problems, rheumatism (Malecite); skin problems (Ojibway); blood purifier, chills, fever, jaundice (Innu); diuretic, cold, scurvy, asthma, kidney problems, tonic, medecine (Mi'qmaq); flu, diarrhea, nervousness, tension, burns, wet eczema (Dene)
Rhododendron tomentosum ssp. subarcticum	Wiisichipukw	Leaf	Diabetes (1/W); Increased thirst (7/W); Heart/chest pain (12/W); Sore and/or swollen limbs (6/W); Foot numbness/sores (7/W); Slow healing infections (7/W); Blurred vision (2/W); Diarrhea (13/W); Abscesses and/or boils (10/W); Headaches (5/W); Back/kidney pain (8/W); Increased urination (2/W); General weakness (1/W); Swelling/ inflammation (4/W); Increased appetite (1/W); Arthritis/rheumatism (1/W) [Throat ache, cold, cough, pulmonary problems, (asthma) stomach ache, pain (knee), sore neck, toothache]	Sore throat, cough, cold, flu, respiratory problems, toothache, stomach ache (Inuit)
Vaccinium angustifolium & *V. myrtilloides*	Miinshe	Fruit	Increased appetite (2/M) General weakness (1/M)	Colic, after miscarriage, induce labour, stop urination (Algonquin); blood purifier (Ojibway), trigger menstruation and blood after childbirth, prevent pregnancy and miscarriage, induce chlidbirth, diaphoretic (Cree)
		Whole	Back/kidney pain (1/W)	Anticancer, prevent pregnancy, prevent miscarriage, bring blood after childbirth, bring menstruation, regulates menstruation (Metis); diaphoretic (Cree); cough (Gwich'in)
		Root	Diabetes (2/M)	Diarrhea (Chippewa); headaches (Dene); induce childbirth, post partum application (Cree); urinary problems (Algonquin)

Latin name	Cree name	Part used	Diabetes symptoms [other uses][1]	Medicinal uses found in the literature[2]
Vaccinium vitis-idaea	Wiishichi-manaanh	Fruit	Increased urination (1/M; 1/W) Blurred vision (10/W) Abscesses and/or boils (2/W) [Toothache, skin problems, snowblindness]	Clean out stomach (Cree); fever (Dene); acne, vomiting (Metis) kidney problems, cold, digestion, to improve appetite (Gwich'in)
		Whole	[Toothache]	Kidney problems (Malecite), tonic tea (Inuit); bladder problems, post-partum application (Cree); bladder problems (Dene), kidney problems (Malecite)
Vaccinium oxyccocus		Leaf		Nausea (Ojibway)

[1] Data obtained through interviews with the Cree Elders of Mistissini (M) and Whapmagoostui (W) regarding traditional medicines used to treat various symptoms of diabetes.

[2] Summarized from reviews of North American ethnobotany by Arnason *et al.* (1981) and Moerman (1998).

considering the well-established efficacy of cranberries (*Vaccinium macrocarpon*) for urinary infections (Kontiokari et al. 2005; Zhang et al. 2005), inflammation, cold and flu, rheumatism and arthritis, kidney problems, diarrhea, headache, cuts and wounds, and blood medicine. Interviews done in two Cree communities based upon 15 symptoms linked to diabetes have shown that the Ericaceae of the boreal forest are widely utilized. Previous studies with the Cree peoples have shown similar uses (see Table 3.2 for a comparison).

Separation of Ericaceae markers

Employing a selection of commercially available phenolic standards known to be present in Ericaceae leaf extracts as a standard mixture, the best separations were achieved using a Synergi RP Polar column (Figure 3.1), which proved most appropriate for further method development. The ether-linked phenyl groups and polar end-capping used in this column offered unique selectivity for polar aromatic compounds particularly when methanol was included as one of the organic modifiers. Special emphasis was on the separation of closely related markers (**15** to **23**) that could not be separated by traditional binary solvent gradient systems comprising of acetonitrile or methanol with water. For this reason, separate HPLC gradient programs comprising of a tri-eluent system [methanol (eluent A) acetonitrile + 0.05% formic acid (eluent B) and water + 0.05% trifluoroacetic acid (eluent C)] were executed and provided suitable initial conditions (especially the reduction in downward baseline drift, presumably due to the difference of the absorbance of solvents) for the separation of all markers.

Although all markers could be detected with our initial conditions, the separation of more closely eluting bands **15** to **18** and **21** to **23** was difficult. A selection of methods that were employed during optimization (Table 3.3), each offering distinctive separation profiles,

is presented in Figure 3.1. In the first run, where methanol was a dominant modifier over acetonitrile (Figure 3.1A), markers **16, 17, 18** showed a partial coelution while **21** and **22** coeluted. In the second run (Figure 3.1B) the gradient was readjusted by introducing an equal percentage of eluent A and eluent B in three steps in 25 min. This change resulted in overall reduction of the Rt by about 2.5 min and the separation of **21** and **22** but **15** and **16** coeluted. In addition, an upward drift of the base line was observed. Selecting a slightly shallower linear gradient of 2 – 15% eluent A and eluent B (without the addition of 0.05% trifluoroacetic acid) for 30 min in the third run (Figure 3.1C) resulted in improved separation of markers **15 – 18** but coelution of **21** and **22**. In the optimized gradient program of 5 –20% eluent A and 5 –10% eluent B in 27 min (Figure 3.1D) resulted in baseline separation of **15, 16** and **21** was obtained while a slight overlap was observed between **17** and **18.** The elution of markers **22** and **23** showed about 37% overlap but was not of major concern since **22** was found in trace amount only in *Kalmia angustifolia.* The final separation method (Figure 3.1E) could not only separate all 14 markers but also offered gaps in the chromatogram to accommodate the other expected markers; the 6 min gap before **3** (Rt 5.9 min) accommodated **1** (Rt 2.0 min) and **2** (Rt 3.9 min), the 2.6 min gap between **6** (Rt 8.8 min) and **8** (Rt 11.4 min) accommodated **7** (Rt 10.1 min), the 5 min gap between **8** (Rt 11.4 min) and **13** (Rt 16.4 min) accommodated **9** (11.8 min) to **12** (Rt 15.9 min), and the 3.8 min gap between **18** (Rt 20.5 min) and **21** (Rt 24.3 min) accommodated **19** (21.7 min) and **20** (Rt 23.3 min).

Table 3.3 highlights the gradient programs and their performance in separating 12 selected markers available as standards (arbutin was not included due to the presence of an impurity). Application of optimal separation conditions (Run optimal, Table 3.3) along with suitable choice of detection conditions facilitated impressive retention factors of 3.85 for **2**

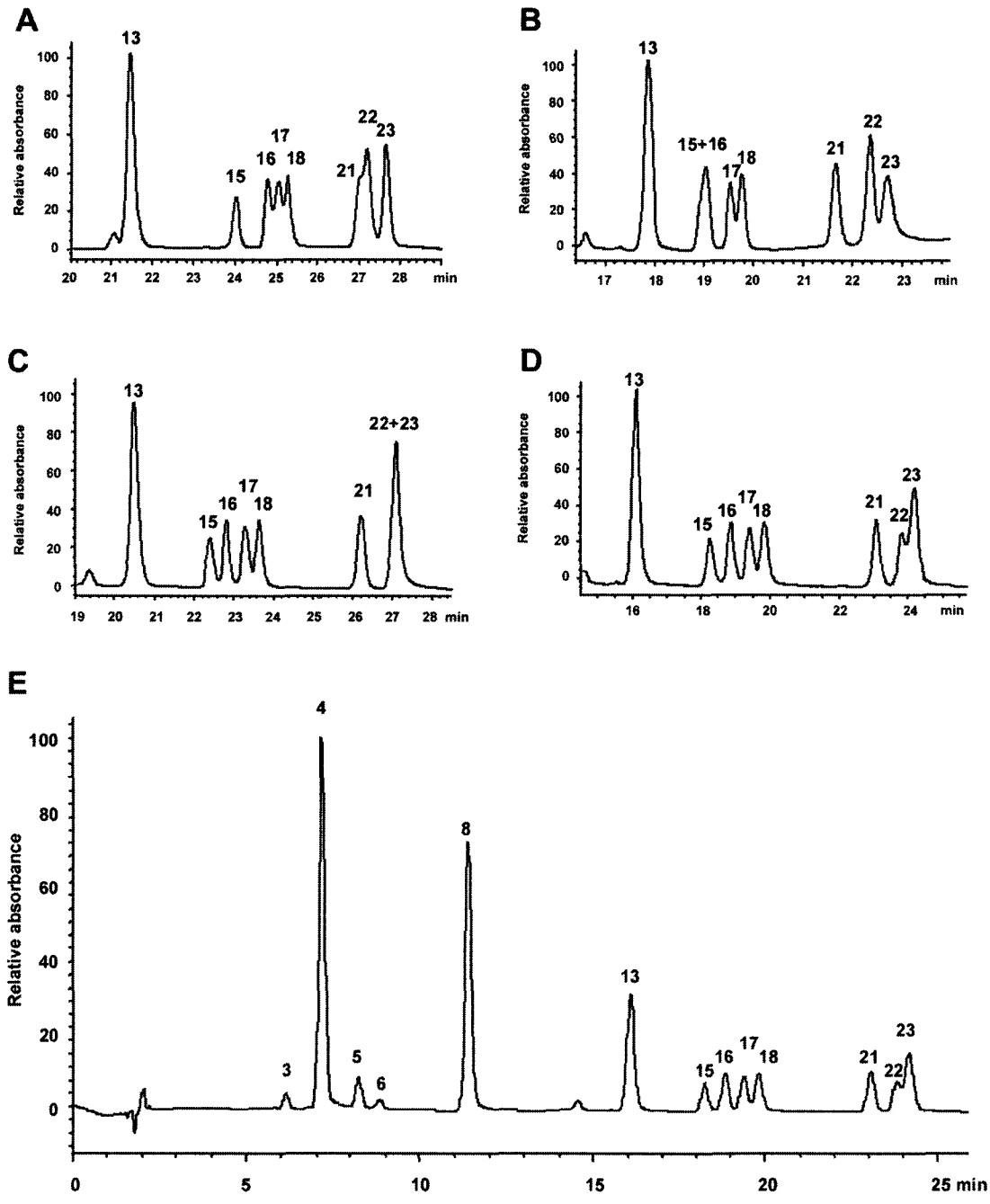

Figure 3.1 – Optimization of HPLC gradient method for separation of phenolic standards. The distinct separation profiles produced by three different HPLC gradient programs (A-C) developed to optimize separation of closely related flavonols, which was achieved with the final method (D-E). Presented chromatographs were obtained PAD monitoring at 280 nm. For the detailed elution gradients of the three developmental and optimized methods, please refer to Table 3.3. The identities of numbered peaks representing phenolic standards are listed in Table 3.4.

Table 3.3 – The HPLC gradient method development steps for the separation of selected marker compounds of Ericaceae leaf extracts. The separations were performed on a Synergi Polar-RP C-18, 150 X 3 mm I.D., particle size 3 micron. The flow rate was maintained at 0.5 mL / min. The column temperature was 50 °C in Run-1 and Run-2, 52 °C in Run-3, 53 °C in Run-optimal.

Gradient time (min)[a]	**Eluent A Methanol**	**Eluent B Acetonitrile + 0.05%TFA[b]**	**Eluent C Water + 0.05%TFA**	**Co-eluting peaks**
Run-1				
0	5	0	95	
7.5	12	0	88	
17	28	0	72	21+22
35	40	20	40	
Run-2				
0	4	4	94	
7.5	6	6	88	15+16
25	15	15	70	
Run-3				
0	2	2	96	22+23
30	15	15	70	
Run-optimal				
0	5	5	90	–
27	20	10	70	

[a] Column washing and equilibration times are not shown

[b] In Run-3, Eluent C = acetonitrile without 0.05% TFA

(Rt 5.9 min) to 18.04 of **23** (Rt 25.3 min). The peak asymmetry of the majority of markers remained within acceptable limits (0.84 for **18** to 1.22 for **22)** indicating good peak shapes of early as well as late eluting markers. Peak width remained equal or below 0.2 min for early eluting markers (**2, 4**, and **8**) and increased from 0.21 – 0.3 min for markers eluting after **8** (Rt 11.4 min) but still stayed within an acceptable range. The observed trend in plate numbers, where the first three markers showed lower plate number values than markers eluting after **13** (Rt 16.4 min), indicated a significant improvement in the resolving power of the stationary phase for late eluting markers that were difficult to separate.

For LOD and LOQ calculation, integrations were performed using a 1 min window before and after the peak elution (where ever applicable) in order to obtain sufficient noise data. For closely eluting markers (**15** – **18** and **21** – **23**), a 1 min window was given before and after the groups. This approach not only gives more 'realistic' noise data but also takes into consideration of the overall drift during the chromatographic separation. Overall, the best LODs and LOQs were obtained for middle eluters (**8, 13, 15** and **16**) without substantial loss of sensitivity for later markers (Table 3.4).

Suitable Choice of PAD Wavelength

Despite their chemical similarities, the presence of genus- and species-specific metabolites in Ericaceae extracts can interfere with UV detection at certain wavelengths. In order to minimize these interferences and optimize the UV response of known markers in each extract, PAD data were collected at multiple wavelengths. For most species, including the *Vaccinium* species (Figure 3.2), as well as *Andromeda polifolia* ssp. *glaucophylla*, *Arctostaphylos uva-ursi*, *Gaultheria hispidula* and *Kalmia angustifolia* (Figure 3.3), 290 nm provided the best overall chromatographic response. However, 280 nm offered better peak

Table 3.4 – The performance data of chromatographic separation of selected Ericaceae markers.

#	Marker [a]	Retention Factor (k)[b]	Peak Asymmetry	Peak width (min)	Plate Numbers	LOD (µg/ml ± SEM) [c]	LOQ (µg/ml ± SEM) [c]
2	(+)-Cacechin	3.85	0.88	0.19	6045	0.30 ± 0.03	1.01 ± 0.11
4	Chlorogenic acid	4.67	0.95	0.19	7984	2.61 ± 0.11	8.67 ± 0.36
5	(+)-Epicatechin	5.49	0.93	0.20	9762	0.33 ± 0.15	1.11 ± 0.49
8	Para-coumaric acid	7.98	0.88	0.21	16945	1.61 ± 0.11	5.33 ± 0.37
13	Taxifolin	11.7	0.88	0.25	23906	0.45 ± 0.11	1.50 ± 0.01
15	Myricitrin	13.4	0.98	0.23	34278	0.42 ± 0.10	1.38 ± 0.01
16	Rutin	13.8	0.92	0.23	37331	0.62 ± 0.43	2.07 ± 0.03
17	Quercetin-3-*O*-galactoside	14.3	0.97	0.24	35884	0.56 ± 0.14	1.86 ± 0.03
18	Quercetin-3-*O*-glucoside	14.6	0.84	0.24	37116	1.30 ± 0.35	4.33 ± 0.09
21	Quercetin-3-*O*-arabinoside	17.2	0.94	0.25	46100	1.97 ± 0.83	6.56 ± 0.26
22	Myricetin	17.8	1.22	0.27	44819	1.63 ± 0.14	5.43 ± 0.46
23	Quercetin-3-*O*-rhamnoside	18.0	0.92	0.30	36436	1.92 ± 0.82	6.40 ± 2.70

[a] All markers were analyzed as one mixture at the concentrations of 58.8 µg/ml, 5.88 µg/ml and 0.588 µg/ml while **5** and **16** were analyzed at concentrations of 88.2 µg/ml, 8.22 µg/ml, 0.88 µg/ml.

[b] k is calculated as $k' = \frac{Rt - t_0}{t_0}$ where Rt = retention time of a marker and t_0 (dead volume) = volume of mobile phase inside the column divided by the flow rate (0.5ml / min).

[c] The LOD (3:1 signal to noise ratio) and LOQ (10:1 signal to noise ratio) are average values if three runs calculated from signal to noise ratios obtained from performance + noise data of chemstation B.03.01.

asymmetry data and baseline stability in the 3 *Rhododendron* species (Figure 3.4) and *Gaultheria procumbens* (Figure 3.3). Finally, monitoring at 210 nm was most suitable for *Empetrum nigrum* (Figure 3.3) since 290 nm and 280 nm did not provide a steady baseline after Rt 23 min. This may have been caused by the presence of non-UV absorbing interferences of a relatively non-polar nature in the extract. When analyzing several species, we therefore recommend using several monitoring wavelengths for the purpose of qualitative comparison but only a single wavelength for quantitative comparison.

Compound identification and quantitation

Details of the metabolomics-based identification of 23 markers of Ericaceae chromatographically separated in 25 min are presented in Table 3.5. The match scores indicate that 16 out of 24 markers met the threshold of library match score of 990 as determined by the Chemstation software (i.e. 99% similarity of UV spectrum of an unknown peak with the UV spectrum of a reference endorsed in the library). Positive library matches were confirmed by their m/z (mass to charge ratio) of major fragments obtained from MS data. It is notable that the markers with match scores below the threshold were present in trace amounts, for example taxifolin (**13**) in *R. tomentosum* ssp. *subarcticum* (0.04 mg/g dry weight) and p-coumaric acid (**8**) in *G. procumbens* (0.06 mg/g dw). In such cases, retention time and online mass spectrometric detection played key roles in confirming identification.

Quantification was based on area under the curve of UV chromatograms at monitoring wavelength of 280 nm. Peak purity data of each marker was obtained from the report after the completion of the sequence of 14 extracts. When the purity threshold was below the set criterion, the peak was inspected for coeluting or closely eluting peaks. If coeluters were observed the peak integration was readjusted to eliminate the area under the

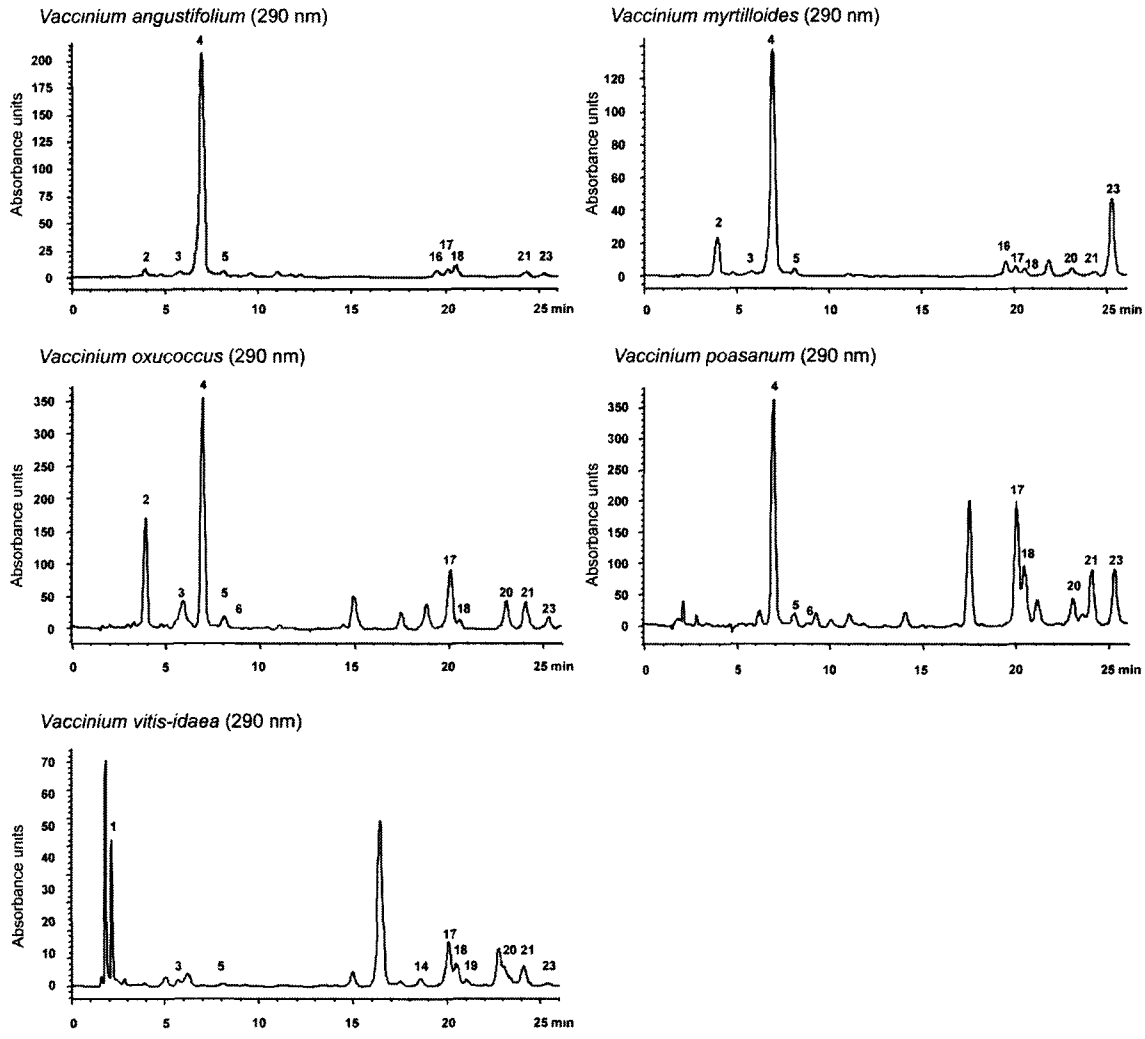

Figure 3.2 – HPLC chromatograms of a leaf extracts of *Vaccinium* species with photodiode array detection (PAD) at 290 nm. Numbered peaks represent identified compounds as reported in Table 3.5.

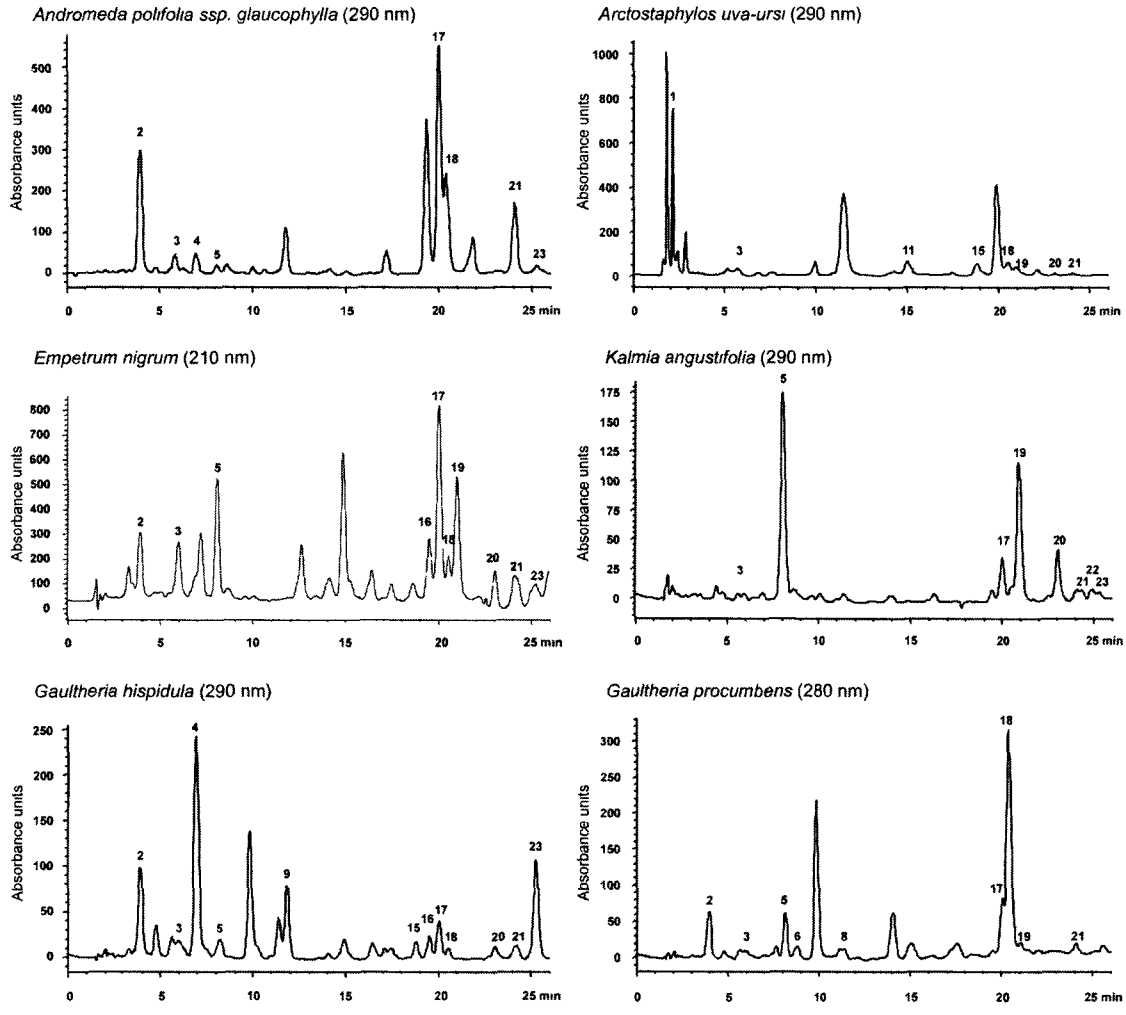

Figure 3.3 – HPLC chromatograms of a leaf extracts of different Ericaceae species with photodiode array detection (PAD) at various wavelengths. Chromatograms of *A. polifolia* ssp. *glaucophylla*, *A. uva-ursi, G. hispidula* and *K. angustifolia* are presented with PAD detection at 290 nm while 280 nm and 210 nm were employed for *G. procumbens* and *E. nigrum*, respectively. Numbered peaks represent identified compounds as reported in Table 3.5.

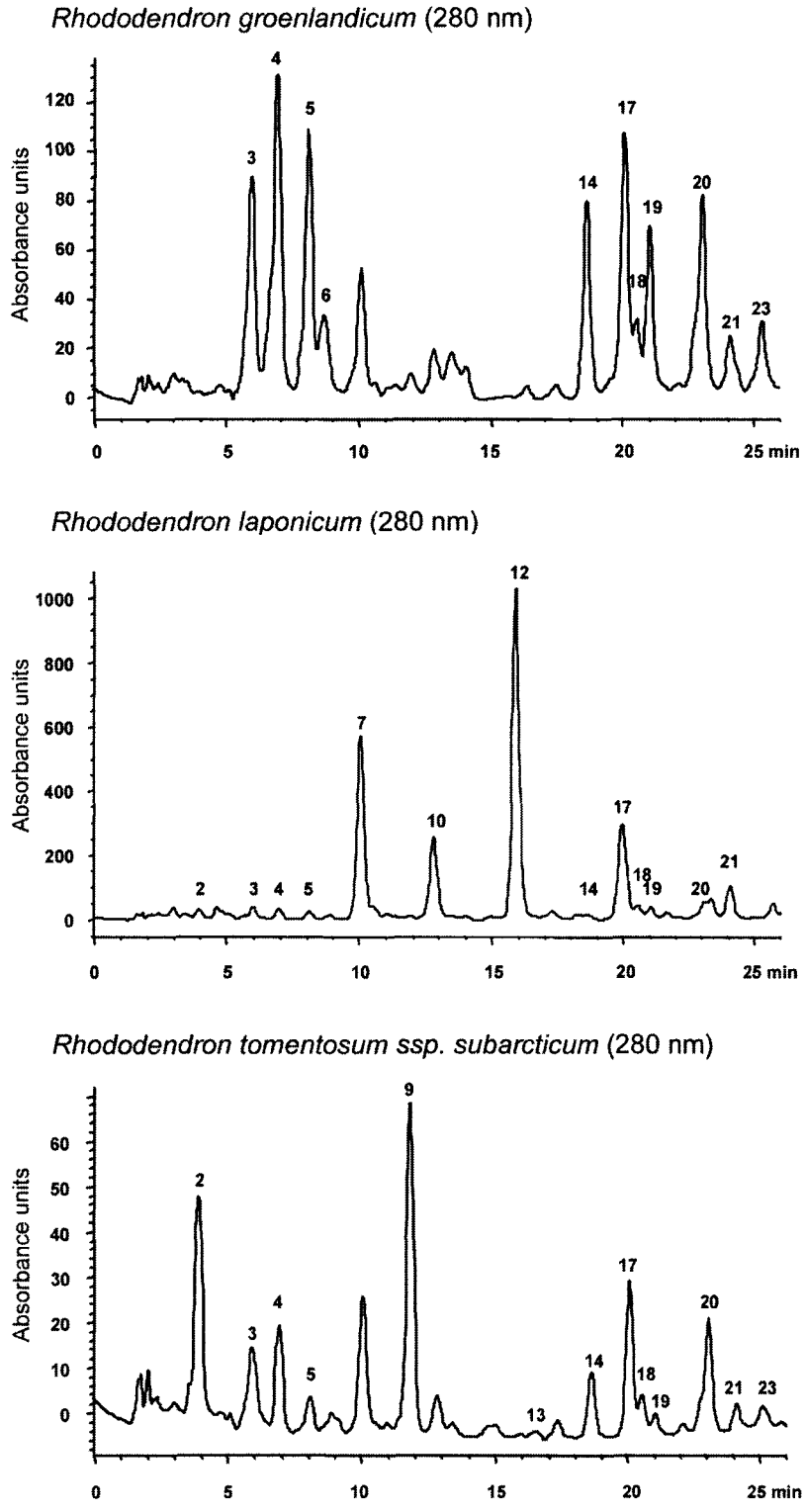

Figure 3.4 – HPLC chromatograms of a leaf extracts of *Rhododendron* species with photodiode array detection (PAD) at 280 nm. Numbered peaks represent identified compounds as reported in Table 3.5.

Table 3.5 – Ericaceae markers identified by HPLC-PAD-MSD.

#	Rt (min)	Marker [a]	Plant	Library match (rank) [b]	Major Mass Fragments (*m/z*)
1	2.2	**Arbutin** [c]	*V. vitis-idaea*	**997.2 (1)**	290.1 $[M-H]^-$
2	4.0	Caffeoylquinic acid	*E. nigrum*	933.0 (3)	353$[M-H]^-$, 191[quinic acid–H]$^-$, 179 [caffeic acid–H]$^-$
3	5.9	**(+)-Catechin**	*E. nigrum*	945.5 (3)	291 $[M+H]^+$
4	7.0	**Chlorogenic acid**	*V. angustofolium*	**998.6 (1)**	181 [M-caffeic]$^+$,355$[M-18]^+$
5	8.1	**(+)-Epicatechin**	*V. myrtilloides*	949.6 (3)	291$[M+H]^+$
6	8.8	**Procyanidin B2**	*G. procumbens*	**995.2 (3)**	579 $[M+H]^+$, 291 [M-catechin]$^+$
7	10.1	Myricetin glycoside 1	*R. laponicum*	**996.3 (2)**	481$[M+H]^+$, 319 [M-glycoside]$^+$
8	11.4	**Para-coumaric acid**	*G. procumbens*	749.4 (5)	165 $[M+H]^+$
9	11.9	Taxifolin glycoside 1	*G. hispidula*	977.7 (2)	467$[M+H]^+$, 305 [M+H–Glu]$^+$
10	12.8	Myricetin glycoside 2	*R. laponicum*	**996.3 (2)**	451$[M+H]^+$, 319[M+H–Glyc]$^+$
11	15.0	Myricetin glycoside 3	*A. uva-ursi*	**996.3 (2)**	481 $[M+H]^+$, 319 [M+H–Glyc]$^+$
12	15.9	Taxifolin glycoside 2	*R. laponicum*	979.2 (2)	437$[M+H]^+$, 305[M+H–Glyc]$^+$
13	16.4	**Taxifolin**	*R. tomentosum*	857.7 (5)	305$[M+H]^+$
14	18.7	A-type procyanidin 1	*R. groenlandicum*	**998.0 (2)**	577$[M+H]^+$, 291 [M+H–Catechin]$^+$
15	18.8	**Myricitrin**	*G. hispidula*	**991.5 (1)**	465$[M+H]^+$, 319 [M+H–Rha]$^+$
16	19.6	**Rutin**	*V. myrtilloides*	**998.3 (1)**	611$[M+H]^+$, 303 [M+H–Rut]$^+$
17	20.1	**Quercetin-3-*O*-galactoside**	*V. poasanum*	**994.3 (2)**	465$[M+H]^+$,303 [M+H–Galac]$^+$
18	20.6	**Quercetin-3-*O*-glucoside**	*G. procumbens*	**997.5 (1)**	465 $[M+H]^+$, 303 [M+H–Gluc]$^+$
19	21.1	A-type procyanidin 2	*K. angulstifolia*	**998.5 (3)**	577$[M+H]^+$, 291 [M+H–Catechin]$^+$
20	23.1	Quercetin glycoside	*K. angulstifolia*	**955.0 (2)**	465 $[M+H]^+$, 303 [M+H–Glyc]$^+$
21	24.3	**Quercetin-3-*O*-arabinoside**	*A. polifolia*	**995.8 (1)**	435$[M+H]^+$, 303 [M+H–Arab]$^+$
22	24.9	**Myricetin**	*K. angulstifolia*	785.3 (6)	319$[M+H]^+$
23	25.3	**Quercetin-3-*O*-rhamnoside**	*V. poasanum*	**999.7 (1)**	449$[M+H]^+$, 303 M+H–Rha]$^+$

[a] The markers in bold face were the authenticated compounds purchased commercially

[b] The library rank in bold face indicate authentic match (i.e. match core of 990 corresponding to 99% similarity of UV spectrum of a peak with the reference endorsed in the library). For library match criterion see Metabolic Library Match Conditions sub-section in the Experimental section

[c] The analysis of standard Arbutin showed an impurity

coeluting peak of no interest. Calibration curves of 13 pure standards, all yielding linear response profiles ($R^2 > 0.999$), were used to quantitate 19 of the identified markers. Five of these (**2**, **7**, **10**, **11** and **20**) were calculated as equivalents of closely related standards. The quantities of **1**, **9**, **12**, **14** and **19** were not determined due to lack of appropriate standards but the peaks were nonetheless valuable for fingerprinting purposes.

As illustrated in Figure 3.5, all extracts contained detectable quantities of compounds from each of three classes of metabolites - phenolic acid derivatives, catechins and flavonols. *K. angustifolia* was the sole exception lacking identified phenolic acids. Of the identified phenolic acid derivatives, chlorogenic acid (**4**) and/or its isomer (**2**) were detected in all extracts except *A. uva-ursi, K. angustifolia and V. vitis-idaea.* Though generally found at concentrations of 1-5 mg/g dw, levels of **4** were particularly high in *V. angustifolium* and *V. myrtilloides* extracts (34.3 and 28.2 mg/g dw, respectively, Figure 3.5A). Similarly, catechin (**3**) and epicatechin (**5**) were nearly ubiquitous among species and were consistently detected in extracts at concentrations of 1-5 mg/g dw. The notable exception was *K. angustifolia*, which contained very little of **3** but 23.4 mg/g dw of **5** (5-20 fold more than other species).

With a minimum of five flavonol glycosides found in every extract, flavonols were the most abundant class of metabolites identified in Ericaceae leaves. With the exception of rutin (**16**), which was detected in four extracts, quercetin glycosides (**17**, **18**, **20**, **21** and **23**) appeared in most species though at different concentrations and relative ratios. The summed concentration of quercetin derivatives ranged from 2.6 mg/g dw in *R. tomentosum* ssp. *subarcticum* to 29.5 mg/g dw in *V. myrtilloides* while the concentration of individual glycosides varied from 0-15 mg/g dw (Figure 3.5). Myricetin algycone (**22**) was only seen in *K. angustifolia* but myricetin glycosides (**7**, **10, 11** and **15**) were identified in 5 other extracts.

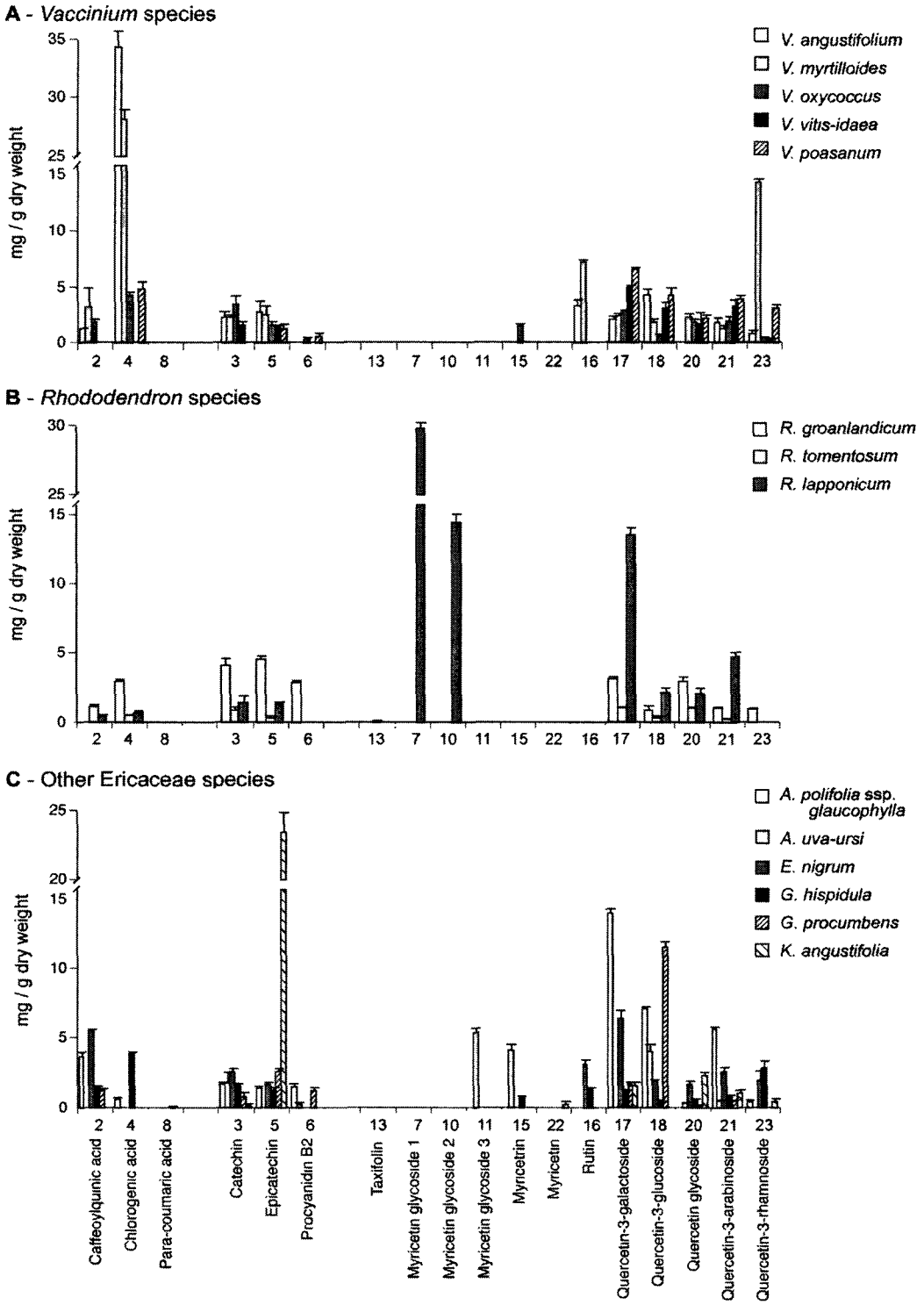

Figure 3.5 – Quantitation of identified phenolic metabolites in leaf extracts of Ericaceae species. Phenolic metabolites, arranged according to structural class, in extracts (A) Vaccinium species, (B) Rhododendron species, and (C) other Ericaceae species were quantified by HPLC based on area under the curve of PAD chromatographs at 280 nm and calculated relative to standard curves produced using standards.

In both *A. uva-ursi* and *R. laponicum*, myricetin derivatives were more abundant than quercetin derivatives (Figure 3.5).

DISCUSSION

The present report offers additional ethnobotanical evidence for useful medicinal activities in Ericaceae species of n North America and represents the first attempt at phytochemical profiling of a significant number of species. While most First Nations inhabiting the boreal forest tend to corroborate the use of Ericaceae by the Cree Nation (Table 3.2), the current study with the Cree focused on treatment of diabetic symptoms so uses unrelated to diabetes were often left unidentified. As shown in this paper, phenolic compounds, which commonly possess biological activities relevant to human health, are ubiquitous in the Ericaceae family and likely contribute their numerous uses among the First Nations. Pharmacological assays are now substantiating those traditional uses with convincing indication that the use of traditional medicine is not a random act, but is based upon experience, observations, and traditional knowledge (Marles 1995; Spoor et al. 2006).

The summary of marker compounds for each Ericaceae species is presented in Table 3.6. In plant extracts where no unique marker/s was identified, a combination of multiple markers provided a unique fingerprint for each individual species. In the case of the five *Vaccinium* species under study, **15** is a unique marker of the *V. oxycoccus* and **14** and **19** are unique to *V. vitis-idaea*, which is also the only *Vaccinium* lacking both **2** and **4.** *V. angustifolium* differs from *V. myrtilloides* in the ratio of **21** to **23** and the presence of **20** (Figures 3.2 and 3.5). In *V. poasanum*, none of the identified compounds were unique within the genus but the presence of **6** and absence of **2** and **3** distinguish the species.

Table 3.6 – Identified markers and unique fingerprint markers for leaf extracts of various Ericaceae species.

Species	Identified Markers *	Fingerprint Marker/s
Andromeda polifolia ssp. *glaucophylla*	2,3,**4**,5,**17**,18,21,23	Quercetin-3-*O*-galactoside (**17**) is a major marker, Chlorogenic acid (**4**) content much lower than *V. angustifolium*
Arctostaphylos uva-ursi	**1**,3,**11**,15,18,19,20,21	Arbutin (**1**),, Myricetin glycoside-3 (**11**)
Epimerium nigrum	2,3,5,**16,17,18,19,20**, 21,23	Rutin (**16**), Quercetin-3-galactoside (**17**), Quercetin-3-glucoside (**18**), A-type procyanidin 2 (**19**), Quercetin glycoside (**20**)
Gaultheria hispidula	2,3,4,5,**9,15,16,17,18,** 20,21,23	Taxifolin glycoside 1 (**9**), Myricitrin (**15**), Rutin (**16**), Quercetin-3-galactoside (**17**), Quercetin-3-glucoside (**18**)
Gaultheria procumbens	2,3,5,6,**8**,17,18,**19**,21	Dominant Quercetin-3-glucoside (**18**), A-type procyanidin 2 (**19**)
Kalmia angustifolia	3,5,17,19,20,21,**22**,23	Myricetin (**22**)
Rhododendron groenlandicum	3,4,5,**6,14**,17,18,19, 20,21,23	Procyanidn B2 (**6**) and A-type procyanidin 1 (**14**)
Rhododendron laponicum	2,3,4,5,**7**,10,**12**,14,17, 18,19,20,21	Myricetin glycoside 1 (**7**) and Taxifolin glycoside 2 (**12**)
Rhododendron tomentosum ssp. *subarcticum*	2,3,4,5,**9,13**,14,17,18, 19,20,21,23	Taxifolin glycoside 1 (**9**) and Taxifolin (**13**)
Vaccinium angustifolium	2,3,**4**,5,16,17,18,**21,23**	Dominant chlorogenic acid (4), quercetin-3-O-rhamnoside (**23**) 2-3 times lower in quantity than in *V. myrtilloides*; no quercetin-glycoside **(20)**
Vaccinium myrtilloides	2,3,**4**,5,16,17,18,**20**, 21,23	Dominant chlorogenic acid peak (4), Quercetin-glycoside (**20**)
Vaccinium oxycoccus	**2**,3,4,5,6,**15**,17,18,20, 21,23	Caffeoylquinic acid (**2**), Myricitrin (**15**)
Vacciniunm poasanm	4,5,**6**,17,18,20,21,23	Procyanidin B2 (**6**), lacks catere echin (**3**)
Vacciniun vitis-idaea	**1**,3,5,**14**,17,18,**19**,20, 21,23	Arbutin (**1**), A-type procyanidin 1 (**14**)

* The codes of fingerprint markers are presented in bold.

The three *Rhododendron* species were more easily differentiated as markers of *R. laponicum* (**9** and **13**) and *R. tomentosum* ssp. *subarcticum* (**7** and **12**) were not found in other Ericaceae leaf extracts. *R. groenlandicum* was the only *Rhododendron* where procyanidins were detected and the only Ericaceae with both **6** and **14** (Table 3.6). The two *Gaultheria* species were quite different in their chemical fingerprints. *G. hispidula* was the only extract with the consecutively eluting markers **15-18** and was further distinguished from *G. procumbens* by the presence of **4, 20** and **23** and absence of **6**, **8** and **19**.

The chromatographic profile of *A. uva-ursi* was similar to that of *V. vitis-idaea*, mainly due to their common characteristic marker arbutin (**1**), but differed by having the unique marker **11**. Interestingly, unlike the majority of Ericaceae, both extracts also lacked **2** and **4,** possibly due to the biosynthetic allocation of quinic acid to the production of arbutin **(1).** The qualitative fingerprint of *A. polifolia* ssp. *glaucophylla* was similar to that of *V. angustifolium* but differed quantitatively; marker **4** was predominant in *V. angustifolium* (34.3 mg/g dw) while **17** was in relatively low amount (2.1 mg.g dw) but, conversely, **4** was present in trace amounts (0.6 mg/g dw) while **17** was predominant (14.0 mg/g dw) in *A. polifolia* ssp. *glaucophylla.* Although no unique markers were identified in *E. nigrum,* it was the only extract that contained markers **16-19**, creating a unique fingerprint. Despite the presence of small amounts of **22,** which was not detected in any other species in our study, *K. angustifolia* was characterized mainly by the lack of common markers **18**, **2** and **4** (Figures 3.3 and 3.5C). For these reasons, authentication of a single sample may in some cases be difficult without additional Ericaceae samples with which to compare.

To date most fingerprinting work in the Ericaceae has concentrated on anthocyanins, flavonoids (Wu and Prior 2005) or, less frequently, procyanidins (Gu *et al.* 2002) in the fruit while the leaf phenolics have been rarely studied. Along with a study by Lhuillier *et al.*

(2007) on *Agauria salicifolia*, our previous work with *Vaccinium angustifolium* (Harris et al. 2007a) is among the first chromatographic studies on leaf phenolics in Ericaceaee. The chromatograph for this species presented here (Figure 3.2) was virtually identical to our previous results with a different genotype except that rutin was not detected and the relative ratio of quercetin glycosides differed (Harris et al. 2007a). Though such populational and/or seasonal variation in marker compounds requires consideration, the fingerprints remain identifiable.

The leaves of *Gaultheria procumbens* are a popular botanical and have therefore received some of attention. Our results corroborated the documented presence of arbutin (**1**), which is also an established marker of *V. vitis-idaea* (Sticher 1979) along with myricitrin (**15**) and quercetin-3-*O*-glucoside (**18**) (Chauhan *et al.* 2007). A variety of simple phenolic acids has been reported in leaves of *A. uva-ursi, K. angustifolia* and *V. vitis-idaea* (Dombrowicz et al. 1991; Zhu 1994) but was not detected in our extracts. Although the UV profiles of many simple phenolics were stored in the metabolomics library, few were identified in this study indicating that alternative extraction and analytical protocols should be considered if targeting these metabolites.

The present describes a novel analytical method for the identification of Ericaceae species by HPLC fingerprinting of phenolic metabolites. While the 14 species studied here represent a small percentage of plant species within the rather large botanical family Ericaceae, which consists of over 350 species, the chromatographic method can be executed on other Ericaceae species to broaden its scope and application. Inclusion and successful analysis of a tropical Ericaceae species (*V. poasanum*) suggests that the method is appropriate for chromatographic separation, identification and fingerprinting of phenolic metabolites from geographically unrelated Ericaceae species. Moreover, with quercetin,

catechin and chlorogenic acid derivatives previously reported in the leaves of two Hawaiian species of *Vaccinium* (Bohm and Koupai-Abyazani 1994), the selected standards appear to represent the major classes of Ericaceae leaf phenolics.

The chromatographic separation was target-oriented in order to reveal the unique marker/s of each species under study. Special emphasis was on authenticity of identification after adequate chromatographic separation by HPLC-PAD/APCI-MSD. Automated metabolomics library matching proved an important first hand software tool for quick identification but online mass spectrometric profiling was mandatory for finalization of trace level identifications.

ACKNOWLEDGEMENTS

Very special thanks are due to the Cree Elders of Mistissini and Whapmagoostui who kindly agreed to be interviewed. They made this article possible by allowing us to use, for the purposes of this research, their knowledge relating to medicinal plants, transmitted to them by their elders. Their trust has also enabled a useful exchange between Traditional knowledge and modern science.

SECTION II

ASSESSMENT OF ANTI-DIABETIC ACTIVITIES

CHAPTER 4 – CASE STUDY: ANTI-DIABETIC ACTIVITIES OF *VACCINIUM ANGUSTIFOLIUM*

4.1 PREFACE

Few boreal plant species have been thoroughly evaluated for anti-diabetic potential, especially with phytochemically characterized material. Taking the same approach used in Chapter 2 and building on the phytochemical analyses conducted therein, the different plant parts of a single species with documented medicinal use for the treatment of diabetes, *V. angustifolium,* were then selected for evaluation in a series of *in vitro* studies. We specifically sought to identify biochemical activities in peripheral tissue cell lines (muscle cells, adipose cells, pancreatic β-cells and nervous system precursor cells) that are relevant to the prevention and management of both diabetes and diabetic complications.

4.2 STATEMENT OF AUTHOR CONTRIBUTIONS

LCM, MP, SALB, JTA and PSH conceived and designed the experiments. CSH developed and performed the PC12 cell experiments (Figure 4.5) and phytochemical analyses (Table 4.1), to which AJB also contributed. LCM, AC, DS, ABA, BM, CL and TV performed the remaining experiments. PML conducted initial extractions and chemical investigations. LCM wrote the manuscript with PSH and contributions from CSH, JTA and SALB in relation to diabetic neuropathy and phytochemistry.

4.3 ANTI-DIABETIC PROPERTIES OF THE CANADIAN LOWBUSH BLUEBERRY VACCINIUM ANGUSTIFOLIUM AIT.

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ABSTRACT

Incidence of type II diabetes is rapidly increasing worldwide. In order to identify complementary or alternative approaches to existing medications, we studied anti-diabetic properties of *Vaccinium angustifolium* Ait., a natural health product recommended for diabetes treatment in Canada. Extracts of root, stem, leaf, and fruit were tested for anti-diabetic activity in peripheral tissues and pancreatic β cells using a variety of cell-based bioassays. Specifically, we assessed: 1) deoxyglucose uptake in differentiated C2C12 muscle cells and 3T3-L1 adipocytes; 2) glucose-stimulated insulin secretion (GSIS) in β TC-tet pancreatic β cells; 3) β cell proliferation in β TC-tet cells; 4) lipid accumulation in differentiating 3T3-L1 cells; 5) protection against glucose toxicity in PC12 cells. Root, stem, and leaf extracts significantly enhanced glucose transport in C2C12 cells by 15-25% in presence and absence of insulin after 20 h of incubation; no enhancement resulted from a 1 h exposure. In 3T3 cells, only the root and stem extracts enhanced uptake, and this effect was greater after 1 h than after 20 h; uptake was increased by up to 75% in absence of insulin. GSIS was potentiated by a small amount in growth-arrested β TC-tet cells incubated overnight with leaf or stem extract. Fruit extracts were found to increase ^{3}H-thymidine incorporation in replicating β TC-tet cells by 2.8-fold. Lipid accumulation in differentiating 3T3-L1 cells was accelerated by root, stem and leaf extracts by as much as 6.5-fold by the end of a 6-day period. Stem, leaf and fruit extracts reduced apoptosis by 20-33% in PC12 cells exposed to elevated glucose for 96 h. These results demonstrate that *V. angustifolium* contains active principles with insulin-like and glitazone-like properties, while conferring protection against glucose toxicity. Enhancement of proliferation in β cells may represent another potential anti-diabetic property. Extracts of the Canadian blueberry thus show promise for use as a complementary anti-diabetic therapy.

INTRODUCTION

The incidence of type 2 diabetes mellitus (T2DM) has reached epidemic proportions in western and developing countries, with an estimated 194 million people afflicted (International-Diabetes-Federation 2005). Peripheral insulin resistance is a key feature of T2DM and results from a combination of sedentary lifestyle, unhealthy dietary habits, and genetic predisposition (Skyler 2004). Insulin resistance is also implicated in a number of life-threatening disorders collectively referred to as the metabolic syndrome (Hansen 1999).

Current medication options for the treatment of diabetes are relatively limited, have non-negligible side-effects, and must often be prescribed in combination (Cheng and Fantus 2005). The use of natural health products as complementary or alternative approaches to existing medications is growing in popularity. While these have been the object of very few rigorous scientific studies, it is clear that many plants possess hypoglycaemic activity, some having considerable anti-diabetic potential (Marles 1995; Yeh et al. 2003). Indeed, the widely prescribed insulin-sensitizer metformin was derived from guanidine, a molecule isolated from *Galega officinalis* L. (French lilac) (Bailey 2004; Witters 2001). Moreover, *Trigonella foenum-graecum* L (Fenugreek) is a plant long-consumed around the world for its anti-diabetic properties. It contains abundant amounts of the unconventional amino acid 4-hydroxy-isoleucine, which, along with its derivatives, is currently being developed as a novel anti-diabetic molecule (Broca et al. 1999; Broca et al. 2004).

Various members of the *Vaccinium* genus, including *Vaccinium myrtillus* L (European blueberry or bilberry) and *Vaccinium macrocarpon* Ait. (American cranberry), possess putative anti-diabetic activity (Blumenthal 1998; Chambers and Camire 2003) and have been used extensively as traditional medicines for the treatment of diabetic symptoms (Jellin 2005). A recent survey identified *Vaccinium angustofolium* Ait. (Canadian lowbush

blueberry) as one of the anti-diabetic plants most highly recommended by Quebec traditional practitioners, alongside fenugreek (Haddad et al. 2001). However, despite such claims and widespread use, the majority of published studies have focussed on the anti-oxidant properties of this species (Kalt et al. 2001; Kay and Holub 2002; Lyons et al. 2003; Rimando et al. 2004; Sweeney et al. 2002; Wang and Jiao 2000) and its anti-diabetic potential has not been the object of rigorous scientific investigation. The purpose of the present study was to test *V. angustifolium* for insulin-like activity, insulinotropic activity, glitazone-like activity, and cytoprotective activity in cell-based bioassays.

EXPERIMENTAL

Plant Material and Preparation

Samples of wild *V. angustifolium* Ait. were harvested near la Vérendrye Wildlife Reserve, Quebec, Canada. Voucher specimens are stored at the University of Ottawa. After collection, the plant was washed and separated into four organs: fruits, leaves, roots and stems. Fruits were frozen at -80°C, leaves were preserved in ethanol (95%), while roots and stems were air-dried and then stored in darkness at room temperature (RT). Preserved leaves were filtered, crushed and re-extracted 3 times with fresh ethanol at RT for 12 h. The 4 ethanolic phases were recovered, pooled, dried at 40°C by rotary evaporator and lyophilized. Dried roots and stems were ground on a Wiley mill to mesh size 40 and extracted 3 times in ethanol at RT for 12 h. The ethanolic phase was then dried and lyophilised, as above. Frozen fruits were crushed and extracted as for roots and stems. Extracts were stored in the dark at 4°C.

HPLC analyses

Root, stem, leaf and fruit extracts were characterized by HPLC-MS analysis. Fruit extracts have also been characterized by others (Kalt et al. 1999; Kalt et al. 2001; Lyons et al. 2003; Rimando et al. 2004; Schmidt et al. 2004). All analyses were performed using a Hewlet-Packard Chemstation series 1100 chromatograph (Agilent, Palo Alto, CA, USA) with a photodiode array detector and an APCI/MS using a validated method as described previously (Chapter 2) with a Waters YMC ODS-AM narrow bore column (100 x 2 mm i.d.; 3 μm particle size) set at 50°C and a flow rate of 0.3 ml/min. Elution conditions included a mobile phase system of methanol (solvent A) and TFA (0.05%) in water (pH 3.4; solvent B) as follows: initial conditions 8:92 (A:B), held for 5 min, then changing to 13:87 in 2 min, then to 30:70 in 14 min, 60:40 in 3 min, 100:0 in 2 min, then washed with 100:0 for 2 min before returning to the initial conditions in 2 min, which was held for 5 min to re-equilibrate the column. The total analysis time was 35 min. Elution profiles were monitored on-line at 325 and 280 nm with the PAD and MS detection was performed in positive ionisation mode with data collection in scan mode for ions from 100 to 800 mass units.

Cell culture

Cell biology reagents were purchased from Invitrogen Life Technologies (Burlington, ON) unless otherwise noted. C2C12 murine skeletal myoblasts and 3T3-L1 murine pre-adipocytes were obtained from American Type Cell Collection (ATCC; Chicago, IL). β TC-tet murine pancreatic β cells were kindly provided by Dr. Shimon Efrat (Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel) (Fleischer et al. 1998; Milo-Landesman and Efrat 2002). In this line derived from transgenic mice expressing the SV40 T antigen (Tag)

under control of the tetracycline gene regulatory system, growth arrest can be induced by shutting off Tag expression in the presence of tetracycline. PC12-AC cells are a clonal derivative of the PC12 pheochromocytoma cell line (ATCC) and can be differentiated into a sympathetic neuronal phenotype with exposure to nerve growth factor.

All cells were cultured at 37°C in a humidified 5% CO_2, 95% air atmosphere. C2C12 myoblasts were cultured in high glucose (2.5 g/L) Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 10% horse serum (HS), and penicillin-streptomycin, until 80% confluence. Myoblasts were then differentiated into myotubes over 6 days in DMEM containing 2% HS. By the end of this period, confluence was attained and all cells were multinucleated. 3T3-L1 cells were proliferated in high-glucose DMEM containing 10% FBS and antibiotics. Upon 80% confluence, differentiation was initiated by adding 250 μM 3-isobutylmethylxanthine (IBMX), 1 μM dexamethasone (DMX), and 670 nM insulin to this medium for two days. Differentiation was then continued in DMEM containing 10% FBS and 670 nM insulin for approximately 10 days. At this point, confluence was attained and over 90% of cells contained lipid droplets visible under phase-contrast microscopy. For adipogenesis assays, proliferating cells were allowed to attain confluence before initiating differentiation as above. Cells were then differentiated for a total of 5-6 days. β TC-tet cells were grown in high-glucose DMEM containing 15% HS, 2.5% FBS, and antibiotics until 80% confluent. PC12-AC cells were cultured in RPMI 1640 containing 11 mM glucose, 10% HS, 5% newborn calf serum, and antibiotics until 80% confluent.

V. angustifolium extracts were solubilized in dimethyl sulfoxide (DMSO) for application to cell cultures; final DMSO concentration was fixed at 0.1% for all assays. All extracts were applied at a dose of 12.5 μg/ml except in the PC12-AC survival assay where

concentrations ranging from 0.5 to 25 μg/ml were tested. The selection of the 12.5 μg/ml dose was based on pilot cytotoxity experiments indicating that root and stem extracts are slightly toxic at 25 μg/ml; at this concentration viability was reduced by approximately 10% as assessed by Trypan blue exclusion (results not shown). Overnight incubation at 12.5 μg/ml was confirmed to have no effect on the morphology or viability of the various cell types employed herein.

Glucose transport assay

To screen for insulin-like activity and for potentiation of insulin action, basal and insulin-stimulated glucose uptake were measured in differentiated C2C12 skeletal myotubes and in differentiated 3T3-L1 adipocytes incubated with extracts of *V. angustifolium*. Both of these cell types exhibit insulin-regulated glucose uptake and possess Glut-1 and Glut-4 glucose transporters (Klip and Paquet 1990). Confluent and differentiated cells, grown in 12 well plates were incubated with either vehicle (DMSO) alone, extract in vehicle, or positive control in vehicle for either 1 h or 18-21 h prior to assay. For 1 h treatments, differentiation medium was replaced with serum-free medium 1 h before the start of incubation period and the incubation was performed in serum-free medium. For 18-21 h treatments, the last three hours of incubation were performed in serum-free medium. Following the incubation period, cells were rinsed twice with a Krebs-phosphate buffer (20 mM HEPES, 4.05 mM Na2HPO4, 0.95 mM NaH2PO4, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1 mM CaCl2, 1 mM MgSO4, 5 mM glucose, 0.5% BSA, pH 7.4) at 37°C. Cells were then treated with 0, 1, or 100 nM insulin in this buffer for 30 min at 37°C, in the presence or absence of extracts. Cells were then washed twice with glucose-free Krebs-phosphate buffer at 37°C and treated with 0.5 μCi/ml 2-deoxy-D-[1-3H]-glucose (TRK-383, Amersham Biosciences, UK) in this buffer for

exactly 10 min at 37° C without extracts. Following this, cells were placed on ice and immediately washed three times with ice-cold Krebs-phosphate buffer, lysed with 0.1 M NaOH for 30 min, and scraped. The lysate was added to 1 ml of liquid scintillation cocktail (Ready-Gel 586601, Beckman Coulter) and incorporated radioactivity was measured in a scintillation counter. Metformin (400 μM) applied for 18 to 21 h was used as a positive control in C2C12 experiments (Klip and Paquet 1990; Kumar and Dey 2002). For 3T3-L1 experiments, fenugreek seed ethanolic extract, a known hypoglycaemic agent (Vats et al. 2002), was used as a positive control at its maximum non-toxic dose of 75 μg/ml (cytotoxicity data not shown).

