The Novel Use of Metabolomics as a Hypothesis Generating Technique for Analysis of Medicinal Plants: *Ligusticum canbyi* Coult. & Rose and *Artemisia tridentata* Nutt.

by

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Abstract

In response to the environment, plants produce a phytochemical arsenal to communicate and to withstand abiotic and biotic pressures. The average plant tissue contains upwards of 30,000 phytochemicals. The vast majority of approaches used to study plant chemistry are reductionist and only target specific classes of compounds which can be easily isolated or detected. Metabolomics is the qualitative and quantitative analysis of all metabolites present in a biological sample. By providing researchers with a phytochemical snapshot of all existing metabolites present in a sample, metabolomics has allowed researchers to study plant primary and secondary metabolism in ways that were never done before.

The first objective of this thesis is to identify candidate species for studying plant neurochemicals. Statistical analysis using residual, bayesian and binomial analysis was applied to the University of Michigan's Native American Ethnobotany Database and revealed that the genera *Artemisa* and *Ligusticum* are used most frequently during ceremony and ritual. Plant melatonin, serotonin, γ-aminobutyric acid, and acetylcholine were quantified in *Artemisia tridentata* Nutt. and *Ligusticum canbyi* Coult. & Rose. Significant variability was observed between tissue types, germplasm line and species. Manipulation of cholinergic signalling in *A. tridentata* led to changes in auxin, melatonin and serotonin levels, and suggests cross-talk between cholinergic and indoleamine pathways could be occurring in plants.

The second objective of this thesis is to develop novel statistical and biochemical tools for analyzing metabolomic datasets and hypothesis generation. Datasets were generated using ultra performance reverse phase chromatography with time of flight mass spectrometry detection. Between 16,000 and 40, 000 metabolites were detected in *L. canbyi* and *A. tridentata* root and leaf tissues respectively. Principal component analysis, synthetic biotransformation, significant ion generation, putative identification and logical algorithms were applied in order to develop hypotheses and research approaches for pthalide biosynthesis, plant responses to stress, and the biological activity of smoke. Metabolomics, as a field of study, is still in its infancy. Thus approaches to effectively mine datasets are still

needed, and provide researchers with new ways to examine the processes which regulate the production of primary and secondary metabolites *in vivo*.

Preface

A version of Chapter 1 has been published in the following manuscripts:

Turi, C.E, Murch, S.J., 2011. The genus *Ligusticum* in North America: An ethnobotanical review with special emphasis upon species commercially known as 'Osha'. HerbalGram 89, 40-51. I conducted the literature review and wrote most of the manuscript.

Turi, C.E., Shipley, P.R., Murch, S.J., 2014a. North American *Artemisia* species from the subgenus *Tridentatae* (Sagebrush): A phytochemical, botanical and pharmacological review. Phytochemistry. I conducted the literature review and wrote most of the manuscript. The section on phytochemistry was revised by Dr. Paul Shipley, all structures were drawn by him.

A version of chapter 2 has been published. Turi, C.E., Murch, S.J., 2013b. Spiritual and ceremonial plants in North America: An assessment of Moerman's Ethnobotanical Database comparing residual, binomial, bayesian and imprecise dirichlet model (IDM) analysis. J. Ethnopharmacol. 148, 386-394. doi: 10.1016/j.jep.2013.03.029. I accessed Moerman's database and conducted all the reported statistical analysis. A majority of the manuscript was written by me.

A version of chapter 3 has been published. Turi, C.E., Murch, S.J., 2013a. Targeted and untargeted phytochemistry of *Ligusticum canbyi*: Indoleamines, phthalides, antioxidant potential, and use of metabolomics as a hypothesis-generating technique for compound discovery. Planta Med. 79, 1370-1379. doi: 10.1055/s-0033-1350618. I conducted targeted, untargeted and antioxidant analysis and wrote the majority of the manuscript.

A version of chapter 4 has been published. Turi, C.E., Axwik, K.E., Murch, S.J., 2014b. *In vitro* conservation, phytochemistry, and medicinal activity of *Artemisia tridentata* Nutt.: metabolomics as a hypothesis-generating tool for plant tissue culture. Plant Growth Regulation. Doi: 10.1007/s10725-014-9915-y. I wrote the majority of this manuscript and conducted both untargeted and anti-AchE analysis. Germplasm lines were established with assistance from Katie Axwik.

A version of chapter 5 has been published. Turi, C.E., Axwik, K.E., Smith, A., Jones, M.P., Saxena, P.K., Murch, S.J., 2014c. Doi: 10.4161/psb.28645. Galanthamine an anticholinesterase drug effects plant growth and development in *Artemisia tridentata* Nutt. through modulation of auxin and neutrotransmitter signaling. Plant Signaling and Behavior. I performed all targeted and untargeted analysis. Katie Axwik helped to keep the germplasm lines viable for the duration of the experiment, while Anderson Smith provided technical assistance for the instruments. Dr. A.M.P Jones kindly ran the standard auxin bioassay at the University of Guleph.

Chapter 6 is based on work I conducted in Dr. Susan Murch's lab at UBC Okanagan. All experimental work and data analysis was performed by me.

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List of Abbreviations

5HT - Serotonin

Ach - Acetylcholine

AchE - Acetylcholinesterase

EtOH - Ethanol

GABA - γ-aminobutyric acid

HPLC - High performance liquid chromatography

IDM - Imprecise dirichlet model

MEL - Melatonin

MeOH - Methanol

OPLS-DA - orthogonal partial least squares discriminant analysis

PCA - Principal component analysis

PLS-DA - Partial least squares discriminant analysis

TLC - Thin layer chromatography

UPLC - Ultra performance liquid chromatography

Glossary

Over-utilized: A plant family or genus whose use is over-represented within the North American flora.

Under-utilized: A plant family or genus whose use is under-represented within the North American flora.

Plant neurochemicals: Phytochemicals which are produced by both humans and plants, and which are known to mediate human brain function (i.e. Ach, GABA, MEL, 5HT).

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Susan Murch for her brilliance, generosity and mentoring over the last four years. Your ability to move swiftly and with such precision in and out of various disciplines in order to produce truly multi-disciplinary research is alchemy. Thank you for making research so magical!

