uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Renée Leduc

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Biology)

GRADE / DEGREE

Department of Biology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Phytochemical Variation in Canadian *Hydrastis canadensis* L. (Goldenseal) and the In vitro Inhibition of Human Cytochrome P450-mediated Drug Metabolism by H. *canadensis* and Other Botanicals

TITRE DE LA THÈSE / TITLE OF THESIS

Dr. John T. Arnason

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

Dr. Robin J. Marles

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. Jeremy Kerr

Dr. Paul Caitling

Dr. Naomi Cappuccino

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

PHYTOCHEMICAL VARIATION IN CANADIAN *HYDRASTIS CANADENSIS* L. (GOLDENSEAL) AND THE IN VITRO INHIBITION OF HUMAN CYTOCHROME P450-MEDIATED DRUG METABOLISM BY *H. CANADENSIS* AND OTHER BOTANICALS

RENÉE IRENE LEDUC

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa
in partial fulfillment of the requirements for the
M.Sc. degree in the

Ottawa-Carleton Institute of Biology

Thèse soumise à
Faculté des études supérieures et postdoctorales
Université d'Ottawa
en vue de l'obtention de la maîtrise ès sciences

L'Institut de biologie d'Ottawa-Carleton

Your file Votre référence
ISBN: 978-0-494-49233-8
Our file Notre référence
ISBN: 978-0-494-49233-8

DEDICATION

This research is dedicated to my husband, my parents and family, and to the memory of my father, Donald Leduc, my uncle, Wayne Lavigne and my grandparents, Irene & Roger Lavigne.

ABSTRACT

To promote recovery and crop potential of *Hydrastis canadensis* – a botanical threatened in Canada – wild populations were phytochemically surveyed. Berberine, hydrastine and canadine were characterized in root-rhizome, stem-leaf, and berry pulp. Comparisons with cultivated material showed no difference in alkaloid content, thus the medicinal value of cultivated material is not likely increased with addition of wild plants. Quantitative analyses suggested genetic diversity among wild populations. Regression analyses indicated a minor relationship between latitude and alkaloid yield.

Because *H. canadensis* and other botanicals may cause adverse botanical-drug interactions, 22 botanicals were assayed for cytochrome P450 (CYP) 2C19, CYP3A4 and CYP19 inhibition. Eight botanicals inhibited CYP2C19 by $\geq$ 57%, 17 inhibited CYP3A4 by $\geq$ 40% and CYP19 by $\geq$ 50%, of which *Arctostaphylos uva-ursi*, *H. canadensis*, *Oenothera biennis*, *Rhodiola rosea* and *Sanguinaria canadensis* were most potent. Regression analyses indicated berberine concentration was a significant factor in CYP3A4 and CYP19 inhibition.

RÉSUMÉ

Le profil phytochimique de 10 populations d'*Hydrastis canadensis* sauvages a été déterminé et comparé à celui de plantes cultivées. Les alcaloïdes berberine, hydrastine et canadine ont été caractérisés. Le contenu d'alcaloïdes de la plante sauvage n'était pas différent de celui de la plante cultivée. Des analyses de régression ont indiqué que la latitude influence le rendement des alcaloïdes.

Puisque plusieurs plantes médicinales peuvent influencer le métabolisme des produits pharmaceutiques, l'inhibition de l'activité métabolique du cytochrome P450 (CYP) 2C19, CYP3A4 et CYP19 a été analysé in vitro chez 22 plantes. Huit plantes ont inhibité l'activité de CYP2C19 de $\geq$ 57%, 17 celle de CYP3A4 de $\geq$ 40% et celle de CYP19 de $\geq$ 50%. *Arctostaphylos uva-ursi*, *H. canadensis*, *Oenothera biennis*, *Rhodiola rosea* et *Sanguinaria canadensis* étaient les plus efficaces. Des analyses de régression ont indiqué que la concentration de berberine est un facteur déterminant de l'inhibition CYP3A4 et CYP19.

ACKNOWLEDGEMENTS

I would like to thank my loving family and friends, who are and have always been infinitely supportive and graciously sympathetic.

I am grateful to Dr. J. Thor Arnason (Biology professor, University of Ottawa) and Dr. Brian C. Foster (Senior Science Advisor, Health Canada) for providing both the opportunity and the financial means for this study, as well as for their guidance throughout the entire process. I am indebted to Dr. Adrianne Sinclair (Scientific Advisor for Plant Trade, Canadian Wildlife Service) who initiated the idea of surveying wild *H. canadensis*, secured harvesting permissions and personally introduced me to the necessary fieldwork. I would also like to acknowledge Dr. Robin J. Marles (Director of the Bureau of Clinical Trials and Health Science, Health Canada), who co-supervised the study, as well as the other members of my thesis advisory committee, Dr. Naomi Cappuccino (Biology professor, Carleton University) and Dr. Jeremy T. Kerr (Biology professor, University of Ottawa).

Botanical material was generously donated by Gary Krentz of Gkginseng Farm (Tillsonburg, Ontario), Paul Haydon of Grow Wild (Bogcaygeon, Ontario), John Kershaw of Goldcap Farm (Princeton, Ontario), Majella Larochelle (Hull, Quebec), Bob Romaniuk Sr. (Scotland, Ontario), Melanie Slick and Jeff Carpenter of Zack Woods Herb Farm (Hyde Park, Vermont), and the folks at Pacific Botanicals (Grants Pass, Oregon), Rosemary's Garden (Sebastopol, California) and Judy's Organic Herbs (Woodland, Ontario).

I would like to thank Peter Goddard, Park Manager of Baxter Conservation Area, and Bond Strand, Park Manager of Foley Mountain Conservation Area, for allowing me to collect wild samples from their parks and for sharing their botanical knowledge. I would also like to acknowledge scientific permits for Mer Bleue Bog Conservation Area from Brian Gravelle (Greenbelt Land Manager, National Capital Commission), and for Gatineau Park from Isabelle

Beaudoin-Roy (Field Biologist, Gatineau Park) and Line Couillard (Chef de division, Ministère du Développement durable, de l'Environnement et des Parcs).

I would like to acknowledge research collaborations with Dr. B. Foster, Dr. Joseph Lam (Chemical Metrologist, National Research Council), Dr. R. Marles, Dr. A. Sinclair, and Dr. Ian M. Scott (Research Scientist, Agriculture and Agri-Food Canada).

Many thanks to all of my field assistants: Erin Lamont, Andrée and Chantale Leduc, Rick Devereux, Nancy Lavigne, George Rejhon and Joan Scott, Chris Laporte, Sean Collins, Cathy Morris, Chieu Anh Ta, Eric Snyder, Philip Fry, Frederick W. Schueler, Vicky Filion and Kathy Hodgins (Land Sterwarship Specialist, Ausable Bayfield Conservation Authority). The following have also generously volunteered assistance in the lab: Nana Bafi-Yeboa, Louis-Philippe Bonhomme Beaulieu, Andrew Burt, Rick Devereux, France Duval, Cory Harris, Nikia Kearns, Linda Kimpe, Erin Lamont, San Nguyen, Carolina Ogrodowczyk, Melanie Racine, Dr. Ammar Saleem, Dr. I. Scott, Pamela Stroud, and Chieu Anh Ta. I much appreciated the stats advice from Patrick Audet. Special thank you to Keomany Ker and Susanna Wiens.

I would also like to thank Huguette Allard, the greenhouse technician at the University of Ottawa, for her guidance and assistance; Dr. Paul Catling, adjunct professor at the University of Ottawa and curator of the Agriculture and Agri-Food Canada Vascular Plant Herbarium (DAO), Gisèle Mitrow the collections manager and Lynn Black the research technician at the DAO, for their advice and assistance with vouchering botanical samples. Micheline Bouchard, collection technician at the National Herbarium of Canada, for allowing me to consult the herbarium.

Many thanks to Dr. Jeannine M. Davis (Associate Professor and Extension Specialist, North Carolina State University), Richo Cech (author of Growing At-Risk Medicinal and co-founder of Horizon Herbs) and Robert Eidus (from North Carolina Ginseng & Goldenseal Company) for sharing their expertise on *H. canadensis* seed germination.

Special thanks to Dr. Nancy J. Turner for her photographic contributions; and to Rick Devereux, George Rejhon, Dr. B. Foster, Dr. T. Arnason, and my thesis examiners, Dr. N. Cappuccino, Dr. P. Catling and Dr. R. Marles for their editorial advice.

Thank you to all of my friends and colleagues in the Arnason, Foster, Charest & Blais labs.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: GENERAL INTRODUCTION

1.1 General Introduction

Whether they served an alimentary, dyeing, textile, medicinal, divinatory or ritualistic purpose, or were used for construction, clothing, or to make music and art, many of North America's native plants have an undeniably deep legacy in the traditional culture of the people of the continent (*1-9*). Given the range of climatic regions of North America, it is not surprising to find a rich diversity of plants and especially so many with great ethnobotanical value. Of particular contemporary value are the plants traditionally used as medicines (botanicals) from which two significant product types and subsequent economies have arisen: single-ingredient pharmaceutical products and natural health products (NHPs) that are often comprised of one or more botanical components. *Hydrastis canadensis* L. (goldenseal), a popular North American botanical, has contributed to both economies. For example, capsules and tinctures of its rhizome and fibrous roots are commonly sold in drug stores and apothecaries. They are also often found in combinatory formulas with *Echinacea*. In Canada alone, there are currently 14 registered NHPs containing *H. canadensis* approved by the Natural Health Products Directorate (Health Products and Food Branch of Health Canada), the Canadian regulatory authority on the subject (*10*). It is marketed in over 500 medicinal products worldwide (*11 and references within*). On the other hand, many of the active constituents that give *H. canadensis* its medicinal properties, like berberine and hydrastine alkaloids, are readily extractable and purified as single-ingredient products that are also used for medical research purposes.

The longstanding therapeutic success of botanicals like *H. canadensis* often leads to two dilemmas: 1) wild populations are exhaustively harvested and 2) consumers commonly trust the 'natural' designation, possibly resulting in adverse reactions with co-administered pharmaceutical drugs. This study aimed to address both dilemmas through phytochemical analysis of the quality of wildcrafted versus cultivated material, and through toxicological analysis of crude botanical extracts for possible botanical-drug interactions.

1.2 Literature Review for *Hydrastis canadensis* L. (Goldenseal)

"*Goldenseal is the king and queen of the herbs...*" – A.L. Tommie Bass (*12*)

1.2.1 Nomenclature & morphology: *Hydrastis canadensis*, a member of the Ranunculaceae (buttercup) family, was once considered to be one of two species in its genus, the second species being *H. jezoensis* Sieb. *ex* Miquel from northeastern Asia (*13 and 14, and references within*). It is now, however, considered the only species in the genus and *Glaucidium palmatum* Sieb. & Zucc. (commonly known as shiraneaoi), from northern Japan, is its nearest relative (*14 and references within*). *Hydrastis canadensis* is believed to have first evolved 15-20 million years ago in the ancient arctotertiary forest of the northern hemisphere (*13, 14 and references within*).

Hydrastis canadensis is an herbaceous perennial with a hairy unbranched stem that can grow up to two feet in height (*15-17*). At maturity, the stem bears two cauline leaves that are alternate and near the top of the stem. A third basal leaf, with a long petiole, is also present and usually drops early in the development of the plant (*16, 18*). The leaves are palmate with 5 to 7 doubly serrate lobes and can spread up to 25 cm in diameter, particularly at maturity (*14, 17, 18*). *Hydrastis canadensis* produces a solitary flower that is terminal and rests on a peduncle that ranges from 0.5 to 4 cm in length. The flower is white, radially symmetric, 8 to 18 mm wide and, instead of petals and it bears small greenish-white sepals that drop soon after the flower opens, and expose numerous stamens and pistils (*14, 16-18*). The fruit is a subglobose aggregate berry, lustrous dark pinkish-red in colour and resembles an enlarged raspberry (5-8 mm long and 1.5-5 mm wide). Each berry in the cluster has 1 to 2 seeds; the aggregate fruit often carries 10 to 30 shiny, black seeds that are 2 to 2.5 mm long, with unpredictable viability (*14, 16-19*).

The rhizome is horizontal, irregularly knotted and measures 2.5 to 7 cm long and 0.5 to 2 cm thick when freshly harvested (*14, 17, 18*). Fibrous roots project from the ventral portion of the rhizome, sometimes measuring two or three feet in length. Adventitious buds sprout from the

rhizome that give rise to new plants (*20*), a method of reproduction that is more reliable than from seed. Cut or injured, the fibrous roots, rhizome and stem ooze a bright yellow, acrid smelling juice that contains numerous isoquinoline alkaloids, the source of its medicinal properties. These compounds are mostly concentrated in the rhizome and roots but have been detected in all tissues including minute concentrations of berberine in ripe berry pulp, first recorded in this study.

1.2.2 Status & ecology: *Hydrastis canadensis* is indigenous and endemic to eastern North America. Wild colonies typically occur in deciduous woodlands near floodplains and spring-flooded plateaus that range west from southern Vermont to southwestern Ontario and to southern Wisconsin, and south through Arkansas and northern Georgia (*17*).

Hydrastis canadensis is mostly found in shady, deciduous forests and edges of woodland in nutrient rich, loamy soil (*14*, *17*). In late summer or early fall, new buds are formed on the rhizome from which a stem will emerge the following spring. In Ontario, by late April or early May, its leaves unfold and its flower opens as the stem and leaves elongate. The leaves become fully expanded by June and the fruit develops and ripens from July to early August. The fruit drops shortly after ripening and the plant becomes senescent in mid-September to late October at frost (*17*) at which time the rhizome and roots are harvested.

Wildcrafted *H. canadensis* roots and rhizome (root-rhizome) first became a commercially popular phytomedicine in North America and in Europe in the mid 1800s. Interestingly, the oldest herbarium specimen in Ontario appears to have been collected in 1889 by J.A. Morton near Wingham in Huron County (*21*). Today, *H. canadensis* is still a top-selling botanical and a recent survey by Sinclair and Catling (*17*) found that "virtually all *H. canadensis* on the market is supplied from the wild". Harvesting whole roots and rhizome over a long period of time is an indisputably unsustainable harvesting practice and for this reason, in addition to a concurrent loss of natural habitat from rural expansion, the occurrence and distribution of wild *H. canadensis* have been considerably reduced. In 1997, the Convention on International Trade in Endangered Species of

Wild Fauna and Flora (CITES), a multinational treaty, designated *H. canadensis* an Appendices II (threatened) species (*22*). Three years later, the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) assigned a threatened status to the species (*23*) and it is legally protected under the Government of Canada's Species At Risk Act (SARA). Even in the early 1900s medicinal botanists, like J.U. Lloyd, expressed deep concern about the unsustainable harvesting of wild populations:

> "The greatest trouble with natural woodland cultivation comes from the poacher, who considers everything that grows in the woodlands free [for their] improvident disposition and destructive vandalism. The present scarcity is unnecessary, but promises to be cruelly lasting, there being seemingly little prospect of cultivated Hydrastis drifting into market in the very near future... Without a doubt, cultivated Hydrastis must command a good commercial return."(*19*)

Lloyd's sentiments are eerily relevant to this day and many since have been investigating recovery strategies and agricultural measures in aid of the species (*19-21, 24-27*). It is fortunate that wild *H. canadensis* has prevailed, albeit still in a vulnerable state, over the past century.

1.2.3 Traditional usage, pharmacognosy of constituents & other alkaloid-containing botanicals:

Hydrastis canadensis was traditionally used by Aboriginal people and later by early settlers of eastern North America as both a medicinal and dye plant. It was first described as a yellow dye in an article written by H. Martin in 1793 published in the American Philosophical Society (*16, 20*) and a few years later, in 1798, a medicinal botanist named B.S. Barton wrote a book describing the Cherokee using *H. canadensis* root decoction as an eyewash, root tincture as an external antiseptic and root tonic for debility, dyspepsia and cancer-like illnesses (*19, 28, 29*). The Cherokee also mixed *H. canadensis* with bear fat to repel insects (*30*). Among the Iroquois, infusions of the root were taken orally for whooping cough, diarrhea, fevers, pneumonia, tuberculosis, and stomach, heart and liver problems (*4*).

According to van Fleet (*19*) there was little commercial demand for the root outside of its aboriginal context until about the 1860s. That changed largely due to two publications: in the 1852 edition of the 'Eclectic Dispensatory of the United States', J. King and R.S. Newton cited *H. canadensis* as one of the most reliable phytomedicines for gastrointestinal and uterine pain, jaundice, infections, and to regularize bile production (*16 and references within*). Four years later, L.E. Jones and J.M. Scudder described the use of *H. canadensis* for mouth ulcers and thrush, for inflammation of mucous membranes and for chronic gonorrhea in 'The American Eclectic Materia Medica and Therapeutics" (*16 and references within*). These publications were widely distributed in America as well as in Europe and *H. canadensis* became widely sought after, particularly as an antimicrobial. Root-rhizome preparations were applied externally as an antiseptic wash and taken internally for the treatment of upper respiratory infections and gastrointestinal disorders (*16*). During the mid 20th century interest in medicinal plant therapies declined as a result of widespread availability of synthetic antibiotics. However, since the 1990's there has been renewed interest in botanicals in the context of complementary and alternative medicine (CAM) and *H. canadensis* continues to be a popular botanical in contemporary NHPs (*11*, *16*).

The medicinal properties of *H. canadensis* are attributed to the highly concentrated bioactive isoquinoline alkaloids berberine, hydrastine and canadine, all of which bear a methylenedioxyphenyl moiety. The compounds are biosynthesized and stored in the root-rhizome and are also present in the stem and leaves, and to a lesser extent, in the fruit. Berberine is the main pharmaceutical component, however hydrastine is also extractable in considerable amounts and contributes to the pharmacological activities of *H. canadensis*. Berberine has demonstrated a broad range of pharmacologically relevant activities, for example as an antimicrobial (*31, 32*), antidiarrheal (*33, 34*), antiarrhythmia (*35*), anti-inflammatory (*36*), and antitumour (*37*) agent. It also acts as a cholesterol-lowering drug (*38, 39*) and has been proposed recently as a treatment for type 2 diabetes and obesity (*40*). Hydrastine has also demonstrated antimicrobial (*31*), antifungal (*41*) and antibacterial (*42*) properties, and it is a $GABA_A$ receptor antagonist (*43, 44*). Canadine is reportedly antibacterial (*42*),

upregulates the expression of hepatic low density lipoprotein receptors (*45*) and is a dopamine receptor antagonist (*46*). Furthermore, all three alkaloids may also interact with adrenergic and adenosinic receptors (*47*).

This study also analyzed the following five alkaloid-containing botanicals, all of which are currently available in conventional NHPs: *Berberis vulgaris* L. (Berberidaceae) common barberry; *Coptis trifolia* var. *groenlandica* (L.) Salisb. (Ranunculaceae) threeleaf goldthread; *Eschscholzia californica* Cham. (Papaveraceae) California poppy; *Mahonia aquifolium* (Pursh) Nutt. (Berberidaceae) Oregon grape; and *Sanguinaria canadensis* L. (Papaveraceae) bloodroot. For full descriptions of the ethnobotany of these species see Moerman (*4*).

1.2.4 Agricultural & commercial aspects: A survey conducted in 1999 by the American Herbal Products Association revealed a noticeable transition from wild-harvested to cultivated *H. canadensis* material being traded in the botanical market (*48*). There are two cultivation practices for *H. canadensis*, conventional crop farming and micropropagation (in vitro tissue culturing), both of which are described below.

Hydrastis canadensis seed dormancy has been characterized as both deep simple and deep simple epicotyl morphophysiological (*49*) and, as such, the complex nature of its seed dormancy has invariably made propagation by seed difficult. Agricultural practices such as soil nutrient regimens, soil type and shade requirements, particularly of transplanted or vegetatively propagated individuals have been studied. J.U. Lloyd (*20*) and W. van Fleet (*19*), among others, published articles on the cultivation of *H. canadensis* in the early 1900s, both describing successful indoor 'hothouse' and outdoor garden cultivation methods. More recently, R. Cech (*50*), an American herb farmer, A. Sinclair (Ph.D.) and P.M. Catling (Ph.D.) (*48*), interested in the recovery and conservation of wild *H. canadensis*, and Professor J.M. Davis (Ph.D.) from North Carolina State University (*51*, *52*), have all contributed significantly to increasing the productivity of *H. canadensis* cultivation.

An alternative area of research that has garnered interest is micropropogation of whole regenerated plants in vitro from isolated tissues. These 'plantlets' yield pharmacologically relevant secondary metabolites that can be obtained under controlled and optimized environments that are independent of climate and soil conditions. Furthermore, there is little to no impact on wild populations and the process can be achieved in a relatively short period of time (shorter than natural development) (*53*, *54*). The cellular derivation of pharmacologically relevant alkaloids similar to those found in *H. canadensis* have been intensively characterized in *E. californica* and *C. japonica* (*55-58*), and recent investigative efforts have focused on the feasibility of artificially cultivating *H. canadensis* cells. Both Bedir et al. (*59*) and Lui et al. (*60*) reported successful micropropagative methods from stem and leaf explants, and HPLC analyses revealed comparable berberine concentrations between the leaves of the mother plant and those of the plantlets, however a greater berberine concentration was measured in the roots of the mother plant (*59*).

1.3. Literature Review of Botanical-CYP Inhibition

"*All in all, it is as if with unlimited faith our use of plant Medicines is limitless.*" – Ted Williams, (*61*)

1.3.1 Natural health products (NHPs) & botanical-drug interactions: Botanicals are increasingly accessible in commercialized products such as NHPs and herbal teas. There is an increased global interest in traditional medicines like botanicals (*62*) and they are often used to complement or replace mainstream medicine (*63-64*). Naturopathic doctors, homeopaths and massage therapists recommend botanicals, while physicians and nurses have also begun to integrate phytomedicines into their practice (*66*).

Not only are botanicals abundantly available, they are also widely perceived, in an alarmingly casual manner, as completely safe. A survey conducted in 2005 by Health Canada (HC) and the Natural Health Products Directorate (NHPD) (*67*) found that of a sample of 2,004 Canadian adults, three in ten felt that NHPs are "natural and safe or better than conventional medications" and one half of those surveyed (52%) thought that NHPs are safe because "they are made from natural ingredients". They also reported that as many as 70% of those surveyed had previously used an NHP at least once and up to 38% of them used them on a daily basis. More importantly, as many as 12% conceded they had experienced at least one unwanted side effect or reaction associated with the NHP and 59% did not report this adverse reaction. The incidence of adverse reactions from botanical-drug interactions is probably underestimated and, despite the widespread use of phytomedicines, baseline and clinical data are limited (*68*). Many have reviewed the safety risks associated with the use of botanicals (*65, 68-75*).

Adverse reactions from concurrent botanical and drug use are grouped into two types of interactions: pharmacodynamic and pharmacokinetic. Pharmacodynamic interactions occur at the active (catalytic) site(s) of an enzyme or receptor whereas pharmacokinetic interactions occur when

the bioactive constituents of the botanical interfere with the processes that are involved in the normal disposition of the drug through the body. These processes include absorption, distribution, metabolism and excretion (*75-78*). Botanical interactions specifically with cytochrome P450 (CYP)-mediated drug metabolism are discussed below.

1.3.2 Botanical-mediated cytochrome P450 (CYP) inhibition & CYP isozymes: As noted by Williamson (*77*) "drug metabolism is the most important mechanism [for interaction] so far reported for herbs"; more specifically, this refers to the interaction between the bioactive constituents of botanicals and drug-metabolizing CYP enzymes (*79*). Human CYPs are primarily expressed in the smooth endoplasmic reticulum of the liver, with lower levels of expression in the lungs, kidneys, intestine and brain, and are responsible for the metabolism of both exogenous and endogenous substrates (*80*). CYP activity can be inhibited by the presence of one or more phytochemicals or it can be overexpressed (induced) in the tissue (*68*). CYP induction increases its metabolizing capacity, eliminating the drug prematurely from the body, while CYP inhibition leads to increased drug levels and the non-activation of prodrugs. CYP inhibition can be harmful when drug concentration in the blood reaches a toxic level (*76, 77*). The CYP2C19, CYP3A4 and CYP19 isozymes were chosen in this study for in vitro CYP-inhibition assays with 22 botanicals and are described below.

CYP2C19 is only expressed in the liver where it constitutes approximately 5% of the total CYP content (*81*). It is one of four isoforms (the other isoforms include CYP2C8, CYP2C9 and CYP2C18) (*80*) and is responsible for metabolizing approximately 20% of all known drugs (*82*). CYP2C19 is associated with the 4′-hydroxylation of (*S*)-mephenytoin and the metabolism of omeprazole, an ulcer drug (*83*). CYP2C19 also catalyzes the 8-hydroxylation of (*R*)-warfarin (*84*) and is responsible for the 5- and 5′-hydroxylation of thalidomide, a drug that is teratogenic in pregnant women but has valid therapeutic uses in multiple myeloma and leprosy (*85*). Progesterone 21-hydroxylation and testosterone 17-oxidation are also catalyzed by CYP2C19 (*86*). The polymorphism poor metabolizer (PM) is associated with CYP2C19, a phenotype that occurs in

approximately 20% of the Asian population (*87*). A study by Chau et al. (88) suggested that a higher incidence of hepatocellular cancer occurs in PMs, but Roddam et al. (*89*) did not find a relationship between the incidence of leukemia and CYP2C19 PMs.

CYP3A4 is mostly expressed in the liver and small intestine, and is the most abundant CYP in the body (*81*). It is one of three isoforms (CYP3A5 and CYP3A7 are the others) (*80*) and it accounts for 25-30% of the total CYPs in the liver (*90*) and as much as 60% in the small intestine (*91*). CYP3A4 is also expressed to a lesser extent in the lungs, stomach, colon and adrenal tissues (*81*). It contributes to the metabolism of approximately 50% of all of the drugs on the market (*92*), including lovastatin (Mevacor®), an HMG-CoA reductase inhibitor for reduction of blood cholesterol levels (*93*); finasteride (Proscar®, Propecia®), a prostate hypertrophy inhibitor (*94*); cyclosporine, an immune suppressant (*95*); as well as indinavir (*96*) and sidenafil (Viagra®) (*97*), two protease inhibitors. Reactions typically conducted by CYP3A4 include testosterone 6β-hydroxylation (*86, 98*) and the conversion of cholesterol into 4β-hydroxycholesterol, a major circulating oxysterol (*99, 100*); however, like CYP2C19, its substrates consist of mostly exogenous compounds (*81*). CYP3A4 is also involved in the activation of 17β-estradiol and of carcinogens like aflatoxin B_1 and aflatoxin G_1, among others (*81*).

CYP19, also referred to as aromatase and CYP19A1, is responsible for the aromatization of androgens in vertebrates (*79*), a three-step process whereby androstandione and testosterone are oxidized into the estrogens estrone and 17β-estradiol, respectively (*81, 101*). Human CYP19 is expressed in the ovaries, testes, placenta, fetal liver, adipose tissue, chondrocytes and osteoblasts of bone, vascular smooth muscle, and in the hypothalamus, limbic system and cerebral cortex of the brain (*102*). CYP19 is also expressed in some tumors, particularly those derived from the tissues where it occurs. The CYP19-inhibitor exemestane is being investigated for the treatment of estrogen-dependent cancers like certain forms of breast cancer (*103, 104*) and is currently in Phase III clinical trials. Exemestane efficacy is being compared to that of tamoxifen, the most popular anticancer drug

for breast cancer on the market (*81 and references within*; *105*, *106*), despite tamoxifen's associated risk of transforming into carcinogenic DNA adducts by CYP3A4 metabolism (*107*).

1.4. Project Overview, Rationale & Objectives

1.4.1 Chapter 2

Overview: Characterization was done of the phytochemical variation observed among ten wild Canadian *H. canadensis* populations, among commercial material sourced from six North American farms and apothecaries, and between wild and cultivated materials.

Rationale: Focus in the literature has been given to cultivated material, but there are no baseline data or previous work dedicated to the phytochemistry of wild Canadian populations. This work aimed to fill that void, while determining if any significant difference exists between wild and cultivated material. This information can help further understand the phytochemical diversity and crop potential of *H. canadensis*, thus promoting recovery of wild stands.

Objectives & corresponding null hypotheses (H_o):

1- Identify quantitative chemical markers unique to wild stock, for each tissue (below-ground, above-ground and berry pulp). *H_o: No significant difference in alkaloid concentration (each alkaloid individually and their sum) and composition among tissues.*

2- Compare and contrast the chemical profiles (composition and quantity) of wild and cultivated material. *H_o: No significant difference in alkaloid concentration (each alkaloid individually and their sum) and composition between wild and cultivated root-rhizome material.*

3- Characterize the phytochemical variation among wild populations. *H_o: No significant difference in alkaloid concentration (each alkaloid individually and their sum) among wild populations.*

4- Determine the genotypic contribution to phytochemical variation among wild populations. *H_o: No significant difference in alkaloid concentration (each alkaloid individually and their sum) among wild populations cultivated under uniform environmental conditions.*

5- Investigate the possible significance of latitude as a determining factor of alkaloid concentration (each alkaloid individually and their sum) in wild populations. *H_o: No relationship between alkaloid concentration and latitude.*

1.4.2 Chapter 3

Overview: The in vitro CYP-inhibiting activity of crude botanical extracts prepared from up to seven different accessions of a total of 22 species (spanning 13 families) using CYP2C19, CYP3A4 and CYP19 was determined. The relationship between berberine concentration in crude extracts and CYP inhibition was assessed.

Rationale: Using the same in vitro high-throughput method, crude extracts prepared from popular botanicals like *Ginkgo biloba* L. (Ginkgoaceae) maidenhair tree (*108*), *H. canadensis* (*109-111*), *Hypericum perforatum* L. (Hypericaceae) common St. John's wort (*110, 111*), *Allium sativum* L. (Alliaceae) garlic (*112*), *Echinacea angustifolia* DC. (Asteraceae) coneflower, *Matricaria recutita* L. (Asteraceae) chamomile and *Glycyrrhiza glabra* L. (Fabaceae) licorice (*111*) have previously been found to inhibit CYP-mediated drug metabolism. Furthermore, purified berberine and other botanically derived methylenedioxyphenyl compounds have been shown to inhibit CYP-dependent metabolism (*109, 111, 113*), and the methylenedioxy functionality reportedly binds with CYP enzymes to form an inactive complex (*113*). CYP inhibition changes the disposition of co-administered drugs, which can lead to adverse reactions. Baseline data generated from high-throughput methods identify candidate species that warrant further in-depth in vitro and clinical investigation (*68, 114, 115*). Many other popular North American botanicals have yet to be screened.

Objectives & corresponding null hypotheses:

1- Identify crude extracts that inhibit in vitro CYP-mediated metabolism. H_o: *No significant difference between test treatment (T, the mean activity of the extract incubated with the isozyme) and the control treatment (C, the mean activity of 55% ethanol at a threshold concentration previously used for distinguishing active extracts incubated with the same isozyme).*

2- For each isozyme separately, identify variations in CYP inhibition among accessions of the same species. H_o: *No significant difference in mean CYP inhibition among accessions of the same species.*

3- For each isozyme separately, identify variations in CYP inhibition among species. H_o: *No significant difference in mean CYP inhibition among species.*

4- For each species separately, determine variations in CYP inhibition among isozymes. H_o: *No significant difference in overall mean CYP inhibition among isozymes.*

5- Identify variations in overall CYP inhibition among species. H_o: *No significant difference in overall mean CYP inhibition (CYP2C19, CYP3A4 and CYP19 inclusively) among species.*

6- For each berberine-containing species separately, identify the quantitative variation in berberine concentration among accessions. H_o: *No significant difference in mean berberine concentration among accessions of the same species.*

7- Identify the quantitative variation in berberine concentration among berberine-containing species. H_o: *No significant difference in mean berberine concentration among berberine-containing species (including all accession data).*

8- For each isozyme, determine the existence of a relationship between berberine concentration in crude extracts and CYP inhibition. H_o: *No relationship between mean berberine concentration in crude extracts and mean CYP inhibition.*

CHAPTER 2: ALKALOID CONCENTRATION AND VARIATION AMONG WILD CANADIAN *HYDRASTIS CANADENSIS* L. (GOLDENSEAL) POPULATIONS AND MATERIAL CULTIVATED IN NORTH AMERICA

This chapter was written as an article for publication in the *Journal of Agricultural and Food Chemistry* in collaboration with R.J. Marles, B.C. Foster, P. Catling, A. Sinclair and J.T. Arnason.

Parts of this chapter have been published:

Sinclair A.; **Leduc, R.I**. Recovery Strategy for Goldenseal (*Hydrastis canadensis*) in Ontario. *Environment Canada*. 2007 (*submitted for publication*)

2.1 Introduction

Hydrastis canadensis L. (goldenseal), a member of the Ranunculaceae (buttercup family), is a woodland herbaceous medicinal plant indigenous to eastern North America. Its natural range extends west from Vermont to southwestern Ontario and southern areas of Wisconsin, and south to Arkansas and northern Georgia (*48*). The medicinal properties of *H. canadensis* are due to the presence of several bioactive isoquinoline alkaloids, primarily berberine, hydrastine and canadine, which are mostly concentrated in the roots and rhizome (root-rhizome) (*116, 117*).

The medicinal properties of *H. canadensis* were first recognized by eastern North American Aboriginals and by the mid-19th century the plant became widely popular as a phytomedicine in North America and Europe (*4, 19*). Root-rhizome extracts were traditionally used as an external antiseptic, as an eyewash and to treat chapped lips, and were taken orally for debility, dyspepsia, cancer-like illnesses, whooping cough, diarrhea, fevers, pneumonia, tuberculosis, and stomach, heart and liver problems (*4, 19, 28, 29*). Today, root-rhizome capsules and tinctures, as well as teas made with stems and leaves (stem-leaf), are marketed for their antimicrobial properties and they are often sold in combination formulas with *Echinacea* products for the treatment of cold and flu symptoms (*11, 16, 118*).

In response to a severe decline in wild populations from excessive harvesting and loss of natural habitat, *H. canadensis* is protected under the Species At Risk Act (SARA) in Canada (*22*). The Committee on the Status of Endangered Wildlife in Canada (COSEWIC) and the Ministry of Natural Resources (MNR) both rank wild populations as threatened (*119*). It is thought that the risk of extirpation is mostly dependent on market demand for the plant, so much effort has been put forth in defining ethical and reliable methods of cultivation (*15, 19, 20, 48, 50-52, 59, 60*).

In July 2003 and 2004, permission was granted to survey and characterize the phytochemical variation among ten naturally occurring populations in southwestern Ontario. At that time, there was no information available on alkaloid concentration and variation in wild Canadian populations, and

the overall purpose of this study was to provide baseline phytochemical data on authentic, wild material.

The quantity of three alkaloids accumulated in aerial (stem-leaves and berry pulp) and below-ground (root-rhizome) tissues from a range of wild populations as well as in root-rhizome tissue from a variety of commercially available cultivated material was evaluated. The following quantification parameters for berberine, hydrastine, canadine and the sum of the three were measured: frequency distribution, tissue distribution and composition. Possible environmental factors for alkaloid variation among populations were considered, including latitude. A practical outcome of these findings is that they provide *H. canadensis* cultivators with authentic levels that can help further the crop potential, thus lessening the commercial desire for wild material.

2.2 Materials & Methods

2.2.1 Plant material: In July 2003 and July 2004, whole *H. canadensis* individuals were collected from ten naturally occurring populations in southwestern Ontario, each of which was given a code number, HC1 to HC10 (Table 2.1). The site locations range from the northern shores of Lake Erie, along the eastern borders of the Detroit River and the St. Clair River, up to the southeastern shores of Lake Huron (Figure 2.1). The exact locations of these populations are not provided, as per our agreement with the Canadian Wildlife Service (CWS), and are only available from COSEWIC. Figure 2.2 shows a typical healthy population of wild fruiting *H. canadensis*.

Hydrastis canadensis was harvested in July when its fruit was ripe and bright red. Only individuals with two mature-sized leaves were collected for analysis since they were considered to be 4 to 7 years old (Figures 2.2 and 2.3). At least four individuals were collected per site each year and depending on the size and health of the population, up to eight individuals were collected from certain sites. In addition to the samples that were collected for alkaloid analysis, a voucher specimen was collected from six of the ten populations and was deposited in the Vascular Plant Herbarium of the Department of Agriculture in Ottawa (DAO), Canada (Table 2.1 and Figure 2.3). Voucher specimens were only collected where population size was large enough. In the field, the approximate size of each population was recorded as well as the width of the largest leaf, the length of the stem and rhizome for each sample (data not shown). For a full description of the physical characteristics of each population site (e.g. population density, canopy size, soil type and chemistry) see Sinclair and Catling (*48*).

Below-ground tissues (root fibers and the rhizome) were removed from above-ground tissues (stem and leaves) and were stored whole in separate Nalgene bottles filled with 95% ethanol (EtOH). Berries were collected from three populations (Table 2.1 and Figure 2.1). The pulp of the berries was separated from the seeds, combined by site and stored at 4°C in Nalgene bottles filled with 95%

Table 2.1 Collection data for wild Canadian *Hydrastis canadensis* L. (goldenseal)

Leduc pop. no.	COSEWIC pop. no.[a]	Sinc. pop. no.[b]	Site county, city[c]	DAO voucher no.[d]	July 2003			July 2004			Total number of collected individuals
					Number of collected individuals	Root-rhizome extract code	Stem-leaf extract code	Number of collected individuals	Root-rhizome extract code	Stem-leaf extract code	
HC1	1A	1	County of Essex, Cedarhurst Park	824201	5	81-85	191-195	5	91-95	196-200	10
HC2	1B	2	County of Essex, Cedarhurst Park	824198	5	86-90	201-205	7	96-102	206-212	12[e]
HC3	2	3	County of Lambton, Duthill	N/A	5	129-133	213-217	4	134-137	218-221	9
HC4	3	4	County of Essex, McGregor	824203	6	103-108	227-232	8	110, 112, 114-116	233-240	14
HC5	5	6	County of Essex, Arner	824178	5	69-73	179-183	7	74-80	184-188, 190	12[f]
HC6	6	7	County of Essex, Cedarhurst Park	N/A	5	59-63	169-173	5	64-68	174-178	10

Table 2.1 continued

HC7	7	8	County of Lambton, Uttoxeter	824199	5	117-121	241-245	7	122-128	246-252	12
HC8	17	11	County of Lambton, Arkona	N/A	5	159-163	266-270	8	146-153	271-278	13[g]
HC9	18	12	County of Huron, Benmiller	824197	5	164-168	253-257	8	138-145	258-265	13
HC10	20	14	Municipality of Chatham-Kent, Moraviantown	N/A	5	154-158	222-226	0	N/A	N/A	5
Total number of individuals or extracts					51	51	51	59	56	58	110 [h](107;109)

[a] From COSEWIC Confidential Site Summary Record; [b] From Sinclair and Catling (*48*); [c] Exact location information is not provided, as it is restricted information and only available from COSEWIC; [d] Voucher number of specimen deposited in the Vascular Plant Herbarium of the Department of Agriculture in Ottawa (DAO); [e] Eight berries containing a total of 75 seeds were collected from population HC2 in July 2004; [f] Nine berries containing a total of 143 seeds were collected from population HC5 in July 2004; [g] Ten berries containing a total of 6 seeds were collected from population HC8 in July 2004; [h] Total number of root-rhizome extracts; total number of stem-leaf extracts

Figure 2.1 Distribution map of ten collection sites for wild Canadian *Hydrastis canadensis* L. (goldenseal). Their respective Leduc population number is indicated on the map. An asterisk (*) indicates sites where berries were harvested. The map was generated using Generic Point Mapper (*120*) with Global Positioning System data provided by COSEWIC. The map was modified from its original format by the author.

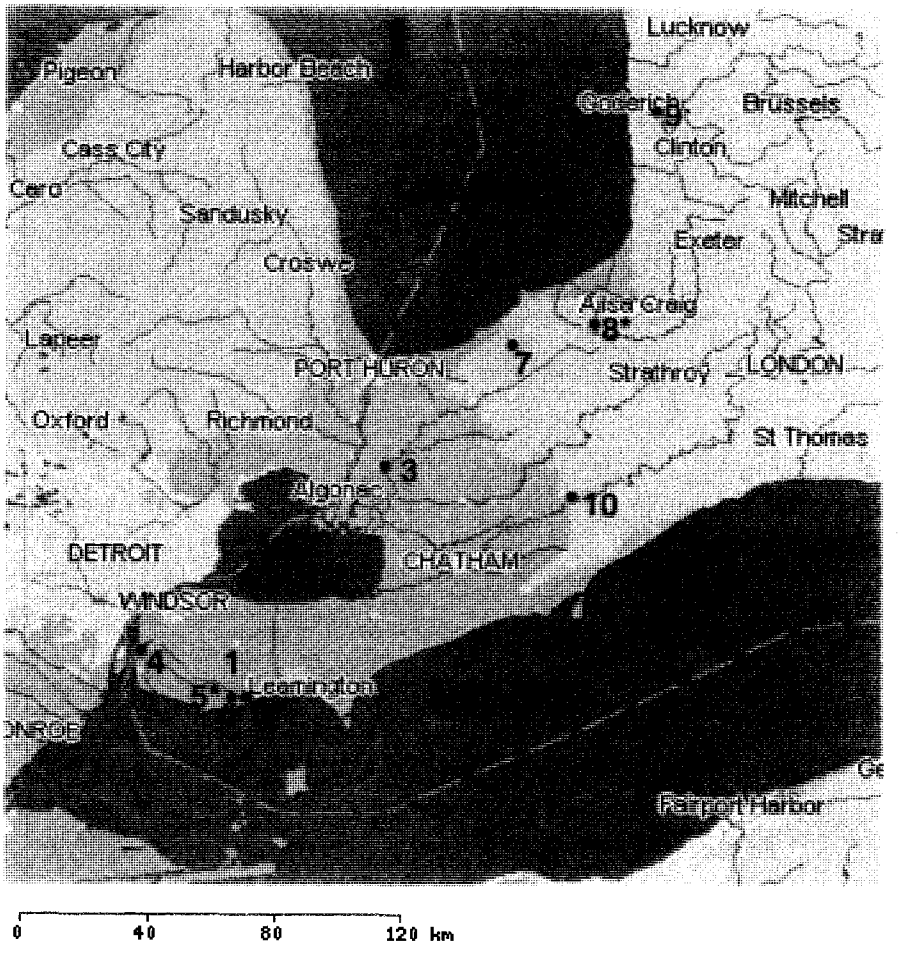

Lucknow
Pigeon
Harbor Beach
Brussels
Cass City
Clinton
Cera
Mitchell
Sandusky
Exeter
Croswe
Ailsa Craig
8
Lapeer
PORT HURON
7
Strathroy
LONDON
Oxford
Richmond
St Thomas
3
Algonac
10
DETROIT
CHATHAM
WINDSOR
4
1
5
Leamington
Fairport Harbor
0
40
80
120 km

Figure 2.2 Photographs of healthy, mature, fruiting, wild plants of *Hydrastis canadensis* L. (goldenseal). Showing A) stem length relative to leaf size, B) rhizome and root fibers, C) a ripe berry, and D) a healthy patch at population site HC8. The author took the photograph in Panel A in July 2003 and the photographs in Panels B, C and D were taken by E. Lamont in July 2004.

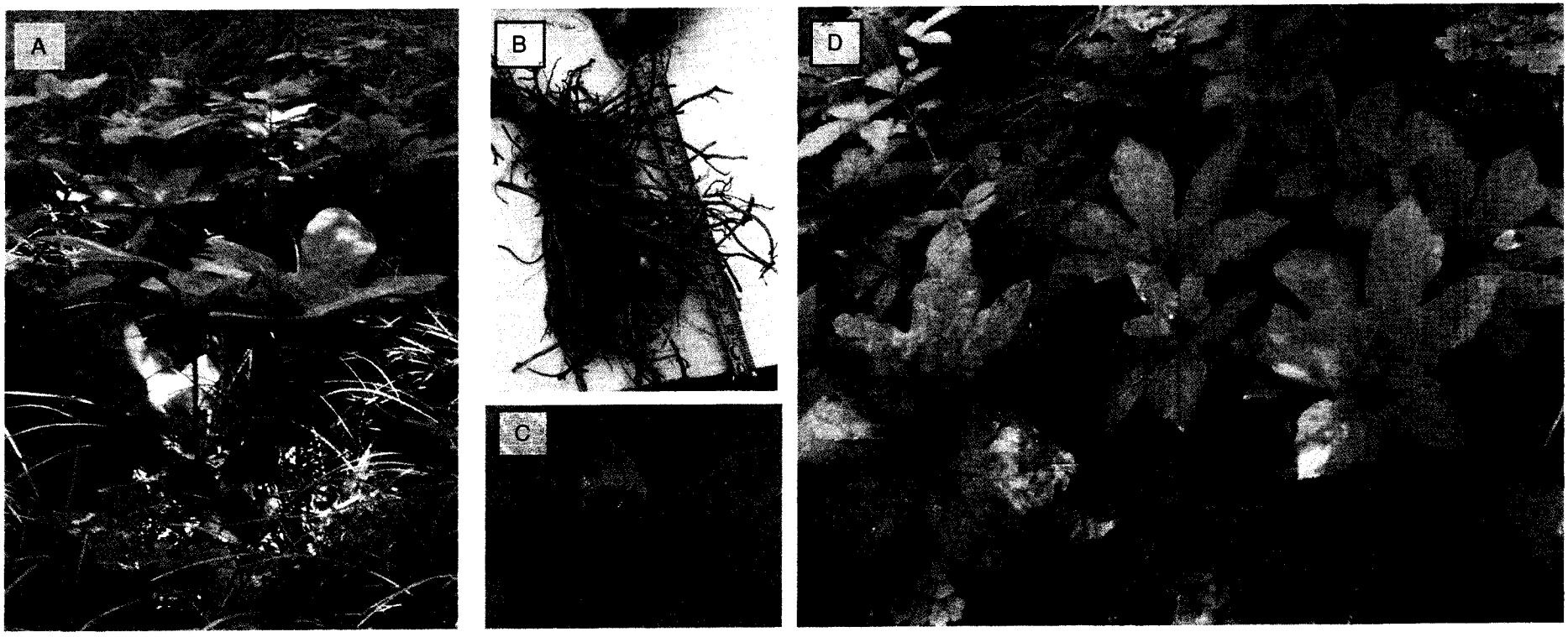
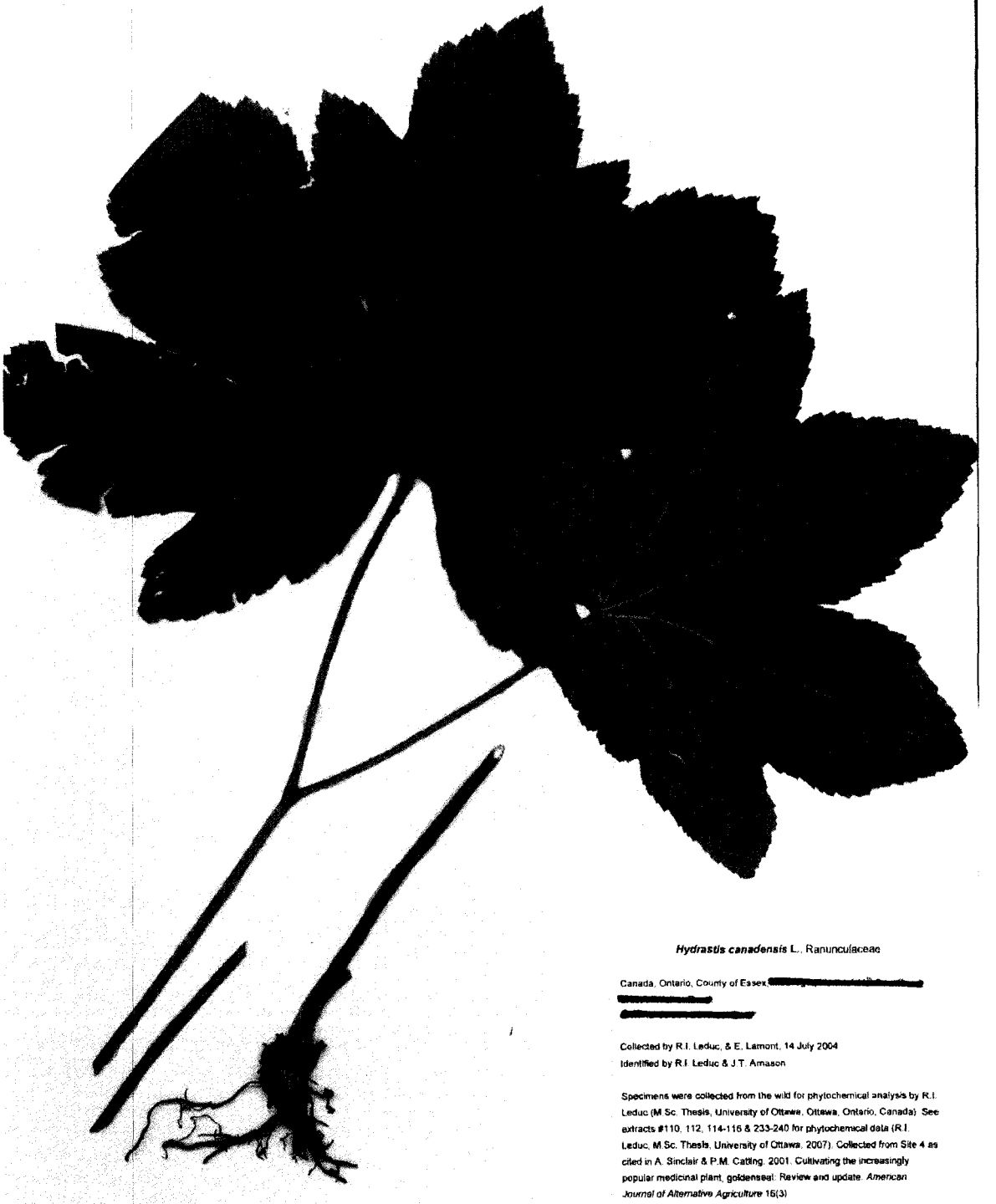

A
B
C
D

Figure 2.3 Vascular Plant Herbarium of the Department of Agriculture in Ottawa (DAO) voucher 824203: a mature wild Canadian *Hydrastis canadensis* L. (goldenseal) individual collected from population HC4 in July 2004. Below-ground and above-ground tissues are shown.



Hydrastis canadensis L., Ranunculaceae

Canada, Ontario, County of Essex [redacted]

Collected by R.I. Leduc, & E. Lamont, 14 July 2004
Identified by R.I. Leduc & J.T. Arnason

Specimens were collected from the wild for phytochemical analysis by R.I. Leduc (M.Sc. Thesis, University of Ottawa, Ottawa, Ontario, Canada). See extracts #110, 112, 114-116 & 233-240 for phytochemical data (R.I. Leduc, M.Sc. Thesis, University of Ottawa, 2007). Collected from Site 4 as cited in A. Sinclair & P.M. Catling. 2001. Cultivating the increasingly popular medicinal plant, goldenseal: Review and update. *American Journal of Alternative Agriculture* 16(3)

EtOH. The seeds were also combined by site, kept moist and stored at 6°C until they were moved into a greenhouse chamber.

Samples of cultivated crude root-rhizome material were donated by or purchased from six different sources (accessions) (Table 2.2). To respect the privacy of each contributor, accessions were randomly assigned a code between RIL1 and RIL6, and these codes are used throughout the study.

Gkginseng Farm, Goldcap Farm and Northfield Agri Products Inc. provided material that had been harvested on-site. In 2004, 13 voucher specimens harvested from Gkginseng Farm (Waterford, Ontario) were pressed, mounted and deposited in the DAO (Table 2.2, Figures 2.4 and 2.5). Crude bulk root-rhizome, from the same farm, was dried and ground by Dr. J. Lam, National Research Council, who provided our laboratory with a sub-sample for analysis. A bulk crude mixture of fresh mature root-rhizome material was also received from Goldcap Farm in Princeton, Ontario. The material was stored at –20°C and sub-samples were dried at 50°C for three days before being processed for HPLC analysis. All other cultivated root-rhizome material was received dry.

Two of the three dry accessions were received from American sources: Pacific Botanicals, an herb farm in Grants Pass, Oregon and Rosemary's Garden, an herbal apothecary in Sebastopol, California. Samples were also purchased from Judy's Organic Herbs, an herb farm in Woodlawn, Ontario. Samples from all three of these companies were not harvested on site but rather purchased from external suppliers. Only Pacific Botanicals was able to disclose the origin of their material, which was from Washington State (Table 2.2).

With the exception of the fresh samples received from Goldcap Farm, all whole plant and crude material samples were stored at 4°C.