Insulin secretion assay

The β TC-tet cell line was used to screen extracts of *V. angustifolium* for the potentiation of glucose-stimulated insulin secretion (GSIS). This pancreatic β-cell line releases insulin in response to physiological concentrations of glucose in a dose-response manner. Changes in cell secretory properties (basal secretion, GSIS, and a shift in glucose-sensitivity) can be detected by measuring insulin secretion released into the medium in response to incremental concentrations of glucose. β TC-tet cells were seeded in 12 well plates at a density of 2.5 x 105. Upon reaching 60-80% confluence, growth medium was supplemented with 1 μg/ml tetracycline for 48 hours. Growth arrested cells were then incubated 18 h with or without 12.5 μg/ml of plant extracts in growth medium. Cells were rinsed with and pre-incubated for 1 h at 37°C in a Krebs-Ringer buffer (10 mM HEPES, 25 mM NaHCO3, 2mM NaH_2PO4, pH 7.4, 118 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM MgSO4,, 0.1% fatty-acid-free BSA, pH 7.4) containing no glucose in presence or absence of

extracts. Insulin secretion was then assessed over a 2 h period in the presence or absence of extracts in buffer containing 0.5 mM IBMX and either 2 mM glucose (basal secretion), 6, 10 or 16 mM glucose (GSIS), or 2 mM glucose and 50 mM K+ (non-fuel secretagogue) (buffer adjusted to 50 mM KCl and 73 NaCl). The insulin released into the medium at the end of the secretion period was determined by radioimmunoassay (RIA), as described below.

Three replicates were performed for each experimental condition. Samples of incubation media were centrifuged 3 min at 4° C at 3,000 g to remove any cells, and supernatants were stored at -20° C until assayed for insulin. Cellular insulin content was measured in cells exposed to 2 mM glucose (basal secretion). Intracellular insulin was extracted overnight at 4°C in 0.2 mM HCl - 75% ethanol. These samples were briefly sonicated and centrifuged at 30,000 g for 5 min before measurement of insulin in the supernatant by RIA. All insulin concentrations were expressed per well of growth-arrested cells. RIA was performed on samples diluted between 100 and 1000 times using a rat insulin RIA kit (# RI-13K, Linco Research, St-Charles, MO) according to the manufacturer's instructions.

β cell proliferation assay

To test for a proliferative effect of *V. angustifolium* extracts on β cells, extracts were applied to replicating (non-growth arrested) β TC-tet cells and incorporation of ^{3}H-thymidine was evaluated. Cells were seeded in 24 well plates at a density of 1.0×10^{5} and incubated in growth medium for 24 h. Incubation was continued for another 48 h in growth medium while one group was treated with tetracycline (1 μg/ml) in order to arrest growth. Replicating cells were then incubated 24 h in the presence or absence of extracts. A measure

of 1 μCi/ml of methyl ^{3}H-thymidine (4 Ci/mmol; # 2404105, MP Biomedicals, Irvine, CA) was added to medium over the last 6 h of treatment. Cells were then rinsed 3 times in PBS and lysed with 0.1 M NaOH for 30 min and scraped. The lysate was added to 1 ml of liquid scintillation cocktail and incorporated radioactivity was measured in a scintillation counter. Four replicates were performed for each experimental condition. Average counts from tetracycline-treated (growth-arrested) wells were considered as background and were subtracted from all other measures.

Adipocyte differentiation assay

As an indirect screen for glitazone (thiazolidinedione)-like activity in *V. angustifolium* extracts, 3T3-L1 pre-adipocytes differentiating in the presence of extracts were assessed for accelerated differentiation over non-treated cells by measuring the accumulation of triglycerides at the end of the treatment period, as is often done to determine PPAR-γ agonist activity (Harmon and Harp 2001; Ljung et al. 2002; Norisada et al. 2004; Tontonoz et al. 1995). Cells were grown in 24-well plates. One day after attaining confluence, proliferation medium was replaced with differentiation medium containing IBMX, DXM, and insulin, as described above, with either vehicle (DMSO) alone, extract in vehicle, or positive control in vehicle. This medium was changed after 24 h. After 48 h, medium was replaced with differentiation medium containing only insulin, as above, with or without plant extracts or controls. This medium was changed every 24 h. Rosiglitazone (10 μM; Alexis Biochemicals, Hornby, ON) was used as a positive control, while vehicle in proliferation medium was used as a negative control. Experiments were terminated after the first visual detection of intracellular lipid droplets by phase-contrast microscopy in vehicle-treated cells, typically by day 5 or 6 of the incubation period. At this time, micrographs were taken of live

cells with a 40 x objective. Intracellular lipids in live cells were then stained with AdipoRed fluorescent reagent (Cambrex Bio Science, Walkersville, MD), a Nile red derivative, as per manufacturer's protocol. Briefly, cells were washed in PBS (8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, 137 mM NaCl, 2.68 mM KCl, pH 7.4), then 1 ml of PBS was added to each well followed by 30 µl of reagent. After a 15 min incubation at ambient temperature, fluorescence was measured with a plate reader (Wallac Victor 2, Perkin-Elmer, St-Laurent, QC) with a 485 nm excitation filter and a 572 nm emission filter. Four replicates were performed for each condition. The mean value obtained from the negative control condition was considered as the background and subtracted from all other readings.

Protection of PC12-AC cells from glucose toxicity

To test for cytoprotective activities against glucose toxicity, viability assays were performed on PC12-AC cells subjected to chronically elevated glucose in the presence of extracts or vehicle (0.1% DMSO). Cells were seeded in 96-well plates at a density of $6.25x10^3$ cells/well and cultured for 24 h at 37°C. Complete medium was replaced with serum-free medium adjusted to 150 mM glucose (approx. 14-fold greater than normal concentration for this cell line) and supplemented with 0.025% BSA, with extracts or vehicle (DMSO). While these conditions feature a supra-physiological concentration of glucose (the concentration observed in severe unmanaged diabetes can attain levels 7-fold greater than normal), they are necessary to induce death in approximately 40% of cells over the 4-day experimental resolution. Under this paradigm, toxicity is due to glucose *per se* and not osmotic stress since the substitution of L-glucose for R-glucose abolishes toxicity (Koshimura et al. 2002). Viable cell counts were determined by a modified WST-1 viability assay (Cell Proliferation Reagent; Roche, Laval, QC). Ten microlitres of WST-1 tetrazolium

salt reagent was added to each well, as per the manufacturer's instructions, and plates allowed to incubate for 75 min before colorimetric analysis of formazan content was made by measurement of absorbance at $\lambda = 420/620$. Number of live cells per well was calculated from absorbance based on experimentally prepared standard curves of known number of PC12-AC cells. High glucose/vehicle controls were included on every plate and pooled for statistical analysis. Eight replicates were performed for each experimental condition. Data were expressed as a percentage of live cell number measured in normal glucose conditions.

Statistical analysis

Results were analysed by one-way analysis of variance with Fisher, Bonferonni or Dunnett *post hoc* test. Comparisons of insulin secretion dose-response curves were performed using AllFit software (André DeLéan, Université de Montréal (DeLean et al. 1978)). Statistical significance was set at $p \leq 0.05$. All data are reported as mean ± SEM.

RESULTS

Phytochemical characterization of extracts

HPLC-MS methods were developed to characterize the root, stem and leaf crude extracts of *V. angustifolium.* Extracts of the four organ part differed significantly in the composition and content of major phytochemical classes (Table 4.1). Root extract contained significant amounts of vanillic acid, was virtually devoid of flavonols and their glycosides, and exhibited moderate amounts of catechins and procyanidins. Stem extract was richest in catechin, epicatechin and procyanidins. Its content in several flavonols and their glycosides was high, and chlorogenic acid was the most abundant phenolic acid present. Leaf extract was very abundant in chlorogenic acid, several flavonols and glycosides, as well as catechin

and epicatechin. Finally, fruit extracts contained chlorogenic acid and small amounts of flavonols and glycosides. Fruit extracts were the only organ to contain anthocyanins. Fruit extracts have been reported by others to be rich in total phenolics and anthocyanins (Kalt et al. 1999; Kalt et al. 2001; Lyons et al. 2003; Rimando et al. 2004; Schmidt et al. 2004).

Enhancement of deoxyglucose uptake in differentiated C2C12 muscle cells and 3T3-L1 adipocytes

Extracts of *V. angustifolium* were tested for insulin-like or insulin-sensitizing properties by assessing insulin-dependent and -independent glucose uptake in two insulin-responsive and Glut4-containing cell lines: differentiated C2C12 myotubes and differentiated 3T3-L1 adipocytes (Berti and Gammeltoft 1999; Calderhead et al. 1990; Galante et al. 1995; Rosen et al. 1978; Sarabia et al. 1990). In C2C12 cells, 100 nM of insulin induces approximately a 40% increase in uptake of labelled deoxyglucose. Metformin can be used as a positive control, and overnight incubation with 400 μM metformin induces an approximate 40% increase over basal or insulin-stimulated uptake. In 3T3-L1 cells, 100 nM of insulin induces approximately a 400% increase in transport.

Overnight incubation of C2C12 cells with 12.5 μg/ml of root, stem, or leaf extracts prior to the deoxyglucose transport assay resulted in significant ($p<0.05$) enhancement of uptake by 15-25% in the presence or absence of insulin, as compared to vehicle (Figure 4.1A). Incubation with fruit extract only enhanced uptake in the presence of insulin. Reducing extract incubation time to 1 h abolished all enhancement of uptake in C2C12 cells (Figure 4.1B).

Table 4.1 – Phytochemical characterization of root, stem, leaf, and fruit crude ethanolic extracts of *V. angustifolium*.

Compound	Content (μg compound / mg extract ± SEM)			
	Root	Stem	Leaf	Fruit
Caffeic acid	Not detected	0.39 ± 0.04	1.22 ± 0.06	Not detected
Chlorogenic acid	0.64 ± 0.12	1.04 ± 0.08	104.0 ± 1.9	3.08 ± 0.02
Vanillic acid	0.66 ± 0.23	Trace	Not detected	Not detected
Q-3-galactoside	Not detected	4.95 ± 0.13	6.56 ± 0.15	0.62 ±0.03
Q-3-glucoside	Not detected	1.45 ± 0.09	11.65 ± 0.27	0.82 ± 0.03
Q-3-arabinoside	Not detected	7.47 ± 0.24	9.15 ± 0.21	Trace
Q-3-rhamnoside	Not detected	0.11 ± 0.01	4.87 ± 0.16	Trace
Quercetin	Not detected	0.13 ± 0.01	3.07 ± 0.12	0.07 ± 0.01
Catechin	1.02 ± 0.17	13.83 ± 0.52	20.55 ± 1.75	Trace
Epicatechin	14.09 ± 0.85	32.21 ± 1.13	24.15 ± 1.18	Trace
Procyanidin B1	0.73 ± 0.15	8.99 ± 0.27	Not detected	Not detected
Procyanidin B2	4.28 ± 0.27	10.84 ± 0.36	Not detected	Not detected
Anthocyanins	Not detected	Not detected	Not detected	Abundant

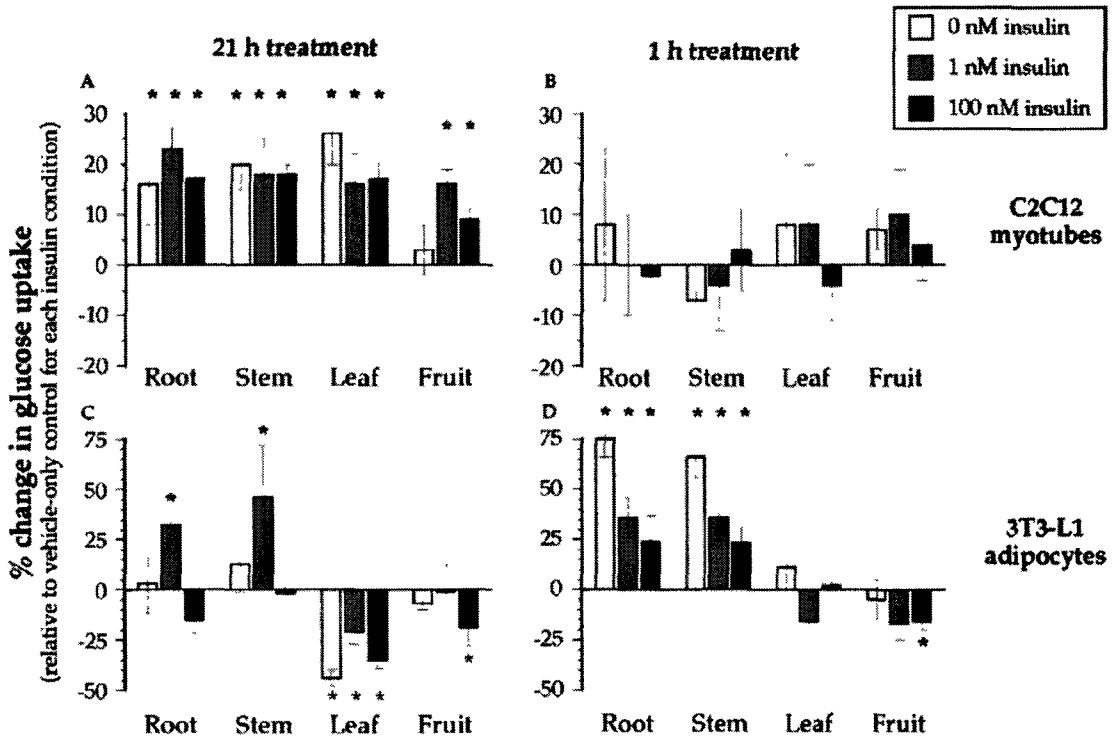

Figure 4.1 – Insulin-like and insulin-sensitizing effects of *V. angustifolium* extracts in differentiated and confluent C2C12 skeletal muscle cells and 3T3-L1 adipocytes. These cell lines express insulin-responsive GLUT-4 glucose transporters. Cells were incubated with 12.5 μg/ml of root, stem, leaf, or fruit extracts or with vehicle only (0.1% DMSO) for 18-21 h or for 1 h and were then subjected to a 3H-deoxyglucose uptake assay in the presence of 0, 1, or 100 nM insulin. For each concentration of insulin, uptake is expressed as mean counts of incorporated radioactivity per well normalized by the mean counts measured in cells treated with vehicle only plus that same concentration of insulin. Means + SEM are presented for 3-6 replicates.
* indicates a significant difference from vehicle only ($p \leq 0.05$).

Overnight incubation of 3T3-L1 cells with root or stem extract significantly enhanced transport at 1 nM insulin, but not at 100 nM or in the absence of insulin (Figure 4.1C). Reducing incubation time with root or stem extract to 1 h resulted in significantly enhanced transport at 0, 1, and 100 nM insulin (Figure 4.1D). Overnight incubation with leaf extract inhibited uptake at all insulin concentrations, while fruit extract had no effect (Figure 4.1C). At 1 h, leaf and fruit extracts did not affect uptake (Figure 4.1D).

Potentiation of GSIS in β TC-tet pancreatic β cells

In order to test whether extracts of V.angustifolium possess insulinotropic activity, tetracycline-treated (growth-arrested) insulin-secreting β TC-tet cells were incubated overnight with or without 12.5 μg/ml of each extract. Insulin secretion was assessed over a 2 h incubation in conditions of basal secretion, glucose-stimulated secretion, or potassium-stimulated secretion, in the continued presence or absence of extract. In this model, insulin secretion increases 2.5-fold above basal levels at 6 mM glucose, 5-fold at 10 mM and 7-fold at 16 mM in cells incubated with vehicle only (DMSO). The use of growth-arrested cells allows for differentiation between proliferative effects (see below) from insulinotropic effects.

Treatment with leaf extract resulted in a small but statistically significant increase in GSIS (Figure 4.2A). While differences in insulin secretion between extract-treated and vehicle-treated cells at each of the four tested glucose concentrations were not significant as analysed by ANOVA, a global comparison of the dose-response curves (modeled as four-parameter sigmoidal curves) using the AllFit method (DeLean et al. 1978) revealed that the curves are significantly different ($F=11.5$; $p<0.001$). Furthermore, this global difference can be explained in large part by a combination of differences in ED50 and maximal response of

the curves (F=5.19; p=0.019); any changes in minimal response did not contribute significantly to the global difference. Therefore, treatment with leaf extract does not significantly affect basal secretion, but slightly increases GSIS magnitude and sensitivity. The dose-response curves for the stem extract treatment and the vehicle treatment were also significantly different (F=3.11; $p<0.05$) when analysed globally (Figure 4.2B).

Secretion stimulated by potassium-induced depolarization was not altered, indicating normal cellular function (results not shown). Likewise, intracellular insulin content was not significantly affected by the treatment (results not shown).

Increase in proliferation of β TC-tet pancreatic β cells

In order to test whether extracts of *V. angustifolium* can stimulate β cell proliferation, geometrically-replicating insulin-secreting β TC-tet cells were incubated overnight with or without 12.5 μg/ml of each extract and ^{3}H-thymidine was added to the medium during the final 6 h of the treatment. Treatment with fruit extract was found to increase radioactivity incorporation by 2.8-fold while other extracts had no effect (Figure 4.3).

Increase in lipid accumulation in differentiating 3T3-L1 cells

A hallmark of glitazone (thiazolidinedione)-like activity is enhanced adipogenesis mediated through PPAR-γ receptor activation. The presence of glitazone-like activity in *V.angustifolium* extracts was indirectly assessed by testing for increased lipid accumulation in differentiating 3T3-L1 pre-adipocytes treated with extracts over a 6-day period. Intracellular triglyceride content was measured using the AdipoRed fluorescent reagent at the end of the treatment period. At this time point, lipid droplets were observable by phase-

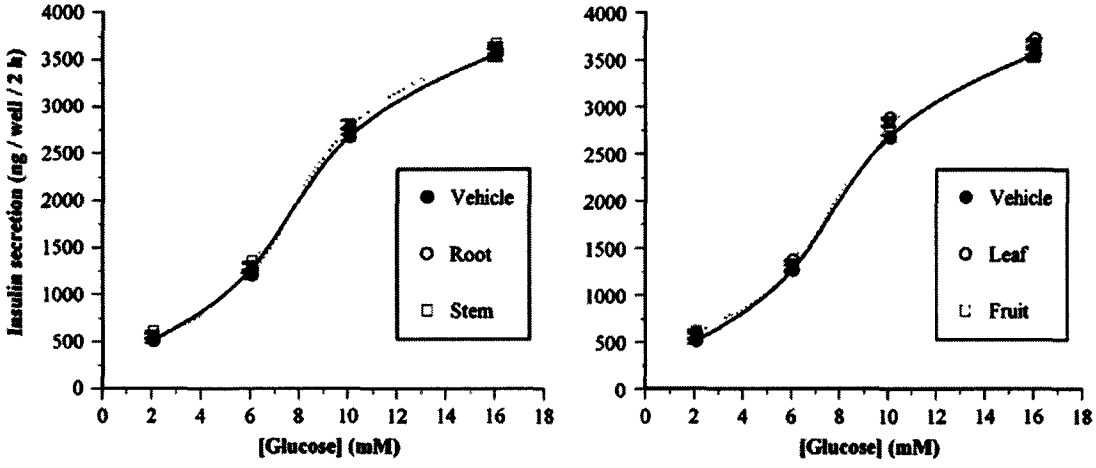

Figure 4.2 – Effects of *V. angustifolium* extracts on glucose-induced insulin secretion were in growth-arrested β TC-tet pancreatic β cells. Cells were incubated with 12.5 μg/ml of root, stem, leaf, or fruit extracts or with vehicle only (0.1% DMSO) for 18 h. Insulin secretion over a 2 h period in response to 2, 6, 10, or 16 mM glucose was measured by RIA. Leaf extract and stem extract dose-response curves were statistically different from the vehicle dose-response curve. No differences were observed in the secretory response to 50 mM potassium or in intracellular insulin content (not illustrated). Means ± SEM are presented for three replicates. Curves were globally analysed using the AllFit method (DeLean et al. 1978).

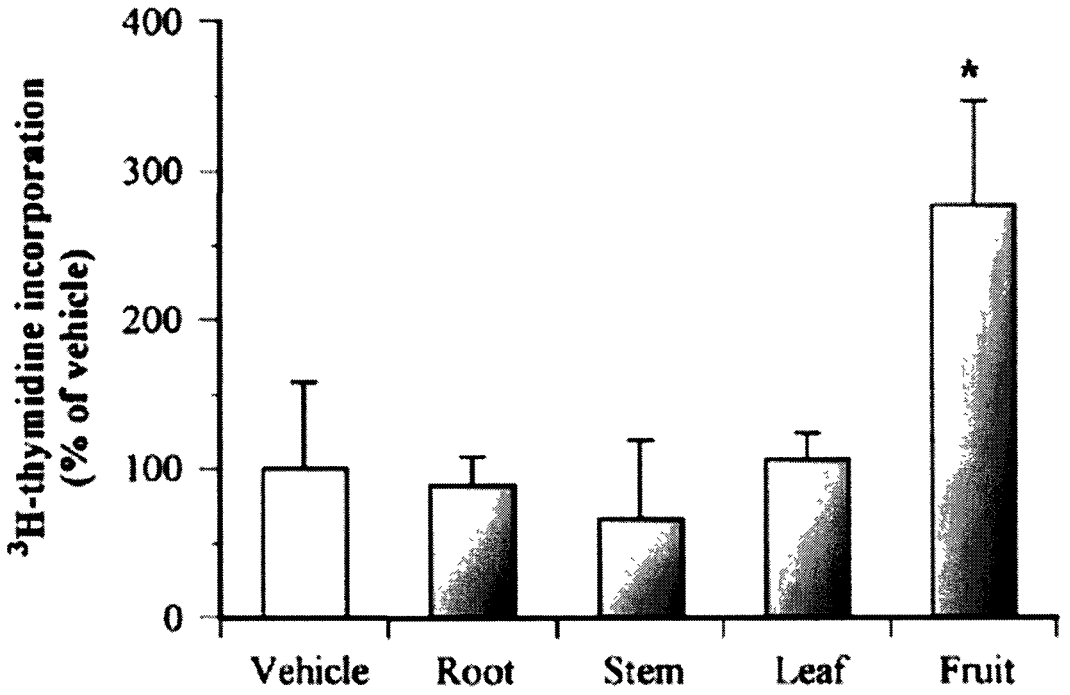

Figure 4.3 – Proliferative effects of *V. angustifolium* extracts on pancreatic β cells in replicating β TC-tet cells. Cells were treated with 12.5 µg/ml of root, stem, leaf, or fruit extracts or with vehicle only (0.1% DMSO) for 24 h. A measure of 1 µCi/ml of ^{3}H-thymidine was added for the final 6 h of treatment and incorporated radioactivity was quantified. Non-specific incorporation (incorporation into growth-arrested cells) was subtracted and results are expressed relative to vehicle treatment. Means + SEM are presented for four replicates. * indicates a significant difference from vehicle only (p?0.05).

contrast microscopy techniques in a small percentage of cells exposed to vehicle only while nearly all cells exposed to 10 μM rosiglitazone contained visible lipid droplets. Rosiglitazone treatment resulted in a 6.8-fold enhancement of lipid accumulation, as compared to vehicle only in differentiation medium. Treatment with *V. angustifolium* extracts resulted in 4-fold increases for root and stem extracts and a 6.5-fold increase for leaf extract (Figure 4.4B). Treatment with fruit extract did not result in a significant increase.

Protection against of glucose toxicity in PC12-AC cells

The ability of extracts of *V.angustifolium* to protect against cell death induced by chronically elevated glucose was tested in PC12-AC cells. In this model, cells are incubated in serum-free medium containing 150 mM glucose for 96 h, effectively inducing apoptosis in approximately 40% of cells. PC12-AC cells were incubated under these conditions in the presence or absence of 0.5, 2.5, 10, and 25 μg/ml of extracts in 0.1% DMSO and number of viable cells was quantified by WST-1 tetrazolium salt assay. Stem, leaf and fruit extracts were found to increase the number of viable cells by 20 – 33%, as compared to vehicle in high glucose (Figure 4.5). However, root extract had no effect. While leaf extract was found to only confer protection at 10 μg/ml, stem and fruit extracts exhibited a dose-response effect. Finally, while protection induced by fruit extracts was highest at 25 μg/ml, root and stem extracts were found to be cytotoxic at this concentration.

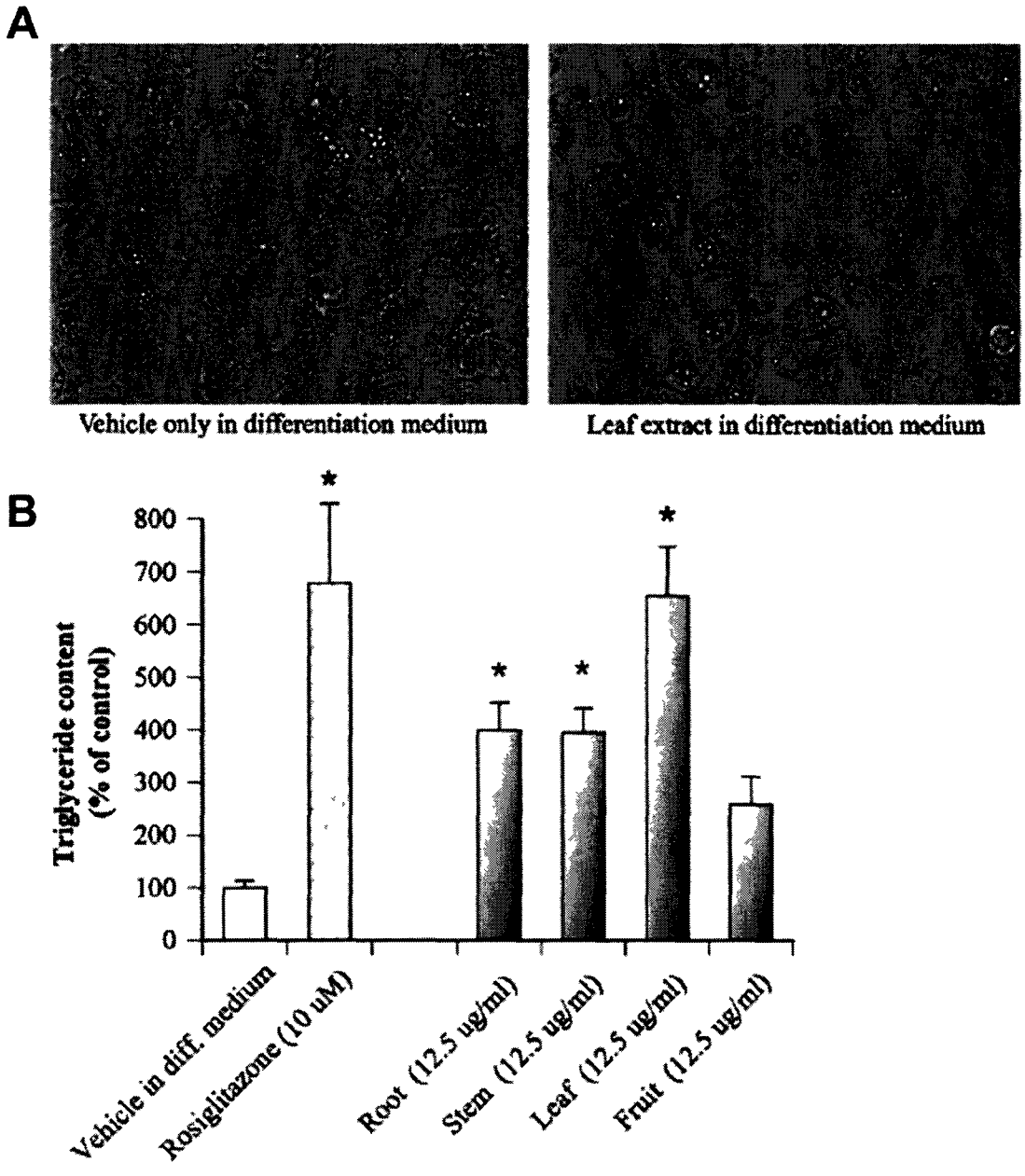

Figure 4.4 – Effects of *V. angustifolium* extracts on lipid accumulation in differentiating 3T3-L1 adipocytes. Cells were treated for 6 days with 12.5 µg/ml of root, stem, leaf, or fruit extracts or with vehicle only (0.1% DMSO in differentiation medium). Rosiglitazone (10 µM) was used as a positive control. (A) Phase-contrast micrograph of vehicle-treated and leaf-treated cells showing lipid droplets at 400x. B) Quantitation of intracellular lipid content in live cells using AdipoRed fluorescent dye. Background (fluorescence in non-differentiated cells treated with proliferation medium only) was subtracted and results expressed relative to the vehicle treated group. Means + SEM are presented for four replicates. * indicates a significant difference from vehicle-only ($p \leq 0.05$).

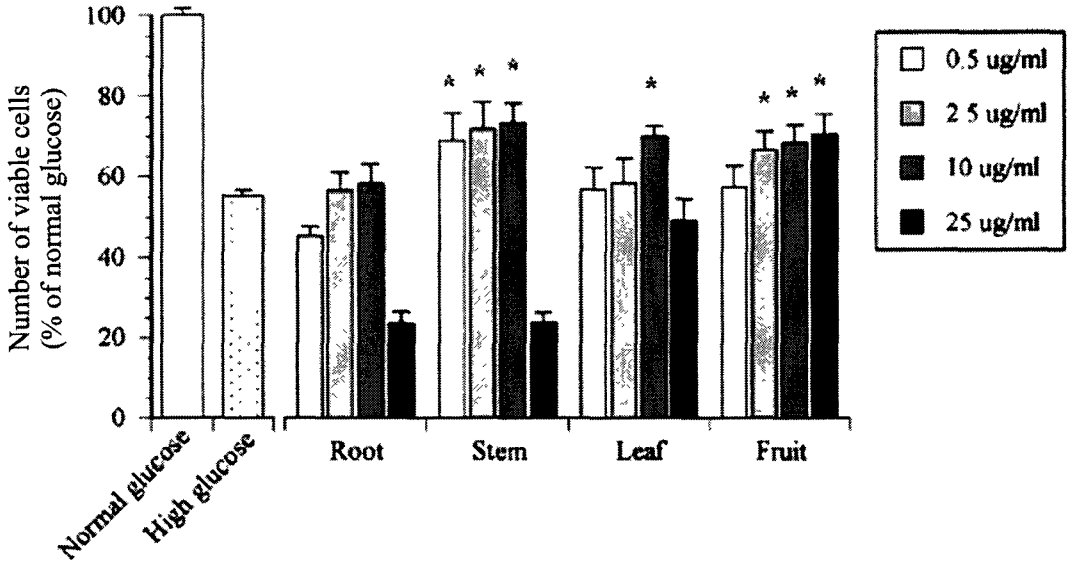

Figure 4.5 – Cytoprotection by *V. angustifolium* extracts against glucose toxicity in PC12-AC cells. Cells were treated with root, stem, leaf, or fruit extracts or with vehicle only (0.1% DMSO) for 96 h in serum-free medium containing 150 mM glucose. These conditions result in a 40% decrease in number of viable cells in control cultures (11 mM glucose). Number of live cells was quantified by WST-1 viability assay. Results are expressed relative to the normal-glucose vehicle-treated group. Means + SEM are presented for eight replicates. * indicates a significant ($p \leq 0.05$) increase over the high-glucose vehicle-treated group.

DISCUSSION

V. angustifolium is reputed to possess anti-diabetic properties (Haddad 2003), and in Canada, extracts of this plant are available as natural health products designed for use as a complementary treatment for diabetes in Canada. While similar claims have been partially validated in other species of the *Vaccinium* genus (Blumenthal 1998; Chambers and Camire 2003), the anti-diabetic potential of *V. angustifolium* has not been subjected to rigorous scientific investigation and only the plant's anti-oxidant properties have been well documented (Kalt et al. 2001; Kay and Holub 2002; Lyons et al. 2003; Rimando et al. 2004; Sweeney et al. 2002; Wang and Jiao 2000). Using multiple cell-based bioassays, the present study has confirmed that *V. angustifolium* possesses insulin-like and glitazone-like activities as well as cytoprotective activities. Furthermore, *V. angustifolium* exhibits potentially anti-diabetic effects in pancreatic β cells.

An important finding of this work is that extracts of *V. angustifolium* possess considerable insulin-like properties, as evidenced by enhancement of insulin-dependent and -independent glucose uptake in cell-based assays. Further work is required to elucidate the mechanism by which this effect is mediated. However, the tissue specific nature of the effect, whereby muscle cells are more greatly affected than adipocytes, combined with the observation that there is no saturation at the highest insulin concentration tested, suggest an insulin-independent mechanism such as an involvement of the AMP-activated protein kinase (AMPK) pathway (Musi and Goodyear 2003). However, the absence of glucose uptake enhancement in muscle following a 1 h incubation with extracts suggests that regulation may occur at the transcriptional or translational level. Alternatively, cellular uptake of the active compound may require facilitative transport and be subject to the kinetics of such a transport mechanism (Strobel et al. 2005).

In addition, *V. angustifolium* extracts possess important glitazone-like activity, as assessed using a screening assay based on 3T3-L1 adipogenesis. While it remains to be confirmed that such glitazone-like activity is mediated through a mechanism involving PPARγ activation, the results from this adipogensis assay suggest PPARγ agonism and potential insulin-sensitization as a result of the upregulation of genes coding for key proteins of glucose transport and metabolism. This will require confirmation using a more direct measurement of PPAR activation, such as a reporter gene assay. While it is difficult to correlate results from the adipogenesis assay with results from the glucose uptake assays due to different time scales, it is possible that PPAR stimulation could account in part for increases in glucose transport observed following 18 h treatment with extracts.

V. angustifolium extracts were found to exert positive effects in pancreatic β cells. The β TC-tet cellular system used here is well suited to distinguish effects on insulin secretion from effects on proliferation. In growth-arrested pancreatic β cells, leaf and stem extracts exerted a subtle anti-diabetic effect on GSIS consisting of both an increase in maximal secretion as well as a leftward shift in the glucose-insulin secretion dose-response curve, without a significant effect on basal secretion. These are highly desirable properties in an anti-diabetic medication, as they minimize the potential for inducing hypoglycaemia, an important concern with sulfonylureas and related insulin secretagogues (Bailey 1999). While the magnitude of this effect is small, it may prove to be more physiologically relevant when the responsible active compounds are purified. Perhaps more important than the effects of leaf and stem extracts on GSIS is the considerable effect of the fruit extract on proliferation of replicating β cells; in light of the existing evidence of β cell mass plasticity in the adult

pancreas (Bouwens and Rooman 2005), such an effect could represent a valuable anti-diabetic activity potentially capable of retarding β cell decompensation in advanced T2DM.

Complications secondary to chronic hyperglycaemia are numerous and include damage to vascular endothelial cells, kidney podocytes, and neural tissue (Ristow 2004; Rojas and Morales 2004; Stas et al. 2004; Wendt et al. 2003). In order to test for anti-diabetic cytoprotection, we chose to use the PC12 cell model and exposed cells to chronically elevated glucose for 4 days. Cell death from hyperglycaemia is thought to be due to oxidative stress, protein glycation, and other insidious mechanisms. In order to accelerate this process so as to achieve a significant amount of cell death in the 96 h experimental, it was necessary to use a supra-physiological concentration of glucose. Nevertheless, *V. angustifolium* extracts exerted a protective effect in PC12 cells under these conditions. While the mechanisms behind these protective effects are unclear, they may occur though a decrease in lipid peroxidative, protein oxidation and/or protein glycation (Kikuchi et al. 2003; McHugh and McHugh 2004), consistent with known anti-oxidant properties of *V. angustifolium* and of flavonoids in general (Wang 2000). *V. angustifolium* has previously been reported to confer neuroprotection in an animal model of ischaemic stroke (Sweeney et al. 2002).

The findings of anti-diabetic properties in cell-based bioassays support the ethnobotanical use of *V. angustifolium* in the context of human T2DM. Nonetheless, these promising *in vitro* results must be ascertained *in vivo* using animal models of insulin resistance and diabetes. The active molecules and mechanisms of action responsible for *V. angustifolium*'s insulin-like, glitazone-like, cytoprotective, and pancreatic effects remain to be identified. However, the observation that different effects are attributable to extracts from different parts of *V. angustifolium* suggests that the plant contains several active molecules

with potential anti-diabetic activity. Since several of these activities are complementary, it may be suitable in the context of diabetes therapy to use the whole plant or a rational combination of various plant parts. Indeed, our results show that significant differences exist in the phytochemical composition of the various parts of the plant. Although it is too early to speculate about active compounds, these differences will guide the future search for the active principles responsible for the various anti-diabetic activities uncovered herein. Our results thus pave the way toward the discovery of novel molecules in various organs of the plant that may contribute to the standardization of preparations of *V. angustifolium* and to the development of novel medications for the treatment of T2DM.

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CHAPTER 5 – EVALUATING CREE MEDICINAL PLANT EXTRACTS IN TWO MODELS OF DIABETIC NEUROPATHY

5.1 PREFACE

Ethnobotanical survey data collected from over 60 Elders in two communties of Eeyou Istchee were compiled and tabulated to provide a syndromic importance value (SIV) for each plant species mentioned during interviews. These SIV scores served to guide the first stage of CIHR-TAAM pharmacological assessment of Cree anti-diabetic plant medicines. Using the same set of *in vitro* assays described in Chapter 4, the 17 most highly ranked species were tested for anti-diabetic activities with the objective of identifying promising samples for further investigation. Using two *in vitro* models of diabetic neuropathy, we sought to identify plant extracts with cytoprotective effects and to investigate possible relationships between observed activity, SIV score, phytochemistry, and/or anti-oxidant capacity. The results have been reported in two manuscripts prepared collaboratively by the CIHR-TAAM (Harbilas unpublished; Spoor et al. 2006) and were included in the theses at the University of Montreal. As such, rather than including both manuscripts, a summary of the results directly related to the current thesis and performed by the author has been provided.

5.2 STATEMENT OF AUTHOR CONTRIBUTIONS

A total of 17 co-authors from the CIHR-TAAM contributed to the project's two screening studies, Spoor et al. (2006) and Harbilas et al. (unpublished). Of the results presented in the current chapter, CSH, JTA, PSH and SALB conceived and designed the

experiments, CSH and JL conducted PC12 cell research. The phytochemical analyses were performed by CSH and AS. As CIHR-TAAM project leader and project co-ordinator, respectively, PSH and LCM have managed and directed the pharmacological assessment of extracts.

5.3 Effects of Cree medicinal plant extracts on PC12 cell viability under conditions of glucose toxicity and glucose deprivation

Authors:

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Publications: This chapter is based primarily on results presented in:

Spoor et al. (2006). Canadian Journal of Physiology and Pharmacology 84: 847-58.
Harbilas et al. (unpublished), under review by the Cree before submission to CJPP.

ABSTRACT

Among the Cree of Eeyou Istchee (CEI) of Northern Quebec, there is a disproportionately high rate of diabetic complications due to the cultural inadequacy of modern type II diabetes therapies. To establish culturally agreeable anti-diabetic treatments, our team identified candidate plant species used by the Elders and Healers of CEI to treat symptoms of diabetes. A subsequent two-part study focusing on 17 medicinal plant species revealed that most possess significant *in vitro* anti-diabetic activity when screened for: 1) potentiation of basal and insulin-stimulated glucose uptake by skeletal muscle cells (C2C12) and adipocytes (3T3-L1); 2) potentiation of glucose-stimulated insulin secretion by pancreatic β cells (βTC); 3) potentiation of adipogenesis in 3T3-L1 cells; 4) protection against glucose toxicity and glucose deprivation in PC12-AC neuronal precursor cells; and 5) diphenylpicrylhydrazyl (DPPH) oxygen free-radical scavenging. While the two-part study provided a summary of results from multiple assays that has already been published (Spoor et al. 2006) or is currently under review by the elders and healers of the CEI for permission to submit to publication (Harbilas et al., unpublished), the purpose of the results presented here was to elaborate on the observed effects of Cree extracts in models of diabetic neuropathy and model possible relationships between observed extract activity, phytochemisty, anti-oxidant capacity and ethnobotanical syndromic importance with respect to diabetic neuropathy. Under conditions of glucose toxicity, nine plant extracts protected PC12-AC cells while three protected cells from glucose deprivation. The only extract effective in both assays was that of *Picea glauca* needles. Few extracts potentiated glucotoxic cell loss but six potentiated death induced by glucose deprivation. Surprisingly, the observed effects on PC12 cells were not correlated with anti-oxidant activity, total phenolic content, or the presence of a particular class of metabolite. These data suggest

additional direct effects on cell survival/death pathways tested in Chapters 9-11. Interestingly, although protection from glucose toxicity did not correlate with syndromic importance values, the most commonly used plant species possessed strong cytoprotective activity, validating both the Traditional Knowledge of the CEI and our novel ethnobotanical approach.

INTRODUCTION

Type II diabetes (T2DM) is a growing global health concern. In Canada, the prevalence of diabetes increased nearly 70% between 1995 and 2005 to approximately 2 million cases, a figure projected to eclipse 3 million by 2030 (WHO 2007). As observed in aboriginal populations around the world, the prevalence of T2DM is even more alarming levels amongst Canadian First Nations, such as the Cree of Eeyou Istchee (CEI) of Northern Quebec. The CEI have an incidence rate that is at least twice as high as the rest of the Canadian population (Brassard et al. 1993; Kuzmina 2004; Légaré 2004). This disparity has been attributed to factors such as recent adoption of a sedentary lifestyle and non-traditional diet (Berkes 1978; Hegele 2001), as well as genetic predisposition towards obesity (Skyler 2004). The impact of diabetes on the CEI is further aggravated by the rate of diabetic complications, which is disproportionately high relative to the rate of T2DM. A contributing factor to increased rates of complications is low compliance with modern drug-based therapies that results in poor management of hyperglycemia within the population. As diabetic complications are becoming a considerable social burden, new more culturally aligned treatment strategies are needed to address this specialized issue (Brassard et al. 1993).

One solution may be found within the rich pharmacopoeia of the Canadian First Nations. Although T2DM is a relatively new disease for this population, products from their own traditional medicine may nonetheless possess anti-diabetic activity. Indeed, anti-hyperglycemic effects and other anti-diabetic activities have been identified in thousands of plant species worldwide (Marles 1995). One such example is *Vaccinium angustifolium*, a medicinal plant widely used in Quebec for treating symptoms of T2DM (Haddad 2003). When tested *in vitro,* extracts of *V. angustifolium* demonstrated insulinotropic activity,

enhancing deoxyglycose transport in myotubes and adipocytes, increased lipid accumulation in adipocytes, and provided cytoprotection against glucose toxicity (Maritneau et al., 2007, Chapter 4). To identify anti-diabetic plant species that are used by the Cree, our team conducted ethnobotanical interviews focusing on traditional medicines used to treat 15 symptoms associated with diabetes. Through this approach, over 35 plant species were identified by Elders and Healers from two Cree communities in Northern Quebec, the Cree Nations of Mistissini (Leduc et al. 2006) and Whapmagoostui (Fraser et al., unpublished). Though many plants were employed in both communities, some species were unique to either Mistissini or Whapmagoostui. The ethnobotanical data from the two studies were combined and identified species were assigned a syndromic importance value (SIV) based on their frequency of citation by informants and the number and specificity of symptoms for which they are used. From this list, 17 species were selected for testing in a series of in vitro bioassays targeting different anti-diabetic effects. The results of this evaluation, reported in two related studies by Spoor et al. (2006) and Harbilas et al. (unpublished) revealed that most species possess significant anti-diabetic activity worthy of further investigation.

To elaborate on some of the results summarized in the two-part screening study conducted in collaboration with multiple TAAM members, the present study provides a more comprehensive analysis of the effects of Cree extracts in models of diabetic peripheral neuropathy generated as part of this thesis including data not presented in the two manuscripts published or under review. The most common type of diabetic peripheral neuropathy, distal symmetric sensorimotor polyneuropathy, occurs to some degree in approximately 50% of all people with diabetes, is characterized by progressive loss of peripheral sensation, pain, and often leads to reduced mobility, unhealing wounds and infections that may ultimately result in amputation of the toes and feet (Dyck et al. 1993).

While the pathophysiology of diabetic neuropathy remains unclear, hyperglycemia and vascular damage are well-documented contributing factors. In addition to direct toxic effects on peripheral neurons (Schmeichel et al. 2003; Vincent et al. 2005a), high levels of glucose elicit indirect effects such as oxidative stress and formation of advanced glycation endproducts that contribute to vascular impairment and subsequent deprivation of glucose, oxygen and neurotrophic factors (Sheetz and King 2002; Sima 2006; Sugimoto et al. 2000). As such, the Cree plant extracts were assessed for cytoprotective activity in two paradigms, glucose toxicity and glucose deprivation in the absence of growth factors. As a peripheral cell line capable of differentiating into a neuronal phenotype, PC12-AC cells, a clonal derivate the rat pheochromocytoma PC12 cell line, were employed as our model system. This system has been used repeatedly by us and others as an in vitro model of diabetic neuropathy in the context of glucotoxicity and glucose deprivation (Koshimura et al. 2002; Liu et al. 2003; Martineau et al. 2006).

Phytochemical analyses were also conducted on all extracts focusing on phenolic metabolites. Total phenolic content was determined using the Folin-Ciocalteau method and the classes of phenolic metabolites present, along with potential marker coumpounds, were determined using high pressure liquid chromatography (HPLC) and mass spectroscopy (MS). To gain insight into possible active constituents and mechanism of action, the observed effects in PC12 cells were considered relative to the phytochemical profiles and anti-oxidant capacities of each extract. As a final step, we evaluated our ethnobotanical approach by investigating the relationship between cytoprotective activity and SIV score.

EXPERIMENTAL

Reagents

All cell culture reagents were obtained from Invitrogen (Burlington, ON Canada) and all chemicals were purchased through Sigma-Aldrich (St. Louis, USA) unless otherwise stated.

Plant material and preparation

The 17 Cree medicinal plant species identified by Cree Elders as relevant to the treatment of symptoms associated with diabetes are listed in Table 1 with the specific organ part tested, in accordance with the ethnobotanical data. The species *Abies balsamea, Alnus incana* ssp. *rugosa, Larix laricina, Picea mariana, Pinus banksiana, Rhododendron groenlandicum, Sarracenia purpurea,* and *Sorbus decora* were the object of a first screening study (Spoor et al. 2006) while *Gaultheria hispidula, Juniperus communis, Kalmia angustifolia, Lycopodium clavatum, Picea glauca, Populus balsamifera, Rhododendron tomentosum, Salix planifolia,* and *Vaccinium vitis-idaea* were the object of the second (Harbilas et al. unpublished). Specimens were collected in a culturally respectful manner in the regions of Mistissini and Whapmagoostui of Northern Quebec, Canada. Dr Alain Cuerrier, taxonomist at the Montreal Botanical Garden, ascertained botanical identity of the plant species, and voucher specimens were deposited in the Marie-Victorin herbarium of the Montreal Botanical Garden in Montreal, Quebec, Canada. The collected plant samples were air dried then sent to the University of Ottawa to be cleaned and separated into plant parts as required. Plant materials were ground using a Wiley Mill (Arthur H. Thomas Co., Swedesboro, N.J.) with a 2 mm filter, and extracted twice in 80 % ethanol (10 mL/g dry material) for 24 h on a mechanical shaker then centrifuged at 1000 g for 15 min at room

temperature before filtering through Whatman paper Number 1. The supernatants were pooled, dried *in vacuo* by rotary evaporation and lyophilization. Dried extracts were conserved in darkness at 4 °C. For cell culture experiments, stock solutions of each extract were prepared in DMSO and serially diluted prior to use to ensure a constant vehicle concentration of 0.1%.

Syndromic importance values

With the help of Cree translators, interviews followed a semi-structured format focusing on traditional medicines used to treat 15 symptoms related to diabetes. Interviews were conducted in two Cree communities, Mistissini and Whapmagoostui, by C. Leduc and M.H. Fraser, respectively. The SIV score for each species was calculated based on frequency of citation during interviews and number and importance of symptoms for which they were cited. Leduc et al. (2006) provides a complete description of ethnobotanical data collection and analysis.

Assessment of total phenolic content

Total phenolics (TPH) were measured by the Folin-Ciocalteau method (Singleton 1965) modified to reduce volumes as previously described (Farsi et al. 2007). Briefly, solubilized extract (1 mg/ml 80% ethanol) was added to freshly diluted Folin-Ciocalteau reagent (BDH, Toronto, Ont.) in clear-bottom 96-well plates (Fisher Scientific, Canada) allowing 5 min for the reaction to proceed to equilibrium for before adding a solution of 7.5 % anhydrous NaHCO3. After 2 h additional incubation at room temperature, the absorbance of the mixture was measured at 725 nm with a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, California). Experiments were conducted in quadruplicate

on three separate occasions. Quercetin (Sigma, Oakville, Canada) was used as a standard with results expressed as μg of quercetin equivalents per mg of extract.

Phytochemical characterization

Extracts were analyzed by high performance liquid chromatography (HPLC) using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) with an autosampler, a photodiode array detector (PAD) and an atmospheric pressure chemical ionization quadrupole mass selective spectrometer (APCI/MS), mass range 50-1500 amu. Extracts of *A. balsamea, A. incana ssp. rugosa, J. communis, L. laricina, L. clavatum, P. glauca, P. mariana, P. banksiana, P. balsamifera, S. purpurea, S. decora, S. planifolia* were separated on a YMC ODS-AM narrow bore column, 100 × 2 mm I.D., 3 micron particle size (Waters, Mississauga, Canada) using methods described by Harris et al. (2007, Chapter 2). Extracts of Ericaceae family members *G. hispidula, K. angustifolia, R. groenlandicum, R. tomentosum* and *V. vitis-idaea* were analyzed on a a Synergi Polar RP-C18 column, 150 × 3.0 mm I.D., 4 micron particle size, (Phenomenex, CA, USA) using methods described in Saleem et al. (unpublished, Chapter 3). Marker compounds were identified by comparing unknown UV spectra to a metabolomic library of over 200 purified phytochemicals using Agilent Chemstation software (version B.03.01) (Harris et al. 2007a) (Chapter 2). Identifications were further corroborated by comparing the fragmentation patterns with reference compounds and performing co-chromatography.

Cell culture, glucose toxicity, and glucose deprivation conditions

PC12-AC cells, a clonal derivate (Brewer et al. 2002) of the PC12 rat adrenal pheochromocytoma cell line (American Tissue Culture Collection), were maintained in

Roswell Park Memorial Institute medium (RPMI 1640) containing 10% horse serum and 5% newborn calf serum under normoglucose (11 mM) conditions. Prior to experimentation, cells were seeded in 96-well plates ($6.25x10^3$ cells/well) and allowed to adhere at 37°C in 5% CO_2 overnight. To establish LD_{50} concentrations for each extract, cultures were treated for 96 h in serum-free RPMI medium at a glucose concentration of 11 mM with 0.1% DMSO (vehicle control) and varying concentrations of plant extract (0-100 μg/ml). The glucose toxicity assay was performed as previously described (Martineau *et al.* 2006) (Chapter 4), replacing serum containing RPMI from seeded cultures with serum-free RPMI supplemented with 150 mM glucose and 0.025% BSA for 96 h of treatment. To elicit glucose deprivation, cells were treated for 96 h with glucose-free RPMI media adjusted to 1 mM glucose, lacking serum and supplemented with 0.025% BSA. Cultures under glucose toxicity or deprivation were co-treated with various concentrations of plant extract below their respective LD_{50}'s to assess for cytoprotective activity.

Following 96 h of treatment in normoglucose, high glucose, or low glucose conditions, viable cell number was determined by a modified WST-1 viability assay (Cell Proliferation Reagent; Roche, Laval, QC) (Martineau et al., 2006, Chapter 4). All experiments were performed in quadruplicate on at least two separate occasions (n = 8-12). Data from control cultures in normoglucose, high, and low glucose treated with 0.1% DMSO were combined across plates (n = 60-64). Percent viability was calculated as follows:

$$\% \text{ viability} = \text{cell number}_{(\text{treatment well})} / \text{mean cell number}_{(\text{normoglucose control})} \times 100$$

Statistical analyses

Cell viability data were analyzed using one-way factorial ANOVA tests followed by *post hoc* Dunnett's *t*-tests on each extract relative to applicable controls. *P* values under 0.05

were considered statistically significant (shown as * or #); *P* values under 0.01 were considered highly significant (shown as ** or ##).

Using these data, each plant was ranked according to their 1) LD_{50} concentrations, 2) cytoprotection under glucose toxicity, and 3) cytoprotection under glucose deprivation. Spearman nonparametric correlation analyses were employed to test associations between effects in PC12 cells, SIV scores, phenolic content, and anti-oxidant activities as reported by Fraser et al. (2007). All statistical analyses were performed using GraphPad InStat version 3.00 (GraphPad Software Inc., San Diego CA).

RESULTS

Based on the SIV scores calculated from ethnobotanical data collected during interviews with the Elders and Healers of CEI, 17 medicinal plant species representing eight plant families were selected for study (Table 5.1). For each species, the organ(s) most commonly mentioned by informants (Table 5.1) were collected and ethanolic extracts were prepared for experimental analyses. Extraction yields ranged from 8.9% to 74.5% of raw material dry weight for *S. decora* and *V. vitis-idaea* respectively (Table 5.2). Assessment of total phenolic content (TPH) similarly revealed large differences between extracts with the highest levels occurring in *P. banksiana* (318 ug of quercetin equivalents per mg of extract), which was more than 20-times more concentrated than the than extract of *L. clavatum* (Table 5.2).

To identify the classes of phenolic metabolites and marker compounds present in each Cree plant extracts, the HPLC-PAD/APCI-MS metabolomics approach applied in Section 1 (Harris et al. 2007a) was again employed. A large diversity of phenolic derivatives was observed, with certain classes common to different families and plant parts and other

Table 5.1 – List of medicinal plant species used by the CEI for the treatment of symptoms of diabetes.

Species	Abbreviation	Common name	Cree name	Family	Plant part	Source[1]	SIV (ranking)
Abies balsamea (L.) Mill.	*A. balsamea*	Balsam fir	Inaast	Pinaceae	Bark	M	0.254 (8)
Alnus incana ssp. rugosa (Du Roi) R.T. Clausen	*A. incana*	Speckled alder	Atuuspiih	Betulaceae	Bark	M	0.163 (11)
Gaultheria hispidula (L.) Muhl.	*G. hispidula*	Creeping snowberry	Piyeumanaan	Ericaceae	Leaf*	M	0.040 (17)
Juniperus communis L.	*J. communis*	Ground juniper	Kaakaachuminatukw	Cupressaceae	Fruit	W	0.353 (4)
Kalmia angustifolia L.	*K. angustifolia*	Sheep laurel	Uischichipukw	Ericaceae	Leaf	M & W	0.247 (9)
Larix laricinia Du Roi (K. Koch)	*L. laricinia*	Tamarack	Waatinaakan	Pinaceae	Bark	M & W	0.409 (3)
Lycopodium clavatum L.	*L. clavatum*	Common clubmoss	Pastinaakwaakin	Lycopodiaceae	Whole plant	M	0.087 (15)
Picea glauca (Moench.) Voss	*P. glauca*	White spruce	Miinhikw	Pinaceae	Needle	M & W	0.275 (6)
Picea mariana (P. Mill) BSP	*P. mariana*	Black spruce	Iinaatikw	Pinaceae	Cone	M & W	0.347 (5)
Pinus banksiana Lamb.	*P. banksiana*	Jack pine	Uschisk	Pinaceae	Cone	M & W	0.088 (14)
Populus balsamifera L.	*P. balsamifera*	Balsam poplar	Mash-mitush	Salicaceae	Bark	M	0.049 (16)
Rhododendron groenlandicum (Oeder) Kron & Judd	*R. groenlandicum*	Labrador tea	Kaachepukw	Ericaceae	Leaf	M & W	0.507 (2)
Rhododendron tomentosum ssp. *subarcticum* (Harjama) G. Wallace	*R. tomentosum*	Northern Labrador tea	Wiisichipukw	Ericaceae	Leaf	W	0.656 (1)
Salix planifolia Pursh	*S. planifolia*	Tealeaf willow	Piyeuwaatikw	Salicaceae	Bark	M & W	0.188 (10)
Sarracenia purpurea L.	*S. purpurea*	Pitcher plant	Aygadash	Sarraceniaceae	Leaf	M	0.092 (13)
Sorbus decora (Sarg.) C.K. Schneid.	*S. decora*	Showy mountain ash	Muskumanaatikw	Rosaceae	Bark	M & W	0.262 (7)
Vaccinium vitis-idaea L.	*V. vitis-idaea*	Bog cranberry	Wiishichimanaanh	Ericaceae	Fruit	M & W	0.112 (12)

M = Mistissini; W = Whapmagoostui; SIV = Syndromic importance value

* Berries not available

[1] Source(s) of ethnobotanical plant use

Table 5.2 – Phytochemical characterization of extracts.

Species (part)	Collection site	Extraction yield [1]	Total phenolic content [2]	Classes of phenolic metabolites (others)	Identified phenolic marker compounds
A. balsamea (bark)	M	15.3 %	97.6 ± 1.9	Procyanidins (saponins)	Gallocatechin derivatives
A. incana (bark)	M	26.1 %	305.9 ± 5.4	Diarylheptanoid derivatives	Oregonins, Rubranoside A & B, hirsutanone
G. hispidula (leaf)	M	33.5 %	132.8 ± 4.7	PAs, procyanidins , flavonones, flavonols	Chlorogenic acid, catechin, epicatechin, taxifolin glycoside, myricitrin, quercetin glycosides (rut, glu, gal, ara & rham)
J. communis (fruit)	W	46.4 %	98.5 ± 1.7	Procyanidins, flavonols	Catechin, 2 kaempferol glycosides, quercetin glycosides (gal, glu & ara)
K. angustifolia (leaf)	M	41.8 %	268.5 ± 5.7	PAs, procyanidins, flavonols	Catechin, epicatechin, A-type procyanidin dimer, myricetin, quercetin glycosides (gal, ara & rham)
L. laricinia (bark)	M	23.8 %	208.0 ± 4.7	Procyanidins, stilbenes	Catechin, epicatechin, 5 piceatannol-like hydroxystilbenes
L. clavatum (whole)	M	18.8 %	14.0 ± 1.9	PAs, flavonones	Ferulic acid derivatives, apigenin derivatives
P. glauca (needle)	W	30.2 %	109.1 ± 3.1	Procyanidins, flavonones, flavonols, stilbenes	Catechin, taxifolin, kaempferol-, quercetin- and isorhamnetin glycosides, 2 piceatannol-like hydroxystilbenes
P. mariana (cone)	M	21.0 %	163.7 ± 2.2	Stilbenes	4 piceatannol-like hydroxystilbenes
P. banksiana (cone)	M	9.0 %	318.0 ± 5.7	Procyanidins, flavonones	Catechin, B-type procyanidin, taxifolin
P. balsamifera (bark)	M	26.5 %	94.5 ± 7.7	PA glycosides	Salicin, salicortins, salireposide, populoside
R. groenlandicum (leaf)	M	31.0 %	188.5 ± 1.9	PAs, procyanidins, flavonols	Chlorogenic acid, catechin, epicatechin, procyanidin B2, quercetin glycosides (glu, gal, ara & rham)
R. tomentosum (leaf)	W	31.2 %	153.5 ± 6.4	PAs, procyanidins, flavonones, flavonols	Chlorogenic acid, catechin, epicatechin, taxifolin glycoside, quercetin glycosides (glu, gal & ara)
S. planifolia (bark)	W	35.0 %	240.3 ± 6.5	PA glycosides	Salicin, isosalireposide derivatives, tremulacin
S. purpurea (leaf)	M	25.2 %	85.4 ± 1.9	Flavonols, anthocyanins	Kaempferol, quercetin and cyaniding glycosides
S. decora (bark)	M	8.9 %	59.6 ± 2.9	Procyanidins (triterpenes)	Epicatechin, hydroxybetulin derivatives
V. vitis-idaea (fruit)	M	74.5 %	59.9 ± 4.2	PAs, procyanidins, flavonols, anthocyanins	p-coumaric acid, catechin, quercetin glycosides (gal & ara) and cyanidin glycosides (gal, glu & ara)

M = Mistissini; W = Whapmagoostui; Pas= phenolic acid derivatives; gal = galactoside; glu = glucosides; ara = arabinoside; rham = rhamnoside; rut = rutinoside

[1] Yield is expressed as (mass of recovered extract / mass dry plant material) X 100%

[2] Total phenolics expressed as quercetin equivalents (μg) / mg extract

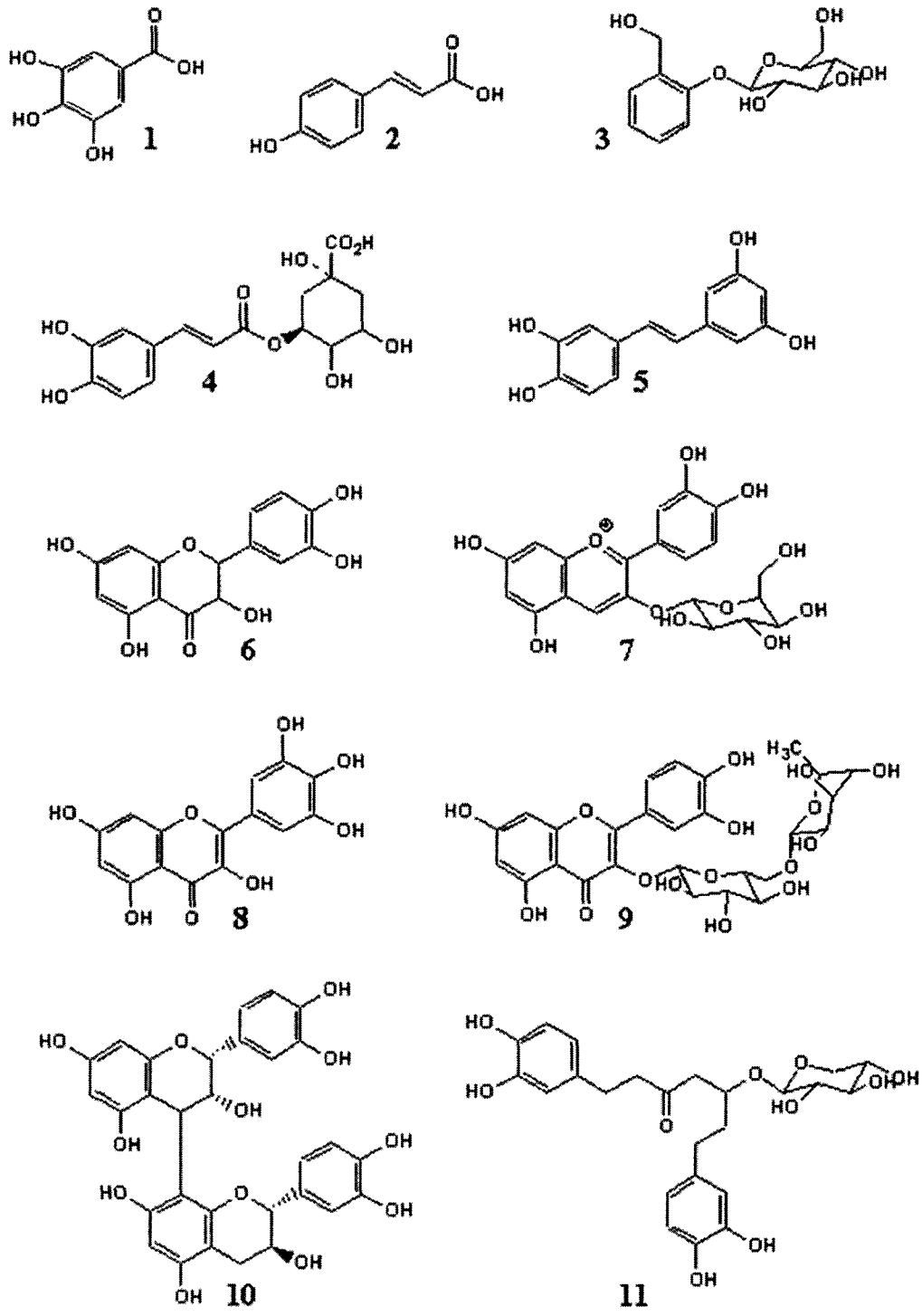

Figure 5.1 – Chemical structures of representative compounds from the classes of phenolic metabolites identified in Cree plant extracts. The phenolic acid derivatives (**1**) gallic acid, (**2**) para-coumaric acid, (**3**) salicin, and (**4**) chlorogenic acid, the stilbene (**5**) piceatannol, the flavonoids (**6**) taxifolin, (**7**) cyaniding-3-*O*-glucoside, (**8**) myricetin, and (**9**) rutin, (**10**) procyanidin B1 and the diarylheptanoid (**11**) oregonin are illustrated (ChemDraw Pro version 8.0).

classes restricted to specific families (Table 5.2, Figure 5.1). Of former group, procyanidins, including monomeric procyanidins catechin and epicatechin (also known as flavan-3-ols) and both A-type and B-type dimers, were identified in 11 extracts while flavonols, mainly quercetin glycosides, were observed in eight extracts, flavonones in five and anthocyanins, namely cyanidin glycosides, in two (Table 5.2). Although the majority of extracts contained phenolic acid derivatives, different sub-classes were limited to certain families. For instance, salicylic acid derivatives were only detected in Salicaceous species (*P. balsamifera* and *S. planifolia*) and ferulic acid derivatives were only detected in *L. clavatum* (Table 5.2). Diarylheptanoid derivatives were exclusively identified in *A. incana* and have been reported in a number of other *Alnus* species (Gonzalez-laredo 1999; Lee et al. 2005c). Hydroxylated stilbenes were observed in three of the five Pinaceae extracts but no others and, similarly, chlorogenic acid was solely found in the leaves of Ericaceous species.