I am also grateful to my supervisory committee Dr. Mahmoud, Dr. Neeland, and Dr. Shipley for their support and feedback throughout this process, particularly Dr. Shipley for taking time out from his busy day to answer questions and teach me the 'tricks of the trade'. I am immensely grateful to the entire Murch-Shipley lab for their assistance with experiments, especially to Alex Lane and Broc Glover for their endless feedback and advice inside and outside of the lab. I would also like to extend my gratitude to my peers Jennifer Forsythe, Kristin Aleklett, and Roger Sherwood for their kindness and support, Dr. Paula Brown from BCIT for opening my eyes to the exciting world of analytical chemistry, and Dr. Max Jones from the University of Guelph for his help and expertise with plant tissue culture.

Last but not least, I would like to acknowledge all the wonderful friends and family who have stood by me during my entire academic journey. Thank you from the bottom of my heart for not only walking me off the edge, but for throwing me right back into battle and giving me the courage, support and wine to push through all the hardships…. time…after time…after time….

WE DID IT!

This thesis is dedicated to my mother Inge Turi

Chapter 1 Introduction

1.1 Phytochemistry

"Plants are all chemists, tirelessly assembling the molecules of the world"

– Gary Snyder

Plants are not mobile. They must remain in one place for an entire lifetime, and withstand all which their surrounding environment presents. As a result, they possess a chemical arsenal for survival that is made up of secondary metabolites, which are synthesized in response to abiotic and biotic elicitors (Wink, 2003). Unlike carbohydrates, amino acids or growth hormones which are necessary for plant survival and subsequently present in all species, secondary metabolites (sometimes called "specialized metabolites") are chemicals produced by plants (phytochemicals) that are non-essential for plant growth and development (Taiz and Zeiger, 2006).

At times, the distinction between primary and secondary metabolite is not apparent. For example, human neurotransmitters acetylcholine (Ach), melatonin (MEL) and serotonin (5HT) have all previously been identified and quantified in numerous plant species (also known as plant neurochemicals) (Murch et al., 2000; Murch et al., 2001; Jones et al., 2007; Cole et al., 2008; Hartmann and Gupta, 1989), however their exact role and function during plant growth and development is yet to be fully elucidated. Current research indicates that MEL and 5HT may be important in plant metabolic systems such as photosynthesis (Lazár et al., 2013) and physiological responses to environmental stresses such as tolerance to cold stress (Murch et al., 2009; Zhao et al., 2011; Uchendu et al., 2013). Similarly, Ach occurs in all parts of plants, but higher concentrations of Ach have been quantified in roots and dark adapted tissues than in shoots or light exposed cells (Hartmann and Gupta, 1989; Tretyn and Kendrick, 1991; Roshchina, 2001; Hartmann and Kilbinger, 1974). The hypothesized roles of Ach in plants include almost all aspects of growth and development (Roshchina, 2001) including phytochrome-linked signalling (Jaffe, 1970), phytochrome-modulated calcium signaling (Mehta et al., 1993), induction of primary and secondary rooting (BaMEL et al., 2007), regulation of phloem transport (Yang et al., 2007) and regulation of pollen tube development (Tezuka et al., 2007).

The presence or absence of specific phytochemicals within a species is directly linked to how specific taxa evolved over time by specializing their metabolism to certain classes of compounds in order to protect themselves from UV exposure, attract pollinators, deter herbivory, avoid microbial infestation, or emit chemical signals (Wink, 2003; Pichersky and Lewinsohn, 2011; Wink, 2008; Wink, 2010). Consequently, secondary metabolites play a crucial role in maintaining plant-animal, plant-microbe, and plant-plant interactions in nature. Given these relationships are often mediated through agonist or antagonist effects upon specific receptors, humans have exploited secondary metabolites and their associated biological activities for medicine (Wink, 2008; Balick and Cox, 1999).

1.2 Ethnobotany, Ethnopharmacology and Drug Discovery

Ethnobotany is the study of the interactions between plants and people (Balick and Cox, 1999). Ethnobotanical investigation towards the traditional use of plants by humans has led to the discovery of a plethora of economically valuable plant-based resources including: indigo, rubber, aspirin, and hemp (Balick and Cox, 1999). Ethnopharmacology is an interdisciplinary sub-discipline to ethnobotany which aims to discover novel biologically active compounds from leads provided by traditional indigenous knowledge. In the last 40 odd years, plants have contributed significantly towards drug discovery. It is estimated that between the years 2000 to 2010, natural sources were actively involved in 50% of all approved new chemical entities (Newman and Cragg, 2012). Thus, continued investigation of plant secondary metabolism is still needed today.

1.2.1 Traditional Approaches for the Study of Medicinal Plants

Scientists have isolated and identified approximately 200,000 secondary metabolites from plants (Dixon and Strack, 2003) and it is estimated that up to 1,000,000 novel structures are still awaiting discovery (Afendi et al., 2012). The isolation and development of valuable drugs from plants classically follows a well-established pipeline consisting of the following steps: ethnobotanical research, collection of plant material, extraction of phytochemicals, bioassay, fractionation, isolation, and pharmaceutical testing (Balick and Cox, 1999) (Figure 1.1). Using this method important drugs such as galanthamine and prostratin have all been discovered (Newman and Cragg, 2012; Cox, 2001; Heinrich and Teoh, 2004).