Table 2.2 Summary of commercially available cultivated *Hydrastis canadensis* L. (goldenseal) material

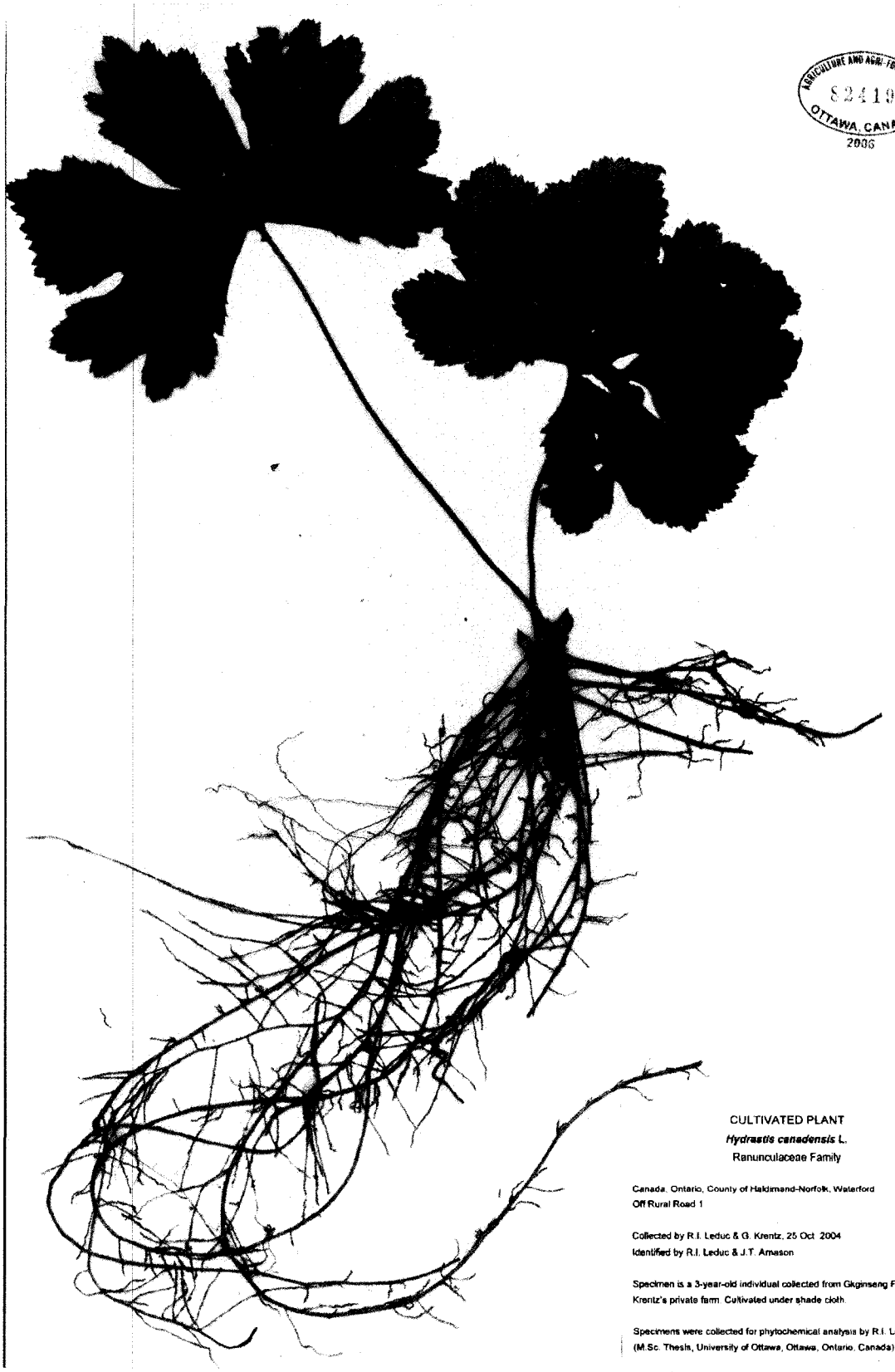
Grower or Distributor	Company type	Contact person and information	Harvest location (date)	Sample description as indicated on label	Extract code and no.	Total no. of extracts
Gkginseng Farm[a]	Herb farm	Gary Krentz R. R. 6, Tillsonburg, ON, N4G 4G4 Phone 519-688-5447 Fax 519-688-5448[b]	On-site: Waterford, ON (25 October 2004)	[c]Dry, bulk, whole root-rhizome mix; cultivated under artificial shade	HCGK, A-F, 1-11, 53-58, 282-284	26
Goldcap Farm	Herb farm	John Kershaw 66 Third Conc., RR#3, Princeton, ON Phone 519-449-2312	On-site: Princeton, ON (2004)	[c]Fresh, bulk, whole root-rhizome mix (stored in freezer)	HCF	5
Northfield Agri Products Inc.	Herb farm	Bob Romaniuk Sr and Jr. 298 Thirteenth Concession Rd, Scotland, ON, N0E 1R0 Phone 519-446-3985 or 519-446-3721	On-site: Scotland, ON (2003)	[c]Dry, bulk, whole root-rhizome mix	HCD	9
Judy's Organic Herbs	Herb farm	Judy PO Box 258, Woodlawn, ON, K0A 3M0 Phone 613-832-8241 Email herbs@earthmedicinie.ca www.earthmedicine.ca	Not available (2005)	1.2 oz, dry, cut [d]root-rhizome mix; Lot # 184111131207	HCJOH, 18-20	5

Table 2.2 continued

Pacific Botanicals, LLC	Herb farm	Toni Corrente-Evans 4840 Fish Hatchery Rd, Grants Pass, OR, 97527, USA Phone 541-479-7777 www.pacificbotanicals.com	Washington State (2005)	1 lb root powder; Oregon Tilth certified organic; Lot # 0806K-TCP	HCPBLLC	3
Rosemary's Garden	Herbal apothecary	Lena Moffat 132 North Main St, Sebastopol, CA, 95472 USA Phone 707-829-2539 Fax 707-829-5613 Email info@rosemarysgarden.com www.rosemarysgarden.com	Not available	Dry, cut root	HCRG, 38-40	5

[a] Thirteen specimens collected from Gkginseng Farm were deposited in the Vascular Plant Herbarium of the Department of Agriculture in Ottawa (DAO). Their respective voucher numbers are listed here: 824184, 824185, 824186, 824187, 824188, 824189, 824190, 824191, 824192, 824193, 824194, 824195 and 824196; [b] From http://www.ces.ncsu.edu/depts/hort/hil/hil-123.html (viewed November 2006); [c] Material was harvested on-site and was not received with a descriptive label; [d] Tissue type not indicated on label

Figure 2.4 Vascular Plant Herbarium of the Department of Agriculture in Ottawa (DAO) voucher 824191: a 3-year-old *Hydrastis canadensis* L. (goldenseal) individual harvested October 2004 from Gkginseng Farm in Waterford, Ontario. The crop, from which the individual originated, was cultivated under artificial shade. Below-ground and above-ground tissues are shown.



CULTIVATED PLANT

***Hydrastis canadensis* L.**

Ranunculaceae Family

Canada, Ontario, County of Haldimand-Norfolk, Waterford
Off Rural Road 1

Collected by R.I. Leduc & G. Krentz, 25 Oct. 2004
Identified by R.I. Leduc & J.T. Arnason

Specimen is a 3-year-old individual collected from Gkginseng Farm. G. Krentz's private farm. Cultivated under shade cloth.

Specimens were collected for phytochemical analysis by R.I. Leduc (M.Sc. Thesis, University of Ottawa, Ottawa, Ontario, Canada)

Figure 2.5 Photographs of cultivated *Hydrastis canadensis* L. (goldenseal) crops at Gkginseng Farm in Waterford, Ontario. Note the wooden structures providing artificial shade to the crops. All photographs were taken by author in October 2004. Showing A) size and health of crop, B) G. Krentz holding freshly harvested mature *H. canadensis* with long fibrous roots, C) row of *H. canadensis*, D) young seedlings, E) fruiting *H. canadensis* individual with large leaves, F) healthy, mature patch and G) G. Krentz showing the colour and size of freshly harvested rhizomes and roots.


A


B


C


D


E


F


G

2.2.2 Mapping: The map in Figure 2.1 was generated using Generic Point Mapper (GPM) with Global Positioning System (GPS) data. GPM is an online mapping service provided by the Government of Canada's Canadian Biodiversity Information Facility (*120*).

2.2.3 Sample preparation & extraction: For wet samples (e.g. fresh, frozen or stored in solvent) and all wild root-rhizome and stem-leaf material, each sample was carefully removed from its storage solvent (e.g. a 60 mL, 250 mL or 1 L Nalgene bottle filled with 95% EtOH), treated with liquid nitrogen and ground into a fine powder using a mortar and pestle. The ground material was dried at room temperature, weighed and returned to its storage solvent. For below-ground tissues, the extraction volume was adjusted with 95% EtOH to a 1 g : 70 mL (dry powder : solvent) extraction ratio. For above-ground tissues, the extraction volume was adjusted with 95% EtOH when the extraction ratio was greater than 1 g : 100 mL. The powder-solvent mixture was mechanically agitated on a shaker at 3 x *g* (150 rpm, 12.7 mm rotor radius, New Brunswick Scientific Corp.) for 24 hours before being filtered through a Buchner filter using a 42 size (0.2 μm) Whatman filter paper. The below-ground plant residue was re-extracted with a 1 g : 45 mL extraction ratio while the above-ground plant residue was re-extracted with an extraction ratio of 1 g : 25 mL. The second powder-solvent mixture was mechanically agitated on the same shaker as before, for 1 hour and filtered through a Buchner filter as above. The filtrates were combined and rotary-evaporated (40°C) in vacuo to < 25 mL. The remaining extract was transferred into a 25 mL volumetric flask and the volume was adjusted to 25 mL with 95% EtOH. A 1 mL sample from the final extract was filtered through a 0.2 μm PTFE nylon filter for HPLC analysis. All extracts were stored at 4°C.

For berry pulp samples, the pulp was pulverized in its storage solvent (approximately 60 mL of 95% EtOH) using a Polytron (Brinkman Instruments, Westbury, NY). The pulp-solvent slurry was transferred into an Erlenmeyer flask and mechanically agitated on a shaker at 3 x *g* (150 rpm, 12.7 mm rotor radius, New Brunswick Scientific Cor.) for 24 hours and filtered through a Buchner filter using a 42 size (0.2 μm) Whatman filter paper. The pulp residue was re-extracted with 60 mL of

95% EtOH, shaken at the same speed as before, for 1 hour and filtered through a Buchner filter using the same filter size as above. The filtrates were combined and rotary-evaporated (40°C) in vacuo to < 25 mL. The remaining extract was transferred into a 25 mL volumetric flask and the volume was adjusted to 25 mL with 95% EtOH. A 1 mL sample from the final extract was filtered through a 0.2 µm PTFE nylon filter for HPLC analysis. The dry weight of the pulp was estimated by the weight of the pulp residue that was dried after extraction. All extracts were stored at 4°C.

All root-rhizome material that was received dry was finely ground using a Wiley mill or a mortar and pestle, and filtered through 0.5 mm mesh screen. Twenty-five milligrams of powder was weighed and extracted with 1 mL 55% EtOH, vortexed on high for 5 minutes and centrifuged at 8.0 x 10^5 x *g* (1.2 x 10^5 rpm, 50 mm rotor radius, Fisher Scientific Micro centrifuge) for 15 minutes. The supernatant was filtered through a 0.2 µm PTFE filter before HPLC analysis. All extracts were stored at 4°C.

2.2.4 Materials for HPLC analyses: High performance liquid chromatography (HPLC) solvents and analytical grade trifluoroacetic acid (TFA) were purchased from EMD Biosciences Inc. (Germany) and JT Baker (Phillipsburg, NJ, USA), respectively. Berberine chloride and (1R, 9S)-β-hydrastine standards were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) and a canadine standard was purchased from Fluka AG (Buchs, SG, Switzerland).

2.2.5 HPLC-DAD analyses: HPLC with diode-array detector (HPLC-DAD) analyses of alkaloid concentrations were conducted on extracts of root-rhizome mixtures, stem-leaf mixtures and berry pulp prepared from wild *H. canadensis* individuals as well as extracts of cultivated *H. canadensis* root-rhizome material. Described below are the HPLC method adapted from Li and Fitzloff (*121*) and its validation.

An Agilent Technologies Inc. HPLC 1100 system was used for alkaloid analysis. The system consisted of an in-line degasser, a quaternary pump, an autosampler, a DAD, and a computer

equipped with Chemstation software (Rev. A.09.01). The separation of berberine, hydrastine, and canadine was achieved with a reverse-phase C-18 Waters YMC ODS-AM (2 x 100 mm; 5 µm particle size, S-3, 120 Å) column that was maintained at 50°C and used at a flow rate of 1 mL/min. The mobile phases were (A) acetonitrile and (B) 0.05 % aqueous TFA and the elution conditions of the mobile phases were the following: initial conditions of 15% A and 85% B, followed by a linear gradient of 15-70 % A in 12 minutes and returned to initial conditions 70-15 % A in 2.5 minutes; re-equilibrated 15 % A for 0.5 minute followed by a post-run time of 1 minute, resulting in a total run time of 15 minutes. A 1-µL injection volume was used for the quantification of both berberine and hydrastine, while a 5-µL injection volume was used to quantify canadine. Alkaloids were quantified at the wavelength of greatest absorbance. The DAD was set at 225 nm for hydrastine and canadine quantification, and at 350 nm for berberine quantification. Typically hydrastine eluted after 5.3 minutes, followed by canadine at 6.3 minutes and berberine at 6.7 minutes.

Each sample was analyzed in triplicate and the mean percent alkaloid weight per dry powder weight (% w/dw) and standard error of the mean (SEM) were calculated for each alkaloid. Analyses consisted of both the identification and quantification of alkaloids. All alkaloids were identified based on their relative retention time to the compound standard and online photodiode array UV spectra. Matches were defined by at least 95% similarity between the two. Each alkaloid was quantified (ng/µL) using peak area multiplied by the response factor, calculated from its respective standard curve. This value was multiplied by the extract's final volume (µL) to powder weight (g) ratio and divided by 10^3 for a final value for % w/dw (mg/g).

2.2.6 Method validation for HPLC-DAD alkaloid analysis: Validation was performed for HPLC-DAD analyses using the mobile solvent system described above. Recovery experiments, modified from Weber et al. (*122*), were undertaken by injecting (spiking) aliquots of berberine (0.2, 0.4 and 0.7 mg/mL), hydrastine (0.1, 0.2 and 0.4 mg/mL) and canadine (0.03, 0.06 and 0.1 mg/mL) standards to

cultivated root-rhizome powder (RIL1). Spiked and unspiked samples were extracted as described above. Experiments were carried out in triplicate and coefficients of variation determined accordingly.

2.2.7 HPLC-DAD/APCI-MS analyses: HPLC-mass spectrometry (HPLC-MS) analyses of alkaloid concentration were conducted on three extracts prepared from bulk mixtures of cultivated crude root-rhizome material from accession RIL1. The purposes of these analyses were to confirm the identity of all three alkaloid peaks in the HPLC elution profile of *H. canadensis* and to screen for phenolic compounds. The method described below was modified from Harris et al. (*123*).

Analyses were performed on 1100 series liquid-chromatography (LC) /MSD Trap VL atmospheric pressure chemical ionization (APCI) system consisting of a quaternary pump, an autosampler, a DAD, an online APCI/MS with mass range of 50-15000 Da (Agilent, Palo Alto, CA, USA) and a computer equipped with Chemstation software. A Waters YMC ODS-AM column (100 x 2mm 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 (A) methanol and (B) 0.05 % aqueous TFA as follows: initial conditions 8% A, 92% B, maintained 0-5 minutes followed by four linear gradients of 8-13% A in 2 minutes, 13-30% A in 14 minutes, 30-60% A in 3 minutes, and 60-100% A in 2 minutes. The column was then washed, 100% A for 2 minutes, returned to initial conditions, 100-8% A in 2 minutes, and re-equilibrated for 6 minutes, resulting in a total run time of 36 minutes. One microliter of each extract was injected through the autosampler for each analysis and the subsequent elution profiles were monitored on-line at 225 and 350 nm.

The MS 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 100-800 amu with fragmentation voltages of 20 V and –160 V for positive and negative ionization respectively.

Each sample was analyzed in triplicate and, in order to confirm the identification of each major peak, the UV spectra for each of the sample's absorbance peaks were compared with those of the standard's as well as with those saved in a library of reference standards. Matches with ≥ 95% similarity were further corroborated by the presence of a major ion in the peak's mass spectrum corresponding to the parent molecule or its major ion fragment.

2.2.8 Statistical analyses: All HPLC results were expressed as a mean percent alkaloid weight per plant powder weight (% w/dw) ± standard error of the mean (SEM) of three replicate analyses, where the coefficient of variance (CV) of the mean was ≤ 5%.

The following null hypotheses were tested in order to address the research objectives of this study: 1) no significant difference in alkaloid concentration (each alkaloid individually and their sum) and composition among tissues (including wild root-rhizome, cultivated root-rhizome, cultivated root-rhizome in the literature, wild stem-leaf and wild berry pulp); 2) no significant difference in alkaloid concentration (each alkaloid individually and their sum) and composition (separately) among accessions of cultivated root-rhizome material; 3) no significant difference in alkaloid concentration (each alkaloid individually and their sum) and composition between wild and cultivated root-rhizome material (also including cultivated root-rhizome from the literature); 4) no significant difference in alkaloid concentration (each alkaloid individually and their sum) among wild populations; 5) no significant difference in alkaloid concentration (each alkaloid individually

and their sum) and composition among individuals from different wild populations propagated from seed under uniform environmental conditions.

These null hypotheses were assessed by two-tailed one-way analysis of variance (ANOVA) and when significant p statistics resulted ($p < 0.05$), pairwise differences among samples were determined by Scheffe's post hoc multiple comparison analysis. Data sets were transformed by the logarithm base 10 when necessary to achieve normality and homoscedasticity; however, when these ANOVA assumptions could not be met even after transformations, the data set was analyzed using the Kruskal-Wallis non-parametric test.

The relationship between latitude of wild populations and alkaloid concentration in wild root-rhizome was determined using simple linear regression of the population means for each alkaloid and their sum total (separately) on the latitude of corresponding material source.

All statistical analyses were conducted using S-PLUS 7.0 for Windows, Academic Site Edition (*124*).

2.3 Results & Discussion

2.3.1 Plant collection & seed germination: In July 2003, a total of 51 whole *H. canadensis* individuals were collected from ten wild populations in southwestern Ontario and an additional 59 individuals were collected the following summer from the same sites, for a total of 110 individuals (Table 2.1, Figures 2.1, 2.2 and 2.3). This was the first sampling of Canadian wild *H. canadensis* for analytical research; previous samplings had been used for ecological and conservational research (*24, 25*).

Measurements of stem length and leaf width of 100 of the sampled individuals demonstrated approximate age homogeneity among samples ($p > 0.05$). The overall average stem height was 30.3 ± 0.50 cm and the overall average leaf width was 18.6 ± 0.46 cm. According to Sharp et al. (*125*), the largest cauline leaf of *H. canadensis* measures between 12 and 20 cm wide at maturity and Sinclair and Catling (*24*) note that stems generally reach up to 30 cm in height, thus confirming that the samples collected for this study were within maturity and approximately the same age.

In July 2004, an additional individual was collected from populations HC1, HC2, HC4, HC5, HC7 and HC9, pressed, mounted and donated to the DAO as a voucher specimen of its respective population (Table 2.1 and Figure 2.3). Cultivated specimens from Gkginseng Farm in Waterford, Ontario, were also donated to the DAO (Table 2.2 and Figure 2.4). The latter are discussed below.

As listed in Table 2.1, the population sites were located within four counties: populations HC1, HC2, HC4, HC5 and HC6 are situated in Essex County; populations HC3, HC7 and HC8 in Lambton County; population HC9 in Huron County and population HC10 in the Municipality of Chatham-Kent. Figure 2.1 shows the approximate distances between population sites. The largest distance (128 km) was estimated between populations in Essex County (HC1, HC2, HC4, HC5 and HC6) and population HC9. The most proximate sites (≤ 0.4 km) were HC1, HC2 and HC6 in Essex County. The relationship between alkaloid concentration and latitude is discussed in section 2.5.

Bulk crude samples of cultivated root-rhizome were received from five herb farms and one herbal apothecary. Three of the five herb farms were located in southwestern Ontario and provided root-rhizome material from their own crop while the other two were distributors (Table 2.2). Figure 2.5 shows mature *H. canadensis* crops and seedlings cultivated under shade structures at Gkginseng Farm. As for the herb farms that distributed cultivated *H. canadensis*, the material received from Pacific Botanicals was harvested from a farm in Washington State. Neither Judy's Organic Farm and Rosemary's Garden was able to provide the harvest location of their samples. All commercial samples were purportedly comprised of root fibers and rhizomes harvested from mature and cultivated *H. canadensis* individuals exclusively.

Twenty-seven ripe berries containing a total of 224 seeds were collected in July 2004 from populations HC2 and HC5, both in Essex County, and population HC8 in Lambton County (Table 2.1, and Figures 2.1 and 2.2). Like the plant samples, this was the first seed and berry sampling of wild Canadian populations for phytochemical research. Previously, in 2003, seeds were collected for ecological germination studies. Results indicated a low germination rate (9.2%) but that germination can occur in a wide range of light availability (0, 30, 50 and 80% shade), with soil moisture being a critical factor (*126*).

Since germination trials in the current study were unsuccessful the genetic component of phytochemical diversity in wild *H. canadensis* populations was not evaluated. The seeds that survived were transferred to the experimental laboratories at the National Wildlife Research Centre (Environment Canada) in Ottawa in order to allow further investigation into the germination ecology of *H. canadensis*. The germination trials and the results from a seed viability test are further described in the Appendix. All the following population analyses were based on field-collected tissue.

2.3.2 Method validation of alkaloid analysis: Following criteria for single laboratory validation, a dual DAD monitoring of UV absorption at wavelengths 225 nm and 350 nm was developed in order

to optimize the sensitivity of detection and quantification of four alkaloids: hydrastine and canadine at 225 nm, and berberine and hydrastinine at 350 nm. Hydrastinine eluted approximately at 2.1 minutes and was not detected in any of the *H. canadensis* samples (data not shown). Hydrastinine is a degradation product of hydrastine and its presence in *H. canadensis* is an indicator of aged material (i.e. the time span since collection) or improper drying or handling procedures (*16*). Its absence confirmed the approximate age homogeneity of the material among all of the samples (wild and cultivated) as well as good manufacturing practices with respect to the commercial samples. The dual DAD monitoring program provided the advantage of optimized and selective detection of each respective alkaloid. For example, hydrastine and canadine peaks were not detected when monitoring at 350 nm, thus simplifying the detection of berberine and hydrastinine (Figures 2.6, 2.7 and 2.8). The former was exclusively identified at a wavelength of 350 nm (data not shown). Given the absence of hydrastinine from the samples and the greater pharmacological relevance of berberine, hydrastine and canadine, the method was validated for the analysis of the three alkaloids only, with the exception of the method's parameters for linearity.

The method was validated for linearity, reproducibility and recovery. The linearity of the standard curves, produced for quantification purposes, revealed linear response profiles where the correlation coefficient (R^2) was $\geq$ 0.999 for all four alkaloids, at ranges of 0.03 –1.98 μg/mL berberine; 0.03 – 0.87 μg/mL hydrastine; 0.03 – 1.64 μg/mL canadine; and 0.02 – 1.16 μg/mL hydrastinine (data not shown).

Measuring the variation in quantification results within and between days assessed the reproducibility of the method. Expressed as the coefficient of variance (CV, standard deviation / mean x 100%), variation between successive trials (intraday) and between interday trials, for all three alkaloids quantified in all samples, was calculated to be $\leq$ 6% (data not shown).

Using the method described by Weber et al. (*122*), recovery was assessed for the three main alkaloids. Pure standards of berberine, hydrastine and canadine were spiked into cultivated root-

Figure 2.6 Representative HPLC chromatograms of wild Canadian *Hydrastis canadensis* L. (goldenseal) root-rhizome extracts showing the absorbance peaks and the retention times of A) hydrastine and canadine at 225 nm and B) berberine at 350 nm. The UV spectra and chemical structures of each alkaloid are shown.

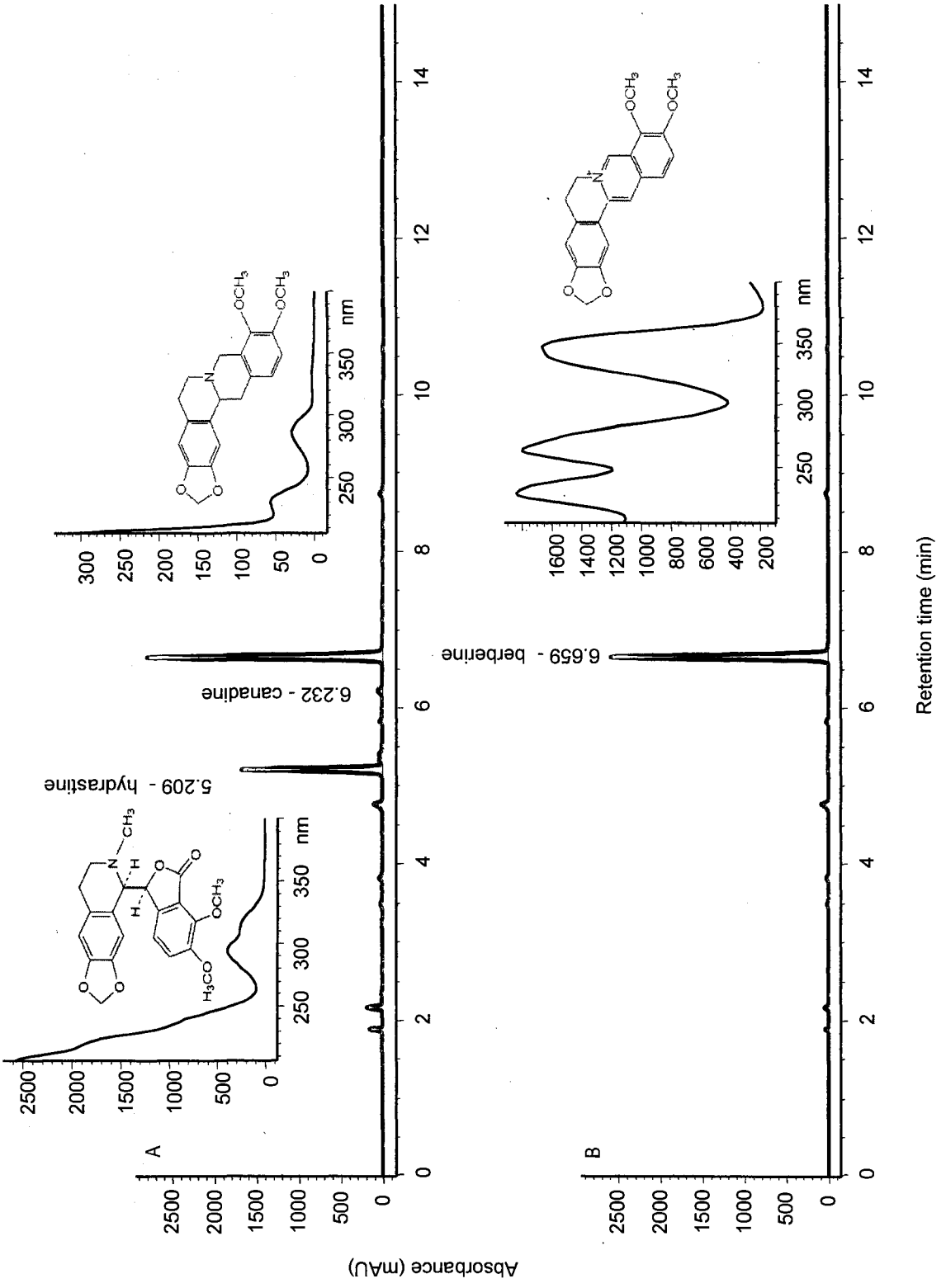

A
B
5.209 - hydrastine
6.232 - canadine
6.659 - berberine
Absorbance (mAU)
Retention time (min)
nm

Figure 2.7 Representative HPLC chromatograms of wild Canadian *Hydrastis canadensis* L. (goldenseal) stem-leaf extracts showing the absorbance peaks and the retention times of A) hydrastine and canadine at 225 nm and B) berberine at 350 nm.

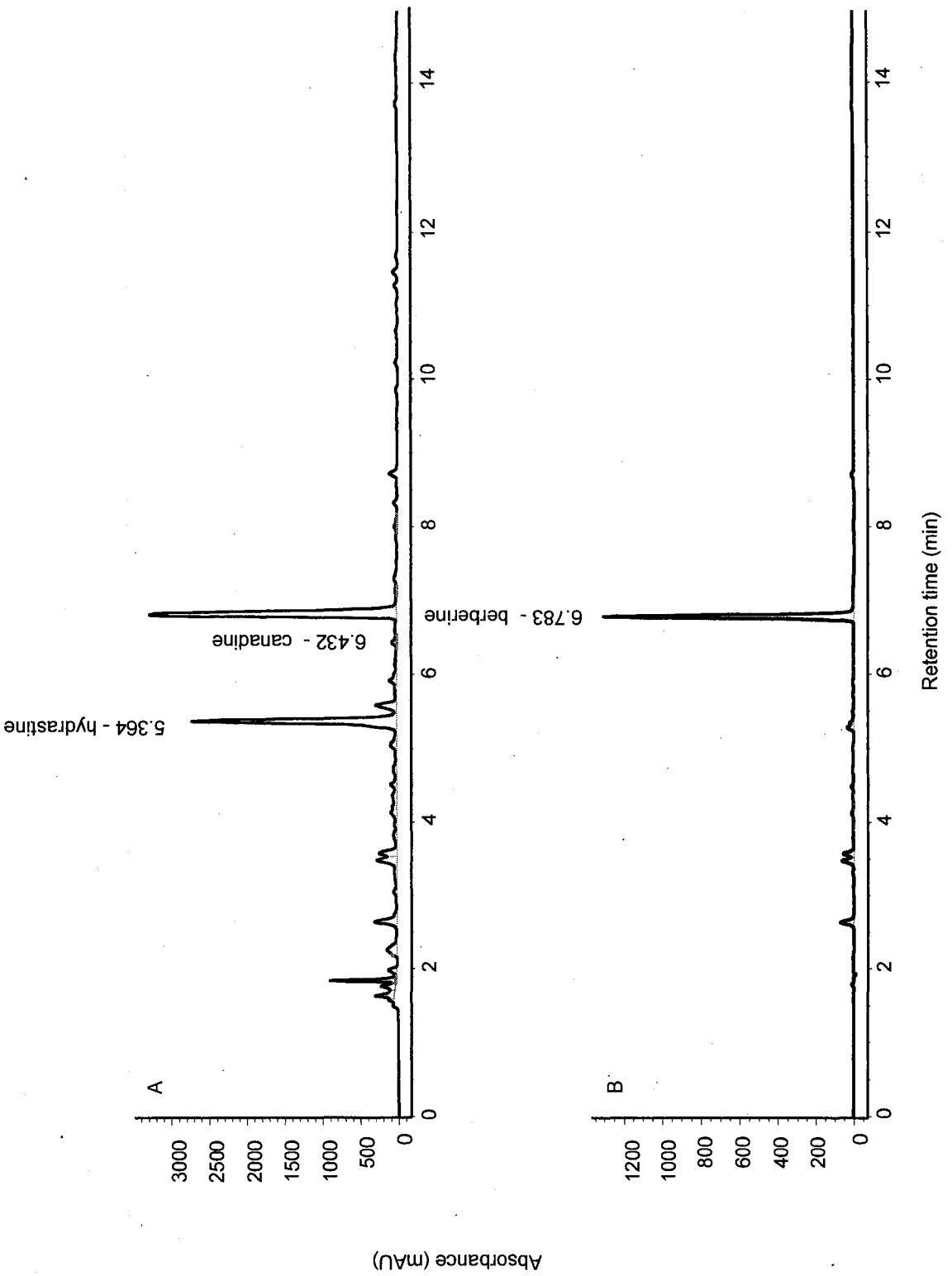
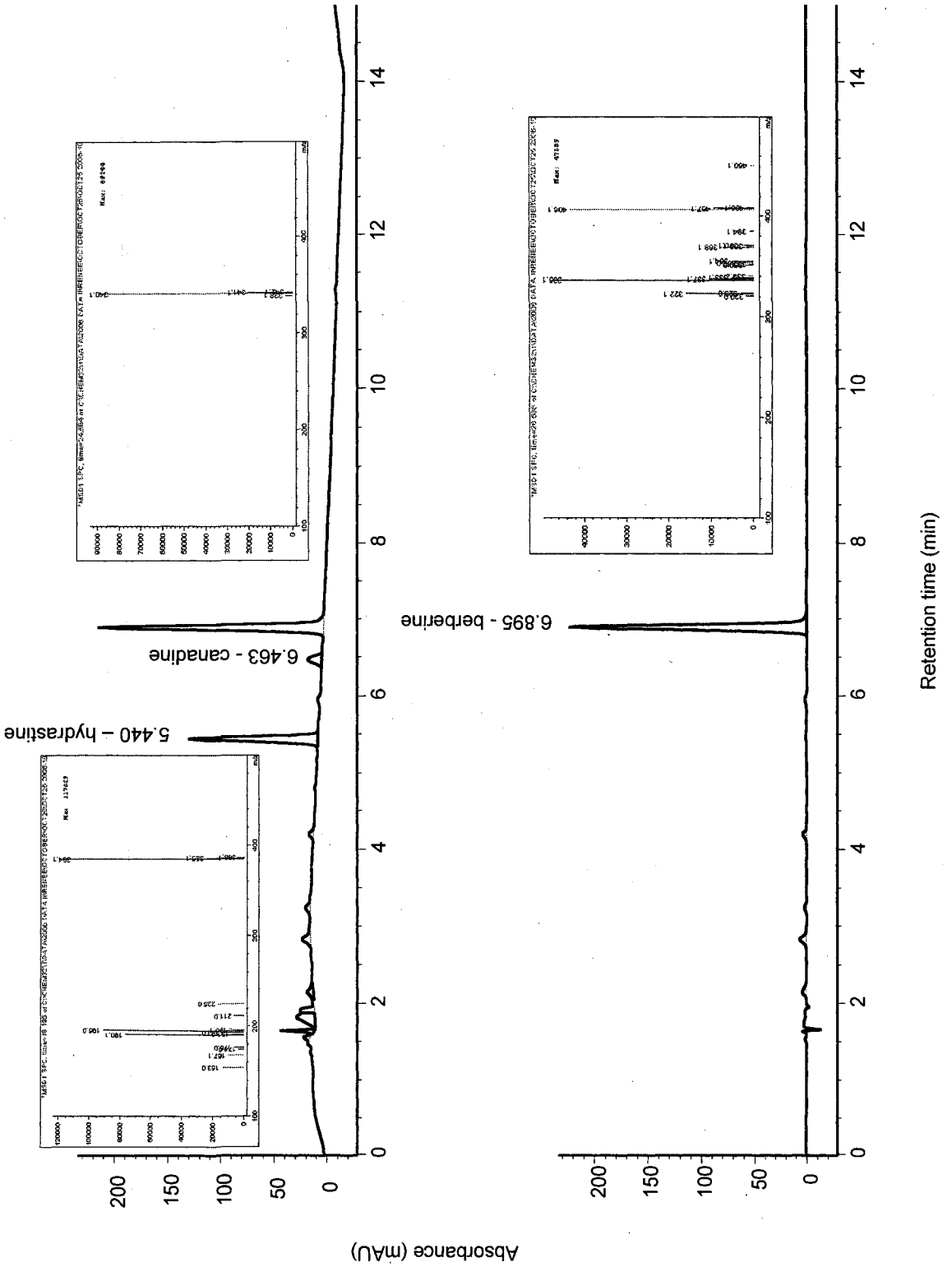

A
B
5.364 - hydrastine
6.432 - canadine
6.783 - berberine
Absorbance (mAU)
Retention time (min)

Figure 2.8 Representative HPLC chromatograms of cultivated *Hydrastis canadensis* L. (goldenseal) root-rhizome extracts showing the absorbance peak, the retention time and the mass spectrometry (MS) profile of A) hydrastine and canadine at 225 nm and B) berberine at 350 nm. The molecular ions (*m/z*) are shown for each alkaloid in the MS profiles.


5.440 – hydrastine
6.463 - canadine
6.895 - berberine
Absorbance (mAU)
Retention time (min)

rhizome powder (RIL1); the powder-standard mixture was extracted and analyzed as described above. The recoveries ± SEM (N = 3) were 84.8% ± 0.80% for berberine, 82.0% ± 2.42% for hydrastine and 72.3% ± 1.15% for canadine; and the overall alkaloid recovery was calculated to be 79.7% ± 2.06% (Table 2.3).

2.3.3 Alkaloid analysis: Reverse-phase HPLC-DAD analyses were completed on 107 wild Canadian *H. canadensis* root-rhizome samples; 24 samples of bulk North American cultivated root-rhizome material; 109 wild Canadian stem-leaf samples and on three samples of wild Canadian berry pulp (Tables 2.1 and 2.2). The results have been compared to the concentration of alkaloids in 28 commercial root-rhizome samples and five commercial stem-leaf samples described in the literature (Tables 2.4 and 2.5).

All analyzed *H. canadensis* material, whether wild, cultivated, root-rhizome, stem-leaf or berry pulp and regardless of origin, consistently gave the same chemical profile (Figures 2.6, 2.7 and 2.8). The chemical profile consisted of two major peaks, hydrastine followed by berberine, and one minor peak, canadine, all of which were confirmed by MS analysis. This method was also used to analyze berberine in other chemically related species and found their chemical profile differed from that of *H. canadensis* (Chapter 3: Figure 3.8), thus confirming the reliability of the chemical profile as a fingerprint of the species.

Figures 2.6 and 2.7 show chromatograms of typical wild root-rhizome extracts and wild stem-leaf extracts, respectively. Berberine, hydrastine and canadine, as well as their respective retention times, are identified in both figures. The HPLC chromatogram for a typical wild berry extract is not shown since berberine was the only alkaloid detected in this material. Figure 2.8 shows the chromatograms of typical cultivated root-rhizome extracts and identifies the peak, retention time and molecular weight, from MS analysis, for berberine, hydrastine and canadine. The molecular ions (*m/z*) measured by MS for hydrastine was 383.1; for canadine was 339.1 and for berberine was

Table 2.3 Spike and recovery results for berberine, hydrastine and canadine as measured by reverse phase HPLC-DAD.

Alkaloid	Amount of powder extracted (g)	Determined alkaloid (mg)	Theoretical alkaloid in spiked sample (mg)[a]	Recovery (%)
Berberine	3.004	86.4	103.8	83.2
	3.001	89.1	103.8	85.8
	3.004	88.5	103.8	85.3
Overall berberine recovery ± SEM				84.8 ± 0.80
Hydrastine	3.004	35.1	42.3	83.0
	3.001	36.2	42.3	85.6
	3.004	32.7	42.3	77.4
Overall hydrastine recovery ± SEM				82.0 ± 2.42
Canadine	3.004	9.5	13.5	70.1
	3.001	9.8	13.5	72.7
	3.004	10.0	13.5	74.0
Overall canadine recovery ± SEM				72.3 ± 1.15
Overall mean alkaloid recovery ± SEM, N = 9				79.7 ± 2.06

[a] Sum of calculated native (mg, based on non-spiked samples) and spiked alkaloid (mg) weight

Table 2.4 Summary of phytochemical data for *Hydrastis canadensis* L. (goldenseal) material in the literature

Reference	Leduc ref. number	Manufacturer or Distributor	Plant tissue (Product name)	Lot or accession number	Extraction solvent	Analytical method	Alkaloid concentration[a]					Hydra /Berb	Can/ Berb
							Berb	Hydra	Can	Hdst	Tot		
Abidi et al. (*45*)	R1	Solgar Vitamin and Herb (Leonia, NJ)	Root powder 60% grain alcohol extract	Lot 8	Diluted with MeOH	HPLC/UV-DAD	6.87[b]	5.07[b]	0.26[b]	/	/	0.7	0.04
	R2	Now Foods (Bloomingdale, IL)	Root powder EtOH extract	Lot 3	/	HPLC/UV-DAD	1.48[b]	2.42[b]	0.09[b]	/	/	1.6	0.1
	R3		Root powder EtOH extract	Lot 6	/	HPLC/UV-DAD	1.50[b]	3.08[b]	0.14[b]	/	/	2.1	0.1
	R4	Gaia Herbs (Brevard, NC)	Root powder 60% grain alcohol extract	Lot 7	Diluted with MeOH	HPLC/UV-DAD	4.47[b]	3.28[b]	0.11[b]	/	/	0.7	0.02
Abourashed and Khan (*144*)	R5	Nature's Way Products, Inc (Spingville, UT)	Capsule (Herbal Single Goldenseal Root)	Sample 1	MeOH	HPLC	4.35% (0.75% RSD)	2.51% (1.46% RSD)	/	/	/	0.6	/
	R6	Thompson Nutritional Products (Boca Raton, FL)	Goldenseal root capsule	Sample 2	MeOH	HPLC	3.41% (0.62% RSD)	2.27% (1.22% RSD)	/	/	/	0.7	/
	R7	Australasian College of herbal Studies (Lake Oswego, OR)	Crude goldenseal powder	Sample 3	MeOH	HPLC	1.79% (0.34% RSD)	1.35% (0.75% RSD)	/	/	/	0.8	/

Table 2.4 continued

Berkel et al. (*145*)	R8	Nature's Resource Goldenseal	Gelatin capsule with dried ground root	/	MeOH	TLC/DESI-MS	12 ± 0.91 (mg ± SD), N = 4	/	/	< 0.24 mg	/	/	/
Edwards and Draper (*128*)	R9	Solaray, Man. By Nutraceutical Corporation (Park City, UT)	Powdered root	Lot 051201	MeOH	HPLC[c]	3.36%[d]	2.93%[d]	/	/	6.29%	0.9	/
	R10	Solgar (Leonia, NJ)	Powdered root	Lot 3875	MeOH	HPLC[c]	3.48%[d]	2.92%[d]	/	/	6.41%	0.8	/
	R11	Nature's Answer (Hauppauge, NY)	Powdered root	Lot 0011YZ	MeOH	HPLC[c]	2.78%[d]	2.64%[d]	/	/	5.42%	0.9	/
	R12	NOW (Bloomingdale, IL)	Raw root powder	Lot 46639	MeOH	HPLC[c]	3.39%[d]	2.47%[d]	/	/	5.86%	0.7	/
	R13	Nature's Resource (Mission Hills, CA)	Powdered root	Lot IG10915	MeOH	HPLC[c]	3.51%[d]	2.45%[d]	/	/	5.96%	0.7	/
	R14	GNC Herbal Plus (Pittsburgh, PW)	Powdered root	Lot 77839	MeOH	HPLC[c]	3.01%[d]	2.45%[d]	/	/	5.46%	0.8	/

Table 2.4 continued

R15	Thompson, Man. by Nutraceutical Corporation (Melville, NY)	Powdered root	Lot 042010	MeOH	HPLC[c]	3.45%[d]	2.31%[d]	/	/	5.76%	0.7	/
R16	Veg Life, Man. by Nutraceutical Corporation (Park City, UT)	Compressed root caplet	Lot 041108	MeOH	HPLC[c]	3.36%[d]	2.18%[d]	/	/	5.54%	0.6	/
R17	Nature's Herbs (American Fork, UT)	Herb	Lot 804241A	MeOH	HPLC[c]	2.91%[d]	2.27%[d]	/	/	5.18%	0.8	/
R18	Nature's way (Springville, UT)	Herb	Lot 914866	MeOH	HPLC[c]	2.96%[d]	2.00%[d]	/	/	4.96%	0.7	/
R19	Hsu's Root to Health (Wausau, WI)	Powdered root	Lot 703HGS	MeOH	HPLC[c]	1.84%[d]	1.57%[d]	/	/	3.41%	0.9	/
R20	Good 'N Natural (Holbrook, NY)	Powdered root	Lot 49951-01C	MeOH	HPLC[c]	1.51%[d]	1.10%[d]	/	/	2.61%	0.7	/
R21	Herbal Select (Guelph, ON)	Powdered root	Lot 010868	MeOH	HPLC[c]	1.68%[d]	0.53%[d]	/	/	2.21%	0.3	/
R22	Mason Natural (Miami Lakes, FL)	Powdered root	Lot 2637	MeOH	HPLC[c]	2.11%[d]	0.29%[d]	/	/	2.40%	0.1	/
R23	Life Time (Anaheim, CA)	Powdered root	Lot 991212	MeOH	HPLC[c]	5.86%[d]	0.00%[d]	/	/	5.86%	N/A[g]	/

Table 2.4 continued

	R24	Country Life (Hauppauge, NY)	Powdered root	Lot N9C043A	MeOH	HPLC[c]	2.01%[d]	0.00%[d]	/	/	2.01%	N/A	/
	R25	Pacific Trends (Chatsworth, CA)	Raw powdered root	Lot 4028	MeOH	HPLC[c]	0.82%[d]	0.00%[d]	/	/	0.82%	N/A	/
Govindan and Govindan (*146*)	R26	American Herbal Pharmacopoeia	Bulk powder or coarsely chopped	GS-2	70% EtOH	HPLC	4.27%[f]	2.68%[f]	/	/	/	0.6	/
	R27	American Herbal Pharmacopoeia	Bulk powder or coarsely chopped	GS-3	70% EtOH	HPLC	0.56%[f]	0.00%[f]	/	/	/	N/A	/
	R28	American Herbal Pharmacopoeia	Bulk powder or coarsely chopped	GS-4	70% EtOH	HPLC	3.48%[f]	2.34%[f]	/	/	/	0.7	/
	R29	American Herbal Pharmacopoeia	Bulk powder or coarsely chopped	GS-5	70% EtOH	HPLC	4.54%[f]	3.22%[f]	/	/	/	0.7	/
	R30	American Herbal Pharmacopoeia	Bulk powder or coarsely chopped	GS-6	70% EtOH	HPLC	0.56%[f]	0.60%[f]	/	/	/	1.1	/
	R31	American Herbal Pharmacopoeia	Bulk powder or coarsely chopped	GS-7	70% EtOH	HPLC	0.28%[f]	0.00%[f]	/	/	/	N/A	/
	R32	ND[e], local commercial sample (Maine)	Fine powder capsule	GS-9	70% EtOH	HPLC	1.51%[f]	0.00%[f]	/	/	/	N/A	/

Table 2.4 continued

	R33	ND[e], local commercial sample (Maine)	Fine powder capsule	GS-10	70% EtOH	HPLC	5.31%[f]	0.00%[f]	/	/	/	N/A	/
Gurley et al. (*147*)	R34	Nature's Resource Products (Mission Hills, CA)	Hard gelatin capsule	Lot OI10184	50% MeOH	HPLC[c]	12.8 mg/capsule, n = 20 capsules	22.0 mg/capsule, n = 20 capsules	/	/	34.8 mg/capsule, n = 20 capsules	1.7	/
Kim et al. (*148*)	R35	ND	Plant powder	ND	MeOH	HPLC	5.54% ± 0.31% (mean w/w ± SD)[f]	/	/	/	/	/	/
McNamara et al. (*127*)	R36	[9]Whole plants grown at Ruakura, New Zealand	Root	/	MeOH	HPLC	3.78% w/w[d]	1.90% w/w[d]	0.26% w/w[d]	0.080% w/w[d]	/	0.5	0.1
	R37		Rhizome	/	MeOH	HPLC	4.62% w/w[d]	2.77% w/w[d]	0.20%w/w[d]	0.00% w/w[d]	/	0.6	0.04
	R38		Lower stem	/	MeOH	HPLC	1.83% w/w[d]	0.43% w/w[d]	0.26% w/w[d]	0.018% w/w[d]	/	0.2	0.1
	R39		Upper stem	/	MeOH	HPLC	1.25% w/w[d]	0.31% w/w[d]	0.07% w/w[d]	0.010% w/w[d]	/	0.2	0.1
	R40		Leaf	/	MeOH	HPLC	1.50% w/w[d]	1.01% w/w[d]	0.43% w/w[d]	0.030% w/w[d]	/	0.7	0.3
Weber et al. (*149*)	R41	ND, bulk commercial supplier	Commercial root powder	Supplier A	ACN-H_2O-H_3PO_4 (70+30+0.1, v/v/v)	LC/MS	3.35 ± 0.01 (% w/w ± SD)[d]	2.47 ± 0.01 (% w/w ± SD)[d]	0.07 ± 0.00 (% w/w ± SD)[d]	/	/	0.7	0.02

Table 2.4 continued

	R42	ND, bulk commercial supplier	Commercial root powder	Supplier C	$ACN/H_2O/H_3PO_4$ (70:30:0.1, v/v/v)	LC/MS	3.97 ± 0.01 (% w/w ± SD)[d]	2.74 ± 0.01 (% w/w ± SD)[d]	0.19 ± 0.01 (% w/w ± SD)[d]	/	/	0.7	0.05
Weber et al. (*122*)	R43	ND, bulk commercial supplier	Root powder	Supplier B	$ACN/H_2O/H_3PO_4$ (70:30:0.1, v/v/v)	HPLC	3.35 ± 0.01 (% w/w ± CV)[d]	2.47 ± 0.01 (% w/w ± CV)[d]	0.07 ± 0.00 (% w/w ± CV)[d]	/	/	0.7	0.02
	R44	ND, bulk commercial supplier	Root powder	Supplier C	$ACN/H_2O/H_3PO_4$ (70:30:0.1, v/v/v)	HPLC	3.97 ± 0.01 (% w/w ± CV)[d]	2.74 ± 0.01 (% w/w ± CV)[d]	0.19 ± 0.01 (% w/w ± CV)[d]	/	/	0.7	0.05
Mean alkaloid concentration (% w/w) in root samples only ± SEM[h]							3.3 ± 0.26, N = 28	2.0 ± 0.23, N = 27	0.5 ± 0.31, N = 10	0.1 ± 0.03, N = 3	/	0.8 ± 0.06, N = 35	0.1 ± 0.01, N = 35
Range of alkaloid concentration (% w/w) in root samples only[h]							0.8 – 6.9	0.0 – 5.1	0.1 – 3.3	0.0 – 0.1	/	/	/

[a] Alkaloids: Berb = berberine, Hydra = hydrastine, Can = canadine, Hdst = Hydrastinine, Tot = Total; [b] Units not indicated; [c] HPLC method described by Abourashed and Khan (*144*); [d] Mean of three replicate analyses; [e] Product name or manufacturer was not disclosed (ND); [f] Number of replicate analyses not indicated; [g] Plants were grown from 1996-2001, sourced from North America in 1990, Voucher 820 (Invermay, AgResearch, Dunedin, New Zealand) [h] Samples R1-R6, R8-R16, R19-R25, R36, R37 and R41-R44 only

Table 2.5 Summary of quantitative results from HPLC-DAD analysis of all wild and cultivated samples, including those cited in the literature

Parameter	Alkaloid(s)	Material and tissue					
		Cultivated root-rhizome, N = varies	[a]Cultivated root-rhizome (literature), N = varies	Wild root-rhizome, N = 107	Wild stem-leaf, N = 109	[b]Cultivated stem-leaf (literature), N = varies	Wild berry pulp, N = 3
[c]Distrubution frequency (% w/dw)	Berberine	1.0 – 5.9% (Δ 4.9%), N = 24; 33%: 1.0-1.9%, 29%: 4.0-4.9%	0.6 – 6.9% (Δ 6.1%), N = 28; 46%: 3.0 – 3.9%	1.0 – 7.9% (Δ 5.9%); 48%: 3.0-3.9%	0.6 – 3.9% (Δ 3.3%); 59%: 1.0-1.9%	/	0.0 – 0.3% (Δ 0.3%)
	Hydrastine	1.0 – 2.9% (Δ 1.9%), N = 24; 63%: 2.0-2.9%	0.0 – 5.9% (Δ 5.1%), N = 27; 59%: 2.0 – 2.9%	0.6 – 4.9% (Δ 4.3%); 51%: 1.0-1.9%, 47%: 2.0-2.9	0.0 – 1.9% (Δ1.9%); 42%: 0.6 – 0.9%	/	0.0%
	Canadine	0.6 – 2.9% (Δ 2.3%), N = 18; 44%: 1.0 – 1.9%, 39%; 2.0-2.9%	0.01 – 3.9% (Δ 3.2%), N = 10; 90%: 0.01 – 0.5%	0.1 – 2.9% (Δ 2.8%); 56%: 0.1 – 0.5%	0.0 – 0.5% (Δ 0.5%); 15%: 0.1 – 0.5%	/	0.0%

Table 2.5 continued

	Total	/	/	2.0 – 13.9% (Δ 9.9%); 27%: 5.0 – 5.9%; 25%: 6.0 – 6.9%	0.6 – 5.9% (Δ 5.3%); 58%: 2.0 – 2.9%	/	0.0 – 0.3% (Δ 0.3%)
Mean distribution (% w/dw ± SEM)	[d]Berberine	3.5% ± 0.09%, N = 159	3.5% ± 0.09%, N = 159	3.5% ± 0.09%, N = 159	2.0% ± 0.04%	3.5% ± 0.55%, N = 3	0.1 ± 0.10%
	[d]Hydrastine	2.0% ± 0.06%, N = 158	2.0% ± 0.06%, N = 158	2.0% ± 0.06%, N = 158	0.7% ± 0.03%	2.0% ± 0.15%, N = 3	0.0%
	Canadine	1.6% ± 0.12%, N = 18	0.5% ± 0.3%, N = 10	0.6% ± 0.03%	0.0% ± 0.00%	0.8%, N = 1	0.0%
	Total	/	/	6.2% ± 0.20%	2.8% ± 0.08%	/	0.1 ± 0.10%
Mean composition (% w/dw / % w/dw ± SEM)	Hydrastine/ Berberine	0.6 ± 0.04, N = 6	0.8 ± 0.06, N = 35	0.6 ± 0.01	0.4 ± 0.02	0.6 ± 0.12, N = 3	/
	Canadine/ Berberine	0.6 ± 0.18, N = 6	0.1 ± 0.01, N = 35	0.2 ± 0.01	0.02 ± 0.009	0.2, N = 1	/

Table 2.5 continued

Canadine/ Hydrastine	1.0 ± 0.24, N = 6	0.1 ± 0.03, N = 35	0.3 ± 0.01	0.04 ± 0.009	0.4, N = 1	/

[a] See Table 2.4, R1-R6, R8-R16, R19-R25, R36, R37 and R41-R44; [b] See Table 2.4, R17-R18 and R38-R40; [c] Frequency distribution data from Figures 2.9, 2.10 and 2.11, where X% - Y% = total range, Δ = range width, N = total sample size, Z%: V% - W% = range (V - W) of ≈50% (Z) of total samples; [d] No significant difference ($p > 0.05$) between cultivated (including literature data) and wild root-rhizome, Kruskal-Wallis analysis followed by Scheffe's post hoc multiple comparison analysis

336.1. In addition to the alkaloids, minute impure peaks of chlorogenic acid were also identified in the extracts between 2.2 and 4.1 minutes (Figure 2.8). McNamara et al. (*127*) have previously reported the presence of chlorogenic acid by HPLC analysis of cultivated *H. canadensis* root, rhizome, lower stem, upper stem and leaf tissue samples.

Since the chromatographic profile is the same for all samples and is unique when compared to chemically related species, chemical profiling can be considered an effective method for identifying *H. canadensis* material. However, for the same reasons, it cannot be considered a viable method for distinguishing among different *H. canadensis* tissues and material types. On the other hand, there are observable quantitative differences in alkaloid concentration (measured by frequency distribution, tissue distribution and composition parameters) that make for reliable markers of the different sample types, in particular for hydrastine and canadine concentration between below-ground and above-ground tissues.

Figure 2.9 shows the frequency distribution of total alkaloid concentration (sum of berberine, hydrastine and canadine concentration) among 107 wild root-rhizomes and 109 wild stem-leaves. A broader range in total alkaloid concentration was observed in root-rhizomes (Δ 9.9% w/dw) compared to stem-leaves (Δ 5.3% w/dw). Root-rhizomes had no less than 2.0% total alkaloids whereas stem-leaf tissues had a maximum of 5.9% w/dw. Total alkaloid concentration was not measured for cultivated material since most of the alkaloids were quantified separately per analysis rather than all together.

The frequency distribution of berberine, hydrastine and canadine concentrations among 107 wild root-rhizomes, 19-24 samples of cultivated root-rhizome and 109 wild stem-leaves is displayed in Figure 2.10. Berberine is the most concentrated alkaloid in *H. canadensis* and canadine the least, particularly in wild stem-leaf samples where it is virtually non-existent. In general, alkaloid concentration was approximately equal between wild and cultivated root-rhizome, and stem-leaf material contained less alkaloid than below-ground samples. Furthermore, the frequency distribution

Figure 2.9 Frequency distribution of total alkaloid concentration among 107 wild Canadian *Hydrastis canadensis* L. (goldenseal) root-rhizomes and 109 wild stem-leaves as measured by reverse phase HPLC-DAD.