Prior to testing in PC12 cell models of diabetic neuropathy, the toxicity of each extract was determined by culturing cells in serum-free, normoglucose conditions for 96 h in the presence of increasing concentrations (3-100 μg/ml) of extract. Following treatment, viable cell number was determined spectrophotometrically based on mitochondrial dehydrogenase cleavage of WST, a formazan dye, relative to concomitantly analyzed cultures of known cell density. LD_{50} concentrations, defined as the amount of extract (μg/ml) that reduced viable cell number by 50% relative to vehicle-treated control cultures, were established to reveal that the majority of extracts were well tolerated by PC12 cells over the treatment period. Although the LD_{50} concentrations of *J. communis*, *P. mariana* and *S. decora* extracts were below 10 μg/ml, those of the remaining extracts exceeded 30 μg/ml (Table 5.3). From these data, a suitable range of non-toxic concentrations was identified for

Table 5.3 – Cytotoxicity of Cree medicinal plant extracts.

Species (part)	LD_{50} (μg/ml)
A. balsamea (bark)	37.8
A. incana (bark)	43.2
G. hispidula (leaf)	60.1
J. communis (fruit)	< 3
K. angustifolia (leaf)	> 100
L. laricinia (bark)	49.3
L. clavatum (whole)	36.6
P. glauca (needle)	> 100
P. mariana (cone)	8.0
P. banksiana (cone)	36.6
P. balsamifera (bark)	81.2
R. groenlandicum (leaf)	30.8
R. tomentosum (leaf)	61.0
S. planifolia (bark)	74.3
S. purpurea (leaf)	> 100
S. decora (bark)	3.5
V. vitis-idaea (fruit)	> 100

further experimentation with each extract.

The first model of diabetic neuropathy was designed to simulate cellular stress associated with chronic hyperglycemia and direct glucose toxicity. As reported previously by us and others (Koshimura et al. 2002; Martineau et al. 2006), 96 h of exposure to high glucose (150 mM) in serum-free conditions resulted in a loss of viable PC12 cells by approximately 50% relative to vehicle-treated normoglucose control cultures (Figure 5.2A). To evaluate the effects of Cree medicinal plant extracts in this model, extracts were administered at concentrations approaching but not exceeding their LD_{50} concentrations established under normoglucose conditions. Of the 17 extracts, nine significantly reduced high glucose-induced cell death with *P. glauca, R. groenlandicum, R. tomentosum, S. purpurea* and *S. decora* fully restoring viability (statistically similar to normoglucose controls) and *A. balsamea, L. laricina, P. mariana* and *P. balsamifera* providing partial protection (statistically greater than high glucose controls) (Figure 5.2B). Two extracts, *G. hispidula* and *S. planifolia*, did not alter cell number at any concentration (Figure 5.3A) while the remaining extracts had no effect at low concentrations but intensified death at higher concentrations (Figure 3B). *J. communis* was toxic at all tested concentrations (Figure 5.3).

The second model of diabetic neuropathy, glucose deprivation, was selected to represent neuropathic damage secondary to microvascular impairment. Although total glucose deprivation, which resulted in > 90% death of PC12 cells over the treatment period (data not shown), may occur with vascular damage, it is usually preceded by reduced blood flow and nutrients. To model this earlier stage, cells were cultured for 96 h in low glucose (1 mM) serum-free media. This less severe glucose deprivation caused a 40% reduction in viable cell number relative to normoglucose (11 mM) control cultures (Figure 5.3). Upon

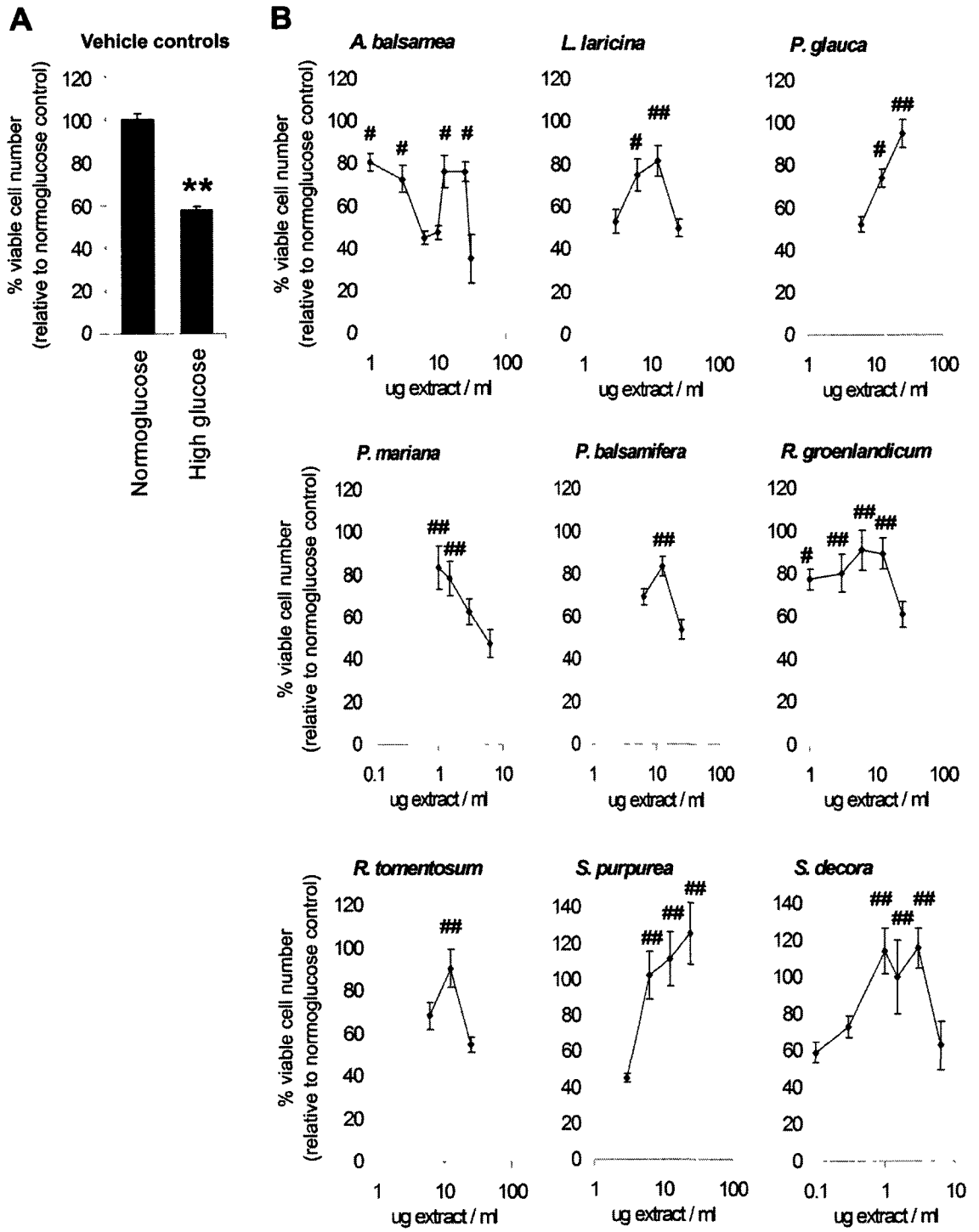

Figure 5.2 – Cree plant extracts that significantly protected PC12 cells from glucose toxicity. (Complete caption on following page).

Figure 5.2 – Cree plant extracts that significantly protected PC12 cells from glucose toxicity. The viability of vehicle-treated cultures in high glucose conditions was significantly reduced by 43% relative to cultures grown under normoglucose conditions (left panel, ANOVA, post-hoc Dunnett's *t*-test relative to vehicle-treated normoglucose control, ** $p < 0.01$). Nine extracts significantly reduced high glucose-induced cell death (ANOVA, post-hoc Dunnett's *t*-test relative to vehicle-treated high glucose control, # $p < 0.05$; ## $p < 0.01$). The maximum viable cell numbers of cultures treated with *P. glauca, R. groenlandicum, R. tomentosum, S. purpurea and S. decora* were statistically similar to normoglucose controls whereas those of the remaining extracts were significantly lower (ANOVA, post-hoc Dunnett's *t*-test relative to vehicle-treated normoglucose control, symbols not shown). Data are reported as mean + SEM (n = 8-64). Extracts were tested at concentrations below their LD_{50} values as established under normoglucose conditions.

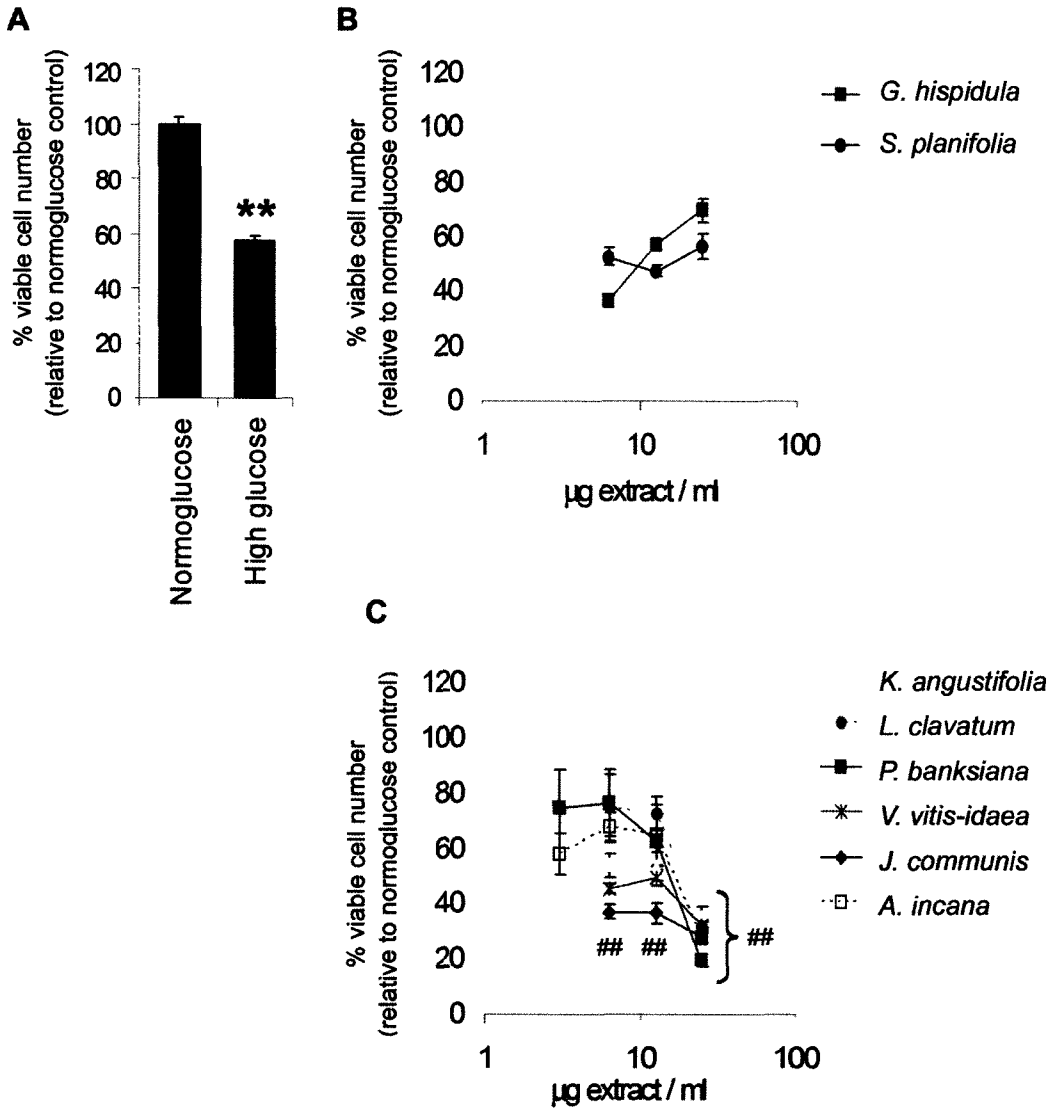

Figure 5.3 – Cree plant extracts that do not protect PC12 cells from glucose toxicity. (A) The viability of vehicle-treated cultures in high glucose conditions was significantly reduced by 43% relative to cultures grown under normoglucose conditions (left panel, ANOVA, post-hoc Dunnett's *t*-test relative to vehicle-treated normoglucose control, ** $p < 0.01$). (B) Extracts that did not alter viable cell number at any concentration. (C) Extracts that intensified high glucose-induced cell loss (ANOVA, post-hoc Dunnett's *t*-test relative to vehicle-treated high glucose control, # $p < 0.05$; ## $p < 0.01$). Data are reported as mean + SEM (n = 8-64). Extracts were tested at concentrations below their LD_{50} concentrations established under normoglucose conditions.

administration to PC12 cells, three Cree extracts, *G. hispidula, P. glauca* and *S. planifolia,* significantly reduced glucose deprivation-induced death (Figure 5.4A). *R. tomentosum* extract displayed an insignificant concentration-dependent trend toward protection and extracts of *L. laricina, L. clavatum* and *P. mariana* produced no change relative to vehicle-treated controls (Figure 5.4B). As seen in the high glucose model, some extracts had no effect at low concentrations but exacerbated cell loss at high concentrations. These were *K. angustifolia, P. balsamifera, R. groenlandicum* and *V. vitis-idaea* (Figure 5.4C). The remaining six extracts elicited toxic effects, intensifying death at concentrations well below their respective LD_{50} values (Figure 5.4D).

Correlation analyses of the observed effects of each extract on PC12 cells cultured under normal, high and low glucose conditions revealed no clear relationship with extract TPH. When comparing the detected classes of phenolic metabolites to observed effects, few trends could be deciphered as most classes were detected in extracts that yielded distinct results. For example, although four of the most protective extracts in the glucose toxicity model contained quercetin glycosides, these metabolites were also detected in extracts that had no activity. The presence of stilbenes may represent an exception since all three extracts containing these metabolites significantly reduced cell loss elicited by glucose toxicity. To further investigate possible factors contributing to bioactivities, effects in PC12 cells were analyzed relative to the anti-oxidant properties of each extract as reported by Fraser et al. (2007) from a parallel study conducted by using the same set of plant extracts. As observed with TPH, no statistically significant relationships were detected. SIV values likewise showed no significant correlation with LD_{50} concentrations or cytoprotection in either model of diabetic neuropathy.

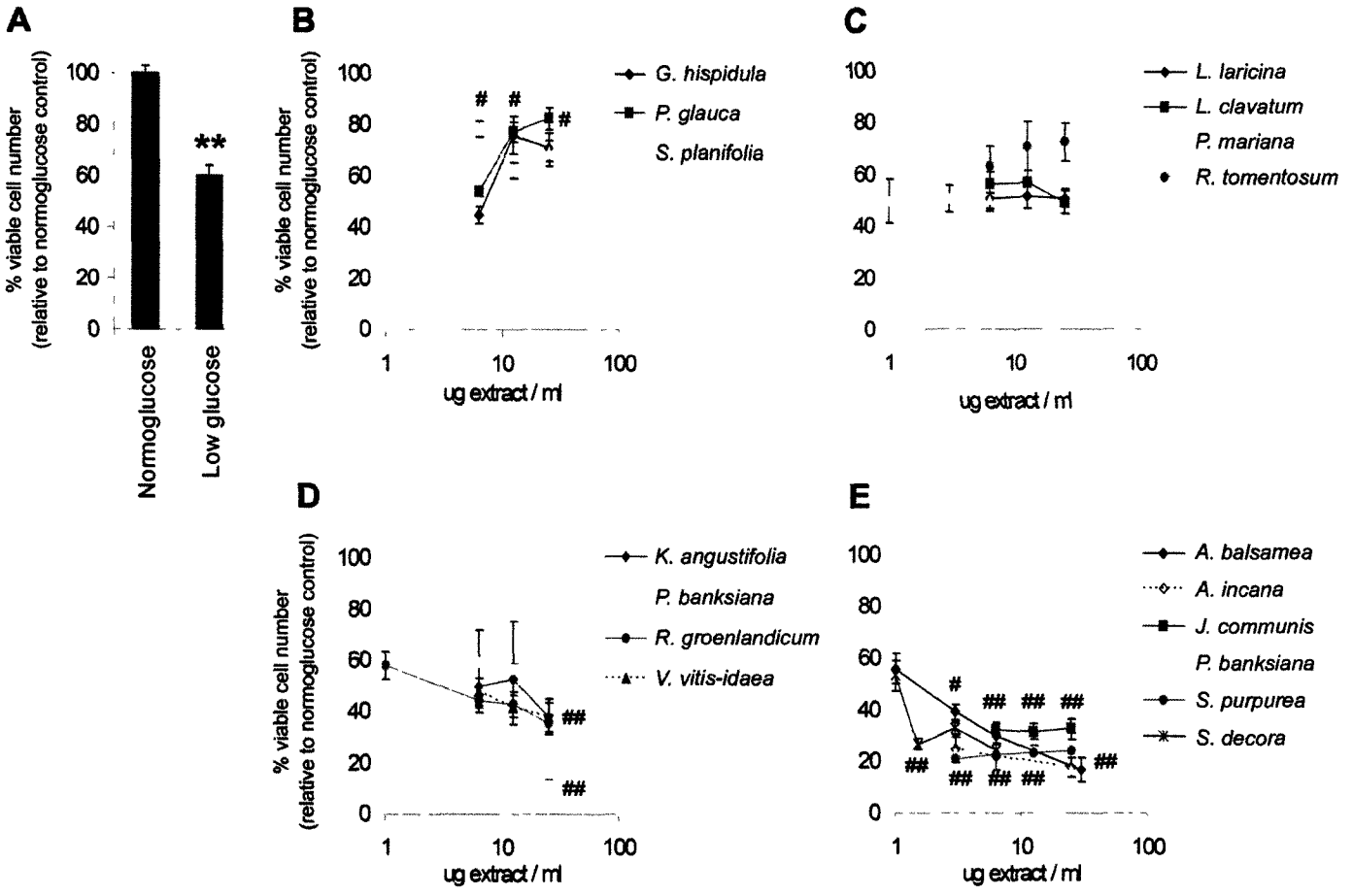

Figure 5.4 – Effects of Cree plant extracts on PC12 cells exposed to glucose deprivation. (Complete caption on following page).

Figure 5.4 – Effects of Cree plant extracts on PC12 cells exposed to glucose deprivation. (A) The viability of vehicle-treated cultures under glucose deprivation was significantly reduced by 40% relative to cultures grown under normoglucose conditions (left panel, ANOVA, *post-hoc* Dunnett's *t*-test relative to vehicle-treated normoglucose control, ** $p < 0.01$). (B) Extracts that significantly reduced low glucose-induced cell death (ANOVA, *post-hoc* Dunnett's *t*-test relative to vehicle-treated high glucose control, # $p < 0.05$; ## $p < 0.01$). (C) Extracts that did not alter viable cell number at any concentration. (D) Extracts that intensified low glucose-induced cell loss only at high concentrations. (E) Extracts that significantly exacerbated cell death at concentrations well below their LD_{50} concentrations (ANOVA, *post-hoc* Dunnett's *t*-test relative to vehicle-treated low glucose control, # $p < 0.05$; ## $p < 0.01$). Data are reported as mean $\pm$ standard error of measurement (SEM) (n = 8-64). Extracts were tested at concentrations below their LD_{50} concentrations established under normoglucose conditions.

DISCUSSION

The current chapter summarizes and elaborates upon the phytochemical characterizations and cytoprotective activities of 17 Cree medicinal plant extracts as reported in two sequential screening studies by Spoor et al. (2006) and Harbilas et al. (unpublished). Though both studies tested extracts in the same PC12 models of glucose toxicity and glucose deprivation, the data were expressed differently in each and the first study (set of 8 extracts) assessed a single concentration while the second used a range of concentrations. To provide a more comprehensive data set allowing comparison across all extracts, the first set was re-evaluated alongside the second set using identical protocols. These results, though largely congruent with those reported by Spoor et al., revealed some inconsistencies that will be discussed further but can be partially attributed to the use of more stringent statistical methods in the current study. When administered to PC12 cells under normoglucose conditions in the absence of serum, the majority of extracts were well tolerated beyond 25 μg/ml, which is likely higher than physiological concentrations attainable through consumption or external application. Since each of the classes of phenolics identified within the entire collection are represented by the four extracts exhibiting LD_{50} concentrations above 100 μg/ml (Table 5.3), it can be assumed that all are relatively non-toxic (i.e., though individual class members may have toxic effects, toxicity is not a general property of the class). Moreover, the toxicities of the two extracts with lowest LD_{50} concentrations are likely due to the presence of non-phenolic metabolites with known cytotoxic effects, thujone in *J. communis* (Millet et al. 1981) and hydroxybetulin derivatives in *S. decora* (unpublished data).

Here, we report that nine of the 17 extracts recommended by the healers and elders of the CEI provided varying degrees of protection to PC12 cells exposed to glucose toxicity. While the extracts of *P. glauca*, *R. groenlandicum* and *S. purpurea* completely reversed high glucose-induced death over a wide range of concentrations, those of *P. mariana* and *S. decora*, both possessing low LD_{50} concentrations, exhibited a narrower window of protection (Figure 5.2). Similarly, extracts of *L. laricina, P. balsamifera* and *R. tomentosum* displayed narrow ranges of activity but higher concentrations were required to elicit significant effects. Only by evaluating a range of extract concentrations (rather than a single concentration) could the nature of each extract's bioactivity be deduced. The observed biphasic activity of *A. balsamea* (Figure 5.2), which was only assessed at 10 μg/ml in our previous study and correspondingly deemed inactive (Spoor et al. 2006), illustrates how failure to consider multiple concentrations can lead to incorrect conclusions. The protective effects of *L. laricina* and *R. groenlandicum* were similarly unidentified in Spoor et al. (2006).

Compared to the effectiveness of Cree extracts in the first model, few were capable of reducing PC12 death under conditions of glucose deprivation. When bioactivity was observed, protection was only partial (Figure 5.4A). Conversely, the frequency of toxic effects was higher in the second model with six extracts intensifying low glucose-induced death well below their respective LD_{50} concentrations (Figure 5.4D). Whereas re-evaluation of the first set of extracts revealed unidentified cytoprotection toward glucose toxicity, the previously reported protective effects of *L. laricina, P. banksiana* and *S, purpurea* toward glucose deprivation (Spoor et al. 2006) were not confirmed when a range of concentrations were tested. The observation that many extracts exacerbated cell loss death at concentrations approaching their LD_{50} values in both models was likely due to the stress imposed by

experimental conditions that, in turn, reduced cellular defenses and increased susceptibility to toxins (Kachadourian et al. 2007; Lamensdorf et al. 2000).

Given that the diabetes crisis among aboriginal populations such as the CEI is characterized by a preponderance of T2DM commonly accompanied by poor compliance with therapeutic interventions (Mak et al. 2004; Young et al. 2000), the observed activity profile of Cree medicinal plants is quite promising. With chronic hyperglycemia likely contributing to the development of diabetic neuropathy in such patients, extracts with protective activity against glucose toxicity may provide therapeutic benefit, particularly during the early stages of neuropathic and microvascular damage. The overall lack of protective effects and presence of toxic effects elicited by Cree extracts in the glucose deprivation model is less significant since such conditions only occur in patients who are heavily medicated with hypoglycemic agents (which is unlikely is the context of the CEI) or who have already developed significant microvascular impairment that would limit extract bioavailability and corresponding effects). The extracts that did prevent low glucose-induced cell loss are nonetheless of potential value in reducing the progression neuropathic damage once blood flow has been restricted.

Because oxidative stress has been implicated in cell death induced by both hypoglycemia and hyperglycemia, the quantity of phenolic metabolites, many of which possess well-established anti-oxidant properties, was hypothesized to correlate with bioactivity. To test this hypothesis, extracts were ranked according to their effects on PC12 cells (maximum viability, viability at 6.25 & 12.5 μg/ml and degree of protection/toxicity) in both cell death paradigms and the results were considered relative to extract TPH using Spearman correlation tests. No relationships were discovered indicating that phenolics as a

heterogeneous group were not responsible for activity. The method of TPH measurements provides an approximation of phenolic content but sensitivity varies between and within classes of phenolics based on their specific chemical structure. As such, it is difficult to compare TPH values from diverse plant sources such as the current collection (Table 5.2). To explore whether a certain activity resides within an individual class of phenolic metabolites, cellular effects were compared to the classes of phenolics identified in each extract. With the exception of the hydroxystilbenes, a group that includes resveratrol and possess well described cytoprotective effects (Brito et al. 2006; Lekli et al. 2008), no class was exclusively identified among extracts with either protective or toxic effects. While this observation eliminates the prospect of any broad class effects, it does not preclude the possibility that individual phenolics contribute to activity.

To further examine the potential role of anti-oxidant capacity in extract cytoprotection, the cytoprotection data were analysed relative to the results of Fraser et al. (2007), who tested the same 17 extracts for radical scavenging activity and the ability to prevent lipid peroxidation and formation of conjugated dienes. As seen with TPH data, no relationship was identified suggesting that anti-oxidant activity does not represent the primary mode of action. Again, this does not mean that anti-oxidant mechanisms are not contributing to the effects of individual extracts but simply that other biochemical mechanisms, notably impact on downstream signalling pathways may also be at play. This hypothesis was further tested in Chapters 9-11.

The employed ethnobotanical approach not only enabled the identification of traditional Cree phytotherapies for symptoms of diabetes but also allowed the plants to be ranked according to SIV scores and subsequently analyzed relative to *in vitro* activity as done with TPH and anti-oxidant data. We hypothesized that anti-diabetic SIV scores would

be positively correlated with cytoprotective activity but were unable to identify a relationship with regards to either experimental model. However, the observation that seven of the eight most highly ranked species in terms of SIV exhibited significant cytoprotection towards glucose toxicity suggests that our approach was nonetheless fruitful, as previously exemplified by the range of other anti-diabetic activities ascribed to these extracts in Spoor et al (2006) and Harbilas et al (unpublished). The lack of correlation between activity and SIV may have resulted from only testing anti-diabetic plants without considering non-medicinal species as controls. Indeed, when the anti-oxidant potential of the Cree extracts was determined along with those from a larger collection of boreal plants, the more commonly used medicinal species possessed significantly greater activity (Fraser 2007).

A secondary objective of this study was to provide a profile of phenolic metabolites for each Cree extract in hopes of identifying marker compounds or a unique HPLC fingerprint that could be used for purposes of quality control. While the metabolomics-based approach yielded sufficient identifications to meet this objective, the results are somewhat limited to differentiating between the 17 studied samples. Unlike the collection of Ericaceae leaves examined in Chapter 3, the current set of extracts represent different plant parts, families and growth habits. In order to identify definitive marker compounds and fingerprints, additional samples, such as closely related (or resembling) species that may be accidentally harvested, need to be characterized. The data listed in Table 5.2 nonetheless provide a basis for the development of validated HPLC methods for species authentication and extract standardization.

Together, our results demonstrate that plants from the traditional Cree pharmacopoeia possess *in vitro* activities relevant to the treatment and prevention of diabetic neuropathy. Although several candidate species have been identified, the activity profiles of *P. glauca, R.*

groenlandicum and *S. purpurea* were exceptionally promising and warrant for further investigation. The mechanism(s) of *in vitro* cytoprotection are currently being investigated while the *in vivo* effects of selected Cree species are being tested in murine models of Type 2 diabetes.

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CHAPTER 6 – DETECTION OF ORGAN-SPECIFIC ACTIVITIES IN A MEDICINAL PLANT USED BY CREE HEALERS

6.1 PREFACE

Once screened for cytoprotective activity in *in vitro* models of diabetic neuropathy (Chapter 5), Cree extracts with promising effects were selected for closer examination. We therefore proceeded with *Picea glauca* since the species was ranked 4th in SIV score and the needle extract reduced cell loss in both models. Considering that organ-specific activities were observed in *V. angustifolium* extracts (Chapter 4), we first wanted to determine whether the cytoprotective properties were restricted to needles or present in extracts of other plant parts. Our next objective was to confirm protective activities utilizing a second, more direct measure of cell survival than that employed for initial screening. Finally, we sought to characterize the phenolic profile of each extract to allow for qualitative and quantitative comparisons.

6.2 STATEMENT OF AUTHOR CONTRIBUTIONS

CSH, JTA, and SALB conceived and designed the experiments. CSH performed all experiments with JL assisting in WST experiments (Figure 2.2) and AS contributing to phytochemical analyses (Figure 2.1, Table 2.2). CSH wrote the manuscript with SALB. AC organized and supervised the supporting ethnobotanical studies. JC aided in plant collection and facilitated the community-based ethnobotanical studies. As CIHR-TAAM project leader

and project co-ordinator, respectively, PSH and LCM have managed and directed the pharmacological assessment of Cree extracts and contributed to manuscript preparation.

6.3 ANTI-DIABETIC ACTIVITY OF EXTRACTS FROM NEEDLE, BARK, AND CONE OF PICEA GLAUCA: ORGAN-SPECIFIC PROTECTION FROM GLUCOSE TOXICITY AND GLUCOSE DEPRIVATION

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ABSTRACT

The incidence of Type 2 diabetes mellitus has reached epidemic proportions worldwide. Canadian aboriginal communities, particularly the Cree Nation of Eeyou Istchee, have been identified as a high-risk population. Culturally acceptable treatment options are limited notably for diabetic complications resulting in peripheral neuropathy. Here, we describe results of an ongoing collaborative research project with the Cree of Eeyou Istchee to identify botanicals capable of protecting peripheral neuronal precursors from glucose toxicity and glucose deprivation *in vitro*. Polar fractions of three plant organs (needles, cone, and bark) collected from *Picea glauca* (Moench) Voss (Pinaceae), were tested for toxicity under normoglucose, glucotoxicity, and glucose deprivation conditions. The profile of phenolic metabolites in each extract was characterized by high-performance liquid chromatography-diode array detection-atmospheric pressure chemical ionization mass spectrometry (HPLC-PAD-APCI/MS). We report here that these fractions are well-tolerated by PC12 neuronal precursors under normoglucose conditions. LD_{50} concentrations of needle extracts exceeded 100 μg/ml, whereas the LD_{50} of bark and cone extracts was 40 and 36.4 μg/ml respectively. We further show that the cytoprotective properties of *P. glauca* following glucose challenge are concentration-dependent and organ-specific. Needle extracts protected PC12 cells from both glucotoxicity and glucose deprivation. Bark extracts had negligible activity. Cone extracts further impaired PC12 cell glucose tolerance. This study provides the first validation of anti-diabetic activity of *P. glauca* organs at the cellular level relevant to the management of diabetic peripheral neuropathy.

INTRODUCTION

The incidence of Type 2 Diabetes mellitus (T2DM) has reached epidemic proportions afflicting over 171 million persons world-wide (WHO 2004). Prevalence rates are predicted to double over the next 30 years (WHO 2004). Aboriginal populations are particularly susceptible with incidence 20-30% higher than dominant national cultures (for a review see Yu and Zinman (2007b)). In Canada, First Nations communities are recognized as among the highest at-risk populations in the world (Pollex et al. 2006). This increased disease incidence is influenced, in part, by specific genetic predispositions whose effects are emerging at alarming rates given changes in diet and lifestyle (Hegele 2001). With the devastating health impact of "acculturation and adoption of a 'Westernized' lifestyle" (Yu and Zinman 2007b), it is not surprising that poor disease prognosis is compounded by a low compliance rate with modern pharmaceuticals (Young et al. 2000). Arguably, most T2DM treatment strategies are designed by professionals unfamiliar with the needs of the local community and without consideration of cultural awareness and accepted practice (Boston et al. 1997).

To address these problems, we have entered into a collaborative research program between basic scientists and elders and healers of the Cree of Eeyou Istchee (CEI). The Cree are the largest First Nations group in Canada with nearly 78,000 persons (Statistics-Canada 2006). The Cree of Eeyou Istchee (CEI) make up nine communities in the northern regions of the province of Quebec, Canada, represented by approximately 13,500 members. These communities are experiencing a dramatic increase in T2DM incidence with few strategies designed to manage peripheral neuropathies associated with chronic pain, infection, paralysis, and loss of sensation (Kuzmina 2004; Légaré 2004). To evaluate alternative and complementary treatment strategies that promote culturally acceptable T2DM management,

we conducted ethnobotanical surveys targeting natural medicines used by CEI for the treatment of diabetic symptoms. Two CEI communities, representing inland and coastal communities, participated (Fraser 2007; Leduc et al. 2006). Multiple, potentially anti-diabetic plants were identified in this survey and subsequently screened for a variety of *in vitro* anti-diabetic activities, including the ability to protect peripheral neuronal precursors from glucose toxicity and glucose deprivation (Spoor et al. 2006). When the bioactivities of extracts made from separate organs of the same species (i.e., root, stem, leaf, and fruit extracts) were compared, certain plants exhibited organ-specific activities (Martineau et al. 2006), some of which could be attributed to differences in phenolic constituents within the plant organs (Harris et al. 2007a). Here, we extend these findings to an analysis of minhikw, the Cree term for *Picea glauca* (Moench) Voss, a plant reported useful for controlling diverse diabetic complications among several First Nations groups (Arnason 1981; Leduc et al. 2006), particularly the Cree of Whapmagoostui. We report that polar fractions of *P. glauca* needle are more effective than bark and cone extracts at protecting PC12 peripheral neuronal precursors from both glucose toxicity and glucose deprivation. The profile of phenolic metabolites in each extract was characterized by HPLC-PAD-APCI/MS to identify marker compounds within the different organs and to gain insight into the identity of potential active metabolites.

EXPERIMENTAL

Reagents

All cell culture reagents were obtained from Invitrogen (Burlington, ON Canada) and all chemicals were purchased through Sigma-Aldrich (St. Louis, USA) unless otherwise stated.

Plant materials

Samples of *Picea glauca* were harvested in Mistissini and Whapmagoostui, Quebec, Canada, as per the instructions of the elders and healers from these communities (Leduc et al. 2006). Plants were identified by Dr. A. Cuerrier (Plant Biology Research Institute, Montréal Botanical Garden) and voucher specimens were deposited in the Marie-Victorin herbarium (MT) of the Montréal Botanical Garden. Whole plants were air dried and transported to the University of Ottawa for separation into bark, cones, and needles. Plant parts were processed using a Wiley Mill with a 2 mm filter and were subsequently extracted twice with 10 ml of 80% ethanol per gram dry material on a mechanical shaker for 24 h and then vacuum-filtered using Whatman paper no. 1. The supernatants of first and second extracts were pooled and dried by rotary evaporation and lyophilization. Prior to experimental use, the lyophilized extracts were prepared in DMSO at a concentration of 100 mg/ml and filtered through a 0.2 μm nylon membrane filter (Chromatographic Specialties Inc., Brockville, ON, Canada). Required serial dilutions were made in DMSO on the day of use to ensure all cultures were exposed to a final concentration of 0.1% DMSO (vehicle).

Assessment of total phenolic content

The total phenolic content of bark, cone, and needle extracts was determined using the Folin-Ciocalteau method previously described (Singleton 1965) and modified for reduced volumes as previously described (Spoor et al. 2006). Each extract was dissolved in 80% methanol at 1 mg/ml and combined with freshly diluted Folin-Ciocalteau reagent then incubated at room temperature for 5 min prior to mixing with 7.5% anhydrous $NaHCO_3$ solution. After an additional 2 h incubation, absorbances were measured at 725 nm with a

SpectraMax Gemini XS microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA). Total phenolic content of each plant extract was calculated relative to serial dilutions of concurrently analyzed quercetin and are expressed as quercetin equivalents. Each sample was tested in duplicate or triplicate on two separate occasions (n=5).

Phytochemical characterization

Bark, cone, and needle extracts were characterized by HPLC-PAD-APCI/MS analysis using a validated method for the identification of phenolic metabolites (Harris et al., 2007). Analyses were performed on an Agilent 1100 LC MS system (Palo Alto, CA, USA) comprising of an autosampler, a quaternary pump, a column thermostat, a photodiode array detector (PAD), and an online APCI/MS. Separations were performed on a YMC ODS-AM column (100 × 2 mm I.D.; 3 μm particle size) (Waters, Mississauga, Canada) at 50°C and a flow rate of 0.3 ml/min. The elution conditions consisted of aqueous trifluoroacetic acid (TFA, 0.05%), pH 3.4 (solvent A) and methanol (solvent B) as mobile phase with initial conditions 92%:8% (A:B) maintained for 0-5 min, 8 – 13% B in 2 min, 13 – 30% B in 14 min, 30 – 60% B in 3 min, and 60 – 100% B in 2 min. After a 2 min wash at 100% B, solvent composition was returned to 92%:8% in 2 min followed by 6 min of post-run equilibration, resulting in a total run time of 36 min. The ethanol extracts were filtered through 0.2 μm PTFE membrane filter (Chromatographic Specialities Inc., Brockville, ON)) and 1 μl of extract individually injected through a 100 μl loop of the autosampler. The chromatographic separation was monitored on-line at 325 nm and at 280 nm by PAD. The mass spectrometric detection was performed in positive ionization mode with the following optimized conditions: drying gas flow rate at 6.0 L/min, nebulizer pressure at 40 psig, drying

gas temperature at 300 °C, vaporizer temperature at 400 °C, capillary voltage at 3000 V, and corona current at 3.0 μA. The total ion chromatogram was obtained in scan mode within the mass range of 100 to 800 amu with a fragmentation voltage at 20V.

Compound identifications were performed by matching the UV spectra of each eluted peak with those of standards registered in an on-line Chemstation library comprising over 120 reference spectra. The threshold for an authenticated match was set at 95% spectral similarity within a specified UV absorbance range of 190-400 nm. Confirmation of identity was achieved by comparing the fragmentation patterns and relative retention time with those of standards and/or reported literature (Harris et al. 2007a; Slimestad 2003).

Cell culture, glucose toxicity, and glucose deprivation conditions

PC12-AC cells, a clonal derivate (Brewer et al. 2002) of the PC12 rat adrenal pheochromocytoma cell line (American Tissue Culture Collection), were maintained in complete media (RPMI 1640 containing 10% horse serum and 5% newborn calf serum) under normoglucose (11 mM) conditions. Cells were seeded in 96-well plates ($6.25x10^3$ cells/well) and allowed to adhere at 37°C in 5% CO_2 overnight. To establish LD_{50} concentrations, cultures were treated for 96 h in serum-free RPMI media at a glucose concentration of 11 mM with 0.1% DMSO (vehicle control) or varying concentrations of plant extract (0-100 μg/ml). Media was supplemented with 0.025% bovine serum albumin (BSA) to facilitate intracellular passage of hydrophobic compounds. To elicit glucose toxicity (hyperglycemia), complete medium was replaced with serum-free medium supplemented with 150 mM glucose and 0.025% BSA. Substitution of D-glucose for L-glucose abolishes toxicity indicating that PC12 cell death is glucose-specific and not the

result of osmotic stress under these treatment conditions (Koshimura et al. 2002). To elicit glucose deprivation (hypoglycemia), glucose (1 mM) was added to glucose-free RPMI media lacking serum and supplemented with 0.025% BSA as we have described previously (Spoor et al. 2006). Cultures were exposed to various concentrations of plant extract below the relevant LD_{50} for each preparation.

Cell viability assay

Following 96 h of treatment in normoglucose, high glucose, or low glucose conditions, the formazan dye WST (Roche Diagnostics, Laval, QC) was added to each well. Cultures were incubated for 60 min before spectrophotometric analysis at 420 nm (formazan) and at 620 nm (reference). Experimental samples were blanked against cell-free treatment media incubated for the same period. Cell number/well was calculated from standard curves of known cell density prepared for each assay. Compounds were tested in multiple wells over two or three independent experiments (n=7-10 wells/condition). Data from control cultures in normoglucose, high, and low glucose treated with 0.1% DMSO were combined across plates (n=55-65 wells). Percent viability was calculated as follows:

$$\% \text{ viability} = \text{cell number}_{(\text{treatment well})} / \text{mean cell number}_{(\text{normoglucose control})} \times 100$$

Cell survival assay

Cell survival was directly assessed by Live/Dead viability/cytotoxicity assay (Invitrogen). Viable cells were identified by the enzymatic conversion by intracellular esterases of non-fluorescent calcein-AM to fluorescent calcein. Dead cells were identified by uptake of ethidium homodimer (ET) indicative of a loss of membrane integrity. Cells

were imaged using a DMIR epifluorescent inverted microscope (Leica, Richmond Hill, Canada) equipped with a QICAM digital camera (Quorum Technologies, Guelph, Canada) and captured using OpenLab software v5.05 (Improvision, Lexington, USA). Percent survival was calculated as follows:

$$\% \text{ survival} = \frac{\text{Viable cell number}^{(\text{calcein}^+ - \text{calcein}^+/\text{ET}^+)}}{\text{Mean viable cells in vehicle control}^{(\text{calcein}^+ - \text{calcein}^+/\text{ET}^+)}} \times 100$$

Statistical analyses

Data were analyzed using unpaired Student's *t*-tests or one-way factorial ANOVA tests followed by *post hoc* Dunnett's *t*-tests on each organ as applicable. *P* values under 0.05 were considered statistically significant (shown as * or #); *P* values under 0.01 were considered highly significant (shown as ** or ##).

RESULTS

We found that the ethanol extraction of *Picea glauca* bark, cones, and needles produced 10%, 24%, and 31% yields, respectively. Employing the Folin-Ciocalteau method to approximate the total content of phenolic compounds in each extract, the bark extract proved to be the richest in phenolics, containing 50% more than the cone extract and 200% more than needle extract (Table 6.1). On the basis of dry weight, however, the cones contain more phenolics than the bark (Table 6.1). To identify marker compounds and characterize the classes of phenolic compounds present in each organ, the extracts were analyzed by HPLC-PAD-APCI/MS. In total 12 compounds were identified, representing three phenolic acids (PA_{1-3}), four stilbenes (S_{1-4}), and five flavonoids (F_{1-5}) (Table 6.2). In contrast to the bulk phenolic content of the three extracts, the HPLC chromatogram of the bark extract

Table 6.1 – Extraction yield and total phenolic content of *Picea glauca* bark, cone and needle extracts.

Organ	Yield[a]	Total phenolic content[b]	
		mg / g dry material	µg / per mg extract
Bark	10.4 %	32.1 ± 0.4	308.7 ± 3.7
Cone	24.0 %	49.8 ± 0.7	207.4 ± 2.8
Needle	13.1 %	12.4 ± 1.2	109.8 ± 9.3

[a] Yield was calculated as (mass recovered extract / mass dry material) x 100%

[b] Total phenolics expressed as quercetin equivalents

Table 6.2 – List of phenolic metabolites detected by HPLC-PAD-APCI/MS in *Picea glauca* extracts.

Identified metabolites	Peak	R_t (mins)	λ_{max} (nm)	M^+ (*m/z*)	Detected in: Needle	Cone	Bark
Benzoic acid derivative	PA_1	6.1	220, 280, 300sh	153	✓		
Benzoic acid derivative	PA_2	9.9	220, 280	137	✓		
Phenylpropanoid derivative	PA_3	13.5	210, 230sh, 320	179		✓	
Tetrahydroxystilbene	S_1	15.3	200, 220, 300	245	✓	✓	✓
Trihydroxytilbene	S_2	18.9	200, 220, 300	229			✓
Methoxytrihydroxystilbene	S_3	20.9	200, 220, 300	259	✓		✓
Methoxytrihydroxystilbene	S_4	25.6	200, 220, 300	259			✓
Catechin	F_1	5.8	210, 230sh, 280	139, 291	✓	✓	
Taxifolin	F_2	16.4	195, 230sh, 290	305	✓		
Quercetin glycoside	F_3	24.5	255, 350	303, 465	✓		
Kaempferol glycoside	F_4	26.4	200, 265, 350	287, 449	✓	✓	
Isorhamnetin glycoside	F_5	26.8	255, 360	317, 479	✓	✓	

revealed relatively few major peaks, all of which were identified as hydroxylated stilbenes (Figure 6.1A). After performing the spectral library match, it was found that the UV absorption spectra of these peaks, as well as the minor peaks eluting after S_3, are very similar to that of standard piceatannol. The UV and MS data obtained from both cone and needle extracts showed a higher diversity of phenolic compounds, containing phenolic acid, stilbene, and flavonoid derivatives (Figure 6.1B-C, Table 6.2). While the cone and bark extracts appeared relatively distinct, sharing only the major stilbene peak (S_1) common to all organs, the observed phytochemical profile of needle extract was intermediate between bark and cone, containing three stilbenes found in the bark (S_{1-3}) and three flavonoids found in the cones (F_1, F_{4-5}) (Figure 6.1).

PC12-AC cells are a clonal derivate of the established adrenal pheochromocytoma cell line that can be differentiated to a peripheral neuron phenotype by the combination of serum deprivation and treatment with nerve growth factor. As such, these cells represent an accessible model for assessment of anti-diabetic activity with respect to peripheral neuropathies. To establish LD_{50} concentrations of *P. glauca* bark, cone, and needle extracts, cultures were treated with various concentrations of extract or vehicle (0.1% DMSO) in serum-free media for 96 h under normoglucose (11 mM) conditions. Viable cell number was established by mitochondrial dehydrogenase cleavage of the formazan dye WST relative to standardized controls of known cell densities. We found that cell viability was not compromised over the 96 h treatment period in vehicle-treated cultures (data not shown). LD_{50} concentrations were defined as the amount of extract in μg/ml eliciting death of 50% of cultured cells relative to control cultures. Needle extracts were not toxic at any of the concentrations tested exhibiting an $LD_{50} > 100$ μg/ml. LD_{50} concentrations of bark and cone

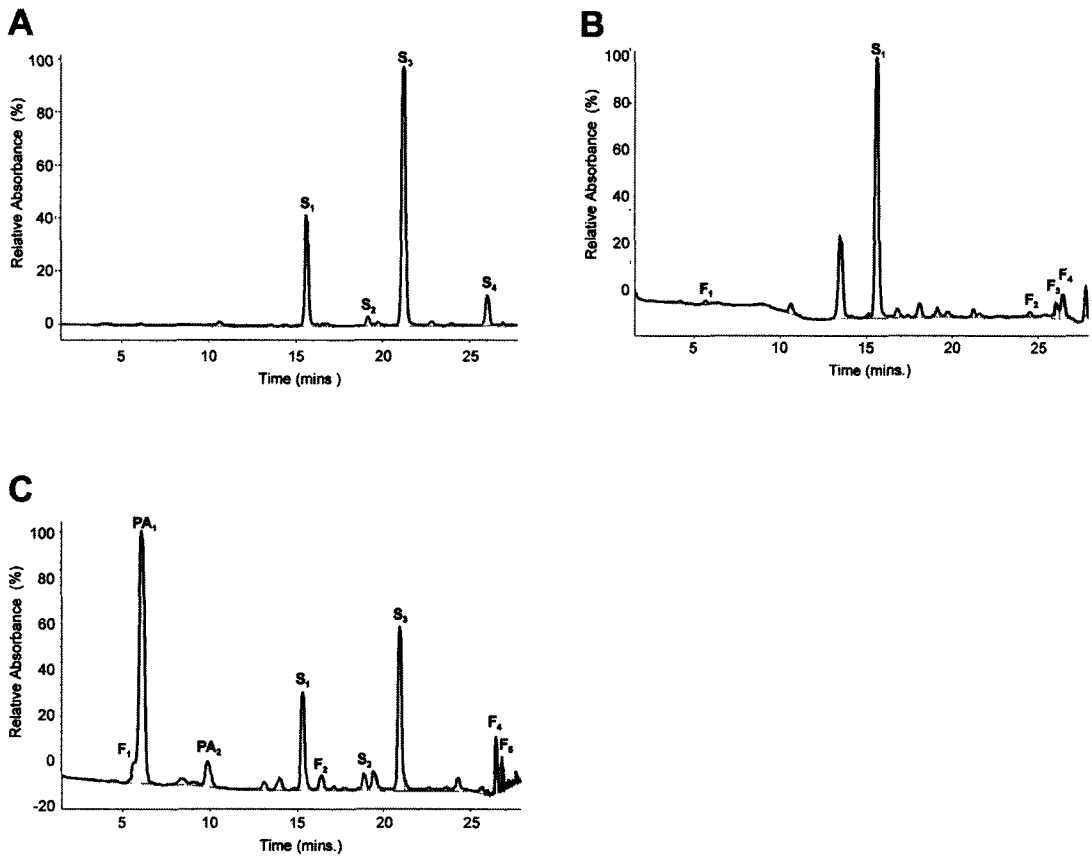

Figure 6.1 – HPLC chromatograms of *Picea glauca* extracts. HPLC-PAD chromatograms of (A) bark, (B) cone, and (C) needle extracts with photodiode array detection (PAD) at 325 nm. Labelled peaks represent identified metabolites as reported in Table 2. Three classes of phenolic compounds were detected, flavonoids (F), phenolic acids (PA), and stilbenes (S).

extracts were comparable at 40 μg/ml and 36.4 μg/ml respectively.

To screen for bioactivity relevant to glucose toxicity, PC12-AC cells were exposed to elevated glucose concentrations (150 mM) under conditions of serum deprivation (Figure 6.2). We have previously demonstrated that this protocol elicits ~50% cell death relative to normoglucose concentrations (Spoor et al. 2006). Consistent with this earlier study, a statistically significant 43% loss in cell viability was detected in DMSO-treated control cultures as a result of glucose toxicity (Figure 6.2A, left panel). We next tested the ability of *P. glauca* extracts to alter cell viability under hyperglycemic conditions. Extracts were tested at concentrations approaching but not exceeding their LD_{50} concentrations established under normoglucose conditions (i.e., 0-30 μg/ml). We found that bark extracts had no signficant effect on glucose toxicity whereas cone extracts significantly exacerbated cell loss when concentrations approached the LD_{50} concentration of 36.4 μg/ml (Figure 6.2A, left panel). By contrast, significant concentration-dependent cytoprotection was observed when PC12-AC cells were exposed to high glucose in the presence of *P. glauca* needle extracts (Figure 6.2A, right panel).

To assess protection from glucose deprivation, PC12-AC cultures were exposed to low glucose (1 mM) and treated with 0.1% DMSO or *P. glauca* extracts (Figure 6.2B). As previously reported (Spoor et al., 2006), glucose deprivation elicited ~40% loss of viable cell number (Figure 6.2B, left panel). We found that bark extract did not confer cytoprotection under low glucose conditions, that cone extracts elicited significant toxicity at all concentrations tested and that needle extracts were cytoprotective, inhibiting cell death associated with glucose deprivation at the highest concentrations tested (Figure 6.2B, right panel).

Because mitochondrial dehydrogenase activity can increase in cells undergoing

mitochondrial-dependent apoptosis (Vogt et al. 2004), it was necessary to confirm cytoprotection observed in *P. glauca* needle extracts by directly assessing viable cell number. Serum-deprived cultures were treated for 96 h with DMSO (control) or increasing concentrations of needle extracts and cell survival quantified by Live/Dead assay. Viable cells were identified by cleavage of calcein AM by intracellular esterases to its fluorogenic product. Dead cells were identified by uptake of the membrane-impermeant ethidium bromide homodimer. As observed using the WST assay, 0-25 μg/ml *Picea glauca* needle extract had no effect on cell viability in serum-deprived cultures under normoglucose conditions (data not shown). Culture in high glucose (Figure 6.3A, left panel) and low glucose-containing media (Figure 6.3B, left panel) in the presence of vehicle (DMSO) significantly compromised cell survival. Needle extracts enhanced PC12-AC cell survival at concentrations of 12.5 μg/ml and 25 μg/ml under hyperglycemic (Figure 6.3A, right panel) and hypoglycemic (Figure 6.3B, right panel) conditions.

DISCUSSION

This study compared the activity of *P. glauca* bark, cone, and needle extracts administered to PC12 cells in two *in vitro* paradigms of diabetic neuropathy (glucotoxicity and glucose deprivation). This study was designed to model cellular effects of impaired glucose regulation associated with T2DM. The observed effects of each extract were consistent in both models and revealed organ-specific anti-diabetic activity. Here, we show that the needle extract displayed concentration-dependent protection whereas the cone extract intensified cell loss and the bark extract failed to affect cell viability.

These findings are consistent with the ethonobotanical uses of *P. glauca* among Native Peoples of North America indicating that, where available, organs of this species are

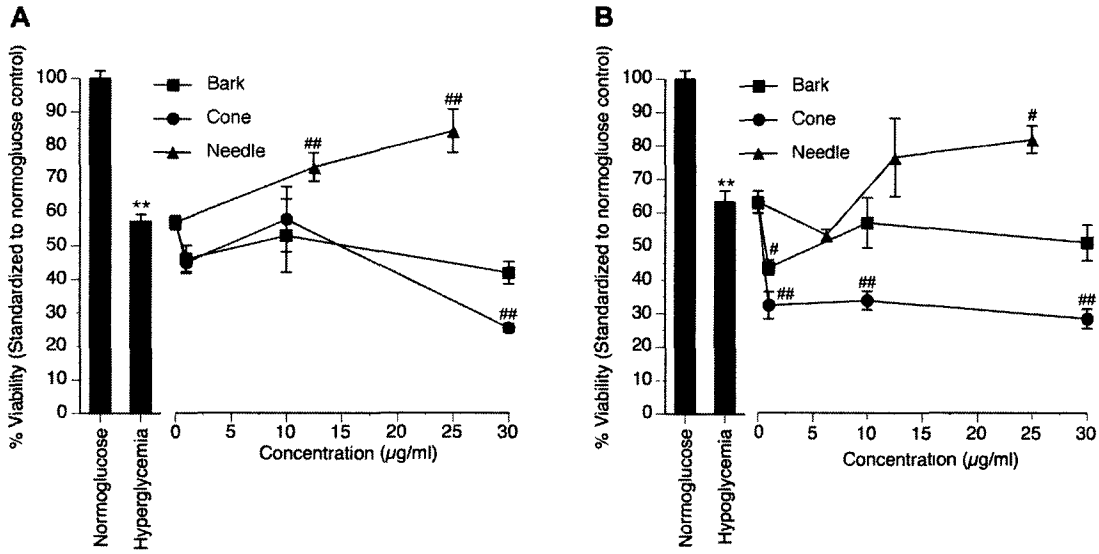

Figure 6.2 – Dissociation of cytoprotection and cytotoxicity in high and low glucose media between bark, cone, and needle extracts of *Picea glauca*.
(A) Exposure to hyperglycemic media in the presence of 0.1% DMSO elicited loss of cell viability as assessed by mitochondrial dehydrogenase activity measured by cleavage of the formazan dye WST (left panel, ** $p < 0.01$ Student's *t*-test, n=55-65 wells/condition). A concentration-dependent increase in cell viability was detected in cultures treated with needle extracts. Bark extracts had no statistical effect on PC12 viability in high glucose media. Cone extracts decreased cell viability in high glucose media at 30 µg/ml (right panel, ## $p < 0.01$ versus DMSO control (0 h), ANOVA, post-hoc Dunnett's *t*-test, n=7-55 wells/condition). (B) Culture in hypoglycemic media in the presence of vehicle (0.1% DMSO) significantly reduced cell viability (left panel, ** $p < 0.01$ Student's *t*-test, n=55-65 wells/condition). Needle extracts protected PC12-AC cells in concentration-dependent manner. Bark extracts had no significant effect on cell viability in low glucose media. Cone extracts decreased cell viability following glucose deprivation at all concentrations tested (right panel, # $p < 0.05$, ## $p < 0.01$ versus DMSO control (0 h), ANOVA, post-hoc Dunnett's *t*-test, n=7-55 wells/condition). Data are reported as mean + SEM. Extracts were tested at concentrations below their LD_{50} values as established under normoglucose conditions.

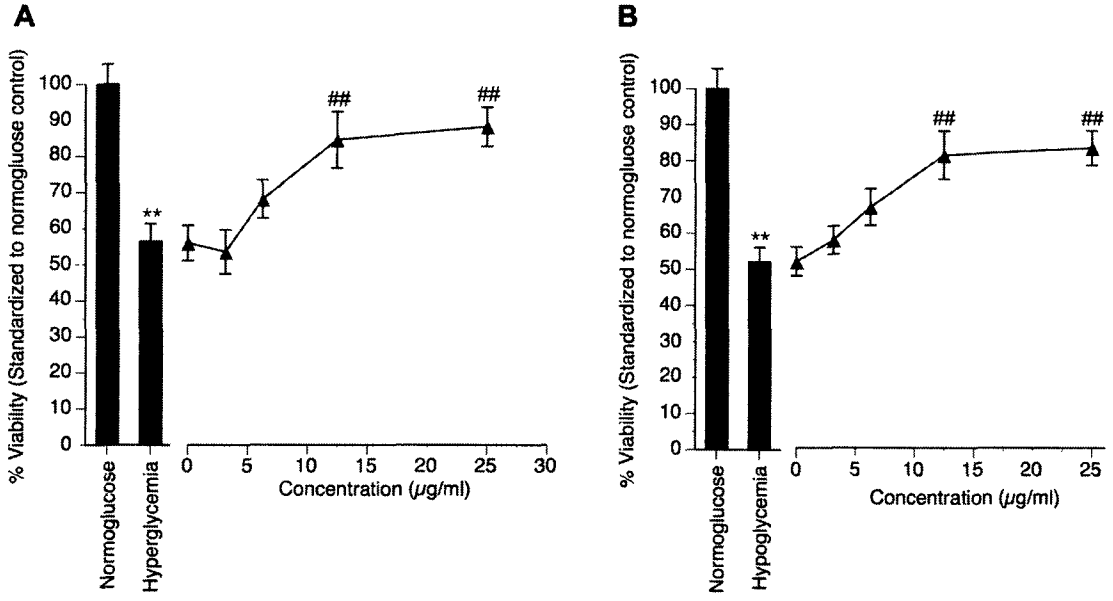

Figure 6.3 – Concentration-dependent protection of PC12-AC cells from glucose toxicity and glucose deprivation by *Picea glauca* needle extract. Cell survival was directly assessed by Live/Dead assay. (A) Culture in high glucose media in the presence of DMSO (vehicle) significantly compromised cell survival (left panel, ** $p < 0.01$ Student's *t*-test, n=6-11 wells/condition). Needle extracts enhanced cell survival (right panel, ## $p < 0.01$ versus DMSO control (0 h), ANOVA, post-hoc Dunnett's *t*-test, n=6-11 wells/condition). (B) Culture in low glucose media in the presence of DMSO (vehicle) significantly compromised cell survival (left panel, ** $p < 0.01$ Student's *t*-test, n=6-11 wells/condition). Needle extracts concentration-dependently enhanced cell survival (right panel, ## $p < 0.01$ versus DMSO control (0 h), ANOVA, post-hoc Dunnett's *t*-test, n=6-11 wells/condition). Data are reported as mean + SEM. Extracts were tested at concentrations below their LD_{50} concentrations established under normoglucose conditions.

exploited for a wide variety of medicinal purposes. As reviewed by Arnason et al. as well as by Moerman, the needles, cones, and bark are used separately by various First Nations inhabiting Canada to treat different symptoms, including those associated with diabetes and its related complications (Arnason 1981; McCune and Johns 2002; Moerman 1998). For instance, the cone and bark (gum) are prepared by the Abenaki for frequent urination while the Mi'kmaq and Atikamekw employ the bark to treat sores and wounds (Arnason 1981). Though the needles are made as a tea by the Algonquin to "heal the insides" (Black 1980), until our recent work with the CEI, the bark, twigs, and cones appeared to be more commonly used than the needles. But old statements stemming from anthropological/ethnological studies do not differentiate between twigs *sensu stricto* and twigs *sensu lato*, which also comprises the needles. Indeed, strong *in vitro* activities against a wide range of microbes have been demonstrated by *P. glauca* (Richardson MD 1992; Ritch-Krc et al. 1996). Taken together, these results clearly validate the Traditional Knowledge shared by the Healers and Elders of CEI at a cellular level.