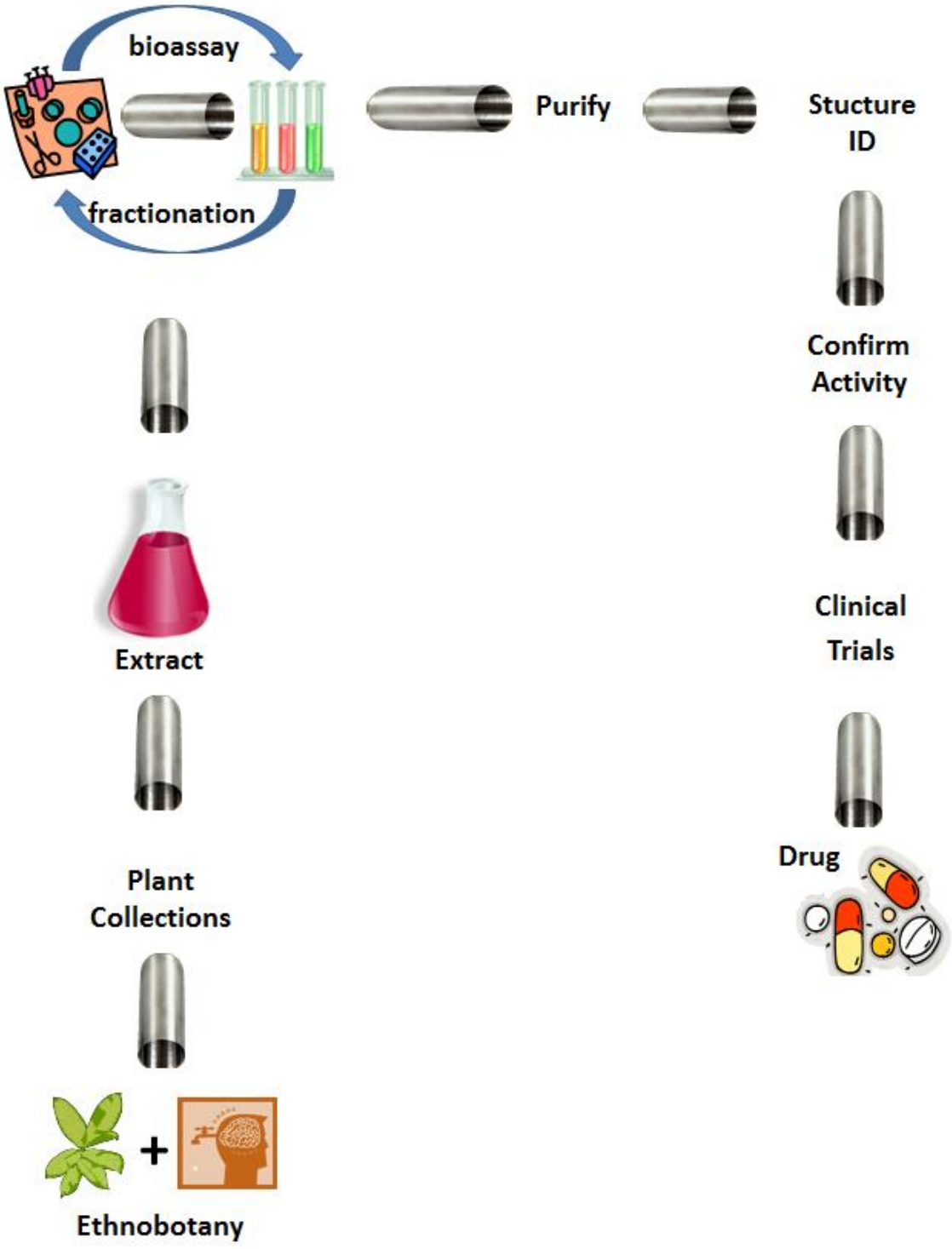


Figure 1.1 Pipeline for traditional drug discovery. Picture generated using Microsoft PowerPoint.

1.2.2 Plant Collection and Extraction

Ethnobotanically guided plant collections aim to acquire plant species from a given environment which have a long history of use by the indigenous peoples (Balick and Cox, 1999; Miller, 2011). It is crucial that collections are done properly by having ethics and prior informed consent from indigenous people, while also obtaining permits, having prior knowledge of what plant part and stage of development is required, and properly vouchering and identifying collected specimens by collaborating with a herbarium (Balick and Cox, 1999; Miller, 2011; Bennett and Balick, 2014). Once adequate collections have been performed, phytochemicals present within tissues should be preserved by storing at conditions which favor chemical stability of active compounds. Furthermore, it is crucial that extracts made from collected tissues reflect the traditional knowledge of preparation, otherwise false negatives or toxicity may be observed during screening (Balick and Cox, 1999; Cox, 2001; Brown and Murch, 2012).

1.2.3 Bioassay

A bioassay is a scientific approach used to measure the concentration or potency of a substance by observing its effect on a biological system. For many years, bioassays were performed by injecting animals with plant extracts and observing their effects. This is otherwise known as “Hippocratic screening” (Stromstedt et al., 2014). Although this method has been widely employed and facilitated the discovery of many biologically active compounds, economic and social pressures led to the development of cell, enzyme and receptor based high throughput screening programs in the 1980s (Miller, 2011; Stromstedt et al., 2014). Consequently, automated screening of plant extracts using *in vitro* models which are customized for 96, 384 or 1536 well microplates (working volume 250 µL) are now standard protocol (Mishra et al., 2008). Despite the application of high throughput screening for over three decades, discovery of new drugs from natural sources remains low (Potterat and Hamburger, 2013) and can likely be attributed to the following : 1) biologically active compounds are in low abundance within complex mixtures and consequently not detected; 2) false positives or negatives are observed due to the presence of compounds which display non-specific activities; 3) interference is caused by compounds which interact with light-based detection methods; 4) adequate drug targets are unavailable or yet to be developed; 5)

synergy of multiple active compounds; and 6) elaborate and costly methods are required to isolate biologically active constituents (Miller, 2011; Potterat and Hamburger, 2013).

1.2.4 Chromatography and Elucidation of Structures

In simplest of terms, chromatography is the separation of chemical constituents present within a complex mixture through physical or chemical means (Marston and Hostettmann, 2009). Once biological activity has been observed from a crude extract, separation into smaller chemical fractions is performed and these are re-screened for biological activity until a single biologically active compound is found (Balick and Cox, 1999; Miller, 2011). Methods routinely used to isolate biologically active compounds present within complex phytochemical mixtures include: thin layer chromatography (TLC), high or ultra performance liquid chromatograph (HPLC or UPLC), supercirtical fluid chromatography (SFC), ion-exchange chromatography (IEC), and size exclusion chromatography (SEC) (Marston and Hostettmann, 2009). Furthermore, by coupling the above separation techniques to spectroscopic methods such as ultraviolet (UV), infrared (IR), mass spectrometry (MS) or nuclear magnetic resonance (NMR) (Marston and Hostettmann, 2009), scientists have been able to elucidate the chemical structures of biologically active constituents. At times, the above approaches can be challenging given 1) the majority of secondary metabolites are present at such low concentrations that they can avoid detection; 2) conventional extraction/separation methods do not permit detection of unstable compounds; and 3) identified compounds may be artifacts or degradative products caused by the isolation technique (Pichersky and Lewinsohn, 2011).