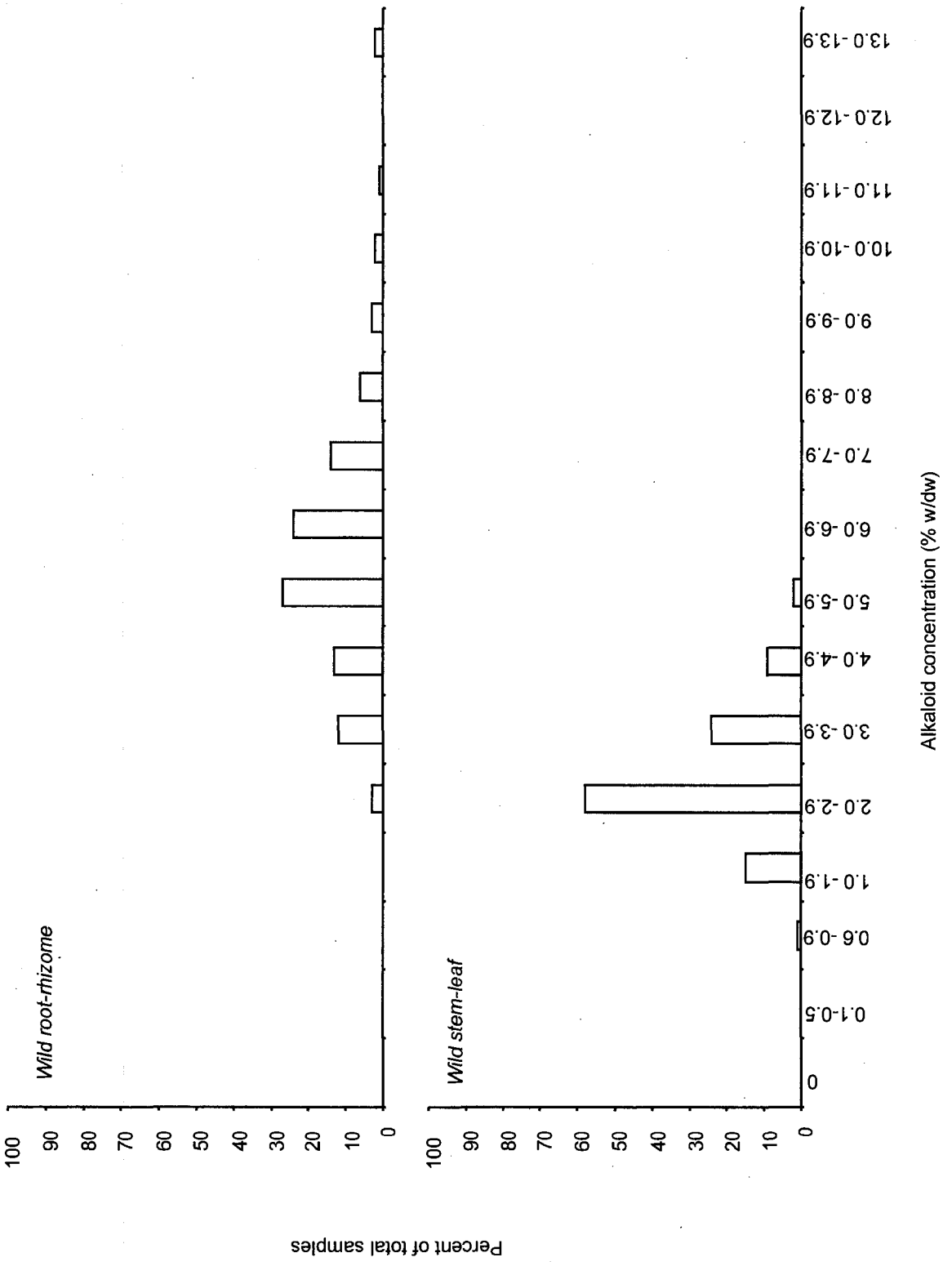
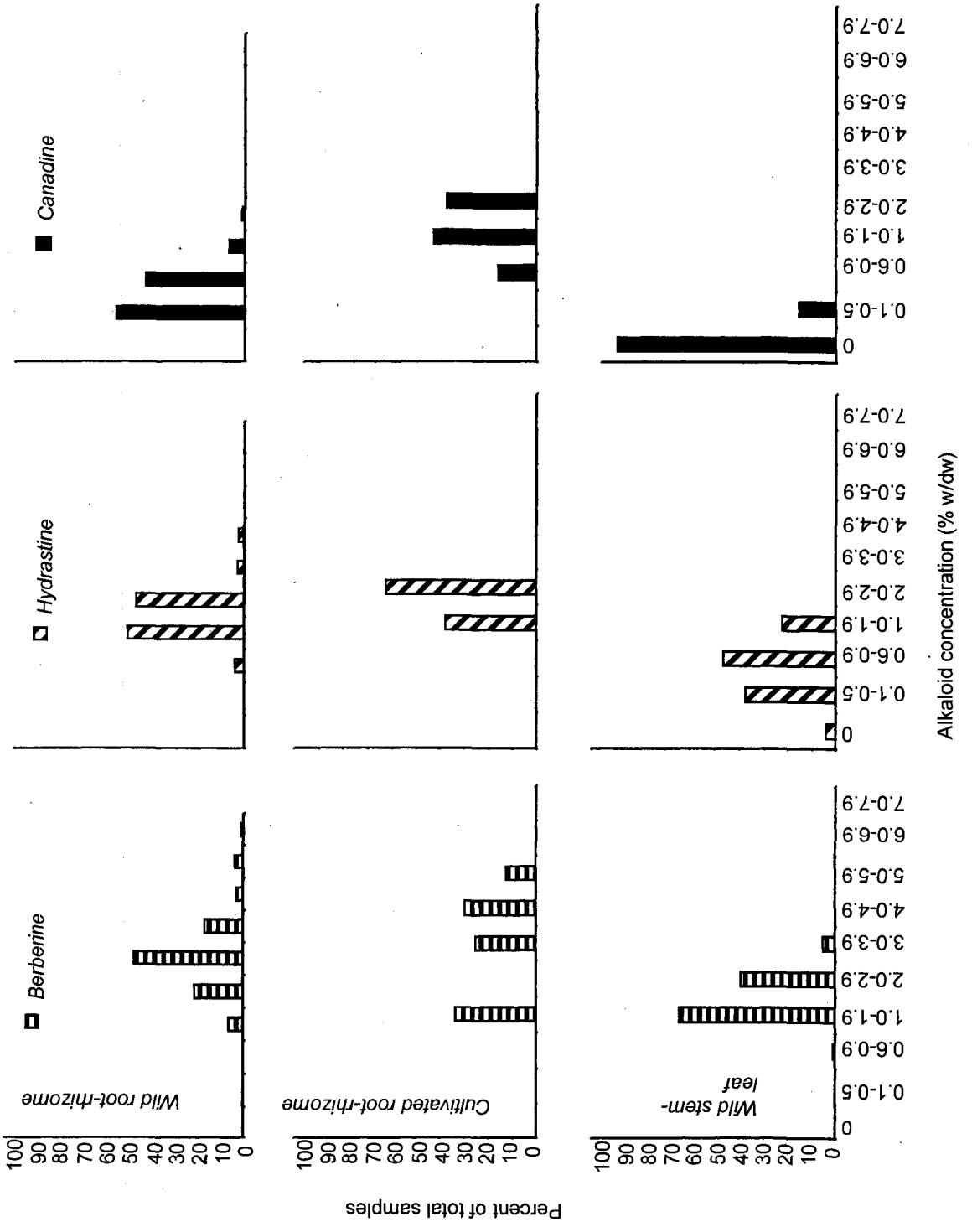

Wild root-rhizome
Wild stem-leaf
Percent of total samples
100
90
80
70
60
50
40
30
20
10
0
0
0.1-0.5
0.6-0.9
1.0-1.9
2.0-2.9
3.0-3.9
4.0-4.9
5.0-5.9
6.0-6.9
7.0-7.9
8.0-8.9
9.0-9.9
10.0-10.9
11.0-11.9
12.0-12.9
13.0-13.9
Alkaloid concentration (% w/dw)

Figure 2.10 Frequency distribution of berberine, hydrastine, and canadine concentration (% w/dw) among 107 wild Canadian *Hydrastis canadensis* L. (goldenseal) root-rhizomes, 18-24 samples of cultivated root-rhizome and 109 wild stem-leaves as measured by reverse phase HPLC-DAD.


Canadine
Hydrastine
Berberine
Wild root-rhizome
Cultivated root-rhizome
Wild stem-leaf
Percent of total samples
Alkaloid concentration (% w/dw)
0
10
20
30
40
50
60
70
80
90
100
0-0.1
0.1-0.5
0.6-0.9
1.0-1.9
2.0-2.9
3.0-3.9
4.0-4.9
5.0-5.9
6.0-6.9
7.0-7.9

was different between tissues (root-rhizome and stem-leaf) and to a lesser extent between material type (wild and cultivated).

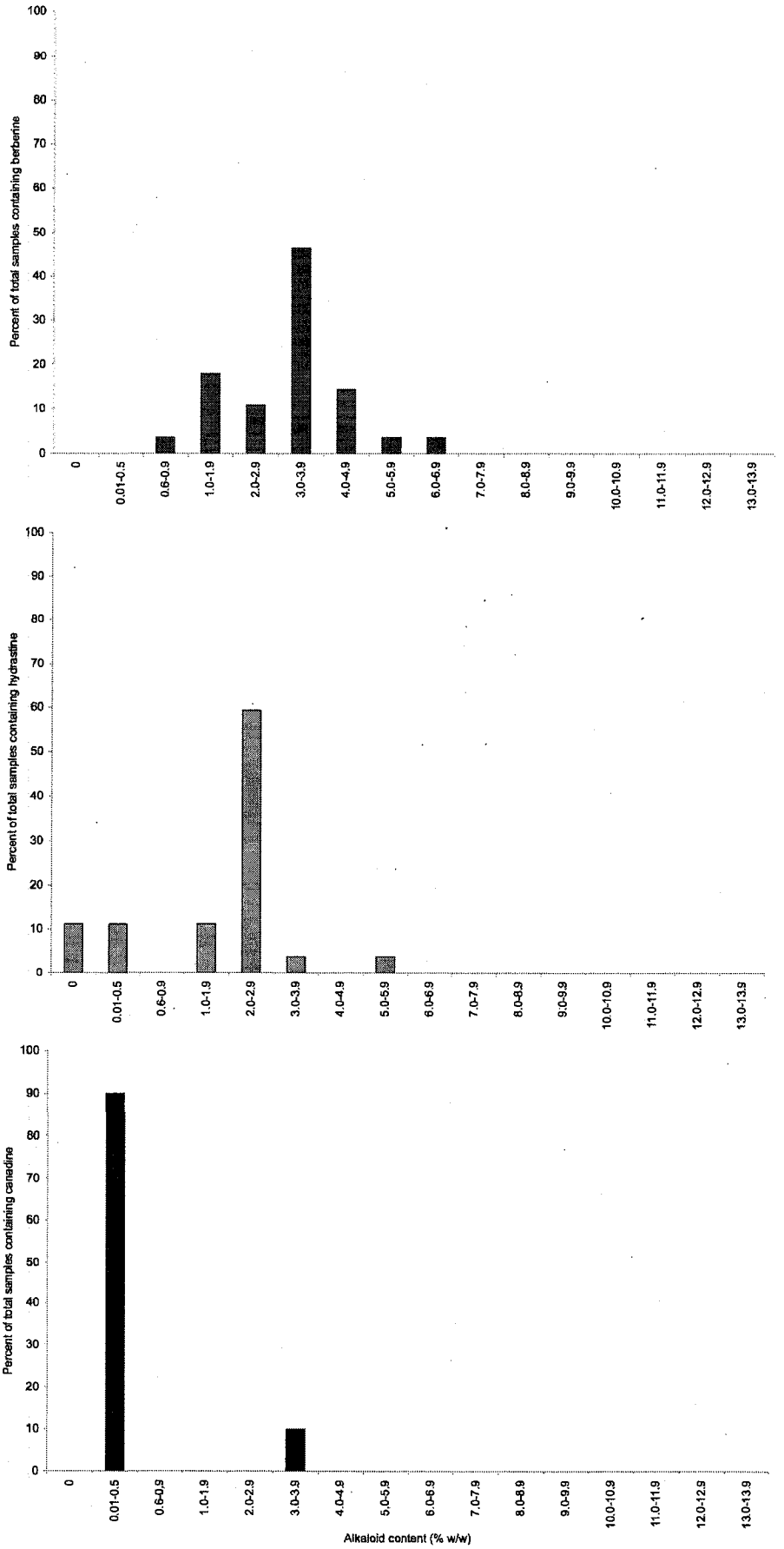
The broadest range in berberine concentration was observed in wild root-rhizome (Δ 5.9 % w/dw) followed by cultivated root-rhizome (Δ 4.9% w/dw) and wild stem-leaf (Δ 3.3% w/dw) where wild and cultivated root had no less than 1.0% w/dw berberine and wild stem had no less than 0.6% w/dw. Wild root contained no more than 7.9% w/dw berberine, cultivated root contained no more than 5.9% w/dw and wild stem-leaf had no more than 3.9% w/dw. Furthermore, since the distribution of cultivated samples is more uniform, this suggested a greater variability in berberine concentration among this sample type.

A broad range in hydrastine concentration was measured among wild root-rhizome (Δ 4.3% w/dw), while a narrow range was measured in both cultivated root-rhizome and wild stem-leaf (Δ 1.9% w/dw, for both). Cultivated root contained no less than 1.0% hydrastine while wild root contained no less than 0.6% w/dw. Hydrastine was not detected in 4% of the wild stem-leaf samples.

The range in canadine concentration was approximately equal for wild and cultivated root-rhizome (Δ 2.8% and 2.3% w/dw, respectively) and this range was approximately five to six times greater than the range measured in wild stem-leaf samples (Δ 0.5% w/dw). Cultivated root contained no less than 0.6% w/dw, wild root contained no less than 0.1% w/dw and 85% of the stem-leaf samples did not contain canadine.

In comparing the frequency distribution of the three alkaloids reported in 28 commercial root-rhizome products in the literature (Figure 2.11) with the frequency distributions of root-rhizome (wild and cultivated) in Figure 2.10 (see also Table 2.5), a broader range in all three alkaloids and a smaller minimum amount of berberine was reported in the literature. A maximum of 6.9% w/w berberine was reported in the literature, which was greater than what was measured in the cultivated samples and less than the amount measured in wild samples. Unlike the wild and cultivated samples, 11% of the samples reported in the literature did not contain hydrastine; and they also contained a

Figure 2.11 Frequency distribution of berberine, hydrastine and canadine concentration (% w/w) among 10-28 commercially available cultivated *Hydrastis canadensis* L. (goldenseal) root-rhizome samples described in the literature (Table 2.4).


Percent of total samples containing berberine
Percent of total samples containing hydrastine
Percent of total samples containing canadine
0
10
20
30
40
50
60
70
80
90
100
0
0.01-0.5
0.6-0.9
1.0-1.9
2.0-2.9
3.0-3.9
4.0-4.9
5.0-5.9
6.0-6.9
7.0-7.9
8.0-8.9
9.0-9.9
10.0-10.9
11.0-11.9
12.0-12.9
13.0-13.9
Alkaloid content (% w/w)

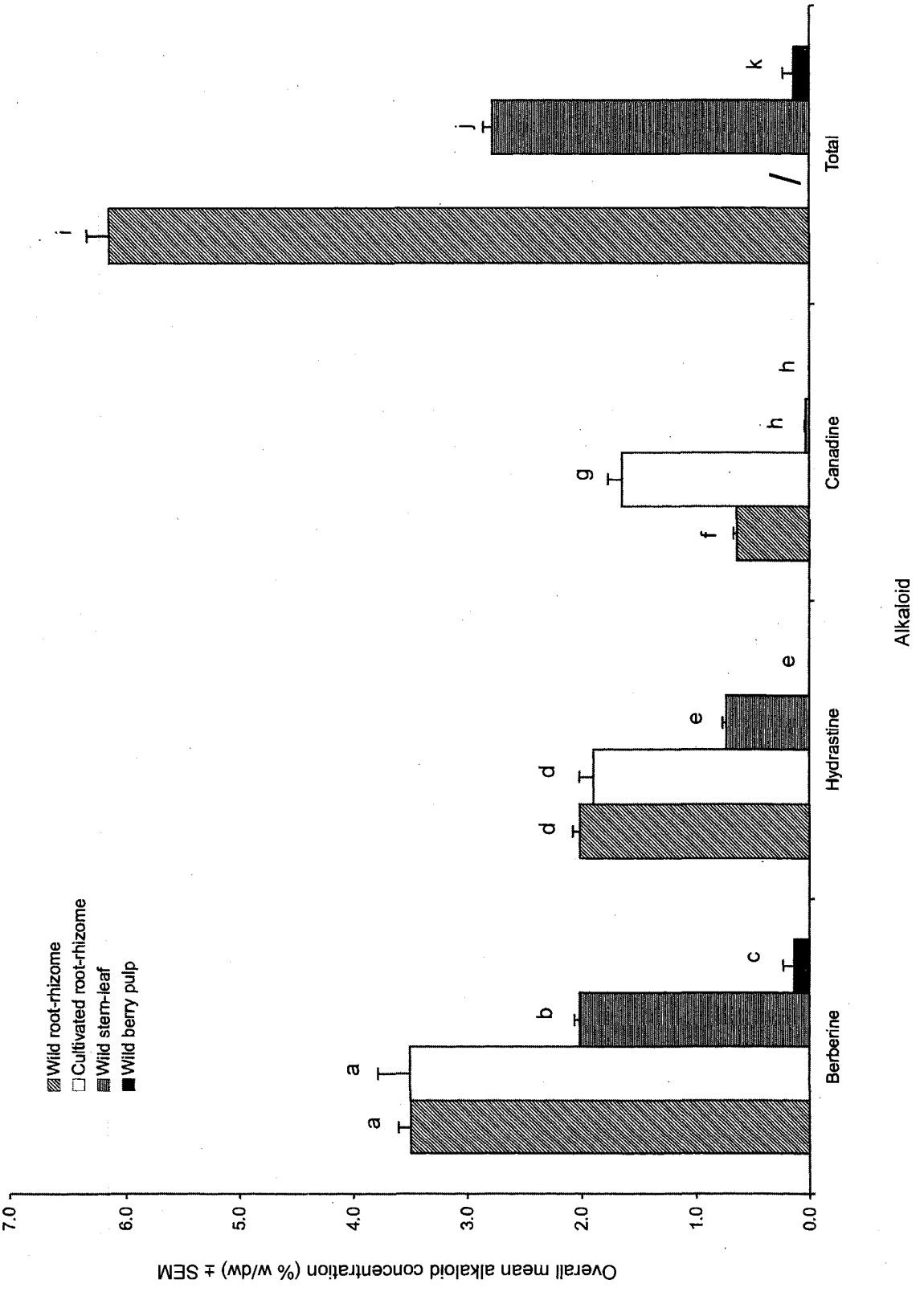
greater maximum hydrastine concentration (5.9%w/w). A greater range in canadine concentration was also reported in the literature.

The following highlights the observations described in Figures 2.9 and 2.10 that help distinguish the different sample types: root-rhizome (wild and cultivated) contained no less than 1.0% w/dw berberine while cultivated root-rhizome contained no more than 6.9% and stem-leaf material contained no more than 3.9% w/dw. Cultivated root-rhizome contained between 1.0% and 2.9% w/dw hydrastine while stem-leaf material contained no more than 1.9% w/dw and was the only sample type that occasionally did not contain any hydrastine. Cultivated root-rhizome contained no less than 0.6% w/dw canadine while wild stem-leaf contained no more than 0.5% and over 90% of the stem-leaf samples did not contain canadine compared to root-rhizome material. Given the relatively uniform frequency distributions for cultivated root-rhizome, it is important to note that the distributions may extend wider with a larger sample size. Wild root-rhizomes contained no less than 2.0% w/dw total alkaloids and stem-leaves contained no more than 5.9% w/dw. These data are important benchmarks for product identification, and commercial products outside these limits may be an indication of adulteration.

Figure 2.12 shows the distribution of berberine, hydrastine, canadine and their sum total between 107 wild root-rhizomes, 18 to 24 samples of cultivated root-rhizomes, 109 wild stem-leaves and three wild berry samples. The alkaloids produced by *H. canadensis* were most concentrated in root-rhizome material and least concentrated in berries. Furthermore the results also confirmed that berberine is the most concentrated alkaloid and canadine the least.

For berberine, equal amounts were measured in wild and cultivated root-rhizomes (3.5% ± 0.10% w/dw, N = 131), less than 3/5 of that amount was measured in wild stem-leaves (2.0% ± 0.04% w/dw, N = 107) and less than 1/25 was measured in wild berries (0.1% ± 0.10% w/dw, N = 3). For hydrastine, equal amounts were found in wild and cultivated root-rhizome (2.0% ± 0.05%

Figure 2.12 Distribution of overall mean berberine, hydrastine, canadine and total alkaloid concentration (% w/dw ± SEM) in wild Canadian *Hydrastis canadensis* L. (goldenseal) root-rhizomes (N = 107), in cultivated root-rhizome samples (N= 18-24), in wild stem-leaves (N = 109) and wild-harvested berry pulp samples (N = 3) as measured by reverse-phase HPLC-DAD. Note that overall mean total alkaloid concentration was not measured for the cultivated material samples. Significant differences in alkaloid concentration among tissues ($p < 0.05$) were determined by Kruskal-Wallis analysis followed by Scheffe's post hoc multiple comparison analysis and are designated by the following distinct letters: (a, b, c) for berberine, (d, e) for hydrastine, (f, g, h) for canadine and (i, j, k) for total alkaloid.


Overall mean alkaloid concentration (% w/dw) ± SEM
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Wild root-rhizome
Cultivated root-rhizome
Wild stem-leaf
Wild berry pulp
a
a
b
c
d
d
e
e
f
g
h
h
i
j
k
Berberine
Hydrastine
Canadine
Total
Alkaloid

w/dw, N = 131), less than 9/25 of that amount was measured in wild stem-leaves and berries (0.7% ± 0.03% w/dw, N = 112). For canadine, the greatest amount was measured in cultivated root-rhizome (1.6% ± 0.11% w/dw, N = 18), less than 2/5 of that amount was measured in wild root-rhizomes (0.6% ± 0.03% w/dw, N = 107) and only 1/50 was measured in wild stem-leaves and berries (3.0 x 10^{-2}% ± 0.01% w/dw, N = 112). Hydrastine and canadine were not detected in berries. Total alkaloid concentration was compared among wild tissues only. The greatest amount of total alkaloids was measured in wild root-rhizomes (6.2% ± 0.20% w/dw, N = 107), 9/20 of that amount was measured in stem-leaves (2.8% ± 0.08% w/dw, N = 109) and only 1/50 in berries (0.1% ± 0.10% w/dw, N = 3).

Alkaloid concentration in commercial *H. canadensis* samples reported in the literature is summarized in Table 2.4 and compared to other material and tissue types in Table 2.5. No significant differences ($p > 0.05$) were found in mean berberine concentration (3.5% ± 0.09 % w/w, N = 159) and in mean hydrastine concentration (2.0 ± 0.06% w/w, N = 158) between root-rhizome samples of this study (Figure 2.12) and the root-rhizome data in Table 2.4. However, there was a significant difference ($p < 0.05$) in canadine concentration where canadine was nearly 1.5x more concentrated in the cultivated root-rhizome samples analyzed in this study (1.6% ± 0.12% w/dw, N = 18) compared to both the wild samples of this study (0.6% ± 0.03% w/dw, N = 107) and the commercial samples described in the literature (0.5% ± 0.3% w/w, N = 10). Possible explanations for this difference in concentration are errors in experimental procedure such as processing (e.g. inefficient milling and/or choice of solvent for extraction) that may have compromised the extractability of canadine from the material or cultivation practices resulting in higher yields. In either case, it is advised that the cultivated samples be re-analyzed for canadine.

Two previous studies analyzed *H. canadensis* above-ground tissues, but both analyzed cultivated material exclusively. Wild stem-leaf samples contained up to half the amount of berberine and a third the amount of hydrastine compared to the samples described in the literature (R17, R18, R38-40 in Table 2.4; and Table 2.5). Combining alkaloid concentration for R38, R39 and R40 (lower

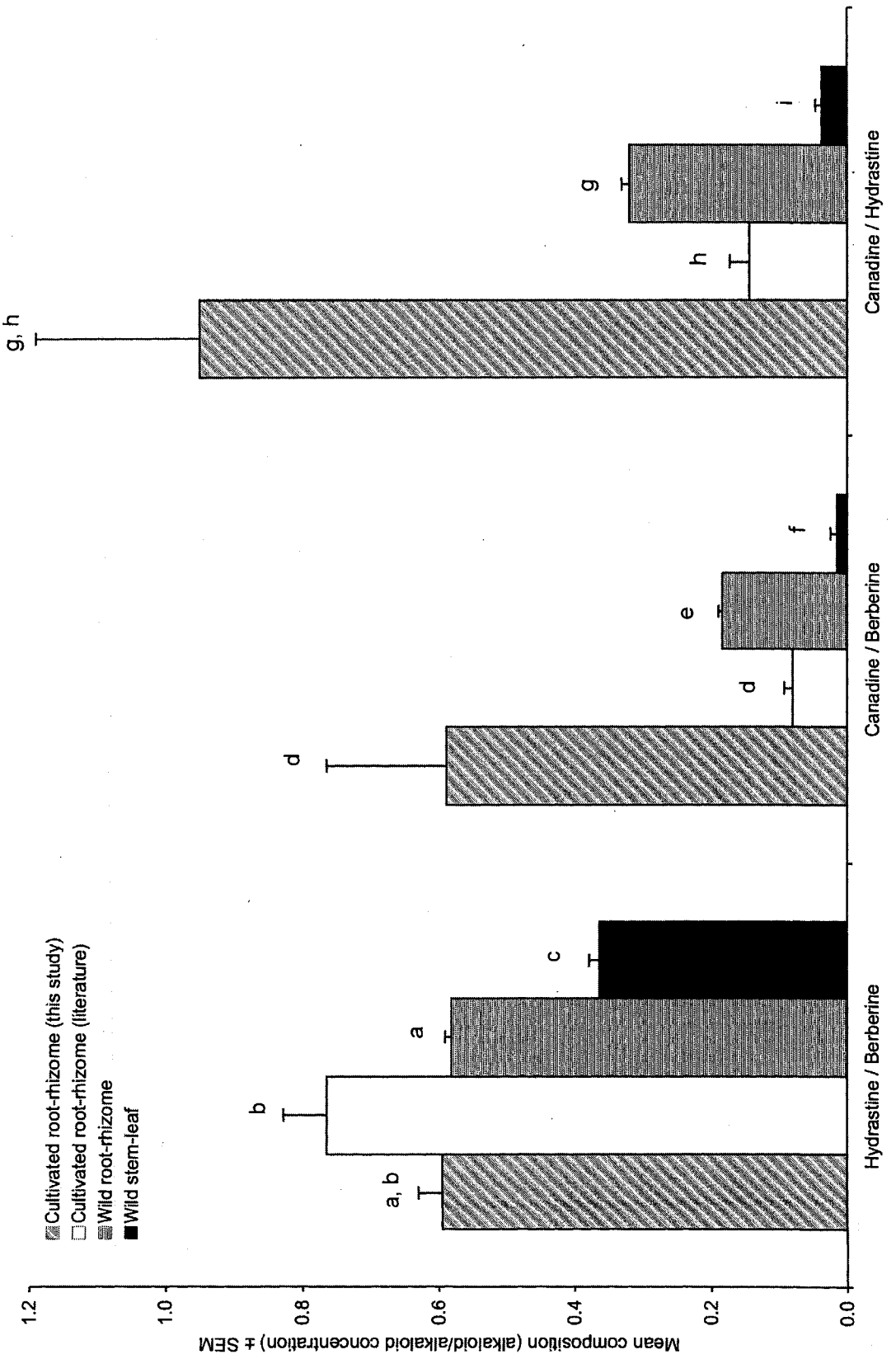
stem, upper stem and leaf samples, respectively in Table 2.4) analyzed by McNamara et al. (*127*) gave 4.6% w/w berberine and 1.8% w/w hydrastine. Edwards and Draper (*128*) reported 2.9% berberine and 2.3% hydrastine in R17 and 3.0% berberine and 2.0% hydrastine in R18 (authors did not specify units). These concentrations are comparable to the alkaloid concentrations measured in wild and cultivated root-rhizome and may represent a sustainable source for these alkaloids, thus warranting further investigation.

Figure 2.13 illustrates the variation in alkaloid composition (ratios) among the different sample types. Overall mean hydrastine concentration relative to berberine concentration (hydrastine / berberine), canadine relative to berberine (canadine / berberine) and canadine relative to hydrastine (canadine / hydrastine) were calculated for all analyzed root-rhizome samples (cultivated and wild, separately) and wild stem-leaf samples as well as root-rhizome samples from published results (Table 2.4). In general, alkaloid composition was significantly different among all sample types, except for cultivated root-rhizome and root-rhizome samples described in the literature (see also Table 2.5).

For hydrastine / berberine and canadine / hydrastine composition, cultivated root-rhizome from this study and those from the literature had the greatest ratios, followed by wild root-rhizome and wild stem-leaf. For canadine / berberine composition, cultivated root-rhizome from this study had the greatest ratio, followed by wild root-rhizome, the root-rhizome described in the literature and wild stem-leaf.

Given the significant difference in canadine concentration between the cultivated root-rhizome samples and those in the literature, the statistical analyses of the results in Figure 2.13 were repeated without including the data for cultivated root-rhizome samples of this study. For all three ratios, individually, the three sample types (cultivated root-rhizome samples from published results, wild root-rhizome and wild stem-leaf sample results) were found to be significantly different ($p < 0.05$, data not shown) from each other, thus suggesting that alkaloid composition may be a quantitative feature that could distinguish wild-harvested root-rhizome from cultivated material.

Figure 2.13 Mean alkaloid composition (concentration ratios) ± SEM in different *Hydrastis canadensis* L. (goldenseal) material types. Samples included cultivated root-rhizome analyzed in this study (N = 18-24), cultivated root-rhizome reported in the literature (N = 35, Table 2.4), and wild root-rhizomes (N = 107) and stem-leaves (N = 109) analyzed in this study by reverse-phase HPLC-DAD. Significant differences in alkaloid ratios among tissue types ($p < 0.05$) were determined by Kruskal-Wallis analysis followed by Scheffe's post hoc multiple comparison analysis and are designated by the following distinct letters: (a, b, c) for hydrastine / berberine, (d, e, f) for canadine / berberine and (g, h, i) for canadine / hydrastine.


Mean composition (alkaloid/alkaloid concentration) ± SEM
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Cultivated root-rhizome (this study)
Cultivated root-rhizome (literature)
Wild root-rhizome
Wild stem-leaf
a, b
b
a
c
d
d
e
f
g, h
h
g
i
Hydrastine / Berberine
Canadine / Berberine
Canadine / Hydrastine

Alkaloid composition analysis is also an additional method for distinguishing between above-ground and below-ground tissues.

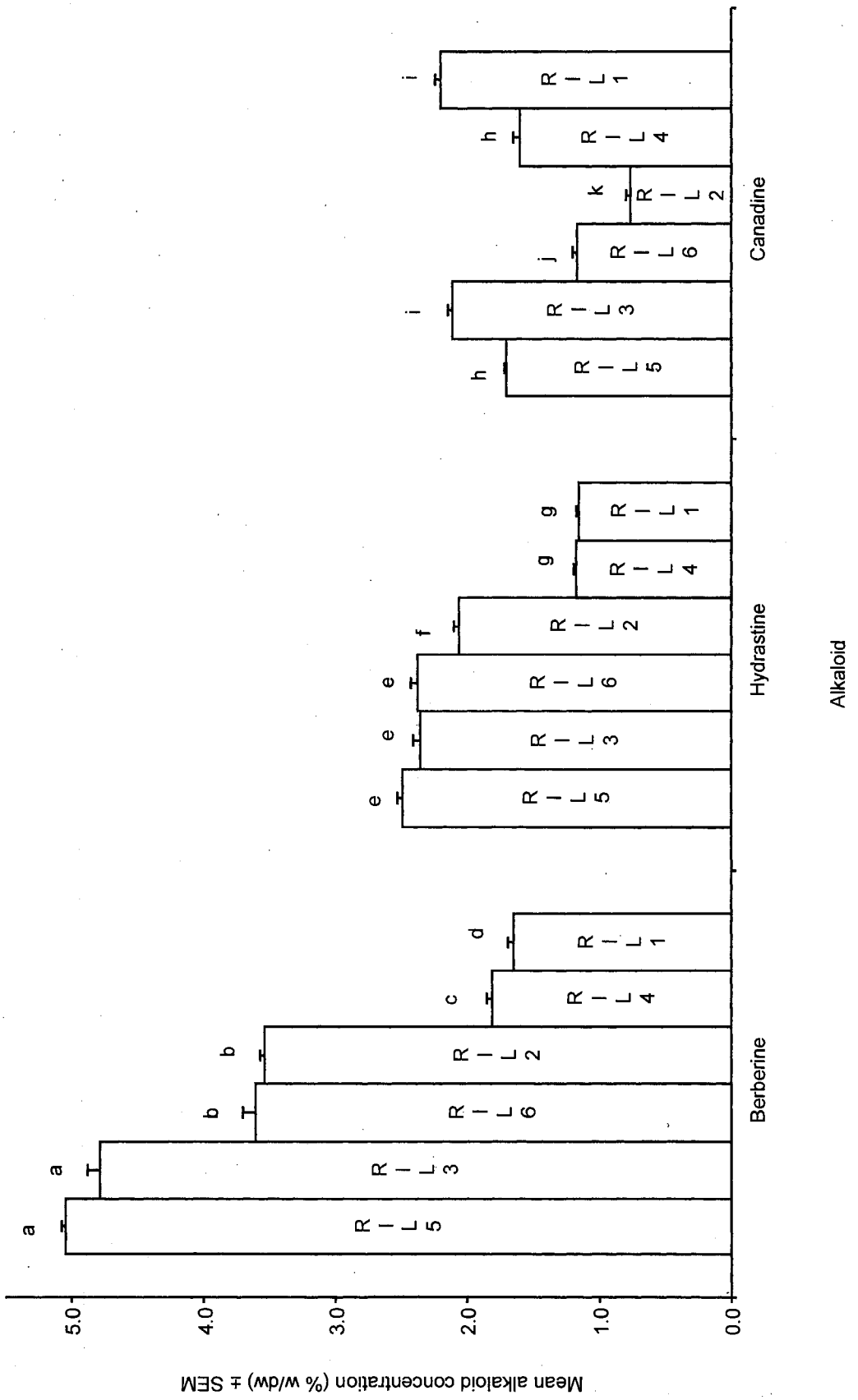
This was the first study to analyze alkaloid concentration in berries and wild Canadian root-rhizomes and stem-leaves. The following overall conclusions were made from all of the results above: 1) the chemical profile for *H. canadensis* was consistent throughout all of the samples and served as a reliable fingerprint of the species for product identification purposes; 2) all three alkaloids were most concentrated in below-ground tissues; 3) differences in frequency distribution and alkaloid composition were mostly observed between above-ground and below-ground samples; 4) alkaloid concentration can serve to distinguish between above-ground and below-ground tissues but not between wild and cultivated root-rhizome samples; 5) with the exception of canadine, wild and cultivated root-rhizome samples contained equal amounts of alkaloids, thus the use of wild plant material in addition to or as an alternative to cultivated material in NHPs may not improve the pharmacological activities of the product.

2.3.4 Phytochemical variation among accessions of cultivated material: Significant differences in berberine, hydrastine and canadine concentrations were detected among six accessions of cultivated root-rhizome (Figure 2.14).

Berberine was the most concentrated alkaloid in the samples, followed by hydrastine and canadine. Berberine was greatest in samples from RIL3 and RIL5 (4.9% ± 0.07% w/dw, N = 10), followed by the samples from RIL2 and RIL6 (3.6% ± 0.03% w/dw, N = 6). The least amount of berberine was measured in the samples from RIL4 (1.8% ± 0.04% w/dw, N = 5) and RIL1 (1.7% ± 0.04%, N = 3). The overall mean berberine concentration was 3.3% ± 0.27% (N = 24).

Hydrastine was most concentrated in RIL3, RIL5 and RIL6 inclusively (2.4% ± 0.03% w/dw, N = 11), followed by RIL2 (2.1% ± 0.03% w/dw, N = 3), and RIL1 and RIL4 inclusively (1.2% ± 0.02% w/dw, N = 9). The overall mean hydrastine concentration was 1.9% ± 0.12% (N = 24).

Figure 2.14 Mean berberine, hydrastine and canadine concentration (± SEM) in six accessions of cultivated *Hydrastis canadensis* L. (goldenseal) root-rhizome material (N = 19-24). Significant differences ($p < 0.05$) in alkaloid concentration among accessions, as determined by one-way analysis of variance followed by Scheffe's post hoc multiple comparison analysis, are designated by the following distinct letters: (a, b, c, d) for berberine, (e, f, g) for hydrastine and (h, i, j, k) for canadine. Leduc accession codes (RIL#) were randomly assigned to each accession to protect the identity of the farmers and distributors listed in Table 2.2.


Mean alkaloid concentration (% w/dw) ± SEM
0.0
1.0
2.0
3.0
4.0
5.0
Alkaloid
Berberine
Hydrastine
Canadine
R – L 5
R – L 3
R – L 6
R – L 2
R – L 4
R – L 1
R – L 5
R – L 3
R – L 6
R – L 2
R – L 4
R – L 1
R – L 5
R – L 3
R – L 2
R – L 4
R – L 1
a
a
b
b
c
d
e
e
e
f
g
g
h
i
j
k
h
i

Canadine was most concentrated in the samples from RIL1 and RIL3 inclusively (2.1% ± 0.03% w/dw, N = 7), followed by the samples from RIL4 and RIL5 (1.6% ± 0.03% w/dw, N = 6) and RIL6 (1.2% ± 0.03% w/dw, N = 3). The least amount of canadine was measured in the material received from RIL2 (0.8% ± 0.02% w/dw, N = 3). The overall mean canadine concentration was 1.6% ± 0.12% (N = 19).

With the exception of canadine, overall mean alkaloid concentration measured in this study is comparable to the literature. Overall canadine concentration measured in the samples was greater than the concentration in cultivated root-rhizome reported in the literature (Table 2.5 and Figure 2.12). Possible factors contributing to this difference were discussed in section 2.3.3.

The observed variation in alkaloid concentration among accessions may be explained by environmental factors, plant genotype and the interactions between the two (*129, 130*). Previous studies have shown that a variety of environmental factors like herbivory and disease (*131, 132*), climate and shade (*131, 133*), as well as moisture regime and nutrient availability (*131, 134, 135*) may affect the production of secondary metabolites (*136, 137, 138*). The influence of genotype on secondary metabolism has also been well documented (*139*). Within the context of this study, it was not possible to explain the trend in alkaloid concentration by these factors. Genotypic influences could not be investigated since seed origin was not disclosed to the author and samples consisted of mixtures of bulk root-rhizome exclusively, most of which were received dried and cut. As for the environmental conditions of the growth sites, it was only possible to visit and document one of the farms, only four of the six companies provided some (mostly limited) details of their growth site location and as such, details of growth conditions like the occurrence of herbivory, climate, shade, soil characteristics (e.g. fertility, pH, texture and chemistry) and nutrient availability (e.g. use of fertilizers and watering frequency) could not be assessed.

Although alkaloid concentration varied significantly among accessions, it is important to note that their frequency distribution and overall mean concentration, with the exception of canadine, were comparable to cultivated samples cited in the literature (Table 2.5). However, the occurrence of

variation in such a popular botanical on the market further justifies the need for product quality control programs and labelling standards to ensure the safety and effectiveness of NHPs in Canada.

2.3.5 Phytochemical variation among wild populations: The distribution of berberine, hydrastine, canadine and their sum concentrations in *H. canadensis* root-rhizome, stem-leaf and berry pulp samples collected from ten wild Canadian populations (HC1-HC10) were compared by population (Figures 2.15 and 2.16). The relationship between alkaloid yield in root-rhizomes and population site latitude is also discussed.

In Figure 2.15 total alkaloid concentration in root-rhizome and stem-leaf were compared separately among populations. For root-rhizome samples, populations HC6 and HC9 contained the greatest amount of total alkaloids (7.7 ± 0.53% w/dw, N = 23) and population HC4 contained the least (4.3 ± 0.35% w/dw, N = 11). The remaining seven populations contained an average of 5.9 ± 0.16% w/dw (N = 73). For stem-leaf samples, population HC6 contained the greatest amount (3.7 ± 0.30% w/dw, N = 10) and populations HC1, HC3 and HC7 contained the least (2.5 ± 0.09% w/dw, N = 31). The remaining six populations contained an average of 2.8 ± 0.08% w/dw (N = 68).

In Figure 2.16 berberine, hydrastine and canadine in root-rhizome, stem-leaf and berry pulp are compared. For root-rhizome samples, berberine was most concentrated in population HC9 and least concentrated in populations HC2, HC4 and HC5. Hydrastine was most concentrated in population HC6 and least concentrated in populations HC2, HC4 and HC5. Canadine was most concentrated in population HC6 and least concentrated in populations HC4 and HC8. For stem-leaf samples, berberine was most concentrated in population HC6 and equal in all other populations. Hydrastine did not vary among populations nor did canadine. Of the three berry samples, berberine was the only alkaloid detected and it was most concentrated in the sample from population HC5, followed by HC2 and it was not detected in the sample from population HC8.

The rate of alkaloid production is reportedly influenced by plant genotype, environmental factors and the interactions between the two (*129-139*). The influence of genotype on the chemical

Figure 2.15 Distribution of mean total alkaloid (berberine, hydrastine and canadine) concentration (% w/dw ± SEM) in *Hydrastis canadensis* L. (goldenseal) root-rhizomes and stem-leaves collected from ten wild Canadian populations (HC1-HC10) as measured using reverse-phase HPLC-DAD. N = 5 - 14 samples per population and each sample was analyzed in triplicate. Significant differences ($p < 0.05$) in mean total alkaloid concentration of root-rhizome and stem-leaf tissues (separately) among populations, as determined by one-way analysis of variance followed by Scheffe's post hoc multiple comparison analysis, are designated by the following distinct letters: (a, b) for total alkaloids in root-rhizomes and (c, d) in stem-leaves. Note populations HC1, HC2, HC4, HC5 and HC6 were located within the most southwestern range of the sampled Canadian populations and population HC9 was the most northeastern population.

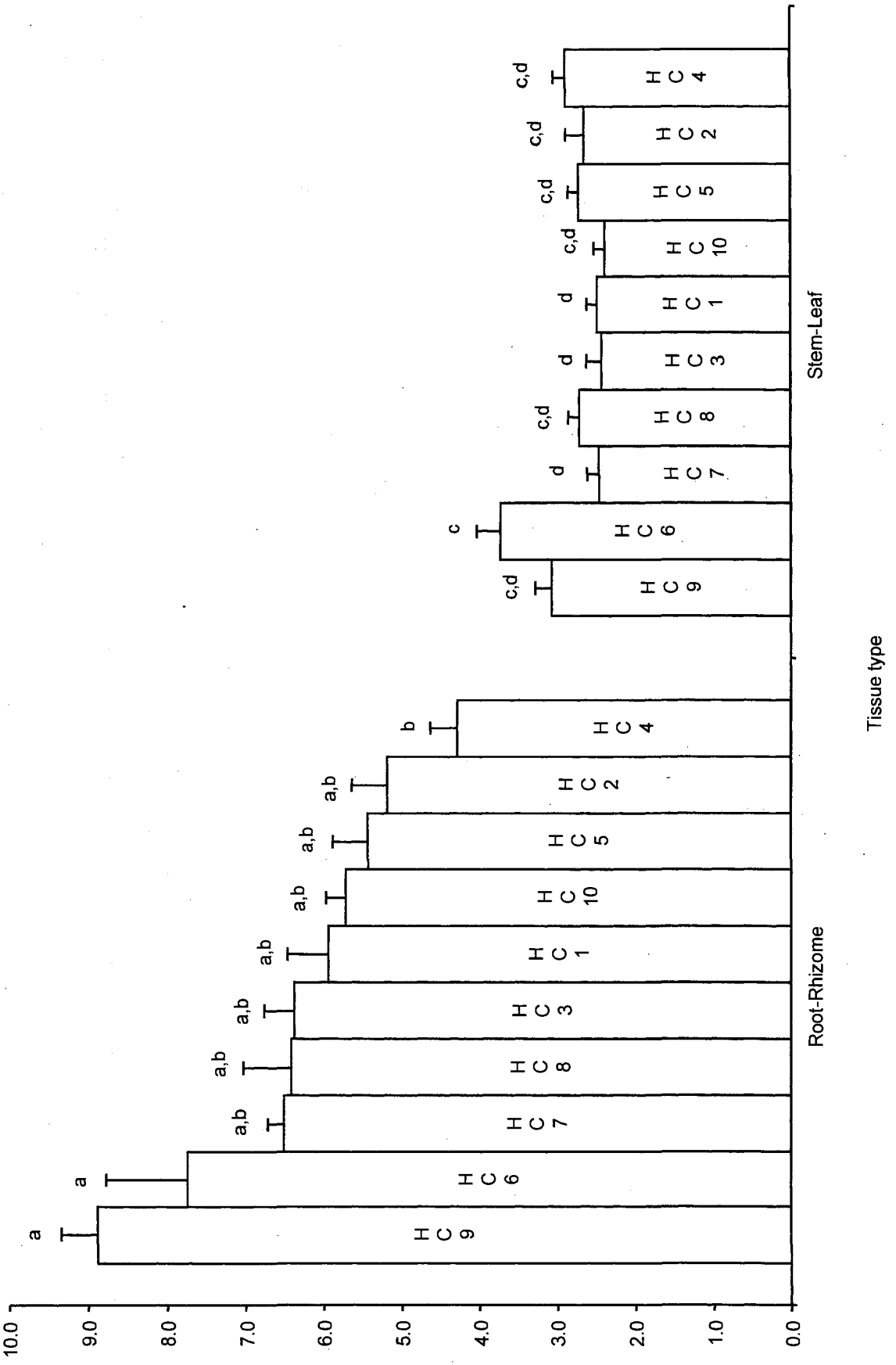
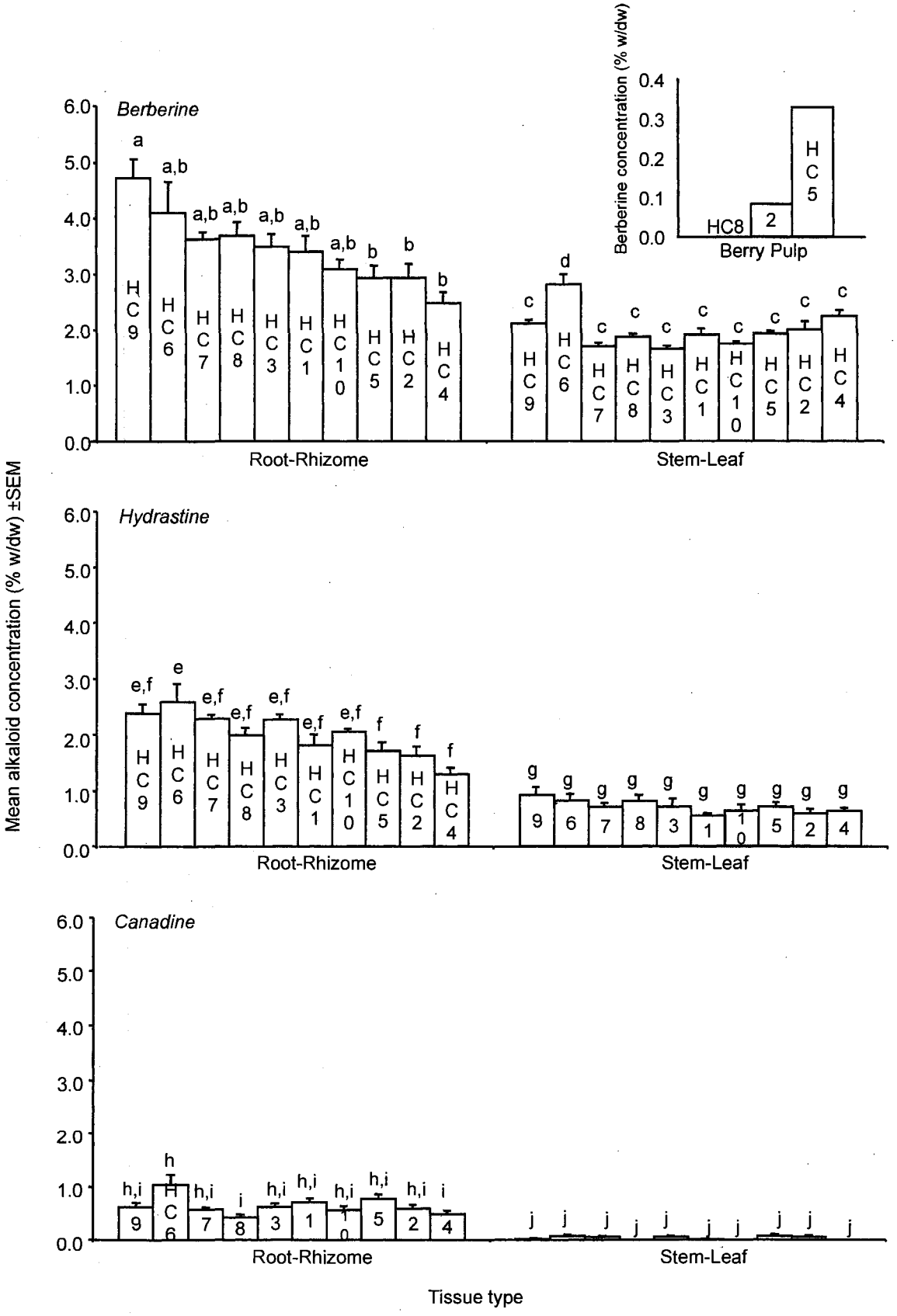

Mean total alkaloid concentration (% w/dw) ± SEM
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Root-Rhizome
Stem-Leaf
Tissue type
HC 9
HC 6
HC 7
HC 8
HC 3
HC 1
HC 10
HC 5
HC 2
HC 4
a
a
a,b
a,b
a,b
a,b
a,b
a,b
b
c,d
c
d
c,d
d
d
c,d
c,d
c,d

Figure 2.16 Distribution of mean berberine, hydrastine and canadine concentration (% w/dw ± SEM) in *Hydrastis canadensis* L. (goldenseal) root-rhizomes and stem-leaves collected from ten wild Canadian populations (HC1-HC10) as measured using reverse-phase HPLC-DAD. N = 5 - 14 samples per population and each sample was analyzed in triplicate. Berberine was also quantified in berry pulp collected from three populations where N = 1 sample per population and each sample was analyzed in triplicate. Significant differences ($p < 0.05$) in alkaloid concentration in root-rhizome and stem-leaf tissues (separately) among populations, as determined by one-way analysis of variance or Kruskal-Wallis analysis followed by Scheffe's post hoc multiple comparison analysis, are designated by the following distinct letters: (a, b) for berberine in root-rhizome and (c, d) in stem-leaves; (e, f) for hydrastine in root-rhizomes and (g) in stem-leaves; and (h, i) for canadine in root-rhizomes and (j) in stem-leaves. Note phytochemical concentration in berry pulp was not statistically analyzed among populations since only one sample was collected per population.


Berberine
Hydrastine
Canadine
Mean alkaloid concentration (% w/dw) ±SEM
Root-Rhizome
Stem-Leaf
Tissue type
Berberine concentration (% w/dw)
Berry Pulp
HC8
2
HC5

phenotype of a plant has been extremely well documented (e.g. *139*) and heritability is commonly used as a quantitative index for the proportion of phenotypic variation attributable to genetic variation. Heritability is defined as the ratio of the genetic variation to the sum of genetic and environmental variation and its value ranges from 0 to 1, where an index of 1 indicates that all variation among a phenotype is attributable to genetic variation (*135*). A previous study reported that alkaloid metabolism, as a whole, has a broad-sense heritability index that ranges from 0.19 to 0.7 (*140*). An original objective of the current study was to phytochemically analyze wild *H. canadensis* seedlings propagated under a uniform environment as to isolate the genetic contribution to alkaloid yield. Since the seeds used for this component of the study failed to germinate, there are no conclusive data in this area with respect to *H. canadensis*.

Environmental factors, including soil nutrients, herbivory and disease as well as climate (temperature and precipitation) and shade, relate to many well documented, albeit controversial, theories that account for allocation of chemical defenses in plants; for example the carbon-nutrient balance (CNB) theory, the resource availability theory and the plant stress and defense theories (*135*, *141*). Population density and soil chemistry for each wild population were obtained (*48*, Table 2.6) and hypothetical correlations with alkaloid concentration are presented and discussed below, followed by the possible contributions of climate, shade, herbivory and disease.

Population HC6 appeared to consistently contain the greatest amount of all three alkaloids in all tissue types. It was located in Essex County, the most southern county within the natural range of *H. canadensis* in Canada, near Cedarhurst Park along the northern shores of Lake Erie (Figure 2.1). Although samples were also collected from nearby populations within the same county (namely HC1 and HC2), alkaloid production in HC6 was superior to neighboring populations that existed under relatively similar environmental conditions. HC6 contained a relatively greater number of stems (2,597) compared to HC1 (1,153) and HC2 (768); greater soil acidity (pH 5.7 compared to pH 6.3 for HC1 and pH 6.4 for HC2); less soil potassium (123 ppm compared to 140 ppm for HC1 and 176 ppm for HC2); greater soil magnesium (375 ppm compared to 323 for HC2 and 266 ppm for HC1);

Table 2.6 Population and soil characteristics for ten wild Canadian *Hydrastis canadensis* L. (goldenseal) populations. Data originally from Sinclair and Catling (*48*)

	Site									
Parameter	HC4	HC5	HC6	HC2	HC1	HC3	HC10	HC7	HC8	HC9
# stems	4,329	2,324	2,597	768	1,153	106	648	455	37	241
# patches	3	2	8	10	8	1	4	2	1	1
Canopy[a]	Or, I	I, A	Hs, Or	W	Hs	I	A	Msu	Msu	Hb
Shade (%)	65	60	70	30	80	70	65	60	85	60
pH	6.3	6.1	5.7	6.4	6.3	7.5	7.3	5.4	7.4	7.8
P (ppm)	7	12	12	8	14	9	30	10	12	7
K (ppm)	100	93	123	176	140	179	114	72	230	77
Mg (ppm)	300	221	375	323	266	528	203	114	191	169
Na (ppm)	72	81	77	80	129	73	62	73	73	81
Ca (ppm)	2,730	2,360	2,710	2,780	2,270	8,240	3,990	1,290	8,300	6,960
Mn (index)	20	24	38	22	30	16	17	42	16	17
Zn (index)	26	60	48	43	27	26	30	30	36	18
OM[b]	8.2	9.4	11.1	8.9	9.7	12.1	6.9	5.8	23.1	10.7
Sand	39	75	33	33	33	70	33	33	70	33
Silt (%)	37	12	33	33	33	20	33	33	20	33
Clay	24	12	33	33	33	10	33	33	10	33

[a] A = *Fraxinus* spp (ash), C = *Populus deltoides* Marshall (cottonwood), Hb = *Carya cordiformis* Wangenh. K.Koch (bitternut hickory), Hs = *Carya ovata* (Miller) K. Koch (shagbark hickory, I = *Ostrya virginiana* (Miller) K. Koch (ironwood), M = *Acer* spp. (maple), Msu = *Acer saccharum* Marshall (sugar maple), Or = *Quercus rubra* L. (red oak), W = *Juglans nigra* L. (walnut); [b] Organic matter

less soil sodium (77 ppm compared to 80 ppm for HC2 and 129 for ppm HC1); greater soil manganese (38 index compared to 30 for HC1 and 22 for HC2); greater soil zinc (48 index compared to 43 for HC2 and 27 for HC1); and greater amounts of organic matter (11.1 compared to 9.7 for HC1 and 8.9 for HC2). It is important to note that these differences are relative to each other; they are not all distinct from other populations and may not be statistically relevant.