Although bark and cone extracts did not exhibit cytoprotective activity on peripheral neuronal precursors in the presence of high (150 mM) or low glucose (1 mM), these extracts have proven important in other studies to reduce other diabetic-associated symptoms. When collections of boreal plants used for treating diabetes were compared, the bark/twig extract proved effective as an anti-oxidant (McCune and Johns 2002; McCune and Johns 2003). Oxidative stress is implicated in the etiology of diabetes and many of its complications (Fridlyand and Philipson 2005). Moreover, we have recently reported promising anti-diabetic activity in the bark and cone extracts of other Pinaceae species (*P. mariana* (Mill.) B.S.P., *Pinus banksiana* Lamb. and *Abies balsamea* (L.) Mill.) (Spoor et al. 2006). The data presented here begin to dissociate the different bioactivities of boreal plant organs relevant to

treatment of T2DM-associated peripheral neuropathies versus other diabetic complications.

To provide further insight, and as expected by the distinct activities elicited by the three extracts, characterization of the phenolic metabolites detected considerable variation in the total phenolic content and the classes of identified compounds among plant parts. These differences in phytochemical composition likely underlie not only the distinct biological activity profiles of bark, cones, and needles in PC12 cells but also their varied medicinal uses as well. Phenolic metabolites were targeted for analysis as these compounds have frequently been associated with both anti-diabetic and neuroprotective abilities (Coskun et al. 2005; Dajas et al. 2003b; Galli et al. 2002; Rodriguez de Sotillo and Hadley 2002). Many of the identified phenolic compounds have been previously reported in closely related species. As the name implies, piceatannol derivatives and other hydroxylated stilbenes are found in multiple *Picea* species and Pinaceae members (Pan and Lundgren 1995; Spoor et al. 2006). The common occurrence of this type of compounds in all plant parts was therefore not surprising. The five detected flavonoids have been identified in *Picea abies* needles (taxifolin, kaempferol, and isorhamnetin glycosides) (Slimestad 2003) or bark (catechin, taxifolin) (Lieutier et al. 2003) but were only detected in the needle and cone extracts of *P. glauca*. While the LC-MS data provided the identification of marker compounds allowing the extracts to be differentiated on a phytochemical basis, speculation on possible active metabolites is difficult at this stage and requires further investigation. Furthermore, additional phytochemicals left unidentified and/or undetected using the current methods, such as terpenes or tannins, may equally possess the biological activity of interest. Biossay-guided fractionation of the needle extract is therefore being pursued to gain further insight into the active constituent(s) and their mechanism(s) of action.

In summary, this study identifies a novel cytoprotective activity promoting peripheral

neuronal precursor survival under conditions of both glucotoxicity and glucose deprivation in needle extracts of *P. glauca.* Bark and cone extracts were not cytoprotective. As such, *P. glauca* needles represent an ideal candidate for further investigation as adjuvant therapy in the treatment and prevention of diabetic neuropathy for the CEI and other communities. As a natural health product, *P. glauca* preparations may be accepted more readily than prescription drugs for some patient groups, particularly First Nations communities, afflicted with rising incidence of T2DM and a paucity of culturally acceptable treatment options.

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CHAPTER 7 – IDENTIFICATION OF ACTIVE METABOLITES IN SARRACENIA PURPUREA BY ASSAY-GUIDED FRACTIONATION

7.1 PREFACE

In Chapter 5, we determined that the cytoprotective activities of Cree medicinal plant extracts were not correlated with their total phenolic contents and, in Chapter 6, we found that only the needle extract of *Picea glauca* protected PC12 cells despite containing fewer phenolics than bark or cone extracts. Together, these observations suggest that, unlike anti-oxidant activity, cytoprotection is not a generalized phenolic class-effect but likely due to the presence of specific active metabolites. Like *P. glauca*, *Sarracenia purpurea* exhibited strong cytoprotective activity in PC12 cells, particularly under high glucose conditions (Chapter 5). As in Chapter 6, we sought to identify organ-specific activity, to confirm activity with a second assay and to expand our analytical methods for extract characterization. As an additional objective, we conducted assay-guided fractionation of the active extract to identify metabolites contributing to *S. purpurea*'s effects.

7.2 STATEMENT OF AUTHOR CONTRIBUTIONS

CSH, JTA, and SALB conceived and designed the experiments. CSH conducted all PC12 experiments and wrote the manuscript with JTA and SALB. AS provided support and guidance throughout assay-guided fractionation, particularly the semi-preparative HPLC stage. MA performed all NMR analyses and assisted with fractionation by Sephadex L20 column chromatography. The Elders of CEI shared their Traditional Knowledge, which served as the basis for this study. PSH and LCM managed and directed the CIHR-TAAM and contributed to manuscript preparation.

7.3 BIOASSAY GUIDED ISOLATION OF NEUROPROTECTIVE CONSTITUENTS OF SARRACENIA PURPUREA, A CREE MEDICINAL PLANT THAT INHIBITS GLUCOTOXICITY IN PC12 CELLS.

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Manuscript is in review by our Cree collaborators.

ABSTRACT

The purple pitcher plant, *Sarracenia purpurea*, is a widely distributed species in North America that is used as traditional medicine by the Cree of Eeyou Istchee in northern Québec to treat symptoms of diabetes. When assessed for biological activity, the leaf extract of *S. purpurea* demonstrated multiple anti-diabetic effects including cytoprotection in an *in vitro* model of diabetic neuropathy. The purpose of the current study was to further investigate this cytoprotective activity by identifying which plant parts and secondary metabolites contribute to protective effects. First, ethanolic extracts of *S. purpurea* leaves and roots were separately assessed in PC12 cells exposed to glucose toxicity revealing that the leaf extract, but not that of the root, prevented high glucose-mediated cell loss in a concentration dependent manner. Assay-guided fractionation of the leaf extract was then conducted to identify active principles. Several fractions elicited protective effects, indicative of multiple active metabolites, and, following subfractionation of the polar fraction, quercetin-3-*O*-galactoside was isolated and identified as an active principle of the leaf extract. Phytochemical analyses confirmed the presence of quercetin-3-*O*-galactoside as well as other flavonol glycosides in the leaf extract but not in the root. Evaluation of different flavonoids in the PC12 cell model of diabetic neuropathy revealed that quercetin derivatives effectively reduced glucotoxic cell death while others were ineffective. These results not only provide support for further study into the therapeutic potential of *S. purpurea* as an alternative and complementary treatment for diabetic neuropathy but also define active principles and analytical methods that can be used for purposes of quality control.

INTRODUCTION

The purple pitcher plant, *Sarracenia purpurea* L. (Sarraceniaceae), is a perennial carnivorous plant widely distributed across northern North America. By consuming nitrogen from insects trapped within their pitchers (fused leaves), they adapt to nitrogen-poor environments such as bogs and peatlands. This unusual natural history has received considerable attention from an ecological perspective. The pitcher plant has a long history of use as a traditional medicine among indigenous peoples across the continent, and the therapeutic potential of the plant has been investigated for a wide variety of therapeutic effects, from treatment of small pox during the 19th century (Grieve 1931; Moerman 1998) and, recently, an alkaline extract of the root, Sarapin®, has been marketed as an injected pain reliever that specifically blocks C-fibre excitation (Manchikanti et al. 2001; Rask 1984).

Here, as part of a collaborative research program between the Cree of Eeyou Istchee (CEI), basic scientists, and community health professionals, we have set out to evaluate traditional Cree medicines as potential culturally acceptable alternative and complementary treatments for Type 2 diabetes mellitus (T2DM). The incidence of T2DM is increasing prevalent among the CEI and other Canadian First Nations communities with First Nations communities recognized as among the highest at-risk populations in the world (Bruce 2003; Pollex et al. 2006). The Elders of CEI, who refer to *S. purpurea* as "aygadash", which translates to 'frog's socks' in reference to the plant's long slender pitchers, identified preparations from *S. purpurea* leaves as beneficial for the treatment of diabetic symptoms, slow healing infections in particular (Leduc et al. 2006). We have previously shown that the ethanolic extract of dry *S. purpurea* leaf material significantly increased glucose uptake in both muscle cells and adipocytes and, in addition to these insulinotropic effects, the extract

also demonstrated cytoprotective activity in two models of diabetic neuropathy, PC12 cells exposed to glucose toxicity or glucose deprivation (Spoor, 2006).

Currently, evidence exists supporting the involvement of high, low, and fluctuating glucose concentrations in the pathophysiology of diabetic peripheral neuropathy (Dyck et al. 1999; Honma et al. 2003; Sugimoto et al. 2000). While glucose deprivation may contribute to the development of diabetic neuropathy in Type 1 diabetics and T2DM patients on intense pharmacotherapy, high levels of circulating glucose are more likely to contribute to neuronal injury in Aboriginal populations such as the CEI where T2DM is far more prevalent and compliance with modern pharmaceuticals is generally low (Young et al. 2000). Thus, culturally acceptable treatments would be of significant benefit in the control of glucotoxic neuropathic complications within the community. Here, we sought to identify the plant organ sources and the active constituents contributing to the cytoprotective effects of *S. purpurea* under conditions of glucose toxicity. In comparing activities of root and leaf extracts, we demostrate enhanced cytoprotective activity in the leaf extract as predicted by the traditional usage. Through subsequent bioassay-guided fractionation and phytochemical analysis of both root and leaf extracts, we identified and quantified marker compounds, including the biologically active phenolics responsible for these anti-neuropathic actions.

EXPERIMENTAL

Reagents

All cell culture reagents were obtained from Invitrogen (Burlington, ON Canada) and all chemicals were purchased through Sigma-Aldrich (St. Louis, USA) unless otherwise stated. Quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside were purchased from Extrasynthase (Lyon, France).

Plant materials

In August 2006, wild samples were collected near Mistissini, Quebec, Canada, as per the instructions of community elders and healers. The specimens were identified as *Sarracenia purpurea* L. by Dr. A. Cuerrier (Plant Biology Research Institute, Montréal Botanical Garden) and voucher specimens were deposited in the Marie-Victorin herbarium (MT) of the Montréal Botanical Garden. Upon collection, loose debris (such as peatmoss) was removed from the plants, which were subsequently partially dried by air (25°C) and transported to the University of Ottawa. Whole plants were separated into leaves (pitchers), roots and flowers then dehydrated using an electric food dehydrator (Nesco/American Hervest WI, USA) set to 40°C. Leaf and root materials were processed using a Wiley Mill (2 mm filter) prior to extraction with 80% ethanol as previously described (Harris 2008). Dried extracts were prepared for experimental use in DMSO at a concentration of 100 mg/ml, filtered through a 0.2 μm nylon membrane filter (Chromatographic Specialties Inc., Brockville, ON, Canada), and serially diluted as required on the day of use to ensure all cultures were exposed to a final concentration of 0.1% DMSO (vehicle).

Cell culture and glucose toxicity assay

PC12-AC cells, a clonal derivative of the PC12 rat adrenal pheochromocytoma cell line (American Tissue Culture Collection), were cultured in Roswell Park Memorial Institute medium (RPMI 1640) containing 11 mM glucose and supplemented with 10% horse serum and 5% newborn calf serum. Prior to experimental use, cells were seeded in 96-well plates at a density of 6.25×10^3 cells/well and incubated overnight at 37°C in 5% CO_2 to allow

adherence. As described in Chapter 6, extract toxicity (LD_{50}) was determined by treating cultures for 96 h in serum-free RPMI 1640 containing 11 mM glucose, 0.025% bovine serum albumin (BSA) to facilitate intracellular passage of hydrophobic compounds, and 0.1% DMSO (vehicle control) or increasing concentrations of plant extract (0-100 μg/ml). Similarly, for the glucose toxicity assay, cells were treated for 96 h in serum-free medium supplemented with 150 mM glucose, 0.025% BSA, and vehicle control or various concentrations of plant extract below its determined LD_{50} value.

Cell viability assay

To assess viable cell number, the formazan dye WST (Roche Diagnostics, Laval, QC) was added to each well following 96 h of treatment in normoglucose (11 mM) or high glucose (150 mM), as described in Chapter 6. After a 60 min incubation with WST, spectrophotometric analysis at 420 nm (formazan) and at 620 nm (reference) was performed using a Tecan Spectra Shell platereader model A-5082 (Durham, NC) and WinSelect software (Tecan US, Inc.). Viable cell number per well was calculated from standard curves produced from wells of known cell density. All treatments were tested in multiple wells over two or three independent experiments (n = 10-15). Data from vehicle-treated control cultures in normoglucose and high glucose were included on all plates and combined across plates when applicable (n = 12-24). Percent viability was calculated as follows:

$$\% \text{ viability} = \text{cell number}_{(\text{treatment well})} / \text{mean cell number}_{(\text{normoglucose control})} \times 100$$

Cell survival assay

Cell survival was directly assessed by Live/Dead viability/cytotoxicity assay

(Invitrogen) as described previously (Harris 2008). Viable cells, identified by the conversion non-fluorescent calcein-AM to fluorescent calcein by intracellular esterases, and dead cells, identified by nuclear incorporation of cell-impermeable ethidium homodimer (ET), were imaged using a DMIR epifluorescent inverted microscope (Leica, Richmond Hill, Canada) coupled with a QICAM digital camera (Quorum Technologies, Guelph, Canada). Images were captured and analysed using OpenLab software v5.05 (Improvision, Lexington, USA). Percent survival was calculated as:

$$\% \text{ survival} = \frac{\text{Viable cell number}^{(\text{calcein}^+ - \text{calcein}^+/\text{ET}^+)}}{\text{Mean viable cells in vehicle control}^{(\text{calcein}^+ - \text{calcein}^+/\text{ET}^+)}} \times 100$$

Phytochemical characterization

The total phenolic content of root and leaf extracts was determined using the Folin-Ciocalteau method described in Chapter 5. Total phenolic content, calculated relative to serially diluted quercetin standard analyzed concurrently with extracts, were expressed as quercetin equivalents. Chromatographic analyses of root and leaf extracts were performed on an Agilent 1100 high pressure liquid chromatography (HPLC) system (Palo Alto, CA, USA) comprising of an autosampler, a quaternary pump, a column thermostat, a photodiode array detector (PAD), and an online atmospheric pressure chemical ionazation mass spectrometer (APCI/MS). Separations were executed using validated method on a YMC ODS-AM column (100 × 2 mm I.D.; 3 μm particle size) (Waters, Mississauga, Canada) as previously described (Harris et al. 2007a) with the minor modifications. Using aqueous trifluoroacetic acid (TFA, 0.05%), pH 3.4 (solvent A) and methanol (solvent B) as mobile phase, initial conditions 92%:8% (A:B) were held for 2 min followed by four linear gradients

of 8 – 13% B in 3 min, 13 – 30% B in 15 min, 30 – 60% B in 5 min, and 60 – 100% B in 4 min. Solvent composition was then returned to initial conditions, which were maintained for 7 min to allow re-equilibration. Solubilized samples were filtered through 0.2 μm PTFE membrane filter (Chromatographic Specialities Inc., Brockville, ON) prior to analysis. Chromatographic separations were monitored on-line at 325 nm, 230 nm and at 520 nm by PAD and MS detection was performed in positive ionization mode as optimized previously (Harris et al. 2007a).

Initial compound identifications were performed by matching the UV spectra of each eluted peak with those of standards registered in an on-line Chemstation library and confirmation of identity was achieved through comparison of fragmentation patterns and relative retention time with those of reference standards and/or isolated compounds identified by NMR. Identified metabolites were quantified on the basis of area under the peak of HPLC-PAD chromatograms (230 nm) using calibration curves produced with pure compounds analyzed on the same day.

Isolation of active compounds

Prior to column-based fractionation, the crude leaf extract was washed with hexane to remove the lipophilic fraction then dried before being solubilized in methanol. To separate the more polar compounds, distilled water was added to the methanolic solution and the resulting precipitate was collected by centrifugation (the methanol fraction) and the remaining soluble fraction (water-methanol fraction) dried by lyophilization. Once the methanol-water fraction had been selected for fractionation, 3 g were loaded onto a 100 x 5 cm Sephadex L20-packed glass column and separated using a step-wise gradient from 100% water to 100% methanol in 10% increments every 100 min. Collected samples were pooled

into 10 fractions based on HPLC chromatographs, each of which was subsequently tested for cytoprotective activity. Subfractionation of the active fraction was conducted using an Agilent 1200 Series semi-preparative HPLC with online PAD and automated time-based fraction collection. In total, 6 subfractions were collected. Additional purification of major compounds within subfractions was achieved by re-running the collected subfractions using high-threshold peak-based collection. The identity of the isolated active was first determined by comparing retention time, UV and MS data with pure those of pure standards, NMR was also used to confirm the structure.

Statistical analyses

Data were analyzed using one-way factorial ANOVA tests followed by *post hoc* Dunnett's *t*-tests on each extract, fraction or pure compound as applicable. *P* values under 0.05 were considered statistically significant and were represented as * (relative to normoglucose control) or # (relative to high glucose control). *P* values under 0.01 were considered highly significant (shown as ** or ##).

RESULTS

LD_{50} concentrations of *S. purpurea* root and leaf extracts were established following 96 h exposure to cells in serum-free, normoglucose (11 mM) conditions. Cell viability was determined by mitochondrial dehydrogenase cleavage of the formazan dye WST relative to standard curves produced from wells of known cell density and expressed as relative to cells treated with vehicle (0.1% DMSO) alone. LD_{50} concentrations were defined as the extract concentration eliciting a 50% loss of viable cells relative to control cultures. Although both extracts were tolerated by cells well beyond physiological concentrations, we found that the

leaf extract was less toxic than that of the root, with LD_{50} concentrations of 129 μg/ml and 56 μg/ml, respectively.

PC12-AC cells, a clonal derivate of the established adrenal pheochromocytoma cell line, can be differentiated to a peripheral neuron phenotype by the combination of serum deprivation and treatment with nerve growth factor. As such, these cells have commonly been used to model neuronal stress and serve as an accessible model of diabetic peripheral neuropathy repeatedly used by us and others (Spoor et al. 2006; Tie et al. 2008; Woronowicz et al. 2007). Consistent with previous reports using the current protocol (Martineau et al. 2006; Spoor et al. 2006), the viability of vehicle-treated PC12-AC cells exposed to elevated glucose concentrations (150 mM) for 96 h was reduced by 40-50% relative to vehicle-treated cells under normoglucose conditions (Figure 7.1, left panel). This cell loss is glucose-specific and not due to osmotic stress as substitution of D-glucose for L-glucose abolishes toxicity (Koshimura et al. 2002). To ascertain whether the protective activity of *S. purpurea* in the glucotoxicity model is organ-specific, root and leaf extracts were evaluated at various concentrations below their respective LD_{50} concentrations (0-30 μg/ml for root and 0-100 μg/ml for leaf). The leaf extract reduced glucose-induced cell loss in a concentration-dependent manner up to 30 μg/ml but failed to provide protection at 100 μg/ml. Conversely, the root had no appreciable effect on glucose toxicity at low concentrations but significantly exacerbated cell loss when concentrations approached the LD_{50} value (Figure 7.1A, right panel).

In order to confirm the cytoprotection offered by *S. purpurea* leaf extracts, a more direct measure of cell survival was employed since mitochondrial dehydrogenase activity can be elevated in cells undergoing mitochondrial-dependent apoptosis (Vogt et al. 2004) and

can lead to erroneous interpretation of results from cell viability assays dependent on the enzyme's activity. Following the same 96 h treatment protocol, serum-deprived cultures were treated for 96 h with DMSO (control) or increasing concentrations of leaf extract and cell survival quantified by Live/Dead assay. This assay allows for direct assessment of viable and dead (or dying) cells; viable cells were identified by cleavage of calcein AM to its fluorogenic product by intracellular esterases and dead or dying cells were identified by uptake of the membrane-impermeant ethidium bromide homodimer. As observed using the WST assay, exposure to leaf extract (0-25 μg/ml) did not effect the survival of cells under serum-free normoglucose conditions (data not shown). Upon exposure to high glucose (Figure 7.2A, left panel), the survival of cells treated with vehicle (DMSO) was compromised by more than 50%. Cell loss was significantly reversed in the presence of leaf extract with concentration-dependent protection (Figure 7.2).

The 80% ethanol extraction of dry leaf material produced a yield of 24.4%. As an initial fractionation step, the leaf extract was washed 3 times with hexane to separate highly lipophilic compounds into one 'hexane' fraction, which represented 6.5% of the crude extract by weight. The resulting defatted fraction was difficult to solubilize at high concentration and, when a methanolic sample was loaded on a Sephadex L20 column, significant precipitation would occur at the interface with the aqueous mobile phase and resulted in a high back pressure. To resolve these problems, the concentrated methanolic extract was mixed with an equal part of cold water and, after centrifugation, resulted in two fractions, the precipitate (methanol) consisting of over 30% by weight of the crude extract and the supernatant (methanol-water). The dried hexane, methanol and methanol-water fractions were each dissolved in DMSO and assessed for protective effects in the high

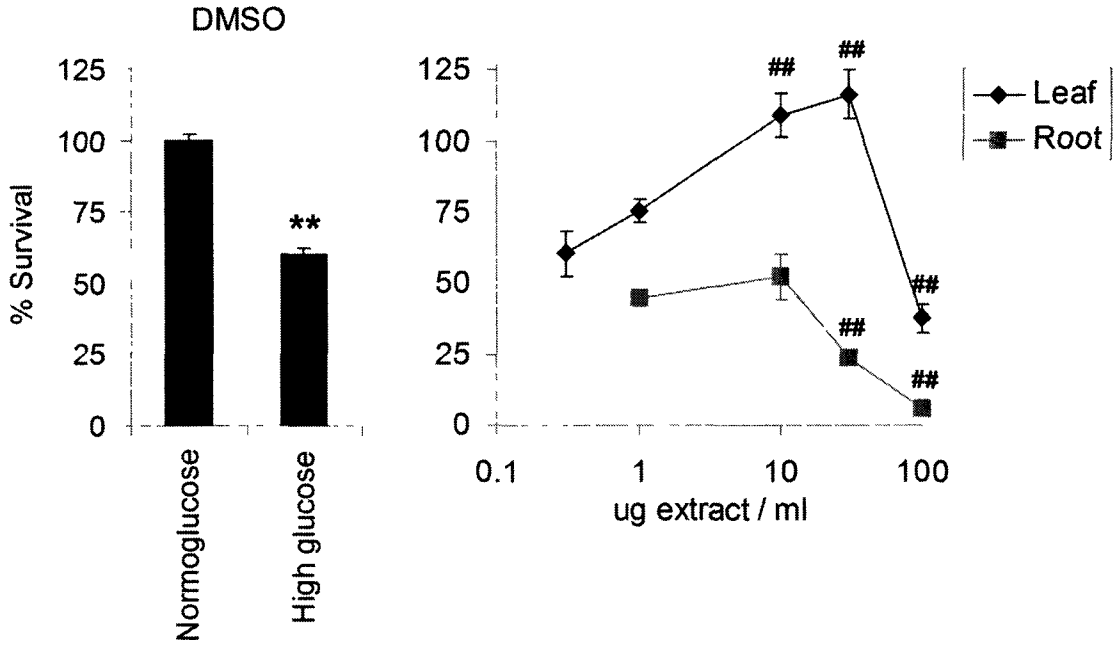

Figure 7.1 – Leaf but not root extract of *Sarracenia purpurea* protects PC12 cells from high glucose-mediated death. Exposure to glucose toxicity elicited a significant loss in cell viability as assessed by mitochondrial dehydrogenase activity measured by cleavage of the formazan dye WST (left panel, ** $p < 0.01$ Student's *t*-test, n=24 wells/condition). A concentration-dependent increase in cell viability was detected in cultures treated with leaf extracts whereas root extract had no protective effect (right panel, ## $p < 0.01$ versus DMSO control, ANOVA, post-hoc Dunnett's *t*-test, n=8-12 wells/condition). Data are reported as mean + SEM. Extracts were tested at concentrations below their LD_{50} concentrations established under normo-glucose conditions.

glucose WST viability assay (Figure 7.3A). Remarkably, all three extracts significantly reduced cell death in a concentration-dependent manner.

Because the methanol-water fraction was the most active, represented the largest fraction of the crude extract, and is likely most similar in chemical content to traditional Cree preparations, this fraction was selected for further fractionation by Sephadex L20 column chromatography. Samples were reconstituted in methanol and fractionated using a step-wise gradient from 100% water to 100% methanol. The collected fractions were analyzed by HPLC-PAD and pooled accordingly into 10 subfractions. When tested in the high glucose paradigm, 3 of the 10 subfractions significantly improved cell viability (Figure 7.3B). Of the three active fractions, fractions 3 and 5 were low in yield and, upon HPLC analysis, revealed a number of minor consitutents while fraction 10 was the second largest subfraction (9.0% of crude extract) and appeared less chemically complex (data not shown). Following confirmation of activity using the Live/Dead assay (Figure 7.3D), fraction 10 was chosen for a third round of fractionation by semi-preparative HPLC with an automated fraction collector. Using time-based peak collection, 7 subfractions (A-G) were collected and prepared for administration to PC12-AC cells. As illustrated in Figure 7.3C and 7.3E, subfraction 10-C provided significant protection from glucose toxicity as determined by both WST and Live/Dead assays.

To identify the active compound(s), subfraction 10-C was analysed by HPLC-PAD/APCI-MS revealing a single peak accounting for > 90% of the total area under the chromatograph with UV and mass spectra consistent with a quercetin glycoside. Using pure quercetin standards for reference and ^{1}H NMR for confirmation, the unknown peak was identified as quercetin-3-*O*-galactoside (Q-3-gal). To verify that this compound was indeed responsible for the effects elicited by the subfraction, the Q-3-gal standard was administered

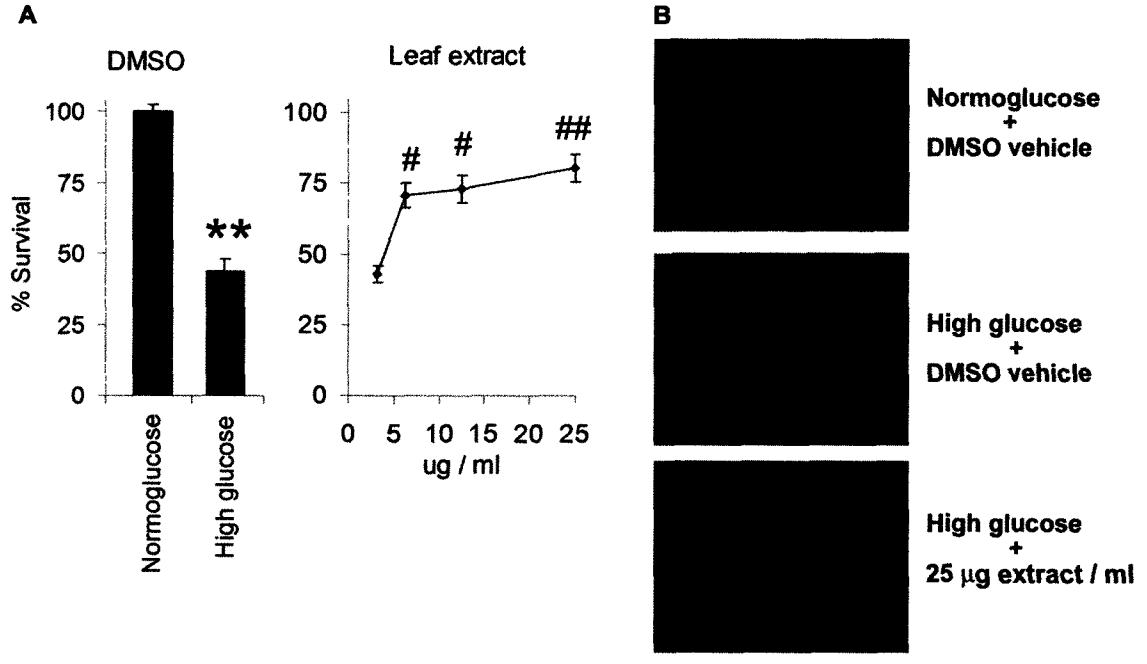

Figure 7.2 – Leaf extract of *Sarracenia purpurea* prevents high glucose-mediated cytotoxicity. (A) Exposure to glucose toxicity elicited a loss in cell survival of approximately 60% as assessed by Live/Dead assay (left panel, ** $p < 0.01$, Student's *t*-test, n=8 wells/condition). A concentration-dependent increase in viable cells was detected in cultures treated with leaf extracts (right panel, # $p < 0.05$, ## $p < 0.01$ versus DMSO control, ANOVA, post-hoc Dunnett's *t*-test, n=8 wells/condition). (B) Treatment with 25 g/ml of leaf extract reversed the high glucose-induced loss of surviving PC12 cells, which were identified by the intracellular cleavage of calcein-AM to its fluorescent product (green cells) without loss of membrane integrity (red cells). Data are reported as mean + SEM. Extracts were tested at concentrations below their LD_{50} concentrations established under normoglucose conditions.

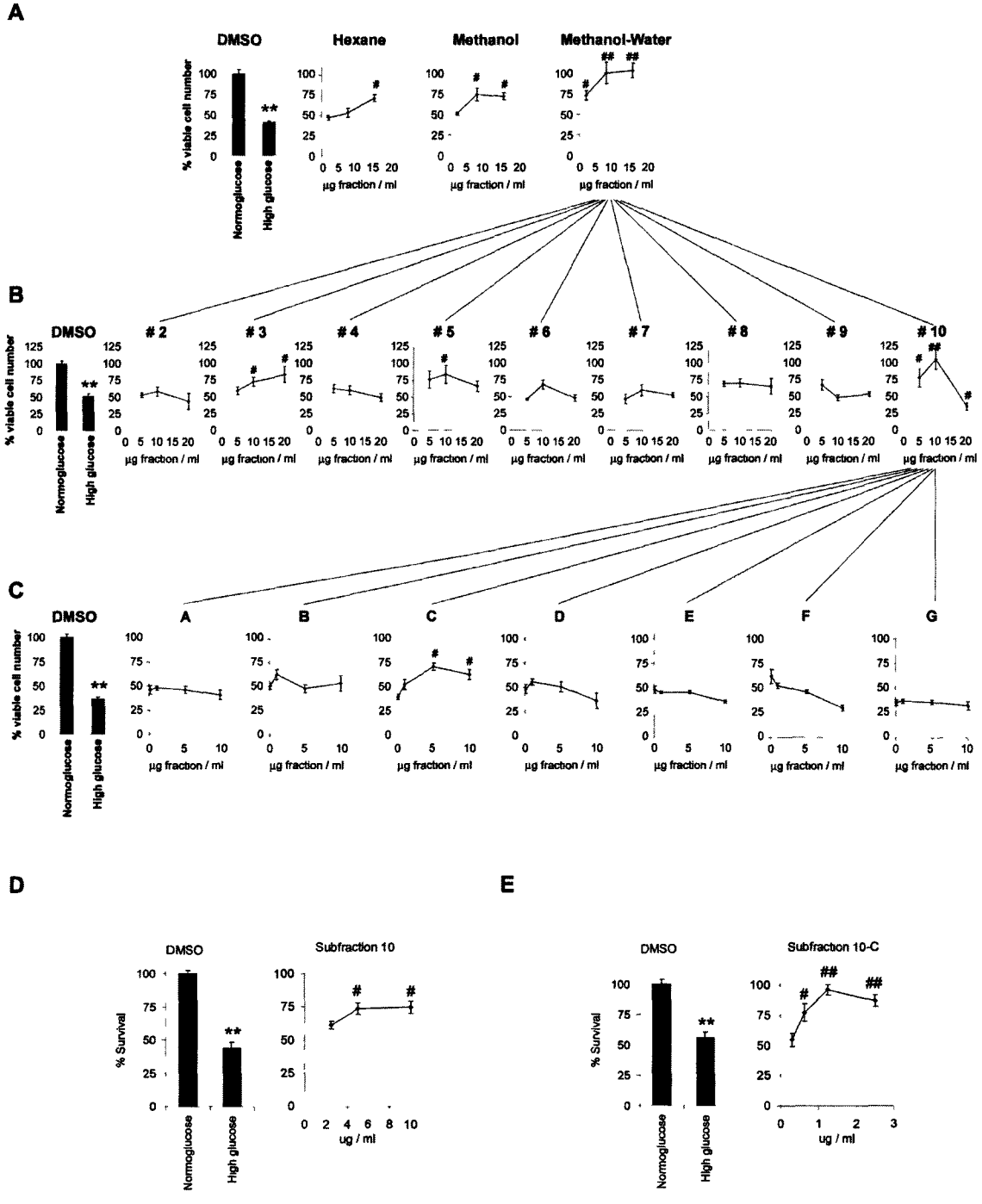

Figure 7.3 – Assay-guided fractionation of active metabolites from *Sarracenia purpurea* leaf extract. (Complete caption on following page)

Figure 7.3 – Assay-guided fractionation of active metabolites from *Sarracenia purpurea* leaf extract. Crude leaf extract was first divided into three fractions based on solubility in hexane, methanol and water. (A) Each of the solvent fractions was evaluated in the glucose toxicity cell viability assay using WST and the methanol-water fraction was selected for further fractionation by Sephadex L20 column chromatography. (B) Each of the subsequent subfractions 1-10 was similarly evaluated using WST and #10 was selected for further fractionation by semi-preparative HPLC. (C) The subsequent subfractions A-G were then evaluated using the WST viability assay. The concentration dependent protective activities of subfractions #10 (D) and #10-C (E) were confirmed using the Live/Dead cell survival assay.

to cells in high glucose resulting in significantly improved viability (Figure 7.4). We next sought to identify and quantitate phytochemical markers of the crude leaf and root extracts. HPLC-PAD analysis revealed distinguishing differences between leaf and root, most notably the absence of the large Q-3-gal peak and surrounding minor peaks representing additional flavonol glycosides (Figure 7.5), as determined by UV spectral analysis. Three common markers, goodyeroside, epicatechin, and morroniside, were identified in both extracts. Goodyeroside and morroniside were isolated during the fractionation process while epicatechin was identified relative to a pure reference standard. For quantitative comparison, the concentration of epicatechin, along with Q-3-gal, in leaf and root extracts were established by HPLC-PAD (Table 7.1). Both goodyeroside, which was only detected by MS, and morroniside appeared to be more concentrated in the leaf extract but the root extract possessed a higher levels of epicatechin and total phenolics (Table 7.1). As a final step, a series of pure flavonoids, were evaluated for activity in high glucose-treated PC12-AC cells. Of these compounds, only quercetin glycosides, as well as the aglycone, were cytoprotective while hesperetin, myricitrin and malvidin diglucoside had no significant effect (Figure 7.4). Goodyeroside, which was detected in both leaf and root extracts, was likewise inactive (data not shown).

DISCUSSION

The objective of the current study was to identify cytoprotective compounds derived from *S. purpurea*, a plant currently used in Cree medicine to treat symptoms of diabetes in a high glucose model of diabetic neuropathy. Here, we show that that anti-diabetic activity in the PC12 model of glucotoxicity is restricted to leaf extracts consistent with reported medicinal usage by the healers and elders of the CEI with the root extract producing no

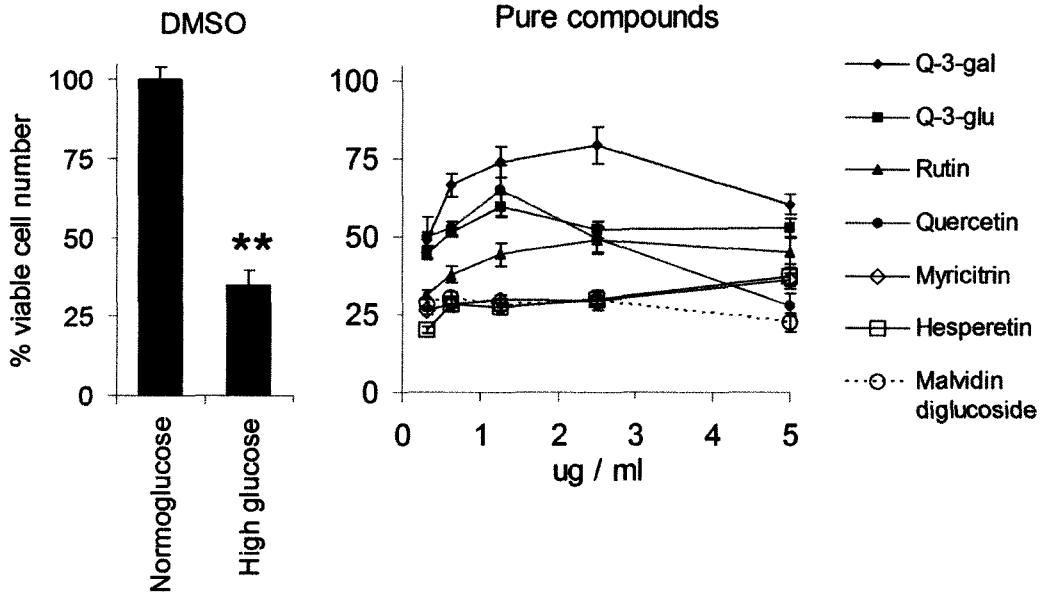

Figure 7.4 – Quercetin derivatives, but not other flavonols, protect PC12 cells from high glucose-mediated death. Exposure to glucose toxicity elicited a significant loss in cell viability as assessed by mitochondrial dehydrogenase activity measured by cleavage of the formazan dye WST (left panel, ** $p < 0.01$ Student's *t*-test, n=24 wells/condition). A concentration-dependent increase in cell viability was detected in cultures treated with quercetin derivatives while other flavonoids had no protective effects (right panel, # $p < 0.05$, ## $p < 0.01$ versus DMSO control, ANOVA, post-hoc Dunnett's *t*-test, n=8-12 wells/condition). Data are reported as mean + SEM. Q-3-gal = quercetin-3-*O*-galactoside; Q-3-glu = quercetin-3-*O*-glucoside; malvidin diglucoside = malvidin-3,5-*O*-diglucoside..

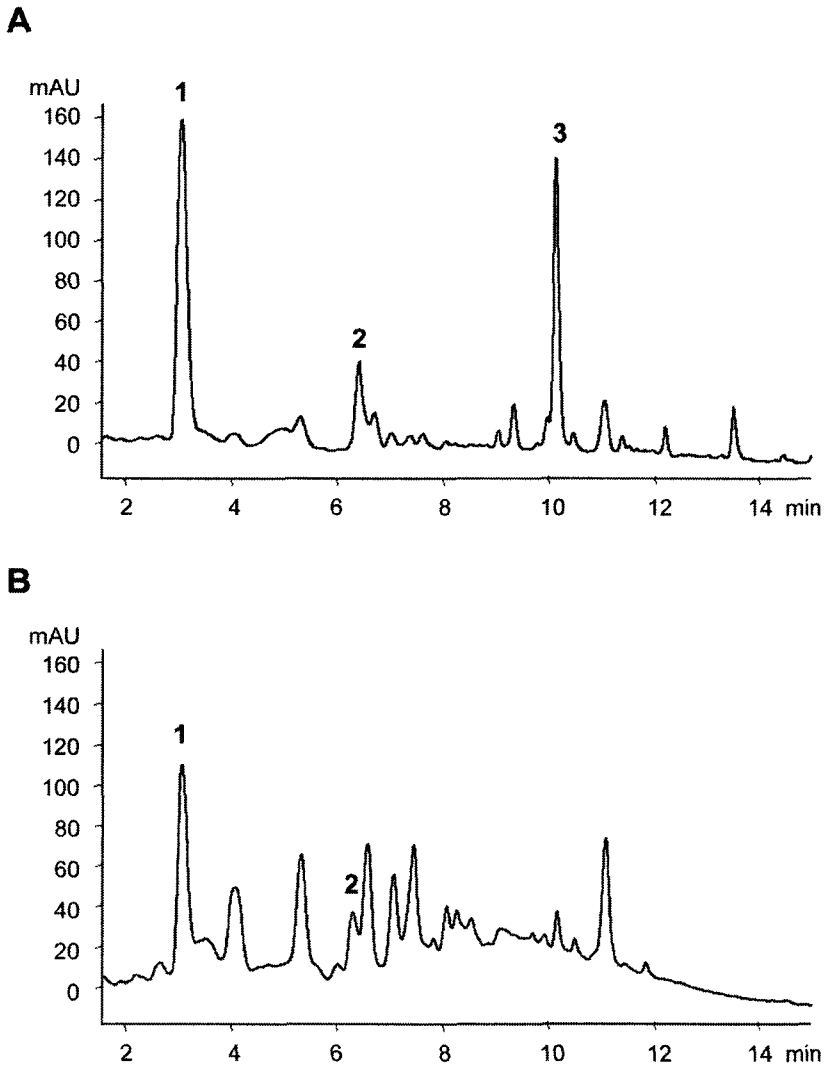

Figure 7.5 – HPLC chromatograms of *Sarracenia purpurea* leaf and root extracts. Chromatograms of (A) leaf and (B) root extracts with photodiode array detection (PAD) at 230 nm. Labelled peaks represent identified metabolites as reported in Table 7.1.

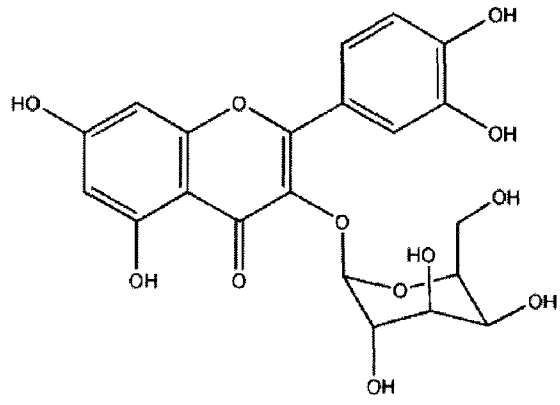

Figure 7.6 – Quercetin-3-*O*-galactoside.

Table 7.1 – Quantitation of phenolic metabolites in leaf and root extracts.

	Leaf		Root	
Yield [a]	24.4%		14.6%	
	mg / g extract	mg / g dry plant tissue	mg / g extract	mg / g dry plant tissue
Total phenolic content [b]	74.5 ± 6.1	18.2 ± 1.5	146.1 ± 6.2	21.3 ± 0.9
1 – morroniside [c]	✓	✓	✓	✓
2 – epicatechin	35.2 ± 1.8	8.6 ± 0.4	48.1 ± 1.7	7.0 ± 0.2
3 – quercetin-3-*O*-galactoside	62.5 ± 1.0	15.3 ± 0.3	n/d	n/d

n/d = not detected

[a] Yield was calculated as (mass recovered extract / mass dry material) x 100%.

[b] Total phenolics expressed as mg quercetin equivalents / g extract.

[c] Morroniside derivative not quantified due to impurities in isolated compound.

positive effect. This organ-specific activity, representing phytochemical differences between extracts, not only provides biochemical validation of the Cree Elders' Traditional Knowledge but also reflects the usage of the plant by other Aboriginal peoples, who generally separate roots from leaves for distinct medicinal purposes (Leighton, 1985; Black, 1980; Chandler, 1979). Through assay-guided fractionation, we demonstrate that all three leaf fractions showed significant activity and therefore contribute to the protective effects of the crude extract. The hexane fraction, though active, was not further pursued as it represented only a small portion of the crude extract and its chemical contents are likely poorly extracted by Cree traditional methods. Similarly, the methanol-water fraction was selected for fractionation as it accounted for more than 60% of the crude extract by weight and is likely more representative of Cree extracts than the methanol fraction. HPLC analysis detected Q-3-gal in the methanol fraction, which may explain some of the fraction's protective effect.

Three active compounds were identified: quercetin, Q-3-gal, and Q-3-glu and while each reduced PC12 cell loss (Figure 7.5), none was as active as the fractions or crude extract. While these findings suggest that additive or synergistics effects may be involved, further investigation is required. Considering the wide range of biological activities ascribed to quercetin and its glycosides, the isolation of Q-3-gal as one of the active metabolites responsible for *S. purpurea*'s cytoprotective effects is not surprising. These data also provide additional insight into the protective activity of other CEI medicinal plants. In our recent study of *Picea glauca,* another Cree medicinal plant, we found that the needle extract, but not those of the bark or cone, protected PC12 cells from high glucose elicited death (Harris 2008). Although assay-guided fractionation was not attempted with *P. glauca* extracts, UV and MS data identified a major quercetin glycoside peak present in the needle but absent from bark and cone extracts (Harris 2008). Moreover, quercetin glycosides such

as Q-3-gal are produced by many plant species, including numerous food, beverage and medicinal plants, and can be found in a variety of natural health products (sometimes referred to as vitamin Q) suggesting a number of potential adjuvants in management of neuropathy complications associated with T2DM.

In addition to the cytoprotective effects observed in the current study, our data are consistent with previous findings indicating that quercetin derivatives display anti-diabetic and neuroprotective activities relevant to the treatment or prevention of diabetic neuropathy. Three of the major factors contributing to microvascular complications of diabetes including neuropathy are oxidative stress, the formation of advanced glycation endproducts (AGEs), and increased flux through the polyol pathway (Aronson 2008; Brownlee 2005). As established anti-oxidants that inhibit both AGE formation (Wu and Yen 2005) and aldose reductase activity (Varma 1986), quercetin derivatives potentially provide a number of defence mechanisms. For this reason, quercetin has been formulated with ascorbyl palmitate and vitamin D_3 and clinically evaluated as a treatment for diabetic neuropathy (Valensi et al. 2005) again validating the traditional usage of *S. purpurea* as an adjuvant anti-diabetic treatment. Although this study was not conducted in primary neurons, we have previously confirmed the neuroprotective activity of plant compounds identified through preliminary testing in PC12 cells (Ryan et al. 2007). Moreover, since quercetin and quercetin glycosides prevent neuronal death in several *in vitro* and *in vivo* models of neurodegeneration (Ha et al. 2003; Spencer et al. 2003), these compounds are neuronally active suggesting that the observed protection in PC12 cells is likely relevant to primary neurons.

The observed effects of *S. purpurea* leaf extract are of increased interest considering that Sarapin, a root extract, is used to relieve pain, a common problem for many individuals with diabetic peripheral neuropathy. Although clinical evidence supporting Sarapin is

incomplete and the active constituents remain unidentified, it has been used for a variety of pain-related ailments (Manchikanti et al. 2001; Rask 1984). While further investigation is clearly required, *S. purpurea* could potentially provide both symptomatic relief and slowed progression of diabetic neuropathy through the preparation of two separate medicines, a leaf tincture and an alkaline root extract.

In summary, we have demonstrated organ-specific cytoprotective activity toward glucose toxicity by the leaf extract of the Cree medicinal plant *S. purpurea.* Beyond providing experimental validation for the traditional knowledge by the CEI, the study also identified quercetin-3-*O*-galactoside as one of the active metabolites, which can serve as a phytochemical marker and guide further investigations into the extract's mechanism(s) of protective activity and *in vivo* pharmacology.

ACKNOWLEDGEMENTS

This work was supported by a CIHR Team Grant to PSH, JTA, AC, and SALB. Very special thanks are due to those Cree Elders from Mistissini and Whapmagoostui who kindly agreed to be interviewed and identified their use of *S. purpurea* for diabetes-related symptoms. They made this article possible by allowing us to use, for the purposes of this research, their knowledge relating to medicinal plants, transmitted to them by their elders. Their trust has also enabled a useful exchange between Indigenous knowledge and Western science.

CHAPTER 8 – ASSESSMENT OF CREE MEDICINAL PLANT EXTRACTS FOR ANTI-GLYCATION ACTIVITY

8.1 PREFACE

In searching for extracts with therapeutic potential in the management of diabetic neuropathy over the previous three chapters, we have focused on preventing cell death directly associated with the glycemic environment. Although glucose toxicity plays an important role in the pathophysiology of diabetic neuropathy, indirect effects of hyperglycemia also contribute to neurodegeneration. Two consequences of unmanaged hyperglycemia are oxidative stress and the formation of advanced glycation endproducts (AGEs). A collaborating lab has recently completed an assessment of the Cree extracts' anti-oxidant capacities, revealing significant correlations between observed activity and both total phenolic content and ethnobotanical anti-diabetes SIV score. In the current chapter, using two assays targeting separate classes of AGEs, we sought to evaluate Cree extracts for their ability to inhibit the *in vitro* formation of AGEs and to explore possible relationships between anti-glycation activity, anti-oxidant capacity, total phenolic content and/or SIV score.

8.2 STATEMENT OF AUTHOR CONTRIBUTIONS

CSH, JTA, and SALB conceived and designed the experiments. CSH and LPBB performed the glycation experiments and fluorescence analyses. The immunochemical analyses were completed by CSH, who wrote the manuscript with JTA and SALB. All anti-oxidant assays were conducted by MHF at McGill University under the supervision of TJ and with the collaboraton of AC. The Elders of CEI shared their Traditional Knowledge,

which served as the basis for this study. PSH and LCM managed and directed the CIHR-TAAM and contributed to project design and manuscript preparation.

8.3 CHARACTERIZING THE INHIBITORY EFFECTS OF CREE MEDICINAL PLANT EXTRACTS ON IN VITRO FORMATION OF ADVANCED GLYCATION ENDPRODUCTS

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ABSTRACT

Like many indigenous peoples worldwide, the First Nations of Canada, including the Cree of Eeyou Istchee, are experiencing devastating rates of Type 2 diabetes mellitus (T2DM) and related complications. Under hyperglycemic conditions characteristic of T2DM, the non-enzymatic formation of advanced glycation endproducts (AGEs) is accelerated and contribute to the development of vascular complications. Consequently, inhibitors of AGE formation are currently receiving considerable attention as therapeutic agents for the prevention and treatment of diabetic complication. In the present study, ethanolic extracts of Cree medicinal plants were assessed for inhibitory effects on AGE formation using two methods, fluorometric detection of fluorescent AGEs and immunochemical detection of N^{ε}-(carboxymethyl)lysine-protein adducts of albumin. Most extracts (76%) demonstrated concentration-dependent inhibition of fluorescent AGE formation allowing for calculation of IC_{50} values. Efficacy of some of these compounds was restricted to low concentrations with efficacy abrogated or even reversed stimulating AGE production as extract concentration surpassed threshold. Immunochemical validation of selected extract-treated proteins revealed similar concentration-dependent reductions in AGE formation. When considered relative to previously published data using the same plant extracts, significant positive correlations were observed between anti-glycation activity, total phenolic content and anti-oxidant activity. Together, our results demonstrate that the phenolic metabolites in Cree medicinal plant extracts prevent *in vitro* AGE formation through anti-oxidant mechanisms and provide pharmacological validation for the traditional anti-diabetic use of these plants.

INTRODUCTION

The global prevalence of Type 2 diabetes mellitus (T2DM) was estimated to be 246 million in 2007, an increase of 79% since 2003 (WHO), positioning the disease as one of the most ominous threats facing human health in the 21st century. As a result, diabetic complications, the main cause of morbidity and mortality among diabetics, have also become an international health concern (Meetoo et al. 2007). While the negative impacts of T2DM are felt throughout developed and developing nations alike, the risk of diabetes is consistently higher among indigenous populations of the world. Canada is no exception with First Nations men and women experiencing 3.6 and 5.3 times higher rates of T2DM than non-native Canadians, respectively (Young et al. 2000). Because access to medical services, daily monitoring of blood-glucose levels and/or compliance with modern T2DM management regimens may be limited in First Nations communities (Assembly-of-First-Nations 2006), hyperglycemia remains uncontrolled, leading to increased incidence and severity of diabetic complications (Young et al. 2000; Yu and Zinman 2007a).

The traditional use of indigenous plants for food and medicine by aboriginal people worldwide offers a unique opportunity to provide T2DM management options that are both culturally and pharmacologically relevant. In addition to representing an integral part of traditional diet and healing, two elements repeatedly identified by aboriginal peoples as central to personal and community health (Ferreira 2006), these plants also represent a source of ethnobotanically selected bioactive compounds. Traditional medicine systems around the world employ thousands of plant species for the treatment of diabetic symptoms and complications and, when evaluated in a laboratory environment, many demonstrate anti-diabetic properties (Kar et al. 2003; Shane-McWhorter 2007). For instance, in a comprehensive review of anti-diabetic plants where more than 1200 plants were screened for

hypoglycemic effects, over 80% of those traditionally used for anti-diabetic purposes were active (Marles 1995). The identification of indigenous traditional plants and assessment of their anti-diabetic potential thus represents a promising approach to developing complementary therapeutic options that are both community-specific and locally accessible.

Since 2003, the Canadian Institute of Health Research Team in Aboriginal Anti-diabetic Medicines, a collaborative project between basic researchers, health professionals and the Cree of Eeyou Istchee (CEI) of Northern Quebec, has followed this approach and, through ethnobotanical survey, identified a number plant medicines traditionally used by the Cree for treating symptoms of T2DM (Fraser 2007; Leduc et al. 2006). Subsequent *in vitro* analysis of the 17 most relevant species revealed a variety of anti-diabetic effects including insulinomimetic, insulin sensitizing, cytoprotective and anti-oxidant activities (See Chapters 5-7 of this thesis and (Fraser 2007; Spoor et al. 2006).

Building on these previous studies, the aim of the current investigation was to assess the protective potential of Cree medicinal plants in relation to vascular complications of diabetes. A common mechanism implicated in the development of both macro- and microvascular complications such as cardiovascular disease, nephropathy, neuropathy, retinopathy, cataract development and atherosclerosis is the increased formation of advanced glycation end products (AGEs) initiated by hyperglycemic conditions (Ramasamy 2006; Rojas and Morales 2004; Sheetz and King 2002). AGEs, a diverse class of biomolecules formed through non-enzymatic reactions between lipids, nucleic acids, or proteins and reducing carbohydrates such as glucose and fructose, accumulate more readily in diabetics and result in protein-protein crosslinkages that contribute to cell and tissue damage by impairing protein function (Smit and Lutgers 2004; Ulrich and Cerami 2001). Certain AGEs, such as Nε-(carboxymethyl)lysine (CML), activate the cell surface receptor for AGE

(RAGE) which in turn activates NF-κB and subsequent downstream proinflammatory pathways implicated in the initiation and progression of vascular complications of T2DM (Bierhaus et al. 2005; Ulrich and Cerami 2001). The reduction of AGE formation through therapeutic intervention has thus emerged as a promising target for the prevention and management diabetic complications.

Many of the plants identified by the Elders of CEI demonstrated strong anti-oxidant capacity that correlated positively with the bulk content of phenolic metabolites. Certain phenolic compounds produced by plants, such as flavonoids, are not only potent anti-oxidants but also exhibit additional activities relevant to diabetic complications, including the ability to inhibit AGE formation (Wu and Yen 2005). Reactive oxygen species (ROS), which are generally elevated in T2DM, promote glycation reactions that often involve oxidative transformations resulting in additional oxidative stress (Cerami 1986; Thornalley et al. 1999). Dietary anti-oxidants such as plant phenolics may therefore offer protection by reducing oxidative formation of AGEs and scavenging ROS produced during glycation reactions (Wu and Yen 2005). We therefore hypothesize that the anti-glycation activity of Cree medicinal plants is mediated through the anti-oxidant properties of their phenolic metabolites. Here, we tested whether the inhibitory effects of Cree medicinal plants on AGE formation will be positively correlated with extract anti-oxidant capacity and total phenolic content.

EXPERIMENTAL

Plant materials and extraction

Seventeen medicinal plant extracts employed by the Cree for treating symptoms of diabetes were collected and extracted for analysis (Table 8.1). Specimens were collected in

the regions of Mistissini and Whapmagoostui of Northern Quebec, Canada, in accordance the instructions provided by CEI Elders. Dr Alain Cuerrier, taxonomist at the Montreal Botanical Garden, ascertained botanical identity of the plant species, and voucher specimens were deposited in the Marie-Victorin herbarium of the Montreal Botanical Garden in Montreal, Quebec, Canada. Dried plant samples were processed and extracted twice with 80% ethanol (10 ml/g dry material) as described in Chapter 5. Dried extracts were conserved in darkness at 4°C and stock solutions (40 mg/ml) prepared in 80 % ethanol were stored at -20°C.

AGE formation assay

Extracts were assessed for effects on AGE formation as previously described (Farsi et al. 2007; Suzuki et al. 2003) with the following modifications. Bovine serum albumin (BSA, Sigma, St. Louis, MO) (1 mg/ml) was incubated with 100 mM glucose/ 100 mM fructose (BDH Chemicals Ltd, Toronto, ON) in sodium phosphate monobasic monohydrate buffer (pH 7.4) with extract (experimental treatment), 80% ethanol vehicle (negative control), or quercetin (positive control). Replicates of each extract treatment without BSA was included to control for analyte autofluorescence (extract control) and a treatment with BSA and vehicle was included to control for fluorescence of BSA (BSA control). On the day of experimentation, stock solutions of each extract were serially diluted and tested at five concentrations (n = 4) in sterile opaque polystyrene 96-well clear bottom plates (Corning Inc., Corning, NY). Plates were covered, sealed with parafilm, and incubated for 7 days at 37 °C in darkness on a mechanical shaker (Series 25 Incubator Shaker, New Brunswick Scientific Co. Inc., Edison, NJ).

Fluorescence-based quantitation of AGE formation

Following incubation, fluorescence was measured using a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, US) at excitation and emission wavelengths of 355 nm and 460 nm, respectively. Plates were then immediately placed at -20°C and stored for immunochemical analyses. Percent inhibition and IC_{50} values were calculated as previously described (Farsi et al. 2007). Fluorescence readings for the negative (vehicle) control and experimental treatments (containing BSA, sugar and extract) and were blanked against the BSA control and appropriate extract controls. The corrected fluorescence readings (F) for the negative control ($F_{\text{negative control}}$) and for the experimental treatments ($F_{\text{experimental corrected}}$) were used to determine the percent inhibition of AGE formation as follows:

$$\text{Percent inhibition} = \frac{(F_{\text{negative control}} - F_{\text{experimental corrected}})}{F_{\text{negative control}}} \times 100\ \%$$

IC_{50} concentrations were defined as the amount of extract in μg/ml required to reduce AGE formation by 50% relative to the negative control as determined by regression analysis (% inhibition vs. log (concentration)).

Immunochemical detection of CML-BSA adducts

Samples (500 μL) collected from all controls and each extract treatment (1.56, 6.25, 25, 100 μg/mL) were first concentrated using Ultracel YM-10 membrane centrifugal filters (Millipore, Billerica, MA, USA) then quantified using a Bio-Rad DC Protein assay Kit (Bio-Rad Laboratories Ltd., Mississauga, ON, CAN) according to manufacturers' instructions.

Proteins were separated by SDS-PAGE under reducing conditions. After the electrophoresis step, one of the gels was stained with Coomassie Blue for use as a loading control. Reversible Ponceau S staining was performed on the second gel prior to immunochemical analysis to confirm consistent protein loading. Western blot analysis was performed using mouse monoclonal antibodies targeted against CML-BSA adducts (Clone 318003, R & D Systems, Minneapolis, MN, USA) and goat anti-mouse IgG polyclonal antibodies conjugated with horseradish peroxidase (R & D Systems) as primary and secondary antibodies, respectively. CML-BSA adducts were visualized with SuperSignal West Pico (MJS BioLynx Inc). To confirm the effects observed by fluorometric measurement, proteins from multiple concentrations of a single extract treatment were analysed simultaneously on the same gel to observe concentration-dependent trends. Immunoreactive bands were quantitated by image densitometry using ImageJ software (version 1.38X, National Institutes of Health, USA).

Statistical analyses

IC_{50} concentrations were determined by log-linear regression of percent inhibition of fluorescent AGE formation and extract concentration. Spearman nonparametric correlation analyses were employed to test associations between anti-glycation activity and previously reported chemical and biological and ethnobotanical data (Fraser et al. (2007) (Harris et al. (unpublished, Chapter 5). All statistical analyses were performed using GraphPad InStat (version 3).