1.3 North American Biodiversity as a Source for Drugs

North America (north of Mexico) is home to a mosaic of healing practices used by Native American and First Nations peoples. Generally, treatments involve some combination of the following: teas, purification ceremonies, herbs, special foods, prayer, chants, dancing, sand painting, and therapeutic activities such as singing (Portman and Garrett, 2006) . Much of the traditional knowledge of the North American indigenous peoples has been summarized in Moerman's Ethnobotanical Database which can be accessed online at the following link http://herb.umd.umich.edu/.

Discovering candidate species for further investigations of complex neurochemistry requires correlating data from many different sources and methods to identify clusters of over-utilized taxa of activity in the traditional knowledge base. Several different research approaches and statistical methods have been used to analyze ethnobotanical databases including residual, binomial, bayesian and imprecise dirichlet model (IDM) analysis (Moerman, 1991;Bennett and Husby, 2008; Weckerle et al., 2011; Frei et al., 1998; Leduc et al., 2006; Moerman and Estabrook, 2003). Residual analysis is used to calculate the expected number of species to be utilized for a specific family size, found within a specific plant population (Moerman, 1991; Moerman, 1996). The method is based on the calculation of the deviation from the expected frequency (residual) in order to describe over or under-utilization within a given plant family. Alternately, binomial (Bennett and Husby, 2008), bayesian (Weckerle et al., 2011), and IDM statistics have been suggested (Weckerle et al., 2012). All of these methods hypothesize that the proportion of species utilized in the total flora is equal to the proportion of species utilized for a given family, and that any deviation from this value will lead to statistical significance. However, bayesian and IDM analysis do not consider the proportion of utilized species as fixed, instead they consider uncertainty surrounding counts for medicinal flora and the total flora, and use probability distribution to determine the proportion of utilized species (Weckerle et al., 2011; Weckerle et al., 2012).

1.3.1 Spiritual and Ceremonial Plants as a Source for Drugs

The ritual and spiritual use of plants in ceremonies, the use of plants to contact the spirit or enhance religious experiences and the use of plants to protect against evil spirits or ghosts is common among peoples in many parts of the world and many cultures. Species identified as spiritual plants convey a sense of well-being, are hallucinogens, or psychotics and can be used to treat a wide spectrum of neurological disorders (O'Connor and Roth, 2005; McKenna, 1995; McKenna et al., 2011). Classic ethnobotany describes a strong connection between plants used to treat neurological diseases and those associated with metaphysical practices (Balick and Cox, 1999). In 1924, Louis Lewin described 28 mind-altering plants and suggested that plants identified as having a role in spirituality are a rich source of modern medicines to treat psychological and/or neurological ailments (Lewin, 1998). Since then, many well-known examples have been studied in detail such as *Cannabis sativa* L. (Cannabaceae) *Erythroxylum coca* Lam. (Erythroxylaceae), and *Papaver*

somniferum L. (Papaveraceae), while other species with subtler effects are less understood (McKenna et al., 2011). Ethnopharmacological investigation of Moerman's database by Turi and Murch (2013a) identified the Apiaceae and Asteraceae as important plant families for medicinal use during ceremony (see Chapter 2). Further analysis at the genus level revealed that members belonging to the genera *Ligusticum* (Osha) and *Artemisia* (Sagebrush) are sought after significantly by First Nations and Native Americans peoples, and may provide promising leads for the discovery of novel neurologically active compounds (Chapter 2, Turi and Murch, 2013a).

1.3.2 Ceremonial Smokes as a Source for Drugs

Ritual use of plant derived smokes is wide-spread. Pennacchio et al. (2010), estimates that 400 species are used world-wide for magico-religious and ceremonial purposes. Ethnopharmacolgical investigation of ceremonial smokes suggests that plants burned in ritual may provide novel therapies for treating neurological disorders. For example, the spiritual use of Cannabis (*Cannabis sativa*) has a long history of use with one of the earliest works from China stating that if taken over time it "makes one communicate with spirits and lightens one's body" (Touw, 1981). Since then, isolated compounds from *Cannabis sativa* have been under extensive investigation as alternative treatments for Parkinson's and Huntington's disease (Fernandez-Ruiz et al., 2013). Similar to the above, ethnopharmacological investigation of Moerman's database revealed that certain plant in the Apiaceae and Asteraceae are sought after as ceremonial smokes. Further analysis at the genus level revealed that members belonging to the genus *Artemisia* (Sagebrush) are good candidates for investigating biological activity of smoke (Chapter 6).

1.4 Model Species 1: *Ligusticum*

The genus *Ligusticum* consists of 40-60 perennial species found within boreal, coastal and mountainous regions of the world (Ben shu et al., 2005; Mabberly, 1997) (Figure 1.2). In Asia, native species of *Ligusticum* are held with great esteem for their therapeutic actions (Beck and Chou, 2007). Likewise in North America, endemic species collectively known as 'Osha' are sought after by consumers within the natural health products industry, while also being revered among indigenous groups (The Scientific Authority of the United States of America, 2000). Although research investigating medicinal attributes of *Ligusticum* species commonly used within Traditional Chinese medicine has been on-going since the 1960's

(Beck and Chou, 2007), research into the medicinal efficacy of North American species is more recent. A literature search of '*Ligusticum*' in Web of Science™ on September 30, 2010 found a total of 257 articles in the scientific literature. Among these articles, approximately 85% pertained to species endemic to Asia (Turi C.E, 2011) (Table 1.1).