Population HC9 followed close behind HC6 with respect to alkaloid concentration. It contained an equal amount of total alkaloids and a greater amount of berberine in its root-rhizomes compared to HC6. HC9 is the most northeastern population and was located in Huron County, near the southeastern shores of Lake Huron (Figure 2.1). The following similarities in population and soil characteristics between HC6 and HC9, as listed in Table 2.6, were found: percent shade (60% for HC9 and 70% for HC6); soil sodium (77 ppm for HC6 and 81 ppm for HC9); organic matter (10.7 for HC9 and 11.1 for HC6); sand, silt and clay in soil (33% for all three for both populations).

Population HC4 contained the least amount of alkaloids overall. Like HC6, HC4 was located in Essex County, but further west where Lake St. Clair empties into Detroit River (Figure 2.1). From the data listed in Table 2.6, the only parameters that distinguished HC4 from all of the other populations were population density (number of stems) and the percentage of silt in the soil; HC4 contained the greatest number of stems (4,329) and its soil contained the greatest percentage of silt (37%).

To determine the contribution of temperature and precipitation to alkaloid production, climate data were obtained from Environment Canada (*142*) for each population or area (HC1, HC2, HC5 and HC6 were grouped as an area, as well as HC7 and HC8). The estimated overall average maximum temperature, mean temperature, minimum temperature and total precipitation from 2003-2004 were 13°C, 9°C, 4°C and 75 mm, respectively. The greatest average maximum temperature was measured at population HC9 (14°C), the lowest average minimum temperature was measured within the area of populations HC7 and HC8 (3°C), the lowest mean temperature was measured at population HC9 (8°C) and the greatest mean temperature was measured at population HC4. The least

average amount of precipitation was recorded within the area of populations HC1, HC2, HC5 and HC6 (71 mm) and the greatest amount was recorded at population HC4 (83 mm). In general, mean maximum temperature, mean temperature and mean total precipitation for population HC4 are relatively different from the other populations; and mean minimum temperature and mean total precipitation for populations HC1, HC2, HC5 and HC6 are also relatively different from the others.

Shade varied among populations but not in a fashion that suggested a relationship with alkaloid concentration. In 2001, Sinclair and Catling (*48*) recorded the average percent shade for all ten populations to be approximately 65%, where the least amount of shade was measured at population HC2 (30%) and the greatest amount was measured at population HC8 (85%) (Table 2.6).

Regarding pests, none of the wild populations showed evidence of herbivory and disease during the collecting periods in 2003 and 2004, and the same was reported in previous years (*48*). Therefore in this case there was no evidence of a link between herbivory and alkaloid production.

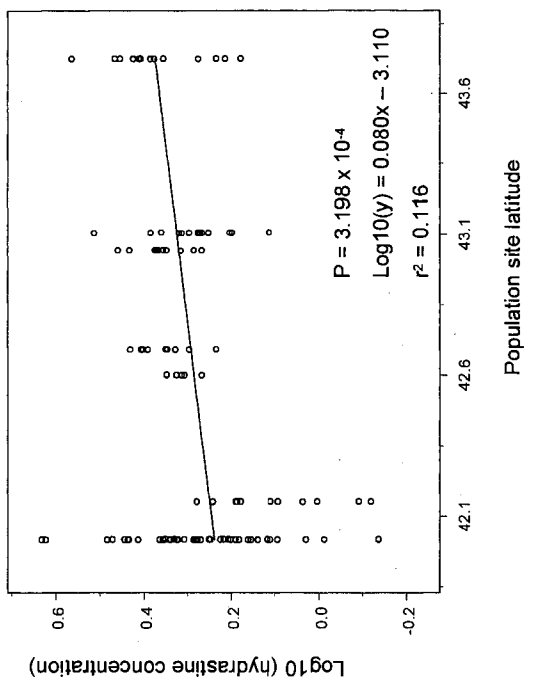
With respect to the environmental data described above and in Table 2.6, the following inferences could be made concerning the possible relationships between environmental factors and alkaloid yield in wild *H. canadensis*: population HC4's low alkaloid concentration may be characteristic of a warm and wet climate, high silt content and large population size. Though population HC9 had the overall coolest climate, there were no shared unique traits between HC6 and HC9 that could be attributed to their high alkaloid concentration. These inferences are both supported and contradicted by the literature and are related to the CNB theory. The CNB theory attempts to explain the concentration of secondary metabolites in plant tissues as a function of the relative abundance of plant resources, in particular the availability of light, water, and nutrients as essentials for growth (*135*, *143*). Since as much research supports the theory as contradicts it, Hamilton et al. (*135*) have proposed a more modern applicability of the CNB theory where it is not the sole determinant for secondary metabolism, but rather one of many contributing factors and may only be relevant in the production of certain secondary metabolites. For example norditerpenoid alkaloid concentrations in *Delphinium barbeyi* (Huth) Huth (subalpine larkspur) foliage grown under

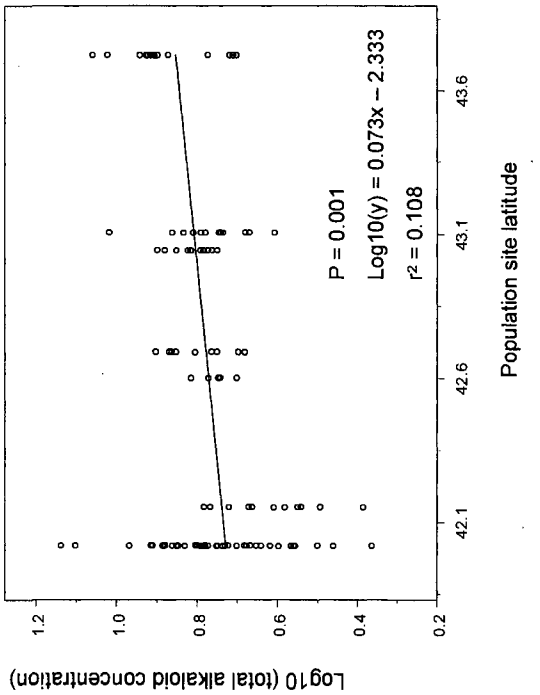
a long-term shade treatment (70% shade for 21 days) were reported lower than those harvested from individuals growing in open sunlight (*133*). Salmore and Hunter (*130*) found alkaloid concentrations in *S. canadensis* increased with decreasing light intensity and fertilizer levels. Another study found that concentrations of phenolic glycosides in the foliage of *Populus tremuloides* Michx. (Quaking aspen) grown under two levels of light and three levels of nutrient availability were moderately influenced by these resources (*131*). However the same study also reported that levels of both water and nitrogen were significantly and inversely related to phenolic glycoside concentrations.

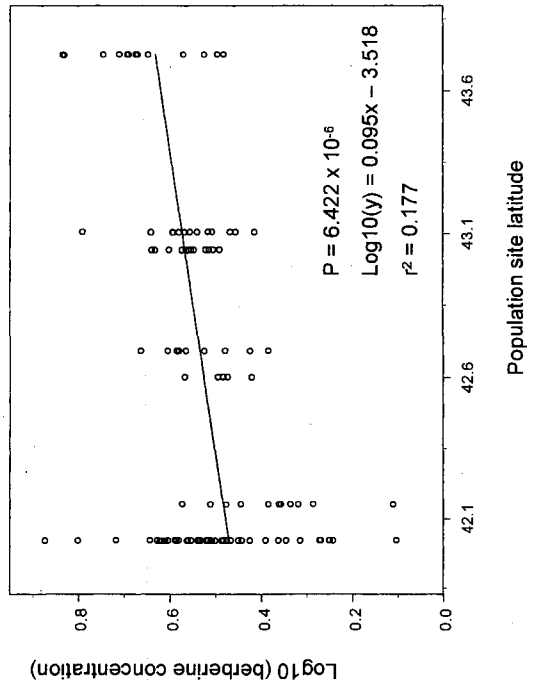
Another potential contributing factor is latitude; according to Levin and York (*138*) alkaloid toxicity (concentration) was found to increase with decreasing latitudes. While this was found to be true for canadine, the converse was observed in the case of berberine, hydrastine and total alkaloid. Figure 2.17 illustrates the linear regression analyses for berberine, hydrastine, canadine and total alkaloid concentration (individually) in root-rhizomes and population latitude. A positive relationship between increasing alkaloid concentration in root-rhizome samples and increasing latitude was statistically significant ($p \leq 0.001$) for berberine, hydrastine and total alkaloid whereas a significant ($p < 0.05$) inverse relationship between increasing canadine and increasing latitude was observed. While in all cases a relationship between latitude and alkaloid concentration was found to exist, it was also found in all cases to be of minimal effect (e.g. multiple $r^2 \leq 0.18$). This may likely be attributed to the fact that goldenseal is not pressured by disease and herbivores (alkaloids are constitutively biosynthesized), and that the range in latitude was minimal; in both cases the opposite is true for the subjects in the study by Levin and York (*138*).

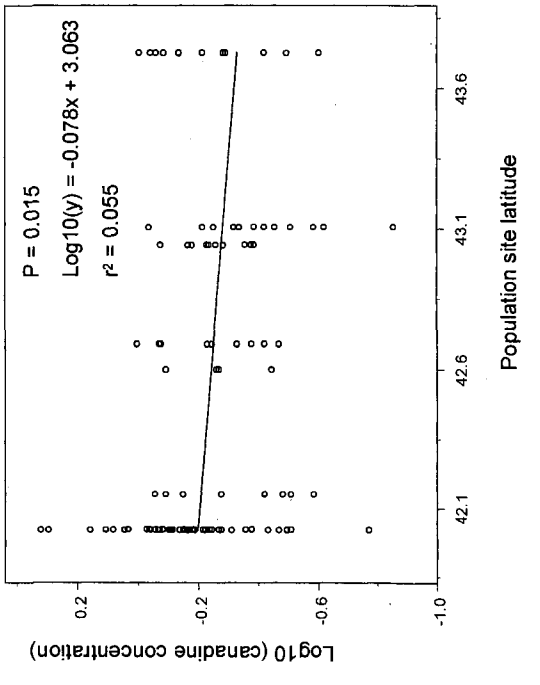
Overall, the variation in alkaloid concentration among wild populations may be attributed to population size, temperature, precipitation and soil fertility; it was not a result of herbivory or disease and, in root-rhizome samples, was minimally dictated by latitude. Further studies should be focused on each of these characteristics with a greater range in each case to further isolate their respective effect on alkaloid yield.

Figure 2.17 Linear regression analysis of mean alkaloid concentration (berberine, hydrastine, canadine and total) and population site latitude for root-rhizome samples collected from ten wild Canadian *Hydrastis canadensis* L. (goldenseal) populations.


P = 3.198 x 10-4
Log10(y) = 0.080x – 3.110
r2 = 0.116
Log10 (hydrastine concentration)
Population site latitude


P = 0.001
Log10(y) = 0.073x – 2.333
r2 = 0.108
Log10 (total alkaloid concentration)
Population site latitude


P = 6.422 x 10-6
Log10(y) = 0.095x – 3.518
r2 = 0.177
Log10 (berberine concentration)
Population site latitude


P = 0.015
Log10(y) = -0.078x + 3.063
r2 = 0.055
Log10 (canadine concentration)
Population site latitude

2.4 Conclusion

The validated analytical method used in this study successfully identified and quantified berberine, hydrastine and canadine in wild and cultivated *H. canadensis* tissues. The chemical profiles derived from the method served as a fingerprint for the species; there were differences in alkaloid frequency distribution and composition among the different tissue types but mostly between above-ground and below-ground tissues; and with the exception of canadine, there were no significant differences in alkaloid concentration between wild and cultivated material. These results support Canada's ban on wild-harvesting *H. canadensis*. There is no chemical advantage to using wild plants together with or instead of cultivated material in commercial products, and when publicized, this knowledge should have a positive impact on *H. canadensis* conservation.

Alkaloid concentration varied significantly among accessions of cultivated root-rhizome material and among wild populations. Although it was not possible to determine the cause of variation, in particular among cultivated samples, the variation in alkaloid concentration among wild populations may not have been driven by herbivory or disease, but rather may have been attributed to population size, climate (temperature and precipitation) and soil composition and fertility. Regression analyses indicated that latitude played a small role in influencing alkaloid yield; however further studies should focus on characterizing intrinsic environmental and genetic factors within a greater habitat range.

Furthermore, the differences in alkaloid concentration observed among wild populations may be an indication of genetic diversity. If this is true, over time it may be possible to increase the potency of cultivated material by cross breading with a potentially superior genotype.

CHAPTER 3: THE IN VITRO INHIBITION OF HUMAN CYTOCHROME P450 (CYP) 2C19-, CYP3A4 AND CYP19-MEDIATED DRUG METABOLISM BY EXTRACTS OF 22 NORTH AMERICAN BOTANICALS AND A FOCUSED INVESTIGATION ON THE INHIBITING EFFECT OF SIX ALKALOID-CONTAINING SPECIES ON CYP3A4 ACTIVITY

This chapter was written as an article for publication in the Journal of *Pharmaceutical Biology* in collaboration with I.M. Scott, R.J. Marles, J.T. Arnason and B.C. Foster.

Parts of this chapter have been published:

Leduc R.I.; Scott, I.M.; Saleem A.; Arnason J.T.; Marles, R.; Foster B.C. Botanical Extracts Containing Berberine and Related Alkaloids Interfere with the Activity of Human Cytochrome P450-Mediated Drug-Metabolism. *J. Pharm. Pharmaceut. Sci.* **2006**; *9*, 198.

Scott I.M.; **Leduc R.I.**; Burt A.J.; Marles R.J.; Arnason J.T.; Foster B.C. The Inhibition of Human Cytochrome P450 by Ethanol Extracts of North American Botanicals. *Pharm. Biol.* **2006**; *44*, 315-327.

Foster B.C.; Arnason J.T.; Briggs C.J. Natural Health Products and Drug Disposition. *Annu. Rev. Pharmacol. Toxicol.* **2005**; *45*, 203-226.

3.1 Introduction

In addition to their traditional context, the use of botanicals as complementary medicines to pharmaceutical drug therapies, or as their alternative, has become an increasingly global trend (*63-65, 150*). Their popularity is partially due to 1) the increase in accessibility of local botanical markets as well as of imported exotic botanicals, and to 2) the widespread notion that botanicals are completely safe.

In 2005, HC and the NHPD (*67*) estimated that a little over one half of the Canadian adult population does not associate risks to consuming NHPs because "they are made from natural ingredients" despite the fact that more and more studies have found numerous phytochemicals in NHPs can change the disposition of drugs in the body by inhibiting the activity of drug metabolizing CYP isozymes, which can lead to adverse reactions (*68, 75-78*). For example, in vitro studies have found extracts of the following popular botanicals to be inhibitory towards certain human CYP isozymes: *Ginkgo biloba* L. (maidenhair tree) (*108*), *Hydrastis canadensis* L. (goldenseal) (*109-111*), *Hypericum perforatum* L. (common St. John's wort) (*110, 111*), *Allium sativum* L. (cultivated garlic) (*112*), *Echinacea angustifolia* DC., *Matricaria recutita* L. (German chamomile) and *Glycyrrhiza glabra* L. (licorice) (*111*).

Baseline botanical-drug interactions data generated from high-throughput in vitro studies, such as those cited above, identify candidate species that warrant further in vitro and clinical investigation (*68, 114, 115*), for example the in vivo studies conducted on *H. perforatum* (*151*). To date many popular North American botanicals have yet to be screened.

As such, extracts of 22 North American botanical species, spanning 13 families, were assayed in vitro for CYP2C19, CYP3A4 and CYP19 inhibition. For accurate representation of the material consumers are exposed to, up to seven North American accessions of a species were assayed. Samples included the seven following Canadian crop species (*14*): *Achillea millefolium* L. (yarrow), *Arctostaphylos uva-ursi* (L.) Spreng. (kinnikinnick), *H. canadensis*, *Oenothera biennis* L. (common evening primrose), *Polygala senega* L. (Seneca snakeroot), *Rhodiola rosea* L. (roseroot

stonecrop) and *Sanguinaria canadensis* L. (bloodroot). A subset of six alkaloid-containing botanicals with varying CYP inhibiting activity and varying berberine concentration were also included to assess the extent to which berberine concentration in extracts influenced the degree of inhibition.

Berberine is an isoquinoline alkaloid whose methylenedioxy moiety, also occurring in other phytochemicals, has previously been shown to inhibit CYP-dependent metabolism (*109-111*) by binding to CYP enzymes to form an inactive complex (*109, 113*).

CYPs are membrane bound and are localized in the endoplasmic reticulum of several different tissues (*81*). CYP2C19 is responsible for metabolizing approximately 20% of all drugs (*82*), whereas CYP3A4, the most abundant CYP isozyme in the body, contributes to the metabolism of as much as 50% of all drugs on the market (*77, 92*). CYP19, also known as aromatase, is involved in steroid conversion of testosterone to estradiol, and androstenedione to estrone (*79, 101*).

The purpose of this study was to provide baseline CYP inhibition data for a large selection of commercially available North American botanicals, with special interest in the relationship between berberine concentration in crude extracts and the degree to which they inhibit CYP activity.

3.2 Materials & Methods

3.2.1 Plant material & extraction: A total of 65 sample accessions of bulk, crude material harvested from 22 botanical species were analyzed and are described in Tables 3.1 and 3.2. Nineteen species were sampled from commercial material obtained from three Canadian and two American herbal apothecaries as well as from seven Canadian and one American herb farms. The three following species were wild-harvested near Ottawa, Ontario: *Prunus serotina* Ehrh (black cherry) bark; *Zanthoxylum americanum* P. Mill. (common prickly ash) bark; and one accession of *Coptis trifolia* (L.) Salisb. var. *groenlandica* (Oeder) Fassett. (threeleaf goldthread) above-ground tissues. Each sample accession was given a Nutraceutical Research Programme (NRP) number by the Office of Science Laboratory (Ottawa Health Sciences Centre, University of Ottawa) and/or was given a code by the author (RIL). Although the seed origin of each sample accession is unknown for all samples, the harvest locations for the following species were disclosed to the author: *Berberis vulgaris* L. (common barberry) accession RIL11, *Eschscholzia californica* Cham. (California poppy) accession RIL14 and *H. canadensis* accessions RIL1-NRP273, RIL2, RIL3 and RIL5. The taxonomic identity of all samples was confirmed by phytochemical analysis (*152*) or by comparison with a herbarium specimen at the University of Ottawa.

All samples were received dry and finely chopped or as a powder; they were finely ground into a powder using a Wiley mill or a mortar and pestle, and filtered through a 0.5 mm mesh screen. All extracts were prepared at room temperature under reduced lighting.

Preliminary CYP2C19, CYP3A4 and CYP19 assays were conducted with crude extracts prepared from 5 g powder sample extracted with 25 mL of 55% ethanol (EtOH), for a final concentration of 200 mg/mL. The powder-solvent mixture was mechanically agitated on a shaker at 3.0 x *g* (12.7 mm rotor radius at 150 rpm, New Brunswick Scientific Corp.) for 24 hrs and filtered through a Buchner filter affixed with a 42 size (0.2 μm) Whatman filter paper. A 1 mL aliquot from the filtrate was passed through a 0.2 μm PTFE filter before CYP and HPLC analysis.

Table 3.1 Summary of the commercial botanical material used for preliminary analyses

[a]Species, Family (common name)	[b]NRP no.	Leduc accession no.	[c]Company or harvest location	Company type	Tissue type	CYP inhibition assay		
						CYP2C19	CYP3A4	CYP19
Achillea millefolium L., Asteraceae (common yarrow)	230	/	Ottawa, ON	Herbal apothecary	Leaf	√[d]	√[d]	√[d]
	231	/	Phippen, SK	Herb farm	Leaf	√[d]	√[d]	√[d]
	232	/	Golden Lake, ON	Herb farm	Leaf	√	√	√
Acorus americanus (Raf.) Raf., Acoraceae (sweetflag)	205	/	Golden Lake, ON	Herb farm	Root-rhizome		√	√
	206	/	Manitoba	*Unknown*	Root-rhizome		√	√
Actaea racemosa L. syn. *Cimicifuga racemosa* L. (Nutt.), Ranunculaceae (black cohosh)	197	/	Ottawa, ON	Herbal apothecary	Root-rhizome	√	√	√
	198	/	Toronto, ON	Herbal apothecary	Root-rhizome		√	√
Arctium lappa L., Asteraceae (greater burdock)	199	/	Ottawa, ON	Herbal apothecary	Root	√	√	√
	201	/	Golden Lake, ON	Herb farm	Root	√[d]	√[d]	√[d]
	202	/	Toronto, ON	Herbal apothecary	Root	√[d]	√[d]	√[d]
Arctostaphylos uva-uris (L.) Spreng., Ericaceae (kinnikinnick)	191	/	Ottawa, ON	Herbal apothecary	Leaf		√	√
	192	/	Phippen, SK	Herb farm	Leaf		√	√
	193	/	Manitoba	*Unknown*	Leaf		√	√

Table 3.1 continued

Berberis vulgaris L., Berberidaceae (common barberry)	189	RIL9	Ottawa, ON	Herbal apothecary	Bark	√[e]	√[e]	√[e]
	190	RIL8	Toronto, ON	Herbal apothecary	Bark	√[e]	√[e]	√[e]
Coptis trifolia (L.) Salisb. var. *groenlandica* (Oeder) Fassett., Ranunculaceae (threeleaf goldthread)	187	/	Ottawa-Rideau Valley, ON	*Wild-harvested*	Aerial	√[e]	√[e]	√[e]
Equisetum arvense L., Equisetaceae (field horsetail)	213	/	Ottawa, ON	Herbal apothecary	Stem		√[d]	√[d]
	214	/	Golden Lake, ON	Herb farm	Stem		√	√
	215	/	Toronto, ON	Herbal apothecary	Stem		√[d]	√[d]
Gaultheria procumbens L., Ericaceae (eastern teaberry)	228	/	Ottawa, ON	Herbal apothecary	Leaf		√[d]	√[d]
	229	/	Toronto, ON	Herbal apothecary	Leaf		√[d]	√[d]
Hydrastis canadensis L., Ranunculaceae (goldenseal)	/	RIL3	Scotland, ON	Herb farm; *harvested onsite*	Root-rhizome	√[e]	√[e]	√[e]
	/	RIL2	Princeton, ON	Herb farm; *harvested onsite*	Root-rhizome	√[e]	√[e]	√[e]
Ledum sp., Ericaceae (Labrador tea)	216	/	Phippen, SK	Herb farm	Leaf		√[d]	√[d]
	217	/	Golden Lake, ON	Herb farm	Leaf		√[d]	√[d]
Mahonia aquifolium (Pursh.) Nutt., Berberidaceae (Oregon grape or hollyleaved barberry)	185	RIL19	Phippen, SK	Herb farm	Root	√[e]	√[e]	√[e]
	186	RIL18	Toronto, ON	Herbal apothecary	Root	√[e]	√[e]	√[e]

Table 3.1 continued

Oenothera biennis L., Onagraceae (common evening-primrose)	210	/	Ottawa, ON	Herbal apothecary	Leaf	√	√	√
	211	/	Toronto, ON	Herbal apothecary	Leaf	√	√	√
Polygala senega L., Polygalaceae (Seneca snakeroot)	222	/	Ottawa, ON	Herbal apothecary	Root	√[d]	√[d]	√[d]
	223	/	Phippen, SK	Herb farm	Root	√	√	√
	224	/	MB	*Unknown*	Root	√[d]	√[d]	√[d]
Prunus serotina Ehrh., Rosaceae (black cherry)	207	/	?	*Wild-harvested*	Bark	√	√	√
Rhodiola rosea L., Crassulaceae (roseroot stonecrop)	220	/	Toronto, ON	Herbal apothecary	Root	√	√[d]	√[d]
	221	/	Trout Lake Farm (Trout Lake, WA)	Herb farm	Root	√	√[d]	√[d]
Rumex acetosella L., Polygonaceae (common sheep sorrel)	225	/	Ottawa, ON	Herbal apothecary	Aerial		√[d]	√[d]
	226	/	Golden Lake, ON	Herb farm	Aerial		√	√
	227	/	Toronto, ON	Herbal apothecary	Aerial		√[d]	√[d]
Sambucus sp., Caprifoliaceae (elderberry)	208	/	Ottawa, ON	Herbal apothecary	Flower		√	√
	209	/	Toronto, ON	Herbal apothecary	Flower		√	√
Vaccinium sp., Ericaceae (blueberry)	194	/	Ottawa, ON	Herbal apothecary	Leaf	√[d]	√[d]	√[d]
	195	/	Phippen, SK	Herb farm	Leaf	√	√	√
	196	/	Golden Lake, ON	Herb farm	Leaf	√[d]	√[d]	√[d]

Table 3.1 continued

Zanthoxylum americanum P. Mill., Rutaceae (common prickly ash)	219	/	?		*Wild-harvested*	Bark	√	√	√

[a] Scientific name according to the United States Department of Agriculture's Natural Resources Conservation Service Plants Database (*192*); [b] Nutraceutical Research Programme (NRP) number assigned by the Office of Science Laboratory, Ottawa Health Sciences Centre, University of Ottawa; [c] Company names are not indicated in order to maintain anonymity; [d] Results published by Scott, Leduc et al. (*152*); [e] Preliminary results published by Foster et al. (*75*).

Table 3.2 Summary of the commercial alkaloid-containing botanical material used for analyses

[a]Species, Family (common name)	[b]NRP no.	Leduc accession no.	[c]Company location	Company type	Sample description as indicated on label	[d]Experimental use
Berberis vulgaris L., Berberidaceae (common barberry)	/	RIL7	Sebastopol, CA, USA	Herbal apothecary	Barberry bark	CYP3A4[f]
	190	RIL8	Toronto, ON	Herbal apothecary	Wildcrafted barberry bark	CYP2C19, CYP3A4[e], CYP3A4[f], CYP19
	189	RIL9	Ottawa, ON	Herbal apothecary	Barberry bark	CYP2C19, CYP3A4[e], CYP3A4[f], CYP19
	/	RIL10	Woodlawn, ON	Herb farm	Barberry root (2002)	CYP3A4[f]
	/	RIL11	Grants Pass, OR	Herb farm	Wildcrafted barberry root powder; Lot# 0216K-WIP; [i]harvested in Minnesota.	CYP3A4[f]
Coptis trifolia (L.) Salisb. var. *groenlandica* (Oeder) Fassett., Ranunculaceae (threeleaf goldthread)	/	RIL12	Sebastopol, CA, USA	Herbal apothecary	Rhizome	CYP3A4[f]

Table 3.2 continued

Eschscholzia californica Cham., Papaveraceae (California poppy)	/	RIL13	Oakham, MA, USA	Herbal apothecary	Certified organic California poppy herb; Lot# L9464W0C	CYP3A4[f]
	/	RIL14	Grants Pass, OR, USA	Herb farm	[h]Oregon tilth certified organic California poppy [g]herb; Lot# 0604K-OU; harvested on-site	CYP3A4[f]
	/	RIL15	Goodwood, ON	Herbal apothecary	Dried herb, cut and sifted; Lot# 21649	CYP3A4[f]
	/	RIL16	Sebastopol, CA, USA	Herbal apothecary	California poppy root/leaf	CYP3A4[f]
*Hydrastis canad*ensis L., Ranunculaceae (goldenseal)	/	RIL5	Grants Pass, OR, USA	Herb farm	Oregon tilth certified organic root powder; Lot# 0806K-TCP; [g]grown in Washington State.	CYP3A4[f]
	/	RIL3	Scotland, ON	Herb farm	[h]Dry, bulk, whole root-rhizome mix; harvested on-site	CYP2C19, CYP3A4[e], CYP3A4[f], CYP19
	273	RIL1	Waterford, ON	Herb farm	[h]Dry, bulk, whole root-rhizome mix; cultivated under shade structures; harvested on-site	CYP2C19, CYP3A4[e], CYP3A4[f], CYP19
	/	RIL6	Sebastopol, CA, USA	Herbal apothecary	Dry, cut root	CYP3A4[f]
	/	RIL4	Woodlawn, ON	Herb farm	Dry, cut [g]root-rhizome mix; Lot# 184111131207	CYP3A4[f]

Table 3.2 continued

Mahonia aquifolium (Pursh.) Nutt., Berberidaceae (Oregon grape or hollyleaved barberry)	/	RIL17	Woodlawn, ON	Herb farm	Oregon grape [g]root; Lot# 007021251081	CYP3A4[f]
	186	RIL18	Toronto, On	Herbal apothecary	[g]root	CYP2C19, CYP3A4[e], CYP3A4[f], CYP19
	185	RIL19	Phippen, SK	Herb farm	Oregon grape root cut	CYP2C19, CYP3A4[e], CYP3A4[f], CYP19
	/	RIL20	Grants Pass, OR, USA	Herb farm	Wildcrafted Oregon grape root powder; Lot# 0110K-WIP; [i]grown in the U.S.	CYP3A4[f]
	/	RIL21	Goodwood, ON	Herbal apothecary	Oregon grape dried root, cut and sifted; Lot# 22011	CYP3A4[f]
	/	RIL22	Sebastopol, CA, USA	Herbal apothecary	Oregon grape root	CYP3A4[f]
	/	RIL23	Oakham, MA, USA	Herbal apothecary	Wildcrafted Oregon grape root; Lot# L6664WC	CYP3A4[f]
Sanguinaria canadensis L., Papaveraceae (bloodroot)	/	RIL24	Oakham, MA, USA	Herbal apothecary	Wildcrafted blood root (*Sanguinaria canadensis*) [g]root-rhizome mix; Lot# L9875WC	CYP3A4[f]
	/	RIL25	Woodlawn, ON	Herb farm	Bloodroot [g]root-rhizome mix, 2002	CYP3A4[f]

[a] Scientific name according to the United States Department of Agriculture's Natural Resources Conservation Service Plants Database (*192*); [b] Nutraceutical Research Programme (NRP) number assigned by the Office of Science Laboratory, Ottawa Health Sciences Centre, University of Ottawa; [c] Company names are not indicated in order to maintain anonymity; [d] CYP2C19, CYP3A4[e] and CYP2C19 correspond with Table 3.1, preliminary assays; [e] Preliminary CYP3A4 assay using a 4.0 mg/mL final extract concentration, corresponds with Table 3.1; [f] Follow-up CYP3A4 assays using a 0.5 mg/ mL final extract concentration; [g] Not indicated on label, tissue type determined by visual inspection; [h] Harvested on-site; [i] Personal communications with company

Follow-up CYP3A4 assays were conducted with crude extracts prepared from 25 mg powder sample extracted with 1 mL 55% EtOH, vortexed on high for five minutes and centrifuged at 8.0 x 10^5 x *g* (50 mm rotor radius at 1.2 x 10^5 rpm Fisher Scientific Micro Centrifuge) for 15 minutes. The supernatant was filtered through a 0.2 μm PTFE filter before CYP and HPLC analysis.

3.2.2 Materials for in vitro CYP inhibition assays: The following materials were purchased for the preparation of reagents: monobasic and dibasic potassium phosphate from Mallinckrodt (Mississauga, ON) and Sigma-Aldrich (Oakville, ON); β-nicotinamide adenine dinucleotide phosphate from Sigma-Aldrich (Oakville, ON); and dibenzylfluorescein (DBF) from Gentest, BD Biosciences (Mississauga, ON). The human drug metabolizing isozymes CYP2C19, CYP3A4 and CYP19 were purchased from BD Biosciences (Mississauga, ON).

3.2.3 In vitro CYP inhibition assays: Crude extracts prepared from commercial and wild-harvested samples of a total of 22 botanical species were assayed for their ability to inhibit CYP-mediated metabolism of DBF marker substrate by three human CYP isozymes using a 200 μL in vitro fluometric microtiter plate assay. The method described below was modified from Health Canada's Health Protection Branch Standard Operating Procedures OoS-3A4-000 (*154*) and Foster et al. (*112*).

Two sets of assays were performed: a preliminary set where extracts prepared from samples of as many as 20 different species were assayed with CYP2C19, CYP3A4 and CYP19 separately; and a follow-up set where extracts of six species were assayed with CYP3A4. For both sets a total extract aliquot volume of 4 μL was assayed, giving a final extract concentration of 4.0 mg/mL for the preliminary and 0.5 mg/mL for the follow-up assays. All extracts were prepared fresh before each assay. The assays were conducted under gold fluorescent lighting (Industrial Lighting, Ottawa, ON) and clear-bottom, white-welled microtiter plates (96 well, Corning Costar. Model # CS00-3632, Corning, NY, USA) were used. Two microliters per well of ketoconazole solution (1 μg/mL) was used as the positive control (data not shown) and 55% EtOH was used as the control blank (Cblk).

Enzyme inhibition was measured by the formation of fluorescent product. For CYP3A4 and CYP2C19 assays, fluorescence was measured after a 20-minute incubation period, whereas for CYP19, fluorescence was measured after 40 minutes. A Cytofluor 4000 Fluorescence Measurement System plate reader (Applied Biosystems, Foster City, CA, USA) was used to measure fluorescence, with excitation set to 485/20, emission set to 535/25, and gain set to 50.

All assay treatments were run in triplicate wells and repeated at least once with a freshly prepared extract. Mean percent inhibition for each extract was calculated after each assay using the following equation:

$$[1 - (T\text{-}Tblk) / (C\text{-}Cblk)] \times 100\%$$

where T, test treatment, is the mean fluorescence of the extract incubated with the isozyme; Tblk, test blank treatment, is the mean fluorescence of the corresponding extract incubated with the corresponding denatured isozyme; C, control treatment, is the mean fluorescence of 55% EtOH incubated with isozyme; and Cblk, control blank treatment, is the mean fluorescence of 55% EtOH incubated with the corresponding inactive isozyme.

The results are given as the mean ± the standard error of the mean (SEM) of all of the mean percent inhibition data for each sample accession, where N, the sample size, is the number of repeated assays per sample accession.

3.2.4 Materials for quantitative analyses: HPLC solvents and analytical grade trifluoroacetic acid (TFA) were purchased from EMD Biosciences Inc. (Germany) and JT Baker (Phillipsburg, NJ, USA), respectively. A pure berberine chloride standard was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

3.2.5 Quantitative analysis of berberine: High performance liquid chromatography coupled with diode array detection (HPLC-DAD) was used to quantify berberine in crude extracts prepared from commercial samples of *B. vulgaris* root and bark; wild samples of *C. trifolia* var. *groenlandica* aerial tissues and commercial samples of its rhizome; commercial samples of *E. californica* dried herb; commercial samples of *H. canadensis* root-rhizome; commercial samples of *Mahonia aquifolium* (Pursh.) Nutt. (Oregon grape) root; and commercial samples of *S. canadensis* root-rhizome, all of which were prepared for CYP analysis. The HPLC-DAD method described below was modified from Li and Fitzloff (*121*) and was validated as described in Chapter 2.

Berberine analysis was performed on a 1100 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) comprising of an in-line degasser, a quaternary pump, an autosampler, a DAD, and a computer equipped with Chemstation software (Rev. A.09.01). The separation of berberine was achieved with a reverse-phase C-18 Waters YMC ODS-AM (2 x 100 mm; 5 µm particle size, S-3, 120 Å) column that was maintained at 50°C and used at a flow rate of 1 mL/min. The mobile phases were (A) acetonitrile and (B) 0.05 % aqueous TFA, and the elution conditions of the mobile phases were as follows: initial conditions of 15% A and 85% B, followed by a linear gradient of 15-70 % A in 12 minutes and returned to initial conditions 70 – 15 % A in 2.5 minutes; re-equilibrated 15 % A for 0.5 minute followed by a post-run time of 1 minute, resulting in a total run time of 15 minutes. The injection volume was set at 1 µL for the analysis of *C. trifolia* var. *groenlandica* and *H. canadensis*, and set at 5 µL for the analysis *B. vulgaris, E. californica, M. aquifolium,* and *S. canadensis*. The DAD was set at 350 nm for maximum absorbance. Berberine typically eluted at 6.7 minutes.

Each extract was injected in triplicate and the mean percent berberine concentration as the weight per dry powder weight (% w/dw) was calculated. Analysis consisted of both identification and quantification of berberine. The compound's peak in each sample's chemical profile was identified based on its relative retention time to the compound standard and to the online photodiode array UV spectrum. Matches were defined by at least 95% similarity between the two. Berberine was

quantified (ng/μL) using peak area multiplied by the response factor, calculated from their respective standard curve. This value was multiplied by the extract's final volume (μL) to powder weight (g) ratio and divided by 10^3 to reach % weight / dry weight (% w/dw).

The results are given as the mean ± SEM of all of the mean percent berberine concentration data for each sample accession, where N is the number of repeated analyses per sample accession.

3.2.6 Statistical analysis: All assay results were expressed as the mean percent CYP inhibition ± SEM of at least two replicate analyses. All HPLC results for each sample accession were expressed as the mean percent berberine weight per plant powder weight (% w/dw) ± SEM of three replicate analyses, where the coefficient of variance (CV) of the mean was ≤ 5%. Each analysis consisted of the mean of three replicate injections.

In order to address the research objectives of this study, the following null hypotheses were tested: 1) no difference between test treatment (T, the mean fluorescence of the extract incubated with the isozyme) and the control treatment (C, the mean fluorescence of 55% ethanol incubated with the same isozyme); 2) no significant difference in mean CYP inhibition among accessions of the same species for each isozyme separately; 3) no significant difference in mean CYP inhibition among species for each isozyme separately; 4) no significant difference in overall CYP inhibition among isozymes for each species separately; 5) no significant difference in overall mean percent CYP inhibition (CYP2C19, CYP3A4 and CYP19 inclusively) among species; 6) no significant difference in mean berberine concentration among accessions of the same species; 7) no significant difference in mean berberine concentration among berberine-containing species (including all accession data); 8) no relationship between mean berberine concentration in crude extracts and mean CYP inhibition, for each isozyme separately.

The null hypotheses were assessed by one-way analysis of variance (ANOVA). When significant p statistics resulted ($p < 0.05$), multiple comparison pairwise differences were determined by Scheffe's post hoc analysis. Data sets were transformed by the logarithm base 10 when necessary

to achieve normality and homoscedasticity. However, when these ANOVA assumptions could not be met even after transformations, the data set was analyzed using the Kruskal-Wallis non-parametric test.

The relationship between CYP inhibition and berberine concentration was determined for each isozyme separately by performing simple linear regressions of mean percent CYP inhibition by berberine-containing species on its corresponding berberine concentration. Data sets were transformed by the logarithm base 10 when necessary to achieve normality and homoscedasticity.

All statistical analyses were conducted using S-PLUS 7.0 for Windows (*124*).

3.3 Results & Discussion

The inhibitory activity of each botanical described below should only be considered to represent its particular extract conditions (e.g. 9 H_2O : 11 EtOH extraction solvent and specified extract concentration) and final experimental concentration (e.g. 0.5 and 4.0 mg/mL). For example *H. canadensis* root-rhizome extract, one of the top CYP inhibitors in this study, may be equally, less or more inhibitory towards none, one or all CYPs if it had been extracted with a different ratio of water and alcohol, or with altogether different solvent(s). However, what can be said with certainty is whether or not the botanicals (and more specifically their respective tissues) were inhibitory using 55% EtOH in water with two different extract concentrations under in vitro conditions.

The CYP inhibition assay results and their relationship with the phytochemical data obtained from this study for the extracts listed below were previously published by Scott, Leduc et al. (*152*): *Arctium lappa* L. (greater burdock) root; *A. millefolium* leaf; *A. uva-ursi* leaf (for CYP3A4 and CYP19 only); *Equisetum arvense* L. (field horsetail) stem (CYP3A4 and CYP19 only); *Gaultheria procumbens* L. (eastern teaberry) leaf (CYP3A4 and CYP19 only); *P. senega* root; *Rumex acetosella* L. (common sheep sorrel) aerial tissues (CYP3A4 and CYP19 only); *R. rosea* root (CYP3A4 and CYP19 only); and *Vaccinium* sp. (blueberry) leaf. In addition, preliminary CYP inhibition and berberine concentration data for the extracts prepared with wild *C. trifolia* var. *groenlandica* material and commercial *B. vulgaris* bark; *H. canadensis* root-rhizome; and *M. aquifolium* root material have also been published (*75*). For more details see Table 3.1.

3.3.1 Preliminary CYP2C19, CYP3A4 & CYP19 inhibition assays: A total of 52 ethanolic extracts (4.0 mg/mL 55% EtOH) prepared from samples of eleven species were assayed with human CYP2C19. In addition, a total of 122 and 123 extracts (of the same concentration) prepared from samples of 20 species were assayed with human CYP19 and CYP3A4, respectively. The extracts were assayed for their ability to inhibit the in vitro CYP-mediated metabolism of DBF. The variation in CYP inhibition among species and among accessions of the same species (for each CYP

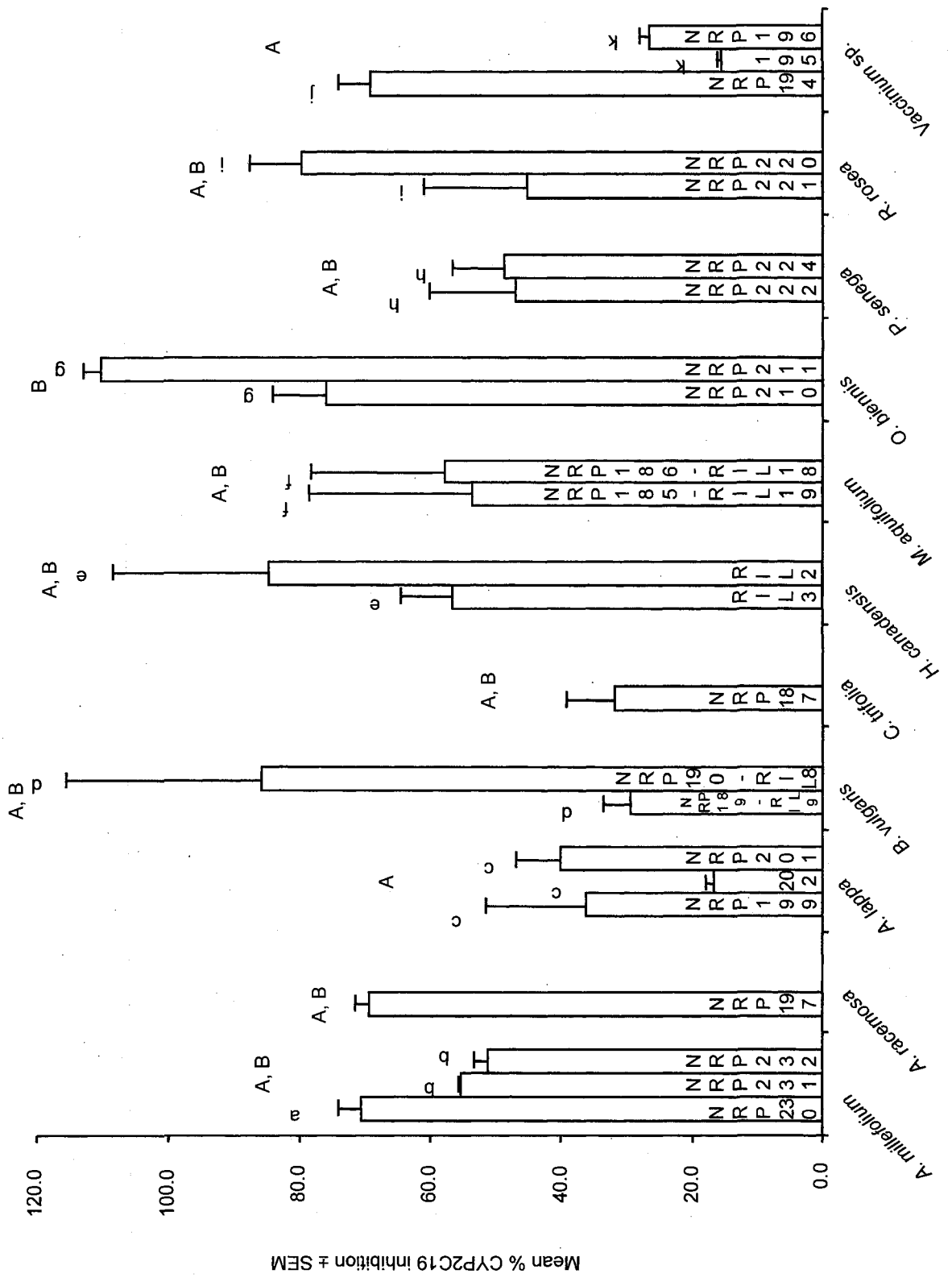
separately) is discussed. The species have also been ranked based on their overall (all isozymes inclusively) and relative (among isozymes) inhibiting capacities. The overall top inhibiting species are discussed as well as the observed relationship between berberine extract concentration and CYP inhibition.

Figure 3.1 shows the mean percent inhibition of CYP2C19-mediated metabolism by extracts prepared from samples of eleven different species. The most inhibitory extract towards CYP2C19 was *O. biennis* L. leaf (93.1% ± 10.54%, N = 4) and the least inhibiting extracts were *A. lappa* root and *Vaccinium* sp. leaf inclusively (34.2% ± 5.95%, N = 13). The remaining seven species inhibited CYP2C19 metabolism by an average of 57.0% ± 4.00% (N = 35). Mean percent CYP2C19 inhibition for all assayed extracts ranged from 16.8% ± 1.10% to 110.25% ± 2.75% and the overall mean was calculated to be 54.0% ± 3.76% (N = 52).

CYP2C19 is one of the major CYPs involved in drug metabolism and some of its substrates include celocoxib (Celebrex®), a cyclooxygenase (COX)-2 inhibitor (*81 and references within*); tolbutamide (Orinase®), a potassium channel blocker, sulfonylurea oral hypoglycemic drug used by patients with type II diabetes; naproxen (Aleve®, Anaprox®, Naprogesic®, Naprosyn®, Naprelan® and Synflex®), a non-steroidal anti-inflammatory drug (NSAID) commonly used for relieving pain, fever, inflammation and stiffness caused by osteoarthritis, rheumatoid arthritis, psoriatic arthritis, fractures, menstrual cramps and tendonitis; ibuprofen (Act-3®, Advil®, Brufen®, Dorival®, Herron Blue®, Panafen®, Motrin®, Nuprin®, Ipren®, Ibumetin®, Ibuprom®, and Moment®), a NSAID used for relief from arthritis, primary dysmenorrhoea, fever, and inflammation (*153 and references within*). Some of the endogenous compounds that are oxidized by CYP2C19 include linoleic acid, an omega-6 fatty acid found in lipids of cell membranes, and vitamin A, an essential human nutrient associated with vision and cornea health (*81 and references within*).

Significant differences in CYP2C19 inhibition observed among accessions of *A. millefolium* leaf (NRP231, NRP231 and NRP232) and of *Vaccinium* sp. leaf (NRP194, NRP195 and NRP196)

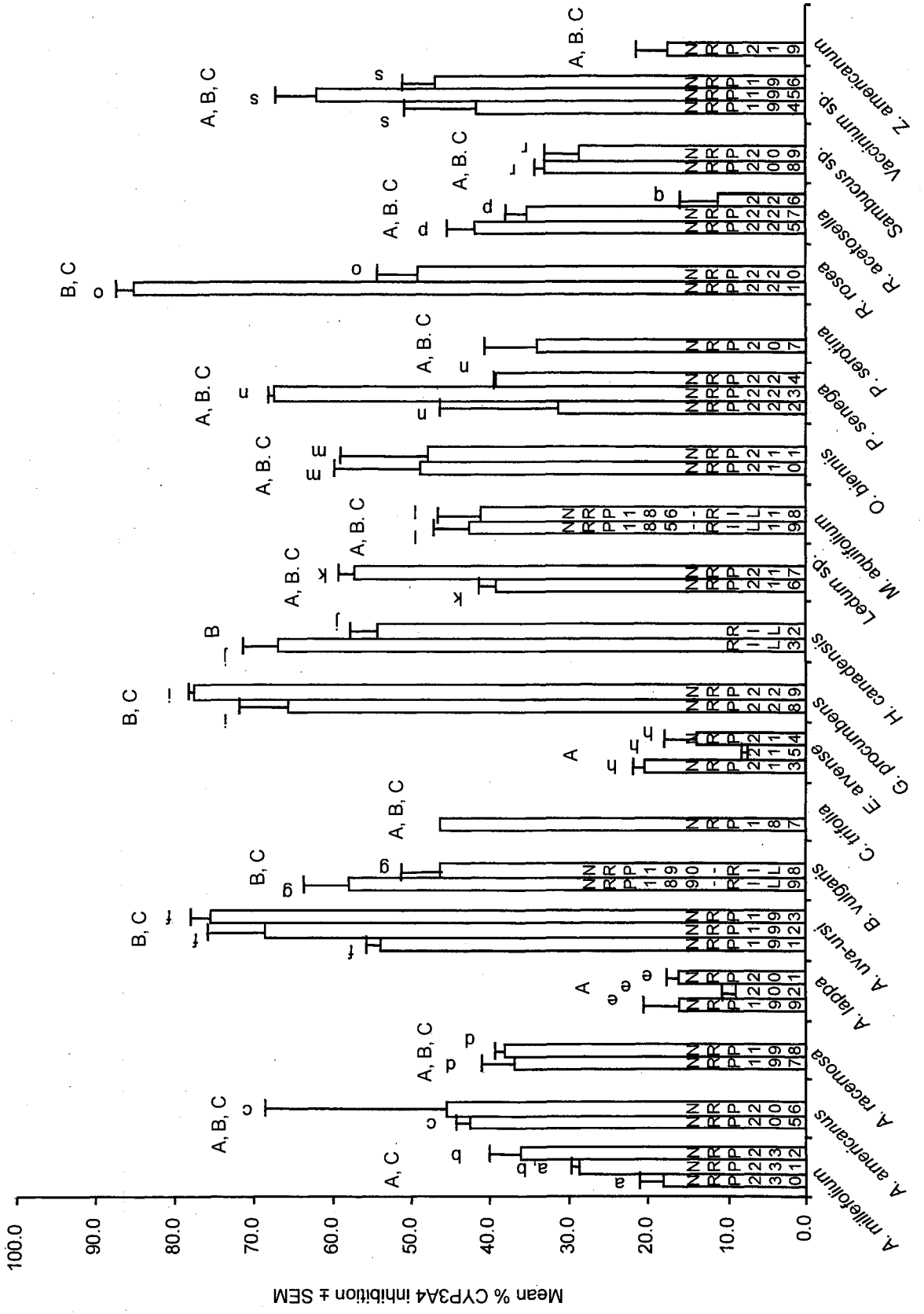
Figure 3.1 Mean percent inhibition (± SEM) of human CYP2C19-mediated metabolism in vitro by 4.0 mg/mL 55% ethanol extracts of commercial samples from one to three accessions of *Achillea millefolium* L. (common yarrow) leaf; *Actaea racemosa* L. (black cohosh) root-rhizome; *Arctium lappa* L. (greater burdock) root; wild-harvested *Coptis trifolia* (L.) Salisb. var. *groenlandica* (Oeder) Fassett. (threeleaf goldthread) aerial tissues; *Oenothera biennis* L. (common evening-primrose) leaf; *Polygala senega* L. (Seneca snakeroot) root; *Rhodiola rosea* L. (roseroot stonecrop) root; and *Vaccinium* sp. (blueberry) leaf material. Each bar represents one accession where N = 2 – 3 replicate analyses per accession. The Nutraceutical Research Programme (NRP) number and the Leduc (RIL) accession number are indicated for each accession and correspond with Table 3.1. Significant differences ($p < 0.05$) in mean percent CYP19 inhibition among accessions of the same species and among species were determined by analysis of variance or Kruskal-Wallis analysis followed by Scheffe's post hoc multiple comparison analysis. Significant differences among accessions of the same species are indicated by sets of distinct letters (a–k) for each species and those among species are indicated by capital letters (A, B).


Mean % CYP2C19 inhibition ± SEM
120.0
100.0
80.0
60.0
40.0
20.0
0.0
A. millefolium
A. racemosa
A. lappa
B. vulgaris
C. thiolla
H. canadensis
M. aquifolium
O. biennis
P. senega
R. rosea
Vaccinium sp.

are illustrated in Figure 3.1. For *A. millefolium*, NRP230 was significantly more inhibiting than NRP231 and NRP232 ($p < 0.05$). For *Vaccinium* sp., NRP194 was significantly more inhibiting than NRP195 and NRP196 ($p < 0.05$). The phytochemical constituents for only two accessions of these species were analyzed (*152*): 13 essential oils (EOs) were quantified in NRP230 and NRP231 (*A. millefolium*) and six flavonoids as well as chlorogenic acid were quantified in NRP194 and NRP196 (*Vaccinium* sp.). The concentration of these compounds was found to be significantly different between the respective accessions ($0.001 \leq p \leq 0.048$). Furthermore, EOs concentration in *A. millefolium* had a positive relationship with CYP3A4 and CYP19 inhibition, except for carvone, which had a negative relationship. In contrast, variations in chlorogenic acid concentration between the *Vaccinium* sp. accessions did not appear to influence CYP inhibition (*152*). These findings suggest that for *A. millefolium* leaf extract, the extent to which it inhibits CYP metabolism may be dependent upon its phytochemical composition.