RESULTS

Inhibition of Fluorescent AGE formation by Cree extracts

The current study evaluated the anti-glycation potential of ethanolic extracts of medicinal plants previously identified by Elders and Healers of the CEI as useful for the treatment of diabetic symptoms. Using a standard *in vitro* model of non-enzymatic AGE formation and established methods of detection and quantitation (Farsi et al. 2007; Suzuki et al. 2003), 17 extracts, prepared from the most commonly used organ(s) of the most frequently cited anti-diabetic plant species (Fraser 2007; Leduc et al. 2006) (Table 8.1), were assessed for the ability to prevent the formation of fluorescent and non-fluorescent AGEs. Each extract was tested at multiple concentrations (0.39 – 200 μg/ml) and, where concentration-dependent inhibition was observed, IC_{50} values were calculated.

Though fluorometric analysis yielded data that allowed IC_{50} determination for 13 of the 17 extracts (Table 8.2), the nature of the effects varied between extracts. Whereas certain extracts (*A. balsamea, J. communis, P. banksiana, R. tomentosum, S. planifolia* and *V. vitis-idaea*) elicited concentration-dependent inhibition that plateaued at a maximum effect, other extracts produced biphasic effects, reducing fluorescent AGE formation at lower concentrations yet losing their inhibitory effects or promoting AGE production as extract concentrations increased (Figure 8.1A-B). These extracts included *G. hispidula, K. angustifolia, P. glauca, P. mariana, P. balsamifera, R. groenlandicum, S. purpurea.* While a similar response was seen with *L. laricina*, no IC_{50} was established as maximum inhibition did not reach 50%. Neither *L. clavatum* nor *S. decora* extracts prevented glycation at any concentration tested yet both appeared to intensify the formation of AGEs at high concentrations (Figure 8.1C). The data collected for *A. incana* revealed an unexpected U-shaped biphasic response with an initial concentration-dependent stimulation of AGE formation that was reversed as extract concentrations exceeded 25 μg/ml (Figure 8.1D).

Table 8.1 – Cree medicinal plant species used for the treatment of diabetic symptoms *

Species	Abbreviation	Cree name	Family	Plant part [a]	SIV Rank [b]
Abies balsamea (L.) Mill.	*A. balsamea*	Inaast	Pinaceae	Bark	8
Alnus incana ssp. *rugosa* (Du Roi) R.T. Clausen	*A. incana*	Atuuspiih	Betulaceae	Bark	11
Gaultheria hispidula (L.) Muhl.	*G. hispidula*	Piyeumanaan	Ericaceae	Leaf	17
Juniperus communis L.	*J. communis*	Kaakaachuminatukw	Cupressaceae	Fruit	4
Kalmia angustifolia L.	*K. angustifolia*	Uischichipukw	Ericaceae	Leaf	9
Larix laricinia Du Roi (K. Koch)	*L. laricinia*	Waatinaakan	Pinaceae	Bark	3
Lycopodium clavatum L.	*L. clavatum*	Pastinaakwaakin	Lycopodiaeae	Whole plant	15
Picea glauca (Moench.) Voss	*P. glauca*	Miinhikw	Pinaceae	Needle	6
Picea mariana (P. Mill) BSP	*P. mariana*	Iinaatikw	Pinaceae	Cone	5
Pinus banksiana Lamb.	*P. banksiana*	Uschisk	Pinaceae	Cone	14
Populus balsamifera L.	*P. balsamifera*	Mash-mitush	Salicaceae	Bark	16
Rhododendron groenlandicum (Oeder) Kron & Judd	*R. groenlandicum*	Kaachepukw	Ericaceae	Leaf	2
Rhododendron tomentosum ssp. *subarcticum* (Harjama) G. Wallace	*R. tomentosum*	Wiisichipukw	Ericaceae	Leaf	1
Salix planifolia Pursh	*S. planifolia*	Piyeuwaatikw	Salicaceae	Bark	10
Sarracenia purpurea L.	*S. purpurea*	Aygadash	Sarraceniaceae	Leaf	13
Sorbus decora (Sarg.) C.K. Schneid.	*S. decora*	Muskumanaatikw	Rosaceae	Bark	7
Vaccinium vitis-idaea L.	*V. vitis-idaea*	Wiishichimanaanh	Ericaceae	Fruit	12

* Table adapted from Chapter 5.

[a] The plant part that was most commonly cited by Elders and Healers of CEI and subsequently extracted for experimental use.

[b] Species were ranked according to syndromic importance value (SIV), a relative measure of species' medicinal use by Elders and Healers of CEI for treating different symptoms of T2DM.

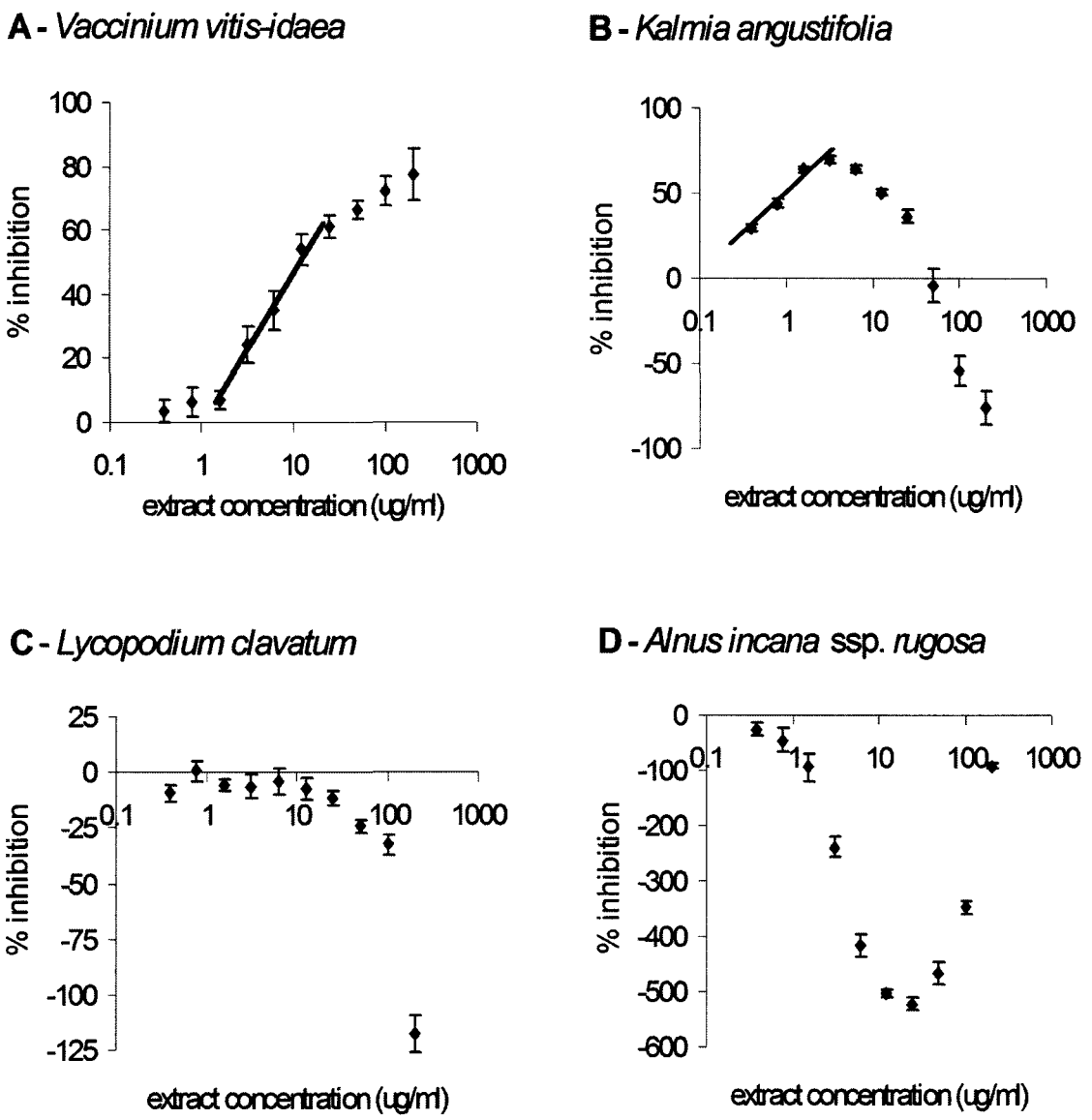

Figure 8.1 – Concentration-dependent effects of representative Cree medicinal plant extracts on in vitro formation of fluorescent AGEs. Results are expressed as the percent inhibition relative to negative control versus extract concentrations of (A) *Vaccinium vitis-idaea*, (B) *Kalmia angustifolia*, (C) *Lycopodium clavatum*, and (D) *Alnus incana* ssp. *rugosa* (means ± SEM for n = 3-4). Where applicable, a logarithmic regression was fitted to the data with IC_{50} values calculated from the corresponding regression equation.

Table 8.2 – Summary of the anti-glycation activities of Cree medicinal plant extracts and their relative rankings.

Species	Anti-glycation activity			Anti-oxidant activity [c]			
	IC_{50} (μg/ml) [a]	IC_{50} Rank	Inhibition of CML [b]	DPPH IC_{50} Rank	Lag time to CDs Rank [d]	TBARS (4h) Rank [e]	TPH Rank [f]
A. balsamea	34.2 ± 12.5	11	Concentration-dependent	11	13	12	12
A. incana	N / A	–	Concentration-dependent	3	2	2	2
G. hispidula	1.5 ± 0.2	4	Concentration-dependent	9	1	10	9
J. communis	5.4 ± 1.6	7	Concentration-dependent	12	8	13	11
K. angustifolia	1.5 ± 0.5	4	Concentration-dependent	5	4	6.5	3
L. laricinia	N / A	–	Concentration-dependent	13	17	14	5
L. clavatum	inactive	13.5	Concentration-dependent	17	16	16	17
P. glauca	6.2 ± 0.6	7	Concentration-dependent	8	14	8	10
P. mariana	2.1 ± 0.22	6	Concentration-dependent	2	3	3	7
P. banksiana	1.5 ± 0.2	4	Concentration-dependent	1	5	1	1
P. balsamifera	21.9 ± 9.7	10	Concentration-dependent	15	11	15	13
R. groenlandicum	7.0 ± 1.7	8	Concentration-dependent	6	7	5	6
R. tomentosum	1.2 ± 0.4	2	Concentration-dependent	7	10	6.5	8
S. planifolia	0.4 ± 0.2	1	Concentration-dependent	4	9	4	4
S. purpurea	38.6 ± 1.9	12	Concentration-dependent	10	6	9	14
S. decora	inactive	13.5	Concentration-dependent	14	15	11	15.5
V. vitis-idaea	10.8 ± 2.1	9	Concentration-dependent	16	12	17	15.5

CML = Nε-(carboxymethyl)lysine; DPPH = 1,1-diphenyl-2-picrylhydrazyl radical; CDs = conjugated dienes; TBARS = thiobarbituric acid-reactive substances; TPH = Total phenolic content; n/d = not determined

[a] IC_{50} values were determined using fluorometric quantitation of fluorescent AGEs.

[b] Inhibition of CML-BSA adduct formation was determined qualitatively using immunochemistry (refer to Figure 8.3).

[c] Anti-oxidant rankings summarized from data presented in Fraser et al. (2007).

[d] Lag time to appearance of CDs with Cu^{2+}-mediated oxidation of human low-density lipoprotein.

[e] Accumulation of TABRS after 4 hours of Cu^{2+}-mediated oxidation of human low-density lipoprotein.

[f] TPH rankings summarized from data presented in Chapter 5.

Inhibition of N^{ε}-(carboxymethyl)lysine adduct formation by Cree extracts

Following quantitation of fluorescent AGEs, immunochemical detection of CML-BSA adducts was conducted on SDS-PAGE separated proteins following control and experimental treatments. As expected, no bands were observed when BSA was excluded from the glycation reaction (BSA control) but two major bands were seen in the vehicle-treated negative control, the larger one of 66 kD representing CML-BSA and the weaker, high-molecular weight band corresponding to cross-linked BSA molecules with at least one CML residue (Figure 8.2). Note that in the positive control containing the flavonoid quercetin (5 μg/ml, 16.5 μM), the intensity of the CML-BSA bands were significantly reduced (Figure 8.2).

While the primary objective of the immunochemical analyses was to provide data regarding a non-fluorescent AGE directly relevant to T2DM, this secondary measure of AGE formation also served to validate the biphasic responses observed through fluorescence-based detection. Proteins collected from samples treated with each extract at different concentrations were purified then separated by SDS-PAGE before probing for CML-BSA adducts using appropriate antibodies (refer to Experimental section). In all cases, Cree extracts inhibited CML formation in a concentration-dependent manner (Figure 8.3). For *A. incana* ssp. *rugosa*, this protective response was not detected fluorometrically (Figure 8.1), either due to autofluorescence or AGE-specific inhibitory effects. The observed effect of *K. angustifolia* extract suggested a similar IC_{50} for fluorescent and CML AGEs but the loss of inhibition seen at high concentrations using the fluorescence assay was not detected immunochemically (Figure 8.1B and 8.3B). Extracts of *L. clavatum* and *L. laricina*, which did not appear active through fluorometry, also showed inhibitory effects (data not shown). Although the relative efficacy of different extracts cannot be compared as analyses were

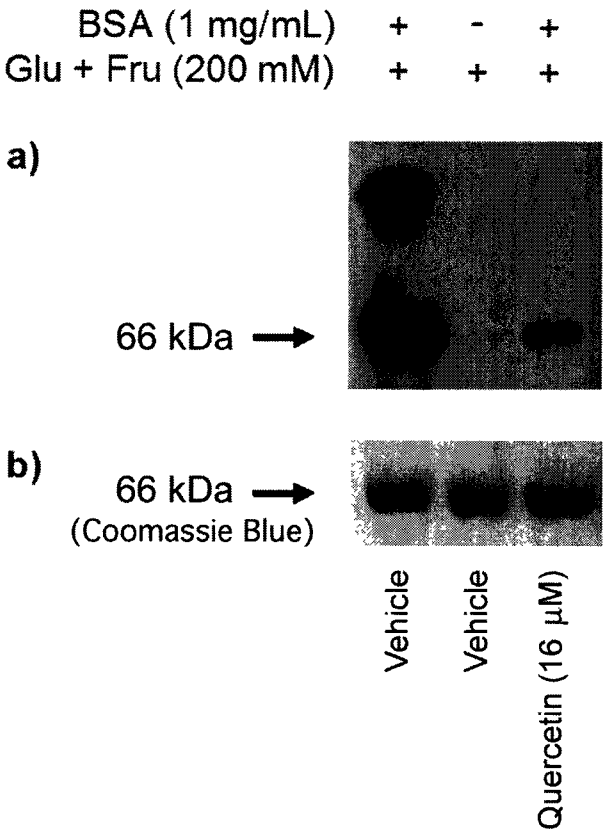

Figure 8.2 – Quercetin reduces the formation of Nε-(carboxymethyl)lysine-protein adducts. Western blot of CML-BSA adducts after 7 days of incubating BSA with glucose/ fructose and vehicle (0.4% ethanol, negative control) or quercetin (16 µg/ml, positive control) at 37°C with agitation. Two simultaneous SDS-PAGE separations were performed, in which 1.9 µg of protein was loaded. The first gel (A) was used for Western blotting with a primary monoclonal anti-CML-BSA antibody and the second (B) was used to verify equal protein loading with Coomassie Blue staining.

performed on different gels, qualitative comparison of immunochemical results corresponded well with the IC_{50} values observed for fluorescent AGE formation (Table 8.2, Figure 8.3).

Inhibition of AGE formation by Cree extracts is correlated with phenolic content and anti-oxidant activity

To test our hypothesis that anti-glycation activity is mediated by the total phenolic content and anti-oxidant effects of a given extract, the plants were ranked according to their IC_{50} values obtained by fluorometry (Table 8.2). Spearman non-parametric correlation analyses were then conducted on anti-glycation ranking relative to the total phenolic content of each extract, previously reported in Chapter 5 (Spoor, 2006) and their anti-oxidant activities as reported by Fraser et al. (2007). The results from this study, which used the same extracts and assessed free radical scavenging activity as well as the ability to delay the appearance of conjugated dienes and thiobarbituric acid-reactive substances induced by Cu^{2+} oxidation of human low-density lipoprotein (LDL), are summarized in Table 8.2 (Fraser 2007). As predicted, inhibition of fluorescent AGE formation was significantly correlated with phenolic content ($r^2 = 0.706$, $p < 0.0001$), phenolic-rich extracts generally yielding lower IC_{50} values (Table 8.3, Figure 8.4A). Similar positive correlations were observed between anti-glycation and anti-oxidant activities, particularly with regards to radical scavenging ($r^2 = 0.572$, $p = 0.0012$), but these relationships were weaker than that observed for total phenolics (Table 8.3, Figure 8.4B-D). No relationships were identified between anti-glycation activity and the traditional anti-diabetic use of the plant species, which was represented by a SIV calculated according to its frequency of citation by informants and the number and specificity of diabetic symptoms for which it is used (Table 8.3).

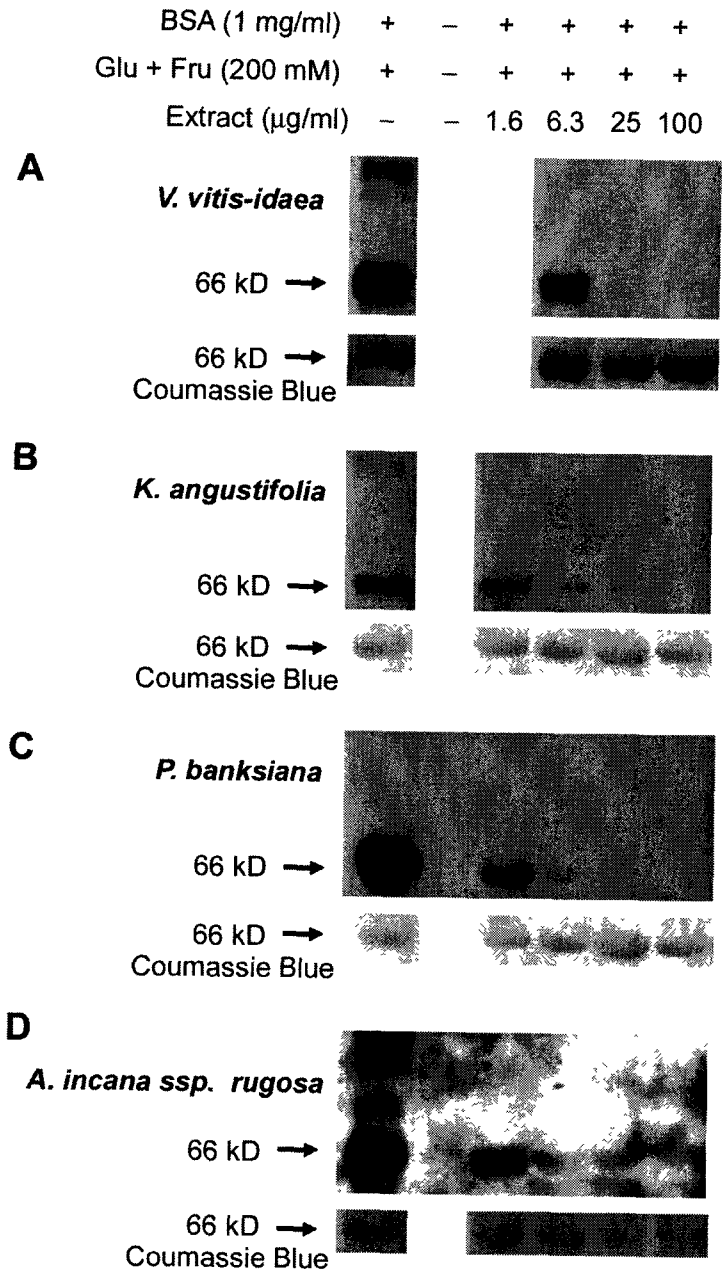

Figure 8.3 – Concentration-dependent effects of representative Cree medicinal plant extracts on in vitro formation of Nε-(carboxymethyl)lysine-protein adducts. Western blot analyses of CML-BSA adducts after 7 days of incubating BSA with glucose/fructose and vehicle or various concentrations of (A) *Vaccinium vitis-idaea*, (B) *Kalmia angustifolia*, (C) *Pinus banksiana*, and (D) *Alnus incana* ssp. *rugosa* at 37°C with agitation. Two simultaneous SDS-PAGE separations were performed, in which 1.9 µg of protein was loaded. The first gel was used for Western blotting with a primary monoclonal anti-CML-BSA antibody (upper panels) and the second was used to verify equal protein loading with Coomassie Blue staining (lower panels).

DISCUSSION

In the current study, 17 ethanolic extracts of medicinal plants used by the CEI to treat T2DM were evaluated for their inhibitory effects on *in vitro* formation of AGEs, which, along with downstream RAGE signaling, have been implicated in the pathophysiology of macro- and microvascular complication of diabetes as well as other age-related cardiovascular and neurodegenerative conditions (Kilhovd et al. 2007; Munch et al. 1997; Simm et al. 2004). Considering the abundance of phenolic metabolites identified within the various extracts (Chapter 5) and the known anti-glycation activities of many phenolic acids, flavonoids and proanthocyanidins (Farrar et al. 2007; Lee et al. 2007; Peng et al. 2008; Wu and Yen 2005), it was not surprising that the majority of extracts were effective. With IC_{50} values below 10 μg/ml in eight extracts and below 2.5 μg/ml in six (Table 2) and given the low micromolar concentrations of some individual anti-glycative plant phenolics reported in plasma of people consuming high-phenolic diets (Erlund et al. 2001; Lotito and Frei 2006; Milbury et al. 2002), these effects likely occur at physiologically relevant concentratons *in vivo*. Indeed, when administered in animal models of diabetes, certain phenolic-containing medicinal plants have effectively reduced AGE formation (Lee et al. 2007; Sohn et al. 2007). However, as many of these plants also reduced glycemia, the anti-glycation activity may not have been direct (Lee et al. 2007; Sohn et al. 2007) and thus, this study, represents one of the first direct demonstrations that Cree plant extracts rich in phenolics exhibit significant anti-AGE activity.

Overall, the majority of published studies investigating AGE-inhibiting plant compounds rely on traditional fluorometric assays. As shown here, while inhibition of fluorescent AGE formation at low concentrations was generally representative of effects on

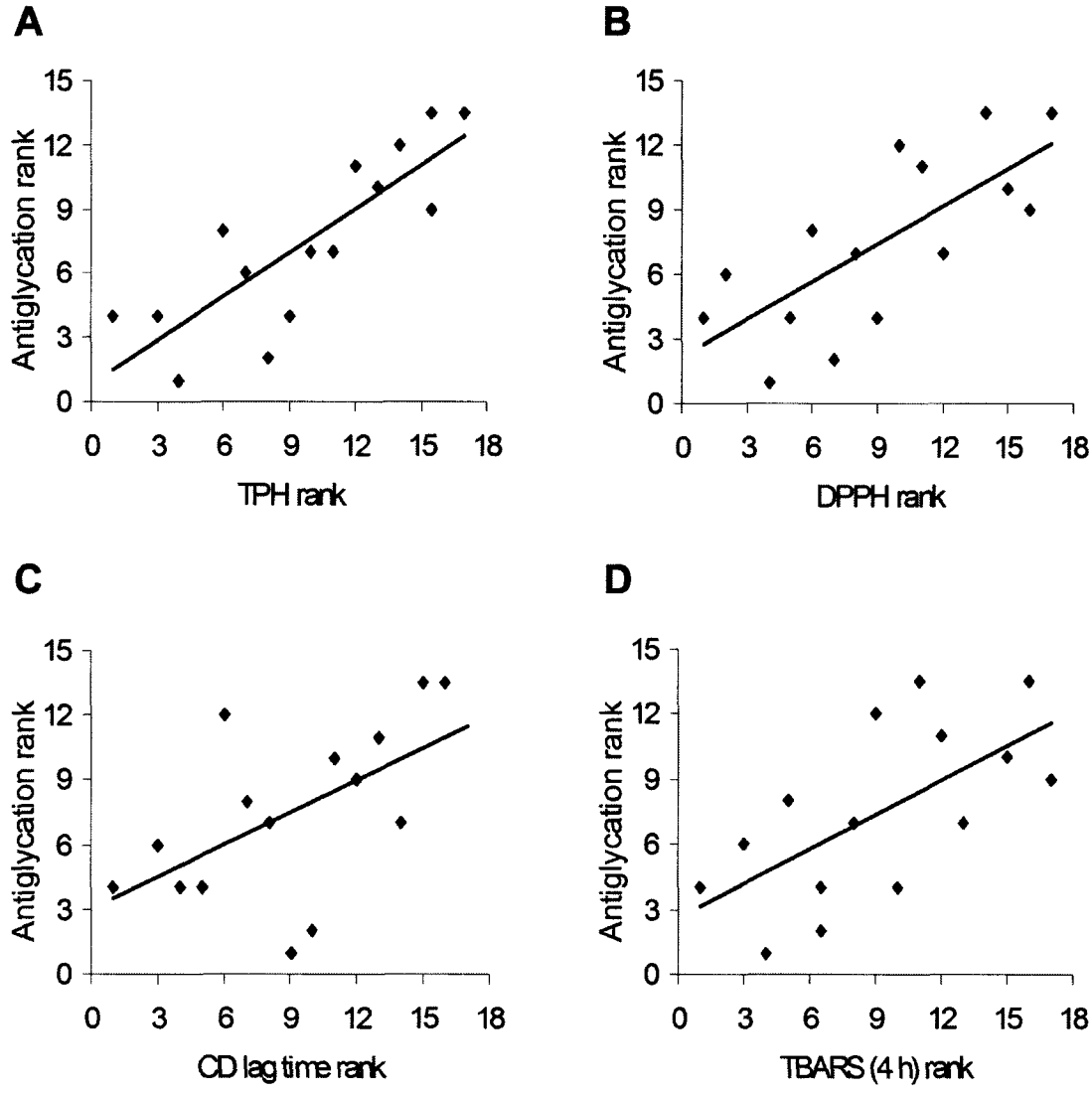

Figure 8.4 – Inhibition of AGE formation by Cree plant extracts were positively correlated with total phenolic content and anti-oxidant activity. Spearman's correlation between anti-glycation ranking as determined by IC_{50} values of individual extracts and their ranking in terms of (A) total phenolic content (TPH), (B) 1,1-di-phenyl-2-picrylhydrazyl (DPPH) radical scavenging activity; and inhibition of Cu2+-mediated formation of (C) conjugated dienes (CD) and (D) thiobarbituric acid-reactive substances (TBARS). All presented relationships were significant ($p < 0.05$). Statistical details are provided in Table 8.3.

Table 8.3 – Results of Spearman correlation analyses of ant-glycation activity, anti-oxidant, activity, total phenolics and ethnobotanical SIV scores.

Parameters	Relationship	Spearman r^2	*p*	DF
IC_{50} - fluorescent AGEs vs. TPH	Positive correlation	0.706	<0.0001	13
IC_{50} - fluorescent AGEs vs. IC_{50} - DPPH scavenging[*]	Positive correlation	0.572	0.0011	13
IC_{50} - fluorescent AGEs vs. Lag time to CD formation [a,*]	Positive correlation	0.345	0.0214	13
IC_{50} - fluorescent AGEs vs. TBARS [a,*]	Positive correlation	0.436	0.0075	13
IC_{50} - fluorescent AGEs vs. SIV [b,*]	No relationship	0.014	0.5262	13

DF = degrees of freedom; DPPH = 1,1-diphenyl-2-picrylhydrazyl radical; TPH = Total phenolic content

[*] Complete data set presented in Fraser *et al.* (2007)

[a] Conjugated diene (CD) and thiobarbituric acid-reactive substances (TBARS) formation was initiated by Cu^{2+}-mediated oxidization of low density lipoproteins.

[b] SIV = Syndromic importance value (Leduc et al. 2006).

CML-BSA adduct formation, readouts appeared to be complicated by problems with extract autofluorescence. The complementary assays performed here demonstrate that the anti-glycative actions of Cree medicinal plants are not limited to fluorescent AGEs, which account for small percentage of physiologically occurring cross-linking structures (Dyer et al. 1991), but extend to CML and possibly other non-fluorescent AGEs. CML levels are drastically elevated in many diabetic patients and have been correlated with the development of diabetic complications (Kislinger et al. 1999; Schleicher et al. 1997; Wautier et al. 2003). Recently, Yamabe *et al.* (2007) reported that AGE inhibition, namely CMLs, reducing downstream signaling through RAGE contributed to the renoprotective effects of *Cornus officinalis* Sieb. & Zucc. on streptozotocin-treated diabetic rats. Further insight into the relative inhibititory effects of extracts on different classes of AGEs could be gained immunochemically with additional AGE-specific antibodies or through analysis by tandem mass spectrometry (Ahmed and Thornalley 2003; Thornalley et al. 2003).

In a previous study on the inhibitory effects of corn silk extracts on *in vitro* AGE formation, a significant positive relationship was observed between extract IC_{50} values and their total phenolic content (Farsi et al. 2007). While the oxidative mechanisms promoting AGE production are well established (Peyroux and Sternberg 2006; Sheetz and King 2002) and potential of dietary anti-oxidant such as polyphenols has been well documented (Matsuda et al. 2003; Urios et al. 2007), the corn silk study was the first to correlate anti-glycation activity with total phenolic content within a collection of crude plant extracts. Here, despite the more diverse collection of plant extracts, our results not only support a positive relationship between the phenolic content of extracts and their AGE inhibiting effects but extend this relationship to include anti-oxidant capacity (Figure 8.4).

As reported by Fraser et al. (2007), the total phenolic content of Cree plant extracts were significantly correlated with their anti-oxidant properties, radical scavenging activity more so than the prevention of lipid peroxidation. The same was observed with regards to anti-glycation activity, which was more significantly related to effects on DPPH radicals than on the formation of either conjugated dienes or TBARS from oxidized LDL (Figure 8.4). This stronger relationship suggests that radical scavenging activity is more directly involved in the anti-oxidant mechanism of Cree extract AGE inhibition, as is possible considering the reactive oxygen and carbonyl species known to promote AGE formation (Peyroux and Sternberg 2006; Ulrich and Cerami 2001). However, the weaker relationship to anti-peroxidative activity likely also reflects the lack of lipids in the experimental glycation mixture. Perhaps evaluation of Cree extracts for inhibitory effects on lipid glycation would reveal more robust relationships with LDL-related anti-oxidant activities. As lipid glycation is also accelerated under the hyperlipidemic conditions of T2DM and, like protein glycation, is believe to contribute to diabetic complications, such a study is warranted. The fact that AGE inhibition was more significantly correlated with total phenolics than anti-oxidant activity suggests that, while anti-oxidants in general may prevent AGE formation, phenolics, which represent one of many types of plant derived anti-oxidants, are particularly effective.

The anti-glycation effects of Cree extracts were not significantly correlated with the ethnobotanically derived SIV scores, which are reflective of the plant species' frequency of citation as treatment for various symptoms of diabetes. One simple explanation for this lack of correlation between activity and ethnobotanical data is that only anti-diabetic plants were evaluated with no control group of non-medicinal or "non-anti-diabetic" plants. A comparison of medicinal and non-medicinal plants is indeed the approach taken by most studies attempting to pharmacological validation of Traditional medicines (Jones et al. 2000;

Marles 1995). This rationalization, however, fails since SIV values were positively correlated with extract anti-oxidant activity as well as total phenolic content (Fraser 2007). An alternative reason for this disparity may be that the positive health impacts of anti-oxidant medicinal plants are selectively or more readily observed compared to anti-glycation activity. Such an explanation is not likely as as oxidative stress is generally implicated in a broader range of pathologies than AGE formation and RAGE signalling while diabetes, the most predominant AGE-related disease, is relatively new to the Cree community.

Current strategies targeting AGE production have met with limited success, notably due to side-effects associated with synthetic inhibitors (Peyroux and Sternberg 2006). A nutritional approach based on foods or herbal supplements with high phenolic content represents an alternative approach to AGE inhibition with a reduced risk of adverse effects. Considering that, in addition to anti-oxidant and anti-glycation effects, certain plant phenolics present in Cree extracts prevent glucotoxic cell death (Chapters 5-7) and inhibit aldose reductase, protein kinase C activation and inflammatory signalling, activities that are all being pursued pharmaceutically as treatments of vascular complications of diabetes, Cree medicinal plants are certainly worthy of the similar consideration.

In the context of integrative management strategies for diabetic patients of Aboriginal ancestry or patients who are simply inclined toward Traditional healing practices, Cree medicinal plants represent a promising, culturally appropriate treatment option. Importantly, as many of the active phenolic metabolites found in boreal plants can be found in plants from around the world, Traditional food and medicine plants of other indigenous societies would likely possess similar therapeutic potential, local relevance and economically accessibility.

SECTION III

ASSESSMENT OF NEUROPROTECTIVE ACTIVITIES

CHAPTER 9: THE EFFECTS OF PHYSIOLOGICAL CONCENTRATIONS OF ISOLATED PHENOLICS ON CELL VIABILITY

9.1 PREFACE

As seen in Section 1, many of the boreal plants employed by the Cree for medicinal purposes, including members of the Ericaceae family, contain a variety of phenolic metabolites. Following *in vitro* assessment of the plant extracts in Section 2, most displayed some degree of anti-diabetic activity. In some cases, such as cytoprotection, anti-glycation and anti-oxidant, phenolic compounds were found to contribute to activity. While plant phenolics have a variety of putative activities, the concentrations employed in many studies are higher than those likely achieved in humans ingesting phenolic-containing foods, medicines or supplements. Because we identified significant effects of individual phenolics at concentrations of physiological relevance, we sought to characterize the effects of structurally diverse phenolic compounds, at low concentration (1 μg/ml), on PC12 cell viability. To investigate potential structure-activity relationships within structural classes, we calculated values for the physicochemical properties of each compound and analysed them relative to observed effects on cell viability.

9.2 STATEMENT OF AUTHOR CONTRIBUTIONS

CSH, SALB, and JTA conceived and designed the experiments. CSH contributed to all experimental and theoretical work and wrote the manuscript with JTA and SALB. FM and LM performed the PC12 cell screening in Table 9.2. FM performed Western blot analysis in Figure 9.5 and contributed to calculations of log P, molecular refractivity and bond dissociation enthalpy (Table 9.1) in collaboration with LC, JSW and WGW. As the

CIHR-TAAM project leader, PSH directed the pharmacological assessment of Cree extracts and pure phenolics and contributed to manuscript preparation.

9.3 PLANT PHENOLICS REGULATE NEOPLASTIC CELL GROWTH AND SURVIVAL: A QUANTITATIVE STRUCTURE-ACTIVITY AND BIOCHEMICAL ANALYSIS

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ABSTRACT

The anti-tumour activities of many plant phenolics at high concentrations (> 100 μM) suggest potential use as dietary supplements in cancer chemoprevention and cancer chemotherapy. However, it is not clear what impact phenolic compounds have on neoplastic cells when present at physiological concentrations obtained through consumption of high phenolic diets. In the present study, 54 naturally occurring phenolics were evaluated at physiologically relevant concentrations for their capacity to alter PC12 cell viability in response to serum deprivation, the chemotherepeutic agent etoposide, and the apoptogen C2-ceramide. Surprisingly, novel mitogenic, cytoprotective, and anti-apoptotic activities were detected. Quantitative structure-activity relationship (QSAR) modelling indicated that many of these activities could be predicted by compound lipophilicity, steric bulk, and/or anti-oxidant capacity, with the exception of inhibition of ceramide-induced apoptosis. Where QSAR analysis was insufficient, biochemical assessment demonstrated that the benzoate orsellinic acid blocked downstream caspase 12 activation following ceramide challenge. These findings demonstrate substantive mitogenic, cytoprotective, and anti-apoptotic biological activities of plant phenolics on neoplastic cells at physiologically relevant dietary concentrations that should be considered in chemopreventive and chemotherapeutic strategies.

INTRODUCTION

Plant-derived phenolic compounds have received considerable attention as dietary anti-oxidants, particularly with regard to aging and its associated health problems (Middleton et al. 2000). Yet, individual compounds trigger a plethora of other biologically relevant effects in mammalian cells, including proliferation, cell cycle inhibitory, anti-apoptotic, and cytotoxic actions not necessarily attributable to their anti-oxidant capacities (Tsuda et al. 2004). As multiple (and sometimes conflicting) therapeutic potentials are elucidated, it becomes increasingly important to identify structure-activity relationships at the circulating concentrations that would be obtained through consumption of phenolic-rich diets (Balunas and Kinghorn 2005; Ji and Peterson 2004; Karakaya 2004; Spencer 2003).

In the current study, 54 phenolic compounds, many with proven cytostatic or cytotoxic effects at concentrations > 100 μM, were screened for mitogenic, cytoprotective, anti-apoptotic, cytotoxic, and pro-apoptotic actions at concentrations between 0.6 and 7.3 μM (Table 10.1). This range was chosen to fall within the physiologically relevant concentration of plasma phenolic concentrations detected in individuals on high phenolic diets (Lotito and Frei 2006). Bioactivities of plant-derived phenolics were assessed in response to the following: (*i*) growth factor-deprivation, (*ii*) DNA damage induced by the topoisomerase II inhibitor etoposide and (*iii*) endoplasmic reticulum stress triggered by the lipid second messenger C2-ceramide (Beck et al. 2001; Darios et al. 2003; Ogretmen and Hannun 2004). These assays tested effects of dietary phenolics on neoplastic cells cultured under conditions designed to promote either growth arrest or apoptosis characteristic of cancer chemopreventive or chemotherepeutic strategies. PC12 cells were used as they respond to growth factor deprivation by growth arrest and, in the presence of nerve growth factor, differentiation to a neuronal phenotype (Eaton and Duplan 2004). Unlike

untransformed adrenal cells, phenotypic differentiation is not terminal and is reversed by exposure to exogenous mitogens (Eaton and Duplan 2004). As such, this cell line offers an ideal model system to rapidly screen the impact of dietary phenolics on neoplastic cell growth and survival under physiological conditions as compared to untransformed or tumour cell types.

To identify underlying structural determinants within classes of phenolics, bioactivities were modelled by quantitative structure-activity relationship (QSAR) analyses. Bond dissociation enthalpy (BDE) was calculated as a measure of oxidant reactivity (Cheng et al. 2003; Wright et al. 2001). Physiochemical parameters of steric bulk and lipophilicity were used to predict compound bioavailability at the cellular level. Molecular refractivity (MR) was used to estimate steric bulk (Hansch et al. 2003). Lipophilicity was determined as the log value of the compound's partition coefficient (log P). A second term, $[\log P]^2$, was also introduced to account for the parabolic relationship between biological activity and lipophilicity relative to partitioning within the cellular environs (Marles et al. 1991; Rosenkranz and Thampatty 2003). Where QSAR analysis was insufficient to account for the variance in observed results, the ability of anti-apoptotic phenolics to inhibit executioner caspase activity was assessed. Surprisingly, we detected significant proliferative and pro-survival activities in 78 percent of the 54 compounds tested that could impact upon their use as cancer chemopreventive or cancer therapeutic adjuvants.

EXPERIMENTAL

Cell culture

PC12-AC cells, a clonal derivate of the PC12 rat adrenal pheochromocytoma cell line (American Tissue Culture Collection), were maintained in complete media (RPMI 1640

containing 10% horse serum and 5% newborn calf serum) and treated in serum-free RPMI 1640 supplemented with 0.025% BSA (treatment media) at 37°C in a 5% CO_2 atmosphere. Cell culture reagents were obtained from Invitrogen. The PC12-AC cell line is a homogenous subclone of the parental PC12 line generated in the Bennett laboratory to ensure genetic homogeneity within a neoplastic background (Bonin et al. 2004; Brewer et al. 2002). This line undergoes growth arrest in serum-deprived media and is responsive to nerve growth factor but is more adherent than the original population and thus more amenable to 96-well bioactivity assays.

Primary Bioactivity Screen

Phenolic compounds were from the phytochemical collections of C Nozzilillo (University of Ottawa, Canada), T Yoshida (Okayama University, Japan) and JT Arnason (University of Ottawa, Canada). Compounds were solubilized in dimethylsulfoxide (DMSO) and used at a final concentration of 1 μg/ml. Ceramide (C2-ceramide, Biomol) and etoposide (Sigma) were prepared in ethanol (EtOH) and used at a final concentration of 20 μM or 25 μM, respectively, based on dose-response analysis establishing ~50% cell loss in PC12-AC cells after a 24 h treatment. Cells were seeded in 96-well plates ($1.25x10^4$ cells/well) and allowed to adhere at 37°C in 5% CO_2 overnight. Cultures were deprived of growth factors in the presence of plant phenolics (1 μg/ml) or vehicle (0.1% DMSO/0.1% EtOH), or challenged with apoptogen and plant phenolic (1 μg/ml) or vehicle. Negative controls included cultures incubated in treatment media only. Following 24 h of treatment, the cell proliferation agent (formazan dye) WST (Roche Diagnostics) was added to each well and incubated for 60 min before spectrophotometric analysis at 420 nm (formazan) and at

620 nm (reference). Cultures were blanked against cell-free treatment media incubated for the same period. Cell number per well was calculated from standard curves derived from wells containing known cell densities. Standardization allowed data to be compared across replicates. Each compound was tested in quintuplicate over two or three independent experiments (n=10-15). Data from control cultures were combined across plates (n=25-80). Percent viability was calculated as follows:

$$\% \text{ viability} = [\text{cell number}_{(\text{treatment well})} / \text{mean cell number}_{(\text{negative control})}] \times 100\%$$

Validation of cell survival, cell death, and mitogenicity

For the Live/Dead Viability/Cytotoxicity assay (Molecular Probes), cells were grown in 96-well black plates with clear bottoms. Viability was determined by the ability of intracellular esterases to cleave calcein-AM, producing a fluorescent wavelength of 515 nm. Cytotoxicity was measured by the ability of ethidium homodimer-1 to enter cells with damaged membranes and intercalate between DNA strands, emitting a fluorescent wavelength of 635 nm. Following 24 h treatments as described above, cells were incubated for 5 min in calcein AM (1.25 μM) and ethidium homodimer-1 (0.75 μM). Counts were performed on a Leica DMIL inverted epifluorescent microscope equipped with a Qimaging QICAM fast 1394 digital camera (Quorum Technologies). Phase contrast and fluorescent photos were taken of five fields per well and counts averaged to yield a single data point per well in duplicate experiments (n = 4-6).

In bromodeoxyuridine (BrdU) incorporation experiments, PC12-AC cells were seeded overnight on 0.1% gelatin-coated glass coverslips in complete media. Cells were subsequently synchronized for 36 hours in serum-free treatment media. BrdU (20 μg/ml,

Boehringer, Germany) was co-administered with 0.1% DMSO or compounds 4, 38, 39, 43 to cells over a 30-minute pulse followed by a 2.5 h chase with DMSO or phenolics only. Incorporated BrdU was labelled with anti-BrdU fluorescein isothiocyanate (Roche). Hoechst 33258 was used as a nuclear counterstain at 2 μg/mL. Cells were photographed (9 shots per coverslip) using a Leica DMXRA2 epifluorescent microscope. BrdU+ cells were counted from fluorescence images and expressed as a percentage of total cells per field. Each compound was tested in triplicate in replicate experiments (n = 6).

Western Analysis of caspase activation

Proteins were collected in RIPA buffer (10 mM PBS, 1% Nonidet P-40, 0.5% Na-deoxycholate, 0.1% SDS, 30 μl/ml aprotonin, 10 mM Na-orthovanadate, 1 μl/ml phenylmethane-sulfonyl fluoride, 1 μl/ml NaF). Protein samples (30 μg) were separated under reducing conditions using SDS-PAGE. Western analyses were performed with monoclonal anti-poly(ADP-ribose) polymerase (PARP, 1:10000, Clontech), detecting both the uncleaved 116 kD fragment and the cleaved 85 kD PARP fragment or polyclonal anti-caspase-12 (1:1000, Stressgen) detecting both uncleaved (55 kD) and cleaved (17 kD) caspase fragments. Monoclonal actin (1:1000, Sigma) was used as a loading control. Secondary antibodies were horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (1:2000, Jackson Immunolabs).

Calculation of physicochemical properties of test compounds

Using ChemDraw Pro Volume 7.0 (CambridgeSoft Laboratory Solutions), the molecular weight, log partition coefficient (log *P*) and molecular refractivity (MR) of test compounds were determined. Due to computational limitations, no log *P* or MR values were

determined for malvidin 3,5-diglucoside and the more structurally complex tannins. Bond dissociation enthalpies (BDE) were predicted by analysis of phenolic substituent effects on proton donating ability as previously described (Wright et al. 2001). BDE values for all hydroxyl groups located on phenolic rings were calculated and the lowest, corresponding to the most stable radical conformation and strongest anti-oxidant capacity, was recorded for use in QSAR analyses. In compounds with hydroxyl-free benzoic rings, BDE could not be estimated. Because the effects of some substituent patterns present in our collection have not been described previously, estimated values were used for calculation as follows: in the case of cinnamic acids, the combined effects of the vinyl and carboxyl groups were considered additive whereas, because of reduced communication with the phenolic ring by saturation of the vinyl group, the three carbon chain in phenyl propanoic acids was considered as a methyl substituent. For hydroxyls located on flavonoids, glycoside groups were regarded as methoxy groups while the C-ring substituent of the B-ring was considered a methyl group for flavanone-type molecules or as a vinyl group for flavone-types. These assumptions were validated according to the lowest level method using Gaussian software as we have previously described (Wright et al. 2001). No additive effects were calculated through conjugated C-rings because the A-ring generally provides additional stability that can be generalized to the group. Similarly, the oxygen atom in the C-ring in most flavonoids provides increased stabilization (not in dihydrochalcones or positively charged anthocyanins) and was not included in our calculations. Thus, our predicted BDEs are conservative estimates.

Table 9.1 – Structures, molecular weights, physicochemical properties and test concentrations of phenolics screened for biological activity.

Class	#	Compound	Substitutions	Mol. Wt.	log P[b]	MR[b] (m^3/ mol)	BDE[b] (kcal/ mol)	[C][c] µM
Benzoic acids	1	Salicylic acid	C_2 = OH	138.1	2.27	33.90	95.1	7.2
	2	3-hydroxybenzoic acid	C_3 = OH	138.1	1.49	33.90	89.5	7.2
	3	Resorcyclic acid	C_2,C_4 = OH	154.1	1.50	35.72	89.2	6.5
	4	Gentisic acid	C_2,C_5 = OH	154.1	1.37	35.72	83.6	6.5
	5	Protocatechuic acid	C_3,C_4 = OH	154.1	0.81	35.72	80.3	6.5
	6	Vanillic acid	C_3 = OMe, C_4 = OH	168.2	1.35	41.15	88.2	6.0
	7	Orsellinic acid	C_2 = Me, C_4,C_6 = OH	168.2	1.30	41.62	88.8	6.0
	8	Gallic acid	C_3,C_4,C_5 = OH	170.1	0.05	37.53	75.5	5.9
	9	Veratric acid	C_3,C_4 = OMe	182.2	1.34	46.59	N/A	5.5
- Benzoids	10	3,5-diOH-4-O-Me benzoic acid	C_3,C_5 = OH C_4 = OMe	184.2	0.58	42.67	87.7	5.4
	11	Syringic acid	C_3,C_5 = OMe C_4 = OH	198.2	0.95	48.40	86.8	5.0
	12	*m*-digallic acid	C_3 = 0-galloyl C_4,C_5 = OH	322.2	1.13	72.49	75.5	3.1
	13	Bergenin	$C_{14}H_{16}O_9$ – see Figure 1	328.3	-1.40	72.29	85.2	3.0
Cinnamic acids	14	Cinnamic acid		148.1	1.00	42.85	N/A	6.8
	15	Trans-cinnamic acid		148.1	1.93	42.85	N/A	6.8
	16	o-coumaric acid	C_2 = OH	164.2	1.57	44.75	91.1	6.1
	17	m-coumaric acid	C_3 = OH	164.2	1.54	44.67	89.3	6.1
	18	p-coumaric acid	C_4 = OH	164.2	1.54	44.76	84.9	6.1
	19	Caffeic acid	C_3,C_4 = OH	180.2	1.15	46.48	75.7	5.6
	20	Ferulic acid	C_3 = OMe, $C_{4'}$ = OH	194.2	1.42	51.92	83.5	5.1
	21	Sinapic acid	C_3,C_5 = OMe C_4 = OH	224.2	1.29	59.17	78.1	4.5
- Phenyl propanoic acids (PPA)	22	3(m-hydroxy)-PPA	C_3 = OH	166.2	1.57	44.75	86.6	6.0
	23	3(p-hydroxy)-PPA	C_4 = OH	166.2	1.56	44.75	84.5	6.0
	24	Hydrocaffeic acid	C_3,C_4 = OH	182.2	1.15	46.45	75.3	5.5
- Phenylacetic acids (PAA)	25	2(m-hydroxy)-PAA	C_3 = OH	152.2	1.15	38.22	86.6	6.5
Coumarins	26	Coumarin		146.1	1.82	42.09	N/A	6.8
	27	Umbelliferone	C_7 = OH	162.1	1.44	43.91	83.9	6.2
	28	Esculetin	C_6,C_7 = OH	178.1	1.05	45.72	73.9	5.6
Flavonoids	29	7-hydroxyflavone	C_7 = OH	240.3	2.41	68.04	88.8	4.2
- Flavones	30	Apigenin	C_5,C_7,$C_{4'}$ = OH	270.2	1.00	73.51	82.3	3.7

Class	#	Compound	Substitutions	Mol. Wt.	log P[b]	MR[b] (m^3/ mol)	BDE[b] (kcal/ mol)	[C][c] µM
	31	Cosmetin[a]	$C_5,C_{4'}$ = OH C_7 = O-glucose	432.4	0.06	107.46	82.3	2.3
	32	Lucenin[a]	$C_5,C_7,C_{3'},C_{4'}$ = OH C_6,C_8 = C-glucose	610.5	-2.94	141.98	73.1	1.6
- Flavonols (C_3 = OH)	33	Quercetin	$C_3,C_5,C_7,C_{3'},C_{4'}$ = OH	302.2	0.35	76.51	73.1	3.3
	34	5-methoxy-quercetin	C_5 = OMe $C_3,C_7,C_{3'},C_{4'}$ = OH	316.2	0.61	80.26	73.1	3.2
	35	Quercitrin[a]	$C_5,C_7,C_{3'},C_{4'}$ = OH C_3 = O-rhamnose	448.4	-0.54	106.36	73.1	2.2
	36	Myricitrin[a]	$C_5,C_7,C_{3'},C_{4'},C_{5'}$= OH C_3 = O-rhamnose	464.4	-0.92	110.23	68.2	2.2
	37	Kaempferitrin[a]	$C_5,C_{4'}$ = OH C_3,C_7 = O-rhamnose	578.5	-1.13	135.56	82.3	1.7
	38	Rutin[a]	$C_5,C_7,C_{3'},C_{4'}$ = OH C3 = O-rutinose	610.5	-2.28	141.69	73.1	1.6
- Flavanones (saturated C_2-C_3 bond)	39	Flavanone		224.3	2.80	66.23	N/A	4.5
	40	Naringenin	$C_5,C_7,C_{4'}$ = OH	272.3	1.90	71.67	84.5	3.7
	41	Hesperetin	$C_5,C_7,C_{3'}$ = OH $C_{4'}$ = Ome	302.3	1.5	78.92	85.2	3.3
	42	Naringin[a]	$C_5,C_{4'}$ = OH C_7 = O-rutinose	580.5	-1.10	137.52	84.5	1.7
- Flavanonols (C_3 = OH)	43	Taxifolin	$C_3,C_5,C_7,C_{3'},C_{4'}$ = OH	304.3	0.58	75.43	75.3	3.3
- Flavan-3-ols (saturated C_2-C_3 no O at C_4)	44	Epicatechin	$C_3,C_5,C_7,C_{3'},C_{4'}$ = OH	290.3	1.50	74.05	75.3	3.4
	45	Epigallocatechin gallate	C_3 = O-galloyl $C_5,C_7,C_{3'},C_{4'},C_{5'}$ = OH	458.4	2.07	110.79	70.4	2.2
- Isoflavones (B-ring at C_3)	46	Daidzein	$C_7,C_{4'}$ = OH	254.2	2.13	69.13	82.3	3.9
- Anthocyanins (no O at C_4)	47	Malvidin-3,5-diglucoside[a]	$C_7,C_{4'}$ = OH $C_{3'},C_{5'}$ = OMe C_3,C_5 = O-glucose	655.6	N/A	N/A	81.2	1.5
- Dihydro-chalcones (open C-ring)	48	Phloridzin[a]	C_5 = O-glucose $C_7,C_9,C_{4'}$ = OH	436.4	-0.48	109.73	83.5	2.3
Tannins	49	Geraniin	See Figure 1	952	0.51	205.53	75.3	1.1
	50	Strictinin	See Figure 1	618	0.14	138.22	75.3	1.6
	51	Pedunculagin	See Figure 1	918	0.74	171.17	75.3	1.1
	52	Oenothein B	See Figure 1	1569	N/A	N/A	74.9	0.6
	53	Casuarinin	See Figure 1	936	0.82	206.82	74.9	1.1
	54	Tannic acid	See Figure 1	1701	N/A	N/A	75.5	0.6

[a] Flavonoid glycoside

[b] Log partition coefficient (log *P*), molecular refractivity (MR), and bond dissociation enthalpy (BDE) values were calculated as described in Experimental Procedures.

[c] Screening concentration of 1 ppm phenolic in µM.

Statistics

All statistics were performed with SYSTAT software (version 10). Biological data were analysed by analysis of variance (ANOVA) followed by Fisher's Least Squared Differences (LSD) *post hoc* tests in screens or Dunnett's *t* tests for validation studies. QSAR modelling was conducted by simple regression between individual physicochemical parameters and measured biological activity. Multiple linear regressions were employed to identify more complex relationships between multiple parameters confirmed by backward stepwise regression analysis. Models with P values below 0.05 were considered significant while models with P values <0.08 were subjected to further manipulations in search of stronger relationships.

RESULTS

Fifty-four compounds, representing five classes of plant phenolics, were screened at a standard concentration of 1 ppm for their ability to alter viable cell number under conditions of growth factor deprivation or in response to etoposide- or ceramide-induced apoptosis. This protocol allowed us to test multiple phenolics within a physiological range falling between 0.6 and 7.3 μM (Table 9.1) consistent with the circulating levels of multiple phenolic compounds obtained in plasma through consumption of a high phenolic diet (Lotito and Frei 2006). Viable cell number was initially established by mitochondrial dehydrogenase cleavage of the formazan dye WST relative to standardized controls of known cell densities and then validated by direct assessment of mitogenicity and cell death. The five phenolic classes studied were: (*i*) benzoic acid derivatives (n=13), (*ii*) cinnamic acid derivatives, including phenylpropanoic acid (PPA) and phenylacetic acid (PAA) derivatives

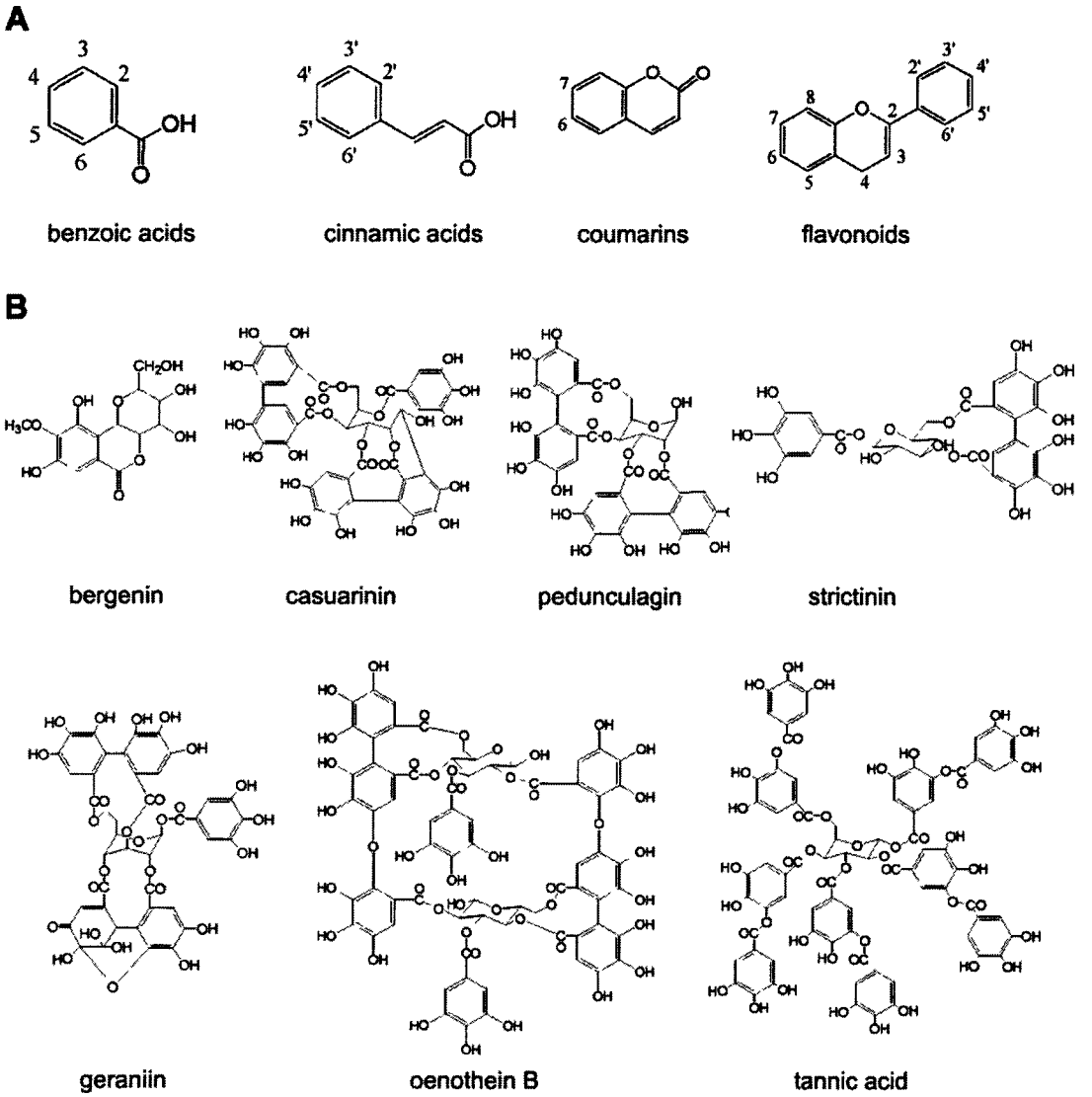

Figure 9.1 – Chemical structures of the plant phenolic compounds. The structure and substitution locations of parental molecules for benzoic acids, cinnamic acids, coumarins, and flavonoids (A). The structures of bergenin and the 6 hydrolysable tannins (B) are indicated. Structures were made using ChemDraw.

(n=12), (*iii*) coumarins (n=3), (*iv*) flavonoids (n=20), and (*v*) hydrolysable tannins (n=6) (Table 9.1, Figure 9.1). The members of each class differed in terms of the type and position of benzyl side groups and the saturation of the vinyl group in the cinnamic acids or the C-ring in the flavonoids (Table 9.1, Figure 9.1). In tannins (all gallotannins, Figure 10.1), the number, position, and binding of gallic and ellagic acid residues varied among members. Purity of compounds was >95% based on HPLC analysis verified by HPLC/MS as described in Harris et al. (2007a). Two different phenolic collections from the Yoshida and the Arnason/Nozzolillo laboratories were assessed separately revealing no differences in biological activity of compounds common to both collections.

Effects on cell viability

Viability of cells treated for 24 h in serum-deprived media without vehicle was comparable to cells treated with 0.1% DMSO (phenolic vehicle) and 0.1% EtOH (apoptogen vehicle) and was standardized to 100% (Table 9.2). Twenty-four compounds (44%) increased viable cell number (*, $p < 0.01$) compared to vehicle-treated cultures in serum-free media, 26 had no effect and four decreased viable cell number (**, $p < 0.01$) (Table 9.2). In etoposide-treated cells, 28 phenolics (52%) reduced etoposide-induced cell loss, 23 had no effect and three potentiated toxicity. Similarly, 24 compounds increased cell number in ceramide treated cultures while 28 had no effect and only two increased cell loss (Table 9.2).

Compounds with no effect on cell viability under all growth conditions tested

Seven percent of tested compounds (three benzoic acids and one flavonoid) had no effect on cell viability under any of the test conditions. These were compounds 1, 2, 5, and

Table 9.2 – Bioactivity of 54 plant phenolics in PC12-AC cells exposed to serum deprivation, etoposide, or ceramide.