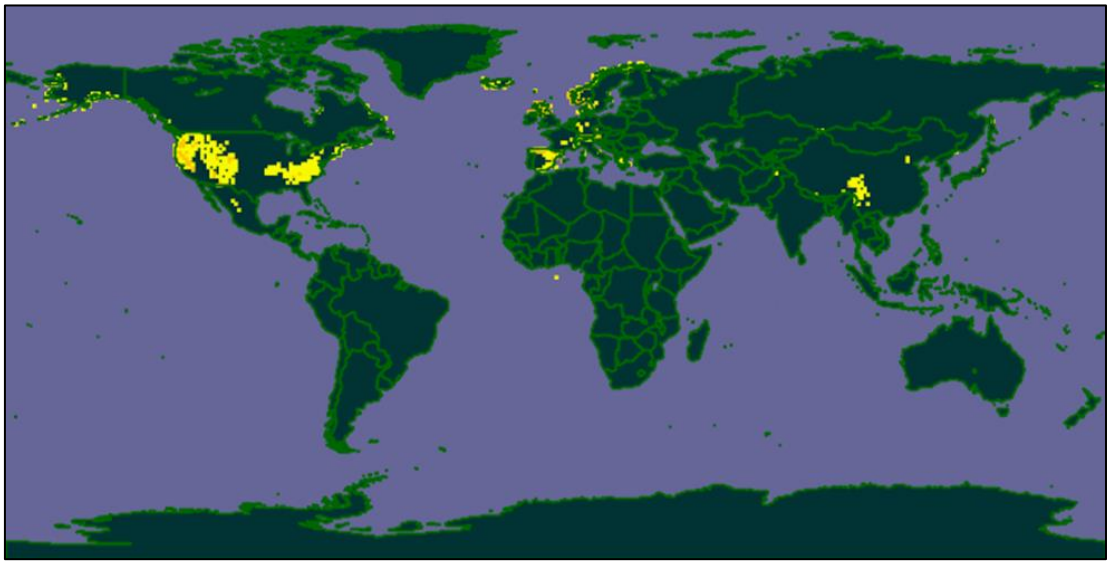


Figure 1.2 Distribution and knowledge of *Ligusticum* species around the world. Biodiversity occurrence data according to the Global Biodiversity Information Facility (GBIF, 2010). Picture generated using GBIF data portal.

In North America, First Nations' uses of *Ligusticum* species include food, hunting, spirituality and medicine (Moreman, 2009) (Table 1.2). They are found ranging from Northeastern parts of Quebec, the Maritimes, Ontario, and the Eastern United States as south as Florida. *Ligusticum* species are also found in the Mexican Sierra Madre and through regions of New Mexico, Colorado, Wyoming, Montana, Alaska, British Columbia, and Western coastal States or Provinces (GBIF, 2010) (Figure 1.2). Ecological zones where *Ligusticum* species known as 'Osha' are found in North America include: montane to subalpine meadows (Scotter and Flygare, 2007), spruce and aspen belts, windswept parks, oak and mature stands of conifers, (Moore, 2003) pine-oak forests of the Northern Sierra Madre Occidental and central to southern Rocky Mountains of the United States (Linares and Bye, 1987) mountain ridges, within dry or moist slopes (Kershaw, 2000), moist forests from the spruce-fir vegetation zone to foothills, (Dodson and Dunmire, 2007) Sagebrush meadows on rocky soils (The Scientific Authority of the United States of America, 2000) and amongst

subalpine zones and altitudes up to 10,000 feet (Tilford, 1997). *Ligusticum porteri* was also identified as a dominant species among Aspen communities in Gunnison County, Colorado (Crawford et al., 1998) (Figure 1.3).

Table 1.1 Summary of reported literature indexed in Web of Science™ as of September 30th, 2010.

Common Name	Species Name	Number of Articles	Endemic to
Szechwan Lovage	*Ligusticum chuanxiong* S.H. Qiu et al.	130	China
Chuānxiōng	*Ligusticum wallichi* Franch	59	China
Gao ben	*Ligusticum sinensis* Oliv	5	China
Xi ye gao ben	*Ligusticum tenuissimum* (Nakai) Kittag	2	China
Duo guan gao ben	*Ligusticum multivittatum* Franch	1	China
Duo bao gao ben	*Ligusticum involucratum* Franch	1	China
	Ligusticum spp.	14	Asia
	Ligusticum marginatum C.B. Clarke	1	W. Pakistan to Himalayas
Gao shen gao ben	*Ligusticum elatum* C.B. Clarke	2	Nepal, India, Pakistan
Dong-Dang-Gui	*Ligusticum acutilobum* Siebold & Zucc.	2	Japan
True Osha	*Ligusticum porteri* Coult. & Rose	17	Mexico, United States
Canby's Lovage	*Ligusticum canbyi* Coult. & Rose	0	Canada, United States
Scottish Lovage and Hulten's Licorice	*Ligusticum scoticum* L (subsp. *scoticum* and subsp. *hultenii* (Fernald) Calder & Roy L. Taylor)	7	Canada, United States
Fern-leaf Licorice	*Ligusticum filicinum* S. Watson	1	Canada, United States
Idaho Licorice	*Ligusticum tenuifolium* S. Wats	1	United States
Gray's Lovage	*Ligusticum grayi* Coult. & Rose	1	Canada, United States
	Ligusticum seguieri Koch	2	Europe
	Ligusticum pyrenaicum Gauan	1	Europe
Alpine Lovage	*Ligusticum mutellina* Crantz	3	Europe

Table 1.2 Ethnobotanical uses of North American species of *Ligusticum* as per the University of Michigan's Native American Ethnobotany Database, Bye and Linares 1987, and Henriette's Herbal Website (www.henriettesherbal.com/)

Species	**Wounds, Skin and Ear Infections, Ticks, Lice or hair rinse**	**Anti-rheumatic**	**Anti-convulsive, Analgesic and Fever**	**Gastrointestinal** (StomAch upset, flatulance, colic….etc) **Dietary aid** (digestion, apperitif..etc)	**Colds, Coughs, Soar Throat, Pulmonary or Respiratory aid** (hemorrahages, tuberculosis, bronchitis, lung infections, sinus infection, congestion…etc.)	**Heart Drug, Circulation problems, Diabetes, Anemia**	**Ceremonial drug or Protection**	**Smoked or Incence**	**Food**	**Poison**
Ligusticum apifolium				**11,26**	**10, 11**	**10**				
Ligusticum californicum									**4**	
Ligusticum canadense				**28**				**28**	**12,21, 31**	
Ligusticum canbyi	**13, 30**		**13**		**13,30**	**13**	**30**	**13,30**		
Ligusticum filicinum					**8, 22, 27,29**					
Ligusticum grayi			**9**	**9**	**9**				**9**	**9**

Table 1.2 Ethnobotanical uses of North American species of *Ligusticum* as per the University of Michigan's Native American Ethnobotany Database, Bye and Linares 1987, and Henriette's Herbal Website (www.henriettesherbal.com/).