Figure 3.2 shows the mean percent inhibition of CYP3A4 by extracts prepared from 20 different species. CYP3A4 metabolism of DBF was most inhibited by *H. canadensis* root-rhizome extracts (62.3% ± 3.47%, N = 11) and least inhibited by *A. lappa* root extracts and *E. arvense* stem extracts inclusively (14.0% ± 1.54%, N = 16). The extracts prepared with *A. uva-ursi* leaf, *B. vulgaris* stem bark, *G. procumbens* leaf and *R. rosea* root-rhizome inhibited CYP3A4 metabolism by 60.5% ± 3.40% (N = 23) and half of all of the species inhibited CYP3A4 by 40.1% ± 2.00% (N = 60). *Achillea millefolium* leaf extracts inhibited metabolism by 26.3% ± 4.09% (N = 5) and ranked the second least CYP3A4 inhibiting species; it was also the only species where mean percent inhibition differed significantly among accessions, in particular between NRP230 < NRP232. Since NRP232 was not phytochemically analyzed, it was not possible to explain the variation in inhibition, however since EO concentration in NRP230 and NRP231 previously found to be positively correlated with CYP3A4 and CYP19 inhibition (and carvone concentration being inversely proportional) (*152*), it was possible that NRP232 had a greater concentration of EOs than NRP230. The total range in mean percent CYP3A4 inhibition for all assayed extracts was 7.4% ± 0.80% to

Figure 3.2 Mean percent inhibition (± SEM) of human CYP3A4-mediated metabolism in vitro by 4.0 mg/mL 55% ethanol extracts of commercial samples from one to three accessions of *Achillea millefolium* L. (common yarrow) leaf; *Acorus americanus* (Raf.) Raf. (sweetflag) root-rhizome; *Actaea racemosa* L. (black cohosh) root-rhizome; *Arctium lappa* L. (greater burdock) root; *Arctostaphylos uva-ursi* (L.) Spreng. (kinnikinnick) leaf; *Berberis vulgaris* L. (common barberry) bark; wild-harvested *Coptis trifolia* (L.) Salisb. var. *groenlandica* (Oeder) Fassett. (threeleaf goldthread) aerial tissues; *Equisetum arvense* L. (field horsetail) stem; *Gaultheria procumbens* L. (eastern teaberry) leaf; *Hydrastis canadensis* L. (goldenseal) root-rhizome; *Ledum* sp. (Labrador tea) leaf; *Mahonia aquifolium* (Pursh.) Nutt. (Oregon grape) root; *Oenothera biennis* L. (common evening-primrose) leaf; *Polygala senega* L. (Seneca snakeroot) root; wild-harvested *Prunus serotina* Ehrh. (black cherry) bark; *Rhodiola rosea* L. (roseroot stonecrop) root; *Rumex acetosella* L. (common sheep sorrel) aerial tissues; *Sambucus* sp. (elderberry) flower; *Vaccinium* sp. (blueberry) leaf; and wild-harvested *Zanthoxylum americanum* P. Mill. (common prickly ash) bark material. Each bar represents one accession where N = 2 – 3 replicate analyses per accession. The Nutraceutical Research Programme (NRP) number and the Leduc (RIL) accession number are indicated for each accession and correspond with Table 3.1. Significant differences ($p < 0.05$) in mean percent CYP3A4 inhibition among accessions of the same species and among species were determined by analysis of variance or Kruskal-Wallis analysis followed by Scheffe's post hoc multiple comparison analysis. Significant differences among accessions are indicated by sets of distinct letters (a – s) for each species and those among species are indicated by capital letters (A, B, C).


Mean % CYP3A4 inhibition ± SEM
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
A. millefolium
A. americanum
A. racemosa
A. lappa
A. uva-ursi
B. vulgaris
C. trifolia
E. arvense
G. procumbens
H. canadensis
Ledum sp.
M. aquifolium
O. biennis
P. senega
P. serotina
R. rosea
R. acetosella
Sambucus sp.
Vaccinium sp.
Z. americanum

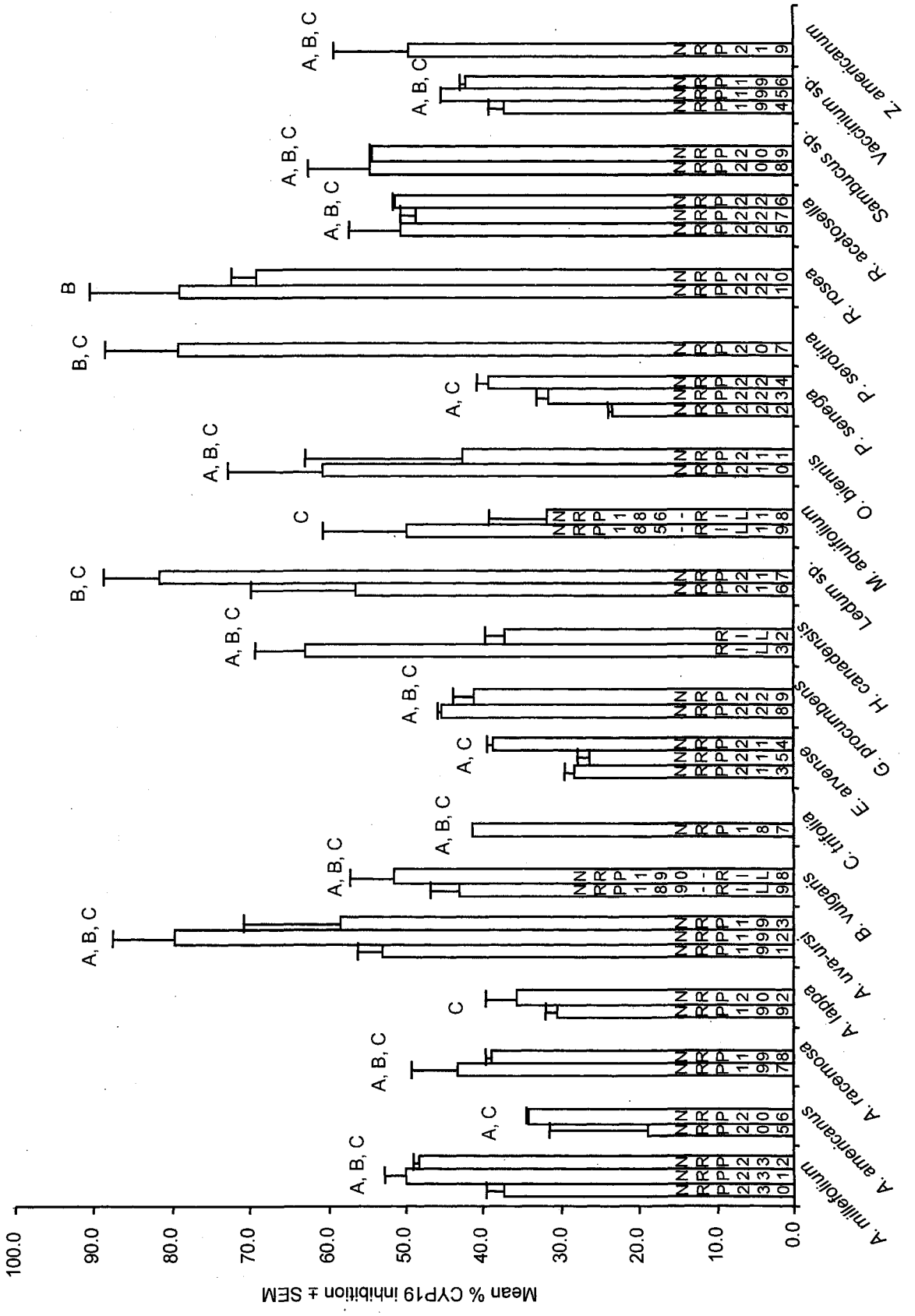
77.6 % ± 0.75%, with an overall mean percent inhibition of 42.4% ± 1.84% (N = 123).

CYP3A4 contributes to the metabolism of approximately 50% of the drugs currently on the market (*92*) including lovastatin (Mevacor®), an HMG-CoA reductase inhibitor for reduction of blood cholesterol levels (*93*); finasteride (Proscar®, Propecia®), a prostate hypertrophy inhibitor (*94*); cyclosporine, an immune suppressant (*95*); and the protease inhibitors indinavir (*96*) and sildenafil (Viagra®) (*97*). CYP3A4 functions in the metabolism of cancer chemotherapeutic drugs (*81*) as well as in the activation of certain drugs and chemical carcinogens (*155*). For example CYP3A4 activated tamoxifen, an estrogen receptor antagonist used as a popular breast cancer therapy, results in the production of carcinogenic DNA adducts (*107*). Other typical CYP3A4 substrates include testosterone, midazolam, quinidine, verapamil, ondansetron, omeprazole, diazepam and niferipine (*153*).

Figure 3.3 shows the mean percent inhibition of CYP19-mediated metabolism by the same species and accessions assayed with CYP3A4, less one accession of *A. lappa* root (NRP201). When comparing among species, the most inhibiting extracts were of *R. rosea* root-rhizome (75.6% ± 7.77%, N = 6), followed by those of *Ledum* sp. leaf and *P. serotina* bark inclusively (70.9% ± 6.92%, N = 10). Twelve species inhibited CYP19-mediated metabolism by 50.4% ± 1.88% (N = 76). *Arctium lappa* root inhibited CYP19 by 38.8% ± 5.18% (N = 16) and the extracts prepared from *A. americanus* root-rhizome, *E. arvense* stem and *P. senega* root were the least inhibiting (30.0% ± 2.11%, N = 16). There were no significant differences in percent CYP19 inhibition among accessions of the same species ($p > 0.05$) and the overall range in mean percent inhibition for 122 assayed extracts was 18.9% ± 12.60 to 81.8% ± 7.10%, with an overall mean of 49.1% ± 1.84%.

CYP19 oxidizes the androgens androstandione and testosterone to the estrogens estrone and 17β-estradiol, respectively. This process is critical in normal physiology and is also a target for anticancer drugs, particularly for breast cancers occurring in postmenopausal women (*81*). Examples of these CYP19-inhibiting anticancer drugs include anastrozole, letrozole and exemestrane (*156*).

Figure 3.3 Mean percent inhibition (± SEM) of human CYP19-mediated metabolism in vitro by 4.0 mg/mL 55% ethanol extracts of commercial samples from one to three accessions of *Achillea millefolium* L. (common yarrow) leaf; *Acorus americanus* (Raf.) Raf. (sweetflag) root-rhizome; *Actaea racemosa* L. (black cohosh) root-rhizome; *Arctium lappa* L. (greater burdock) root; *Arctostaphylos uva-ursi* (L.) Spreng. (kinnikinnick) leaf; *Berberis vulgaris* L. (common barberry) bark; wild-harvested *Coptis trifolia* (L.) Salisb. var. *groenlandica* (Oeder) Fassett. (threeleaf goldthread) aerial tissues; *Equisetum arvense* L. (field horsetail) stem; *Gaultheria procumbens* L. (eastern teaberry) leaf; *Hydrastis canadensis* L. (goldenseal) root-rhizome; *Ledum* sp. (Labrador tea) leaf; *Mahonia aquifolium* (Pursh.) Nutt. (Oregon grape) root; *Oenothera biennis* L. (common evening-primrose) leaf; *Polygala senega* L. (Seneca snakeroot) root; wild-harvested *Prunus serotina* Ehrh. (black cherry) bark; *Rhodiola rosea* L. (roseroot stonecrop) root; *Rumex acetosella* L. (common sheep sorrel) aerial tissues; *Sambucus* sp. (elderberry) flower; *Vaccinium* sp. (blueberry) leaf; and wild-harvested *Zanthoxylum americanum* P. Mill. (common prickly ash) bark material. Each bar represents one accessions where N = 2 – 6 replicate analyses per accession. The Nutraceutical Research Programme (NRP) number and the Leduc (RIL) accession number for each accession are indicated and correspond with Table 3.1. Significant differences ($p < 0.05$) in mean percent CYP19 inhibition among species were determined by analysis of variance or Kruskal-Wallis analysis followed by Scheffe's post hoc multiple comparison analysis and are indicated by capital letters (A, B, C). No significant differences in CYP19 inhibition were observed among accessions of the same species.


Mean % CYP19 inhibition ± SEM
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
A. millefolium
A. americanus
A. racemosa
A. lappa
A. uva-ursi
B. vulgaris
C. trifolia
E. arvense
G. procumbens
H. canadensis
Ledum sp.
M. aquifolium
O. biennis
P. senega
P. serotina
R. rosea
R. acetosella
Sambucus sp.
Vaccinium sp.
Z. americanum

The overall and selective CYP inhibiting capacities of all 20 species are shown in Figure 3.4. Overall inhibition for each species was expressed as the mean of all assay results for all three CYPs inclusively, whereas selective inhibition for each species was the ranking of mean CYP inhibition of all assay results for each isozyme separately.

When comparing the relative sensitivity of each isozyme to inhibition, CYP3A4 was less sensitive (inhibited) than CYP2C19 and CYP19 ($p < 0.05$), and the latter two were inhibited to the same degree ($p > 0.05$). All species showed significant but variable degrees of inhibition towards CYP2C19-, CYP3A4- and CYP19-mediated metabolism of DBF. Overall mean percent inhibition significantly varied ($p < 0.05$) among isozymes for the following species that were assayed with all three isozymes: *A. millefolium* leaf extracts were more inhibitory towards CYP2C19 and least inhibitory towards CYP3A4 (CYP2C19 > CYP19 > CYP3A4); *Actaea racemosa* L. (black cohosh) root-rhizome, CYP2C19 > CYP3A4, CYP19; *A. lappa* root, CYP2C19, CYP19 > CYP3A4; and *O. biennis* leaf, CYP2C19 > CYP3A4, CYP19. When comparing mean inhibition between isozymes for each species that were assayed with two isozymes only (CYP3A4 and CYP19), significant differences in inhibition between isozymes ($p < 0.05$) were found for the following species: *E. arvense* stem, CYP19 > CYP3A4; *G. procumbens* leaf, CYP3A4 > CYP19; *P. serotina* bark, CYP19 > CYP3A4; *R. acetosella* aerial tissues, CYP19 > CYP3A4; and *Sambucus* sp. (elderberry) inflorescence, CYP19 > CYP3A4.

Arctostaphylos uva-ursi leaf, *H. canadensis* root-rhizome, O. *biennis* leaf and *R. rosea* root were the overall top inhibitors of CYP metabolism (63.2% ± 2.52%, N = 72) and are illustrated in Figure 3.5. The least inhibiting species were *A. lappa* root and *E. arvense* stem (23.2% ± 2.30%, N = 33).

Arctostaphylos uva-ursi is native to North America (*157*) and is a traditionally important medicine to the Carrier people of north central British Columbia. *Arctostaphylos uva-ursi* was used as a poultice for wounds and in teas for bladder infections, kidney problems, high blood pressure, diabetes, for menstrual and birthing cramps and for relieving mental stress during menopause (*5*).

Figure 3.4 Overall mean percent inhibition (± SEM) of human CYP2C19-, CYP3A4- and CYP19-mediated metabolism in vitro by 4.0 mg/mL 55% ethanol extracts of commercial samples from one to three accessions of *Achillea millefolium* L. (common yarrow) leaf; *Acorus americanus* (Raf.) Raf. (sweetflag) root-rhizome; *Actaea racemosa* L. (black cohosh) root-rhizome; *Arctium lappa* L. (greater burdock) root; *Arctostaphylos uva-ursi* (L.) Spreng. (kinnikinnick) leaf; *Berberis vulgaris* L. (common barberry) bark; wild-harvested *Coptis trifolia* (L.) Salisb. var. *groenlandica* (Oeder) Fassett. (threeleaf goldthread) aerial tissues; *Equisetum arvense* L. (field horsetail) stem; *Gaultheria procumbens* L. (eastern teaberry) leaf; *Hydrastis canadensis* L. (goldenseal) root-rhizome; *Ledum* sp. (Labrador tea) leaf; *Mahonia aquifolium* (Pursh.) Nutt. (Oregon grape) root; *Oenothera biennis* L. (common evening-primrose) leaf; *Polygala senega* L. (Seneca snakeroot) root; wild-harvested *Prunus serotina* Ehrh. (black cherry) bark; *Rhodiola rosea* L. (roseroot stonecrop) root; *Rumex acetosella* L. (common sheep sorrel) aerial tissues; *Sambucus* sp. (elderberry) flower; *Vaccinium* sp. (blueberry) leaf; and wild-harvested *Zanthoxylum americanum* P. Mill. (common prickly ash) bark material. Each bar represents one isozyme where N = 2 - 6 replicate analyses per species for CYP2C19; N = 2 - 13 for CYP3A4 and N = 2 - 12 for CYP19. Significant differences ($p < 0.05$) in overall mean percent CYP inhibition among isozymes for each species individually and among species were determined using analysis of variance or Kruskal-Wallis analysis followed by Scheffe's post hoc multiple comparison analysis. Significant differences in CYP inhibition among isozymes for each species are indicated by sets of distinct letters (a – z and II - IV) and those among species are indicated by capital letters (A, B).

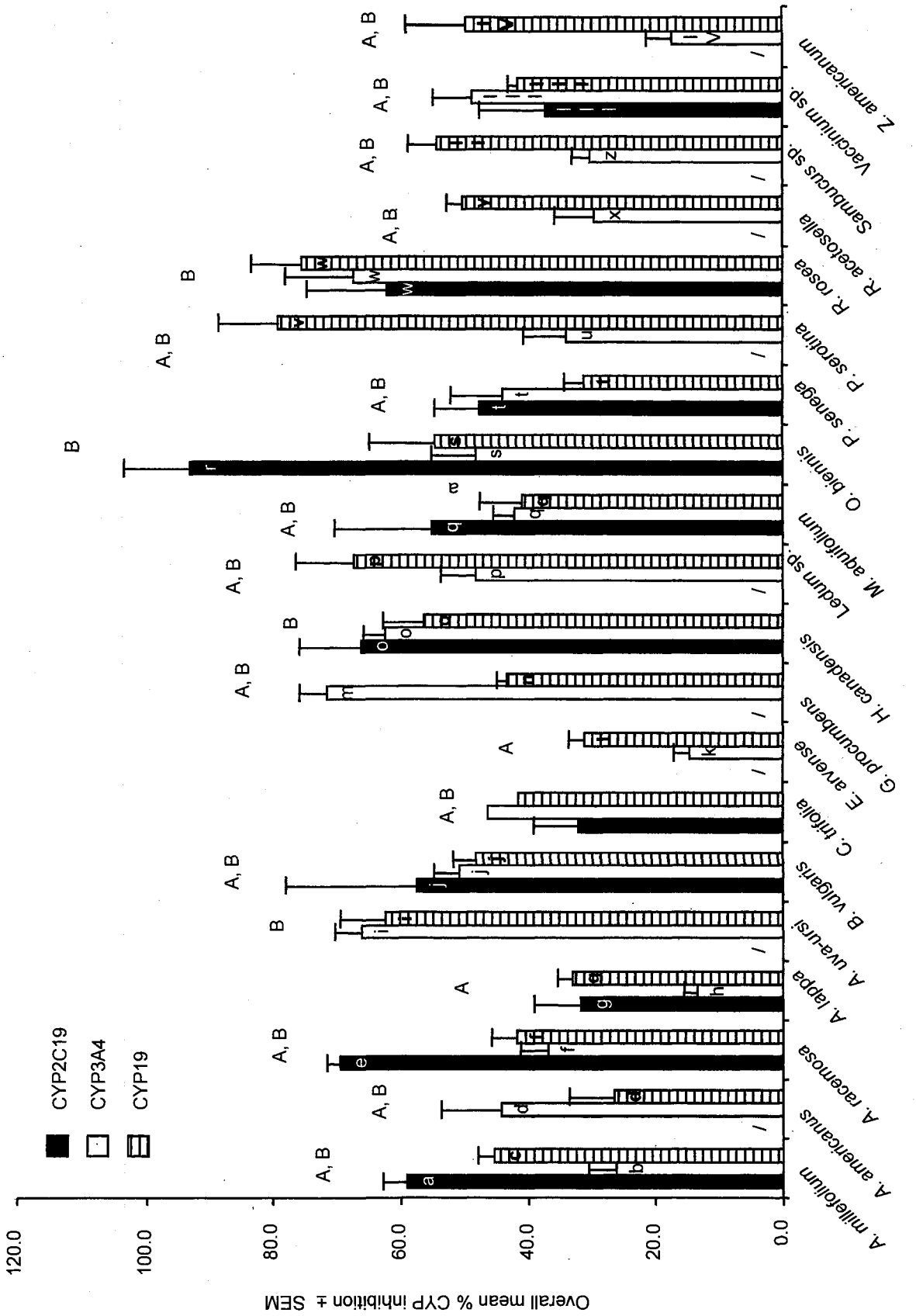
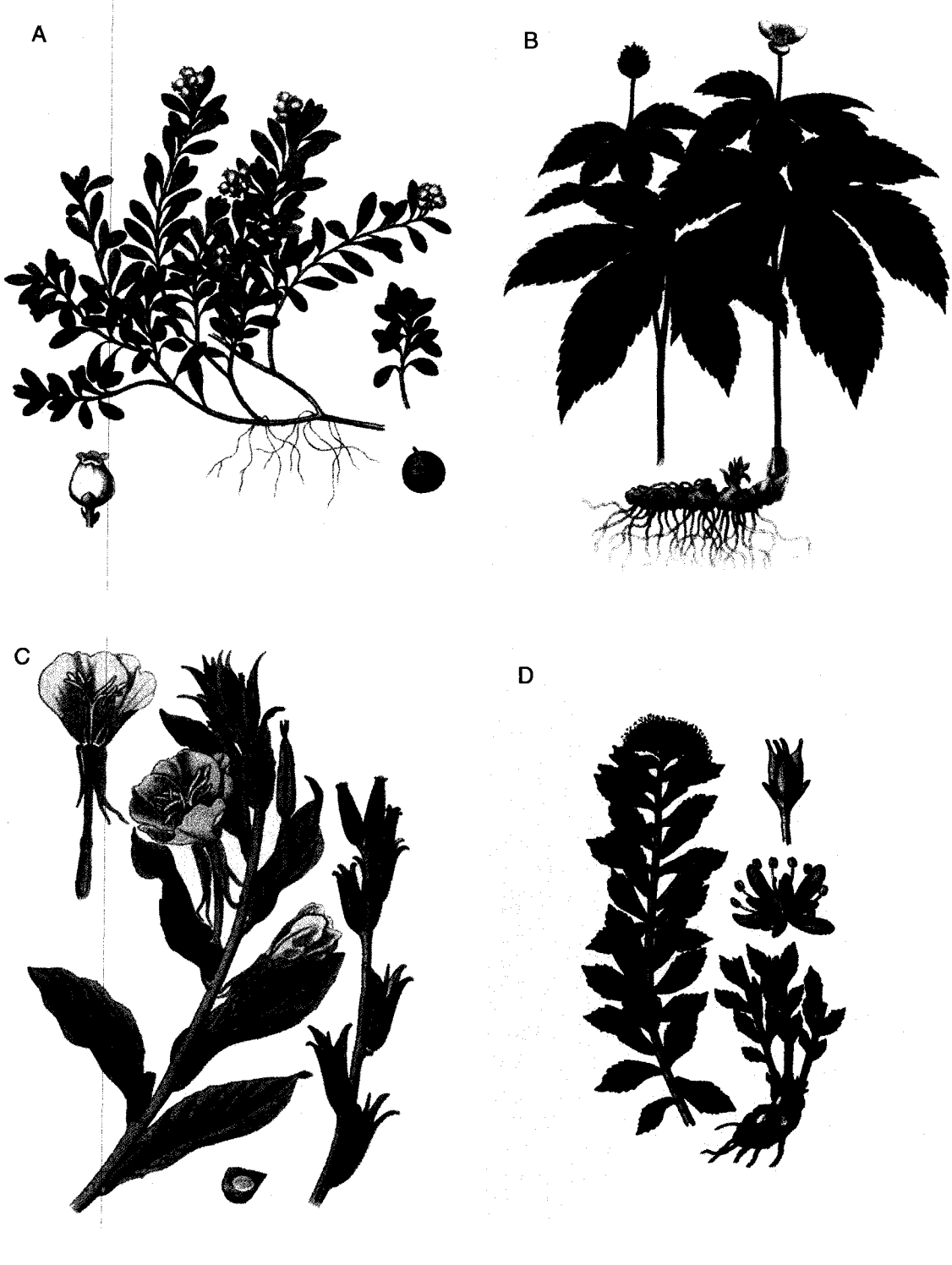

Overall mean % CYP inhibition ± SEM
120.0
100.0
80.0
60.0
40.0
20.0
0.0
CYP2C19
CYP3A4
CYP19
A. millefolium
A. americanus
A. racemosa
A. lappa
A. uva-ursi
B. vulgaris
C. trifolia
E. arvense
G. procumbens
H. canadensis
Ledum sp.
M. aquifolium
O. biennis
P. senega
P. serotina
R. rosea
R. acetosella
Sambucus sp.
Vaccinium sp.
Z. americanum

Figure 3.5 Schematic illustrations of A) *Arctostaphylos uva-ursi* (L.) Spreng (kinnikinnick), B) *Hydrastis canadensis* L. (goldenseal), C) *Oenothera biennis* L. (common evening-primrose) and D) *Rhodiola rosea* L. (roseroot stonecrop), the top four inhibitors of human CYP2C19-, CYP3A4- and CYP19-mediated metabolism in vitro. From Small and Catling (*14*), reproduced with permission of NRC Research Press, pp 20, 82, 96 and 134.


A
B
C
D

Today, *A. uva-ursi* is a Canadian crop harvested for its leaves and is included in many tea preparations, particularly those marketed in Europe, as a urinary antiseptic and as an astringent for the treatment of urethritis and cystitis (*14*, *158*). *Arctostaphylos uva-ursi* crude extract contains three main groups of pharmaceutically relevant compounds: tannins, flavonoids and phenols, with arbutin (hydroquinone-β-D-monoglucopyranoside) being the main phenolic compound (*159*, *160*). The medicinal properties of *A. uva-ursi* are mostly due to hydroquinone, a phenol derived from arbutin. When arbutin is ingested, it is absorbed via a glucose transporter in the lower intestine (*161*) and hydrolyzed in the gut to glucose and hydroquinone (*157*). Hydroquinone is absorbed into the liver where it is glucuronidated and then carried to the kidneys where it is excreted in the urine. If urine pH is less than 7, hydroquinone glucuronide decomposes releasing hydroquinone that acts as a direct antimicrobial agent in the urinary tract (*157*). Although a previous study found *A. uva-ursi* inhibited tyrosinase activity (*162*), the current study is the first to report the inhibitory effect of *A. uva-ursi* towards CYP3A4, CYP19 and CYP2C19.

Oenothera biennis is a wildflower and a Canadian medicinal crop. Whole plant infusions were traditionally consumed to ease pain caused by asthma, gastrointestinal disorders and whooping cough (*14*). Its seed oil is rich in linoleic acid and γ-linolenic acid (*163*) and is currently sold in supplements and specialty foods marketed for infants and seniors (*14*). *Oenothera biennis* is also commonly used for treatment of menopausal symptoms despite findings that have shown it does not offer any measurable benefits over placebo (*164*). A recent study by Pellegrina et al. (*165*), who investigated the anti-tumour potential of *O. biennis* seed, found the phenolic fraction purified from defatted seed inhibitory towards the incorporation of ^{3}H-thymidine during cell growth in human colon carcinoma $CaCo_2$ cells and mouse fibrosarcoma WEHI164 cells. The current study is the first to report CYP inhibition by *O. biennis* leaf.

Rhodiola rosea is a Canadian medicinal crop plant whose wild populations are mostly distributed at high altitudes in the arctic coastal regions of North America. It is also commonly found in Greenland, Iceland and subarctic areas of Eurasia (*14*). At this time a large number of recently

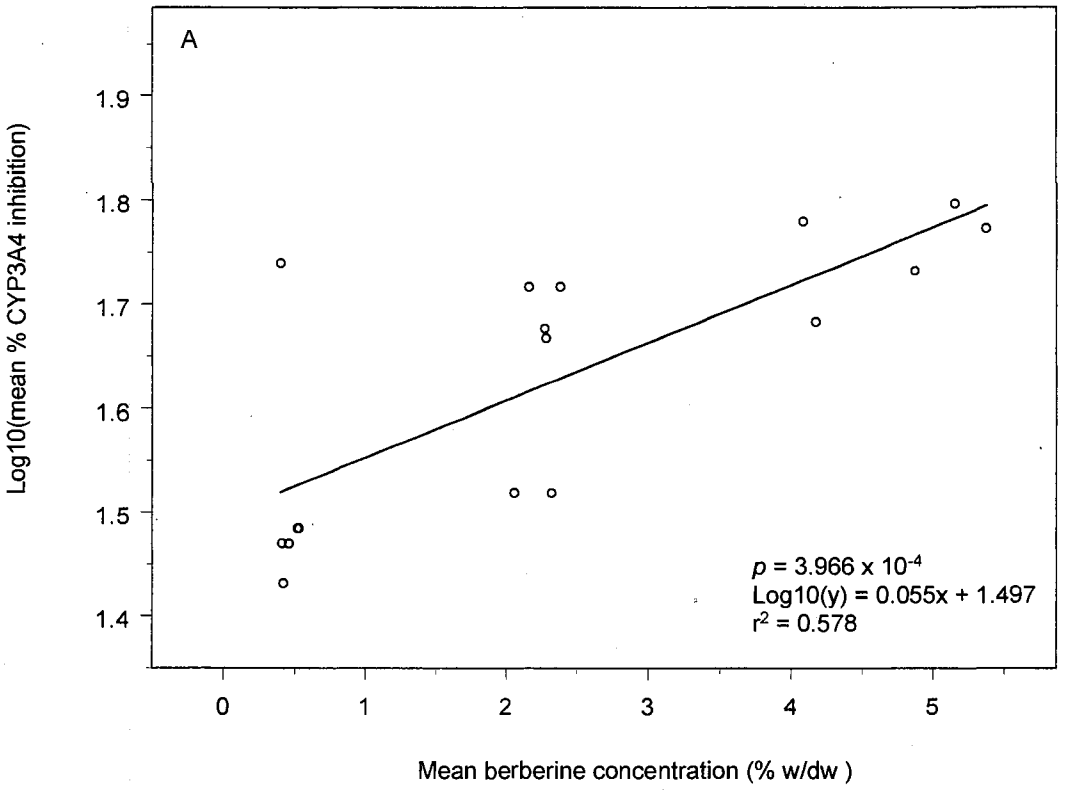
discovered wild populations in Nunavik, Quebec, were sampled by Filion et al. (*166*) for phytochemical analysis. Their findings to date suggest that these populations contain rosavins and salidroside, both marker compounds for the species. *Rhodiola rosea* is a traditional medicinal herb in Asia and Europe, variously believed to help increase physical endurance, work productivity, longevity, resistance to high altitude sickness, fatigue, and ameliorate depression, anemia, impotence, gastrointestinal ailments, infections, and disorders of nervous system. Today, *R. rosea* is used for much of the same reasons, notably as a psychostimulant in psychiatry and neurology, and is widely commercialized as an energy boosting and stress-relieving supplement (*167*). The non-specific, normalizing effects of *R. rosea* on the body are collectively defined as "adaptogenic" (*14*). Much of adaptogenic properties of *R. rosea* derive from readily extractable high concentrations of phenolic compounds like rosarin, a rosavin that is a phenylpropane derivative unique to the species, and salidroside, a phenylethane derivative common to the genus (*168*). Rosarin concentration of the assayed extracts in this study, measured by HPLC-DAD, was found to have a positive relationship with CYP inhibition (*152*). The current study is the first to report data to suggest *R. rosea* root as an inhibitory agent towards CYP-mediated metabolism.

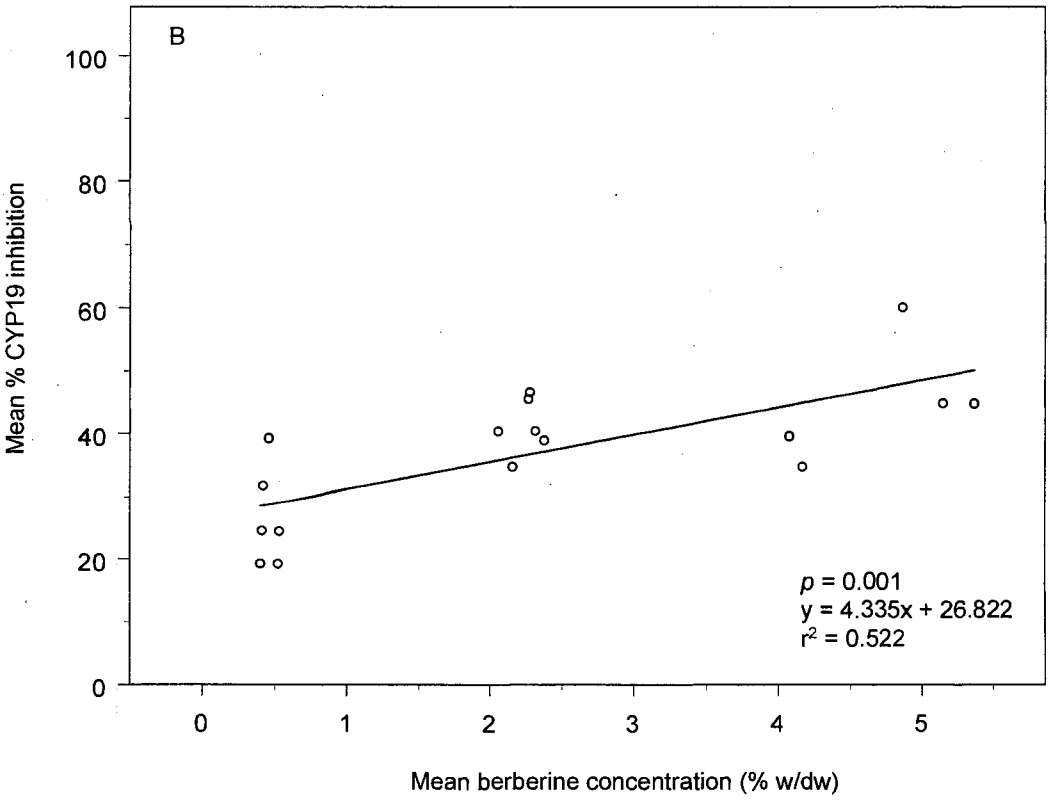
Hydrastis canadensis is another popular Canadian medicinal crop plant, and whose Canadian range is limited to southwestern Ontario. Indigenous people of eastern North America traditionally used *H. canadensis* root-rhizome tinctures as an all-purpose antimicrobial and to treat skin diseases, ulcers, gonorrhea, eye ailments, and cancers (*14*). Currently, the most common clinical use of *H. canadensis* is as an antimicrobial and anti-secretory for mucosal and intestinal infections, cystitis, vaginitis and conjunctivitis (*16*). *Hydrastis canadensis* is also a popular ingredient added to *Echinacea* products since it is thought to help suppress cold and flu symptoms (*47, 118*). Berberine, hydrastine and canadine are the isoquinoline alkaloids responsible for the pharmacological properties of *H. canadensis* and more research has been conducted on the individual alkaloids than on crude extracts of the plant. Berberine alone has demonstrated a broad range of pharmacological properties including significant antimicrobial (*32*); antidiarrheal (*33, 34*); antiarrhythmia (*35*); anti-

inflammatory (*36*); as well as antitumour (*37*) properties. Unlike the other three species, the CYP inhibiting effect of *H. canadensis* and its isoquinoline alkaloids are well documented in the literature. The findings reported here correspond to earlier work conducted in this lab by Budzinski et al. (*111*), who reported ethanolic *H. canadensis* root extracts as being the most potent CYP3A4 inhibitor in vitro compared to 20 other botanicals. Berberine was also found to be significantly inhibitory towards CYP3A4. A more recent study found *H. canadensis* herb tea extracts inhibited CYP3A4-mediated metabolism by 88% (*110*) and a second study reported CYP3A4 most susceptible to inhibition in vitro caused by *H. canadensis* root-rhizome extracts when compared to CYP2C9, CYP2D6 (*109*). CYP3A4 was reported as being 13 times more sensitive to hydrastine compared to berberine, and that hydrastine formed a metabolic-intermediate complex when incubated with the isozymes (*109*). The inhibitory effect of *H. canadensis* towards CYPs has also been documented in vivo by Gurley et al. (*169*) where CYP2D6 and CYP3A4/5 activity were significantly inhibited in twelve healthy volunteers who were administered a 900 mg root extract three times daily for 28 days. However, these findings are contradicted by Sandhu et al. (*170*) who found no statistically significant difference in peak concentration or oral clearance of the CYP3A4 substrate indinavir (a protease inhibitor) in ten healthy volunteers following a fourteen day treatment of 1140 mg of *H. canadensis* root twice daily.

Berberine was quantified by HPLC-DAD in *H. canadensis* root-rhizome extracts, as well as in *B. vulgaris* bark and *M. aquifolium* root extracts. *Hydrastis canadensis* contained the greatest amount of berberine (4.7% ± 0.26% w/dw, N = 5), followed by *B. vulgaris* (2.2% ± 0.05% w/dw, N = 6) and *M. aquifolium* (0.5% ± 0.02% w/dw, N = 6). Given the variation in berberine concentration and in CYP inhibition among the species, the data were analyzed by linear regression and the results are illustrated in Figure 3.6. A positive relationship between increasing berberine concentration and CYP inhibition was statistically significant ($p \leq 0.001$) for CYP3A4 and CYP19, where berberine concentration explained 58% ($r^2 = 0.578$) and 52% ($r^2 = 0.522$) of the variation in CYP inhibition, respectively. These results were supported by the literature cited above. To further verify this

Figure 3.6 Linear regression analyses for mean berberine concentration (% w/dw) in extracts A) versus mean % CYP3A4 inhibition, and B) versus CYP19 inhibition using the preliminary analytical data (Table 3.1, Figures 3.1, 3.2 and 3.3) for the extracts prepared with *Berberis vulgaris* L. (common barberry) bark, *Hydrastis canadensis* L. (goldenseal) root-rhizome and *Mahonia aquifolium* (Pursh) Nutt. (Oregon grape) root. N = 17 extracts for berberine analysis, N = 37 extracts for CYP3A4 inhibition and N = 30 extracts for CYP19 inhibition. Note the data set for CYP3A4 inhibition was transformed by logarithm base 10 in order to meet the requirements of the linear model.


A
Log10(mean % CYP3A4 inhibition)
1.9
1.8
1.7
1.6
1.5
1.4
0
1
2
3
4
5
Mean berberine concentration (% w/dw)
p = 3.966 x 10-4
Log10(y) = 0.055x + 1.497
r2 = 0.578


B
Mean % CYP19 inhibition
100
80
60
40
20
0
0
1
2
3
4
5
Mean berberine concentration (% w/dw)
p = 0.001
y = 4.335x + 26.822
r2 = 0.522

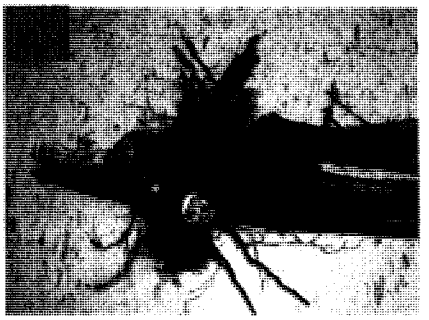
relationship, the analyses were repeated using a lower extract concentration, a greater number of accessions and a greater variety of berberine-containing species (Section 3.3.2).

Since in vitro tests do not holistically represent the conditions found in humans, the findings suggest that there is potential for interactions and that caution should be used when preparations of the botanicals analyzed in this study are taken concomitantly with drugs or other botanicals, in particular those that are primarily metabolized by CYP2C19, CYP3A4 and CYP19. The high CYP inhibitory qualities of these botanicals make them good candidates for further study.

3.3.2 Berberine analysis & follow-up in vitro CYP3A4 inhibition assays: A total of 24 ethanolic extracts (0.5 mg/mL 55% EtOH final concentration) prepared from several accessions of commercially available *B. vulgaris* root and bark, *C. trifolia* var. *groenlandica* rhizome, *E. californica* dried herb, *H. canadensis* root-rhizome, *M. aquifolium* root and *S. canadensis* root-rhizome were assayed for their ability to inhibit the in vitro metabolism of DBF by human CYP3A4. Photographs of each species in the wild are displayed in Figure 3.7, and smaller inset photographs were included to show the medicinal tissues of select species. Source and material details for each sample accession are listed in Table 3.2. Following each assay berberine was quantified in each extract by HPLC-DAD. The variation in berberine concentration and in CYP inhibition among species as well as among their respective accessions is discussed below followed by an analysis of the relationship between berberine concentration and CYP inhibition.

The chromatograms in Figure 3.8 show the chemical profiles for each species. With the exception of *B. vulgaris* bark accession RIL7, all same-species extracts, including those prepared with material from different accessions, produced chromatograms with matching chemical profiles (data not shown); furthermore, all species produced unique chemical profiles. With the exception of RIL7, these results confirmed relative product homogeneity among same-species material and provided phytochemical fingerprints for each species.

Figure 3.7 Photographs of A) wild *Berberis vulgaris* L. (common barberry) and a) its roots and stem; B) wild *Coptis trifolia* (L.) Salisb. var. *groenlandica* (Oeder) Fassett. (threeleaf goldthread) and b) a sample showing its rhizome; C) wild *Eschscholzia californica* Cham. (California poppy); D) wild *Hydrastis canadensis* L. (goldenseal) and d) its rhizome and roots; E) wild *Mahonia aquifolium* (Pursh.) Nutt. (Oregon grape); and F) wild *Sanguinaria canadensis* L. (bloodroot) and f) a sample showing its rhizome and roots. The photographs in panels A, B, D and F were taken by the author and those in panels C and E were taken by Dr. Nancy J. Turner.



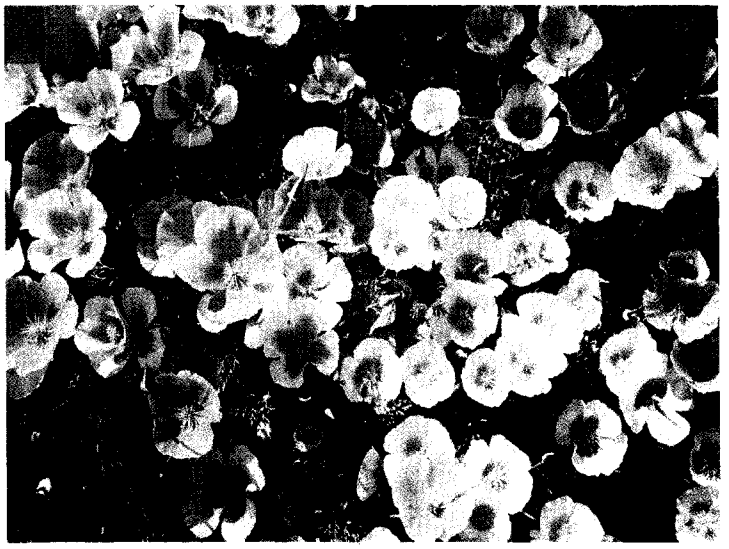
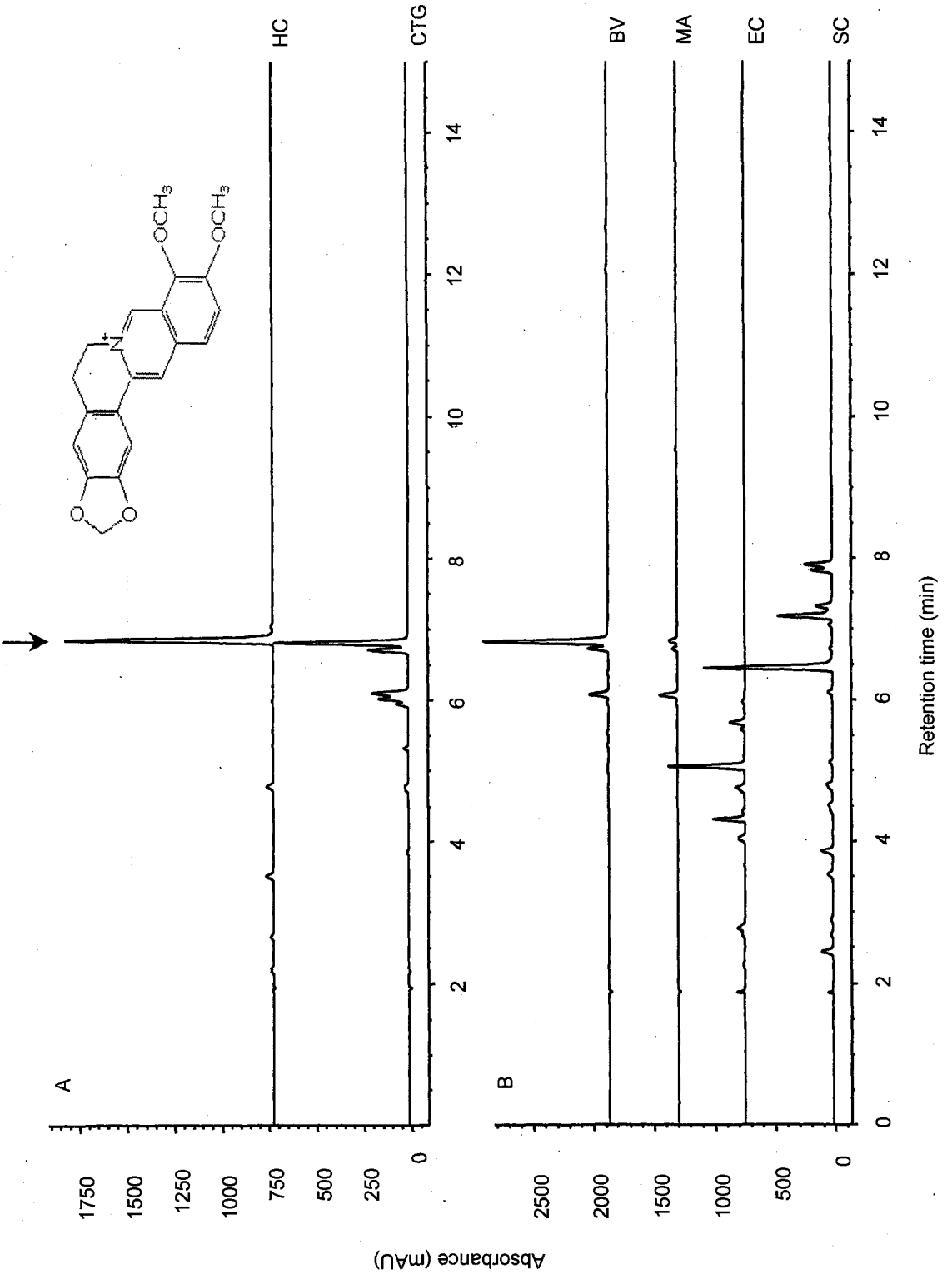


Figure 3.8 Overlap of representative HPLC chromatograms of A) *Coptis trifolia* (L.) Salisb. var. *groenlandica* (Oeder) Fassett (threeleaf goldthread) rhizome (CTG) and *Hydrastis canadensis* L. (goldenseal) root-rhizome (HC); and B) *Berberis vulgaris* L. (common barberry) bark and root (BV), *Eschscholzia californica* Cham. (California poppy) herb (EC), *Mahonia aquifolium* Pursh (Nutt.) (Oregon grape) root (MA) and *Sanguinaria canadensis* L. (bloodroot) root-rhizome (SC) crude extracts prepared with raw commercial material. Arrow indicates berberine peak in the profile detected at 350 nm. The chemical structure of berberine is shown. Note berberine was not detected in *E. californica* and *S. canadensis* extracts.


A
HC
CTG
B
BV
MA
EC
SC
OCH3
OCH3
N+
O
O
O
Absorbance (mAU)
Retention time (min)
0
250
500
750
1000
1250
1500
1750
2000
2500
2
4
6
8
10
12
14

Berberine was identified and quantified in all of the extracts except for those of *E. californica* herb and *S. canadensis* root (N = 13 samples) and it was also not detected in *B. vulgaris* accession RIL7. The unexpected absence of berberine from RIL7 was further confirmed by comparing its chromatographic profile with those of the other *B. vulgaris* accessions in Figure 3.9; the profile varied quite distinctively and as a result, the accession was not considered an accurate representative of the species and was not included in the regression analysis of berberine concentration and CYP inhibition. Nevertheless, RIL7's inhibitory effect on CYP3A4 metabolic activity was included in the discussion below.

There are many possible explanations for RIL7's unique chemical profile. The product may have been mislabeled and/or the sample may have been a related species or a species with similar physical features but from an entirely different genus. If RIL7 was in fact sampled from true *B. vulgaris* individuals, it may be that the sample represented a novel chemotype within the species (*139*) or a product of the influence of unique and/or extreme environmental conditions. Alternatively, the sample may have been harvested very young or old, or its constituents may have experienced degradation from extreme storage or processing conditions (*75*). The seed source and the exact growing location and conditions were not disclosed therefore it was not possible to understand RIL7's unique chemical profile.

The quantitative statistics for berberine concentration and CYP inhibition are found in Figure 3.10. Each bar, in both panels, represents the mean of two or three replicate assays or analyses for one sample accession.

Of the species that contained berberine, significant differences in mean berberine concentration among species and among accessions of the same species were observed (Figure 3.10). Among species, *B. vulgaris* (excluding RIL7), *C. trifolia* var. *groenlandica* and *H. canadensis*, inclusively, contained the greatest concentration of berberine (3.3% ± 0.27% w/dw, N = 24) and *M. aquifolium* contained the least (0.6% ± 0.06% w/dw, N = 19). To our knowledge, this is the first study to survey and compare a number of different North American berberine-containing

Figure 3.9 Overlap of representative HPLC chromatograms of extracts of three accessions of commercial *Berberis vulgaris* L. (common barberry) bark material (RIL7, RIL8 and RIL9) and of two accessions of root material (RIL10 and RIL11). Arrow indicates berberine peak in the profile detected at 350 nm. Note the difference in chromatographic profile between accession RIL7 and accessions RIL8, RIL9, RIL10 and RIL11. Berberine was not detected in accession RIL7 and it was therefore considered non-representative of the species.

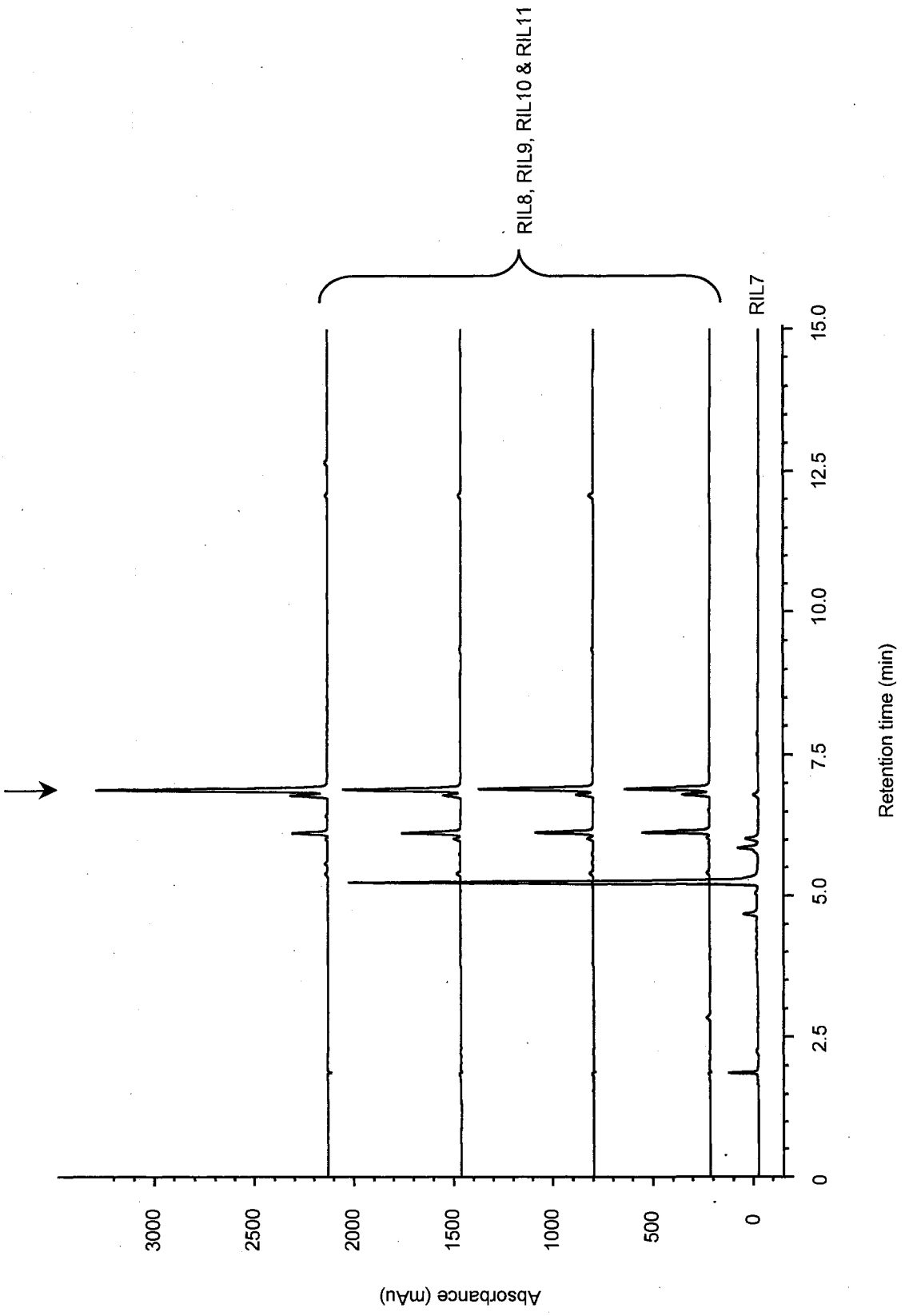
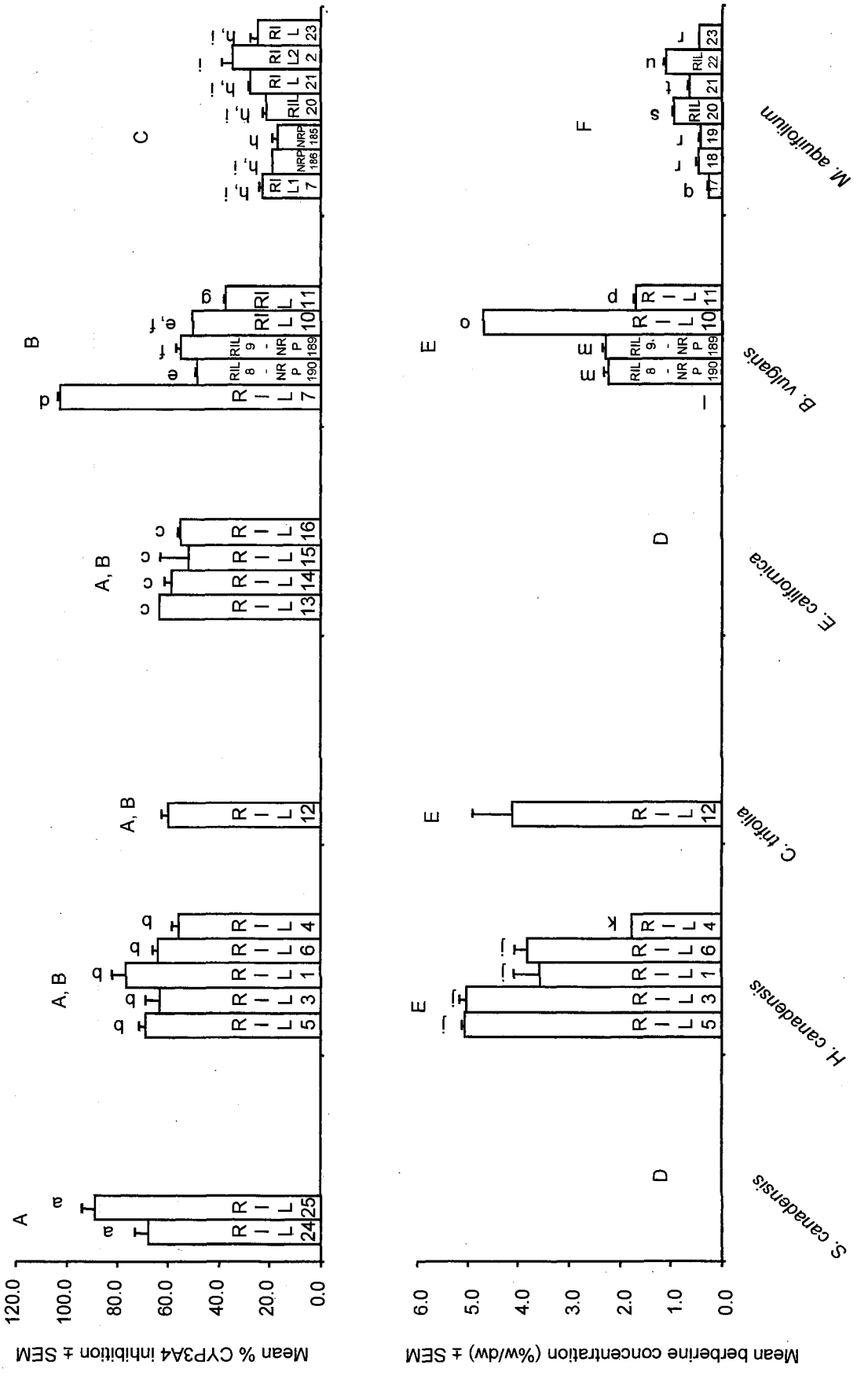

RIL8, RIL9, RIL10 & RIL11
RIL7
Absorbance (mAu)
3000
2500
2000
1500
1000
500
0
0
2.5
5.0
7.5
10.0
12.5
15.0
Retention time (min)

Figure 3.10 Mean percent inhibition (± SEM) of human CYP3A4-mediated metabolism in vitro and mean berberine concentration (% w/dw ± SEM) of 0.5 mg/mL 55% extracts of commercial samples from one to seven accessions of *Sanguinaria canadensis* L. (bloodroot) root-rhizome; *Hydrastis canadensis* L. (goldenseal) root-rhizome; *Coptis trifolia* (L.) Salisb. var. *groenlandica* (Oeder) Fassett (threeleaf goldthread) rhizome; *Eschscholzia californica* Cham. (California poppy) herb; *Berberis vulgaris* L. (common barberry) bark (RIL7, RIL8 and RIL9) and root (RIL10 and RIL11); and *Mahonia aquifolium* Pursh (Nutt.) (Oregon grape) root material. Each bar represents one accession where N = 2 - 13 replicate analyses per accession for both CYP inhibition and berberine analysis. The Nutraceutical Research Programme (NRP) number and the Leduc (RIL) accession number are indicated for each accession and correspond with Table 3.2. Significant differences ($p < 0.05$) in mean percent CYP3A4 inhibition among accessions of the same species and among species, and significant differences in mean berberine concentration among accessions of the same species and among species were determined by one-way analysis of variance or Kruskal-Wallis analysis followed by Scheffe's post hoc multiple comparison analysis. Significant differences in CYP3A4 inhibition among accessions are indicated by sets of distinct letters (a – i) for each species and those among species are indicated by capital letters (A, B, C). Significant differences in berberine concentration among accessions are indicated by sets of distinct letters (j – t) for each species. Note since the chromatographic profile of *B. vulgaris* RIL7 did not match those of other *B. vulgaris* accessions (see Figure 3.9), it was considered non-representative of its species.