#	Compound	Percent viable cell number ± SEM[a]			Identified Biological activity
		Serum withdrawal	Etoposide (25 μM)	Ceramide (20 μM)	
Vehicle Controls					
	DMSO/EtOH (vehicles)[b]	100 ± 1.1	42.8 ± 1.6	44.4± 2.4	---
Benzoic acids					
1	Salicylic acid	110.8 ± 5.0	50.7 ± 2.7	33.6 ± 6.0	No effect
2	3-hydroxybenzoic acid	112.7 ± 5.3	40.9 ± 4.8	64.3 ± 9.6	No effect
3	Resorcyclic acid	91.4 ± 5.5	57.6 ± 4.1[c]	38.7 ± 3.5	Anti-apoptotic
4	Gentisic acid	170.9 ± 13.6[c]	68.6 ± 7.0[c]	104.9 ± 13.4[c]	Mitogenic[f,g]
5	Protocatechuic acid	85.7 ± 7.0	46.4 ± 6.3	29.2 ± 6.6	No effect
6	Vanillic acid	98.9 ± 4.8	68.2 ± 3.0[c]	39. ± 7.8	Anti-apoptotic
7	Orsellinic acid	104.6 ± 4.5	58.3 ± 5.1[c]	67.6 ± 5.4[c]	Anti-apoptotic[f,g]
8	Gallic acid	137.9 ± 2.5[c]	49.5 ± 2.2	32.6 ± 11.0	Cytoprotective
9	Veratric acid	154.5 ± 6.7[c]	63.1 ± 2.2[c]	63.7 ± 12.2[c]	Mitogenic
10	3,5-diOH-4-O-Me BA	96.0 ± 2.4	65.0 ± 2.6[c]	65.7 ± 13.2[c]	Anti-apoptotic
11	Syringic acid	137.7 ± 10.3[c]	74.3 ± 7.7[c]	40.6 ± 8.9	Mitogenic
12	*m*-digallic acid	65.4 ± 7.1[d]	49.2 ± 6.4	75.3 ± 5.4[c]	Cytotoxic / Anti-apoptotic
13	Bergenin	99.8 ± 5.2	78.2 ± 3.7[c]	26.2 ± 6.3	Anti-apoptotic
Cinnamic acids					
14	Cinnamic acid	108.1 ± 9.3	53.4 ± 1.8	84.8 ± 12.3[c]	Anti-apoptotic
15	Trans-cinnamic acid	105.0 ± 6.4	37.4 ± 4.7	69.4 ± 9.6[c]	Anti-apoptotic
16	o-coumaric acid	83.7 ± 2.6[d]	51.2 ± 5.1	98.5 ± 7.1[c]	Cytotoxic / Anti-apoptotic
17	m-coumaric acid	128.6 ± 10.6[c]	66.8 ± 8.6[c]	77.2 ± 16.0[c]	Mitogenic
18	p-coumaric acid	89.3 ± 5.7	17.1 ± 1.1[d]	56.7 ± 5.7	Cytotoxic
19	Caffeic acid	103.7 ± 8.0	30.3 ± 1.1	96.2 ± 6.8[c]	Anti-apoptotic
20	Ferulic acid	120.3 ± 6.5[c]	21.4 ± 1.0[d]	39.1 ± 9.0	Cytotoxic[f]
21	Sinapic acid	117.7 ± 4.1[c]	41.7 ± 1.1	71.9 ± 10.2[c]	Mitogenic
22	3(m-hydroxy)-PPA	109.7 ± 2.7	85.1 ± 6.7[c]	42.1 ± 3.4	Anti-apoptotic
23	3(p-hydroxy)-PPA	57.7 ± 4.2[d]	38.3 ± 3.6	14.9 ± 3.8[d]	Cytotoxic
24	Hydrocaffeic acid	95.0 ± 2.9	64.1 ± 4.4[c]	54.4 ± 4.3	Anti-apoptotic
25	2(m-hydroxy)-PAA	120.4 ± 6.7[c]	53.9 ± 6.4	95.3 ± 8.9[c]	Mitogenic
Coumarins					
26	Coumarin	158.4 ± 13.3[c]	62.8 ± 4.2[c]	127.7 ± 14.2[c]	Mitogenic
27	Umbelliferone	120.5 ± 7.5	58.8 ± 6.8[c]	35.6 ± 4.7	Anti-apoptotic
28	Esculetin	142.2 ± 10.7[c]	59.2 ± 8.1[c]	91.8 ± 10.4[c]	Mitogenic
Flavonoids					
29	7-hydroxyflavone	83.9 ± 5.8[d]	52.9 ± 5.9	41.1 ± 6.1	Cytotoxic

#	Compound	Percent viable cell number ± SEM[a]			Identified Biological activity
		Serum withdrawal	Etoposide (25 μM)	Ceramide (20 μM)	
30	Apigenin	105 1 ± 5 8	23 3 ± 0 7[d]	58 7 ± 5 7[c]	Pro-apoptotic / Anti-apoptotic
31	Cosmetin[h]	169 7 ± 5 6[c]	61 5 ± 4 1[c]	40 6 ± 3 3	Mitogenic
32	Lucenin[h]	139 7 ± 9 1[c]	61 0 ± 5 0[c]	27 0 ± 4 1	Mitogenic
33	Quercetin	94 9 ± 4 6	52 4 ± 5 8	54 0 ± 8 5[c]	Anti-apoptotic[g]
34	5-methoxy-quercetin	150 9 ± 9 5[c]	41 2 ± 2 5	57 0 ± 9 5[c]	Mitogenic
35	Quercitrin[h]	125 5 ± 3 9[c]	84 2 ± 5 7[c]	36 1 ± 3 5	Mitogenic
36	Myricitrin[h]	125 2 ± 4 7[c]	62 7 ± 4 1[c]	50 4 ± 6 8	Mitogenic
37	Kaempferitrin[h]	92 2 ± 6 7	75 1 ± 4 1[c]	26 1 ± 1 7	Anti-apoptotic
38	Rutin[h]	130 4 ± 9 0[c]	74 3 ± 5 1[c]	29 4 ± 4 3	Mitogenic[e f]
39	Flavanone	105 3 ± 11 0	32 1 ± 4 4	26 9 ± 5 9	No effect[e f]
40	Naringenin	102 1 ± 9 7	42 3 ± 3 5	10 2 ± 1 8[d]	Pro-apoptotic
41	Hesperetin	93 7 ± 4 2	60 6 ± 3 4	86 3 ± 7 9[c]	Anti-apoptotic
42	Naringin[h]	117 6 ± 5 1[c]	55 7 ± 3 6	50 6 ± 6 8	Cytoprotective
43	Taxifolin	149 1 ± 7 2[c]	35 4 ± 1 9	103 0 ± 9 0[c]	Mitogenic[e]
44	Epicatechin	138 5 ± 7 7[c]	55 9 ± 4 1	56 8 ± 8 2[c]	Mitogenic
45	Epigallocatechin gallate	116 8 ± 2 2	60 8 ± 3 7[c]	20 9 ± 2 9	Anti-apoptotic
46	Daidzein	85 4 ± 3 1	74 4 ± 4 0[c]	64 3 ± 6 7[c]	Anti-apoptotic
47	Malvidin-3,5-diglucoside[h]	134 0 ± 5 8[c]	65 5 ± 6 8[c]	69 0 ± 11 4[c]	Mitogenic
48	Phloridzin[h]	120 3 ± 4 3[c]	29 6 ± 3 3	43 8 ± 4 4	Cytoprotective
Tannins					
49	Geranin	143 2 ± 9 6[c]	74 0 ± 6 0[c]	45 7 ± 7 2	Mitogenic
50	Strictinin	137 1 ± 10 9[c]	98 3 ± 6 6[c]	84 6 ± 10 8[c]	Mitogenic
51	Pedunculagin	104 7 ± 5 4	100 7 ± 4 6[c]	29 73 ± 5 3	Anti-apoptotic[f]
52	Oenothein B	103 4 ± 7 5	115 1 ± 9 0[c]	66 3 ± 2 1[c]	Anti-apoptotic
53	Casuarinin	73 4 ± 2 1	117 6 ± 6 9[c]	17 6 ± 5 2	Anti-apoptotic
54	Tannic acid	135 7 ± 15 4[c]	66 8 ± 7 2	51 7 ± 3 5	Cytoprotective

[a] Viable cell number was established by WST assay compared to standard curves of known cell number following treatment in 1) growth factor-deprived media, 2) with etoposide in growth factor-deprived media, and 3) with ceramide in growth factor-deprived media as defined in Experimental Procedures

[b] Mean viable cell number for vehicle controls includes all experimental trials (n=110) Note that statistics were performed by phenolic class using only the yoked controls run with each phenolic (n=10-15) per group (n=25-80)

[c] Significantly greater than the vehicle control (ANOVA, *post hoc* Fisher LSD, $p < 0.01$)

[d] Significantly less than the vehicle control (ANOVA, *post hoc* Fisher LSD, $p < 0.01$)

[e] Biological activity was confirmed by quantification of $BrdU^+$ nuclei following serum starvation as described in Experimental Procedures

[f] Biological activity was confirmed by LIVE/DEAD assay as described in Experimental Procedures

[g] Ability to inhibit caspase 3 and 12 cleavage in response to etoposide and ceramide was assessed by Western analysis as described in Experimental Procedures

[h] Flavonoid glycoside

39 (Table 9.2). Compound 39, flavanone, was employed as a negative control for the activity validation experiments.

Cytoprotective and mitogenic activities

Cytoprotective compounds were defined as phenolics that promoted cell viablity in the absence of exogenous growth factors without protecting PC12-AC cells from etoposide or ceramide challenge. Compounds 8, 42, 48, and 54 met these criteria (7% of test compounds) (Table 9.2). To distinguish between cytoprotective and mitogenic activities, we compared effects on viable cell number in the three test paradigms. Mitogenic progression through the G1/S or G2/M checkpoints can protect tumour cell lines, including PC12 cells, from etoposide and ceramide-induced apoptosis (Kurosu et al. 2005; Lee et al. 2005b; Misasi et al. 2001). Nineteen phenolics (35% of test compounds) both increased cell number following serum deprivation and reduced etoposide- and/or ceramide-induced cell death. Compounds 4, 9, 11, 17, 21, 25, 26, 28, 31, 32, 34-36, 38, 43, 44, 47, 49 and 50 were categorized as mitogens (Table 9.2).

Since an increase in mitochondrial dehydrogenase activity and hence WST absorbance can occur in the absence of cell proliferation (Vogt et al. 2004), assigned bioactivities of selected compounds identified in 96-well screens were validated by direct assessment. Growth factor-deprived cultures were treated for 24 h with DMSO/EtOH (control) or compounds 4, 20, 28, and 39 and cell survival quantified directly by Live/Dead assay. Viable cells were capable of cleaving calcein AM to its fluorogenic product. Dead cells were identified by uptake of the membrane-impermeant ethidium bromide homodimer. Consistent with the WST screen (Table 9.2), compound 39 had no effect on cell survival whereas compounds 4 and 38 increased the number of viable cells present in serum-deprived

cultures without affecting the basal rate of cell death (Figure 9.2A). By contrast, compound 20 increased WST absorbance (Table 9.2) but decreased the number of viable calcein^{+}/ethidium bromide^{-} cells (Figure 9.2A). These data pointed to an increase in mitochondrial enzymatic activity in dying cells. We further confirmed this cytotoxicity by quantifying terminally compromised ethidium bromide^{+} cells (Figure 9.2A, inset).

To confirm mitogenicity, PC12-AC cells were cell cycle synchronized by serum starvation for 36 h before stimulation with vehicle or test phenolics. After a 30 min pulse in the presence of the thymidine analog BrdU, cells were chased for 2.5 h with DMSO or phenolics. As predicted by WST results, compound 39 did not affect the number of BrdU^{+} cells while compounds 4, 43, and 48 promoted entry into S-phase (Figure 9.2B). Increased BrdU labelling strongly correlated with WST estimation of increased cell number (Figure 9.2B, inset).

Cytotoxic effects of plant-derived phenolics

Of the 54 test compounds, only 8 (15%) reduced PC12-AC viability under one or more of the three test conditions. Six phenolics, compounds 12, 16, 18, 20, 23 and 29, decreased metabolic activity and/or cell survival in growth factor-deprived media and were classified as cytotoxic (Table 9.2). Of these, 18 and 20 also potentiated etoposide-induced cell loss while 23 increased ceramide-induced cell death. Four compounds exhibited more complex interactions. Compounds 12 and 16 reduced PC12-AC viability in the absence of exogenous growth factors but partially protected cells from ceramide-induced cell death. These phenolics were thus classified as both cytotoxic and anti-apoptotic (Table 9.2). Compound 40 potentiated ceramide-induced cell death without affecting cell survival in serum-free conditions and was classified as pro-apoptotic. Compound 30 did not alter

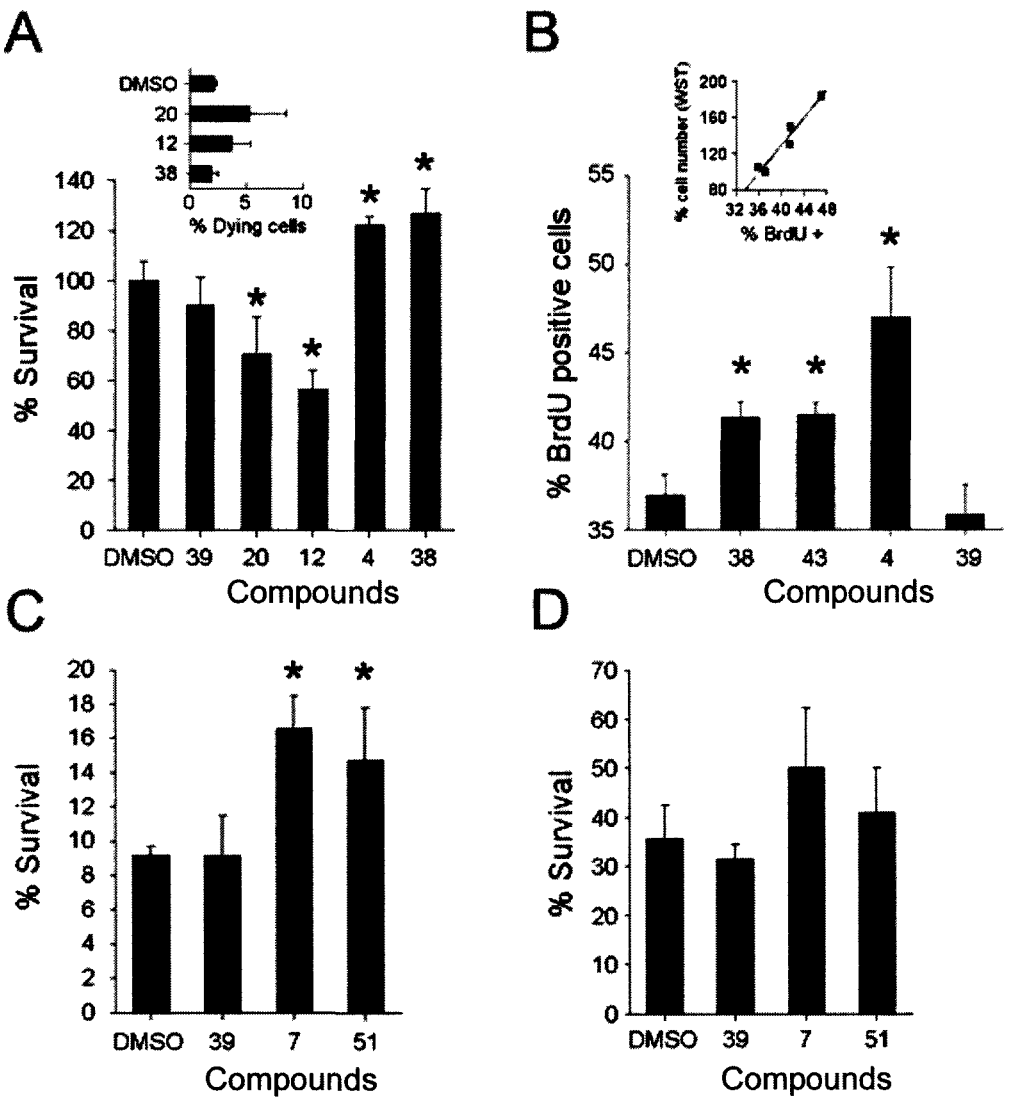

Figure 9.2 – Validation of assigned bioactivities of plant phenolic compounds. (A) PC12-AC cells were cultured in growth factor-deprived media for 24 h in the presence of vehicle control or test phenolics and cell survival was assessed by Live/ Dead assay. Data represent % survival standardized to vehicle-treated cells + SEM. Inset depicts the number of ethidium bromide+ dead cells detected in treated cultures. N=4-6 performed over duplicate experiments. (B) PC12-AC cells were synchronized by serum starvation for 36 h and pulse-chased with BrdU and phenolics. Data represent the % BrdU-labelled cells. N=6-9 performed over duplicate experiments. Inset depicts linear regression comparing WST assay and BrdU labelling validating the WST assay as a measure of cell proliferation. R2=0.947, $p < 0.01$. (C) Cell survival following etoposide or (D) ceramide treatment was assessed by Live/Dead assay. Statistics were ANOVA and *post-hoc* Dunnett's *t*-test (* $p < 0.05$).

viability of growth factor deprived cultures, augmented etoposide-induced toxicity, yet inhibited ceramide-induced apoptosis and therefore was classified as both pro- and anti-apoptotic depending upon the apoptotic pathway initiated (Table 9.2).

Anti-apoptotic activities of plant-derived phenolics

Nineteen compounds inhibited ceramide- or etoposide-induced cell death without altering viability of vehicle-treated cultures. These anti-apoptotic phenolics were: 3, 6, 7, 10, 13-15, 19, 22, 24, 27, 33, 37, 41, 45, 46, and 51-53 (Table 9.2). Including the three phenolics exhibiting more complex interactions (compounds 12, 16, 30), 22 phenolics (41%) exhibited significant anti-apoptotic actions in one or both of the cell death models. The anti-apoptotic activities of compound 7 and 51 were confirmed by Live/Dead assay following etoposide challenge (Fig 9.2C and 2D).

Calculation of physicochemical parameters within phenolic families

To identify structural determinants underlying these phenolic bioactivities, lipophilicity (log *P*) and steric bulk (MR) values were calculated for each compound (Table 9.1). Only phenolics containing sugar moieties, including bergenin (compound 13) and flavonoid glycosides, produced negative log *P* values, indicating preferential partitioning to the hydrophilic phase. The tannins, which also contain sugars, were more lipophilic, producing scores between 0 and 1. All cinnamic acids, coumarins, and most of the benzoic acids and flavonoid aglycones produced values between 1 and 2. Compounds 1, 29, 39, and 46, were the only compounds with log *P* values greater than 2, signifying a 100-fold preference for lipophilic conditions. As expected, calculated MR values were lowest for the simple phenolics (benzoic acids: 34-72 m^3/mol, cinnamic acids: 38-59 m^3/mol, coumarins:

42-46 m^3/mol) and higher for flavonoids (68-141 m^3/mol) and tannins (>138 m^3/mol), particularly those containing glycosidic, galloyl (trihydroxyphenolic), or hexahydroxydiphenolic residues.

We determined theoretical bond dissociation enthalpy (BDE) values as a measure of anti-oxidant capacity as described in Wright et al. (2001). To validate these calculations, we compared our predicted values to empirical measures reported in the literature for the same phenolics (Baderschneider and Winterhalter 2001; Cai et al. 2006; Meyer and Frankel 2001; Pannala and Rice-Evans 2001). Linear regression detected a significant correlation between predicted BDE values and those obtained using the radical ABTS and DPPH radical scavenging assays (data not shown). BDE is an indication of chain-breaking anti-oxidant ability with lower energies providing superior activity. A BDE value below, near, or above 80 kcal/mol designates strong, weak, and poor anti-oxidant potential, respectively, with regards to lipid peroxidation. In general, cinnamic acids were predicted to be better anti-oxidants than benzoic acids, but weaker than flavonoids and other polyphenolics (Table 9.1). Catechol (3,4-dihydroxy) and galloyl (3,4,5-trihydroxy) substitution patterns decreased BDE values. Of the benzoic acids, only compound 8, its condensation product compound 12 and, to a lesser extent, compound 5 possessed BDEs indicative of good anti-oxidant potential. Similarly, compounds 19, 21, and 24 were the only cinnamic acids with BDEs below 80 kcal/mol. Compound 28, a catechol-containing coumarin, presented the lowest BDE among phenolic acid derivatives (73.9 kcal/mol). Flavonoids are recognized anti-oxidants and, correspondingly, many members presented BDE values between 70-79 kcal/mol resulting in family-wide mean of 78.5 kcal/mol. Unlike log *P* and MR values, glycosylation of flavonoids did not strongly influence BDE values. Though the calculated BDEs across tannins remained fairly constant (74.9-75.5 kcal/mol), the number of trihydroxyphenolic

substituents varied from compound to compound, resulting in more or fewer sites of potential radical scavenging. For instance, compounds 50 and 52 respectively contain 9 and 22 benzene-associated hydroxy groups but shared nearly identical BDEs (Table 9.1).

Analysis of covariance between the physicochemical parameters revealed several significant relationships within families. For benzoic acids, steric volume (MR) increased with decreasing log *P* value ($p=0.023$, $r^2=0.386$), as substitution of the phenolic ring with polar side-groups resulted in greater bulk and hydrophilicity. Within the cinnamic acids, log *P* values were all greater than 1 thus $[\log P]^2$ values showed near perfect covariance. Additionally, the calculated BDEs of cinnamic acids decreased with decreasing log *P* values ($p=0.017$, $r^2=0.531$), again due to substitution of the phenolic ring. The flavonoids displayed a similar but much stronger relationship between log *P* and MR ($p=0.000$, $r^2=0.866$) as larger glycosidic compounds were markedly more hydrophilic than aglycones.

QSAR modelling of phenolic bioactivities

Compounds exhibiting similar biological activities within given families were analyzed as subgroups. Subgroups with fewer than six compounds were not considered. Coumarins are derived from the phenylacrylic skeleton of cinnamic acids. As such, the cinnamic acids were assessed with and without the coumarins as analytical subgroups. Owing to limited physicochemical data, no QSAR analyses were conducted on the tannins.

Structure-activity relationships were identified for the flavonoids and benzoic acids but not for the cinnamic acids/coumarins. The physicochemical properties of flavonoids were strongly correlated with their mitogenic and cytoprotective activities. As expected, phenolic capacity to increase PC12-AC viability in growth factor-deprived cultures depended, in part, on relative anti-oxidant potential but, surprisingly, when all 20 flavonoids

were analyzed, only a weak correlation was identified ($p=0.062$, $r^2=0.190$, Figure 9.3A). However, when we restricted our analysis to the aglycones, the anti-oxidant model was significantly strengthened ($p=0.037$, $r^2=0.437$, Figure 9.3B). This separation also revealed a second structure-activity relationship within the glycosides wherein effects on cell viability under conditions of serum deprivation was a function of lipophilicity (Figure 9.3C). According to this model, viability was optimized at log *P* approaching -0.8, suggesting that the glycosides are acting in the aqueous phase.

Anti-apoptotic activities in the etoposide paradigm were strongly correlated with physicochemical properties. Among flavonoids, protection increased with MR (Figure 9.4). Although this model was not improved by separate analysis of flavonoid subgroups, the relationship largely reflects the finding that the bulky glycosides displayed significant activity while the smaller aglycones were generally inactive (Table 9.2). Within the benzoic acids, anti-apoptotic activity following etoposide treatment was associated with lipophilicity and steric bulk.

Interestingly, the anti-apoptotic effects in the ceramide death paradigm could not be modelled with the considered physicochemical parameters for any phenolic class beyond the presence of a catechol group associated with reduced ceramide toxicity among flavonoid aglycones (compounds 33, 34, 43 and 44) (Table 9.2).

Compound 7 (orsellinic acid) inhibits caspase 12 cleavage

In the absence of a strong QSAR model, the ability of select phenolics to protect PC12-AC cells from ceramide-induced apoptotic signalling was assessed biochemically. We chose a catechol-containing anti-apoptotic flavonoid (compound 33) and an anti-apoptotic benzoic acid (compound 7) for mechanistic evaluation. Both etoposide and C2-ceramide

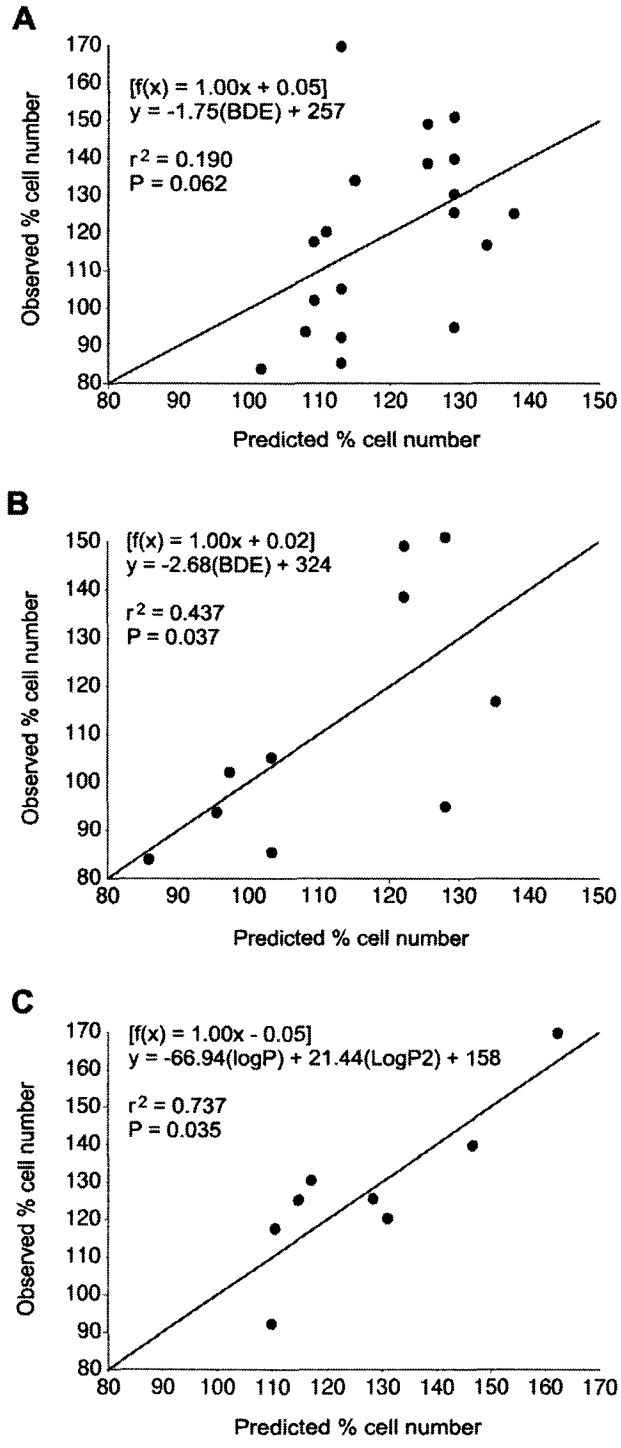

Figure 9.3 – QSAR models of flavonoid activity in growth factor-deprived cells. (A) QSAR analysis of flavonoid activity in growth factor-deprived cells and compound anti-oxidant capacity, BDE. (B) QSAR analysis as in A but of the aglycone subgroup. (C) QSAR as in A but of the glycoside subgroup and lipophilicity, log P. Trend lines represent the expected values predicted by the given QSAR model. Refer to Table 9.1 for individual physicochemical values.

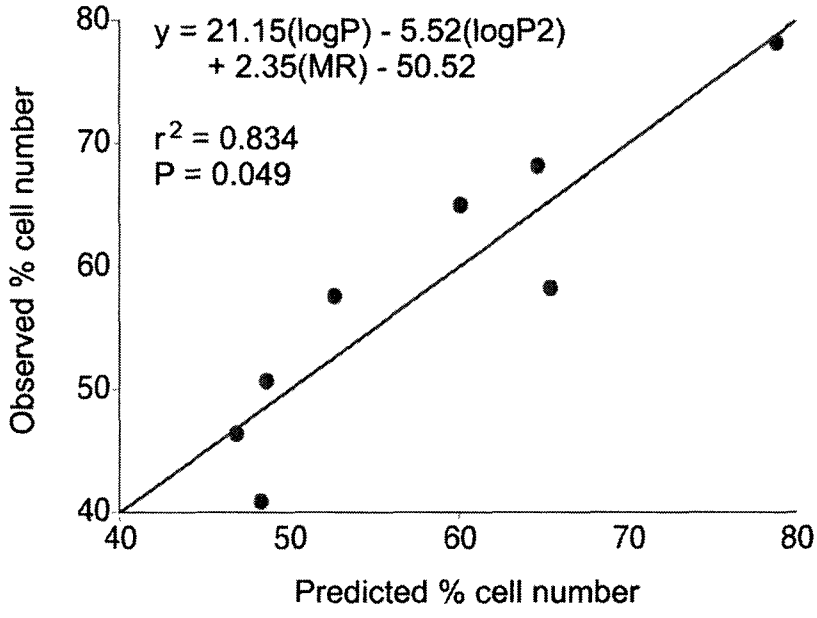

Figure 9.4 – QSAR models of benzoic acid activity in etoposide-treated cells. QSAR analysis of the anti-apoptotic activity of benzoic acids and lipophilicity (log P) and steric bulk (MR). Trend lines represent the expected values predicted by the given QSAR model. Refer to Table 1 for individual physicochemical values.

elicit caspase-dependent apoptosis with caspase 3 activation (Scorrano et al. 2003) but only ceramide triggers an endoplasmic reticulum stress pathway (Darios et al. 2003; Mandic et al. 2003) activating caspase 12 in PC12 cells (Smith et al. 2005). Figure 9.5 confirms that etoposide and ceramide treatment activated caspase 3, indicated by cleavage of the caspase 3 substrate PARP, but only ceramide activated caspase 12, determined by cleavage of pro-caspase 12 to its active form, in PC12-AC cells. Exposure to compounds 7 and 33 had no effect on PARP cleavage following etoposide (data not shown) and ceramide treatment, whereas compound 7, but not 33, inhibited caspase 12 cleavage in ceramide-treated cultures (Figure 9.5).

DISCUSSION

Dietary phenolics have been promoted as an adjuvant means of reducing cancer incidence and sensitising tumour cells to existing chemotherapeutic agents (d'Ischia et al. 2006; Landis-Piwowar et al. 2006; Lee and Lee 2006). Underlying molecular mechanisms have only begun to be elucidated. In this study, we present the results of an *in vitro* screen designed to compare the bioactivities of a panel of natural phenolic compounds at concentrations ranging between 0.6 to 7.3 μM (Table 9.1). Whereas similar studies have focused on the pharmacological activities of phenolics at higher concentrations (> 50 μM), few have examined effects on cell viability at the physiologically relevant dietary concentrations. We compared 54 phenolics for their capacity to enhance or reduce PC12-AC cell viability under conditions of growth-factor deprivation or following challenge with C2-ceramide or etoposide. Our test compounds included compounds previously shown to potentiate tumour cell death in response to chemotherapeutic agents (i.e., epigallocatechin

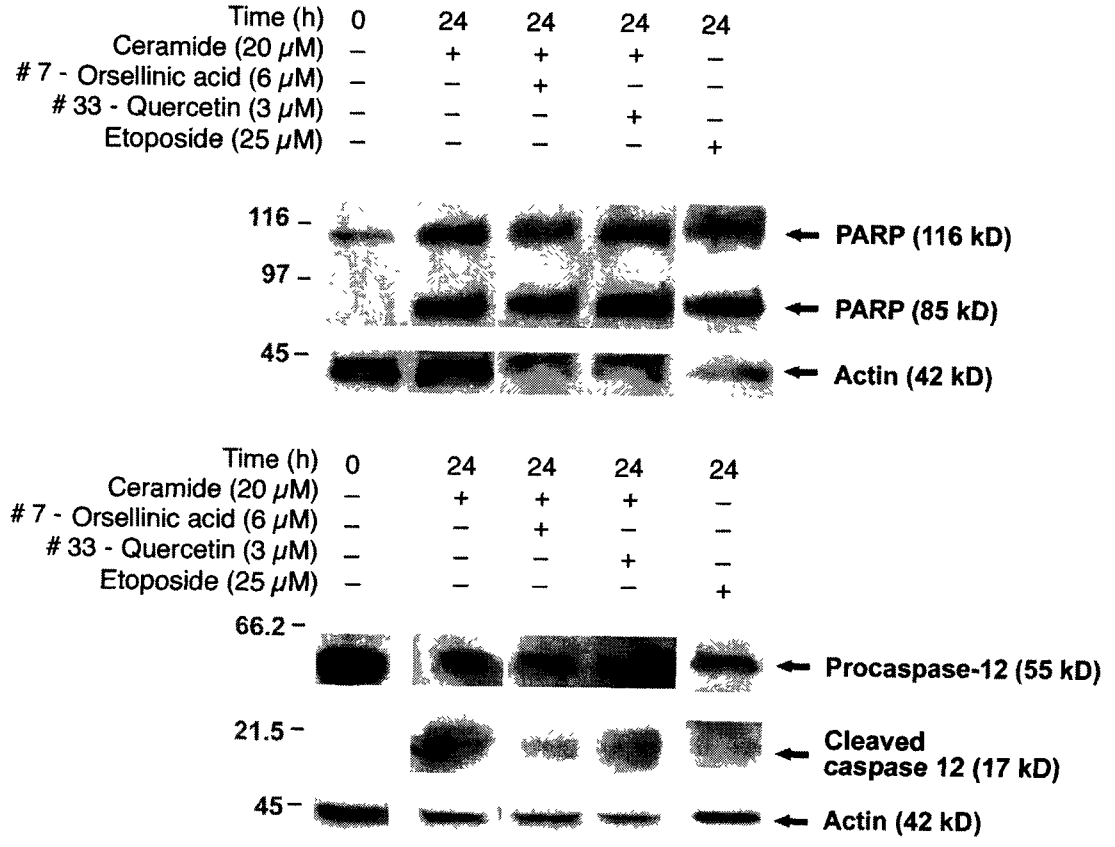

Figure 9.5 – Western analysis of caspase cleavage following etoposide or ceramide treatment in the presence of phenolics. Biochemical assessment was performed for compounds for which QSAR analysis could not predict bioactivity. Etoposide and ceramide activated caspase-3, resulting in the cleavage of PARP, but only ceramide treatment induced cleavage of caspase-12 to its activated form. Neither phenolic prevented PARP cleavage. We identified that orsellinic acid (compound 7) attenuated caspase-12 cleavage but quercetin (compound 33) did not.

gallate, protocatechuic acid) as well as phenolics with purported chemopreventive potential (d'Ischia et al. 2006; Kulkarni et al. 2006; Lee and Lee 2006; Narayanan 2006; Stuart et al. 2006; Tseng and Lee 2006). Anti-apoptotic and, surprisingly, mitogenic activities were most commonly identified for phenolics in the benzoic acid, cinnamic acid, coumarin, flavonoid, and tannin classes. These activities could be modelled in some, but not all, cases by QSAR analysis. As summarized in Table 9.2, 39% of compounds were identified as anti-apoptotic (< 6 μM), reducing cell death triggered by etoposide and/or ceramide, 35% were identified as mitogenic (1.5-7 μM), increasing PC12-AC cell proliferation in the absence of exogenous growth factors, and another 7% were cytoprotective (2-5.5 μM), promoting cell survival during serum-withdrawal without altering cell cycle kinetics. Only eight (15%) of the tested compounds were cytotoxic or pro-apoptotic. Three of these exhibited multiple activities dependent upon the stressor present in the microenvironment.

The high percentage of mitogenic phenolics detected in this study was unexpected. Although consistent with reports that coumarins and flavonoids regulate G1/S and G2/M cell cycle check-point transitions, our data conflict with findings that many of the same phenolics inhibit cell cycle progression and augment the toxicity of chemotherapeutic DNA damaging agents (Finn et al. 2004; Kuo et al. 2006; Lacy and O'Kennedy 2004; Singh and Agarwal 2006; Wang et al. 2002; Yeh et al. 2005). We argue that phenolic bioactivity is dramatically affected by concentration. Previous studies have documented similar dose-dependent activation of pro-survival and cytotoxic pathways in other cell systems. For example, esculetin (compound 28) at concentrations <30 μM inhibits cell death triggered by disruptions in calcium homeostasis or by excitotoxicity (Kim et al. 2000; Wie et al. 2001) yet initiates a dose-dependent mitochondrial apoptotic response when concentrations exceed 100 μM (Yang et al. 2006). Interestingly, salicylic acid (compound 1) and protocatechuic acid

(compound 5) did not exhibit any detectable bioactivity in this study at concentrations <10 μM despite reports of cytotoxicity on carcinoma tumour cells at >100 μM (Nunez et al. 2006; Yip et al. 2006).

The anti-apoptotic interactions detected here also raise important considerations for use of natural health products with phenolic actives as adjuvants with chemotherapeutic agents. Here, two thirds of tannins (<1.6 μM) completely inhibited etoposide-induced death despite reported pro-apoptotic activity at higher concentrations (Wang et al. 2001a). The ability of strictinin (compound 50) and oenothein B (compound 52) to reduce etoposide toxicity is nonetheless consistent with previous reports (Choi et al. 2002; Wei et al. 2002). Interestingly, within the benzoic acids, the aldehyde vanillin, but not its major metabolite vanillic acid (compound 6), has been shown to have growth inhibitory effects on tumour cells at concentrations > 250 μM (Lirdprapamongkol et al. 2005). The anti-apoptotic activity of vanillic acid (6 μM) observed was consistent with reports that vanillic acid derivatives alter NF-κB and Akt kinase signalling in tumour cells (Kumar et al. 2003). Together, these data suggest that the metabolism of potential anti-tumour phenolics may inhibit their efficacy as chemosensitizers.

We found that the protective effects of low dose phenolics could be accurately modelled by their physicochemical properties. The 20 flavonoids included in our collection are composed of several structurally distinct groups with a variety of phenolic ring substituents (Table 9.1). Sub-dividing these compounds into glycosides and aglycones (no sugar moiety) revealed two distinct structure-activity profiles associated with both cytoprotection and mitogenicity. Moreover, the QSAR for flavonoid mitogenicity and cytoprotection differed from the QSAR for anti-apoptotic activity. Within the aglycone

group, lower BDE values (greater anti-oxidant capacity) correlated with increasing viability under conditions of serum-deprivation as previously reported (Lee et al. 2005a; Mandel et al. 2003; Meyer and Frankel 2001). Conversely, glycoside activity in the same conditions did not depend on anti-oxidant capacity but on lipophilicity. Nearly all the glycosides tested in this study enhanced viability; those with log *P* values close to -1 had the greatest effect. Such compounds are unlikely to passively diffuse across the cell membrane and therefore are predicted to interact with extracellular receptors or transporters where they may activate signalling cascades or be transported into the cell. In support of this hypothesis, the flavonoid glycosides quercitrin and kaempferitrin (compounds 35 and 37) have been shown to interact directly with glucose transporters or extracellular receptors (Jorge et al. 2004). Thus glycosidic flavonoids are predicted to interact with critical effectors (in addition to their anti-oxidant capacities) whereas the ability of aglycones to promote cell viability appears due, in large part, to their ability to act as effective anti-oxidants at low concentration.

The QSAR for anti-apoptotic flavonoid activity in the etoposide paradigm was positively correlated with increasing MR values. With 7 of 9 glycosides but only 2 of 11 aglycones significantly reducing etoposide-induced death (Table 9.2), this relationship is likely a consequence of added bulk provided by glycosyl groups rather than an accurate model of activity within the entire class. Curiously, no significant model was identified within glycosides or aglycones. The strong association between glycosylation and protection suggests a distinct mode of action with glycoside-specific access to cellular targets or pathways. This activity may depend upon a closed C-ring as suggested by the observation that compound 48, a dihydrochalcone glycoside, failed to protect cells from death (Table 9.2).

Structural correlates underlying the anti-apoptotic activity of benzoic acids revealed an unexpected determinant with potential *in vivo* implications on the efficacy of the chemotherapeutic agent etoposide. QSAR analysis of anti-apoptotic benzoic acids in the etoposide paradigm was highly correlated with MR and log *P*. This model became even stronger with inclusion of syringic acid (compound 11) that together with bergenin (compound 13) represent the largest and most protective benzoic acid derivatives. Moreover, syringic acid and bergenin were the only two phenolic acids in this study that possess a 3,5-dimethoxy 4-hydroxybenzyl structure. Etoposide, a semi-synthetic derivative of the plant lignan podophyllotoxin, contains an exposed syringic acid residue. If this residue is directly involved in topoisomerase II interaction, free syringic acid-like compounds may provide protection by occupying the site of etoposide-topoisomerase association and preventing DNA damage. If such is the case, the lesser (yet significant) protection provided by structurally similar benzoic acids (compounds 6, 9, and 10, Table 9.1) may reflect lower affinities for the site of association, and the involvement of lipophilicity in the QSAR model may reflect the required passage through plasma and nuclear membranes.

The anti-apoptotic activity of benzoic acids in the ceramide model did not correspond to a distinct structural profile but to an activity profile. With the exception of *m*-digallic acid (compound 12), all of the benzoic acids that reduced ceramide toxicity also inhibited etoposide-induced cell death, perhaps indicative of additional direct effects on cell survival and/or death pathways. In comparing the ability of the anti-apoptotic flavonoid quercetin (compound 33) with the anti-apoptotic benzoic acid orsellinic acid (compound 7), we show, for the first time, that orsellinic acid inhibits cleavage of pro-caspase 12 to its active form during endoplasmic reticulum stress triggered by ceramide, providing direct evidence of a direct signalling effect.

In summary, a comparison of 54 phenolics revealed an unexpected degree of cytoprotective, mitogenic, and anti-apoptotic potential at dietary concentrations. QSAR analyses indicate that many of these activities can be predicted *a priori* by compound lipophilicity, steric bulk and/or anti-oxidant capacity. To begin to characterize cellular targets, we identified a novel caspase 12 inhibitor in orsellinic acid (compound 7). These findings highlight the need to continue to mechanistically evaluate the bioactivities of phenolics at physiologically relevant concentrations on normal, neoplastic, and tumour cells to be able to predict potential biological activity under different environmental conditions.

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CHAPTER 10: INHIBITION OF PLATELET-ACTIVATING FACTOR NEUROTOXICITY BY BENZOIC ACID DERIVATIVES

10.1 PREFACE

Having characterized the effects of isolated plant phenolics on PC12 cell viability (and finding low toxicity), the list of potential mechanisms through which phenolics could reduce the severity and progression of diabetic neuropathy includes induction of anti-apoptotic/cytoprotective signaling pathways, inhibition of pro-apoptotic effecters, antioxidant activity, and the prevention of AGE formation. These actions are not only relevant to treating diabetes and related complications but would likely also provide benefit in other conditions, such as neurodegenerative diseases of the CNS, where similar pathogenic mechanisms are involved.

To explore the neuroprotective abilities of plant phenolics in a model of CNS neurodegeneration, we sought to identify individual compounds capable of preventing neuronal death elicited by platelet activating factor (PAF), a lipid messenger associated with multiple neurodegenerative conditions. In a series of experiments not included in this thesis, we have identified a number of PAF isoforms that are altered in the brain of humans with Alzheimer's disease and the peripheral nerve of diabetic rats (in preparation). As such, a series of benzoic acids was first assessed for effects on PAF-treated PC12 cells and the one with the best activity profile was selected for testing in primary neurons with intent of identifying phenolics capable of impinging on PAF pathways in neurodegenerative conditions of the central and peripheral nervous system. We also sought to determine if the compound inhibited PAF-induced apoptotic signalling in both cell types.

10.2 Statement of Author Contributions

SDR, CH, JTA, and SALB conceived and designed the experiments. SDR and CH equally contributed to the experimental work and wrote the manuscript with SALB. FM performed Caspatag assay in WT condition in Figure 3 and assisted with PC12 cell screen of benzoates in Figure 10.5 A and B. HL designed the adenoviral vector used in Figure 10.2 under the guidance of STH. JTA supplied benzoates for screening and NGB provided $PAFR^{-/-}$ mice. As the CIHR-TAAM project leader, PSH directed the pharmacological assessment of Cree extracts and pure phenolics and contributed to manuscript preparation.

10.3 PLATELET ACTIVATING FACTOR-INDUCED NEURONAL APOPTOSIS IS INITIATED INDEPENDENTLY OF ITS G-PROTEIN COUPLED PAF RECEPTOR AND IS INHIBITED BY THE BENZOATE ORSELLINIC ACID

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ABSTRACT

The bioactive lipid mediator platelet activating factor (PAF) is recognized as a key effecter of neuronal apoptosis, yet it is not clear whether its G-protein-coupled receptor (PAFR) initiates or prevents PAF neurotoxicity. Using PAFR null-mutant ($PAFR^{-/-}$) and congenic wild-type mice ($PAFR^{+/+}$), we show that PAF triggers caspase-3/7 activity and neuronal death in $PAFR^{-/-}$ cerebellar granule neurons but not $PAFR^{+/+}$ neurons. Restoring receptor expression by recombinant adenoviral infection protected cells from PAF challenge. Neuronal death was not mediated by nitric oxide or *N*-methyl-D-aspartate receptor signalling given that *N*-nitro-L-arginine methyl ester and MK-801 did not inhibit PAF-induced neuronal loss in $PAFR^{-/-}$ neurons. To intervene in PAFR-independent neurotoxicity, the anti-apoptotic actions of three structurally distinct PAF antagonists were compared to a panel of plant and fungal benzoic acid derivatives. We found that the PAF antagonist BN 52021 but not FR 49175 or CV 3988 inhibited PAFR-independent neurotoxicity. Orsellinic acid, a fungal-derived benzoic acid, blocked PAF-mediated neuronal apoptosis without affecting PAFR-mediated neuroprotection. These findings demonstrate that PAF can transduce apoptotic death in primary neurons independently of its G-protein-coupled receptor, that PAFR activation is neuroprotective, and that orsellinic acid and BN 52021 effectively attenuate PAFR-independent neuronal apoptosis.

INTRODUCTION

The platelet activating factor (PAF: 1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine) family of glycerophospholipids exerts potent pro-inflammatory and neuromodulatory effects in the CNS (Prescott et al. 2000). Under normal physiological conditions, PAF participates in long-term potentiation associated with learning and memory through stimulation of its G-protein coupled receptor (PAFR) (Chen et al. 2001; Izquierdo et al. 1995; Kato 1999; Teather et al. 1998). When concentrations are elevated under pathological conditions, PAF becomes neurotoxic and has been identified as a key mediator of neuronal death following ischemia, encephalitis, epileptic seizure, meningitis, and HIV-1 dementia (Bazan et al. 2002; Birkle et al. 1998; Farooqui and Horrocks 2001; Perry et al. 1998).

Targeting PAF neurotoxicity is complicated by the fact that bioactivity can be signalled through both PAFR-dependent and PAFR-independent pathways. In non-neuronal cells, PAFR expression exacerbates cell death initiated by etoposide and mitomycin C yet protects cells from tumour necrosis factor α, tumour necrosis factor-related apoptosis inducing ligand (TRAIL), and extracellular PAF exposure (Brewer et al. 2002; Li et al. 2003; Southall et al. 2001). PAF also interacts with intracellular binding sites (Bratton et al. 1992; Marcheselli et al. 1990; Sapir et al. 1997) initiating caspase-3-dependent DNA fragmentation in the absence of PAFR (Bonin et al. 2004). Although cytotoxic signalling can be reversed by ectopic PAFR expression (Brewer et al. 2002), in neurons, it is not known whether PAF triggers apoptosis through its G-protein-coupled receptor or whether PAFR activation is neuroprotective.

To address this issue, cerebellar granule cells (CGNs) cultured from $PAFR^{+/+}$ and $PAFR^{-/-}$ mice were treated with apoptotic concentrations of PAF in the absence of serum PAF-

acetylhydrolases (PAF-AHs). Here, we show that PAF elicits caspase-dependent neuronal apoptosis in the absence of PAFR, that endogenous PAFR expression protects cells from PAF neurotoxicity, and that ectopic PAFR expression confers neuroprotection. To intervene in this death pathway without affecting PAFR-mediated neuroprotection, we used a 96-well screening approach to identify anti-apoptotic PAF inhibitors. We found that the non-competitive PAFR antagonist BN 52021 but not the PAF inhibitor FR 49175 or the competitive PAFR antagonist CV 3988 prevented PAF-induced neuronal apoptosis. Within a panel of naturally occurring benzoic acids, we identified a novel PAF inhibitor. Orsellinic acid, a fungal-derived benzoic acid derivative, selectively blocked PAF-induced neuronal apoptosis without inhibiting PAFR-mediated neuronal survival. Together, these data highlight a new anti-apoptotic role for PAFR signalling and a novel pharmacological means of inhibiting PAF-induced neuronal apoptosis.

EXPERIMENTAL

Reagents

All cell culture reagents were obtained from Invitrogen (Burlington, ON Canada) and all chemicals were purchased through Sigma-Aldrich (St Louis, USA) unless otherwise stated. Pure benzoic acid derivatives (>95 % purity as determined by HPLC analysis) were obtained from the phytochemical collections of C Nozzilillo (University of Ottawa, Canada) and JT Arnason (University of Ottawa, Canada). Cultures were treated with a final concentration of 1 μg/ml based on previous dose-response reports of optimal anti-apoptotic activity (unpublished data). Cells were also treated with the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo [a,d] cyclohepten-5,10-imine maleate, 10 μM)], N-nitro-L-arginine methyl ester (L-NAME) (5 mM, Biomol

Research Laboratories, Plymouth Meeting, USA), or the PAF antagonists/inhibitors BN 52021 (21 μg/ml), FR 49175 (4 μg/ml), and CV 3988 (1 μg/ml) (Biomol Research Laboratories). Concentrations employed were based on previous dose-response reports of optimal anti-apoptotic or PAF inhibitory effects (Bonin et al. 2004; Brewer et al. 2002; Hou et al. 2006; Malipiero et al. 1999). Benzoic acids and PAF inhibitors were dissolved in dimethylsulfoxide (DMSO). L-NAME and MK 801 were dissolved in EtOH. Cells were exposed to a final concentration of 0.1% vehicle. All treatments were performed in serum-free media as described below.

Congenic $PAFR^{-/-}$ and $PAFR^{+/+}$ mice

Breeding pairs of $PAFR^{-/-}$ mice in a hybrid C57BL/6J x 129/Ol background (Ishii et al. 1998) were obtained from Dr. Takao Shimizu (University of Tokyo, Japan). To insure uniformity in genetic background, animals were backcrossed to C57BL/6 wild-type mice (Charles River Laboratories, Senneville, Canada) for 10 generations. Congenic 10^{th} generation $PAFR^{+/+}$ and $PAFR^{-/-}$ colonies were established and maintained from $PAFR^{+/-}$ matings. Animals were genotyped as described by (Nagase et al. 1999).

Primary murine cell culture

Cerebellar granule cells were cultured from post-natal day 7-10 mice as previously described (Cregan et al. 2000). Briefly, cerebella were dissected, meninges removed, and tissue minced in ice-cold dissection solution [124 mM NaCl, 5.37 mM KCl, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 14.5 mM d-glucose, 25 mM Hepes, 3 mg/ml bovine serum albumin (BSA), pH.7.4]. Tissue was incubated in dissection solution containing 0.5 mg/ml trypsin (Sigma) at 37°C for 18 min. Trypsin was deactivated by addition of 0.52 mg/ml chicken egg white

trypsin inhibitor and cells were pelleted. Cells were resuspended in dissection solution containing 0.75 mg/ml DNase I (Roche, Mississauga, Canada) and triturated. $CaCl_2$ was added to a final concentration of 15 μM. Following centrifugation, cells were plated in Eagle's minimum essential medium (EMEM) containing 25 mM glucose, 10% fetal bovine serum (FBS), 1% gentamycin, 2mM L-glutamine, and 20 mM KCl at 37°C in a 5% CO_2/95% air atmosphere. CGNs were seeded at a density of 2 x 10^5 cells/cm^2 in 96-well plates coated with laminin (20 μg/ml) and poly-D-lysine (100mg/ml, Sigma-Aldrich, St Louis, USA) and maintained in this serum-free media for 72 h before being exposed to PAF in serum-free media. Cultures were composed of >90% neurons as established by immunocytochemistry (data not shown).

Cell survival assay

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycerol-3-phosphocholine, Biomol Research Laboratories) and *lyso*-PAF (1-*O*-hexadecyl-*sn*-glycerol-3-phosphocholine, Biomol Research Laboratories) were dissolved in treatment media (EMEM containing 25 mM glucose, 0.025% BSA, 1% gentamycin, 2 mM L-glutamine, and 20 mM KCl). CGNs were treated for 24 h with 1 μM PAF, 1 μM *lyso*-PAF or vehicle (treatment media alone). Note that all treatments were performed in serum-free media containing 0.025% BSA to ensure that cultures were not exposed to plasma PAF-AH. Where treatment with L-NAME, MK-801, test compounds or respective vehicles (0.1%) are indicated, cultures were pre-treated for 15 min prior to addition of PAF. CGN survival was assessed by Live/Dead viability/cytotoxicity assay (Invitrogen). Viable cells were identified by the enzymatic conversion by intracellular esterases of non-fluorescent calcein-AM to fluorescent calcein.

Dead cells were identified by uptake of ethidium homodimer (ET) as a result of loss of membrane integrity. Cells were imaged using a DMIR epifluorescent inverted microscope (Leica, Richmond Hill, Canada) equipped with a QICAM digital camera (Quorum Technologies, Guelph, Canada) and captured using OpenLab software v5.05 (Improvision, Lexington, USA). Percent survival was calculated as follows:

$$\% \text{ survival} = \frac{\text{Viable cell number}^{(\text{calcein}^+ - \text{calcein}^+/\text{ET}^+)}}{\text{Mean viable cells in vehicle control}^{(\text{calcein}^+ - \text{calcein}^+/\text{ET}^+)}} \times 100$$

Recombinant adenovirus infection

Recombinant pADTrack-CMV adenoviral vectors carrying a 1 kb Flag-tagged human PAFR cDNA excised from pCDM8PAFR (Kunz et al. 1992) kindly provided by Dr. Norma Gerard (Harvard Medical School, USA) and enhanced green fluorescent protein (EGFP) under separate cytomegalovirus promoters were prepared using the AdEasy adenoviral vector system (QBiogene Inc., Irvine, USA) and titred as we have described previously (Huang et al. 2005). Control adenovirus contained EGFP only. Recombinant adenoviruses were added to cell suspensions at the time of plating. All experiments were performed at a multiplicity of infection (MOI) of 100 pfu/cells. Efficiency of infection of both vectors was comparable as established by counting $EGFP^+$ cells immediately before treatment. Cell survival in serum-free media following PAF (1 μM) treatment was calculated as follows:

$$\% \text{ survival} = \frac{\text{EGFP+ cell number following treatment}}{\text{Mean EGFP+ cell number in the vehicle control}} \times 100$$

Screening assay for PAF inhibitors

PC12-AC cells, a clonal derivative of the PC12 pheochromocytoma cell line derived in our laboratory, were cultured in Roswell Park Memorial Institute medium (RPMI 1640)

supplemented with 10% horse serum and 5% newborn calf serum and maintained at 37°C and 5% atmospheric CO_2 and seeded in 96-well plates (1.25×10^4 cells/well) for treatment. After plating, cells were treated for 24 h with PAF (1 μM, 1-O-alkyl -2-acetyl-*sn*-glycerol-3-phosphocholine) or PAF vehicle (0.1% EtOH) in the presence of each benzoic acid (1 μg/ml), PAF antagonist, or treatment compound vehicle (0.1% DMSO) in RPMI 1640 supplemented with 0.025% BSA. All treatments were carried out in serum-free media. Negative controls included cultures incubated in treatment media only. WST (Roche Diagnostics, Mississauga, Canada) was added to each well at the end of the treatment period and incubated for 60 minutes before spectrophotometric analysis at 420 nm (formazan) and at 620 nm (reference). Cultures were blanked against cell-free treatment media incubated for the same period of time. Cell number per well was calculated from standard curves derived from wells containing known cell density. Percent viability was calculated as follows:

$$\% \text{ viability} = \text{cell number}^{(\text{treatment well})} / \text{ mean cell number}^{(\text{vehicle control})} \times 100$$

Caspase activation

Executioner caspase activation in CGNs was determined using CaspaTag (caspase-3/7) assay (Chemicon, Temecula, USA). CGNs capable of cleaving FAM-DEVD-FMK to its fluorescent product were scored as positive and reported as a percentage of total cell number after a 24 h treatment with PAF (1 μM) or vehicle (treatment media). Cells were imaged using a DMIR epifluorescent inverted microscope (Leica, Richmond Hill, Canada) equipped with a QICAM digital camera (Quorum Technologies) and captured using OpenLab software v3.5 (Improvision). Activation was confirmed by Western analysis. Proteins were isolated in radioimmunoprecipitation buffer (10 mM PBS, 1% NP40, 0.5%

sodium deoxycholate, 0.1% SDS, 30 µl/ml aprotinin, 10 µl/ml Na orthovanadate, 10 µl/ml phenylmethylsulfonyl fluoride, and 1 µl/ml NaF). Protein samples (30 µg) were separated by SDS-PAGE under reducing conditions. Western analyses were performed using monoclonal anti-poly(ADP ribose) polymerase (PARP) (1:10 000, Clonetec) and monoclonal anti-actin (1:1000, Sigma). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse IgG (1:2000, Jackson Immunolabs). Immunoreactive bands were visualized using SuperSignal West Pico (MJS BioLynx Inc).

Statistical analysis

Data were analyzed using one-way factorial ANOVA tests followed by *post hoc* Dunnett's *t*-tests or unpaired Student's *t*-tests, as applicable. *P*-values under 0.05 were considered statistically significant (shown as *); P values under 0.01 were considered highly significant (shown as **).

RESULTS

PAF triggers neuronal apoptosis in the absence of PAFR

To evaluate the role of PAFR in PAF-mediated neuronal cell death, primary CGN cultures derived from $PAFR^{+/+}$ and $PAFR^{-/-}$ mice were treated with 1 µM PAF in treatment media devoid of serum. Cell survival was assessed by Live/Dead assay. $PAFR^{+/+}$ neuronal viability was not affected by 24 h of PAF treatment (Fig. 10.1A and C). In $PAFR^{-/-}$ neurons, PAF reduced cell survival by 40% compared to vehicle controls (Fig. 10.1C and D). To establish PAF specificity, CGNs were treated with the immediate PAF metabolite, *lyso*-PAF. *Lyso*-PAF (1 µM) did not alter $PAFR^{+/+}$ or $PAFR^{-/-}$ neuronal viability demonstrating that

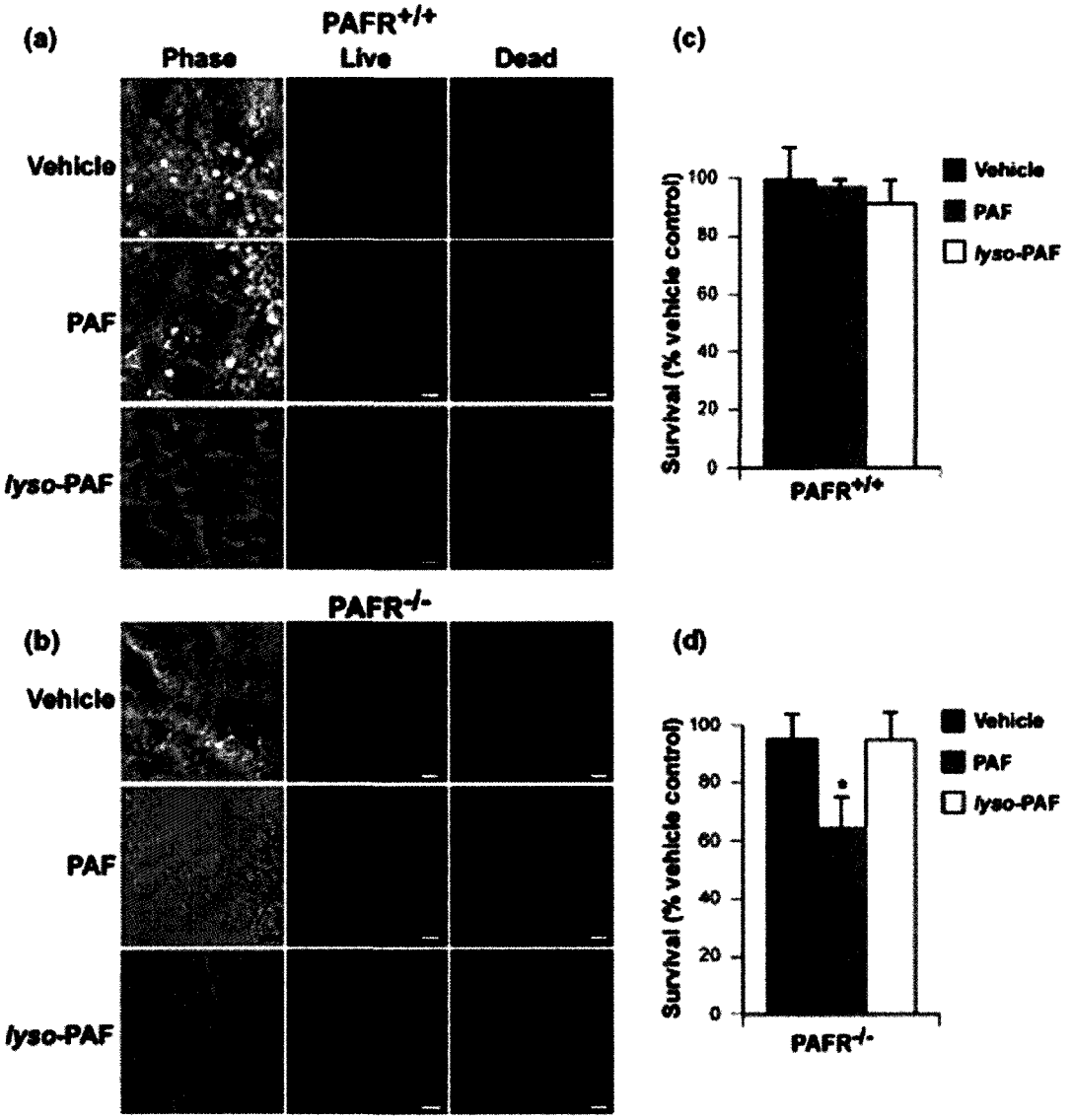

Figure 10.1 – PAF but not lyso-PAF triggers cell death in neurons that do not express PAFR. CGNs derived from PAFR+/+ and PAFR-/- mice were treated for 24 h with vehicle or PAF (1 μM). Cell survival was assessed by Live/Dead staining. (A) PAFR+/+ CGNs were capable of cleaving calcein-AM to its fluorescent product (green cells) without loss of membrane integrity (red cells) following all treatments. (B) Cell viability was only compromised in PAFR-/- cultures treated with PAF. Scale bars, 10 μm. (C, D) Quantification of neuronal survival by Live/Dead assay. * $p < 0.05$, ANOVA, post hoc Dunnett's t-test, n = 10-15 fields per condition performed over triplicate experiments. Data are reported as mean + SEM..