Species	**Wounds, Skin and Ear Infections, Ticks, Lice or hair rinse**	**Anti-rheumatic**	**Anti-convulsive, Analgesic and Fever**	**Gastrointestinal** (StomAch upset, flatulance, colic….etc) **Dietary aid** (digestion, apperitif..etc)	**Colds, Coughs, Soar Throat, Pulmonary or Respiratory aid** (hemorrahages, tuberculosis, bronchitis, lung infections, sinus infection, congestion…etc.)	**Heart Drug, Circulation problems, Diabetes, Anemia**	**Ceremonial drug or Protection**	**Smoked or Incence**	**Food**	**Poison**
Ligusticum porteri	**3,20**	**5,3,20**	**20**	**3,20**	**3,5,20**	**20**	**5,7, 18,20, 24**		**7,20**	**6,20**
Ligusticum scoticum								**15**	**1,2,14 ,16,17 23**	**19**

Legend: 1 (Ager and Ager, 1980); **2** (Anderson, 1939); **3** (Appelt, 1985); **4** (Baker, 1981); **5** (Camazine and Bye, 1980); **6** (Campbell, 1958); **7** (Castetter and Opler, 1936); **8** (Felter and Llyod, 1898); **9** (Garth, 1953); **10** (Goodrich and Lawson, 19980); **11** (Gifford, 1967);**12** (HaMEL and Chiltoskey,); **13** (Hart, 1992); **14** (Hatfield, 2007); **15** (Hedrick, 1919); **16** (Heller, 1953); **17** (Jones, 1983); **18** (Jordan, 2008); **19** (Lantis, 1959); **20** (Linares and Bye, 1987); **21** (Perry, 1975); **22** (Remington and Wood,); **23** (Rousseau, 1946); **24** (Salmon, 2000); **25** (Sayre, 1917); **26** (Schenck and Gifford, 1952); **27** (Smith, 1929); **28** (Sturtevant, 1954); **29** (Train et al., 1941); **30** (Turner et al., 1980); **31** (Withoft, 1977)

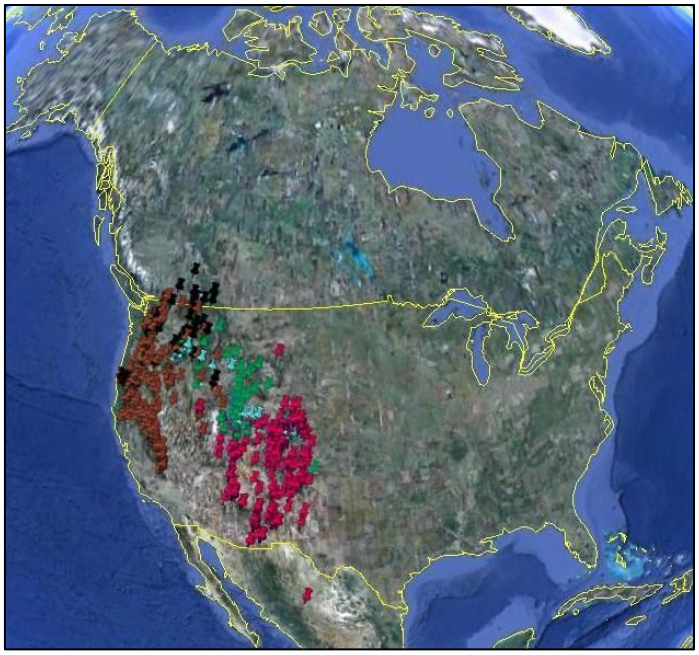


Figure 1.3 Distribution of *Ligusticum porteri* , *Ligusticum filicinum* , *Ligusticum tenuifolium* , *Ligusticum canbyi* , and *Ligusticum grayi* in North America as compiled by the Global Biodiversity Information Facility (GBIF, 2010). Picture generated in Google Earth.

1.4.1 Botany and Taxonomy

Ligusticums belong to the family Apiaceae otherwise known as the Umbelliferae or the carrot family. In North America, there are 12 species of *Ligusticum* (USDA, 2010). Among these, *L. porteri*, *L .canbyi*, *L. grayi*, *L. tenuifolium*, and *L. filicinum* are most commonly known as 'Osha (The Scientific Authority of the United States of America, 2000; Moore, 2003). Like other genera belonging to the Apaiceae, *Ligusticums* are identified by their compound umbels, white or sometimes pinkish flowers, distinctly ribbed-narrowly winged fruits, taproot system, basal leaves, and ternate to pinnately compound or dissected leaves (Applequist, 2005; Hitchcock and Cronquist, 1996). Though challenging at times, differentiation between species collectively known as 'Osha' is accomplished through examination of plant height, leaf morphology and habitat (Table 1.3, Figure 1.4).