Mean % CYP3A4 inhibition ± SEM
Mean berberine concentration (%w/dw) ± SEM
S. canadensis
H. canadensis
C. trifolia
E. californica
B. vulgaris
M. aquifolium

commercial botanicals with *H. canadensis*.

Berberine concentration differed significantly among the accessions of *H. canadensis*, *B. vulgaris* and *M. aquifolium*. For *H. canadensis*, accession RIL4 was found to have significantly less berberine ($p < 0.05$) compared to accessions RIL1, RIL3, RIL5, and RIL6. For *B. vulgaris*, the greatest amount of berberine was detected in accession RIL10 root (4.7% ± 0.01, N = 3) followed by accessions RIL8 bark and RIL9 bark (2.2% ± 0.05%, N = 6) and accession RIL11 root (1.7% ± 0.06%, N = 3). For *M. aquifolium*, accession RIL22 contained the greatest amount of berberine (1.1% ± 0.04, N = 2), followed by accession RIL20 (0.9% ± 0.01, N = 3), accession RIL21 (0.6% ± 0.01, N = 3) and accessions RIL18, RIL19 and RIL23 (0.5% ± 0.02%, N = 8); and the least amount of berberine was measured in extracts of accession RIL17 (0.2% ± 0.02%, N = 3).

As summarized by Foster et al. (*75*), variation in phytochemical yield and production are largely due to a combination of several pre-harvest and post-harvest factors. Pre-harvest factors generally occur at the molecular level (e.g. genetic predispositions, light and nutrient availability during growth, age and season of harvest) whereas post-harvest factors like storage and processing conditions can cause degradation (*130-138*). Given the limited source information provided with the samples, it was not possible to attribute a cause to the observed differences in berberine concentration among accessions.

CYP inhibition assay results illustrated in Figure 3.10 showed significant differences ($p < 0.05$) in mean percent CYP3A4 inhibition among species as well as among the accessions of certain species. When comparing percent inhibition among species, *S. canadensis* root-rhizome extracts were the most potent inhibitors (80.5% ± 6.16%, N = 5) followed by *C. trifolia* var. *groenlandica* rhizome, *E. californica* herb and *H. canadensis* root-rhizome (62.0% ± 1.92%, N = 20). *Berberis vulgaris* (root and bark inclusively) inhibited CYP3A4-mediated metabolism by 55.7% ± 5.57% (N = 14) and *M. aquifolium* root was the least inhibiting species (23.9% ± 1.64%, N = 14). The overall range in mean percent CYP3A4 inhibition was 23.9% ± 1.64% to 80.5% ± 6.16, with an overall mean of 52.0% ± 3.02% (N = 54).

For *B. vulgaris* accessions, there were significant differences in CYP3A4 inhibition. Accession RIL7 (not a confirmed representative of the species) was the top inhibitor (102.4% ± 2.42%, N = 2), followed by RIL9-NRP189 (55.1% ± 2.08%, N = 3), RIL10 (50.4% ± 0.14, N = 3), RIL8-NRP190 (48.9% ± 0.51%, N = 3) and RIL11 (37.3% ± 0.60%, N = 3). Accessions RIL8-NRP190 and RIL9-NRP189 were bark samples and accessions RIL10 and RIL11 were root samples. For *M. aquifolium* root, accession RIL22 was the most potent CYP3A4 inhibitor (34.7% ± 4.35%, N = 2) followed by RIL21 (27.4% ± 0.82%, N = 2). The remaining *M. aquifolium* accessions inhibited CYP3A4 metabolism in the following decreasing order: RIL17 (22.9% ± 1.43%, N = 2) > RIL20 (21.5% ± 1.43%, N = 2) > RIL18, RIL19 and RIL23 (20.2% ± 1.71%, N = 6). All *M. aquifolium* samples consisted of root material with matching chemical profiles (data not shown).

After extensive review of the literature, it was found that with the exception of *H. canadensis* and *E. californica*, this was the first study to assay crude extracts of *B. vulgaris* root and bark, *C. trifolia* var. *groenlandica* rhizome, *M. aquifolium* root and *S. canadensis* root-rhizome with human drug metabolizing CYPs. The existing literature on inhibition is reviewed below for each species in decreasing order of inhibitory potency. Vouchers of wild *B. vulgaris*, *C. trifolia* var. *groenlandica* and *S. canadensis* can be found in the Appendix.

Sanguinaria canadensis root extracts have been shown to be inhibitory towards 5-lipoxygenase synthesis of leukotriene B_4 and cyclooxygenase 1 and 2 synthesis of prostaglandin (*171*, *172*). Furthermore, sanguinarine, extracted from *S. canadensis* root, strongly inhibited Na-K-dependent ATPase isolated from guinea pig brain and myocardium (*173*, *174*), NF-κB phosphorylation and degradation of IκBα protein (*175*) as well as vascular endothelial growth factor-induced Akt phosphorylation (*176*, *177*).

Coptis trifolia var. *groenlandica* water extracts were found to be the most effective against the growth of five human liver-cancer cell lines (HepG2/C3A, HA22T/VGH, SK-HEP-1, Hep3B and PLC/PRF/5) compared to fourteen other botanical species (*178*).

Preliminary research conducted by Tom's of Maine® and the University of Ottawa showed

that *E. californica* tea and ethanolic extracts both inhibited CYP19- and CYP3A4-mediated metabolism, where the ethanolic extracts were more inhibitory than the teas and CYP19 was more sensitive to inhibition than CYP3A4 (*179*). A recent study published by Gafner et al. (*180*) found *E. californica* extracts containing 1.82% total alkaloids were inhibitory towards CYP3A4 activity (IC_{50} value of 128.6 ± 61.8 μg/mL). Escholtzine, a benzylisoquinoline alkaloid, was the most inhibiting constituent in these extracts.

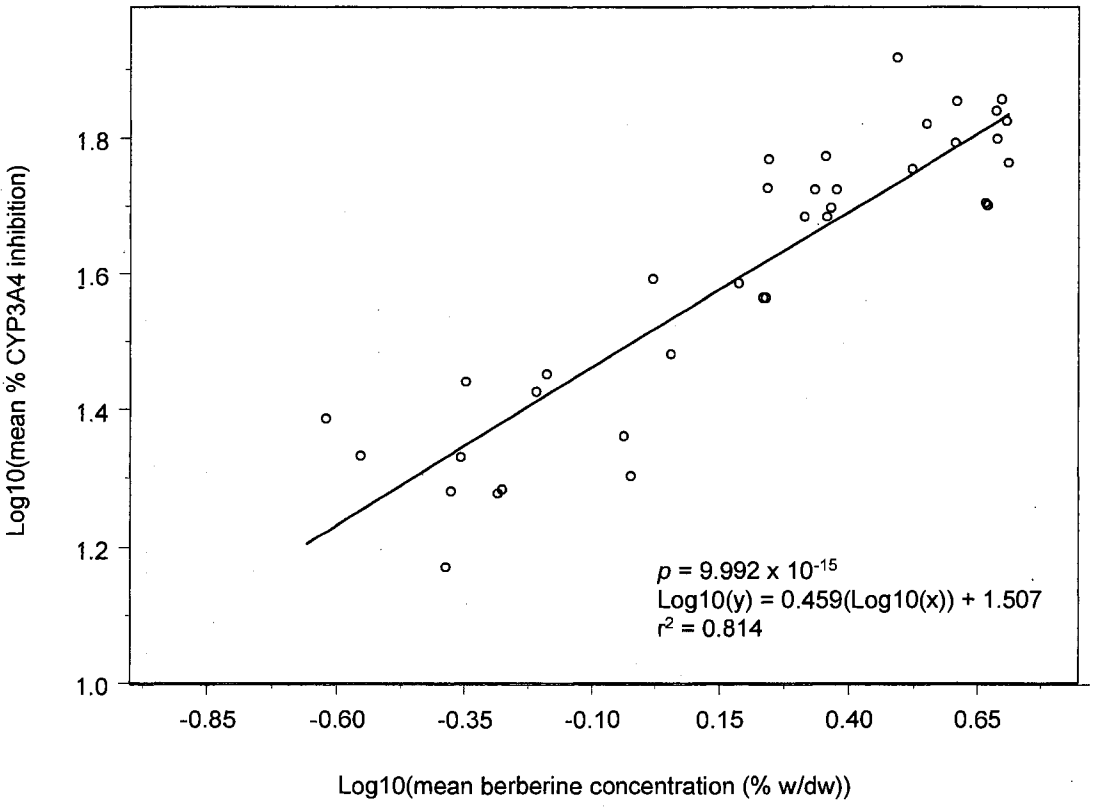
As mentioned earlier, the inhibitory effect of *H. canadensis* on CYP3A4-mediated metabolism has been well documented in the literature and with the exception of an in vivo study by Sandhu et al. (*170*) our findings support those described in the literature.

Research on *B. vulgaris* has focused on the pharmacological effects of the berries rather than the leaves, stems and root. A study by The Local Food-Nutraceuticals Consortium (*181*) identified crude ethanolic extracts of dried *B. vulgaris* leaves and tender stems as one of the top overall inhibitors of xanthine oxidase, acetylcholine esterase and myeloperoxidase-catalysed guaiacol oxidation out of a total of 127 botanical species.

Previous studies with *M. aquifolium* have reported approximate 30% inhibition of interleukin-8 production in cells stimulated with lipopolysaccharide by crude ethanolic extracts (20 μg/ mL) of stem bark (*182*, *183*). The alkaloid fraction isolated from crude ethanolic extracts of roots and stems had a potent inhibitory effect on lipoxygenase and lipid peroxidation (*184-186*). Muller et al. (*187*) found bark extracts particularly inhibiting towards keratinocyte growth with an IC_{50} of 35 μM.

Figure 3.11 illustrates the linear model for overall CYP3A4 inhibition and overall berberine concentration in all of the assayed botanicals except for *E. californica*, *S. canadensis* and *B. vulgaris* (RIL7), where N = 39 for CYP inhibition and N = 43 for berberine analysis. The linear model confirmed a positive relationship between berberine concentration and CYP3A4 inhibition ($p < 0.001$) with 81% of the inhibiting effect being berberine-dependent ($r^2 = 0.814$). Given berberine's pharmacological properties, its significant contribution towards CYP inhibition is not surprising.

Figure 3.11 Linear regression analyses for mean berberine concentration (% w/dw) in extracts versus mean % CYP3A4 inhibition. The regression model was analyzed using follow-up analytical data (Table 3.1 and Figure 3.10) for the extracts prepared with *Berberis vulgaris* L. (common barberry) bark (RIL8 and RIL9) and root (RIL10 and RIL11); *Coptis trifolia* (L.) Salisb. var. *groenlandica* (Oeder) Fassett (threeleaf goldthread) rhizome; *Hydrastis canadensis* L. (goldenseal) root-rhizome; and *Mahonia aquifolium* Pursh (Nutt.) (Oregon grape) root material. N = 45 extracts for berberine concentration and N = 41 extracts for CYP3A4 inhibition. Note the data sets were transformed by logarithm base 10 in order to meet the requirements of the linear model.


Log10(mean % CYP3A4 inhibition)
1.8
1.6
1.4
1.2
1.0
-0.85
-0.60
-0.35
-0.10
0.15
0.40
0.65
Log10(mean berberine concentration (% w/dw))
p = 9.992 x 10-15
Log10(y) = 0.459(Log10(x)) + 1.507
r2 = 0.814

Other studies have also described berberine as a CYP inhibitor, for example Budzinski et al. (*111*) ranked berberine sixth top CYP3A4 inhibitor out of eleven other botanical compounds. Berberine was also found to be more inhibitory than hydrastine towards CYP2D6, but less inhibitory towards CYP2C9 and CYP3A4 (*109*). Other inhibitory properties of berberine include the dose-dependent inhibition of interleukin-1β induced disruption of the barrier function in human retinal pigment epithelial cell line ARPE-19 (*188*); the in vitro inhibition of the peroxisome proliferator-activated receptors γ and α, as well as causing the suppression of 3T3-L1 adipocyte differentiation (*189*); the inhibition of potassium and calcium currents in isolated rat hepatocytes (*190*); the inhibition of human keratinocyte cell line (HaCaT) growth used as a model for epidermal hyperproliferation in psoriasis (*187*); and the inhibition of sortase, a bacterial surface protein anchoring transpeptidase, from *Staphylococcus aureus* ATCC 6538p (*191*).

Our findings, confirmed by the literature cited above, found that berberine concentration in botanical extracts is a reliable indicator for in vitro inhibition of CYP-mediated drug metabolism. While in vitro results are difficult to relate to the clinical setting, caution is advised when berberine-containing botanical products are taken concurrently with pharmaceutical drugs or other botanicals. Given the high CYP inhibitory qualities of the extracts, the species in this study warrant further in-depth investigation.

3.4 Conclusion

The results showed that crude extracts prepared from various botanicals have the potential to inhibit human CYP-mediated drug metabolism, thus potentially modulating both the efficacy and toxicity of co-administered drugs and/or other therapeutic botanicals. These findings were supported by the literature and berberine was found to play a significant role in the inhibiting capacities of the botanicals and may be a marker for inhibition. However, the phytochemical complexity of crude extracts ultimately defines CYP inhibition.

Many plant species are used as medicines but few have been tested. This study extended the number of botanicals found to have the potential to interfere with the disposition of drugs in the body by inhibiting CYP-mediated metabolism.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSION

4.1 General Discussion

The overall objectives of this thesis were to address the lack of phytochemical data on the wild populations of one of Canada's most valued botanicals, *H. canadensis* (Chapter 2), and to extend the baseline in vitro data on the inhibition of CYP-mediated metabolism by North American botanicals, with particular interest in berberine as a marker for inhibition (Chapter 3).

To our knowledge this was the first study to report the phytochemical variation among wild Canadian *H. canadensis* populations, to investigate alkaloid levels in above-ground tissues (stem-leaves and berries) and to compare the data for these wild samples with those of cultivated material.

Of particular significance, alkaloid concentration was found to vary significantly among certain wild populations. This suggests genetic diversity within the species and could lead to the discovery of a superior genotype that could be used to increase the potency of crop plants. Further to this, it was found that cultivated plants are no different in phytochemical concentration and quality compared to those in the wild, thereby supporting the use of cultivated plants as the exclusive source for the medicinal plant market. Similar findings were reported by an independent study by Cech (*50*), who conducted chromatographic comparisons of organically cultivated *H. canadensis* root with wild-harvested samples. Cech (*50*) found no significant quantitative differences between wild and cultivated samples. As with the current study, Cech also reported variation in alkaloid concentration among commercial root sources.

The results also suggest leaf material as an additional and (more importantly) renewable source of isoquinoline alkaloids since measurable amounts of these compounds were detected (e.g. wild leaf contained 60% of the concentration of berberine found in wild root-rhizome).

Moreover, the analytical approach assisted in characterizing quantitative features unique to different tissues (above-ground versus below-ground). Knowledge of these features can be useful in product quality control programs that would identify commercial products adulterated with non-identified material (e.g. the use of stem-leaf material instead of, or in addition to, root-rhizomes).

These results have already served as supporting 'actions' in a recently written Recovery Strategy Plan for *H. canadensis*, a series published by Environment Canada (*27*).

The study of Canada's wild *H. canadensis* first derived from a collaborative effort between the University of Ottawa and CWS, with the purpose of assisting in its recovery from threatened status in Canada. The study's methodology was based on the research conducted by two former University of Ottawa graduate students from our laboratory, V. Assinewe (Ph.D.) and S. Binns (Ph. D). Assinewe (*193*) used analytical approaches in characterizing the natural diversity of the secondary metabolites found in wild *Panax quinquefolius* L. and other Araliaceae species in North America, while Binns (*194*) did similar research on several wild *Echinacea* species. Both studies characterized factors like organ type and plant age that influenced phytochemical yield. For example, Assinewe et al. (*195*) reported no statistical difference in mean ginsenoside content between wild and cultivated *P. quinquefolius* roots harvested at four years of age; while Binns et al. (*196*) found the greatest accumulation of cichoric acid in older, wild inflorescences of *E. pallida* var. *sanguinea*, and the greatest concentrations of certain alkamide constituents in wild *E. purpurea* root. Like *H. canadensis*, these *Panax* and *Echinacea* species are important Canadian medicinal crops with extensive histories in North American medicinal folklore (*4*, *14*). They are mostly harvested for their root-rhizomes and as such, this makes their wild stands extremely vulnerable to 'root digging' practice. Since a COSEWIC assessment in 2000, wild *P. quinquefolius* has been considered endangered in Canada, and like *H. canadensis*, both *P. quinquefolius* and most wild *Echinacea* species have experienced widespread global declines (*13*). The results of this study as well as those from Assinewe and Binns provided significant baseline phytochemical data and chromatographic profiles that are valuable tools for the protection and identification, for NHP quality assurance programs and for encouraging and ameliorating the cultivation for commercial use of otherwise extremely vulnerable botanicals.

The results of Chapter 2 provide a starting point for further characterization of wild *H. canadensis*. Future phytochemical studies of the species in the wild should focus on characterizing

the environmental and genetic factors that drive alkaloid yield. These studies should occur within a greater habitat range in each case to further isolate their respective effect, and this would be the next step in fully characterizing the species. Subsequent work should analyze cultivated stem-leaf material and combine the ecological data published by Sinclair et al. (*17, 24, 25, 48, 197, 198, 199*) with our phytochemical data to seek trends, as well as ameliorate *H. canadensis* cell culturing methods (*59, 60*) for the production of alkaloids and other active secondary metabolites (e.g. chlorogenic acid). Cell culturing is also advantageous in that useful metabolites are obtained under a controlled environment, independent of climate and soil variables, as shown in similar research on *Coptis* species (*53, 200, 201, 202*). A further benefit is that there are no negative impacts on wild populations.

Chapter 3 provides baseline data with respect to in vitro botanical-drug interactions for 22 North American botanical species from a total of 13 families, including the seven following Canadian medicinal crop species: *A. millefolium*, *A. uva-ursi*, *H. canadensis*, *O. biennis*, *P. senega*, *R. rosea* and *S. canadensis* (*14*). The primary objectives of this chapter were to identify which species, when extracted with 55% EtOH, were inhibitory towards CYP2C19, CYP3A4 and CYP19 metabolism, and to characterize the relationship between berberine concentration and their inhibiting capacity. The null hypotheses formulated for these objectives were the following: the extracts are not inhibitory towards CYP2C19, CYP3A4 (using two extract concentrations) and CYP19 isozymes; and no relationship exists between berberine concentration and CYP inhibition. The secondary objective was to determine any phytochemical and inhibitory variation among accessions of the same species; the null hypothesis was that no phytochemical nor inhibitory differences exist among accessions.

All species were inhibitory towards all three isozymes. Of the twenty species that were assayed in the first section of the study, extracts of *O. biennis* leaf, *H. canadensis* root-rhizome and *R. rosea* root-rhizome were the top inhibitors of CYP2C19, CYP3A4 and CYP19, respectively. Overall mean percent inhibition (for all three isozymes inclusively) ranged from approximately 21%

to 66%, where the overall top inhibitors included the three species listed above and *A. uva-ursi* leaf. The overall least inhibiting extracts were of *A. lappa* root and *E. arvense* stem. With respect to differences in CYP inhibition among accessions of the same species, mean CYP2C19 inhibition by extracts prepared from different accessions of *A. millefolium* leaf and *Vaccinium* sp. leaf varied significantly among their respective accessions. The inhibition of CYP3A4 by *A. millefolium* extracts also varied among its accessions. It was later discovered that EO concentration in *A. millefolium* was positively correlated with CYP3A4 inhibition, with the exception of carvone, a bicyclic monoterpene, that was found to have a negative relationship with CYP3A4 inhibition *(152)*. Berberine concentration in *B. vulgaris*, *H. canadensis* and *M. aquifolium* was found to explain 52% and 58% of the extracts' inhibiting capacities towards CYP19 and CY3A4, respectively.

Of the six alkaloid bearing species assayed with CYP3A4 in the second section of the chapter, mean CYP inhibition varied significantly. It was found that extracts of *S. canadensis* root-rhizome were the most inhibiting and *M. aquifolium* stem bark were the least. Mean percent CYP inhibition ranged from approximately 22% to 87% and varied significantly among the sample accessions of *B. vulgaris* and *M. aquifolium*. Berberine concentration was measured in all extracts except for those prepared with *E. californica* and *S. canadensis*. *Berberis vulgaris* root and stem bark, C. *trifolia* var. *groenlandica* rhizome and *H. canadensis* root-rhizome extracts contained the greatest amount and *M. aquifolium* stem bark contained the least. Berberine also varied among the accessions of all four species but it was beyond the scope of this study to determine the factors contributing to this variability. Lastly, berberine concentration in the extracts was again found to have a positive relationship with CYP3A4 inhibition whereby it explained 81% of the variation in inhibition.

In support of the relationship between berberine and CYP inhibition, a simulation study by Zhou et al. *(203)* suggested that botanical-drug metabolic interactions may be predicted by the quantity of inhibiting botanical constituents. They also found that the number of inhibitory botanical constituents, among a number of other factors, could predict the extent to which the plasma

concentration-time curve of a drug would increase when the drug was co-administered with a botanical with known inhibiting properties.

The extracts assayed in this study were prepared to reflect the composition of NHPs on the market and berberine measured in these extracts were found to be of a biologically relevant concentration. Since uptake occurs primarily in the small intestine where the majority of CYPs occur (*204*), the experimental process of assaying the extracts directly with a CYP was a relevant representation of the process in the body. Furthermore the overall range in berberine concentration in the total reaction volume (well) for the preliminary CYP inhibition assays (4.0 mg/mL 55% EtOH extract concentration in well) and the follow-up CYP3A4 inhibition assays (0.5 mg/mL 55% EtOH) was compared to the plasma berberine concentrations measured in 56 adult volunteers, after two weeks of orally administered 1.2 g/day berberine therapy (*205*). Plasma berberine concentrations in all samples were measured by HPLC and ranged from 0.07 ± 0.04 μg/mL to 0.19 ± 0.08 μg/mL. In both sections of this study, the extract volume in each experimental well for each CYP isozyme represented 5% of the total volume. In the first section the concentration of the extracts was 4.0 mg/mL 55% EtOH and berberine concentration ranged from 0.40 to 5.37%. This translates into 0.80 μg/mL and 10.74 μg/mL in the wells, respectively. In the second section, extract concentration was 0.5 mg/mL 55% EtOH and berberine ranged from 0.28 to 5.03%. This range translates into 0.07 μg/mL and 1.26 μg/mL per well, respectively. Although berberine concentration in the experimental wells exceeded those measured in plasma, it matched the overall biologically relevant range.

With the exception of the *E. californica* herb and *H. canadensis* root-rhizome extracts, to our knowledge this was the first study to report the inhibitory potential of the extracts of these 20 species towards CYP activity. As such, the findings reported in this study are a considerable contribution to the field of botanical-CYP interactions and general health safety regarding botanicals. A number of other studies have also used recombinant cDNA-expressed human CYP (supersome) in high-throughput screening assays to successfully explore possible botanical-CYP interactions with subsets of NHPs (*110, 111, 206*), with specific botanicals and their purified isolated bioactive constituents

(*108, 112, 207*). The findings of many of these studies resulted in the identification of more clinically relevant botanical-drug metabolism interactions, which have been reviewed by many (*75, 77, 78, 155, 204, 208-213*).

The method used was proven effective in assessing the likelihood of inhibition (*82, 214-216*) and the results for *E. californica* and *H. canadensis* are supported by the literature (*109-111, 179*). Although much faster data acquisition rates have been achieved by using fluorometric substrates in high throughput assays with supersomes compared to more classical approaches (*217*) (for example: radiolabelled probes whose metabolites are detected by HPLC analysis [*218, 219*]), the methodology has its own shortcomings, particularly when attempting to extrapolate the results to in vivo conditions. A general consensus is that the concentrations of accessory proteins under the experimental conditions (supersome) are not entirely similar to human liver microsomes; moreover, heterologously expressed systems typically contain a single CYP interacting with the accessory proteins, whereas in liver microsomes multiple CYPs may compete for interaction with the accessory proteins (*80, 217, 220*). These discrepancies are of concern when measuring metabolic rates and methods have been developed to correct these discrepancies by transforming them into enzyme kinetic data more relevant to human liver conditions (*80, 221*). In terms of interpreting solely CYP inhibition data, Crespi and Miller (*217*) suggested comparing in vitro potency to pharmacological models; if the latter is similar to the CYP inhibition potency then botanical-drug interactions are much more likely to occur. Even under these criteria, results can vary. For example, when administered to healthy volunteers, *H. canadensis* root-rhizome, one of the consistently top CYP2C19, CYP3A4 and CYP19 inhibitors in this study, was found to strongly inhibit CYP2D6 and CYP3A4/5 activity in vivo (*169*). However, in a second in vivo clinical study it was found not to have any effect on CYP3A4 activity in the liver (*170*). Clearly these results altogether make *H. canadensis* an appropriate candidate for further in-depth analysis.

In vitro models explore possible botanical-CYP interactions, resulting in the identification of more clinically relevant outcomes with respect to drug disposition. The identification of bioactive

constituents in botanical preparations alone warrants further health risk assessment. Further in vitro studies should focus on whether inhibition is competitive, noncompetitive or mechanism-based. Additional focus should be directed towards their effect on CYP gene expression and on more advanced in vivo pharmacokinetic clinical studies. The significant CYP inhibition potential of the extracts screened in this study, combined with their widespread availability in the marketplace and their phytochemical complexity, warrant further investigation into their clinical and toxicological roles.

4.2 Conclusion

The results described in Chapter 2 clearly demonstrated no significant difference between wild and cultivated samples in their level of alkaloid concentration. Knowing this, as the use of NHPs becomes more and more widespread, *H. canadensis* material obtained from cultivated sources provides both a phytochemically equivalent and sustainable alternative to wild-harvesting, thus alleviating economic pressure on the species and aiding its long-term stability in the wild. In Chapter 3, extracts prepared from 22 botanicals where found to inhibit human CYP-mediated drug metabolism in vitro by at least 20% and some as much as 90%. While further in vivo studies and more specific analyses of these botanicals would be beneficial, it is clear that caution must be employed when ingesting these botanicals in NHPs or in any other form to prevent possibly harmful botanical-drug interactions.

REFERENCES

1. Cavender, A. Folk Medical Uses of Plant Foods in Southern Appalachia, United States. *J. Ethnopharmacol.* **2006**; *108*, 74-84.

2. Leduc, C.; Coonishish, J.; Haddad, P.; Cuerrier, A. Plants Used by the Cree Nation of Eeyou Istchee (Quebec, Canada) for the Treatment of Diabetes: A Novel Approach in Quantitative Ethnobotany. *J. Ethnopharmacol.* **2006**; *105*, 55-63.

3. Tuner, N.J. Plants of Haida Gwaii, Xaadaa Gwaay gud gina k'aws (Skidegate) Xaadaa Gwaayee guu gin k'aws (Massett). Sononis Press: British Columbia, Canada. 2004.

4. Moerman, D.E. Native American Ethnobotany. Timber Press: Portland. 1998.

5. Ritch-Krc, E.M.; Thomas, S.; Turner, N.J.; Towers, G.H.N Carrier Herbal Medicine: Traditional and Contemporary Plant Use. *J. Ethnopharmacol.* **1996**; *52*, 85-94.

6. Turner, N.J.; Hebda, R.J. Contemporary Use of Bark for Medicine by Two Salishan Native Elders of Southeast Vancouver Island, Canada. *J. Ethnopharmacol* **1990**; *29*, 59-72.

7. Turner, N.J. Counter-Irritant and Other Medicinal Uses of Plants in Ranunculaceae by Native Peoples in British Columbia and Neighbouring Areas. *J. Ethnopharmacol.* **1984**; *11*, 181-201.

8. Arnason, J.T.; Hebda, R.; Richard J.; Johns, T. Use of Plants for Food and Medicines by Native Peoples of Eastern Canada. *Can. J. Bot.* **1981**; *59*, 2189-2325.

9. Moerman, D.E. Symbols and Selectivity: A Statistical Analysis of Native American Medical Ethnobotany. 1979; *J. Ethnopharmacol.* **1979**, *1*, 111-119.

10. Natural Health Products Directorate. List of Licensed Natural Health Products. Last updated May 11 2007. Website http://www.hc-sc.gc.ca/dhp-mps/prodnatur/applications/licen-prod/lists/listapprnhp-listeapprpsn_e.html (viewed May 2007).

11. Bannerman, J.E. Goldenseal In World Trade: Pressures and Potentials. *HerbalGram.* **1997**; *41*, 51.

12. Crellin, J.K.; Philpott, J. A Reference Guide to Medicinal Plants, Herbal Medicine Past and Present. Duke University Press: London, UK. 1990.

13. NatureServe. NatureServe Explorer: Version 6.1. NatureServe, Arlington, Virginia. 2006. Website http://www.natureserve.org/explorer. (viewed April 2007)

14. Small, E.; Catling, P.M. Canadian Medicinal Crops. NRC Research Press: Ottawa, ON, Canada. 1999.

15. Adam, K.L. Ginseng, Goldenseal, and Other Native Roots, Horticulture Technical Note. National Center for Appropriate Technology: Fayetteville, AR. Website http://attra.ncat.org/attra-pub/ginsgold.html (viewed June 2007).

16. Upton, R. (Ed). American Herbal Pharmacopoeia and Therapeutic Compendium, Goldenseal Root *Hydrastis Canadensis* Standards of Analysis, Quality Control, and Therapeutics. American Herbal Pharmacopoeia: Santa Cruz, California. 2001.

17. Sinclair, A.; Catling, P. M. Ontario Goldenseal, *Hydrastis canadensis*, Populations in Relation to Habitat Size, Paths, and Woodland Edges. *Can. Field Nat.* **2000**; *114*, 652-655.

18. Snow, J.M. *Hydrastis canadensis* L. (Ranunculaceae). *Protocol Journal of Botanical Medicine.* **1997**; *2*, 25-8.

19. van Fleet, W. Goldenseal Under Cultivation. *Farmer's Bulletin.* **1914**; *613*, 1-15.

20. Lloyd, J.U. The Cultivation of Hydrastis. *Journal of the American Pharmaceutical Association.* **1912**; *1*, 5-12.

21. White, D.J. Status Report on the Golden Seal *Hydrastis canadensis* L. Committee on the Status of Endangered Wildlife in Canada (COSEWIC). 1990.

22. Convention On International Trade in Endangered Species of Wild Fauna and Flora (CITES). Appendices I, II and III. 2007. Website http://www.cites.org/eng/app/appendices.shtml (viewed June 2007).

23. Committee on the Status of Endangered Wildlife in Canada (COSEWIC). Species Search, Goldenseal *Hydrastis canadensis* L. July 2006. Website http://www.cosewic.gc.ca/eng/sct1/printresult_e.cfm (viewed June 2007).

24. Sinclair, A.; Catling, P.M. Restoration of *Hydrastis canadensis*: Experimental Test of a Disturbance Hypothesis After Two Growing Seasons. *Restor. Ecol.* **2004**; *12*, 184-189.

25. Sinclair, A.; Catling, P.M. Restoration of *Hydrastis canadensis* by Transplanting with Disturbance Simulation: Results of One Growing Season. *Restor. Ecol.* **2002**; *11*, 217-222.

26. van der Voort, M.E.; Bailey, B.; Samuel, D.E.; McGraw, J.B. Recovery of Populations of Goldenseal (*Hydrastis canadensis* L.) and American Ginseng (*Panax quinquefolius* L.) Following Harvest. *Am. Midl. Nat.* **2003**; *149*, 282-92.

27. (*in review*) Sinclair A.; Leduc, R.I. Recovery Strategy for Goldenseal (*Hydrastis canadensis*) in Ontario. *Environment Canada.* **2007**.

28. Lloyd, J.U.; Lloyd, C.G. *Hydrastis canadensis,* Drugs and Medicines of North America 1884 *In* Bulletin of the Lloyd Library of Botany, Pharmacy and Materia Medica, Bulletin No. 10, Reproduction Series No. 6. J.U. & C.G. Lloyd: Cincinnati, Ohio. 1908.

29. Hutchens, A.R. Indian Herbology of North America. 3rd ed., Merco: Ontario, Canada. 1973.

30. Vogel, V.J. American Indian Medicine. University of Oklahoma Press: Norman, Oklahoma. 1970.

31. Mahady, G.B.; Pendland, S.L.; Stoia, A.; Chadwick, L.R. In Vitro Susceptibility of *Helicobacter pylori* to Isoquinoline Alkaloids from *Sanguinaria canadensis* and *Hydrastis canadensis*. *Phytother. Res*. **2003**; *17*, 217–221.

32. Singh, B.; Srivastava, J.S.; Khosa, R.L.; Singh, U.P. Individual and Combined Effects of Berberine and Santonin on Spore Germination of Some Fungi. *Folia Microbiol.* **2001**; *46*, 137-142.

33. Rabbani, G.H.; Butler, T.; Knight, J.; Sanyal, S.C.; Alam, K. Randomized Controlled Trial of Berberine Sulfate Therapy for Diarrhea Due to Enterotoxigenic *Escherichia coli* and *Vibrio cholerae*. *J. Infect. Dis*. **1987**; *155*, 979-984.

34. Khin-Maung-U; Myo-Khin; Nyunt-Nyunt-Wai; Aye-Kyaw; Tin-U. Clinical Trial of Berberine in Acute Watery Diarrhea. *Brit. Med. J.* **1985**; *291*, 1601-1605.

35. Lau, C.-W.; Yao, X.-Q.; Chen, Z.-Y.; Ko, W.-H.; Huang, Y. Cardiovascular Actions of Berberine. *Cardiovasc. Drug Rev.* **2001**; *19*, 234-244.

36. Yesilada, E.; Kupeli, E. *Berberis crataegina* DC. Root Exhibits Potent Anti-inflammatory, Analgesic and Febrifuge Effects in Mice and Rats. *J. of Enthnopharmacol.* **2002**; 79, *237-248.*

37. Letasiova, S.; Jantova, S.; Cipak, L'ubos, C.; Muckova, M. Berberine-antiproliferative activity

and induction of apoptosis/necrosis of the U937 and B16 cells. *Cancer Letters*. **2006**; *239*, 254-262.

38. Abidi, P.; Zhou, Y.; Jiang, J. D.; Liu, J. W. Extracellular Signal-Regulated Kinase-Dependent Stabilization of Hepatic Low-Density Lipoprotein Receptor mRNA by Herbal Medicine Berberine. *Arterioscler. Thromb. Vasc. Biol.* **2005**; *25*, 2170–2176.

39. Kong, W.; Wei, J.; Abidi, P.; Lin, M.; Inaba, S.; Li, C.; Wang, Y.; Wang, Z.; Si, S.; Pan, H.; Wang, S.; Wu, J.; Wang, Y.; Li, Z.; Liu, J.; Jiang, J.D. Berberine is a Novel Cholesterol-Lowering Drug Working Through a Unique Mechanism Distinct from Statins. *Nat. Med.* **2004**; *10*, 1344–1352.

40. Lee, Y.S.; Kim, W.S.; Kim, K.H.; Yoon, M.J.; Cho, H.J.; Shen, Y.; Ye, J.M.; Lee, C.H.; Oh, W.K.; Kim, C.T.; Hohnen-Behrens, C.; Gosby, A.; Kraegen, E.W.; James, D.E.; Kim, J.B. Berberine, a Natural Plant Product, Activates AMP-Activated Protein Kinase with Beneficial Metabolic Effects in Diabetic and Insulin-Resistant States. *Diabetes*, **2006**; *55*, 2256-64.

41. Goel, M.; Singh, U.P.; Jha, R.N.; Pandey, V.B.; Pandey, M.B. Individual and Combined Effect of (+/-)-α-Hydrastine and (+/-)-β-Hydrastine on Spore Germination of Some Fungi. *Folia Microbiol (Praha)*. **2003**; *48*, 363-8.

42. Scazzocchio, F.; Cometa, M.F.; Tomassini, L.; Palmery, M. In Vitro Susceptibility of *Helicobacter pylori* to Antibacterial Activity of *Hydrastis canadensis* extract and its major isolated alkaloids. *Planta Med.* **2001**; *67*, 561-564.

43. Waters, S.L.; Miller, G.W.; Aleo, M.D.; Schnellmann, R.G. Neurosteroid inhibition of cell death. *Am. J. Physiol. Renal. Physiol.* **1997**; *273*, 869-876.

44. Huang, J.H.; Johnston, G.A. (+)-Hydrastine, a Potent Competitive Antagonist at Mammalian GABAA receptors. *Br. J. Pharmacol.* **1990**; *99*, 727-730.

45. Abidi, P.; Chen, W.; Kraemer, F.B.; Li, H.; Liu, J. The Medicinal Plant Goldenseal is a Natural LDL-Lowering Agent with Multiple Bioactive Components and New Action Mechanisms. *J. Lipid Res.* **2006**; *47*, 2134–2147.

46. Wu, J.; Jin, G.Z. Tetrahydroberberine Blocks Membrane K+ Channels Underlying its Inhibition of Intracellular Message-Mediated Outward Currents in Acutely Dissociated CA1 Neurons From Rat Hippocampus. *Brain Res.* **1997**; *775*, 214-218.

47. Abdel-Haq, H.; Cometa, M. F.; Palmery, M.; Leone, M.G.; Silvestrini, B.; Saso, L. Relaxant Effects of *Hydrastis canadensis* L. and its Major Alkaloids on Guinea Pig Isolated Trachea. *Pharmacol. Toxicol.* **2000**; *87*, 218–222.

48. Sinclair, A.; Catling, P.M. Cultivating the Increasingly Popular Medicinal Plant, Goldenseal: Review and Update. *Am. J. of Alternative Agr.* **2001**; *16*, 131-140.

49. Baskin, C.C.; Baskin, J.M. Seeds, Ecology, Biogeography, and Evolution of Dormancy and Germination. Academic Press: New York. 2001.

50. Cech, R. Growing At-Risk Medicinal Herbs, Cultivation, Conservation and Ecology. Horizon Herbs: Oregon. 2002.

51. Davis, J.M.; McCoy, J.-A. Commercial Goldenseal Cultivation. Hort. Info Leaflet 131. Rev. ed. North Carolina Cooperative Extension Service, Raleigh. 2000. Website http://www.ces.ncsu.edu/depts/hort/hil/hil-131.htm (viewed June 2007).

52. Davis, J.M. Forest Production of Goldenseal. Agroforestry Note 16. U.S. Dept. of Agriculture, National Agroforestry Center: Lincoln, NE. 1999. Website http://www.unl.edu/nac/agroforestrynotes/an15ff04.pdf (viewed June 2007).

53. Sato, F.; Hashimoto, T.; Hachiya, A.; Tamura, K.; Choi, K.-B.; Morishige, T.; Fujimoto, H.; Yamada, Y. Metabolic Engineering of Plant Alkaloid Biosynthesis. *PNAS*. **2001**; *98*, 367-372.

54. Roberts, M.F. Production of Alkaloids in Plant Cell Culture. In: Alkaloids Biochemistry, Ecology, and Medicinal Applications. Roberts, M.F.; Wink, M. (eds). Plenum Press: New York. 1998. Pp 159-197.

55. Inui, T.; Tamura, K.-I.; Fujii, N.; Morishige, T.; Sato, F. Overexpression of *Coptis japonica* Norcoclaurine 6-*O*-Methyltransferase Overcomes the Rate-Limiting Step in Benzylisoquinoline Alkaloid Biosynthesis in Cultured *Eschscholzia californica*. *Plant Cell Physiol*. **2007**; *48*, 252-262.

56. Park, S.-U.; Yu, M.; Facchini, P.J. Modulation of Berberine Bridge Enzyme Levels in Transgenic Root Cultures of California Poppy Alters the Accumulation of Benzophenanthridine Alkaloids. *Plant Mol. Biol*. **2003**; *51*, 153-164.

57. Choi, K.-B.; Morishige, T.; Shitan, N.; Yazaki, K.; Sato, F. Molecular Cloning and Characterization of Coclaurine *N*-Methyltransferase from Cultured Cells of *Coptis japonica*. *J. Biol. Chem*. **2002**; *277*, 830-835.

58. Park, S.-U.; Facchini, P.J. *Agrobacterium rhizogenes*-Mediated Transformation of Opium Poppy, *Papaver somniferum* L., and California Poppy, *Eschscholzia californica* Cham., Root Cultures. *J. Exp. Bot*. **2000**; *51*, 1005-1016.

59. Bedir, E.; Lata, H.; Schaneberg, B.; Khan, I.A.; Moraes, R.M. Micropropagation of *Hydrastis canadensis*: Goldenseal a North American Endangered Species. *Planta Med*. **2003**; *69*, 83-86.

60. Lui, C.-Z.; Murch, S.J.; Jain, J.C.; Saxena, P.K. Goldenseal (*Hydrastis canadensis*): In Vitro Regeneration for Germplasm Conservation and Elimination of Heavy Metal Contamination. *In Vitro Cell Dev.-Pl*. **2003**; *40*, 75-79.

61. Williams, T. Big Medicine From Six Nations. Syracuse University Press: New York. 2007.

62. Halberstein, R.A. Medicinal Plants: Historical and Cross-Cultural Usage Patterns. *Ann. Epidemiol.* **2005**; *15*, 686-699.

63. Lahans, T. Integrating Chinese and Conventional Medicine in Colorectal Cancer Treatment. *Integrative Cancer Therapies.* **2007**; *6*, 89-94.

64. Xu, K.T.; Farrell, T.W. The Complementarity and Substitution Between Unconventional and Mainstream Medicine Among Racial and Ethnic Groups in the United States. *Health Services Research.* **2007**; *42*, 811-826.

65. Khan, I.A. Issues Related to Botanicals. *Life Sci.* **2006**; *78*, 2033-2038.

66. Gamus, D.; Pintov, S. Integration of Complementary and Alternative Medicine Services in the Hospital Setting in Israel. *IMAJ.* **2007**; *9*, 169-170.

67. Health Canada; Natural Health Products Directorate. Baseline Natural Health Products Survey Among Consumers. 2005. Website http://www.hc-sc.gc.ca/dhp-mps/alt_formats/hpfb-dgpsa/pdf/pubs/eng_cons_survey_e.pdf (viewed June 2007).

68. Zhou, S;. Gao, Y.; Jiang, W.; Huang, M.; Xu, A.; Paxton, J.P. Interactions of Herbs with Cytochrome P450. *Drug Metab. Rev.* **2003**; *35,* 35-98.

69. van Breemen, R.B.; Fong, H.H.S.; Farnsworth, N.R. The Role of Quality Assurance and Standardization in the Safety of Botanical Dietary Supplements. *Chem. Res. Toxicol.* **2007**; *20*, 577-582.

70. Coppens, P.; Delmulle, L.; Gulati, O.; Richardson, D.; Ruthsatz, M. Use of Botanicals in Food Supplements. *Ann. Nutr. Metab.* **2006**; *50*, 538-554.

71. Gibson, J.E.; Taylor,D .A. Can Claims, Misleading Information, and Manufacturing Issues Regarding Dietary Supplements Be Improved in the United States? *J. Pharmacol. Exp. Ther.* **2005**; *314*, 939-944.

72. Kelly, J.P.; Kaufman, D.W.; Kelley, K.; Rosenberg, L.; Anderson, T.E.; Mitchell, A.A. Recent Trends in Use of Herbal and Other Natural Products. *Arch. Intern. Med.* **2005**; *165*, 281-286.

73. Schilter, B.; Andersson, C.; Anton, R.; Constable, A.; Kleiner, J.; O'Brien, J.; Renwick, A.G.; Korver, O.; Smit, F.; Walker, R. Guidance for the Safety Assessment of Botanicals and Botanical Preparations for Use in Food and Food Supplements. *Food Chem. Toxicol.* **2003**; *41*, 1625-1649.

74. Swanson, C.A. Suggested Guidelines for Articles About Botanical Dietary Supplements. *Am. J. Clin. Nutr.* **2002**; *75*, 8-10.

75. Foster, BC; Arnason, J.T.; Briggs, C.J. Natural Health Products and Drug Disposition. *Annu. Rev. Pharmacol.* **2005**; *45*, 203-226.

76. Renon, K.W. Regulation of Drug Metabolism and Disposition During Inflammation and Infection. *Expert. Opin. Drug Metab. Toxicol.* **2005**; *1*, 629-640.

77. Williamson, EM. Drug Interactions Between Herbal and Prescription Medicines. *Drug Safety.* **2003**; *26,* 1075-1092.

78. Brinker, F. Herb Contraindications and Drug Interactions, 3rd edition. Eclectic Medical Publications: Oregon. 2001.

79. Nelson, D.R.; Koymans, L.; Kamataki, T.; Stegeman, J.J.; Feyereisen, R.; Waxman, D.J.; Waterman, M.R.; Gotoh, O.; Coon, M.J.; Estabrook, R.W.; Gunsalus, I.C.; Nebert, D.W. P450 Superfamily: Update on New Sequences, Gene Mapping, Accession Numbers and Nomenclature. *Pharmacogenetics*. **1996**; *6*, 1-42.

80. Venkatakrishnan, K.; von Moltke, L.L.; Court, M.H.; Harmatz, J.S.; Crespi, C.L.; Greenblatt, D.J. Comparison Between Cytochrome P450 (CYP) Content and Relative Activity Approaches: Ratios of Accessory Proteins as Sources of Discrepancies Between the Approaches. *Drug Metab. Dispos.* **2000**; *28*, 1493-1504.

81. Guengerich, F.P. Human Cytochrome P450 Enzymes. In: Cytochrome P450, Structure, Mechanism, and Biochemistry. 3rd ed. Ortiz de Montellano, P.R. (ed). 2005. pp 377-530.

82. Ghosal, A.; Hapangama, N.; Yuan, Y.; Lu, X.; Horne, D.; Patrick, J.E.; Zbaida, S. Rapid Determination of Enzyme Activities of Recombinant Human Cytochromes P450, Human Liver Microsomes and Hepatocytes. *Biopharm. Drug Dispos.* **2003;** *24*, 375-384.

83. Karam, W.G.; Goldstein, J.A.; Lasker, J.M.; Ghanayem, B.I. Human CYP2C19 is a Major Omeprazole 5-hydroxylase, as Demonstrated with Recombinant Cytochrome P450 Enzymes. *Drug Metab. Dispos.* **1996**; *24*, 1081-1087.

84. Wienkers, L.C.; Wurden, C.J.; Storch, E.; Kunze, K.L.; Rettie, A.E.; Trager, W.F. Formation of (R)-8-Hydroxywarfarin in Human Liver Microsomes. A New Metabolic Marker for the (S)-Mephenytoin Hydroxylase. *Drug Metab. Dispos.* **1996**; *24*, 610-614.

85. Ando, Y.; Fuse, E.; Figg, W.D. Thalidomide Metabolism by the CYP2C Subfamily. *Clin. Cancer Res.* **2002**; *8*, 1964 –1973.

86. Yamazaki, H.; Shimada, T. Progesterone and Testosterone Hydroxylation by Cytochromes P450 2C19, 2C9, and 3A4 in Human Liver Microsomes. Arch. Biochem. Biophys. **1997**; *346*, 161 – 169.

87. Nakamura, K.; Goto, F.; Ray, W.A.; McAllister, C.B.; Jacqz, E.; Wilkinson, G.R.; Branch, R.A. Interethnic Differences in Genetic Polymorphism of Debrisoquin and Mephenytoin Hydroxylation Between Japanese and Caucasian Populations. *Clin. Pharmacol. Ther.* **1985**; *38*, 402-8.

88. Chau, T.K.H.; Murakami, S.; Kawai, B.; Nasu, K.; Kubota, T.; Ohnishi, A. Genotype Analysis of the *CYP2C19* Gene in HCV-Seropositive Patients with Cirrhosis and Hepatocellular Carcinoma. *Life Sci.* **2000**; *67*, 1719-1724.

89. Roddam, P.L.; Rollinson, S.; Kane, E.; Roman, E.; Moorman, A.; Cartwright, R.; Morgan, G.J. Poor Metabolizers at the Cytochrome P450 2D6 and 2C19 Loci Are At Increased Risk of Developing Adult Acute Leukemia. *Pharmacogenetics.* **2000**; *10*, 605-615.

90. Shimada, T.; Yamazaki, H.; Mimura, M.; Inui, Y.; Guengerich, F.P. Interindividual Variations in Human Liver Cytochrome P-450 Enzymes Involved in the Oxidation of Drugs, Carcinogens and Toxic Chemicals: Studies with Liver Microsomes of 30 Japanese and 30 Caucasians. *J. Pharmacol. Exp. Ther.* **1994**; *270*, 414-423.

91. Guengerich, F.P. Mechanism-Based Inactivation of Human Liver Microsomal Cytochrome P-450 IIIA4 by Gestodene. *Chem. Res. Toxicol.* **1990**; *3*, 363-371.

92. Rendic, S. Summary of Information on Human CYP Enzymes: Human P450 Metabolism Data. *Drug Metab. Rev.* **2002**; *34*, 83-448.

93. Wang, R.W.; Kari, P.H.; Lu, A..H.; Thomas, P.E.; Guengerich, F.P.; Vyas, K.P. Biotransformation of Lovastatin, IV. Identification of Cytochrome P450 3A Proteins as the Major Enzymes Responsible for the Oxidative Metabolism of Lovastatin in Rat and Human Liver Microsomes. *Arch. Biochem. Biophys.* **1991**; *290*, 355-361.

94. Huskey, S.W.; Dean, D.C.; Miller, R.R.; Rasmusson, G.H.; Chiu, S.H. Identification of Human Cytochrome P450 Isozymes Responsible for the In Vitro Oxidative Metabolism of Finasteride. *Drug Metab Dispos.* **1995**; *23*, 1126-1135.

95. Combalbert, J.; Fabre, I.; Fabre, G.; Dalet, I.; Derancourt, J.; Cano, J.P.; Maurel, P. Metabolism of Cyclosporin A. IV. Purification and Identification of the Rifampicin-Inducible Human Liver Cytochrome P-450 (Cyclosporin a Oxidase) as a Product of P450IIIA Gene Subfamily. *Drug Metab. Dispos.* **1989**; *17*, 197-207.

96. Koudriakova, T.; Iatsimirskaia, E.; Utkin, I.; Gangl, E.; Vouros, P.; Storozhuk, E.; Orza, D.; Marinina, J.; Gerber, N. Metabolism of the Human Immunodeficiency Virus Protease Inhibitors Indinavir and Ritonavir by Human Intestinal Microsomes and Expressed Cytochrome P4503A4/3A5: Mechanism-Based Inactivation of Cytochrome P4503A by Ritonavir. *Drug Metab. Dispos.* **1998**, *26*, 552-561.

97. Warrington, J.S.; Shader, R.I.; von Moltke, L.L.; Greenblatt, D. J. In Vitro Biotransformation of Sildenafil (Viagra): Identification of Human Cytochromes and Potential Drug Interactions. *Drug Metab. Dispos.* **2000**; *28*, 392-397.

98. Guengerich, F.P.; Martin, M.V.; Beaune, P.H.; Kremers, P.; Wolff, T.; Waxman, D.J. Characterization of Rat and Human Liver Microsomal Cytochrome P-450 Forms Involved in Nifedipine Oxidation, a Prototype for Genetic Polymorphism in Oxidative Drug Metabolism. *J. Biol. Chem.* **1986**; *261*, 5051-5060.

99. Bodin, K.; Bretillon, L.; Aden, Y.; Bertilsson, L.; Broomé, U.; Einarsson, C.; Diczfalusy, U. Antiepileptic Drugs Increase Plasma Levels of 4beta -Hydroxycholesterolin Humans, Evidence For Involvement Of Cytochrome P450 3A4. *J. Biol. Chem.* **2001**; *276*, 38685-38689.