PAF and not its metabolite was responsible for the observed effects (Fig. 10.1). In complementary gain of function studies, $PAFR^{-/-}$ CGNs were infected with a recombinant adenoviral vector expressing EGFP or EGFP and PAFR. PAF significantly reduced survival of $EGFP^{+}/PAFR^{-/-}$ cells (Fig. 10.2A and C) whereas ectopic expression of PAFR inhibited PAF-induced cell death (Fig 10.2B and D). To establish whether PAF triggers caspase-dependent apoptosis in null-mutant neurons, caspase 3/7 activity was evaluated. In $PAFR^{+/+}$ neurons, the percentage of cells exhibiting activated caspase-3/7 was minimal and the basal rate of DEVD substrate cleavage was comparable between vehicle and PAF treatment (Fig 10.3A and C). By contrast, in $PAFR^{-/-}$ neurons, a significant increase in caspase activation was observed after 24 h exposure to PAF (Fig. 10.3B and D).

PAF-induced cortical neuron death has been reported to involve nitric oxide (NO) pathways and glutamate excitotoxicity (Xu and Tao 2004). To determine whether these mechanisms underlie PAFR-independent apoptosis in CGNs, $PAFR^{-/-}$ neurons were exposed to PAF (1 μM) in the presence of the NO synthase inhibitor L-NAME (5 mM) or the NMDA receptor antagonist MK-801 (10 μM). These concentrations have been shown previously to inhibit NO and glutamtate toxicity in primary CGNs (Hou et al. 2006; Malipiero et al. 1999). We found no effect of either inhibitor on PAF-induced toxicity, suggesting that these pathways are not activated by PAF in neurons lacking PAFR (Fig. 10.4).

Screening plant and fungal metabolites for inhibitors of PAF-mediated apoptosis

To identify novel compounds capable of inhibiting PAFR-independent neuronal apoptosis, test compounds were selected from a larger library of ~100 natural products, pure phenolics, and fungal extracts (Harris et al. 2007b; Spoor et al. 2006). Compounds were prioritized based on previously published studies demonstrating that (*i*) benzoic acid

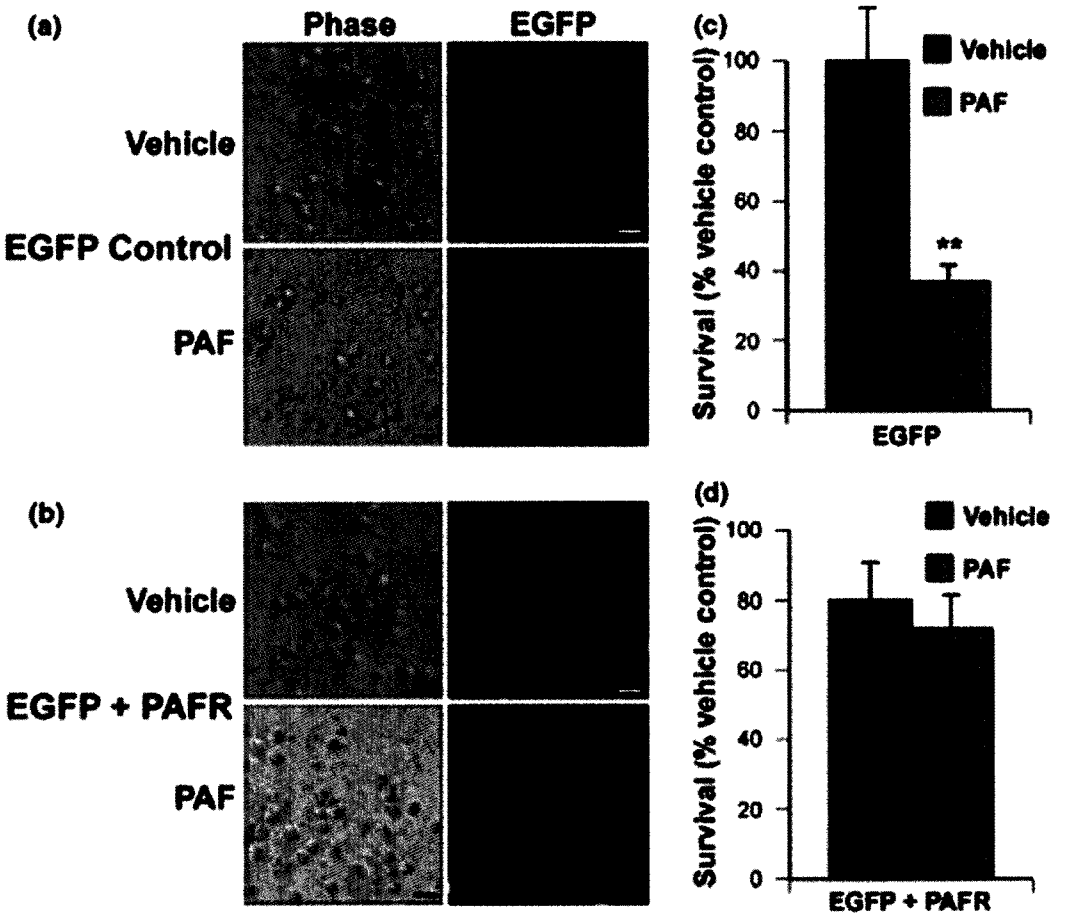

Figure 10.2 – PAFR expression protects PAFR-/- neurons from PAF-induced death. PAFR-/- CGNs were infected with recombinant adenovirus containing EGFP (A,C) or EGFP and PAFR (B,D). The number of EGFP+ cells in both cultures was comparable prior to treatment indicating equivalent infection efficiency. (A, B) Cultures were treated with vehicle or PAF (1 μM) for 24 h prior to Live/Dead assay. (C,D) In quantitative analyses, neuronal survival was expressed as percentage of the total number of EGFP+ cells present in vehicle controls. (** $p < 0.01$, Student *t*-test, n = 5-10 fields per condition performed over duplicate experiments). Data are reported as mean + SEM.

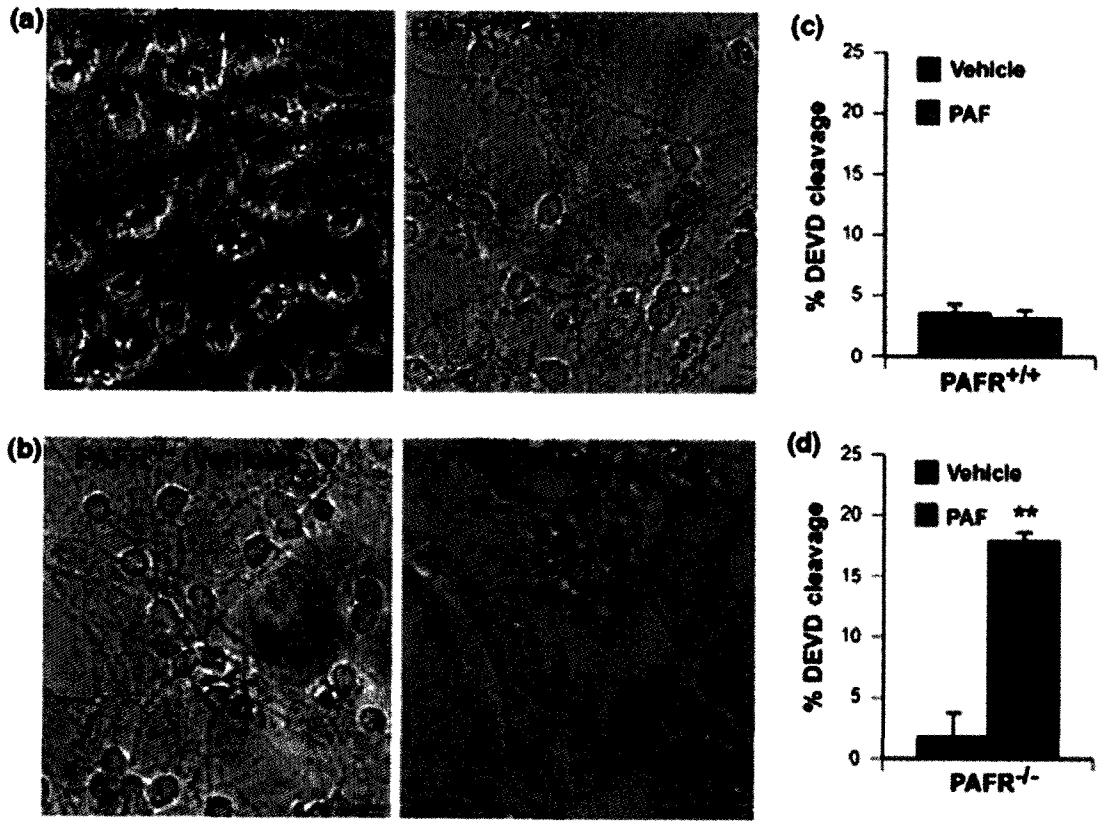

Figure 10.3 – PAF induces a caspase 3/7-dependent apoptotic pathway in PAFR-/-neurons. PAFR+/+ (A,C) and PAFR-/- CGNs (B,D) were treated with vehicle or PAF (1 μM) for 24 h then activated caspase 3/7 was identified by the cleavage of FAM-DEVD-FMK to its fluorescent product. Quantitative data are expressed as the percentage of total cells per field positive for activated caspase 3/7. (** $p < 0.01$, Student's *t*-test, n = 6 performed over duplicate experiments). Data are reported as mean + SEM.

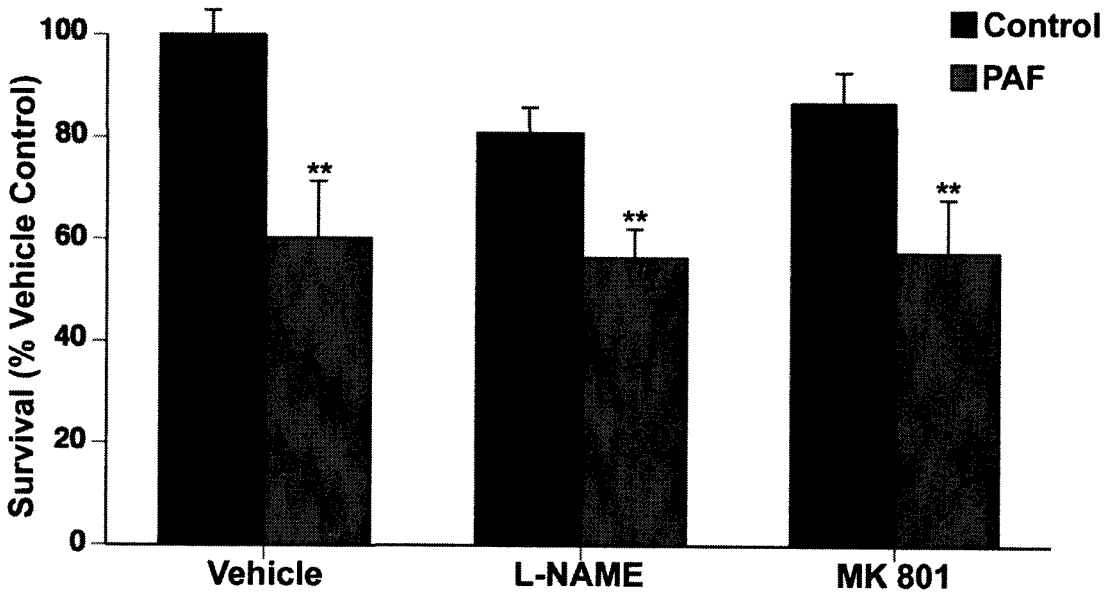

Figure 10.4 – PAFR-/- neurons are not protected by NOS inhibition or NMDA receptor blockade. The effect of L-NAME (5 mM) and MK 801 (10 μM) on PAFR-/- survival was determined in the presence and absence of vehicle or PAF for a 24 h period. Cell survival was assessed by LIVE/DEAD assay. (** $p < 0.01$ relative to EtOH control, *post hoc* Dunnett's *t* test, n=6 performed over duplicate experiments). Data represent mean + SEM.

derivatives can alter PAF catabolism and thereby PAF-mediated biological activity and (*ii*) PAF-like lipids play a role in etoposide-induced apoptosis (Ahlemeyer et al. 2001; Bonin et al. 2004; Hattori et al. 1995; Li et al. 2003; Southall et al. 2001). Based on this evidence, we prioritized 10 benzoic acid derivatives (Table 11.1) to be screened for anti-PAF activity using the PAF-sensitive PC12-AC cell line (Bonin et al. 2004; Brewer et al. 2002). As positive controls, the non-competitive PAF antagonist ginkgolide B (BN 52021) and the fungal derivative, bis(methylthio)gliotoxin (FR 49175) (Bonin et al. 2004; Brewer et al. 2002) were included. As a negative control, we used the competitive PAF antagonist CV 3988 previously shown to block PAFR-dependent but not PAFR-independent PAF signalling in non-neuronal cells (Brewer et al. 2002).

The effects of test compounds on cell viability were established by mitochondrial dehydrogenase cleavage of the formazan dye WST. As expected, both BN 52021 and FR 49175 inhibited PAF-induced death without altering the viability of vehicle-treated cultures while CV 3988 had no significant effect on cell viability in the presence or absence of PAF (Fig. 10.5A and B). Five benzoic acids (gentisic acid, veratric acid, gallic acid, syringic acid, salicylic acid) increased viable cell number in both vehicle- and PAF-treated cultures (Fig. 10.5A and B) likely indicative of mitogenic effects in growth factor-deprived treatment media. Two benzoates (resorcyclic acid and *m*-digallic acid) decreased viable cell number in vehicle-treated cultures (Fig. 10.5A). Interestingly, both compounds significantly increased viable cell number in PAF-treated cultures (Fig. 10.5B). Of the benzoic acids tested, only orsellinic acid significantly inhibited PAF-mediated death without affecting viability of vehicle-treated cells (Fig. 10.5).

We next assessed that ability of orsellinic acid, BN 52021, FR 49175, and CV 3988 to inhibit PAF-induced caspase 3/7 activity. PAF-induced caspase 3/7 activity, denoted by

Table 10.1 – Names and molecular weights of benzoic acids screened for PAF inhibitor activity.

Common Name	IUPAC Name	Mol. Wt.
Salicylic acid	2-hydroxybenzoic acid	138.1
3-hydroxybenzoic acid	3-hydroxybenzoic acid	138.1
Resorcyclic acid	2,4-dihydroxybenzoic acid	154.1
Gentisic acid	2,5-dihydroxybenzoic acid	154.1
Protocatechuic acid	3,4-dihydroxybenzoic acid	154.1
Vanillic acid	4-hydroxy-3-methoxybenzoic acid	168.2
Orsellinic acid	4,6-dihydroxy-2-methylbenzoic acid	168.2
Gallic acid	3,4,5-trihydroxybenzoic acid	170.1
Veratric acid	3,4-dimethoxybenzoic acid	182.2
Syringic acid	4-hydroxy-3,5-dimethoxybenzoic acid	198.2
m-digallic acid	3,4-dihydroxy-5-[(3,4,5-trihydroxy-benzoyl)oxy]benzoic acid	322.2

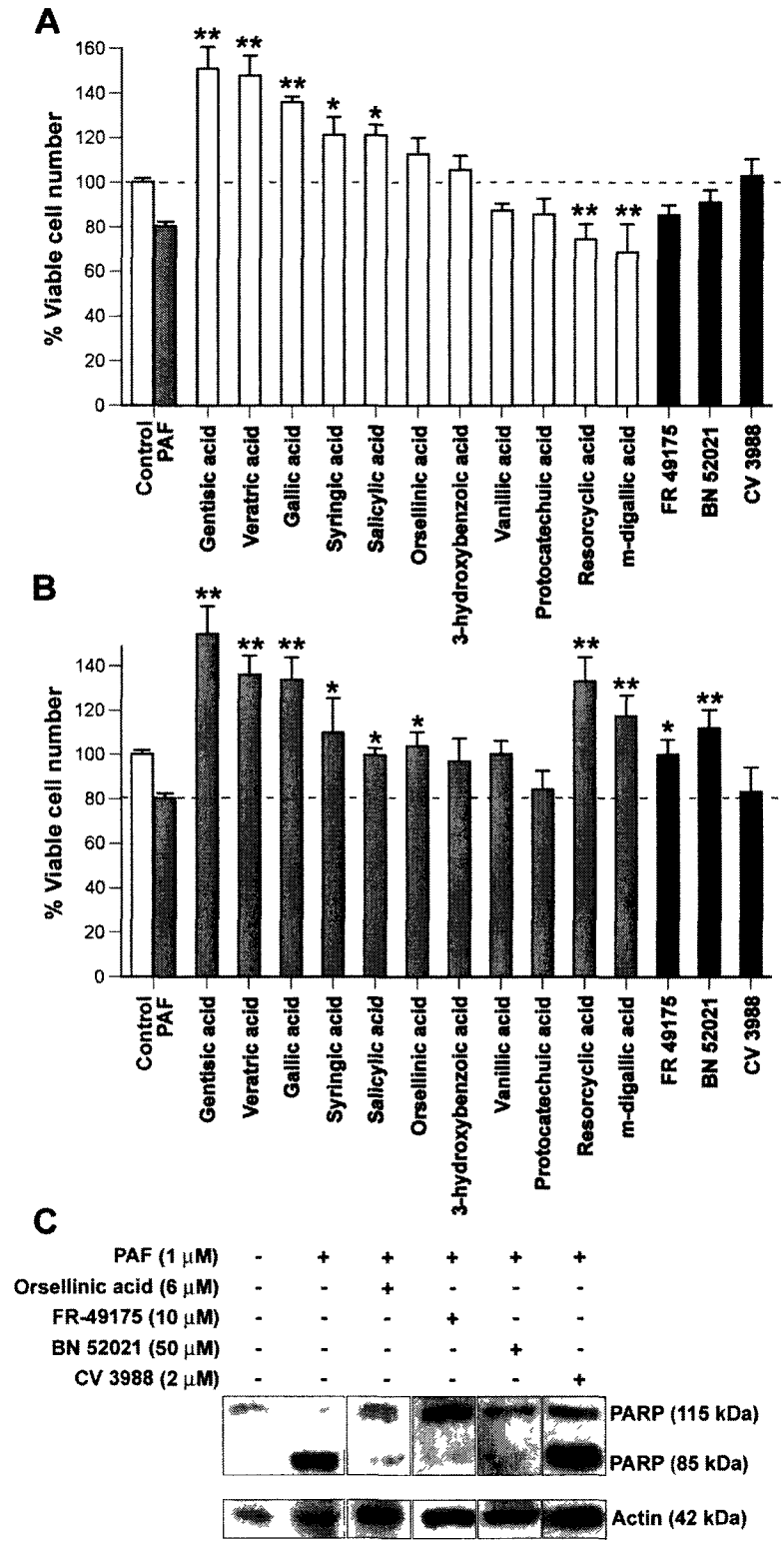

Figure 10.5 – Orsellinic acid, BN 52021, and FR 49175 protect PC12-AC cells from PAF-induced apoptosis without altering viability of vehicle-treated cells. (Complete caption on following page).

Figure 10.5 – Orsellinic acid, BN 52021, and FR 49175 protect PC12-AC cells from PAF-induced apoptosis without altering viability of vehicle-treated cells. Viable cell number was quantified by WST assay and standardized to wells of known cell densities after a 24 h treatment with vehicle (A) or PAF (B) and test compounds. Benzoic acids (A, white bars, B, grey bars), BN 52021, FR 49175, and CV 3988 (black bars) were tested at 1, 21, 4, and 1 μg/ml. (A) Toxic (<100% viability) and proliferative (>100% viability) activities were established by comparing the effects of test compounds on vehicle-treated controls (* $p <$ 0.05, ** $p < 0.01$ relative to EtOH+DMSO (dashed line), *post hoc* Dunnett's *t* test, n=10-20). (B) Effect of test compounds on PAF-induced PC12-AC cell loss (* $p < 0.05$, ** $p < 0.01$ relative to PAF+DMSO (dashed line), *post hoc* Dunnett's *t* test, n = 10-20). PAF + DMSO elicited significant loss in viable cell number compared to EtOH+DMSO treatment (# $p <$ 0.05 in (B) relative to vehicle-treated controls in (A), Student's *t* test n = 10-20). (C) Effect of orsellinic acid, BN 52021, FR 49175 and CV 3988 on cleavage of PARP.

appearance of the 85 kDa PARP cleavage fragment by Western analysis, was reduced by BN 52021, FR 49175, and orsellinic acid but not CV 3988 (Fig. 10.5C).

Orsellinic acid inhibits PAF-mediated neuronal apoptosis

PC12-AC cells are tumour derived pheochromcytoma cells that can be differentiated to a peripheral nervous system neuronal phenotype. As such, their responses may not accurately reflect post-mitotic terminally differentiated neurons of the CNS. To establish whether the PAF inhibitors identified in our screen block PAF-induced apoptosis in CNS neurons without impacting on PAFR-mediated neuroprotection, $PAFR^{+/+}$ and $PAFR^{-/-}$ CGNs were treated with vehicle (treatment media) or PAF (1 μM) in the presence or absence of CV3988 (1 μg/ml), BN 52021 (21 μg/ml), FR 49175 (4 μg/ml), or orsellinic acid (1 μg/ml) (Fig. 10.6). Cell survival was assessed by Live/Dead assay. BN 52021, FR 49175, CV 3988 and orsellinic acid had no effect on $PAFR^{+/+}$ neurons treated with vehicle or PAF indicating that these compounds will likely not impede PAFR-mediated neuroprotection (Fig. 10.6A). Likewise, test compounds had no effect on the viability of vehicle-treated $PAFR^{-/-}$ CGNs (Fig. 11.6B). Only BN 52021 and orsellinic acid increased neuronal survival (Fig. 10.6B) and inhibited caspase 3/7 activation following treatment of $PAFR^{-/-}$ cultures with PAF (Fig. 10.6C).

DISCUSSION

In this study, we show for the first time that PAF triggers PAFR-independent caspase-dependent apoptosis in primary neurons. This toxic response was specific to PAF and not its immediate or subsequent metabolites as *lyso*-PAF did not initiate death. Interestingly, we found that PAFR-independent death does not involve NO or NMDA

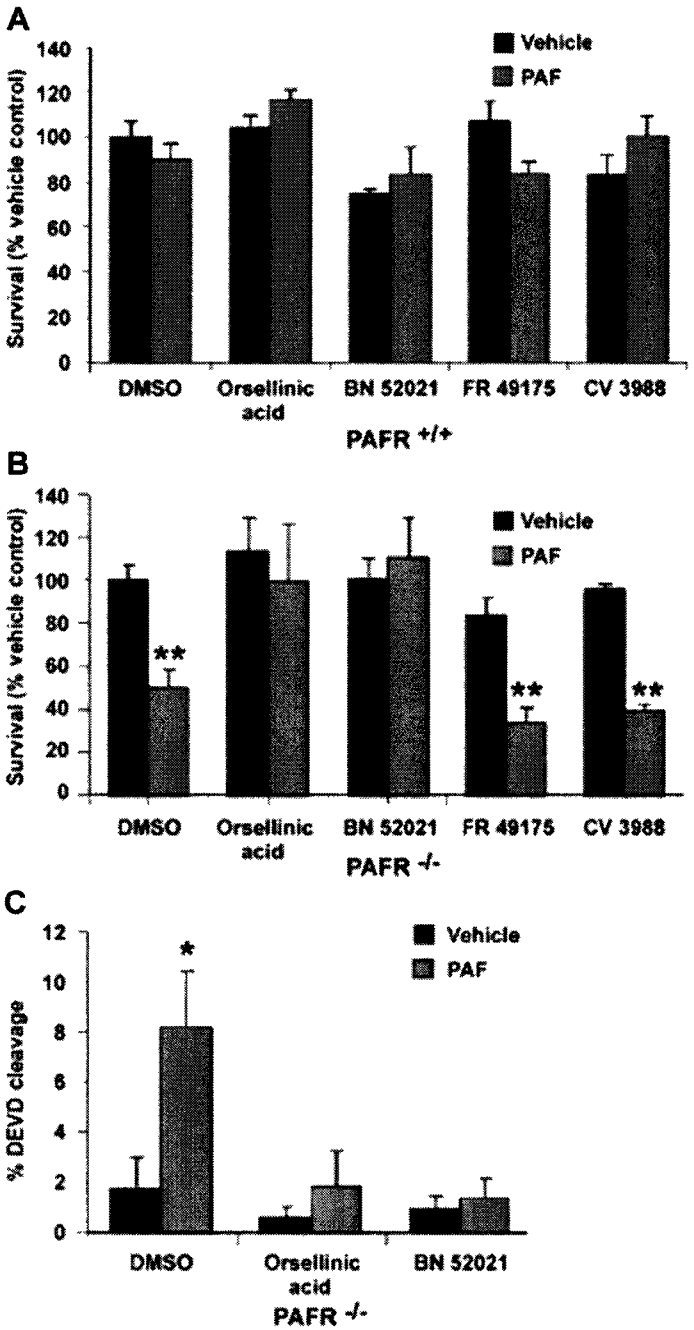

Figure 10.6 – Orsellinic acid and BN 52021 protect PAFR-/- neurons from PAF toxicity without perturbing PAFR-mediated neuroprotection. The effect of 24 h treatment of orsellinic acid (1 μg/ml), BN 52021 (21 μg/ml) FR 49175 (4 μg/ml), and CV 3988 (1 μg/ml) on (A) PAFR+/+ and (B) PAFR-/- survival was determined in the presence and absence of vehicle by Live/Dead assay. (C) PAF-induced executioner caspase activation was determined in PAFR-/- CGNs by CaspaTag assay. (* $p < 0.05$, ** $p < 0.01$ relative to DMSO control, *post hoc* Dunnett's *t* test, n = 5-18). Data represent mean + SEM.

receptor signaling pathways. Neither L-NAME nor MK 801 protected $PAFR^{-/-}$ CGNs from PAF. These results are consistent with previous reports that NO production depends upon PAFR activation (Han et al. 2006; Lopez-Ongil et al. 1999). Furthermore, we demonstrate that expression of the PAF G-protein coupled receptor can elicit neuroprotection and inhibit PAF-mediated cell death. To intervene in PAFR-independent apoptosis, we identified a benzoic acid derivative, orsellinic acid, and a non-competitive PAF antagonist, BN 52021 capable of reducing PAF neurotoxicity in $PAFR^{-/-}$ neurons without impacting upon the neuroprotection provided by PAFR expression.

The significance of these findings lies in converging evidence that PAF plays a key role in transducing neuronal death in multiple neurodegenerative conditions (Bate et al. 2004b; Bate et al. 2004c; Bazan et al. 2002; Birkle et al. 1998; Farooqui and Horrocks 2001; Perry et al. 1998), yet therapeutic strategies targeting PAF signalling have met with limited success (Le Bars et al. 2000; Le Bars et al. 1997; van Dongen et al. 2000; Wettstein 2000). In rodent brain, PAFR is primarily expressed by activated microglia with neuronal expression restricted to subpopulations of cells in the hippocampus, notably the CA3 pyramidal cell field, cortex, and cerebellum (Bennett et al. 1998; Mori et al. 1997; Mori et al. 1996). Earlier studies have hinted at the possibility that PAFR-independent signalling pathways may participate in neuronal loss in regions where PAFR is not detected. For example, striatal neurons synthesize and bind PAF following ischemic insult (Domingo et al. 1994) yet express little to no PAFR mRNA (Mori et al. 1996). Here, we show that PAF triggers executioner caspase activation in $PAFR^{-/-}$ neurons providing conclusive evidence that PAF does not require its G-protein coupled receptor to initiate neuronal apoptosis. Moreover, we show that PAFR expression protects neurons from pathological PAF challenge. These data provide possible insight into why subpopulations of neurons, such as

PAFR-expressing CA3 pyramidal neurons, are spared following ischemic insult (Cronberg et al. 2005). These findings also highlight the need to identify reagents that inhibit PAFR-independent apoptosis without impacting upon PAFR neuroprotection in injured brain.

To this end, our comparison of known PAF inhibitors demonstrates significant differences in their efficacies between cell types that could impact on potential *in vivo* use. First, we found that the PAF antagonist CV 3988 does not protect cells from PAF challenge in the absence of PAFR. Thus, it is likely that not all PAF antagonists will prove useful in inhibiting PAF-induced neuronal loss. Second, FR 49175, BN 52021, and orsellinic acid were found to protect PC12-AC cells from PAF challenge but, in this study, only BN 52021 and orsellinic acid inhibited PAFR-independent apoptosis in primary neurons. We have previously demonstrated that the ginkgolide BN 52021 and the fungal metabolite derivative FR 49175 accelerate PAF catabolism in PC12-AC cells (Bonin et al. 2004). Both compounds inhibit expression of the α_1 catalytic subunit of PAF-AH I complex thereby promoting dimerization of the α_2 subunit (Bonin et al. 2004). Here, we show that this mechanism likely does not impact upon neuronal PAF catabolism. In embryonic brain, as in undifferentiated PC12-AC cells, PAF-AH I α_1 is expressed at higher levels than PAF-AH I α_2 (Bonin et al. 2004; Manya et al. 1998). Transiently reducing α_1 protein levels is sufficient to promote α_2 homodimerization. In mature brain, PAF-AH α_2 predominates over PAF-AH α_1 (Manya et al. 1998). Consequently, any pharmacological reduction in PAF-AH α_1 expression is unlikely to enhance formation of the anti-apoptotic α_2/α_2 homodimer. Thus, we predict that FR 49175 will not prove effective in strategies targeting adult neurons *in vivo* but may protect embryonic brain from pathological exposure to PAF. Third, BN 52021 has been shown to bind two distinct PAF intracellular sites as well as acting as a non-competitive

inhibitor of PAFR and promoting PAF-AH I α_2/α_2 homodimer formation (Bonin et al. 2004; Marcheselli et al. 1990). The finding that BN 52021 but not FR 49175 blocks neuronal apoptosis suggests that PAFR-independent apoptosis may be initiated by one of these intracellular binding sites. The presence of these sites in CGNs is consistent with evidence that subtoxic concentrations of a non-metabolizable PAF analog alters neuronal migration in $PAFR^{-/-}$ CGNs (Tokuoka et al. 2003).

We also characterized a novel PAF inhibitor identified from a panel of benzoic acid derivatives produced by plants and fungi. We found that orsellinic acid selectively inhibits PAFR-independent death in neurons without altering PAFR-neuroprotection following PAF challenge. Orsellinic acid (2-methyl-4,6-dihydroxybenzoic acid) is a fungal metabolite structurally similar to the known anti-inflammatory compound acetylsalicylic acid (aspirin). While we have yet to identify the mechanism through which this benzoate elicits its biological effects, previous studies have shown that other benzoate family members modulate PAF catabolism (Ahlemeyer et al. 2001; Huh et al. 1998).

In summary, these data highlight a novel PAFR-independent pathway leading to caspase-dependent apoptosis in primary neurons following PAF challenge and identify two natural health product inhibitors, orsellinic acid and BN 52021, capable of intervening in this pathway. These findings provide a pharmacological means of differentially targeting distinct PAFR-dependent and independent pathways that can be used to further elucidate underlying mechanisms of neurotoxicity and neuroprotection in PAF-associated disease.

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CHAPTER 11: INHIBITION OF PLATELET-ACTIVATING FACTOR NEUROTOXICITY BY FLAVONOIDS

11.1 PREFACE

Of the five classes of plant phenolics studied in Chapter 10, flavonoids have been most widely studied and possess the greatest diversity of biological activity, demonstrating both neuroprotective and anti-diabetic effects. The identification of quercetin-3-*O*-galactoside as an active constituent of *S. purpurea* in Chapter 7 represents yet another example. With the successful identification of orsellinic acid as a novel inhibitor of PAF neurotoxicity in Chapter 10, we next targeted flavonoids in search of similar activity. These studies were designed to expand of our evaluation of the anti-diabetic actions of active phenolics in Cree plants to a broader use as potential adjuvant dietary supplements in the management of other progressive neurodegenerative disease. Because PAF elicits death independently of the PAF receptor (PAFR) in both PC12 cells and mouse primary neurons (Chapter 10), we specifically sought to identify flavonoids that inhibit PAF toxicity in human neurons devoid of PAFR. For this reason, flavonoids that proved protective in the first two models (PC12 cells and murine CGNs) were then evaluated in human neuronal cultures (hNT neurons) that are PAF-sensitive in the absence of PAFR. Thus, as a final objective, we tested the ability of flavonoids identified as PAF inhibitors in a model of Alzheimer disease, where neurodegeneration has been associated with PAF toxicity and pre-existing T2DM.

11.2 STATEMENT OF AUTHOR CONTRIBUTIONS

CSH, JTA, and SALB conceived and designed the experiments. CSH and FM performed the PC12 experiments with LM and CGN experiments (Figures 11.1-2) with SDR

culturing the neurons. Human neuronal experiments were conducted by CSH, SDR (Figure 11.3) and TM, who completed the amyloid-beta experiment (Figure 11.4). CSH wrote the manuscript with SDR and SALB. JTA supplied flavonoids for screening. As CIHR-TAAM project leader, PSH directed the pharmacological assessment of extracts.

11.3 Flavonoids as Novel Inhibitors of Platelet-Activating Factor-Mediated Neuronal Death

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ABSTRACT

While it is well-recognized that, at pathophysiological concentrations, the platelet-activating factor (PAF) family of glycerophosphocholine second messengers are implicated in neuronal death, our laboratory has recently shown that PAF-induced neuronal death is isoform-specific with C16:0 PAF-induced toxicity proceeding in the absence of the PAF receptor. Because neuronal PAFR expression in the brain is regionally restricted and generally low, PAFR-independent apoptotic signaling likely contributes to neuronal loss in PAF-associated neurodegenerative conditions. Here, we used three *in vitro* models of PAF-mediated apoptosis in the absence of PAFR to identify neuroprotective compounds within a collection of plant flavonoids representative of compounds identified in extracts of Cree medicinal plants. From preliminary results in PC12 cells, the most promising candidates, quercetin and hesperetin, were selected for analysis in primary murine and terminally differentiated human neurons (hNT). Both hesperetin and quercetin significantly inhibited PAF's neurotoxic effects in PAFR null-mutant mouse cerebellar granule neurons (CGNs), preventing executioner caspase activation and neuronal death, while having no effect on the viability of PAFR-expressing CGNs. Upon administration to hNT cells, again, both flavonoids successfully inhibited PAF neurotoxicity. Activity was also assessed in Aβ-treated human neurons associated with enhanced C16:0 PAF production with both preventing Aβ-mediated death. The presented results provide support for continued study of flavonoids, particularly hesperetin and quercetin, in models of PAF-related neurodegeneration, including Alzheimer's disease and diabetic neuropathy.

INTRODUCTION

Under physiological conditions, the platelet activating factor (PAF: 1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine) family of glycerophospholipids exert neuromodulatory effects in the CNS, wherein PAF acts as a retrograde transmitter in memory and learning (Chen et al. 2001; Heusler and Boehmer 2007; Teather et al. 1998). However, under pathological concentrations, PAF becomes neurotoxic and participates in neuronal death associated with ischemia, encephalitis, epileptic seizure, meningitis, HIV-1 dementia, Alzheimer's disease (AD), and diabetic complications (Bazan et al. 2002; Beauchamp et al. 2002; Birkle et al. 1998; Farooqui and Horrocks 2001; Perry et al. 1998).

AD is characterized by progressive memory dysfunction associated with pathological changes that include impairments in multiple neurotransmitter systems, cytoskeletal abnormalities, alterations in mitochondrial energy and oxidation processes precipitated by aberrant processing of amyloid precursor and tau proteins (Hardy and Selkoe 2002). Using HPLC-MS based lipidomic methods, we have recently demonstrated that PAF levels are significantly elevated in the human Alzheimer's brain (Ryan et al., submitted) and that PAF isoforms increase in peripheral neurons in rodent models of diabetes (unpublished results). Moreover, aberrant processing of amyloid precursor and tau proteins has been shown to elicit PAF-mediated neurotoxicity (Bate et al. 2004b) and related glycerophospholipid imbalances (Farooqui and Horrocks 2001; Kanfer et al. 1998; Sweet et al. 2002), changes that may be targeted pharmacologically to prevent associated neuronal loss.

While three putative PAF binding sites have been identified (Marcheselli et al. 1990), most PAF-elicited biological effects are associated with activation of a single PAF-specific G-protein-coupled receptor, PAFR. PAF-sensitivity, however, is not limited to PAFR expressing cells and accumulating evidence points towards additional PAF signalling

pathways independent of PAFR (Bratton 1993; Bratton et al. 1992; Brewer et al. 2002; Marcheselli et al. 1990; Ryan et al. 2008; Ryan et al. 2007; Sapir et al. 1997; Tokuoka et al. 2003). PAFR expression in the rodent brain is primarily restricted to microglia with neuronal expression detected in discrete subpopulations of cells in the hippocampus, cortex, and cerebellum (Bennett et al. 1998; Mori et al. 1997; Mori et al. 1996). Earlier studies have alluded to the possibility that PAFR-independent signaling pathways may participate in neuronal loss in regions where PAFR is not detected. For example, striatal neurons synthesize and bind PAF following ischemic insult (Domingo et al. 1994) yet express little to no PAFR mRNA (Mori et al. 1996). We have shown that PAFR expression protects neurons from PAF challenge while neurons devoid of PAFR are sensitive to PAF (Ryan et al. 2007). Together, these data suggest a role for PAFR-independent signaling in neuronal death associated with elevated PAF concentrations. Identifying novel inhibitors of PAF neurotoxicity in a PAFR-negative background is therefore warranted and of potential clinical relevance in the treatment of neurodegenerative disorders such as AD.

Plant derived flavonoids are common dietary components found in fruits, vegetables, grains, and beverages such as wine and tea. Flavonoids are diverse in chemical structure and exhibit a wide range of biochemical and pharmacological effects including anti-oxidant, anti-thrombotic, and anti-inflammatory activities (Croft 1998; Gryglewski et al. 1987; Havsteen 1983; Middleton and Kandaswami 1992; Middleton et al. 2000). Having also received considerable attention as neuroprotectants in a variety of neurodegenerative models including AD (Galli et al. 2002; Kim et al. 2005; Mandel et al. 2004), flavonoids offer multiple avenues of potential PAF inhibition. In the present study, we sought to identify flavonoids that inhibit PAF-induced apoptosis in PAFR deficient neurons *in vitro*. Rat pheochromocytoma (PC12) cells, representing a transformed source of peripherial neuron

precursors, served as a preliminary model for screening a panel of 15 compounds representative of structurally distinct flavonoids. From these results, two candidates were selected for further investigation in PAFR negative murine and human CNS systems. The relevance of the identified PAF inhibitors was then validated in an *in vitro* AD neurodegenerative model. These data provide the first evidence that flavonoids exert neuroprotective activity, in part, through modulated glycerophospholipid cytotoxicity.

EXPERIMENTAL

Materials and treatment preparation

Cell culture reagents and plates were purchased from Invitrogen (Burlington, ON, Canada) and VWR (Montreal, QC, Canada) unless otherwise noted. Pure flavonoids were selected from the Arnason lab phytochemical collection, which includes commercially and specially isolated compounds. The compounds were checked for purity and freedom from degradation by HPLC/MS analysis >95 % purity as previously described (Harris et al. 2007a). The flavonoids were solubilized in dimethylsulfoxide (DMSO) to a standard concentration of 1 mg/ml before dilution to a final concentration of 1 μg/ml in appropriate serum-free media. Vehicle concentration (DMSO, ethanol) was maintained at 0.1%.

Congenic $PAFR^{-/-}$ and $PAFR^{+/+}$ Mice

Breeding pairs of $PAFR^{-/-}$ mice in a hybrid C57BL/6J x 129/Ol background (Ishii et al. 1998) were obtained from Dr. Takao Shimizu (University of Tokyo, Japan). Animals were backcrossed to C57BL/6 wild-type mice (Charles River Laboratories, Senneville, Canada) for 10 generations (N10) to ensure uniformity in genetic background. Congenic

N10 $PAFR^{+/+}$ and $PAFR^{-/-}$ colonies were established and maintained from $PAFR^{+/-}$ matings. Animal genotyping was performed as previously described (Nagase et al. 1999).

Cell culture

PC12 cells: PC12-AC cells clonally derived from the PC12 pheochromocytoma cell line (American Tissue Culture Collection) were cultured in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% horse serum and 5% newborn calf serum and maintained at 37°C in 5% CO_2/95% air atmosphere. Twenty-four hours before experimental manipulations, cells were seeded in 96-well plates ($1.25e^4$ cells/well) in serum-supplemented media.

Primary murine cell culture: Cerebellar granule neurons (CGNs) were cultured from postnatal day 7 to 10 mice as previously described (Cregan et al. 2000). Briefly, upon dissection of the cerebella, meninges were removed and remaining tissue minced in ice-cold dissection solution (124 mM NaCl, 5.37 mM KCl, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 14.5 mM D-glucose, 25 mM Hepes, 3 mg/ml bovine serum albumin (BSA), pH.7.4). Tissue was incubated in dissection solution containing 0.5 mg/ml trypsin (Sigma) at 37°C for 18 min before addition of 0.52 mg/ml chicken egg white trypsin inhibitor to deactivate the trypsin. Cells were then pelleted, resuspended in dissection solution containing 0.75 mg/ml DNase I (Roche, Mississauga, Canada) and triturated. $CaCl_2$ was added to a final concentration of 15 μM and, following centrifugation, cells were plated in Eagle's minimum essential medium (EMEM) containing 25 mM glucose, 10% fetal bovine serum (FBS), 1% gentamycin, 2 mM L-glutamine, and 20 mM KCl at 37°C in a 5% CO_2/95% air atmosphere. CGNs were seeded

in 96 well plates ($5.7e^4$ cells/well) coated with laminin (20 μg/ml) and poly-D-lysine (100 mg/ml, Sigma-Aldrich, St Louis, USA) and maintained in for 72 h before experimental manipulations, yielding cultures of >90% neurons as established by immunocytochemistry.

Human neuronal cell culture: NT2/D1 cells, obtained from Stratagene, a subclone of the NT2 teratocarcinoma cell line, were cultured in 10 cm tissue culture plates at 37°C in a 5% CO2/95% air atmosphere using Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamine (complete media). Differentiation of NT2/D1 cells into hNTs was performed as previously described (Boucher and Bennett 2003). Briefly, NT2/D1 cells were plated in 75-cm^2 flasks and fed three times weekly for 4-5 weeks with complete media supplemented with 10 μM retinoic acid (RA). Forty-eight hours after replating in the absence of RA, hNT neurons were separated from non-neuronal cells by mechanical force and trypsinization and replated onto tissue culture dishes (1.25×10^5 cells/ cm^2) containing laminin (20 μg/mL) and poly-D-lysine (100 μg/ml)-coated coverslips or on LabTek 4-well microslides. After 24 h, cells were treated with the mitotic inhibitors cytosine β-D-arabinofuranoside (ara-C, 1 μM, Sigma) and 5-fluoro-deoxyuridine (FUdR, 10 μM, Sigma) and maintained as previously described (Boucher and Bennett 2003) generating terminally differentiated hNT cultures after 3 weeks of mitotic inhibition.

Screening assay for PAF inhibitors

As previously described (Ryan et al. 2007), following seeding in 96-well plates, PC12 cells were treated with PAF (1 μM, 1-O-alkyl -2-acetyl-*sn*-glycerol-3-phosphocholine)

or PAF vehicle (0.1% EtOH) in the presence of each flavonoid (1 μg/ml) or vehicle (0.1% DMSO) in serum-free RPMI 1640 supplemented with 0.025% BSA. Note that all treatments were performed in serum-free media containing 0.025% BSA to ensure that cultures were not exposed to plasma PAF-AH. Following 24 h treatment, WST (Roche Diagnostics, Mississauga, Canada) was added to each well and incubated for 60 minutes before spectrophotometric analysis at 420 nm (formazan) and at 620 nm (reference). Cell number per well was calculated from standard curves derived from wells containing known cell densities. Percent cell number was calculated relative to mean cell number in vehicle-treated wells. At minimum, each compound was tested in quadruplicate on two separate occasions (n = 8).

Assessment of CGN survival

Cultured CGNs were treated in serum-free EMEM containing 25 mM glucose, 0.025% BSA, 1% gentamycin, 2 mM L-glutamine, and 20 mM KCl (treatment media) for 24 h at 37°C in a 5% CO_2 atmosphere with 1 μM PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycerol-3-phosphocholine, Biomol Research Laboratories) or PAF vehicle (treatment media alone) in the presence of hesperetin, quercetin or DMSO vehicle. CGN survival was assessed by Live/Dead viability/cytotoxicity assay (Invitrogen). Viable cells, identified by enzymatic conversion of non-fluorescent calcein-AM to fluorescent calcein, and dead cells, identified by uptake of ethidium homodimer (ET) in cells with compromised membrane integrity, were imaged using a DMIR epifluorescent inverted microscope (Leica, Richmond Hill, Canada) equipped with a QICAM digital camera (Quorum Technologies, Guelph, Canada) and captured using OpenLab software v3.5 (Improvision, Lexington, USA). Percent survival

was calculated as viable cell number (cells that are calcein-positive and ET-negative) in treatment wells relative to mean number of viable cells in vehicle control. Each treatment was tested in triplicate on two separate occasions (n = 6).

Assessment of caspase activation

Following the 24 h treatment protocol described above, executioner caspase activation in CGNs was determined using the CaspaTag (caspase-3/7) assay (Chemicon, Temecula, USA). CGNs capable of cleaving FAM-DEVD-FMK to its fluorescent product were scored as positive and reported as a percentage of total cell number after a 24 h treatment with PAF (1 μM) or vehicle (treatment media). Cells were imaged and captured as described above. Each treatment was tested in triplicate on two separate occasions (n = 6).

Assessment of hNT survival

Prior to treatment, hNTs were washed with 10 mM phosphate-buffered saline (PBS; 2.5 mM monobasic sodium phosphate, 7.5 mM dibasic sodium phosphate, 154 mM NaCl, pH 7.2). Cells were treated for 24 h with either PAF (1 μM), $A\beta_{1\text{-}42}$ (25 μM) or EtOH vehicle (0.1%), in the presence of either hesperetin, quercetin or DMSO vehicle (0.1%) in serum-free media consisting of DMEM/F12 supplemented with 1% penicillin/ streptomycin, 2 mM L-glutamine, and 0.025% BSA. $A\beta_{1\text{-}42}$ was prepared according to (Klein, 2002) by dissolving 0.1 mg Aβ (Chemicon) in 100 μl of isopropanol, separating into 10 μg aliquots and drying under steady stream nitrogen. Appropriate volumes of treatment media were then added at 4°C to give a final concentration of 25 μM. Following PAF treatment, hNT survival was determined with the Live/Dead assay as described above. Because neuronal

exposure to $A\beta_{1-42}$ is known to elicit apoptosis, hNT survival was determined by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) following 24 h treatment with $A\beta_{1-42}$. Cells were fixed for 20 min in 3.7% paraformaldehyde diluted in PBS, washed in PBS and permeabilized in ice cold 0.1% sodium citrate / 0.1% Triton-X 100 for 5 min followed by 2 min in 2:1 ethanol:acetic acid on ice. Cultures were rinsed for 2 min in PBS and incubated for 1 hr at 37°C with FITC-labeled dUTP in terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride) and TdT according to the manufacturer's protocol (Roche). Negative controls included sections incubated with FITC-labeled dUTP in the absence of TdT. Cells were washed in PBS, mounted in Vectashield (Vector Laboratories) and analyzed with a Leica DMRXA2 epifluorescent microscope equipped with a Hamamatsu ORCA-ER digital camera and OpenLab 3.5 software.

Statistical analysis

Data were analyzed using SYSTAT version 10.0 ANOVA tests. Subsequent to detection of a statistically significant difference in a given series of treatments, *post hoc* Fisher LSD tests for the PC12-AC data and *post hoc* Dunnett's t-tests on the CGN and hNT data were performed where appropriate. *P* values less than 0.05 were considered statistically significant.

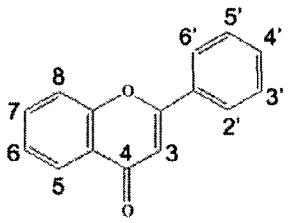
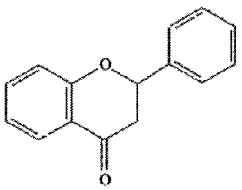
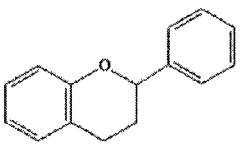
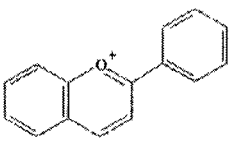
RESULTS

Flavonoid inhibitors of PAF-mediated PC12 cell loss

To identify novel inhibitors of PAF-induced neuronal death in the absence of PAFR, a preliminary screen of 15 plant-derived flavonoids was performed in the PAF-sensitive PC12-AC cell line. This cell system was chosen for preliminary screening as it does not express PAFR (Brewer et al. 2002), can differentiate to a peripheral neuronal phenotype upon treatment with nerve growth factor, and has proven successful in identifying neuroprotective compounds in previous studies (Brewer et al. 2002; Ryan et al. 2007). The selected flavonoids are structurally similar but vary in hydroxylation, methoxylation and glycosylation as well as saturation and oxidation of the C-ring (Table 11.1). Based on previously determined biologically active doses (Harris et al. 2007b), each flavonoid was tested at a concentration of 1 μg/ml (1 ppm) for its ability to alter viable cell number in serum-free media with and without 1 μM PAF.

In serum-deprived conditions, only one compound showed signs of toxicity (7-hydroxyflavone) while nine (cosmetin, 5-methoxyquercetin, taxifolin, lucenin, epicatechin, malvidin-3,5-diglucoside, rutin, myricitrin, naringin) significantly improved viable cell number relative to vehicle-treated controls (Figure 11.1, black bars). Upon PAF co-treatment, 67% of flavonoids, including all glycosides, significantly prevented PAF-induced cell loss (Figure 11.1, white bars). Of the 10 potential PAF-inhibiting compounds, seven also improved viable cell number in the absence of PAF (cosmetin, lucenin, epicatechin, malvidin-3,5-diglucoside, rutin, myricitrin, naringin). Conversely, 7-hydroxyflavone prevented PAF-induced loss but decreased viability in the absence of PAF. Only quercetin and hesperetin significantly protected PC12 cells exposed to PAF without eliciting detectable effects on viability when administered alone. To avoid complications that may arise from co-existing mitogenic or cytotoxic activities, quercetin and hesperetin were selected for

Table 11.1 – Common name, substitution pattern and molecular weight of flavonoids screened for PAF inhibiting activity.

Structures	Common Name	Substitutions	Mol. Wt.
Flavones / Flavonols	7-hydroxyflavone	C_7 = OH	238.2
	Apigenin	C_5,C_7,$C_{4'}$ = OH	270.2
	Cosmetin[a]	C_5,$C_{4'}$ = OH C_7 = O-glucose	432.4
	Lucenin[a]	C_5,C_7,$C_{3'}$,$C_{4'}$ = OH C_6,C_8 = C-glucose	610.5
	Quercetin	C_3,C_5,C_7,$C_{3'}$,$C_{4'}$ = OH	302.2
	5-methoxyquercetin	C_5 = OMe C_3,C_7,$C_{3'}$,$C_{4'}$ = OH	316.2
	Myricitrin[a]	C_5,C_7,$C_{3'}$,$C_{4'}$,$C_{5'}$= OH C_3 = O-rhamnose	464.4
	Rutin[a]	C_5,C_7,$C_{3'}$,$C_{4'}$ = OH C3 = O-rutinose	610.5
Flavonones / Flavononols	Flavanone		224.3
	Hesperetin	C_5,C_7,$C_{3'}$ = OH $C_{4'}$ = Ome	302.3
	Naringenin	C_5,C_7,$C_{4'}$ = OH	272.3
	Naringin[a]	C_5,$C_{4'}$ = OH C_7 = O-rutinose	580.5
	Taxifolin	C_3,C_5,C_7,$C_{3'}$,$C_{4'}$ = OH	304.3
Flavan-3-ols			
	Epicatechin	C_3,C_5,C_7,$C_{3'}$,$C_{4'}$ = OH	290.3
Anthocyanins			
$$			
	Malvidin-3,5-diglucoside[a]	C_7,$C_{4'}$ = OH $C_{3'}$,$C_{5'}$ = OMe C_3,C_5 = O-glucose	655.6

[a] Flavonoid glycoside

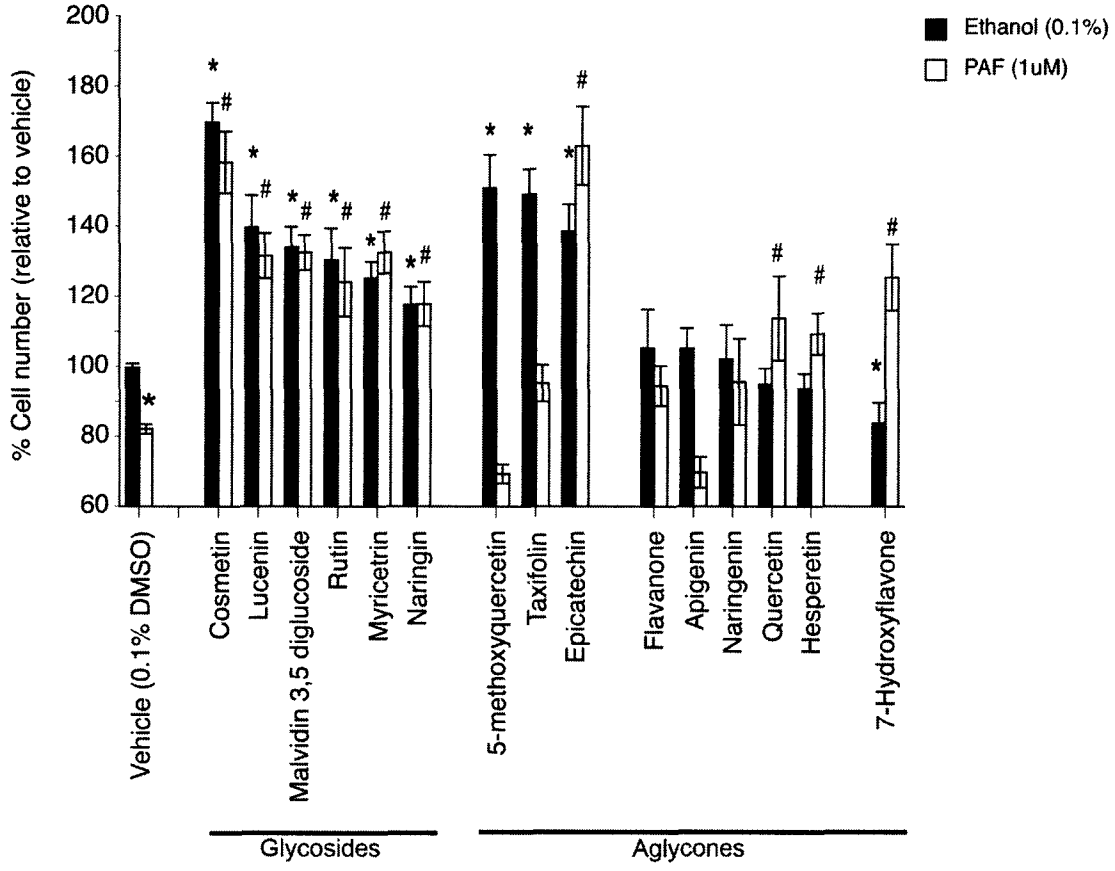


Figure 11.1 – Effects of flavonoids on PC12 cell number under serum-deprived conditions in the absence and presence of PAF. Viable cell number was quantified by WST assay and standardized to wells of known cell densities after a 24 h treatment with vehicle (black bars) or PAF (white bars) and test compounds. Flavonoids were tested at 1 μg/ml. (A) Toxic (<100% viability) and proliferative (>100% viability) activities were established by comparing the effects of flavonoids on vehicle-treated controls (* $p < 0.05$, relative to EtOH/DMSO, Fischer LSD *post hoc*) while protective effects were established by comparing the effects of flavonoids on PAF treatment (# $p < 0.05$ relative to PAF/DMSO). All flavonoids were tested on two occasions (n=8-10) and control data were pooled from all experiments (n=60). Data represent mean + SEM.

further analysis in primary murine neurons.

Quercetin and hesperetin inhibit PAF-mediated apoptosis in PAFR deficient primary mouse neurons

To determine whether the two inhibitors selected from our PC12 screen, quercetin and hesperetin, likewise protect CNS neurons, mouse $PAFR^{-/-}$ CGNs, were exposed to pathophysiological concentratons of PAF in the presence or absence of test compounds. Exposure to flavonoids alone had no significantly effect on PAFR-negative neuronal viability (Figure 11.2A, black bars). In DMSO-treated vehicle controls, exposure to PAF resulted in a 50% loss in CGN survival, an effect that was significantly abated by quercetin and completely inhibited by hesperetin (Figure 11.2A, white bars). Furthermore, both compounds effectively prevented PAF-induced executioner caspase activation (Figure 11.2B).

Because the expression of PAFR blocks PAF-induced death in $PAFR^{+/+}$ CGNs (Ryan et al. 2007), we also determined whether quercetin or hesperetin interfered with PAFR-mediated protection in WT CGNs upon PAF treatment. In these cells, PAF treatment had little effect on cell survival and neither quercetin nor hesperetin elicited significant changes when administered alone (Figure 11.2C, black bars) or in the presence of PAF (Figure 11.2C, white bars).

Hesperetin and quercetin protect human neurons from PAF-mediated death

Upon confirmation of protective activity in PAF-treated PC12 cells and primary murine neurons lacking PAFR, the efficacy of quercetin and hesperetin was assessed in the hNT human neuronal cell line, which similarly does not express PAFR and is susceptible to

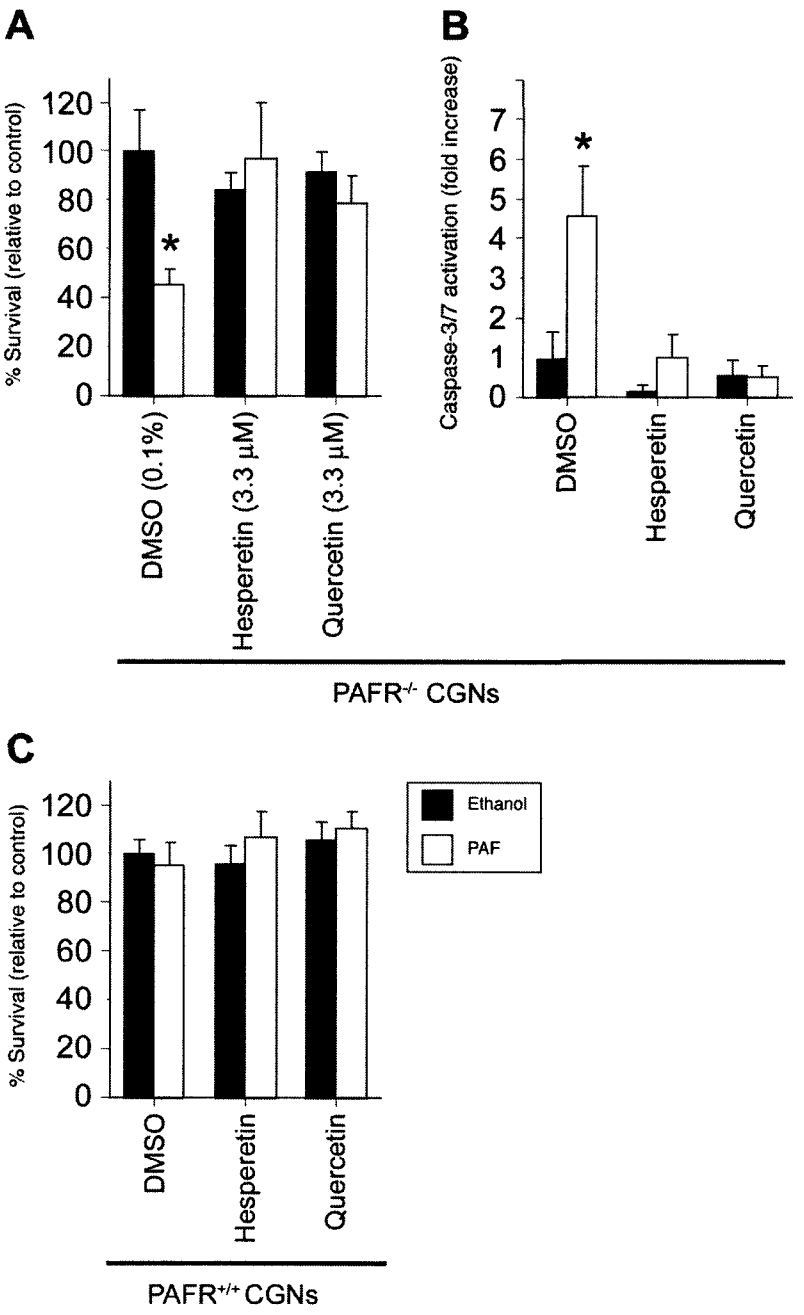


Figure 11.2 – Quercetin and hesperetin protect PAFR-/- neurons from PAF toxicity without compromising PAFR-mediated neuroprotection. Cell survival was assessed by LIVE/DEAD Viability/Cytotoxicity Kit. The effect of hesperetin and quercetin (1 µg/ml) on (A) PAFR-/- and (B) PAFR+/+ survival was determined in the presence of vehicle (black bars) or PAF (white bars) in serum-free media. (C) Executioner caspase activation was determined in PAFR-/- CGNs. (* $p < 0.05$ relative to EtOH/DMSO; # $p < 0.05$ relative to PAF/DMSO, *post hoc* Dunnett's *t* test, n=6). Data represent mean + SEM standardized to EtOH/DMSO control cultures.