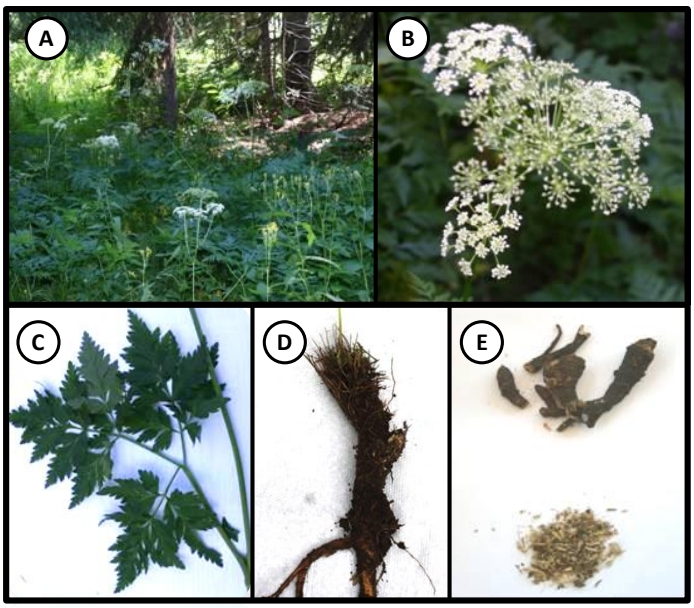


Figure 1.4 Wild collection of North American *Ligusticum* species. A) A stand of *L. canbyi* in British Columbia, Canada; **B)** Umbel of *L. canbyi*; **C)** Leaves of *L. canbyi;* **D)** Roots of wild-harvested *L. canbyi*; **E)** Roots and powder sold commercially as 'Osha'. Pictures taken by Christina E. Turi.

Table 1.3 Botanical descriptors used to differentiate several North American *Ligusticum* species commonly known as 'Osha' in the natural health products industry (The Scientific Authority of the United States of America, 2000; Hitchcock and Cronquist, 1996)**.**

Species	Leaves	Umbel	Plant Height	Fruit	Habitat
Ligusticum filicinum	Leaves dissected into numerous linear segments 1-3 mm wide. Basal leaves 10-25 cm wide.	Rays of main umbel generally 12-20	Robust, 50-100 cm tall	5-7 mm	Open or wooded, moist to dry slopes and ridges in mountains.
Ligusticum tenuifolium	Leaves dissected, usually scapose or with 1 greatly reduced leaf. Basal are usually less then 10 cm wide.	Rays of umbel generally 5-13.	small slender, 10-60 cm tall	3-5 mm	Marshes to wet or moist slopes
Ligusticum canbyi	Leaves less dissected, segments more or less toothed or cleft, leaflets generally 1-5 x 0.5-2cm	Rays of terminal umbel 15-40.	50-120 cm tall	Ribs of fruit narrowly winged.	Wet to moist or occasionally dryish soil.
Ligusticum grayi	Leaves less dissected, segments more or less toothed or cleft. Leaflets generally 1-5 X 0.5-2 cm. Usually scapose or with 1-2 reduced stem leaves.	Rays of terminal umbel 7-14.	20-60 cm tall	Ribs of fruit narrowly winged.	Moist to dry, open or wooded montain slopes and drier meadows.
Ligusticum porteri	Elliptic or lance-shaped 0.5-4 cm in width. Basal leaves 15-30 cm long.	Flat topped umbels	50-100 cm tall	Oblong, ribbed fruits 5-8 mm long.	Fertile ground in upland meadows and ravines.

1.4.2 Ethnobotany

Zoopharmacognosy

Kodiak bears (*Ursus arctos)* have been observed to macerate the roots of *L. porteri* sometimes known as Bear root, and rub the salivated contents over their bodies, possibly to medicate topical wounds suffering from infection (Newton and Wolfe, 1992). The Navajo believe that Bear root's medicinal use was given to them by bear and use the roots for treating many aliments including skin abrasions (Table 1.2) (Huffman, 2003; Cowen, 1990; Grisanzio, 1992).

Poison and Hunting

Among the Eskimo, mature plants from *L. scoticum* are considered mildly poisonous (Lantis, 1959), while the roots of *L. grayi* are used for hunting and poisoning fish among the Atsugewi of northern California (Garth, 1953). Similarly roots from *L. porteri* have served as a fish poison by the Tarahumar of northern Mexico (Table 1.2) (Linares and Bye, 1987; Campbell, 1958).

Spirituality, Protection, Smoke or Incense

Ceremonial uses for *L. porteri* and *L. canbyi* are documented for North American indigenous groups. The Tarahumar were observed using infusions of *L. porteri* during ritual curing ceremonies and to protect individuals against witches or rattlesnakes (Linares and Bye, 1987). Both the patient and medicine man in Zuni traditional healing rituals would chew on the root during healing rituals (Camazine and Bye, 1980). The Arapahoe and Pawnee used *L. porteri* roots during sweat lodge ceremonies and purification rites (Jones, 1983). While staying with the Tonkawa, Opler recounted that one must possess *L. porteri* in order to partake in the peyote ceremony (Opler, 1939). Among the Plains ApAchE, the roots of *L. porteri* also known as "medicine fat", were thrown over fire to help console individuals during rituals associated with mourning.(Jordan, 2008). The roots of *L. canbyi* were used by the Okanagan-Colville as aromatic smoke believed to release individuals from trance, possession by spirits such as the 'bluejay spirit' or unconsciousness (Turner et al., 1980). During the early 1900s, chewing the roots of *L. scoticum* was considered a substitute for tobacco (Hedrick, 1919). Similarly, *L. canbyi* can be added to tobacco (Hart, 1992) and smoked in order to produce a menthol taste. It has been suggested that smoking its roots

provides a relaxant effect, which most likely explains its common name 'Indian marijuana'. *Ligusticum canbyi* can be used as an incense (Hart, 1992). Exposure to excess amounts of smoke is believed to have a strong sedative effect upon children (Turner et al., 1980). Furthermore, roots from *L. canadense* were smoked to treat stomAch problems among the Cherokee (Table 1.2) (Sturtevant, 1954).

Food

Although it is likely that most species have been used for cooking or substituted with other species, *L. californium*, *L. canadense*, *L. grayi*, *L. porteri*, and *L. scoticum* have described culinary uses within the literature (Table 1.2). It is believed that *Ligusticums* produce a chervil, celery, parsley flavor (Moore, 2003), as a result leaves, seeds and roots are used to season meats, beans and chillis (Moore, 2003; Linares and Bye, 1987; Ager and Ager, 1980; Anderson, 1939; Castetter and Opler, 1936; Jones, 1983; Rousseau, 1946). *Ligusticums* can be eaten without preparation or by preparing leaves like greens through boiling, or adding to salads in a way similar to celery (Garth, 1953; Hamel and Chiltoskey, 1975; Perry, 1975; Withoft, 1977). Stalks can be used similar to celery (Heller, 1953), while roots can be boiled, thrown into salads and soups, or eaten raw, by peeling back the stem (Baker, 1981; Hatfield, 2007; Hedrick, 1919).