100. Bodin, K.; Andersson, U.; Rystedt, E.; Ellis, E.; Norlin, M.; Pikuleva||, I.; Eggertsen, G.; Björkhem, I.; Diczfalusy, U. Metabolism of 4β-Hydroxycholesterol in Humans. *J. Biol. Chem.* **2002**; *277*, 31534-31540.

101. Simpson, E.R.; Mahendroo, M.S.; Means, G.M.; Kilgore, M.W.; Hinshelwood, M.M.; Graham-Lorence, S.; Amarneh, B.; Ito, Y.; Fisher, C.R.; Michael, M.D.; Mendelson, C.R.; Bulun, S.E. Aromatase Cytochrome P450, the Enzyme Responsible for Estrogen Biosynthesis. *Endocr. Rev.* **1994**; *15*, 342-355.

102. Simpson, E.R.; Clyne, C.; Rubin, G.; Boon, W.C.; Robertson, K.; Britt, K.; Speed, C.; Jones, M.; Aromatase—A Brief Overview. *Annu. Rev. Physiol.* **2002**; *64*, 93-127.

103. Brodie, A.M.H. Aromatase Inhibition and Its Pharmacologic Implications. *Biochem. Pharmacol.* **1985**; *34*, 3213-3219.

104. Marcotte, P.A.; Robinson, C.H. Design of Mechanism-Based Inactivators of Human Placental Aromatase. *Cancer Res.* **1982**; *42*, 3322s-3326s.

105. Lonning, P.E. The Role Of Aromatase Inactivators in the Treatment of Breast Cancer. *International J. Clin. Oncol.* **2002**; *7*, 265-270.

106. Lonning, P.E. Aromatase Inhibitors and Inactivators for Breast Cancer Treatment. *Eur. J. Cancer.* **2002**; *38*, 47 s -48s.

107. Boocock, D.J.; Brown, K.; Gibbs, A.H.; Sanchez, E.; Turteltaub, K.W.; White, I.N.H. Identification of Human CYP Forms Involved in the Activation of Tamoxifen and Irreversible Binding to DNA. *Carcinogenesis.* **2002**; *23*, 1897–1901.

108. Gaudineau C.; Beckerman, R.; Welbourn, S.; Auclair, K . Inhibition of Human P450 Enzymes by Multiple Constituents of the *Ginkgo biloba* Extract. *Biochem. Biophys. Res. Commun.* **2004**, *318*, 1072–1078.

109. Chatterjee, P.; Franklin, M.R. Human Cytochrome P450 Inhibition and Metabolic-Intermediate Complex Formation by Goldenseal Extract and its Methylenedioxyphenyl Components. *Drug Metab. Dispos.* **2003**; *31*, 1391-1397.

110. Foster, B.C.; Vandenhoek, S.; Hana, J.; Krantis, A.; Akhtar, M.H.; Bryan, M.; Budzinski, J.W.; Ramputh, A.; Arnason, J.T. In Vitro Inhibition of Human Cytochrome P450-mediated Metabolism of Marker Substrates by Natural Products. *Phytomedicine*. **2003**; *10*, 334-342.

111. Budzinski, J.W.; Foster, B.C.; Vandenhoek, S.; Arnason, J.T. An In Vitro Evaluation of Human Cytochrome P450 3A4 Inhibition by Selected Commercial Herbal Extracts and Tinctures. *Phytomedicine*. **2000**; *7*, 273-282.

112. Foster, B.C.; Foster, M.S.; Vandenhoek, S.; Budzinski, J.; Gallicano, K.D.; Choudri, S.; Krantis, A.; Arnason, J.T. An In Vitro Evaluation of Human Cytochrome P450 3A4 and P-Glycoprotein Inhibition by Garlic. *J. Pharm. Pharm. Sci.* **2001**; *4*, 159-167.

113. Franklin, M.R. Methylenedioxyphenyl Insecticide Synergists as Potential Human Health Hazards. *Environ. Health Persp.* **1976**; *14*, 29-37.

114. Tucker, G.T.; Houston, J.B.; Huang, S.-M. Optimizing Drug Development: Strategies to Assess Drug Metabolism/Transporter Interaction Potential – Towards a Consensus. *Pharmaceut. Res.* **2001**; *18*, 1071-1079.

115. Crespi, C.L.; Penman, B.W. Use of cDNA-Expressed Human Cytochrome P450 Enzymes to Study Potential Drug-Drug Interactions. *Advances in Pharmacology*. **1997**; *43*, 171-188.

116. Gentry, E.J.; Hanuman, B.J.; Keshavarz-Shokri, A.; Morton, M.D.; Velde, D.V.; Telikepalli, H.; Mitscher, L.A. Antitubercular Natural Products: Berberine from the Roots of Commercial *Hydrastis*

canadensis Powder. Isolation of Inactive 8-Oxotetrahydrothalidendine, Canadine, β-Hydrastine, and Two New Quinic Acid Esters, Hycandinic Acid Esters-1 and –2. *J. Nat. Prod.* **1998**; *61*, 1187-1193.

117. Blumenthal, M.; Wollschlaeger, B.; Brinckmann, J.A. The American Botanical Council Clinical Guide to Herbs. Haworth Press: Binghamton, New York. 2003.

118. Rehman, J.; Dillow, J.M.; Carter, S.M.; Chou, J.; Le, B.; Maisel, A.S. Increased Production of Antigen-Specific Immunoglobins G and M Following In Vivo Treatment with the Medicinal Plants *Echinacea angustifolia* and *Hydrastis canadensis*. *Immunol. Lett.* **1999**; *68*, 391-395.

119. Oldham MJ. Natural Heritage Resources of Ontario: Rare Vascular Plants. 3rd Edition. Natural Heritage Information Centre, Ontario Ministry of Natural Resources, Peterborough, Ontario. 1999. Website http://www.mnr.gov.on.ca/MNR/nhic/species/lists/rarevascular.pdf (Viewed May 2004).

120. Generic Point Mapper (GPM) online mapping service, Canadian Biodiversity Information Facility, Government of Canada. PHP MapScript and ROSA java applet from D.M. Solutions Group Inc., Ottawa, Canada. Map engine: Mapserver, University of Minnesota. Web Map Service (WMS): Centre for Topographic Information, Sherbrooke, Quebec, Canada and Demis BV, Netherlands. WMS data: Department of Natural Resources. Website www.cbif.gc.ca/mc/index_e.php?p_cancarte=t (Viewed January 2007).

121. Li, W.; Fitzloff, J.F. A Validated High Performance Liquid Chromatographic Method for the Analysis of Goldenseal. *J. Pharm. Pharmacol.* **2002**; *54*, 435-439.

122 Weber, H.A.; Zart, M.K.; Hodges, A.E.; White, K.D.; Barnes, S.M.; Moody, L.A.; Clark, A.P.; Harris, R.K. Method Validation for Determination of Alkaloid Content in Goldenseal Root Powder. *J. AOAC Int.* **2003;** *86*, 476-483.

23. Harris, C.S.; Burt, A.J.; Saleem, A.; Le, P.M.; Martineau, L.; Haddad, P.S.; Bennett, S.A.L.; Arnason, J.T. A single HPLC-DAD/APCI-MS Method for Quantitative Comparison of Phenolic Compounds Found in Leaf, Stem and Fruit Extracts of Lowbush Blueberry, *Vaccinium angustifolium* Ait. *Phytochem. Anal.* **2007**; *18*, 161-169.

124. S-PLUS: Copyright 1988, 2003 Insightful Corp.; S: Copyright Lucent Technologies, Inc.; Portions Copyright 1994-1996 McRae Software Int'l, Inc.; Portions Copyright 1990-1994 Halcyon Software, Inc. Rev. Date: 2 Dec 2003, Build: 6713; Serial # WA001305010666].

125 Sharp, P.C. New England Plant Conservation Program, *Hydrastis canadensis* L. Goldenseal, Conservation and Research Plan for New England. New England Wild Flower Society: Framingham, MA. 2003. Website http://www.newfs.org/conserve/pdf/hydrastiscanadensis.pdf (viewed May 2007).

126. *unpublished data*, Sinclair, A.; Boutin, C.; White, A. 2003.

127. McNamara, C.E.; Perry, N.B.; Follett, J.M.; Parmenter, G. A.; Douglas, J.A. A New Feruloyl Quinic Acid as a Potential Marker for Roots and Rhizomes of Goldenseal, *Hydrastis canadensis*. *J. Nat. Prod.* **2004** *67*, 1818-1822.

128. Edwards, D.J.; Draper, E.J. Variations in Alkaloid Content of Herbal Products Containing Goldenseal. *J. Am. Pharm. Assoc.* **2003** *43*, 419-423.

129. Falconer, D.S. Introduction to Quantitative Genetics; Longman: Essex, UK. 1989.

130. Salmore, A.K.; Hunter, M.D. Environmental and Genotypic Influences on Isoquionoline Alkaloid Content in *Sanguinaria canadensis*. *J. Chem. Ecol.* **2001**; *27*, 1729-1747.

131. Hemming, J.C.; Lindroth, R.L. Effects of Light and Nutrient Availability on Aspen: Growth, Phytochemistry, and Insect Performance. *J. Chem. Ecol.* **1999**; *25*, 1687-1714.

132. Castells, E.; Berhow, M.A.; Vaughn, S.F.; Berenbaum, M.R. Geographic Variation in Alkaloid Production in *Conium maculatum* Populations Experiencing Differential Herbivory by *Agonopterix alstroemeriana*. *J. Chem. Ecol.* **2005**; *31*, 1693-1709.

133. Ralphs, M.H.; Manners, G.D.; Gardner, D.R. Influence of Light and Photosynthesis on Alkaloid Concentration in Larkspur. *J. Chem. Ecol.* **1998**; *24*, 167-182.

134. Ohnmeiss, T.E.; Baldwin, I.T. The Allometry of Nitrogen Allocation to Growth and an Inducible Defense Under Nitrogen-Limited Growth. *Ecology*. **1994**; *75*, 995-1002.

135. Hamilton, J.G.; Zangerl, A.R.; DeLucia, E.H.; Berenbaum, M.R. The Carbon-Nutrient Balance Hypothesis: Its Rise and Fall. *Ecology Letters*. **2001**; *4*, 86-95.

136. Zangerl, A.R.; Berenbaum, M.R. Furanocoumarin Induction in Wild Parsnip: Genetics and Populational Variation. *Ecology*. **1990**; *71*, 1933-1940.

137. Baldwin, I.T.; Preston, C.A. The Eco-Physiological Complexity of Plant Responses to Insect Herbivores. *Planta*. **1999**; *208*, 137-145.

138. Levin, D.A.; York Jr., B.M. The Toxicity of Plant Alkaloids: Ecogeographic Perspective. *Biochem. Syst. Ecol.* **1978**; *6*, 61-76.

139. Marquis, R.J. Selective Impact of Herbivores. In Plant Resistance to Herbivores and Pathogens: Ecology, Evolution, and Genetics; Fritz, R.S.; Simms, E.L., Eds.; The University of Chicago Press, Chicago, IL, 1992; pp301-325.

140. Ostrem, L. Studies on Genetic Variation in Reed Canarygrass, *Phalaris arundinacea* L. I. Alkaloid Type and Concentration. *Hereditas*.**1987**; *107*, 235-248.

141. Berenbaum, M.R. The Chemistry of Defense: Theory and Practice. *Proc. Natl. Acad. Scie. USA.* **1995**; *91*, 2-8.

142. Online Climate Data. Environment Canada. 2005 Webpage http://www.climate.weatheroffice.ec.gc.ca/climateData/canada_e.html (viewed April 2007).

143. Coley, P.D.; Bryant, J.P.; Chapin III, F.S. Resource Availability and Plant Antiherbivore Defense. *Science*. **1985**; *230*, 895-899.

144. Abourashed, E.A.; Khan, I.A. High-Performance Liquid Chromatography Determination of Hydrastine and Berberine in Dietary Supplements Containing Goldenseal. *J. Pharm. Sci.* **2001**; *90*, 817-822.

145. Van Berkel, G.J.; Tomkins, B.A.; Kertesz, V. Thin-Layer Chromatography/Desorption Electrospray Ionization Mass Spectrometry: Investigation of Goldenseal Alkaloids. *Anal Chem.* **2007**; *79*, 2778-2789.

146. Govindan, M.; Govindan, G. A Convenient Method for the Determination of the Quality of Goldenseal. *Fitoterapia*. **2000**; *71*, 232-235.

147. Gurley, B.J.; Swain, A.; Barone, G.W.; Williams, D.K.; Breen, P.; Yates, C.R.; Stuart, L.B.; Hubbard, M.A.; Tong, Y.; Cheboyina, S. Effect of Goldenseal (*Hydrastis canadensis*) and Kava Kava (*Piper methysticum*) Supplementation on Digoxin Pharmacokinetics in Humans. *Drug. Metab Dispos*. **2006**; *34*, 69–74.

148. Kim, J.-S.; Tanaka, H.; Shoyama, Y. Immunoquantitative Analysis for Berberine and its Related Compounds using Monoclonal Antibodies in Herbal Medicines. *Analyst.* **2004**; *129*, 87-91.

149. Weber, H.A.; Zart, M.K.; Hodges, A.E.; Molloy, H.M.; O'Brien, B.M.; Moody, L.A.; Clark A.P.; Harris, R.K.; Overstreet, J.D.; Smith, C.S.; Chemical Comparison of Goldenseal (*Hydrastis*

canadensis L.) Root Powder From Three Commercial Suppliers. *J. Agric. Food. Chem.* **2003**; *51*, 7352-7358.

150. World Health Organization (WHO). Traditional Medicine. Fact sheet No. 134. 2003. Website http://www.who.int/mediacentre/factsheets/fs134/en/print.html (viewed May 2007).

151. Whitten, D.L.; Myers, S.P.; Hawrelak, J.A.; Wohlmuth, H.; The Effect of St John's Wort Extracts on CYP3A: A Systematic Review of Prospective Clinical Trials. *Br. J. Clin. Pharmacol.* **2006**; *62*, 512-526.

152. Scott, I.M.; Leduc, R.I.; Burt, A.J.; Marles, R.J.; Arnason, J.T.; Foster, B.C. The Inhibition of Human Cytochrome P450 by Ethanol Extracts of North American Botanicals. *Pharm. Biol.* **2006**; *44*, 315 – 327.

153. Lewis, D.F.V. Guide to Cytochromes P450 Structure and Function. Taylor and Francis, New York. 2001.

154. Standard Operating Procedures (SOP) OoS-3A4-000, Revision Number 015. Health Protection Branch, Health Canada. 2005.

155. Gonzalez, F.J.; Gelboin, H.V. Role of Human Cytochromes P450 in the Metabolic Activation of Chemical Carcinogens and Toxins. *Drug. Metab. Rev.* **1994**; *26*, 65-83.

156. Briest, S.; Davidson, N.E. Aromatase Inhibitors for Breast Cancer. *Rev. Endocr. Metab. Disord.* **2007** [Epub ahead of print]

157. Yarnell, E. Bontanical Medicines for the Urinary Tract. *Worl. J. Urol.* **2002**; *20*, 285-293.

158. Karikas, G.A.; Euerby, M.R.; Waigh, R.D. Isolation of Piceoside from *Arctostaphylos uva-ursi. Planta Med.* **1987; *53***, 307-308.

159. Dombrowicz, E.; Zadernowski, R.; Swiatek, L. Phenolic Acids in Leaves of *Arctostaphylos uva ursi* L., *Vaccinium vitis idaea* L. and *Vaccinium myrtillus* L. *Pharmazie.* **1991;** *46*, 680-681.

160. Sticher, O.; Soldati, F.; Lehmann, D. High-Performance Liquid Chromatographic Separation and Quantitative Determination of Arbutin, Methylarbutin, Hydroquinone and Hydroquinone-monomethylether in *Arctostaphylos*, *Bergenia*, *Calluna* and *Vacciunium* Species. *Planta Med.* **1979**; *35*, 253-261.

161. Semenza, G.; Bircher, J.; Mulkaupt, E.; Koide, T.; Pfenninger, E.; Marthaler, TH.; Gmunder, U.; Haemmerli, U.P. Arbutin Absorption in Human Small Intestine: A Simple Procedure for the Determination of Active Sugar Uptake in Peroral Biopsy Specimens. *Clin. Chim. Acta.* **1969**; *25*, 213-219.

162. Matsuda, H.; Nakamura, S.; Kubo, M. Studies of Cuticle Drugs from Natural Sources. II. Inhibitory Effects of Prunus Plants on Melanin Biosynthesis. *Biol. Pharm. Bull.* **1994**; *17*, 1417-20.

163. Low Dog, T. Menopause: A Review of Botanical Dietary Supplement. *Am. J. Med.* **2005**; *118*, 985-1085.

164. Chenoy, S.; Hussain, S.; Tayob, Y.; O'Brien, P.M.S.; Moss, M.Y.; Morse, P.F. Effect of Oral Gamolenic Acid from Evening Primrose Oil on Menopausal Flushing. *BMJ* **1994**; *308*, 501-503.

165. Pellegrina, C.D.; Padovani, G.; Mainente, F.; Zoccatelli, G.; Bissoli, G.; Mosconi, S.; Veneri, G.; Peruffo, A.; Andrighetto, G.; Rizzi, C.; Chignola, R. Anti-Tumour Potential of a Gallic Acid-Containing Phenolic Fraction from *Oenothera biennis*. *Cancer Letter*. **2005**; *226*, 17-25.

166. *personal communications* Filion, V.; Archambault, M.; Cuerrier, A.; Arnason, J.T. 2007.

167. Brown, R.P.; Gerbarg, P.L.; Ramazanov, Z. *Rhodiola rosea*: A Phytomedicinal Overview. *HerbalGram.* **2002**; *56*, 40-52.

168. Perfumi, M.; Mattioli, L. Adaptogenic and Central Nervous System Effects of Single Doses of 3% Rosavin and 1% Salidroside *Rhodiola rosea* L. Extract in Mice. *Phytother. Res.* **2006**; *21*, 37-43.

169. Gurley, B.J.; Gardner, S.F.; Hubbard, M.A.; Williams, D.K.; Gentry, W.B.; Khan, I.A.; Shah, A. In Vivo Effects of Goldenseal, Kava Kava, Black Cohosh, and Valerian on Human Cytochrome P450 1A2, 2D6, 2E1, and 3A4/5 Phenotypes. *Clin. Pharmacol. Ther.* **2005**; *77*, 415-26.

170. Sandhu, R.S.; Prescilla, R.P.; Simonelli, T.M.; Edwards, D.J. Influence of Goldenseal Root on the Pharmacokinetics of Indinavir. *Clin. Pharmacol.* **2003**; *43*, 1283-1288.

171. Jeng, J.-H.; Wu, H.-L.; Lin, B.-R.; Lan, W.-H.; Chang, H.-H.; Ho, Y.-S.; Lee, P.-H.; Wang, Y.-J.; Wang, J.-S.; Chen, Y.-J.; Chang, M.-C. Antiplatelet Effect of Sanguinarine is Correlated to Calcium Mobilization, Thromboxane and camp Production. *Atherosclerosis.* **2007**; *191*, 250-258.

172. Jaggi, R.; Wurgler, U.; Grandjean, F.; Weiser, M. Dual Inhibition of 5-Lipoxygenase/cyclooxygenase by a Reconstituted Homeopathic Remedy; Possible Explanation for Clinical Efficacy and Favourable Gastrointestinal Tolerability. *Inflamm. Res.* **2004**; *53*, 150-157.

173. Seifen, E.; Adams, R.J.; Riemer, K. Sanguinarine: A Positive Inotropic Alkaloid which Inhibits Cardiac N^+,K^+-ATPase. *Eur. J. Pharmacol.* **1779**; *60*, 373-377.

174. Cohen, H.G.; Straub, K.D.; Stermitz, F.R. Structural Specificity of the NaK-ATPase Inhibition by Sanguinarine, an isoquinoline benzophenanthridine Alkaloid. *Biochem. Pharmacol.* **1978**; *27*, 2555-2558.

175. Chaturvedi, M.M.; Kumar, A.; Darnay, B.G.; Chainy, G.B.N.; Agarwal, S.; Aggarwal, B.B. Sanguinarine (Pseudochelerythrine) is a Potent Inhibitor of NF-κB Activation, IκBα Phosphorylation and Degradation. *J. Biol. Chem.* **1997**; *272*, 30129-30134.

176. Basini, G.; Santini, S.E.; Bussolati, S.; Grasselli, F. Sanguinarine Inhibits VEGF-Induced Akt

Phosphorylation. *Ann. N.Y. Acad. Sci.* **2007**; *1095*, 371-376.

177. Eun, J.-P.; Koh, G.Y. Suppression of Angiogenesis by the Plant Alkaloid, Sanguinarine. *Biochem. Bioph Res. Co.* **2004**; *317*, 618-624.

178. Lin, L.-T.; Liu, L.-T.; Chiang, L.-C.; Lin, C.-C. In Vitro Anti-hepatoma Activity of Fifteen Natural Medicines from Canada. *Phytother. Res.* **2002**; *16*, 440-444.

179. Bergeron, C.; McCollom, M.; Scott, I.; Glinsky, J.A.; Arnason, J.T.; Foster, B.; Gafner, S. In Vitro Anxiolytic Activity of California Poppy (*Eschscholtzia californica* Cham.). Website http://www.tomsofmaine.com/downloads/pdf/CaliforniaPoppy.pdf (viewed May 2007).

180. Gafner, S.; Dietz, B.M.; McPhail, K.L.; Scott, I.M.; Glinski, J.A.; Russell, F.E.; McCollom, M.M.; Budzinski, J.W.; Foster, B.C.; Bergeron, C.; Rhyu, M.-R.; Bolton, J.L. Alkaloids from *Eschscholzia californica* and Their Capacity to Inhibit Binding of [3H]8-Hydroxy-2-(di-*N*-Propylaminotetralin to 5-HT1A Receptors In Vitro. *J. Nat. Prod.* **2006**; *69*, 432-435.

181. The Local Food-Nutraceuticals Consortium. Understanding Local Mediterranean Diets: a Multidisciplinary Pharmacological and Ethnobotanical Approach. *Pharmacol. Res.* **2005**; *52*, 353-66.

182. Hajnicka, V.; Kost'alova, D.; Svecova, D.; Sochorova, R.; Fuchsberger, N.; Toth, J. Effect of *Mahonia aquifolium* Active Compounds on Interluking-8 Production in the Human Monocytic Cell Line THP-1. *Planta Med.* **2002**; *68*, 266-8.

183. Kost'alova, D.; Kardosova, A.; Hajnicka, V. Effect of *Mahonia aquifolium* Stem Bark Crude Extract and One of its Polysaccharide Components on Production of IL-8. *Fitoterapia.* **2001**; *72*, 802-806.

184. Bezakova, L.; Misik, V.; Malekova, L.; Svajdlenka, E.; Kostalova, D. Lipoxygenase Inhibition

and Antioxidant Properties of Bisbenzylisoquinoline Alkaloids Isolated in *Mahonia aquifolium*. *Pharmazie*. **1996**; *51*, 758-761.

185. Misik, V.; Bezakova, l.; Malekova, l.; Koslalova, D. Lipoxygenase Inhibition and Antioxidant Properties of Protoberberine and Aporphine Alkaloids Isolated from *Mahonia aquifolium*. *Planta Med*. **1995**; *61*, 372-73.

186. Muller, K.; Ziereis, K. The Antipsoriatic *Mahonia aquifolium* and its Active Constituents; I. Pro- and Antioxidant Properties and Inhibition of 5-Lipoxygenase. *Planta Med*. **1994**; *60*, 421-24.

187. Muller, K.; Ziereis, K.; Gawlik, I. The Antipsoriatic *Mahonia aquifolium* and its Active Constituents; II. Antiproliferative Activity Against Cell Growth of Human Keratinocytes. *Planta Med*. **1995**; *61*, 74-75.

188. Cui, H.-S.; Hayasaka, S.; Zhang, X.-Y.; Hayasaka, Y.; Chi, Z.-L.; Zheng, L.-S. Effect of Berberine on Barrier Function in a Human Retinal Pigment Epithelial Cell Line. *Jpn. J. Ophthalmol.* **2007**, *51*, 64-67.

189. Huang, C.; Zhang, Y.; Gong, Z.; Sheng, X.; Li, Z.; Zhang, W.; Qin, Y. Berberine Inhibits 3T3-L1 Adipocyte Differentiation Through the PPARγ Pathway. *Biochem. Bioph.* Res. *Co.* **2006**; *348*, 571-578.

190. Wang, F.; Zhou, H.-Y.; Zhao, G.; Fu, L.-Y.; Cheng, L.; Chen, J.-G.; Yao, W.-X. Inhibitory Effects of Berberine on Ion Channels of Rat Hepatocytes. *World J. Gastroenterol*. **2004**; *10*, 2842-2845.

191. Kim, S.-H.; Shin, D.-S.; Oh, M.-N.; Chung, S.-C.; Lee, J.-S.; Oh, K.-B. Inhibition of the Bacterial Surface Protein Anchoring Transpepetidase Sortase by Isoquinoline Alkaloids. *Biosci. Biotechnol. Biochem*. **2004**; *68*, 421-424.

192. USDA, NRCS. The PLANTS Database (http://plants.usda.gov, 19 June 2007). National Plant Data Center, Baton Rouge, LA 70874-4490 USA. 2007.

193. Assinewe, V.A. Phytochemical Variation and Immunopharmacology of *Panax quinquefolius* L. (American Ginseng). Thesis (Ph.D.). University of Ottawa: Ottawa, Canada, 2001.

194. Binns, S.E. The Taxonomy, Phytochemistry and Biological Activity of the Genus *Echinacea* (Asteraceae). Thesis (Ph.D.). University of Ottawa: Ottawa, Canada, 2001.

195. Assinewe, V.A.; Baum, B.R.; Gagnon, D.; Arnason, J.T. Phytochemistry of Wild Populations of *Panax quinquefolius* L. (North American Ginseng). *J. Agric. Food Chem.* **2003**; *51*, 4549-4553.

196. Binns, S.E.; Livesey, J.F.; Arnason, J.T.; Baum, B.R. Phytochemical Variation in *Echinacea* from Roots and Flowerheads of Wild and Cultivated Populations. *J. Agric. Food Chem.* **2002**; *50*, 3673-3687.

197. Sinclair, A. *Recovery of the threatened economic plant, goldenseal (Hydrastis canadensis).* Thesis (Ph.D.). University of Ottawa: Ottawa, Canada, 2002.

198. Sinclair, A.; Catling, P.M. Recent Trends in Stem Numbers in Goldenseal, *Hydrastis canadensis*, Populations at the Northern Limit of its Range. *Can. Field Nat.* **2002**; *116*, 112 - 115.

199. Sinclair, A.; Catling, P. M.; Dumouchel, L. Notes on the Pollination and Dispersal of Goldenseal, *Hydrastis canadensis* L., in Southwestern Ontario. *Can. Field Nat.* **2000**; *114*, 499-501.

200. Shitan, N.; Bazin, I. Dan, K.; Obata, K.; Kigawa, K.; Ueda, K.; Sato, F. Forestier, C.; Yazaki, K. Involvement of CjMDR1, a Plant Multidrug-Resistance-Type ATP-Binding Cassette Protein, in Alkaloid Transport in *Coptis japonica. PNAS.* **2003;** *100*, 751-756.

201. Yakazi, K.; Shitan, N.; Takamatsu, H.; Ueda, K. Sato, F. A Novel *Coptis japonica* Multidrug-Resistant Protein Preferentially Expressed in the Alkaloid-Accumulating Rhizome. *J. Exp. Bot.* **2001**; *52*, 877-879.

202. Okada, N.; Koizumi, N.; Tanaka, T.; Ohkubo, H.; Nakanishi, S.; Yamada, Y. Isolation, Sequence, and Bacterial Expression of a cDNA for (*S*)-Tetrahydroberberine Oxidase from Cultured Berberine-Producing *Coptis japonica* Cells. *Proc. Natl. Acad. Sci.* **1989**; *86*, 534-538.

203. Zhou, S. Huang, M.; Xu, A.; Yang, H.; Duan, W.; Paxton, J.W. Prediction of Herb-Drug Metabolic Interactions: A Simulation Study. *Phytother. Res.* **2005**; *19*, 464-471.

204. Bailey, D.G.; Dresser, G.K. Natural Products and Adverse Drug Interactions. *CMAJ.* **2004**; *170*, 1531-1532.

205. Zeng, X.; Zeng, X. Relationship Between the Clinical Effects of Berberine on Severe Congestive Heart Failure and its Concentration in Plasma Studied by HPLC. *Biomed. Chromatogr.* **1999**; *13*, 442-444.

206. Foster, B.C.; Vandenhoek, S.; Li, K.Y.; Tang, R.; Budzinski, J.W.; Krantis, A. Effect of Several Chinese Natural Health Products of Human Cytochrome P450 Metabolism. *J. Pharm. Pharmaceut. Sci.* **2002**; *5*, 185-189.

207. Foster, B.C.; Drouin, C.E.; Krantis, A.; Arnason, J.T.; Livesey, J.F.; Jordan, S.A. In Vitro Activity of Commercial Valerian Root Extracts Against Human Cytochrome P450 3A4. *J. Pharm. Pharmaceut. Sci.* **2004**; *7*, 265-273.

208. Lee, L.S.; Andrade, A.S.A.; Flexner, C. Interactions Between Natural Health Products and Antiretroviral Drugs: Pharmacokinetic and Pharmacodynamic Effects. *CID.* **2006**; *43*, 1052-1059.

209. Pal, D.; Mitra, A.K. MDR- and CYP3A4-Mediated Drug-Herbal Interactions. *Life Sci.* **2006**;

78, 2131-2145.

210. Ulbricht, C.; Basch, E.; Weissner, W.; Hackman, D. An Evidence-Based Systematic Review of Herb and Supplement Interactions by the Natural Standard Research Collaboration. *Expert. Opin. Drug Saf.* **2006**; *5*, 719-728.

211. Mills, E.; Montori, V.; Perri, D.; Phillips, E.; Koren, G. Natural Health Product-HIV Drug Interactions: A Systematic Review. *Int. J. Std. Aids*. **2005**; *16*, 181-186.

212. Dresser, G.K.; Bailey, D.G. A Basic Conceptual and Practical Overview of Interactions with Highly Prescribed Drugs. *Can. J. Clin. Pharmacol.* **2002**; *9*, 191-199.

213. Haller, C.A.; Anderson, I.B.; Kim, S.Y.; Blanc, P.D. An Evaluation of Selected Herbal Reference Texts and Comparison to Published Reports of Adverse Herbal Events. *Adv. Drug React. Toxicol. Rev.* **2002**; *21*, 143-150.

214. Modarai, M.; Rahte, S.; Kortenkamp, A.; Heinrich, M. Screening Herbal Medicinal Products for CYP P450 Enzyme Inhibition with the Fast, Robust Supersome Assay. *Z. Phytother.* **2006**; *27*.

215. Walsky, R.L.; Obach, R.S. Validated Assays for Human Cytochrome P450 Activities. *Drug Metab. Dispos.* **2004**; *32*, 647-660.

216. Crespi, C.L.; Miller, V.P.; Penman, B.W. Microtiter Plate Assays for Inhibition of Human, Drug-Metabolizing Cytochromes P450. *Anal. Biochem.* **1997**; *248*, 188-190.

217. Crespi, C.L.; Miller, V.P. The Use of Heterologously Expressed Drug Metabolizing Enzymes-State of the Art and Prospects for the Future. *Pharmacol. Therapeut.* **1999**; *84*, 121-131.

218. Rodrigues, A.D.; Surber, B.W.; Yao, Y.; Wong, S.L.; Roberts, E.M. [*O*-Ethyl ^{14}C]Phenacetin *O*-Deethylase Activity in Human Liver Microsomes. *Drug Metab. Dispos.* **1997**; *25*, 1097-1100.

219. Wynalda, M.A.; Wienkers, L.C. Assessment of Potential Interactions Between Dopamine Receptor Agonists and Various Human Cytochrome P450 Enzymes Using a Simple In Vitro Inhibition Screen. *Drug Metab. Dispos.* **1997**; *25*, 1211-1214.

220. Nomeir, A.A.; Ruegg, C.; Shoemaker, M.; Favreau, L.V.; Palamanda, J.R.; Silber, P.; Lin, C.-C. Inhibition of CYP3A4 in a Rapid Microtiter Plate Assay Using Recombinant Enzyme and in Human Liver Microsomes Using Conventional Substrates. *Drug Metab. Dispos.* **2001**; *29*, 748-753.

221. Stormer, E.; von Moltke, L.L.; Greenblatt, D.J. Scaling Drug Biotransformation Data from cDNA-Expressed Cytochrome P-450 to Human Liver: A Comparison of Relative Activity Factors and Human Liver Abundance in Studies of Mirtazapine Metabolism. *J. Pharmacol. Exp. Ther.* **2000**; *295*, 793-801.

APPENDIX

A1 Phytochemical study of wild *Hydrastis canadensis* L. (goldenseal) germplasm

In reference to Chapter 2, section 2.3.1.

A1.1 Materials & Method

A1.1.1 Seed harvest & germination trials: In July 2004, a total 224 *H. canadensis* seeds were collected from three wild populations. Eight berries containing 75 seeds were collected from site HC2, nine berries containing 143 seeds were collected from site HC5, and ten berries containing six seeds from site HC8 were collected (Chapter 2: Table 2.1, Figure 2.1 and 2.2). Berries were only collected from relatively large populations ($\geq$ 50 individuals) that produced an abundance of ripe berries. In the field, the fruit pulp was removed from the seeds and the seeds were kept moist and cool.

From July to December 2004 the seeds were placed in 550 mL of sand or over moist filter paper in a Petri dish and stored in a ventilated refrigerator at 6°C for vernalization.

From January to May 2005, preliminary germination tests using three different substrates (e.g. moist filter paper in a Petri dish, sand and vermiculite) were performed on a subset of seeds. The seeds were removed from vernalization and held at room temperature for 24 hours before being transferred into a substrate and placed into a greenhouse chamber. A total of six seeds from site HC5 were placed over a moist filter paper, three seeds per Petri dish. A total of ten seeds from site HC2 and ten seeds from site HC8 were used to test sand and vermiculite, where five seeds per site were used for each treatment. On a daily basis the seeds in sand were given approximately 25 mL of distilled water while the seeds in vermiculite were given approximately 40 mL. The seeds tested with filter paper were kept moist with distilled water and condensation that formed on the lid of the Petri dish was removed daily in order to reduce the risk of fungal contamination. All treatments were conducted in the same greenhouse chamber under the following conditions: 30% relative humidity; 14 hour 200 watts/m^2 photoperiod; and 22/20°C for 14/10 hrs a day/night thermoperiod.

In June 2005 all seeds were removed from vernalization and tested for their ability to germinate in vermiculite. The seeds were first held at room temperature for 24 hours and all moldy seeds and those that collapsed when lightly pinched were considered no longer viable and were removed from the experiment. As a control treatment, 10 g of *H. canadensis* seeds (S2920, Lot # 15340) originally from California were purchased from Richters (Goodwood, ON). All seeds were sterilized in a 0.05% Javex solution and were sown individually in standard size (4 x 4 x 6 cm) cell pots. The cells were placed in trays, up to 24 cells in a tray, and placed in the greenhouse chamber. The greenhouse chamber conditions, described in Table A1.1, were adjusted to mimic the daily minimum and maximum temperatures and average humidity in Windsor, Ontario (*A1*), between the months of May and September.

Each cell was misted with distilled water and each tray was filled with approximately 600 mL of distilled water every second day. Since the greenhouse chamber was designed to only permit natural light to enter from a north facing vertical windowpane, the trays were shuffled once a week in order to insure that each seed was exposed to an equal amount of natural sunlight.

The experiment was terminated ten months later in March 2006, and all of the remaining viable seeds were removed from the vermiculite and stored individually in 15 mL vials at room temperature under minimal light. In January 2007, as requested by Dr. A. Sinclair, the remaining 89 wild seeds were given to Andrea White, MSc, a research assistant at the National Wildlife Research Centre (Environment Canada) for further testing. Eleven of the 89 seeds were originally from population HC2 and 78 were from population HC5.

A1.1.2 Seed viability test: The viability of 60 undamaged (with full seed coat) wild *H. canadensis* seeds were tested, following the 10-month germination experiments described above, using 2,3,5-triphenyltetrazolium chloride (TTC). The method was modified from Cottrell (*A2*) and Grabe (*A3*).

Ten seeds were randomly selected and placed overnight in individual moist filter papers. The following morning, the seeds were bisected longitudinally, one half was placed in a 10 mL beaker

Table A1.1 Environmental conditions for germination tests using wild-harvested Canadian *Hydrastis canadensis* L. (goldenseal) seed and cultivated seed

Condition	Range	Description
Photoperiod	14 hours/day (5am-7pm)	24 Nov 2004 to 22 June 2005
	16 hours/day (5am-9pm)	22 June 2005 to 31 March 2006
Mean temperature (°C) ± SEM	20.7 ± 0.35	17 month period; Ranged from 18.5 - 23.5°C
Heating target (°C)	20	Day hours
	18	Night hours
Cooling target (°C)	22	Day hours
	19	Night hours
Mean humidity (%Rh) ± SEM	34.7 ± 4.54	17 month period; Ranged from 14.9 - 64.9 %Rh

and submerged with 0.1% tetrazolium salt solution comprising of TTC (Sigma-Aldrich Inc., St. Louis, MO) dissolved in distilled water. The bisected seeds were incubated in the solution for 2 hours at room temperature and 2 hours at 35°C. The beakers were covered with aluminum foil during the incubation periods in order to reduce exposing the tetrazolium salt solution to light. Following the initial incubation period, the embryonic tissues of the seeds were inspected under a dissection microscope for staining: tetrazolium stains living tissues a reddish-pink colour. The seeds were left to soak in the tetrazolium salt solution at room temperature for an additional 20 hours before being inspected a final time. The seeds were digitally photographed under 100x magnification during each inspection. The percent of viable seeds was calculated by dividing the number of stained seeds after 24 hours by the total number of treated seeds x 100%. The results are reported as the mean of three replicate experiments ± SEM.

A1.2 Results & Discussion

A1.2.1 Seed germination & viability: Twenty-seven ripe berries containing a total of 224 seeds were collected in July 2004 from populations HC2 and HC5, both in Essex County, and population HC8 situated in Lambton County (Table 2.1, Figures 2.1 and 2.2). This was the first seed sampling of wild Canadian populations for phytochemical research. Previously, in 2003, seed was collected for ecological germination studies. Results indicated a low germination rate (9.2%) but that germination can occur in a wide range of light availability (0, 30, 50 and 80% shade), with soil moisture critical (*A4*).

From January to May 2005, preliminary germination tests using moist filter paper, sand and vermiculite were performed on a subset of 26 wild seeds. The environmental conditions are described in Table A1.1. On the 13th day, a radicle appeared from a seed that had been placed on moist filter paper in a sealed Petri dish. Four days later, a radicle emerged from a second seed that had been placed on a moist filter paper in an unsealed Petri dish. Both radicles grew roughly half the

length of the seeds, however germination did not progress and no other seeds showed signs of germination. The test was terminated after five months.

In June 2005 all seeds, including those from a commercial source, were removed from vernalization and allowed to germinate in vermiculite under the same environmental conditions as above (Table A1.1). One cotyledon was observed in November from a cultivated seed, but it did not survive. No other seeds showed signs of germination during the ten-month period.

The results of these tests are similar to those described in the literature. As mentioned above, Sinclair et al. (*A4*) were only able to successfully germinate < 10% of their wild seed collection. Furthermore, as explained by K. Bryce (Program Manager, Crops for Enhanced Human Health, NRC-Plant Biotechnology Institute, National Research Council of Canada) *H. canadensis* was replaced with *Thalictrum flavum* in a Natural Products Genomics (NAPGEN) project since it was too difficult to cultivate (*A5*). Personal communications with, and literature by, Dr. J. Davis, an associate professor at the University of North Carolina who has extensively researched goldenseal cultivation in the U.S. (*A6, A7*) and Richo Cech (*A8*), an American *H. canadensis* farmer, as well as personal communications with R. Eidus and Gary Krentz, both herb farmers, have all stated that *H. canadensis* seed germination is difficult and unpredictable.

The low germination rate of *H. canadensis* seed may be explained by its dormancy pattern. According to Baskin and Baskin (*A9*) *H. canadensis* seed has characteristics of both deep simple and deep simple epicotyle morphophysiological dormancy (MPD). Seeds with deep simple MPD require warm followed by cold stratification before they will germinate, while in seeds with deep simple epicotyle MPD the radicle emerges in autumn and the shoots emerge the following spring. Unlike our study, they observed embryo growth after four months from harvest (July - October), and by January the seeds were approximately six times their original length. In November, the seed coats began splitting open at the radicle end of seeds and the radicle did not emerge until March. Clearly, more research is required with respect to the germination ecology of *H. canadensis*.

Following the germination test, 30 wild seeds were tested for their viability using TTC. When TTC combines with H^+ that has been released from respiring embryonic tissue, TTC stains the tissue reddish pink (*A2*). Figure A1.1 shows staining occurrence and frequency after exposing the seeds to an aqueous TTC solution (tetrazolium salt solution) for 4 and 24 hours. The results are summarized in Table A1.2. A surprisingly high percentage of seeds (67± 8.8%) were stained red after tetrazolium exposure and were considered still viable. However, according to Grabe (*A3*) the tetrazolium test does not differentiate between dormant and non-dormant seeds producing test results to be considerably higher than germination test results in deep-seated dormancy seeds. Given the dormancy patterns of *H. canadensis*, in addition to the germination test results, it is probably that the tetrazolium test results of this study are an overestimation of the true proportion of viable seeds.

Since germination was unsuccessful during the time period of this study, the genetic component of phytochemical diversity in wild *H. canadensis* populations was not evaluated. However, the remaining wild seeds were transferred to the experimental laboratories at the National Wildlife Research Centre (Environment Canada) in Ottawa in order to allow further investigation of the germination ecology of the botanical.

Figure A1.1 Digital photographs of bisected wild Canadian *Hydrastis canadensis* L. (goldenseal) seeds that were incubated in tetrazolium salt solution for 4 and 24 hours. Living cells stained red-pink after incubation. Panels A, B and C show seed staining after incubating for 4 hours, and panels Ai, Bi, Ci show the same seeds stained after a 24 hour incubation period. 100x magnification.

A
A90
A177
A60
A173
A58
A162
A52
A152
A13
A130
Ai
A90
A177
A60
A173
A58
A162
A52
A152
A13
A130
B
B79
B191
B77
B181
B72
B171
B46
B96
B24
B87
Bi
B79
B191
B77
B181
B72
B171
B46
B96
B24
B87
C
C186
C184
C180
C155
C53
C55
C45
C139
C43
Ci
C186
C184
C180
C155
C53
C55
C45
C139
C43

Table A1.2 Summary of results for tetrazolium salt seed viability test after 24-hour incubation

Replicate	Observed seed colour		% Viable seeds[b]
	Stained	Not stained	
1	A52[a], A58, A90, A152, A177	A13, A60, A130, A162, A173	50
2	B46, B77, B79, B87, B96, B171, B181	B24, B72, B191	70
3	C53, C55, C61, C139, C155, C180, C184, C186	C43, C45	80
Mean % viable seeds ± SEM			67 ± 8.8

[a] Letter corresponds with replicate number: A = 1, B = 2 and C = 3; Number corresponds with collection site: population HC8 = 1-4, population HC2 = 5-38, 193-222, and population HC5 = 39-107,129-192; [b] % viable seeds = [number of stained seeds / (total number of seeds)] x 100%

A1.3 References

A1. Online Climate Data. Environment Canada. 2005 Webpage http://www.climate.weatheroffice.ec.gc.ca/climateData/canada_e.html (viewed April 2007).

A2. Cottrell, H.T. Tetrazolium Salt as a Seed Germination Indicator. *Nature*. **1947**; *159*, 748.

A3. Grabe, D.F (ed). Tetrazolium Testing Handbook For Agricultural Seeds. Contribution No. 29 to the Handbook on Seed Testing. Association of Official Seed Analysis. 1970.

A4. *unpublished data*, Sinclair, A.; Boutin, C.; White, A. **2003.**

A5. *personal communications* K. Bryce, Program Manager, Crops for Enhanced Human Health, NRC-Plant Biotechnology Institute, National Research Council of Canada. 2006.

A6. Davis, J.M.; McCoy, J.-A. Commercial Goldenseal Cultivation. Hort. Info Leaflet 131. Rev. ed. North Carolina Cooperative Extension Service, Raleigh. 2000. Website http://www.ces.ncsu.edu/depts/hort/hil/hil-131.htm (viewed June 2007).

A7. Davis, J.M. Forest Production of Goldenseal. Agroforestry Note 16. U.S. Dept. of Agriculture, National Agroforestry Center: Lincoln, NE. 1999. Website http://www.unl.edu/nac/agroforestrynotes/an15ff04.pdf (viewed June 2007).

A8. Cech, R. Growing At-Risk Medicinal Herbs, Cultivation, Conservation and Ecology. Horizon Herbs: Oregon. 2002.

A9. Baskin, C.C.; Baskin, J.M. Seeds, Ecology, Biogeography, and Evolution of Dormancy and Germination. Academic Press: New York. 2001.

A2 Vascular Plant Herbarium of the Department of Agriculture in Ottawa (DAO) Vouchers

A2.1 DAO voucher 824179: wild *Berberis vulgaris* L. (common barberry)

A2.2 DAO voucher 824204: wild *Coptis trifolia* (L.) Salisb. var. *groenlandica* (Oeder) Fassett. (threeleaf goldthread)

A2.3 DAO voucher 824183: wild *Sanguinaria canadensis* L. (bloodroot)

A2.1 Vascular Plant Herbarium of the Department of Agriculture in Ottawa (DAO) voucher 824179: wild *Berberis vulgaris* L. (common barberry) harvested September 2005 in Andrewsville, Ontario, Canada. Root, stem and leaf tissues are shown.

***Berberis vulgaris* L.**

Berberidaceae Family

Canada. Ontario. County of Lanark, Andrewsville
North shore of the Rideau River at Nicholsons Locks, bridge linking Hwy 2 and Hwy 23, approx. 100m West along trail from Dilworth Road
N44.95017, W75.8204

Collected by R.I. Leduc & R.W. Devereux. 25 Sept. 2005
Identified by R.I Leduc & J.T. Arnason

Secondary overgrowth, woodlot.

Specimens were collected from the wild for phytochemical analysis by R.I. Leduc (M.Sc. Thesis, University of Ottawa, Ottawa, Ontario, Canada). See extracts # 414, 415, 426 & 427 for phytochemical data (R.I. Leduc, M.Sc. Thesis, University of Ottawa, 2007).

A2.2 Vascular Plant Herbarium of the Department of Agriculture in Ottawa (DAO) voucher 824204: wild *Coptis trifolia* (L.) Salisb. var. *groenlandica* (Oeder) Fassett. (threeleaf goldthread) harvested May 2004 from Mer Bleue Conservation Area, Ottawa, Ontario, Canada.

***Coptis trifolia* var. *groenlandicum* (L.) Salisb.**

Ranunculaceae Family

Canada, Ontario, Ottawa-Carleton, Notre-Dame-des-Champs
Mer Bleue Conservation Area, end of Dolman Ridge Road. Parking lot
#23, Dewberry Trail
N45 40741, W075 54372

Collected by R.I. Leduc, V. Fillon & R.W. Devereux, 17 May 2004
Identified by R.I. Leduc & J.T. Arnason

Bog forest. Site slightly elevated, near an *Acer rubrum* L.; *Onoclea sensibilis* and *Matteuccia struthiopteris* were also observed.

Specimens were collected from the wild for phytochemical analysis by R.I. Leduc (M.Sc. Thesis, University of Ottawa, Ottawa, Ontario, Canada). See extracts #286 & 379 for phytochemical data (R.I. Leduc, M.Sc. Thesis, University of Ottawa, 2007).

A2.3 Vascular Plant Herbarium of the Department of Agriculture in Ottawa (DAO) voucher 824183: wild *Sanguinaria canadensis* L. (bloodroot) harvested from North Gower, Ontario, Canada. Below-ground and above-ground tissues are shown.

***Sanguinaria canadensis* L.**
Papaveraceae Family

Canada, Ontario, Ottawa-Carleton, North Gower
Near 5884 Third Line Road North
45°10.227'N, 075°44.080' W

Collected by an employee of Gardens North, 23 Sept. 2005
Identified by R.I. Leduc & J.T. Arnason

Private woodlot

Specimens were collected in the wild for phytochemical analysis by R.I. Leduc (M.Sc. Thesis, University of Ottawa, Ottawa, Ontario, Canada). See extracts #334, 335, 336 & 337 for phytochemical data (R.I. Leduc, M.Sc. Thesis, University of Ottawa, 2007).

A3 Reference 152

Scott, I.M.; **Leduc, R.I.**; Burt, A.J.; Marles, R.J.; Arnason, J.T.; Foster, B.C. The Inhibition of Human Cytochrome P450 by Ethanol Extracts of North American Botanicals. *Pharm. Biol.* **2006**; *44*, 315 – 327.

Pharmaceutical Biology
2006, Vol. 44, No. 5, pp. 315–327

Taylor & Francis
Taylor & Francis Group

The Inhibition of Human Cytochrome P450 by Ethanol Extracts of North American Botanicals

I.M. Scott[1], R.I. Leduc[1], A.J. Burt[1], R.J. Marles[2], J.T. Arnason[1], and B.C. Foster[3]

[1]Biology Department, University of Ottawa, Ottawa, Ontario, Canada; [2]Natural Health Products Directorate, Health Canada, Ottawa, Ontario, Canada; [3]Therapeutic Products Directorate, Health Canada, Ottawa, Ontario, Canada

Abstract

High-throughput enzyme inhibition screening assays were used to quantify the effect of ethanol extracts of 2 accessions of 10 North American (NA) botanicals against the activity of the human cytochrome P450s: CYP3A4, CYP19, and CYP2C19. In addition, phytochemical biomarkers within each extract were identified and quantified using HPLC-MS or GC. Extracts containing uncharacterized phytochemicals were identified taxonomically. The overall objective was to describe the relationship between types and quantities of phytochemicals in ethanol extracts and their ability to inhibit CYP activity. The top three inhibitors of CYP3A4 were *Gaultheria procumbens* L. leaf > *Rhodiola rosea* L. root > *Arctostaphylos uva-ursi* L. Spreng leaf; of CYP19 were *R. rosea* root > *Rhododendron groenlandicum* (Oeder) Kron & Judd leaf > *A. uva-ursi* leaf; and of CYP2C19 were *Achillea millefolium* L. leaf and flower > *Vaccinium* sp. L. leaf > *Polygala senega* L. root. *Equisetum arvense* L. leaf, *Arctium lappa* L. root, and *P. senega* root had the least effect on CYP3A4 and CYP19 activity. These results suggest that North American botanicals have the potential to inhibit the metabolism of drug-specific CYPs *in vivo*, causing a direct shift in the availability of drugs and their pharmacokinetics in the body. Furthermore, the concentration of certain phytochemical markers varied significantly between accessions (i.e., rosarin and essential oils), suggesting that the extent of metabolic inhibition is directly dependent upon the concentration of bioactive constituents in an extract.

Keywords: Botanicals, CYP3A4, CYP19, CYP2C19, cytochrome P450 inhibition, P450-dependent drug metabolism, phytochemical biomarker.

Introduction

A large proportion of the world's population relies on the use of traditional medicine (TM), including the use of botanicals, in order to meet primary health care needs (WHO, 1999). Despite the introduction and practice of conventional medicines across the globe, a large number of people still rely on TM for financial, cultural, and traditional reasons. For example, North American (NA) aboriginal peoples still rely on more than 2500 medicinal plants for their traditional healing value (Moerman, 1998). Today, significant numbers of people in the United States (U.S.) and Canada use many of these plants as alternatives or complementary therapies. These plants are readily accessible in nature and continue to be wild crafted and cultivated for their medicinal properties. Given the continued popularity of using botanicals, it is important to recognize their potential to adversely affect the body. For example, many of the phytochemical constituents found in natural health products (NHPs) have the potential to significantly influence the disposition of pharmaceutical drugs in the body by altering their bioavailability, absorption, distribution, and excretion (Williamson, 2003; Zhou et al., 2003; Foster et al., 2005).

Pharmaceutical drugs, xenobiotics, as well as endogenous products are metabolized by a variety of cytochrome P450s (CYPs) that are widely distributed in the body. CYPs consist of numerous isozymes that have diverse metabolic roles and substrate specificities, each of which have a distinct effect on the rate of absorption and bioavailability of pharmaceutical drugs in the body. Products that alter P450-dependent drug metabolism,

Accepted: March 27, 2006

Address corresponding to: Dr. Brian Foster, Office of Science, Bureau of Policy, Therapeutic Products Directorate, Health Products and Food Branch, Health Canada, Holland Cross, Tower B, 1600 Scott Street, A.L. 3102C3, Ottawa, Ontario, K1A 1B6, Canada. Tel.: (613) 957-3506; Fax: (613) 941-5035; E-mail: brian_foster@hc-sc.gc.ca

DOI: 10.1080/13880200600746196

especially those that are not discriminatory, can therefore have a significant cascading impact on health. For example, St. John's wort is a known inducer of CYP3A enzymes, a factor that has been demonstrated to decrease the plasma levels of indinavir and cyclosporine leading to decreased bioavailability (Huang & Leshko, 2004). In contrast, *Echinacea* products have been shown to inhibit liver CYP1A2 and intestinal CYP3A activities. Besides St. John's wort and *Echinacea*, other botanical constituents of NHPs alter the function of CYPs including ginseng, goldenseal, garlic, and grapefruit (Foster et al., 2003, 2005; Huang & Leshko, 2004). Given that CYP enzymes affect the metabolism of endogenous steroids and neurotransmitters as well as their pharmaceutical analogues (Foster et al., 2003), it is important to characterize the extent and specificity of metabolic interference particularly caused by common herbs found in NHPs (Zhou et al., 2003). Such knowledge provides cautionary information regarding the potential health risks of complementing pharmaceutical drug therapies with botanical NHPs.

This study assessed metabolic inhibition by measuring the activity of specific CYPs *in vitro* with isozyme-specific P450-substrate markers (Ghosal et al., 2003). This high-throughput enzyme inhibition assay has proved itself as effective as previous HPLC methods (Bapiro et al., 2001). Three isozymes were screened: CYP3A4, CYP19, and CYP2C19. CYP3A4 is found in the liver, small intestine, and kidney, but it is primarily concentrated in the liver. It is induced by glucocorticoids and phenobarbitol (Yu, 2005) and is involved in metabolizing a broad range (75%) of pharmaceutical drugs, including indinavir and cyclosporine (Williamson, 2003), and the activation of aflatoxin B1 and nitroaromatics. CYP19, also commonly known as aromatase, is involved in steroid conversion of testosterone into estrogen (Simpson et al., 1994). CYP2C19 also is responsible for metabolizing xenobiotics (Ghosal et al., 2003).