PAF-mediated apoptosis. In the absence of PAF, no negative effects were observed with flavonoid treatment relative to vehicle treated cells (Figure 11.3A, black bars). PAF treatment resulted in significant hNT death, reducing survival after 24 h to nearly 50% of vehicle treated control cultures (Figure 11.3, white bars). As observed in both PC12 cells and mouse CGNs, hesperetin and quercetin significantly reduced PAF-mediated apoptotic death (Figures 11.3, black bars).

Hesperetin and quercetin protect human neurons from Aβ-mediated death

To further characterize the neuroprotective potential of quercetin and hesperetin as novel pharmacological agents for treatment against AD, each compound was co-administered to hNTs with β-amyloid$_{1-42}$ (Aβ) as an *in vitro* model of Alzheimer's Disease. Following 24 h of treatment, effects on cell survival were assessed by TUNEL assay. As observed using the Live/Dead assay, TUNEL analysis revealed no effect on cell survival when either flavonoid was administered with treatment medium alone (Figure 11.4, black bars). Similar to PAF treatment, Aβ induced a 50% reduction in surviving cells. Both flavonoids significantly improved hNT survival under Aβ challenge, with hesperetin providing a more pronounced effect (Figure 4, white bars).

DISCUSSION

Using three *in vitro* models of PAF-mediated apoptosis in the absence of PAFR, plant-derived flavonoids were assessed for protective activity, identifying two novel PAF inhibitors. The first model, the PC12 cell line, was selected for preliminary screening because these cells do not express PAFR, are sensitive to PAF toxicity and have previously served successfully in identifying inhibitors of PAF-induced death of primary neurons

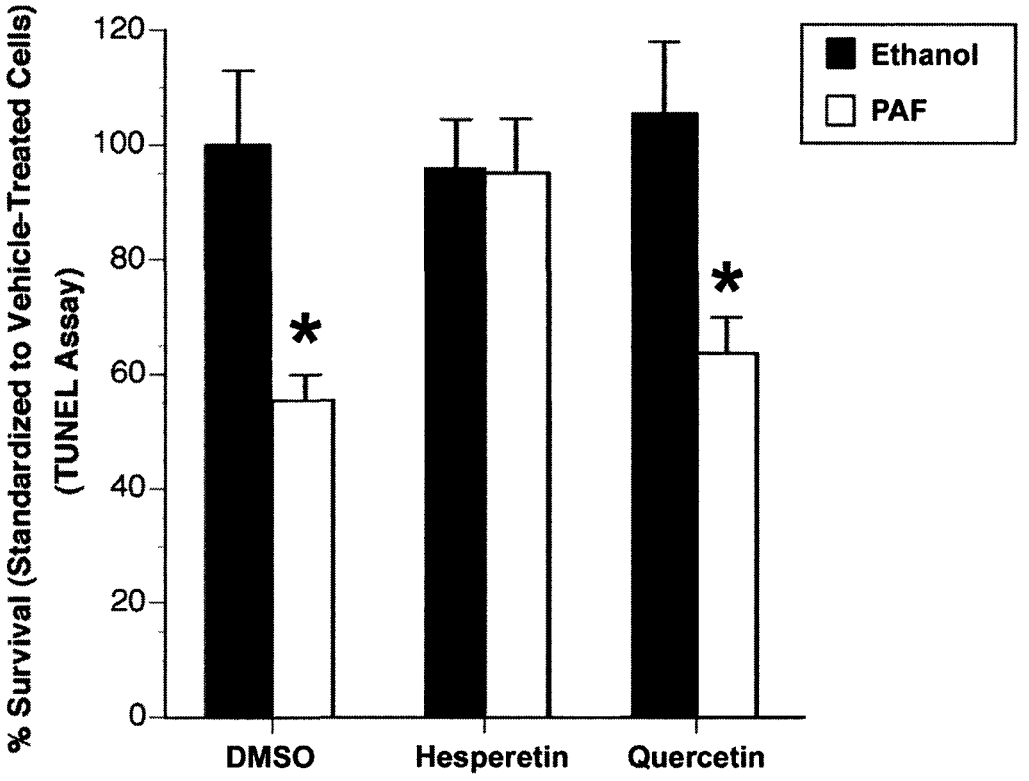


Figure 11.3 – Hesperetin and quercetin prevent PAF-induced death of human neuronal cells. Cell survival was assessed by LIVE/DEAD Viability/Cytotoxicity Kit. The effect of quercetin and hesperetin (1 μg/ml) on hNT survival was determined in the presence of vehicle (black bars) or PAF (white bars) in serum-free media (* $p < 0.05$ relative to EtOH/DMSO; # $p < 0.05$ relative to PAF/DMSO, *post hoc* Dunnett's *t*-test, n=5-6). Data represent mean + SEM standardized to EtOH/ DMSO control cultures.

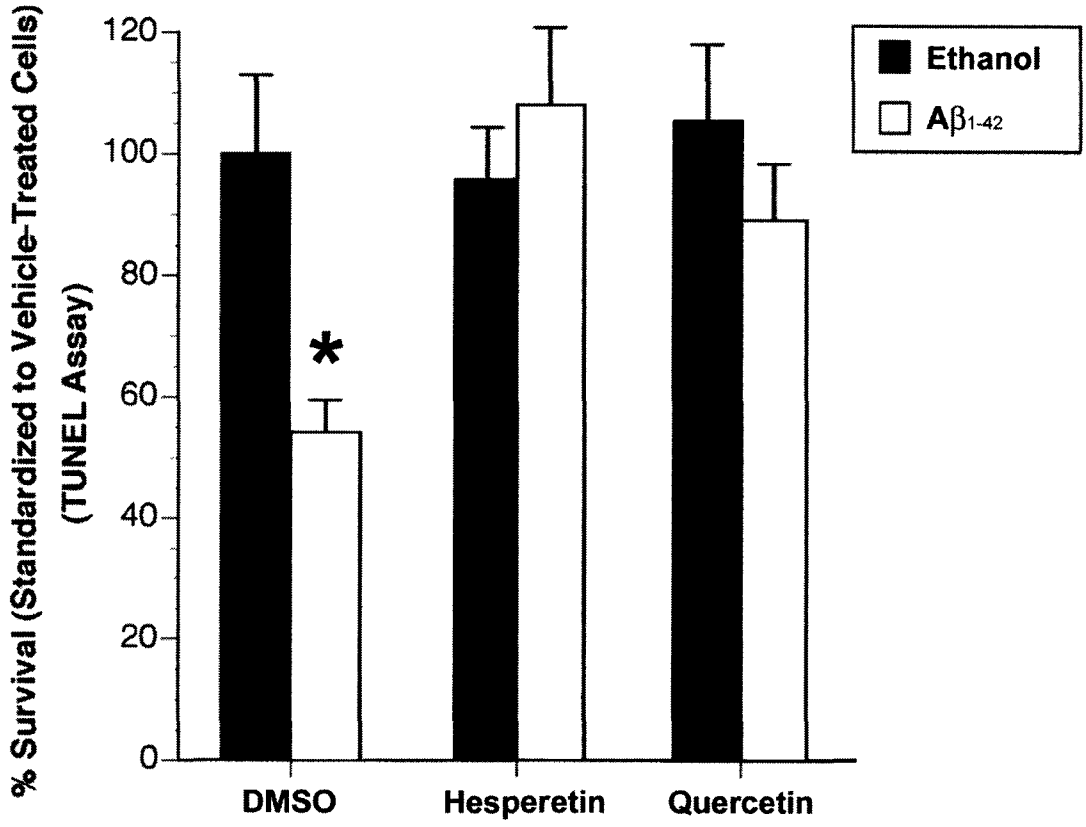


Figure 11.4 – Hesperetin and quercetin inhibit Aβ-mediated death of human neuronal cells. Cell survival was assessed by TUNEL assay. The effect of quercetin and hesperetin (1 μg/ml) on hNT survival was determined in the presence of vehicle (black bars) or $A\beta_{1-42}$ (white bars) in serum-free media (* $p < 0.05$ relative to EtOH/ DMSO; # $p < 0.05$ relative to $A\beta_{1-42}$/DMSO, *post hoc* Dunnett's *t*-test, n=5-6). Data represent mean + SEM standardized to EtOH/DMSO control cultures.

(Bonin et al. 2004; Brewer et al. 2002; Ryan et al. 2008; Ryan et al. 2007). A constant flavonoid concentration of 1 ppm (1 μg/ml) was selected because it reflects previously determined active levels (Harris et al. 2007b) as well as physiologically relevant conditions attainable through dietary consumption (Erlund et al. 2001; Erlund et al. 2006; Nielsen et al. 2006). Although 10 of 15 flavonoids significantly improved viable PC12 cell number following PAF treatment in serum-free media, seven also increased cell number in the absence of PAF, a possible mitogenic effect that may mimic PAF inhibition. As post-mitotic terminally differentiated CNS neurons were our primary target, the effects of putative mitogens were not investigated further. It is nonetheless interesting to note that each of the six tested flavonoid glycosides improved cell viability in the absence of PAF to a similar degree as it did in the presence of PAF. This observation is consistent with a previously reported structure-activity-relationship among glycosides with respect mitogenic activity in P12 cells (Harris et al. 2007b) suggesting that PAF-mediated PC12 cell death could involve exit from the cell cycle and that induced re-entry (by flavonoid glycosides) may inhibit cell death.

The two flavonoids that prevented PAF-treated PC12 cell loss but had no impact on cell number when administered alone were subsequently evaluated in $PAFR^{+/+}$ and $PAFR^{-/-}$ CGNs. Both hesperetin and quercetin significantly inhibited PAF's neurotoxic effects in $PAFR^{-/-}$ CGNs while having no effect on $PAFR^{+/+}$ CGNs under control or PAF conditions, indicating specific PAF inhibition without compromising the viability of PAFR expressing neurons. Confirmation of their protective actions in our third model of PAFR-independent PAF-mediated death, hNT neurons, suggests that common cellular mechanisms of PAF toxicity and flavonoid protection exist in all three cell systems and validates our approach to

identifying novel neuronal PAF inhibitors.

Although similar caspase-dependent mechanisms have been reported in PC12 cells, $PAFR^{-/-}$ CGNs and hNTs (Bonin et al. 2004; Ryan et al. 2008; Ryan et al. 2007), the cellular events leading to PAF-mediated apoptosis remain unclear. In PC12 cells, induced executioner caspase activation and cell death can be prevented by accelerated PAF catabolism (Bonin et al. 2004). As known modulators of PAF metabolising enzymes such as phospholipase A_2, PLA_2 (Lindahl and Tagesson 1997), flavonoids may function in a similar manner. Quercetin, for example, inhibits lysoPAF-acetyltransferase activity and ensuing PAF production in rat white blood cells treated with a calcium ionophore (Yanoshita et al. 1996). Through modification of PAF-dependent transacetylase activity in endothelial cells, quercetin and hesperedin (a glycosylated form of hesperetin) have been shown to reduce hydrogen peroxide-induced production of PAF and simultaneously increase production of acyl-PAF, a natural PAF antagonist (Balestrieri et al. 2003). In various cell types including neurons, signal transduction pathways mediating cell survival and cell death, such as NIK/IKK, ERK and JNK, are modulated by hesperetin and quercetin (Kim et al. 2006; Schroeter et al. 2001; Spencer et al. 2003), thus providing another speculative mechanism of PAF inhibition.

To gain additional insight into the neuroprotective potential of quercetin and hesperetin in a human neuronal system, we assessed their activity in hNTs treated with Aβ, a challenge to which flavonoid-mediated protection has been established (Rezai-Zadeh et al. 2005; Wang et al. 2001b). Quercetin, the most extensively studied flavonoid, has established inhibitory effects on Aβ aggregation, fibril formation, associated oxidative stress (Kim et al. 2005; Matsuzaki et al. 2007; Ono et al. 2003) and Aβ-mediated cell death (Kim et al. 2005; Zhu et al. 2007). In other studies, quercetin has shown little or no protection from Aβ

neurotoxicity (Bate et al. 2004a; Wang et al. 2001b). Quercetin's protective activity toward Aβ in hNTs is the first such report in human neurons. As reported in rat cortical neurons (Cho 2006), hesperetin prevented Aβ-induced hNT loss, providing support for continued analysis of this compound in Aβ-related neurodegeneration. This finding is of further interest because, unlike the mouse CGN model employed here, rat cortical neurons, which express PAFR, undergo PAF-mediated death via NMDA receptor excitotoxicity and nitric oxide formation (Bate et al. 2007; Xu and Tao 2004). As such, while natural and synthetic PAFR antagonists (Bate et al. 2006; Longpre et al. 2006), as well as cholesterol synthesis inhibitors (Bate et al. 2007), have proven effective as *in vitro* inhibitors of both PAF- and Aβ-mediated neuronal death in PAFR-expressing cultures, hesperetin and quercetin are the first to display such activities in PAFR-deficient neurons.

In conclusion, the presented results demonstrate that hesperetin and quercetin inhibit the apoptotic actions of PAF in three separate *in vitro* systems lacking PAFR and and prevent Aβ toxicity in human neurons. Considering that the observed protection was achieved at physiologically relevant concentrations and that both hesperetin and quercetin appear capable of crossing the blood-brain-barrier (Dimpfel 2006; Paulke et al. 2006; Youdim et al. 2003), our findings provide further support for continued study of these flavonoids in PAF-associated neurodegenerative conditions and of PAF inhibitors as candidates for the treatment of AD. Quercetin is commonly produced by plants and is present in large quantities in many food and medinal plants such as blueberry (*Vaccinium angustifolium*) and *Ginkgo biloba* while hesperitin is common in plants such as citrus and mints. This study also highlights the value in comprehensively evaluating PAF sensitivity in multiple neuronal populations (with and without PAFR) and identifying PAF inhibitors with appropriate specificity as candidates for treatment of glycerophosphocholine-associated toxicity in AD.

ACKNOWLEDGEMENTS

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CHAPTER 12 – GENERAL DISCUSSION

12.1 OVERVIEW OF RESULTS AND NOVEL CONTRIBUTIONS

Taking an ethnobotanical approach to identify boreal plants with anti-diabetic properties, I applied techniques in analytical chemistry and pharmacology to characterize the phytochemistry and biological activities of some of Canada's most abundant, medicinally important, yet pooly studied plant species. The phytochemical and *in vitro* pharmacological investigations of lowbush blueberry, *Vaccinium angustifolium*, a common boreal plant that is widely used in Québec to treat T2DM, served as a pilot study for the TAAM project and provided a starting point for further work with other boreal plants.

Because the berries of *V. angustifolium*, cultivated and harvested from the wild, are used in foods, drinks and health supplements throughout North America, berry phytochemistry has been previously characterized and includes several classes of phenolic secondary metabolites (Kalt et al. 2001; Lyons et al. 2003; Schmidt et al. 2004). The analytical method described in Chapter 2, the first developed for comparative analysis of other plant parts, identified a variety of phenolic compounds including phenolic acids, flavonoids and tannins. Phenolic metabolites were a focus for subsequent characterization of other boreal plants because of their abundance in northern plants. In total, 33 extracts representing 27 species were evaluated and over 60 different phenolics were identified. While the profiling of phenolic metabolites alone is but one component of extensive phytochemical characterization, the obtained results were sufficient in terms of identifying marker compounds for species authentication (i.e., between Cree anti-diabetic plants (Chapter 5) as well as other related boreal species (Chapter 3)). The fingerprinting of

medicinal Ericaceae species of the boreal forest (Chapter 3) is the first of its kind, as systematic phytochemical analyses of medicinal plants have generally been limited to economically important plants of southern Canada such as Echinacea (Binns et al. 2002) and Ginseng (Kite et al. 2003). Given the abundance of Ericaceae species across Canada and their widespread use and availability as medicinal plants among aboriginals or natural health products by herbal practitioners and the general public alike, the effort was likely overdue. Overall, although 27 species accounts for only a small fraction of the boreal forest's plant diversity, when considering the limited data available regarding the chemistry of Canada's native flora, the results described herein represent a significant contribution to phytochemistry.

A similar deficiency in data exists with regard to the biological activity and pharmacological potential of Canada's boreal plants and their chemical constituents. To date, much of the research in this field has focused on anti-microbial and allelopathic activities that are relevant to chemical ecology and agriculture but, apart from the most prominent commercial herbal medicines, biological effects pertinent to human health have remained largely unexplored. Prior to the TAAM, the anti-diabetic potential of medicinal boreal plants had only been studied in relation to their anti-oxidant properties and traditional uses (McCune and Johns 2002; McCune and Johns 2003). The TAAM project is not only novel in its ethnobotanical approach centering on symptoms of diabetes but also in its comprehensive evaluation of identified plants in cell-based systems targeting multiple anti-diabetic mechanisms. As such, the majority of biological activities observed in the thesis have not been reported previously in studied species. The bioactivities of many of the phenolic metabolites identified within the Cree medicinal plant extracts, however, have been studied extensively in mammalian systems and include, among others, anti-diabetic, anti-

oxidant, anti-glycative and neuroprotective effects (Dajas et al. 2003b; Galli et al. 2002; Middleton et al. 2000).

For example, the identified activities of *V. angustifolium* (Chapter 4) provide the first pharmacological validation of one of Canada's most popular anti-diabetic herbal remedies (Haddad 2003) and distinguished organ-specific effects that likely underlie, in part, the plant's varied medicinal uses. Working with other anti-diabetic plants identified by the Elders of CEI, we not only discovered that every species exhibited some degree of *in vitro* anti-diabetic activity but also found that many extracts possessed promising activities in more than one assay (Spoor et al. 2006; Harbilas, unpublished; Chapters 5 & 8). While these findings are relevant to the Cree of northern Québec, they also provide support for the medicinal use of widely distributed plants such as Labrador tea (*Rhododendron groenlandicum* and *R. tomentosum*), tamarack (*Larix laricina*) or white and black spruce (*Picea glauca* and *P. mariana*) by other First Nations groups living in the boreal region (Marles 2000). Beyond providing candidate species for further evaluation as potential complementary anti-diabetic therapies, these findings validate the Traditional Knowledge shared by the Elders of CEI thereby establishing a bridge between Traditional Cree healing practices and those of Western medicine. Although unrelated to the stated objectives of the thesis and the TAAM project, the overall high occurrence of modulatory effects on energy metabolism in mammalian cells is relevant to the field of chemical ecology and identifies the production of metabolically disruptive secondary metabolites as a common mechanism of deterring herbivory in boreal plant species (Marles 1995).

The first hypothesis of this thesis, that selection of medicinal plants by Cree Elders is based, in part, on the plant's pharmacological properties, received only partial support from the collected results. Although comparison of different plant parts of the same species

followed the prediction that the parts most frequently used by Elders are more active than those that are seldom used (Chapters 6 & 7), cytoprotection in the PC12 models of diabetic neuropathy did not correlate with ethnobotanial SIV score (Chapter 5). Similarly, no relationship was observed between SIV scores and anti-glycation activity (Chapter 8). One simple explanation for this lack of correlation between activity and ethnobotanical data is that only anti-diabetic plants were evaluated with no control group of non-medicinal or at least non-anti-diabetic plants. Indeed, a comparison of medicinal and non-medicinal plants is a more established means of validating Traditional Knowledge (Jones et al. 2000; Marles 1995). A second explanation may be that the ethnobotanical data and therefore SIV score were based on general symptoms of diabetes rather than specific symptoms of diabetic neuropathy. It is nonetheless noteworthy that, in support of this hypothesis, a significant positive correlation between SIV score and anti-oxidant capacity was observed by our collaborators at McGill University (Fraser 2007).

The second and third hypotheses, that anti-diabetic activities are mediated by extract phenolic content and anti-oxidant activity, are interrelated since the two factors are positively correlated among the Cree extracts (Fraser 2007). This relationship is not unique to Cree medicinal plants but has been consistently identified by anti-oxidant studies involving crude plant extracts (Pellati et al. 2005; Xu et al. 2007). It is therefore not surprising both hypotheses were supported by the results in the anti-glycation assay where increasing phenolic content and anti-oxidant activity were positively correlated with extracts' ability to inhibit AGE formation (Chapter 8). While oxidative processes are known to promote AGE formation (Peyroux and Sternberg 2006) and anti-oxidants such as plant phenolics are known to inhibit this reaction (Wu and Yen 2005), the current study is one the first to report these correlations within a collection of plant samples (Farsi et al. 2007). Interestingly, the relative

anti-glycation activity of different pure phenolics has been attributed to their relative anti-oxidant potential (Matsuda et al. 2003). However, the fact that AGE inhibition was more strongly correlated with total phenolics than anti-oxidant activity suggests that, while anti-oxidants in general may prevent AGE formation, phenolics, which represent one of many types of plant derived anti-oxidants, are particularly effective.

Cytoprotection from glucose toxicity and glucose deprivation was not correlated with either the phenolic content or anti-oxidant effects of extracts as initially predicted (Chapter 5). Whereas the total phenolic content of Cree extracts was significantly correlated with anti-oxidant and anti-glycation activity determined in chemical-based *in vitro* assays, it was not correlated with any of the anti-diabetic effects observed in cell-based assays. This, however, does not preclude sub-classes or individual phenolics (or other anti-oxidants) from exerting protective effects. For instance, glucose toxicity in the peripheral nervous system has only recently been implicated in the development of diabetic neuropathy (Vincent et al. 2005a) and, consequently, few studies have sought to identify inhibitors of glucose-induced cell death. Hypothesizing that oxidative stress generated by glucose toxicity initiated apoptosis in peripheral neurons, Vincent et al. found that anti-oxidants such as lipoic acid and nicotinamide prevented glucotoxic neuronal death (Vincent et al. 2005b). In Section 2, a number of Cree extracts prevented glucotoxic cell death in PC12 cells and quercetin-3-*O*-galactoside, a cytoprotective flavonoid isolated from *S. purpurea*, is likewise a strong anti-oxidant.

Conversely, the lack of identified relationships suggests that cytoprotective activity of certain extracts was mediated by non-phenolic constituents acting through mechanisms independent of oxidative stress. As evidenced by the involvement of bond dissociation enthalpy in the QSAR model of flavonoid cytoprotection outlined in Chapter 9, anti-oxidant

properties no doubt impact upon the overall cellular effects elicited by plant compounds. Such relationships, however, are not always clear or even valid with the added complexity of crude extracts. Unlike chemical reactions occurring in a homogenous liquid phase, living cell cultures are compartmentalized by membranes and, though many cellular responses can be mediated from the extracellular space, exogenously applied compounds often need to cross the cell membrane (passively or actively) to exert their effects. With complex mixtures such as crude extracts, the bioavailability of active compounds may be altered by others in the mixture. Similarly, if a given compound activates or inhibits a targeted pathway, others may directly or indirectly influence the same pathway and thereby modify the final result. Because oxidative stress and AGE formation occur in extracellular spaces such of the lumen of blood vessels (Aronson 2008; Rojas and Morales 2004), the anti-oxidant and anti-glycation activities of Cree extracts would likely be relevant *in vivo* if the actives are capable of entry into the bloodstream.

In addition to their relevance to diabetic neuropathy and other microvascular complication of diabetes, many of the biological activities identified in Cree extracts and otherwise ascribed to their contained phenolic metabolites, including anti-oxidant, anti-glycation, anti-inflammatory and anti-apoptotic are also relevant to neurodegenerative conditions of the CNS, notably given known complications and predispositions in diabetic patients (Haan 2006; Tanne 2008). Moreover, the observed concentrations at which individual phenolics exerted protective effects were within a range that is attainable through dietary consumption of foods rich in phenolics. For these reasons, a library of structurally diverse phenolic compounds representative of those identified in Cree extracts was systematically evaluated in a series of cell-based experiments aimed first at characterizing

their cellular effects at low micromolar levels and second at identifying inhibitors of PAF- and Aβ-mediated neuronal death.

New activities were also identified over the course of this study that impact upon clinical use. In contrast to the anti-tumour effects of many plant phenolics on neoplastic cell growth at high concentrations, when present at physiologically relevant concentrations, 78% of tested phenolics elicited pro-survival (mitogenic or anti-apoptotic) effects while only a small number (15%) were cytotoxic or pro-apoptotic (Chapter 9). Though the wealth of activity was surprising, the appearance of anti-apoptotic activity was not unexpected since many plant phenolics have been shown to protect neurons and neuronal cell models such as PC12 cells from a variety of apoptotic stresses (Dajas et al. 2003a; Ishige et al. 2001; Mandel and Youdim 2004). As the phenolic library contained both extensively and scarcely studied compounds, few of which have been evaluated in cells treated with either etoposide or ceramide, these results add to the growing literature on the bioactivities of plant phenolics in mammalian systems *in vitro* and suggest a possible role for dietary phenolics in the prevention of neuropathic side effects common to cancer treatments with etoposide- or cermaride-like apoptotic mechanisms (Quasthoff and Hartung 2002; Verstappen et al. 2003).

With an abundance of observed anti-apoptotic activities and concurrent evidence from my laboratory that PAF family members are increased in diabetic peripheral nerve and Alzheimer central neurons, the direction of my research shifted to identifying inhibitors of PAF-induced neuronal death. While the neurotoxic effects of PAF are often mediated through its receptor (PAFR), increasing evidence including the results presented in Chapter 10 indicate that neuronal apoptosis initiated independently of PAFR may contribute to PAF-related pathologies (Bonin et al. 2004; Brewer et al. 2002; Ryan et al. 2008). As such,

neuroprotection from PAF in the absence of PAFR represents a novel target for therapeutic intervention. Two classes of plant phenolics, benzoic acid derivatives and flavonoids, were selected for evaluation, in part, because members of both classes alter PAF catabolism (Balestrieri et al. 2003; Huh et al. 1998) and prevent etoposide-induced death, in which PAF-like lipids play a role (Ahlemeyer et al. 2001; Hattori et al. 1995; Li et al. 2003; Southall et al. 2001). In addition to several novel PAF inhibitors effective in PC12 cells, three novel anti-apoptotic inhibitors of PAFR-independent PAF neurotoxicity were identified, orsellinic acid, hesperetin and quercetin (Chapters 10 & 11). Hesperetin and quercetin, two flavonoids commonly found in fruits and vegetables, also prevented neuronal death in a second *in vitro* model of Alzheimer's disease, AB_{1-42}, confirming their previously described neuroprotective activity (Cho 2006; Kim et al. 2005) albeit with a species-specific effects with respect to quercetin. Since activities were generally consistent in PC12 and neuronal cultures alike, these results are also encouraging with regards to the cytoprotective activities of Cree extracts observed in PC12 models of diabetic neuropathy.

During the course of my research, new methodologies were developed or modified from existing protocols to meet experimental needs. In Section 1, the metabolomics-based approach used to characterize the phenolic metabolites of medicinal plant extracts proved effective for different organs of the same plant species (Chapter 2), for the same organ from related species (Chapter 3) and, though less effectively, for different organs from unrelated species (Chapter 5). The cytoprotection and anti-glycation assays employed throughout Section 2 were based on previous protocols but were modified for high-throughput screening of crude extracts and pure compounds and adapted to include secondary more advanced measures to confirm effects observed during screening. In Section 3, the PC12 cell system and cytoprotection assay was further modified for a novel purpose – to rapidly distinguish

between mitogenic, cytopropective, anti-apoptotic, pro-apoptotic and cytotoxic effects elicited by individual plant phenolics.

12.2 BOREAL PLANTS AND THEIR PHENOLIC METABOLITES IN THE MANAGEMENT OF DIABETIC NEUROPATHY

As outlined in Chapter 1, the pathophysiology of diabetic neuropathy is thought to involve multiple interrelated factors promoted by hyperglycemia. Together, these factors result in direct neurotoxicity through oxidative stress, the accumulation of AGEs and activation of apoptotic signaling pathways as well as indirect toxicity by reducing cellular defenses and damaging the vasculature supplying nutrients and growth factors to the peripheral nervous system (Figure 1.4). The DCCT and UKPDS studies, which dealt with the development of complications in patients with T1DM and T2DM, respectively, revealed three important messages; 1) there is a continuous relationship between glycemia and the incidence and severity of diabetic complications, 2) intensive glycemic control reduces the incidence and severity of diabetic complications, and 3) intensive glycemic control is difficult to sustain (DCCT 1993; UKPDS 1998a; UKPDS 1998b). Unfortunately, like many other vascular complications of diabetes, diabetic neuropathy is difficult to treat once developed and no effective pharmacotherapies are currently available beyond those used to control glycemia (Donnelly 2005). A number of the boreal plants studied, such as *V. angustiflium* and *S. purpurea*, demonstrated insulinomimetic or insulin sensitization effects that could assist in maintaining glycemic control and therefore in preventing the development and progression of diabetic neuropathy. Not surprisingly, several phenolic metabolites identified in these extracts possess similar hypoglycemic activities (Liu et al.

2004; Pinent et al. 2004; Su et al. 2006). However, as illustrated in this thesis and supported by TAAM collaborators as well as numerous previous studies, the biological activities of boreal plant species and their phenolic metabolites are particularly relevant to the prevention and treatment of diabetic neuropathy.

The potential mechanisms through which the studied plants and phytochemicals may provide protection are summarized in Figure 12.1. This simplified recreation of Figure 1.4 from the Introduction has been modified to include specific processes that are inhibited (or opposed) by boreal plants (represented as **B** in Figure 12.1) or phenolic metabolites characteristic of boreal plants (**P**) according to evidence provided by the current thesis (in green), collaborating laboratories from the TAAM (in red) or the published literature (in black). In terms of reducing oxidative stress associated with diabetes, many boreal plants, including those used by the CEI, have been shown to effectively scavenge free radicals and to reduce the oxidation of low density lipoproteins (Fraser 2007; McCune and Johns 2002; Spoor et al. 2006). Individual phenolic compounds, for example quercetin derivatives (Chapter 7) and catechins (Igarashi et al. 2007; Oh et al. 2008), have also been shown to reduce oxidative stress and cell death mediated directly by high levels of glucose. Similarly, a number of plant phenolics, particularly flavonoids, prevent neuronal loss through anti-oxidant and anti-apoptotic mechanisms in diverse *in vitro* and *in vivo* disease models (Chapter 10 & 11) (Dajas et al. 2003a; Ishige et al. 2001).

As presented in Chapter 8, the anti-glycation activities of Cree plant extracts were highly correlated with total phenolic content and radical scavenging activity. This not only supports previous reports but also suggests that AGEs formed through high glucose-mediated oxidative processes are specifically reduced. Interestingly, other groups have shown that (-)-epigallocatechin gallate (EGCG) prevents AGE-induced neuronal damage

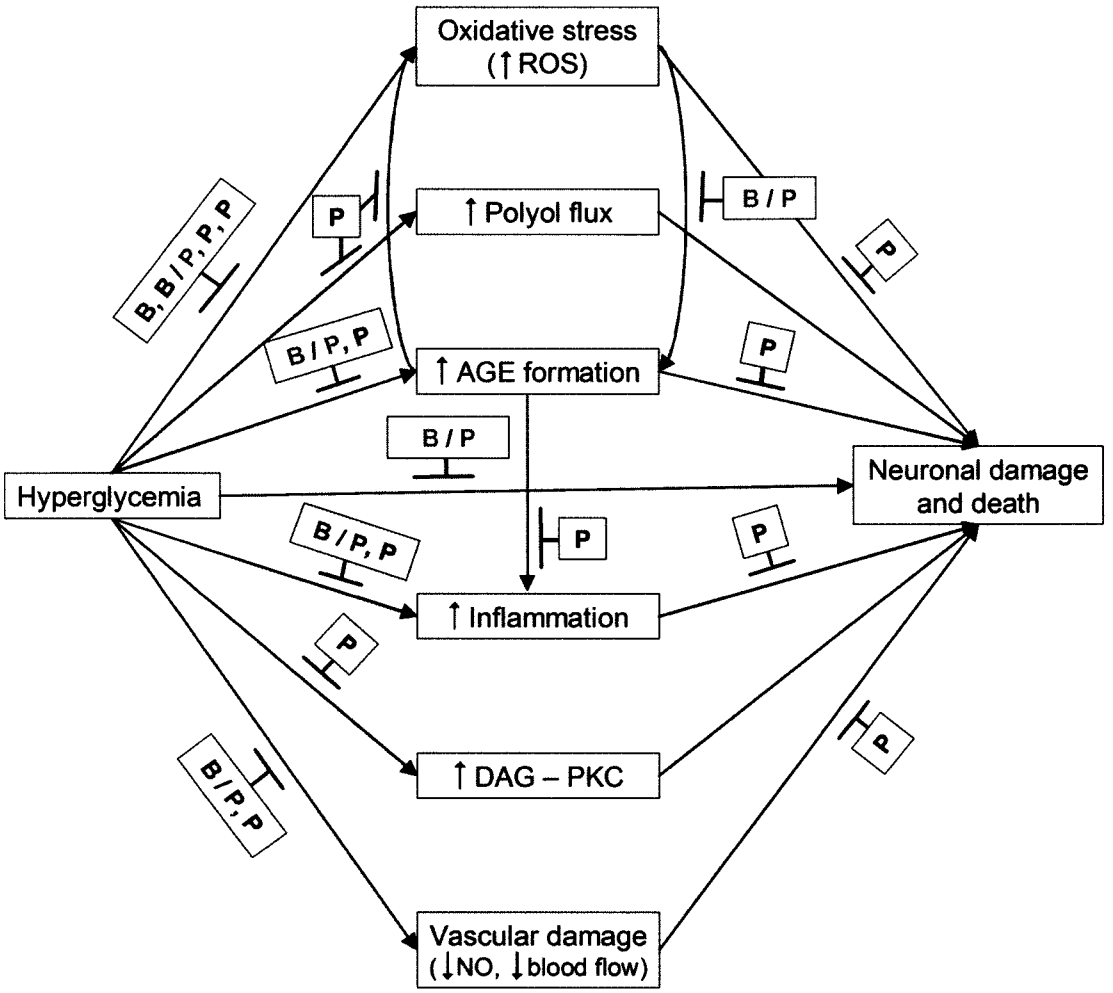

Figure 12.1 – Biological activities of boreal plant extracts and their phenolic metabolites relevant to the prevention and treatment of diabetic neuropathy. As shown in this thesis (green), by TAAM collaborators (red) and otherwise published studies (black), Boreal plants (B) and their associated phenolic metabolites (P) inhibit a large number of the hyperglycemia-mediated biochemical and molecular mechanisms contributing to the development of diabetic neuropathy.

(Lee and Lee 2007), grape seed procyanidins reduce AGE-mediate RAGE expression in endothelial cells (Zhang et al. 2007) and quercetin inhibits RAGE-activated inflammatory signaling in human monocytes at both the gene and protein levels (Huang et al. 2006). Together, these results show how plant phenolics could protect from AGEs both upstream and downstream of their formation. Moreover, different classes of plant phenolics possess anti-inflammatory activities that would likely oppose the pro-inflammatory consequences of AGEs and RAGE activation to some degree (Middleton and Kandaswami 1992). As demonstrated elsewhere by the TAAM, many of the Cree extracts inhibit lipopolysaccharide-induced macrophage activation (unpublished data).

In hyperglycemic conditions, excess glucose is metabolized to sorbitol by aldose reductase, which increases the flux through the polyol pathway and, in turn, perpetuates oxidative stress, non-enzymatic glycation and PKC activation. While aldose reductase inhibiting effects have not yet been evaluated by the TAAM, certain plant phenolics are known to inhibit the enzyme's activity (Aida et al. 1989; Varma 1986). Another consequence of hyperglycemia that has not been directly targeted by the TAAM is the elevated rate of glycolysis that results in accumulation of DAG and activation of PKC. Although evidence exists for phenolic-based inhibition of different PKC isoforms, including those most associated with diabetic complications, in cell types relevant to diabetes (Ferriola et al. 1989; Gamet-Payrastre et al. 1999; Romero et al. 2008), PKC activating effects have also been reported (Vargo et al. 2006).

With the exception of direct glucose neurotoxicity, each of the mechanisms discussed above leads to vascular damage and, as such, not only contributes to diabetic neuropathy but to microvascular complication in general. TAAM collaborators at McGill have recently identified several Cree extracts that modulate endothelial NO levels as well as NO synthase

(NOS) activity (unpublished data). As with PKC, plant phenolics are known to positively and negatively influence NOS expression and activity, depending on both the cell type and the stimulus (Hamalainen et al. 2007; Kim et al. 2007; Mann et al. 2007). Once vascular damage has occurred, however, those Cree extracts that protected against glucose deprivation may offer another line of defense. The fact the plants such as *V. angustifolium* (berries) and individual phenolics have reported neuroprotective effects in *in vitro* and *in vivo* models of ischemia is also promising (Behl and Moosmann 2002; Rivera et al. 2004; Sweeney et al. 2002).

Considering that anti-oxidants, anti-glycation agents, aldose reductase inhibitors, PKC inhibitors and anti-inflammatory drugs have been all pursued pharmaceutically, the combined protective activities of Cree medicinal plants certainly support their further investigation as complementary treatments for diabetic neuropathy and other vascular complications. Importantly, as many of the active phenolic metabolites found in Canadian boreal plants are commonly produced by plants in other regions of the world, traditional food and medicine plants of other indigenous societies would likely possess similar therapeutic potential. As locally available, economically accessible and culturally ingrained, such plants could help ease the burden of T2DM among aboriginal people worldwide.

12.3 FUTURE DIRECTIONS

Due to the interdisciplinary nature of my doctoral work, future research could proceed in several directions, many of which will be followed as part of the TAAM project. In terms of phytochemistry, certain species remain largely uncharacterized and require further investigation into their non-phenolic metabolites. The Pinaceae represents one of the

boreal forest's dominant plant families in terms of both species composition and Traditional medicinal use and, as done for the Ericaceae, systematic evaluation of different Pinaceae species would be valuable to the TAAM but also to other sectors such as forestry. Another important issue is the variation in the chemical composition of extracts following different extraction procedures including Traditional Cree methods. With analytical methods in place, the seasonal and populational variations in the phytochemistry of different extracts can be monitored for purposes of quality control and optimization.

While many of the observed biological activities were confirmed by secondary measures, the mechanisms and active components responsible for these effects remain unclear, as is their *in vivo* efficacy. Although extract anti-glycation activity appears to be mediated by the anti-oxidant properties of their phenolic metabolites, immunochemical detection and quantification of CML-BSA adducts should be performed on protein samples from the entire Cree collection to confirm these relationships. Extracts could also be tested for the ability to break AGE crosslinks or antagonize RAGE-mediated signaling. The cytoprotective activities toward glucose toxicity and deprivation observed in PC12 cells need to be validated in primary neuronal cultures such as dorsal root ganglia and the mechanisms of action require elucidation. Assay-guided fractionation of promising extracts to identify the active compounds behind the various types of anti-diabetic activities is essential for quality control and standardization and also monitoring levels of absorbed plant metabolites from *in vivo* samples. As illustrated in Figure 12.1, additional opportunities for inhibiting pathological mechanisms of diabetic neuropathy have not been explored. Inhibition of polyol and hexosamine pathway enzymes or of hyperglycemia-induced PKC activation are just a few examples.

Finally, a number of *in vivo* experiments remain to be completed, including the pharmacodynamics, pharmacokinetics and tissue-specific bioavailabilities of active phytochemicals or metabolites and the assessment of efficacy (glycemic control and the occurrence of vascular complications), safety and drug interactions. An observational clinical study is currently underway in T2DM patients of the Cree Nation of Mistissini.

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APPENDIX 1 – SUMMARY OF HPLC ANALYSES OF CREE PLANT EXTRACTS

Table A1 – Phenolic markers identified in Cree medicinal plant extracts by HPLC-DAD/APCI-MS.

Species – part (method used)	Identified phenolics	Rt (min)	λmax (nm)	[*m/z*]+
Abies balsamea - bark (method 1)	no markers identified			
Alnus incana ssp. *rugosa* - bark (method 4)	Oregonin 1	9.7	220sh, 280	347, 449
	Oregonin 2	10.1	220sh, 280	347, 449
	Oregonin 3	10.3	220sh, 280	345, 447
	Hirsutanonol	11.2	220sh, 280	327, 445, 469
	Plathylloside derivative	12.9	220, 280	295, 475, 521, 589
	Rubranoside A	13.5	220sh, 280	493
	Rubranoside B	14.1	220sh, 280	463, 577
	Hirsutanone	15.3	220sh, 280	327, 441
Gaultheria hispidula - leaf (method 2)	Refer to Chapter 3			
Juniper communis - fruit (method 1)	Catechin	5.8	210, 230sh, 280	139, 291
	Quercetin-3-galactoside	23.6	250, 360	303, 465
	Quercetin-3-glucoside	24.7	250, 360	303, 465
	Quercetin pentoside	25.5	250, 360	303, 435
	Quercetin-3-arabinoside	26.6	250, 360	303, 435
	Kaempferol glycoside 1	27.1	200, 265, 350	287, 419
	Kaempferol glycoside 2	27.4	200, 265, 350	287, 419
Kalmia angustifolia	Refer to Chapter 3			
Larix laricina - bark (method 1)	Catechin	5.8	210, 230sh, 280	291
	Epicatechin	12.8	210, 230sh, 280	291
	Hydroxystilbene 1	15.3	220, 240sh, 300sh, 320	245
	Hydroxystilbene 2	18.8	220, 240sh, 300sh, 320	229
	Hydroxystilbene 3	19.1	220, 240sh, 300sh, 320	245, 407
	Hydroxystilbene 4	22.7	220, 240sh, 300sh, 320	259
	Hydroxystilbene 5	26.4	220, 240sh, 300sh, 320	259
Lycopodium clavatum - whole (method 1)	Ferulic acid derivative 1	1.7	290sh, 330	193, 3
	Ferulic acid derivative 2	6.0	290, 315	193, 341
	Apigenin derivative 1	10.4	265, 330	269, 473
	Apigenin derivative 2	20.4	265, 330	269, 431
Picea glauca - needle (method 1)	Refer to Chapter 6			
Picea mariana - cone (method 1)	Hydroxystilbene 1	15.3	220, 240sh, 300sh, 320	245
	Hydroxystilbene 2	18.8	220, 240sh, 300sh, 320	229
	Hydroxystilbene 3	19.4	220, 240sh, 300sh, 320	245
	Hydroxystilbene 4	20.9	220, 240sh, 300sh, 320	259

Species – part (method used)	Identified phenolics	Rt (min)	λmax (nm)	[m/z]+
Pinus banksiana	Procyanidin B-type	4.6	210, 230sh, 280	291, 579
- cones (method 1)	Catechin	6.4	210, 230sh, 280	139, 291
	Taxifolin	16.7	195, 230sh, 290	305
Populus balsamifera	Salicin	6.0	210	331
- bark (method 4)	Iso Salicin	7.8	210	285
	Coumaroylglucoside A	9.2	310	325, 351
	Coumaroylglucoside B	10.5	310	325, 351
	Salicortin A	21.1	220, 270	405, 423, 469
	Salireposide	27.8	240(sh), 310	297, 315, 405, 451
	Populoside	28.1	300 (sh), 320	423, 447, 451
	Salicortin B	30.8	270, 300(weak)	423
	Salicortin C	30.9	270, 300(weak)	423
	Salicortin D	31.6	270, 300(weak)	423, 469
	Salicortin E	34.0	270, 300 (weak)	423, 469
Rhododendron groenlandicum - leaf (method 2)	Refer to Chapter 3			
Rhododendron tomentosum - leaf (method 2)	Refer to Chapter 3			
Salix planifolia	Salicin	5.5	215 (sh), 270	285
- bark (method 1)	Tremulacin	14.5	275	527
	Isosalipurposide dimmer	9.0	220, 250	423, 855
Sarracenia purpurea - leaf (method 3)	Refer to Chapter 7			
Sorbus decora	Catechin	8.6	210, 230sh, 280	291
- bark (method 1)	Epicatechin	10.1	210, 230sh, 280	291
Vaccinium vitis-idaea	Catechin	9.6	210, 230sh, 280	139, 291
- fruit (method 5)	para-coumaric acid derivative	10.0	200, 230, 310	147, 165, 179, 434
	Cyanidin-3-O-galactoside	12.6	205, 280, 520	287, 449
	Cyanidin-3-O-glucoside	13.1	205, 280, 520	287, 449
	Cyanidin-3-O-arabinoside	14.2	205, 280, 520	287, 419
	para-coumaric acid	15.1	200sh, 210sh, 230, 310	179, (195)
	Quercetin-3-galactoside	17.6	250, 360	303, 465
	Quercetin-pentoside	19.2	250, 360	303, 435
	Quercetin-3-arabinoside	19.5	250, 360	303, 435

Methods used

Method 1 – Please refer to Experimental section of Chapter 2.

Method 2 – Please refer to Experimental section of Chapter 3.

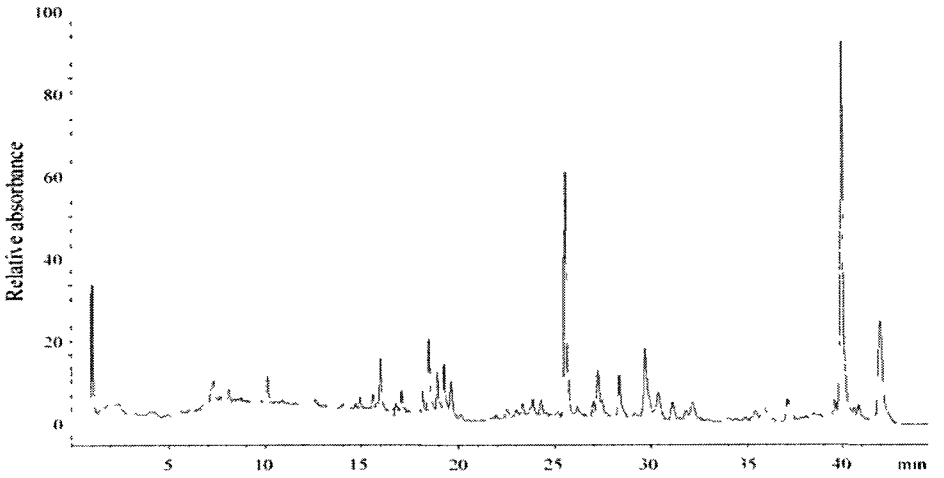
Method 3 – Please refer to Experimental section of Chapter 7.

Method 4 – The HPLC-PAD/APCI-MS analyses were with a narrow bore column YMC-ODS-AM, 100 mm × 2.1 mm, particle size 3.5 μm (YMC Inc. Kyoto, Japan). The mobile phase consisted of A = acetonitrile + 0.1% trifluoroacetic acid and B = trifluoroacetic acid 0.1% (aqueous) and optimal peak separation was achieved with a linear gradient of 5–60% A in 30 min followed by increasing A to 100% in 5 min and maintaining isocratic conditions for 5 min. The column was brought back to initial conditions in 0.1 min and equilibrated for 7 min prior to subsequent injections. The flow rate of 0.4 mL/min and column thermostat temperature of 45°C was maintained throughout the analysis. PAD monitoring wavelengths of 210 nm band width 4, reference off and 280 nm, reference 360, band width 10 were used for storing the spectra.

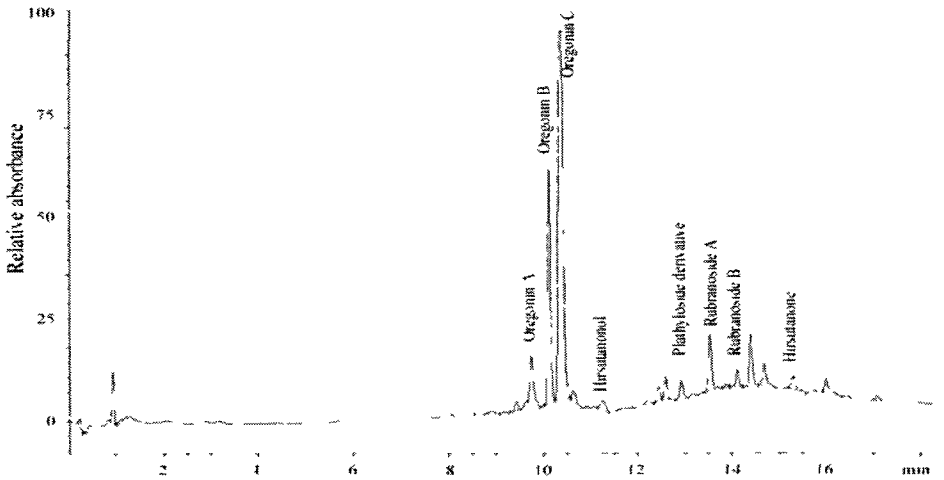
Method 5 – Please refer to Experimental section of Chapter 3. The gradient programme was modified to separate co-eluting flavonol- and cyanidin-glycosides. As in Chapter 3, analyses were performed on a Synergi Polar-RP column (150 mm × 3 mm; 4 μm particle size, Phenomenex, Torrence, CA USA) with a mobile phase system of methanol (solvent A), acetonitrile (solvent B) and aqueous trifluoroacetic acid (0.05% V/V, solvent C). The gradient elution conditions were optimized to a 40 min program: initial conditions 6:6:88 (A:B:C), changing to 25:25:50 in 25 min, then to 50:50:0 in 3 minutes, then isocratic elution with 50:50:0 for 2 minutes, finally returning to initial conditions in 4 minutes, which were held for 6 minutes to re-equilibrate the column. The flow rate of 0.4 mL/min and column thermostat temperature of 45°C was maintained throughout the analysis.PAD profiles were monitored at 325 nm for detecting UV absorbing phenolics and 520 nm for detecting proanthocyanins.

Chromatographs of the remaining Cree medicinal plant extracts

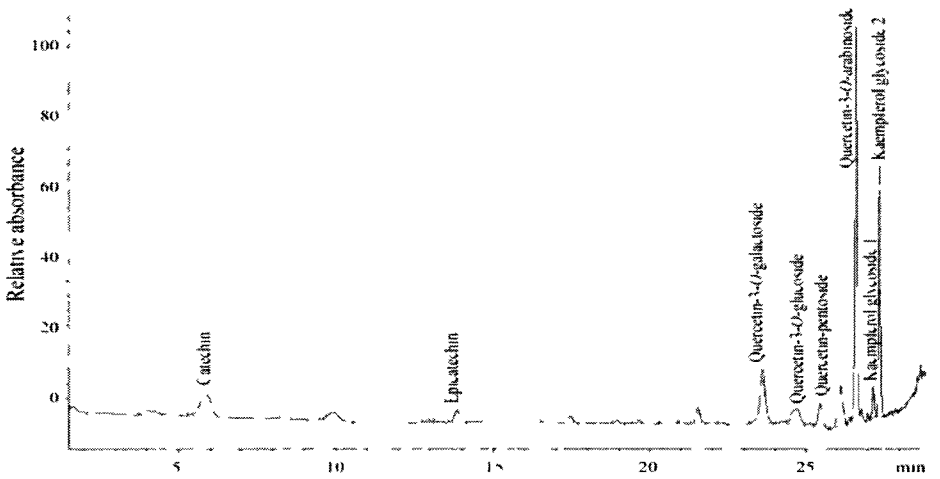
Abies balsamea (bark – 280 nm PAD)



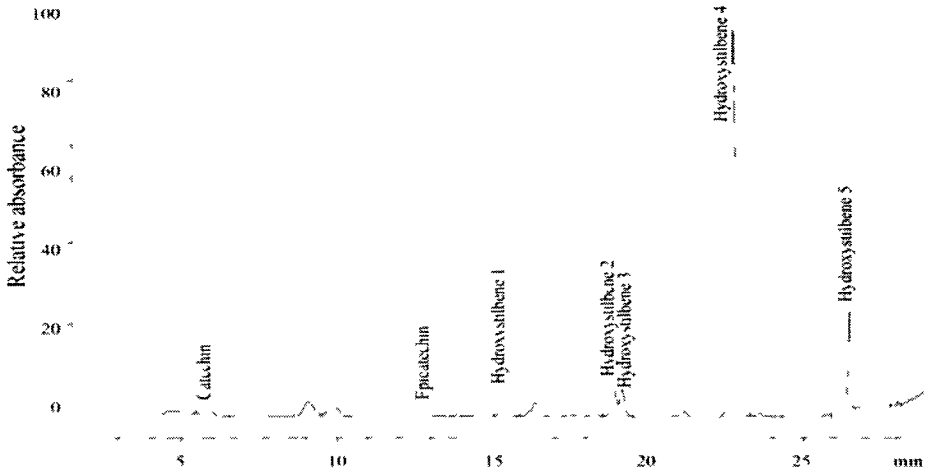
Alnus incana ssp. *rugosa* (bark – 210 nm)



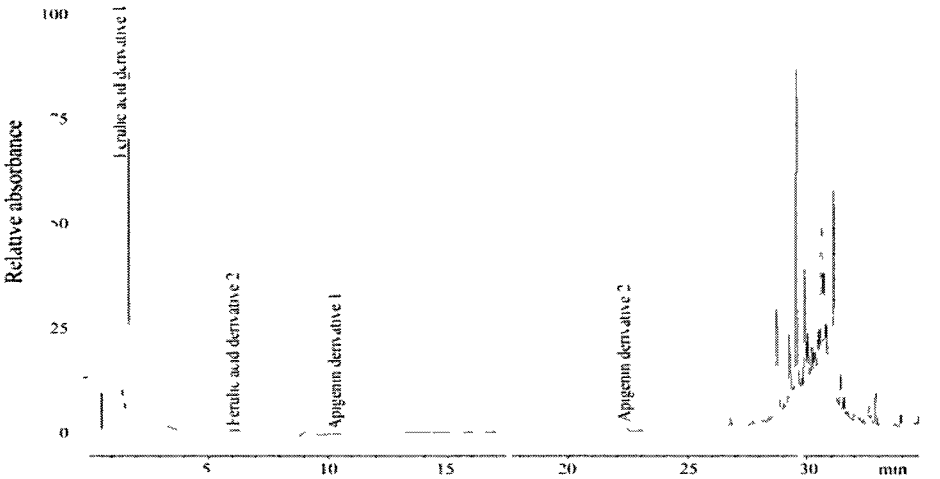
Juniper communis **(fruit – 280 nm)**



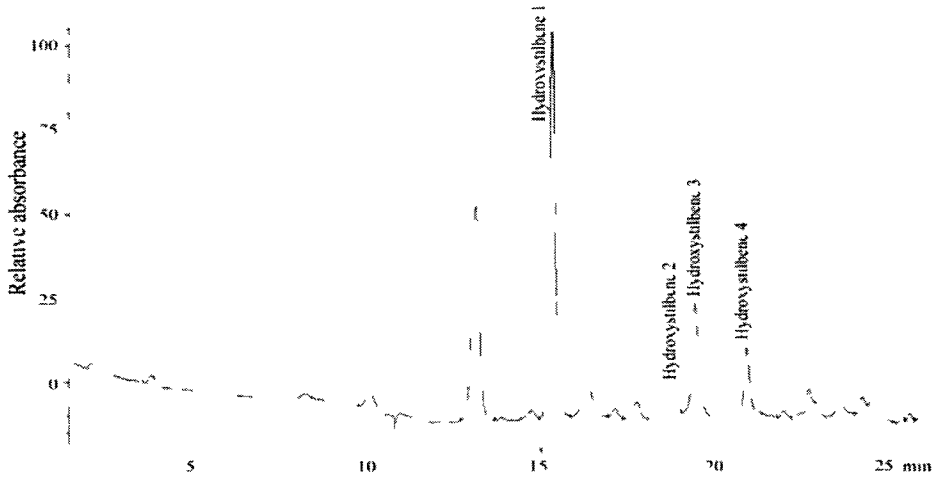
Larix laricina **(bark – 280 nm)**



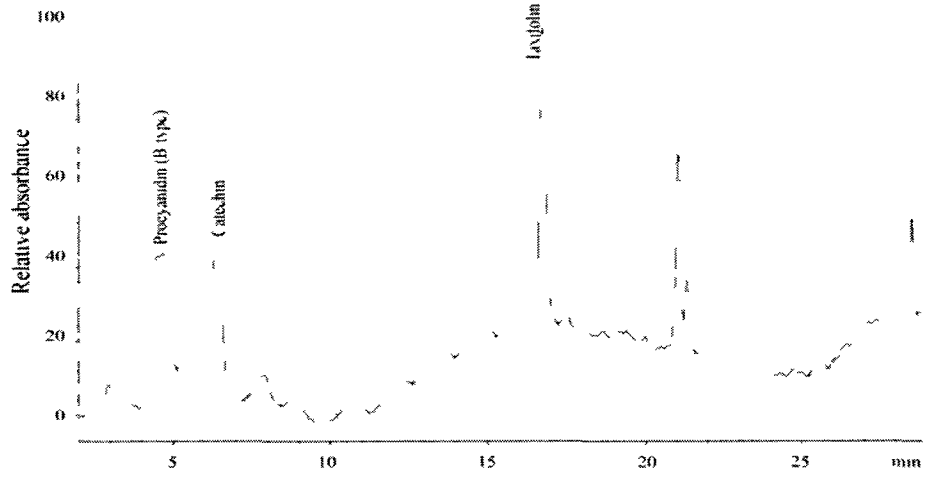
Lycopodium clavatum **(whole plant – 280 nm)**



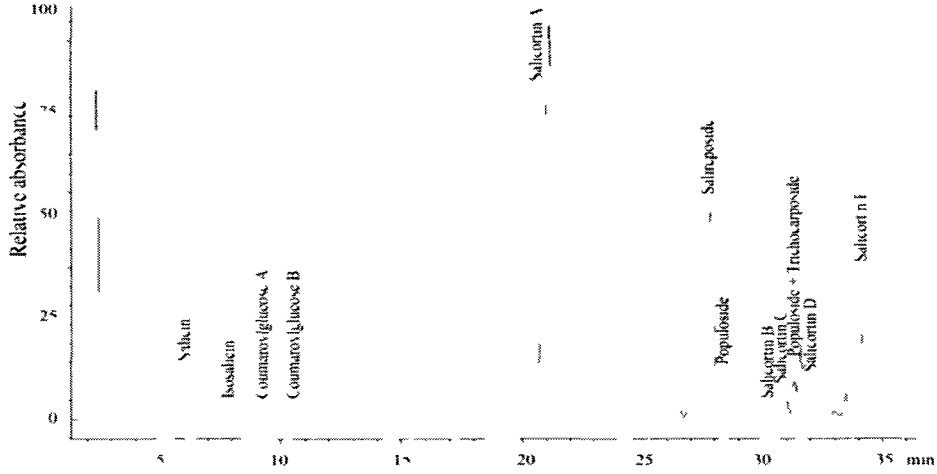
***Picea mariana* (cones – 280 nm)**



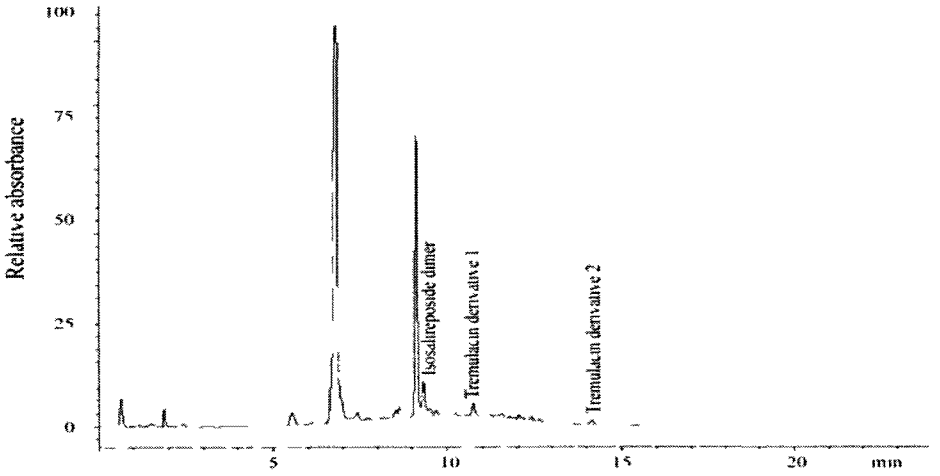
***Pinus banksiana* (cones – 280 nm)**



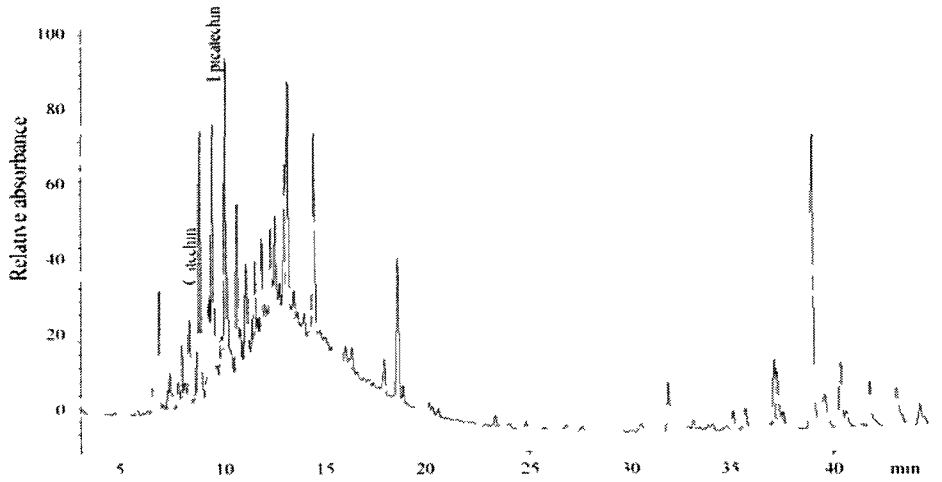
***Populus balsamifera* (bark – MS TIC)**



Salix planifolia **(bark – 280 nm)**



Sorbus decora **(bark – 280 nm)**



Vaccinium vitis-idaea **(fruit – 280 nm; --- 520 nm)**