Besides *Echinacea*, goldenseal, and a few other popular botanicals, there is a shortage of information regarding the ability of botanicals to inhibit CYP-dependent metabolism. In response to this lack of knowledge, the primary objective of this study was to screen ethanol extracts of 10 commonly used NA botanicals for their potential to inhibit CYP3A4, CYP19, and CYP2C19. Ethanol extracts (55% ethanol) of the following 10 botanicals were screened using a high-throughput, commercial *in vitro* assay method: *Vaccinium* sp. L. (Ericaceae) leaf; *Arctium lappa* L. (Asteraceae) root; *Polygala senega* L. (Polygalaceae) root; *Achillea millefolium* L. (Asteraceae) leaf and flower; *Arctostaphylos uva-ursi* L. Spreng (Ericaceae) root; *Equisetum arvense* L. (Equisetaceae) leaf; *Rhododendron groenlandicum* (Oeder) Kron & Judd (Ericaceae) leaf; *Rumex acetosella* L. (Polygonaceae) root; *Gaultheria procumbens* L. (Ericaceae) leaf; and *Rhodiola rosea* L. (Crassulaceae) root.

The secondary objectives of this study were to identify the differing inhibiting capabilities between accessions of a botanical species and to characterize this difference by comparing the phytochemical profile of each accession. Phenol compounds such as flavonoid glycosides and iridoids were analyzed using high-performance liquid chromatography with diode array detector (HPLC/DAD) coupled either with or without mass spectrometry with atmospheric pressure chemical ionization (MS/APCL). Gas chromatography (GC) was used to analyze extracts that contained essential oils. Chemically profiling a botanical extract is also a method of determining its true taxonomic identity and addresses issues of quality control.

Materials and Methods

Botanical sources

Dry bulk plant material was purchased from five Canadian NHP wholesalers and one U.S. wholesaler (Table 1). Although the geographical origin of each botanical is unknown, purchasing botanicals from different Canadian and U.S. wholesalers allowed for a varied source of genetic material. A sample of each botanical was purchased from at least two different wholesalers.

Ethanol fluid extracts

All plant material was finely ground in a Wiley mill and filtered through an 0.5-mm mesh screen to provide a total of 20 g of powder. Twenty-five milliliters of 55% ethanol: water (v:v) was added to 5 g of powder, and the plant-solvent mixtures were mechanically shaken (150 rpm) for 12 to 18 h. Amber glass vials were used in order to reduce the occurrence of photodegradation. Solids were removed using a Buchner filter system (Whatman no. 1 filter paper), and the filtrate was transferred into a 25-mL amber glass vial and stored at 4°C. A 1-mL aliquot of filtrate was dried at 60°C for 72 h and weighed in order to determine the percent dried extract weight (mg extract/g dry plant material). All filtrates were re-filtered with an 0.2-µm PFDE 13-mm membrane syringe filter (Chromatographic Specialties Inc., Brockville, Ontario, Canada) prior to HPLC separation.

Quantitative analyses of phytochemical markers

HLPC/DAD

The HPLC method used to analyze *A. uva-ursi* leaf extracts was modified from the Parejo et al. (2001) method. Four commercial standards in 6% methanol (m) were used: arbutin (hydroquinone-β-D-glucopyranoside) (Figure 1), gallic acid (3,4,5-trihydroxybenzoic acid), isoquercetin (3-glucosylquercetin), and myricitrin

Table 1. List of botanical scientific names, organ type, accession numbers, location from which accessions were purchased, percent recovery of total dried extract ± standard error (SE) (n = 2 to 3 per accession), and the statistical difference between accessions.

Botanical scientific name	Organ	Accession	% dried extract recovery ± SE	Statistical difference
A. millefolium L.	Leaf	230[2]	18.1 ± 2.3	0.01*
		231[4]	41.3 ± 3.2	
A. lappa L.	Root	201[1]	38.7 ± 0.8	0.003*
		202[3]	21.6 ± 0.5	
A. uva-ursi (L.) Spreng.	Leaf	191[2]	50.7 ± 5.52	0.42
		192[4]	40.4 ± 1.65	
E. arvense L.	Leaf	213[2]	14.3 ± 2.1	0.573
		215[3]	12.8 ± 0.2	
G. procumbens L.	Leaf	228[2]	34.6 ± 1.6	0.184
		229[3]	32.8 ± 0.7	
R. groenlandicum Oeder	Leaf	216[4]	29.9 ± 0.6	0.061
		217[1]	24.2 ± 1.8	
P. senega L.	Root	222[2]	18.4 ± 0.8	0.005*
		224[5]	35.4 ± 1.7	
R. rosea L.	Root	220[3]	37.7 ± 0.1	0.638
		221[6]	47.4 ± 3.8	
R. acetosella L.	Root	225[2]	8.0 ± 0.4	0.247
		227[3]	9.1 ± 0.1	
Vaccinium sp. L.	Leaf	194[2]	32.8 ± 0.7	0.019*
		196[1]	36.4 ± 1.0	

[1]Eastern Ontario, [2]Northeastern Ontario, [3]Southern Ontario, [4]Saskatchewan, [5]Manitoba, [6]Washington, USA.
*Indicates significant difference between two accessions (paired sample *t*-test, Bonferroni adjusted, $p < 0.05$).

(ChromaDex, Inc., Santa Ana, CA, USA). The HPLC-DAD was a Hewlett Packard 1100 Series HPLC: G1322A degasser; G1311A quatpump; G1313A ALS detector with ChemStation for LC 3D software (Agilent Technologies Inc., Palo Alto, CA, USA). The HPLC method was as follows: flow rate 1 ml/min; oven temperature 30°C; and initial conditions 0% water (A), 0% acetonitrile (B), 5% m (C), and 95% 10 mM phosphate buffer (D). Over the first 5 min, the conditions were held the same; at 5 min, B increased to 90% by 20 min, C remained constant, and D decreased to 5%. At 21 min, A:B:C:D returned to initial conditions of 0:0:5:95. The column was a YMC pro pack C18 (4.6 × 150 mm 5 µm) (Waters, Milford, MA, USA).

R. rosea L. root extracts were analyzed following the HPLC method of Tolonen et al. (2003). Two standards were used: salidroside (*p*-hydroxyphenethyl *O*-β-D-glucopyranoside) and rosarin [cinnamyl-(6′-*O*-α-L-arabinofuranosyl)-*O*-β-D-glucopyranoside] (Figure 1) (Chromadex, Inc.). HPLC analysis was conducted using an Agilent Technologies 1100 Series LCMS: attached to a G1315B DAD G1322A degasser; G1311A quatpump; G1313A ALS and G1316A with ChemStation for LC 3D software (Agilent Technologies Inc). The HPLC gradient went from initial conditions of 90% water (A), 5% acetonitrile (B), 5% m to 76:12:12 A:B:C in 16 min, 100% acetonitrile for 1 min, and returned to initial conditions after 5 min; the total run time was 22 min and the post-run time was 1 min. The flow rate was 0.3 ml/min, oven temperature 35°C, and the column was the YMC pro pack C18.

Vaccinium sp. L. leaf extracts were also analyzed using the Agilent Technologies HPLC system described above. The following standards were used: quercetin, myricitrin, chlorogenic acid, (Figure 1) catechin, epicatechin, kaempferol, glycosides of quercetin, and the anthocyanins, proanthocyanidin B1 and B2. The method was developed specifically for blueberry analyses as follows: Column separation was achieved using a YMC ODS-AM reverse phase column (3 µm, 120 Å, 2.0 × 100 mm), oven temperature at 50°C, and flow rate of 0.3 ml/min. The binary gradient initial conditions were 8% acetonitrile (A) and 92% 0.05% trifluoroacetic acid (TFA) pH 2.5 (B); 35% A and 65% B (12 min); 100% A (15 min); hold for 0.5 min; return to initial conditions 8% A and 92% B (19.5 min).

HPLC/DAD-MS/APCI

HPLC-MS analyses were conducted with *A. lappa* L. root, *R. acetosella* L. root, and *G. procumbens* L. leaf extracts using a phenolics method developed for the LC/MS whereby peaks were identified through the use of a HPLC-DAD phenolic compound library and then confirmed by MS spectral data. HPLC analysis was conducted using an Agilent Technologies 1100 Series LCMS and the same method described for *Vaccinium* sp. above. The MSD was equipped with APCI source and operated in positive and negative ionization mode. The MS was set on scan-mode, with either positive or negative polarity,

with the following parameter settings: mass range, 100 to 800 ms; fragmentor, 160; gain, 1.0; threshold, 150; step size, 0.1. The N_2 gas flow rate was 6.0 l/min; temperature, 300–350°C; nebulizer pressure, 60 psig; vaporizer temperature, 400–500°C; capillary voltage, 3000 V positive/4000 V negative; corona current, 4 μA positive/15 μA negative.

GC

The following two botanicals contain essential oils (EO) (Figure 1) and were analyzed using GC: *R. groenlandicum* Oeder leaf and *A. millefolium* L. leaf extracts. The EO fraction in the 55% extracts was re-extracted into hexane (1:1) and the hexane evaporated on ice to 1 ml. A 2-μl volume of each hexane-extracted EO sample was injected into the column. The GC used for this analysis was a Hewlett Packard 5890 A with FID detector, Agilent 6890 Series autoinjector (Hewlett Parkard, Avondale, PA, USA) with Peaksimple Version 2.75 software (SRI Instruments, Torrance, CA, USA). The column was a DB-5 60 m × 0.5 mm (J and W Scientific, cat. no. 125-5062, serial no. 2638511). The method was as follows: temperature gradient from initial 75°C; increase by 2°C/min to 125°C (25 min); from 125°C increased by 10°C/min to 300°C (42.5 min), and then hold for 5 min (47.5 min). The carrier gas was helium set for column head pressure of 20 psi at 75°C.

Taxonomic identification of senega and horsetail

Phytochemical constituents were not identified in the chromatographic profiles of *P. senega* L. root and *E. arvense* L. leaf extracts. Commercial standards could not be obtained for these botanicals. Therefore, taxonomic characters were applied to each of the two accessions for both species: samples of the leaf and root material were compared with herbarium specimens and taxonomically identified by J.T. Arnason at the University of Ottawa (Ottawa, Ontario, Canada).

P450 enzyme inhibition assay

Enzyme inhibition assays were conducted with CYP3A4, CYP19, and CYP2C19 isozymes (BD Bioscience, Bedford, MA, USA). The method described by Foster et al. (2001) was used for all three isozymes. All extracts were tested within 2–3 weeks of their preparation.

All extracts were incubated in the presence of an enzyme and the fluorescent substrate dibenzylfluorescein (DBF) (0.2 mmol L^{-1}). Percent inhibition of each extract was determined relative to metabolism in the presence of 55%. Ketoconazole at 1 μg/ml in concentration, 2 μl per well, was used as the positive control. The fluorescence readings were measured by a Cytofluor 4000 Fluorescence Measurement System plate reader (Applied Biosystems, Foster City, CA, USA) with excitation and emission at 485/20 and 535/25 with two gains (50 and 57).

All assay solutions were prepared in 1.5-ml plastic centrifuge tubes. The assays were performed on 96-well microtiter plates (Corning Costar Brand, VWR, Mississauga, Ontario, Canada). The following reagents were prepared for the assays: 0.5 M potassium phosphate buffer, pH 7.4, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) (Sigma, St. Louis, MD, USA), 15 mg/ml of 0.5 M potassium phosphate buffer, and 10 mg ketoconazole/ml in methanol.

Each plate well contained 100 μl of solution A (78 μl NADPH solution + 1222 μl distilled water); 90 μL of solution B [196 μl distilled water + 1036 μl 0.5 M potassium phosphate buffer (pH 7.4) + 14 μl enzyme, 14 μl DBF], or 90 μl of solution C [196 μl distilled water + 1036 μl 0.5 M, potassium phosphate buffer (pH 7.4) + 14 μl denatured enzyme, and 14 μl DBF]; 6 μl distilled water and 4 μl 55% or botanical extract. The positive control wells were similarly prepared except that the amount of distilled water was increased to 8 μl and the control solutions were 2 μl methanol or ketoconazole.

Given CYP19 and CYP2C19's inherently slower rate of metabolism, each well received 28 μl of enzyme, and solutions B and C were prepared with 182 μL of distilled water instead of 196 μL.

Each well was prepared under gold lamps in order to reduce NADPH exposure to natural and fluorescent light. Each extract was tested in triplicate. CYP3A4 assays were incubated for 20 min at 37°C during which fluorescence was recorded at T = 0, 10, and 20 min. CYP 19 and CYP 2C19 assays were incubated for 40 min, at 37°C, and fluorescence readings were recorded at T = 0, 10, 20, 30, and 40 min.

The percent enzyme inhibition was calculated using the following equation:

$$100\%[100 - \{(T - [TB - CB])/C\} \times 100]$$

where T is test, C is control, TB is test blank, and CB is control blank. Both the control and test well blank readings were considered in the formula in order to compensate for endogenous fluorescence and quenching. The mean percent inhibition and coefficient of variance was calculated for two assays of three triplicate well readings.

Statistical analysis

Statistical differences in percent yield, concentration of phytochemical constituents, and percent inhibition of each CYP isozyme between accessions were determined using Bonferroni adjusted, paired sample *t*-tests (SYSTAT Software Inc, Point Richmond, CA, USA) except the yarrow extracts, which were analyzed using a one-way ANOVA and Tukey's multiple comparison of means test. The Labrador tea extracts were statistically analyzed using using Kruskal-Wallis one-way

ANOVA with Mann-Whitney *U*-test (non-parametric data) (SYSTAT).

The relationship between either the dry extract weight and the CYP inhibition or the phytochemical biomarker concentration and the CYP P450 inhibition were tested only when those extracts or biomarkers were significantly different ($p < 0.05$) between accessions of the same botanical. A positive relationship was assumed if one of the two botanical accessions had either a significantly greater extract dry weight or phytochemical biomarker concentration that matched a significantly higher CYP inhibition relative to the other accession.

Results

Ethanol fluid extracts

The percent yield of dried extract for all 10 NA ethanol extracts, including each accession, ranged from 8% to 50% dry weight (mg extract/g plant material) (Table 1). *A. uva-ursi* leaf yielded the highest percent of dried extract (40–50%), followed by *R. rosea* root (38–48%) and *Vaccinium* sp. leaf (33–37%). The botanicals that yielded the least amount of dried extract were *R. acetosella* leaf (8–9%), *E. arvense* leaf (13–15%), and *P. senega* root (18–35%). Six of the 10 botanicals were found to have no significant differences in dried extract recovery between accessions. Significant differences in dried extract recovery between accessions were only found for *Vaccinium* sp. leaf, *A. lappa* root, *P. senega* root, and *A. millefolium* leaf and flower (paired sample *t*-test, Bonferroni adjusted, $p < 0.05$).

Analysis of botanicals by HPLC-DAD and HPLC-MS

The phytochemical markers of 8 out of the 10 botanicals were quantified using HPLC-MS and GC (Table 2). Salidroside and rosarin were identified and quantified in both accessions of *R. rosea* root (Fig. 2). Rosarin was found to be more concentrated in accession 221 compared with accession 220 (paired sample *t*-test, Bonferroni adjusted, $p = 0.035$), but there was no difference in the salidroside concentrations between accessions ($p = 0.092$).

Three phytochemical markers were identified and quantified in both accessions of *A. uva-ursi* leaf extracts: arbutin, gallic acid (Fig. 3; Table 2), and myricitrin (not shown). Arbutin was the most concentrated of the three constituents in both accessions. No significant differences in the total concentration of phytochemical markers between the accessions were found (paired sample *t*-test, Bonferroni adjusted, $p > 0.088$).

Chlorogenic acid was identified and quantified in both *A. lappa* root and *G. procumbens* leaf extracts (Fig. 3).

Table 2. Mean concentration of isolated phytochemical markers (μg/ml) ± standard error (SE) from two accessions of *Arctostaphylos uva-ursi* leaf, *Rhodiola rosea* root, *Arctium lappa* root, *Gaultheria procumbens* leaf, *Rumex acetosella* root, and *Vaccinium* sp. leaf 55% ethanol extracts (n = 2 to 3 per accession) and the statistical difference between accessions.

Botanical and phytochemical marker	Mean concentrations (SE)		Statistical difference
	Accession A	Accession B	
A. lappa root	201	202	
Chlorogenic acid	249 (35)	267 (46)	0.799
A. uva-ursi leaf	191	192	
Arbutin	9831 (163)	8724 (246)	0.226
G. procumbens leaf	228	229	
Chlorogenic acid	127 (5.1)	105 (3.4)	0.009*
R. rosea root	220	221	
Salidroside	417 (1)	1460 (341)	0.092
Rosarin	115 (8)	1443 (253)	0.035*
R. acetosella root	225	227	
Luteolin	0	0.6 (0)	0.022*
Emodin	55 (6)	17 (1)	0.05*
Vaccinium sp. leaf	194	196	
Chlorogenic acid	7269 (266)	8425 (412)	0.017*
Quercetin-3,7-di-*O*-glucoside	3 (0.1)	1.4 (0.1)	0.006*
Myricitrin	23 (2)	107 (3)	0.002*
Quercetin-7-glucoside	12 (2)	479 (15)	0.017*
Quercetin	175 (6)	78 (5)	0.025*
Quercetin-3-glucoside	8 (0.4)	20 (1)	0.02*
Kaempferol	15 (0.7)	23 (2)	0.04*

*Indicates significant difference between two accessions (paired sample *t*-test, Bonferroni adjusted, $p < 0.05$).

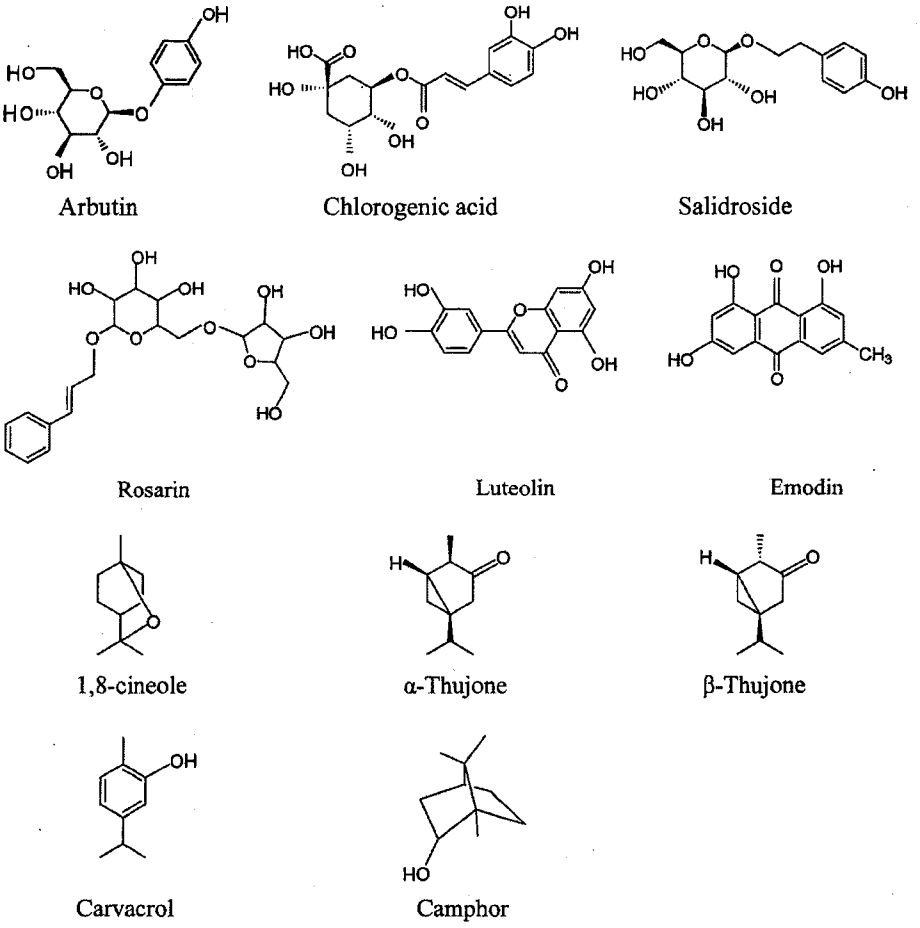


Figure 1. Phenolic compounds and essential oil constituents.

There was no measurable difference in chlorogenic acid concentration between the two *A. lappa* root accessions (paired sample *t*-test, Bonferroni adjusted, $p = 0.799$), however, chlorogenic acid was more concentrated in *G. procumbens* accession 228 than accession 229 (paired sample *t*-test, Bonferroni adjusted, $p = 0.009$) (Table 2).

Emodin and luteolin (Figure 1) were identified and quantified in both *R. acetosella* root accessions. Both phytochemical markers were significantly more concentrated in accession 225 compared with accession 227 (paired sample *t*-test, Bonferroni adjusted, $p \leq 0.05$) (Table 2).

Thirteen phenolic compounds were identified and quantified in both *Vaccinium* sp. leaf accessions (Table 2). Catechin, epicatechin, quercetin-3,7-di-*O*-galactoside, quercetin-3-galactoside, quercetin-3-arabinoside, and quercetin-3-rhamnoside were equally concentrated in both accessions (paired sample *t*-test, Bonferroni adjusted, $p > 0.059$). Quercetin and quercetin-3,7-di-*O*-glucoside were more concentrated in accession 194 ($p < 0.025$) and chlorogenic acid, myricitrin, quercetin-7-glucoside, quercetin-3-glucoside, and kaempferol were more concentrated in accession 196 ($p < 0.04$).

Analysis of botanicals by GC

Twenty-one EO marker compounds were identified and quantified in the *A. millefolium* leaf and flower extracts (chromatograph not shown), 13 of which were found to be significantly different in concentration between accessions (one-way ANOVA, Tukey's test, $p < 0.048$) (Table 3). 1,8-Cineole, camphor, and 4-terpineol were more concentrated in accession 231 ($p = 0.001$). Carvone was more concentrated in accession 230 ($p = 0.002$). The concentration of β-pinene was not significantly different between accessions ($p > 0.493$).

A total of nine EO marker compounds were identified (chromatograph not shown) and quantified in both *R. groenlandicum* leaf accessions (Table 3). No significant differences in concentration of EO marker compounds were found between the two accessions (Kruskal-Wallis one-way ANOVA, Mann-Whitney *U*-test statistic,

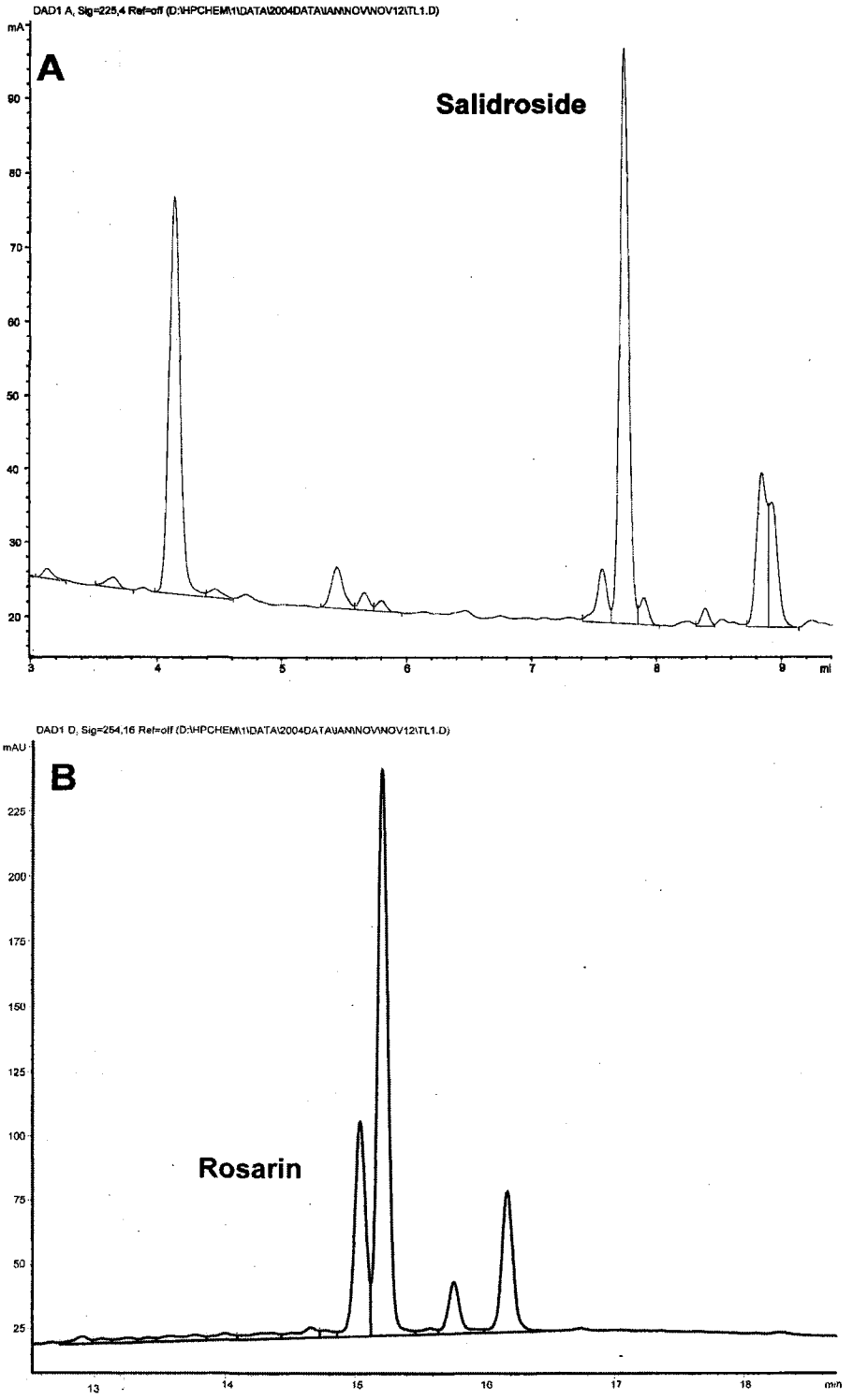


Figure 2. HPLC chromatograph of *Rhodiola rosea* showing salidroside at 225 nm (A) and rosarin at 254 nm (B).

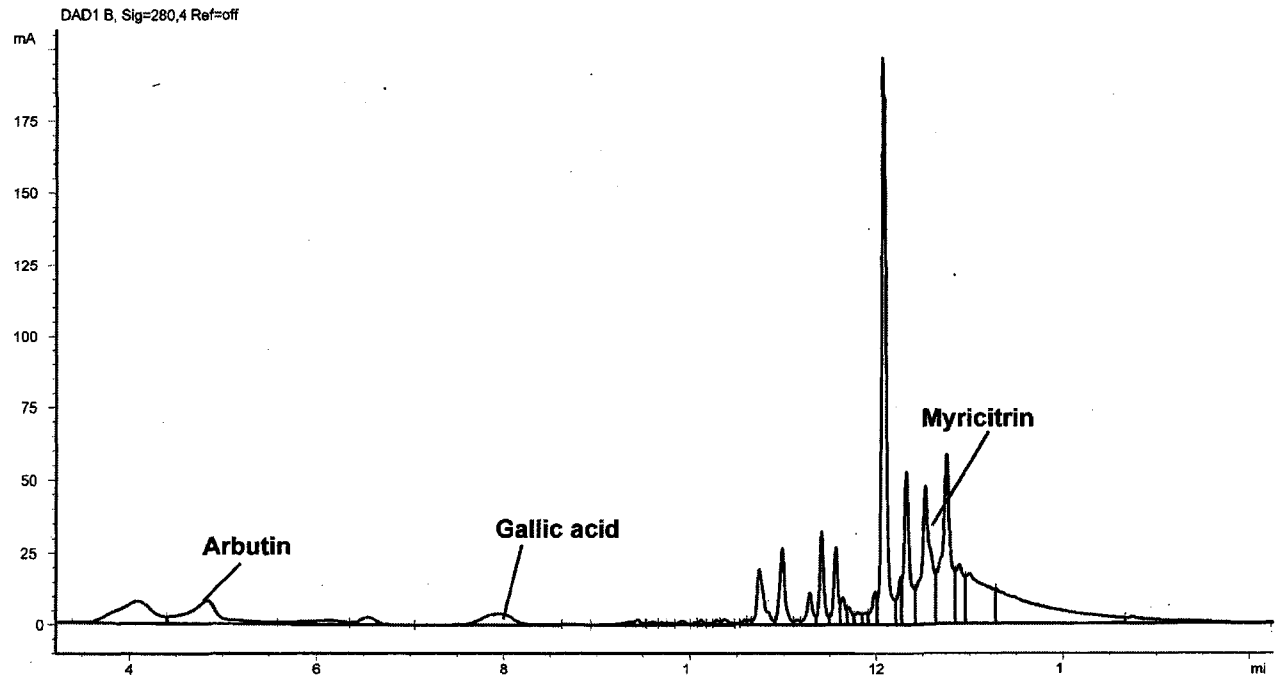


Figure 3. HPLC chromatograph of *Arctostaphylos uva-ursi* showing arbutin, gallic acid, and myricitrin at 280 nm.

$p > 0.114$). Nerolidol was the most concentrated constituent in both accessions.

P450-dependent enzyme inhibition assay

The following 10 botanical extracts inhibited CYP3A4-dependent metabolism in decreasing order of inhibition: *G. procumbens* leaf > *R. rosea* root > *A. uva-ursi* leaf > *R. groenlandicum* leaf > *Vaccinium* sp. leaf > *R. acetosella* root > *P. senega* root > *A. millefolium* leaf and flower > *E. arvense* leaf > *A. lappa* root (Table 4).

CYP19-dependent metabolism was inhibited in decreasing order of inhibition: *R. rosea* root > *R. groenlandicum* leaf > *A. uva-ursi* leaf > *R. acetosella* root > *A. millefolium* leaf and flower > *G. procumbens* leaf > *Vaccinium* sp. leaf > *P. senega* root > *A. lappa* root > *E. arvense* leaf (Table 4).

CYP2C19-dependent metabolism was inhibited by the following four botanicals, in decreasing order of inhibition: *A. millefolium* leaf and flower > *Vaccinium* sp. leaf > *P. senega* root > *A. lappa* root. The remaining six botanical extracts were not screened for their ability to inhibit CYP 2C19-dependent metabolism.

In the majority of cases, no significant differences in percent enzyme inhibition were observed between accessions (paired sample *t*-test, Bonferroni adjusted, $p > 0.05$). Significant differences in CYP19 and CYP2C19 inhibition were observed with *A. lappa* root: accession 202 had greater CYP19 inhibition than 201 ($p = 0.007$), whereas accession 202 had less CYP2C19 inhibition than 201 ($p = 0.046$). The only other extracts where differences between accessions were observed were the following: CYP3A4, *P. senega* root, *E. arvense* leaf, and *R. groenlandicum* leaf ($p \leq 0.018$); CYP19, *R. acetosella* root and *R. rosea* root ($p \leq 0.038$) (Table 4).

Discussion

Quantitative analyses of phytochemical markers

Iridoids and phenol glycosides in the *R. rosea* root and *A. uva-ursi* leaf extracts were quantified based on existing methods (Parejo et al., 2001; Tolonen et al., 2003). Several glycosides, aglycones, catechins, as well as chlorogenic acid were successfully quantified using a method developed by the authors. Chlorogenic acid in *A. lappa* root and *G. procumbens* leaf extracts, as well as luteolin and emodin in *R. acetosella* root extracts were also quantified using this new method. Methyl salicylate is the active component most often associated with *G. procumbens* leaf extracts (Botma et al., 2001; Brinker, 2001), however, it was not identified using this method.

The EO phytochemical markers that were most concentrated in the *A. millefolium* leaf and flower extracts were 1,8-cineole, camphor, 4-terpineol, L-borneol, and β-thujone (Table 3). In contrast, nerolidol 1 and 2, α-terpineol, and carvacrol were the most concentrated EO markers in the *R. groenlandicum* leaf extracts (Table 3). The latter phytochemicals are all relatively less volatile compared with α-pinene and camphene and, thus, may be more easily

Table 3. Mean essential oil (EO) concentration (μg/ml) ± standard error (SE) in two accessions of *Achillea millefolium* and *Rhododendron groenlandicum* leaf 55% ethanol extracts (n = 2 to 3 per accession) and the statistical difference between accessions.

Botanical and phytochemical marker	Mean concentration (SE)		Statistical difference
A. millefolium	230	231	
α-Pinene	0	3 (1)	0.048*
p-Cymene	1 (0)	12 (3)	0.009*
1,8-Cineole	23 (3)	518 (29)	< 0.001*
γ-Terpinene	4 (0.3)	8 (0.4)	0.001*
α-Thujone	1 (0)	3 (0)	< 0.001*
β-Thujone	1 (0)	37 (3)	< 0.001*
Camphor	11 (1)	172 (19)	< 0.001*
4-Terpineol	16 (2)	74 (6)	0.001*
Ascaridol	1 (0)	14 (1)	< 0.001*
Carvone	13 (1)	1 (1)	0.002*
Carvacrol	0.3 (0.3)	17 (4)	0.009*
β-carophyllene	2 (0.3)	7 (1)	0.004*
L-Borneol	3 (2)	56 (9)	0.002*
Nerolidol 2	0	2 (0)	< 0.001*
R. groenlandicum	216	217	
β-Pinene	0	0.6 (0.3)	0.114
Linalool	0.3 (0.3)	0	0.317
Camphor	0.3 (0.2)	0.6 (0.6)	0.817
iso-Borneol	0.3 (0.2)	0.4 (0.4)	0.817
L-Borneol	0.1 (0.1)	0	0.317
α-Terpineol	1.6 (1.6)	0.6 (0.6)	0.796
Carvacrol	1.7 (0.8)	0	1.0
Nerolidol 1	6.1 (3.3)	8.1 (2)	0.658
Nerolidol 2	11.3 (11.3)	0	0.317

*Indicates significant difference in mean concentration of *A. millefolium* essential oil between accessions (one-way ANOVA, Tukey's test, $p < 0.05$) and concentration of *R. groenlandicum* essential oil between accessions (Kruskal-Wallis one-way ANOVA, Mann-Whitney *U*-test, $p > 0.05$).

retained in 55% ethanol, and therefore this might be the reason why they are more concentrated in the EO extract. EO extracts are frequently prepared using steam or hydrodistillation, for example from *R. groenlandicum* leaves (Belleau & Collin, 1993). Because 55% crude extracts were initially prepared, it was necessary to use a hexane extraction of the aqueous/alcohol preparations instead of the original preparation in order to analyze the EO markers.

Phytochemical markers in *E. arvense* leaf and *P. senega* root extracts were not fully characterized due to lack of saponin standards required and resources to complete the multistep method described by Yoshikawa et al. (1995). *E. arvense* leaf extracts are typically characterized by their saponin and high silica content (Duke & Fulton, 2002) but also have many flavanoids (Veit et al., 1995).

Relationship between phytochemical markers and CYP P450 inhibition

Rhodiola rosea root

R. rosea has long been used in Europe and Asia to treat infectious illnesses, to increase mental acuity, and to treat psychiatric and neurological conditions (Brown et al., 2002) and more recently as adoptogens in the west (Ganzera et al., 2001). According to our results, *R. rosea* root extracts significantly inhibited the activities of CYP3A4 and CYP19 (67% and 83%, respectively) and were ranked the most potent extract compared with the nine other botanical species. A positive relationship between rosarin concentration and the level of metabolic inhibition was also found for both accessions. Our findings correspond with those by Tolonen et al. (2003) where glycosidic constituents extracted from *R. rosea* root have inhibited human prolyl endopeptidases. Our findings are unique in that there are no other published data to suggest *R. rosea* root interacts with P450 enzymes.

Arctostaphylos uva-ursi

The principal pharmacological claim associated with *A. uva-ursi* leaf extracts is as a urinary antiseptic (Moerman, 1998; Parejo et al., 2001), but it also has noted antioxidative activity (Amarowicz, 1999). According to our results, *A. uva-ursi* leaf crude extracts were also consistently potent inhibitors of CYP3A4 and CYP19 activity (61% and 67%, respectively) and were ranked the second most potent inhibitors. The most concentrated

Table 4. Mean percent inhibition ± coefficient of variance (c.v.) of CYP3A4, CYP19, and CYP2C19 activity by two accessions of 10 botanical extracts and ketoconazole positive control, total mean percent inhibition, and rank for each botanical per isozyme.

Botanical (organ type)	CYP3A4 inhibition ± c.v. (Accession no.)		Mean/rank	CYP19 inhibition ± c.v. (Accession no.)		Mean/rank	CYP2C19 inhibition ± c.v. (Accession no.)		Mean/rank
A. millefolium (leaf, flower)	21 ± 7 (230)	29 ± 5 (231)	25.0/8	37 ± 8 (230)	*50 ± 8 (231)	43.5/5	71 ± 7 (230)	55 ± 1 (231)	63.0/1
A. lappa (root)	16 ± 13 (201)	11 ± 7 (202)	13.5/10	24 ± 8 (201)	*33 ± 5 (202)	28.5/9	*33 ± 0 (201)	17 ± 9 (202)	25.0/4
A. uva-ursi (leaf)	54 ± 5 (191)	69 ± 15 (192)	61.5/3	53 ± 8 (191)	80 ± 14 (192)	66.5/3			
P. senega (root)	16 ± 6 (222)	*39 ± 1 (224)	27.5/7	23 ± 4 (222)	39 ± 5 (224)	31.0/8	34 ± 7 (222)	41 ± 10 (224)	37.5/3
E. arvense (leaf)	22 ± 4* (213)	7 ± 15 (215)	14.5/9	28 ± 7 (213)	26 ± 8 (215)	27.0/10			
G. procumbens (leaf)	66 ± 13 (228)	78 ± 1 (229)	72.0/1	45 ± 2 (228)	41 ± 10 (229)	43.0/6			
R. groenlandicum (leaf)	39 ± 8 (216)	*57 ± 5 (217)	48.0/4	77 ± 10 (216)	75 ± 9 (217)	76.0/2			
R. rosea (root)	49 ± 15 (220)	85 ± 3 (221)	67.0/2	69 ± 7 (220)	*98 ± 3 (221)	83.5/1			
R. acetosella (root)	42 ± 12 (225)	35 ± 11 (227)	38.5/6	57 ± 2 (225)	49 ± 6 (227)	53.0/4			
Vaccinium sp. (leaf)	32 ± 12 (194)	47 ± 12 (196)	39.5/5	37 ± 8 (194)	42 ± 3 (196)	39.5/7	69 ± 10 (194)	26 ± 9 (196)	47.5/2
Ketoconazole positive control	78 ± 10 (n = 4)			39 ± 27 (n = 7)			50 ± 41 (n = 8)		

*Indicates significant difference between two accessions (paired sample *t*-test, Bonferroni adjusted, $p < 0.05$).

phytochemical identified in *A. uva-ursi* leaf extracts was arbutin (hydroquinone-β-D-monoglucopyranoside). No relationship was found between the extent of CYP inhibition and the concentrations of arbutin or the other two minor constituents, gallic acid or myricitrin. Because arbutin is metabolized in the liver into hydroquinone conjugates (Newton et al., 2001), becoming potent antimicrobials in the urinary tract (Dykes et al., 2003), future studies should investigate the effect of hydroquinone conjugates on the activity of CYP enzymes.

Rhododendron groenlandicum and achillea millefolium

R. groenlandicum is used for treating headcolds, stomach aches, and used as a diuretic and emetic (Moerman, 1998) and to treat gout (Owen & Johns, 1999). Ethanol extracts of *R. groenlandicum* leaves were shown to have moderate inhibitory activity against CYP3A4 (48%), but greater inhibition with CYP19 (76%). However, there was no relationship between the concentration of EOs measured in the extract and the extent of CYP inhibition. Our findings are supported in part by those of two previous studies. First, the tea made from *R. groenlandicum* leaves was determined to be antimutagenic, the explanation being the extract constituents inhibit the CYP-activation system and therefore reduce genotoxin production (Idaomar et al., 2002). Second, *R. groenlandicum* leaf extracts contain quercetin-3-rhamnoside and quercetin-3-arabinoside, two glycosidic derivatives of quercetin (data not shown), two flavonoids among others found in *Gingko biloba*, which are reported to greatly inhibit the activity of CYP3A4 (Gaudineau et al., 2004).

A. millefolium was recognized as having the greatest number of drug-related uses of all NA plants by native peoples, commonly as an analgesic (Moerman, 1998). According to our results, *A. millefolium* leaf and flower extracts moderately inhibited CYP3A4, CYP19, and CYP2C19 (25%, 43%, and 63%, respectively). All EO constituents quantified in the *A. millefolium* extracts had a positive relationship with CYP3A4 and CYP19 inhibition except for carvone, which described a negative relationship (Table 3). Our findings contradict those of Brinker (2001) where *A. millefolium* leaf and flower extracts were thought to stimulate acid secretion in the stomach, reducing effectiveness of gastrointestinal tract drugs, but not because of CYP inhibition. The *A. millefolium*–CYP inhibition shown in the current study indicates a nonspecific activity against a range of human drug-metabolizing enzymes, a fact that might lead to a greater than expected *in vivo* or clinical herb-drug interaction.

Rumex acetosella

R. acetosella is used traditionally in North America for gastrointestinal problems (Moerman, 1998) and in southern Europe as an antidiarrheal and hypotensive, and other *Rumex* spp. are recommended for colds, digestive and heart tonic use (Rivera et al., 2005). According to our results, *R. acetosella* root has moderate inhibitory activity with CYP3A4 and CYP19 (38–53%, respectively). The concentrations of the marker constituents, emodin and luteolin, were found to be significantly different between *R. acetosella* root accessions (Table 3), but there was no positive relationship found between the concentrations and the CYP3A4 or CYP19 inhibition (Table 4). The antimutagenic activity of emodin in *Rheum officinale* Baill. is attributed to its inhibition of CYP1A1, the enzyme responsible for *N*-hydroxylation of Trp-P-2, creating a genotoxic compound (Sun et al., 2000). Therefore, emodin and possibly luteolin may have some CYP-inhibitory activity, as observed in *R. officinale*, but other constituents are likely more responsible for the inhibition observed in *R. acetosella*.

Arctium lappa, gaultheria procumbens and vaccinium sp.

In North America, *A. lappa* has been applied to treat rheumatism, to cleanse the blood, and as a urinary aid; *G. procumbens* infusions are used as an analgesic, a cold remedy, and for gastrointestinal and rheumatism; and *Vaccinium* sp. are used as an antidiarrheal and kidney aid (Moerman, 1998). The *A. lappa* root extract was found to be a weak inhibitor of CYP3A4, CYP19, and CYP2C19 (< 30%), the *Vaccinium* sp. leaf extract moderately inhibited CYP3A4 and CYP19 (39%), and the *G. procumbens* leaf extract highly inhibited CYP3A4 (72%) but not CYP19 (43%). Chlorogenic acid was isolated from all three botanicals, however, no relationship appeared to exist between the concentration of chlorogenic acid (*Vaccinium* sp. leaf > *A. lappa* root > *G. procumbens* leaf) and enzyme inhibition (*G. procumbens* leaf > *Vaccinium* sp. leaf > *A. lappa* root) for all three CYP isozymes. In the case of *Vaccinium* sp. leaf extracts, the dried extract weight (Table 1) and chlorogenic acid content in accession 196 was significantly greater than 194 (Table 2), yet there was no difference in the mean CYP3A4 or CYP19 inhibition (Table 4). It was also found that *G. procumbens* leaf extract's potent inhibiting effect on CYP3A4 metabolism was not related to chlorogenic acid content in the extracts.

In support of these findings, chlorogenic acid in crude St. John's wort extracts was found to have no inhibitory action against other monoxygenases: CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (Obach, 2000). Contradicting these results, however, were findings that chlorogenic acid at 0.5 μM inhibited other monoxygenases: benzyloxyresorufin *O*-dealkylase (BROD) (51%), ethoxyresorufin *O*-demethylase (EROD) (47%), and methoxyresorufin *O*-demethylase (MROD) (54%) (Teel & Huynh, 1998). These results were repeated by Baer-Dubowska et al. (1998), but it was found that chlorogenic acid barely inhibited the activity of EROD ($IC_{50} = 3.5$ mM),

moderately inhibited the activity of MROD (IC_{50} = 33 μM), and potently inhibited the activity of pentoxyresorufin *O*-dealkylase (PROD) (IC_{50} = 18 μM). Chlorogenic acid inhibition of EROD and MROD activity (associated with CYP1A1 and CYP1A2, respectively) was described as noncompetitive and as a mixed competitor of PROD (associated with CYP2B1/2).

The moderate inhibition of the CYP3A4 and CYP19 by *Vaccinium* sp. leaf extract may have less to do with chlorogenic acid and more to do with quercetin-3-rhamnoside, quercetin-3-arabinoside, and other flavonoids mentioned previously to be in *R. groenlandicum* leaf extracts. These flavanoids have high inhibitory activity against CYP3A4 (Gaudineau et al., 2004), as does kaempferol, commonly found in black tea *Camellia sinensis*, and recognized to inhibit CYP3A4 and aromatase (Brinker, 2001). Because *G. procumbens* and *Vaccinium* sp. leaf extracts are used to treat many common ailments, there is a risk that the high to moderate CYP3A4 inhibition associated with each, respectively, may increase the plasma levels of drugs. There may be less concern for *A. lappa* root, commonly used in traditional Japanese cuisine, and as a source of fructofuranan, an insulin-type compound that is used for treating urogenital diseases (Kardošová et al., 2003).

Polygala senega and equisetum arvense

P. senega root decoctions are used to treat respiratory diseases, rheumatism, and as diaretic, and *E. arvense* stem and leaves are used as a kidney and urinary aid (Moerman, 1998). *P. senega* root extracts moderately inhibited the activity of CYP19 (31%) and CYP2C19 (37%) but showed a weaker inhibition of CYP3A4 (27%). Although no phytochemical markers were identified, a positive relationship was measured between the significantly greater dried extract recovery (Table 1) and CYP3A4 inhibition for accession 224 relative to accession 222 (Table 4). Accession 224 had greater CYP19 and CYP2C19 inhibition than accession 222 as well, but this was not statistically different. Because *P. senega* root constituents have shown promise in treating hypoglycemia as evidenced by significantly lowered glucose levels shown *in vivo* (Yoshikawa et al., 1995), the low potential for drug interaction mediated by CYP3A4 inhibition in patients is encouraging. The *E. arvense* leaf and *A. lappa* root extracts demonstrated the lowest inhibition (<15% inhibition) against the CYP3A4 enzyme. Both were twice as active at inhibiting CYP19 (<30%). These botanicals have not previously been reported to inhibit CYP activity. *E. arvense* from NA sources contain flavonoids such as luteolin 5-(6′-*O*-malonyl-glucoside) and quercetin 3-*O*-(6″-*O*-malonylglucoside) (Veit et al., 1995). Although not identified as marker constituents in the current study, these flavanoids may provide some of the CYP inhibition.

In conclusion, it has been shown that several NA botanicals commonly used in TM and to complement pharmaceutical drug therapies can potentially influence the bioavailability and pharmacokinetics of a broad range of important pharmaceutical drugs by inhibiting CYP P450 drug-metabolism. Although it is difficult to extrapolate information from *in vitro* studies to a clinical context, the *in vitro* induction of enzyme activity by St. John's wort and garlic have also been demonstrated with repeated exposure to lead to decreased drug bioavailability *in vivo* (Foster et al., 2005). The findings of this article also indicate that a wide variety of NA botanicals have the potential to affect the major drug-metabolizing enzyme CYP3A4 and steroid-metabolizing enzyme CYP19. This is of concern given (1) the current popularity of NHPs, (2) they are often used to complement pharmaceutical therapies, and (3) the lack of this type of information provided by manufacturers, although it may be required for the cautionary labeling that is mandatory for licensed natural health products under the Canadian Natural Health Products Regulations. Future studies will focus on *A. uva-ursi* and *R. rosea* as these were the botanicals that demonstrated consistently the greatest inhibition against CYP isozymes 3A4 and 19.

Acknowledgments

Funding was provided by Health Canada; the authors would also like to thank L. Ntezurubanza for his analytical assistance as well as S. Nguyen and D. Farsi for their laboratory assistance.

References

Amarowicz R (1999): Potential natural antioxidants from Saskatchewan indigenous plants. *J Food Lipids 6*: 317–329.

Bapiro TE, Egnell A-C, Hasler JA, Masimirembwa CM (2001): Application of higher throughput screening (HTS) inhibition assays to evaluate the interaction of antiparasitic drugs with cytochrome P450s. *Drug Metabol Disposition 29*: 30–35.

Belleau F, Collin G (1993): Composition of the essential oil of *Ledum groenlandicum*. *Phytochemistry 33*: 117–121.

Botma M, Colquhiun-Flannery W, Leighton S (2001): Laryngeal oedema caused by accidental ingestion of oil of wintergreen. *Int J Pediatr Otorhinolaryngol 58*: 229–232.

Brinker F (2001): *Herb Contraindications and Drug Interactions*, 3rd ed. Sandy, OR, Eclectic Medical Publications.

Brown RP, Gerbarg PL, Ramazanov Z (2002): *Rhodiola rosea* A phytomedicinal overview. *Herbal Gram 56*: 40–52.

Duke JA, Fulton M eds. (2002): *Handbook of Medicinal Herbs*, 2nd ed. Boca Raton, CRC Press.

Dykes GA, Amarowicz R, Pegg RB (2003): An antioxidant bearberry (*Arctostaphylos uva-ursi*) extract modulates surface hydrophobicity of a wide range of food-related bacteria: Implications for functional food safety. *Food Control 14*: 515–518.

Fan W, Tezuka Y, Komatsu K, Namba T, Kadota S (1999): Prolyl endopeptidase inhibitors from the underground part of *Rhodiola sacra* S.H. Fu. *Biol Pharm Bull 22*: 157–161.

Foster BC, Foster MS, Vandenhoek S, Budzinski J, Gallicano KD, Choudri S, Krantis A, Arnason JT (2001): An *in vitro* evaluation of human cytochrome P450 3A4 and P-glycoprotein inhibition by garlic. *J Pharm Pharmaceut Sci 4*: 159–167.

Foster BC, Vandenhoek S, Hana J, Krantis A, Akhtar MH, Bryan M, Budzinski JW, Ramputh A, Arnason JT (2003): *In vitro* inhibition of human cytochrome P450-mediated metabolism of marker substrates by natural products. *Phytomedicine 10*: 334–342.

Foster BC, Arnason JT, Briggs CJ (2005): Natural health products and drug disposition. *Annu Rev Pharmacol Toxicol 45*: 203–226.

Ganzera M, Yayla Y, Khan IA (2001): Analysis of the marker compounds of Rhodiola rosea L. (golden root) by reversed phase high-performance liquid chromatography. Chem Pharm Bull (Tokyo) 49: 465–467.

Gaudineau C, Beckerman R, Welbourn S, Auclair K (2004): Inhibition of human P450 enzymes by multiple constituents of the *Ginkgo biloba* extract. *Biochem Biophys Res Commun 318*: 1072–1078.

Ghosal A, Hapangama N, Yuan Y, Lu X, Horne D, Patrick JE, Zbaida S (2003): Rapid determination of enzyme activities of recombinant human cytochromes P450, human liver microsomes and hepatocytes. *Biopharm Drug Dispos 24*: 375–384.

Huang S-M, Leshko LJ (2004): Drug-drug, drug-dietary supplement, and drug-citrus fruit and other food interactions: What have we learned? *J Clin Pharmacol 44*: 559–569.

Idaomar M, El Hamss R, Bakkali F, Mezzoug N, Zhiri A, Baudoux D, Muñoz-Serrano A, Liemans V, Alonso-Moraga A (2002): Genotoxicity and antigenotoxicity of some essential oils evaluated by wing spot test of *Drosophila melanogaster*. *Mutat Res 513*: 61–68.

Kardošová A, Ebringerová A, Alföldi J, Nosál'ová G, Fraňová S, Hříbalová V (2003): A biologically active fructan from the roots of *Arctium lappa* L., var. Herkules. *Int J Biol Marcromol 33*: 135–140.

Moerman DE (1998): *Native American Ethnobotany*. Portland, OR, Timber Press.

Newton M, Combest W, Kosier H (2001): Select herbal remedies used to treat common urological conditions. *Urologic Nursing 21*(3): 232–234.

Obach RS (2000): Inhibition of human cytochrome P450 enzymes by constituents of St. John's wort, an herbal preparation used in the treatment of depression. *J Pharm Exper Ther 294*: 88–95.

Owen PL, Johns T (1999): Xanthine oxidase inhibitory activity of northeastern North American plant remedies used for gout. *J Ethnopharmacol 64*: 149–160.

Parejo I, Viladomat F, Bastida J, Codina C (2001): A single extraction step in the quantitative analysis of arbutin in bearberry (*Arctostaphylos uva-ursi*) leaves by high-performance liquid chromatography. *Phytochem Anal 12*: 336–339.

Rivera D, Obon C, Inocencio C, Heinrich M, Verde A, Fajardo J, Llorach R (2005): The ethnobotanical study of local Mediterranean food plants as medicinal resources in southern Spain. *J Physiol Pharmacol 56*(Suppl. 1): 97–114.

Simpson ER, Mahendroo MS, Means GM, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, Mendelson CR, Bulun SE (1994): Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Rev 15*: 342–355.

Sun M, Sakakibara H, Ashida H, Danno G, Kanazawa K (2000): Cytochrome P4501A1-inhibitory action of antimutagenic anthraquinones in medicinal plants and the structure-activity relationship. *Biosci Biotechnol Biochem 64*: 1373–1378.

Teel RW, Huynh H (1998): Modulation by phytochemicals of cytochrome P450-linked enzyme activity. *Cancer Lett* 133: 135–141.

Tolonen A, Hohtola A, Jalonen J (2003): Comparison of electrospray ionization and atmospheric pressure chemical ionization techniques in the analysis of the main constituents from *Rhodiola rosea* extracts by liquid chromatography/mass spectrometry. *J Mass Spectrom 38*: 845–853.

Veit M, Beckert C, Höhne C, Bauer K, Geiger H (1995): Interspecific and intraspecific variation of phenolics in the genus *Equisetum* subgenus *Equisetum*. *Phytochemistry 38*: 881–891.

WHO (1999): *WHO Monographs on Selected Medicinal Plants*, Vol. 1. Geneva, World Health Organization.

Williamson EM (2003): Drug interactions between herbal and prescription medicines. *Drug Safety 26*: 1075–1092.

Yoshikawa M, Murakami T, Ueno T, Kadoya M, Matsuda H, Yamahara J, Murakami N (1995): Bioactive saponins and glycosides. I. Senega Radix. (1); *E*-Senegasaponins a and b and *Z*-Senegasaponins a and b, their inhibitory effect on alcohol absorption and hypoglycemic activity. *Chem Pharm Bull 43*: 2115–2122.

Yu MH (2005): *Environmental Toxicology. Biological and Health Effects of Pollutants*, 2nd ed. Boca Raton, CRC Press, p. 95.

Zhou S, Gao Y, Jiang W, Huang M, Xu A, Paxton JP (2003): Interactions of herbs with cytochrome P450. *Drug Met Rev 35*: 35–98.