Medicine

The use of 'Osha' to treat sickness and promote 'well-being' has played an important role among North American indigenous cultures for centuries. In 1988, researchers in Utah discovered a medicine bundle containing *L. porteri*, thought to be 200 to 400 years old (Harrison, 2000). After analysing the contents of the package, it was suggested that these items were not simply 'stash items', but rather of great significance (Harrison, 2000). Medicinal applications of *Ligusticum* species include the following: an antirheumatic (Linares and Bye, 1987), treatments for hair, lice, ticks, wounds, and skin and ear infections (Linares and Bye, 1987; Hart, 1992; Turner et al., 1980), anti-convulsive, analgesic and fever (Linares and Bye, 1987; Garth, 1953; Hart, 1992), gastrointestinal problems or dietary aid (Linares and Bye, 1987; Garth, 1953; Schenck and Gifford, 1952; Sturtevant, 1954), colds, coughs, sore throat, pulmonary or respiratory aid (Linares and Bye, 1987; Felter and Llyod,

1898; Garth, 1953; Goodrich and Lawson, 1980; Gifford, 1967; Hart, 1992; Remington and Wood,1918;Sayre, 1917; Smith, 1929; Train et al., 1941; Turner et al., 1980), treatment for anaemia, diabetes, circulation or heart problems (Linares and Bye, 1987; Goodrich and Lawson, 1980; Hart, 1992) (Table 1.2). Personal preference towards a specific *Ligusticum* species varies throughout North America. The dispensatory of the United States indicated in 1918 that *Ligusticum filicinum* 'is a highly prized expectorant' within Utah and surrounding States (Remington and Wood, 1918). The Menomini describe 20 kinds of 'Osha root', the most powerful being the Mani'k (*L. filicinum*) (Smith, 1929). More recently, *L. porteri* was identified as a commonly used herb in Hispanic communities of the San Luis Valley, Colorado (Appelt, 1985).

1.4.3 Medicinal Chemistry

Much of the research that exists investigating the medicinal activity of *Ligusticum* species includes phthalide derivatives such as Z-ligustilide and butylidenphthalide (Beck and Chou, 2007; Zschocke et al., 1998) and phenolic compounds such as ferulic acid (Figure 1.5). Phthalides are compounds which are most commonly found in members belonging to the Apiaceae (carrot family) (Beck and Chou, 2007). Although the exact origin of their biosynthesis is yet to be determined, it is believed that all phthalides stem from Z/E-ligustilide (Beck and Chou, 2007; Kobayashi and Mitsuhashi, 1987; Mitsuhashi and Nomura, 1966). To date, a full biochemical pathway describing phthalide biosynthesis, catabolism and the production of derivatives has not been fully elucidated. Early works using radiolabelled acetatae in cultivated *Levisticum officinale* determined that biogenesis of Z/E-ligustilide occurs through head to tail addition of acetate units (Beck and Chou, 2007; Kobayashi and Mitsuhashi, 1987; Mitsuhashi and Nomura, 1966). Despite uncertainty regarding their biosynthesis, Asian members of the genus *Ligusticum* have facilitated the discovery of many novel phthalide compounds. Approximately 40 phthalides have been isolated from *L. chuanxiong*, 20 from *L. officinale*, and 20 from *L. wallichi*.(Beck and Chou, 2007). Medicinally, Z-ligustilide is used for smooth muscle relaxation, vasodilation, insecticidal, antibacterial, antifungal and inflammation. While antianginal, antihypertensive, antispasmodic, vasodilation, serotonergic activity, and selectivity upon the central cholinergic neuronal system in rats, has been associated with butylidenpthalide (Beck and Chou, 2007). Ferulic acid is an antioxidant with therapeutic applications in inflammation,

cancer, ageing, neurodegeneration, radiation, hypertension and atherosclerosis (Srinivasan et al., 2007).

Figure 1.5 Main biologically active constituents of the genus *Ligusticum*. Structures were made using Chemdraw software.

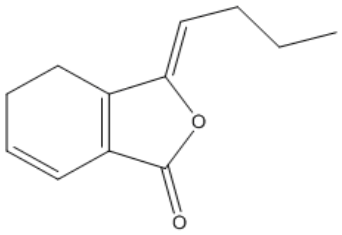
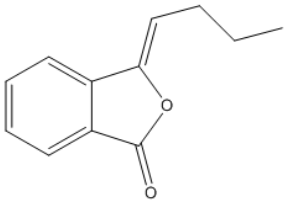
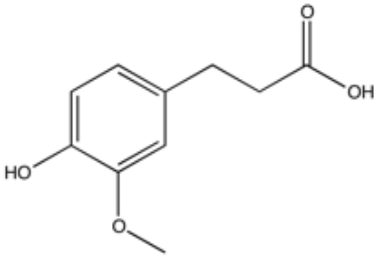
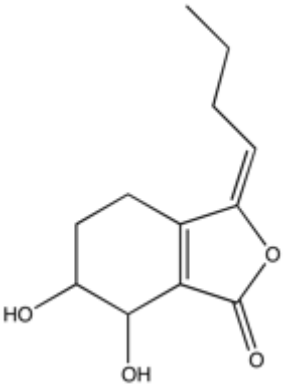
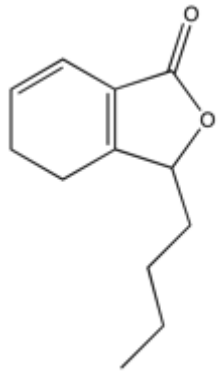
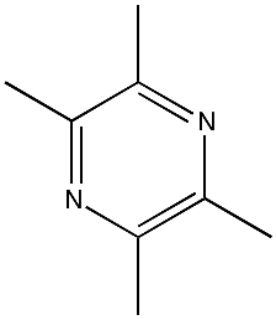
Name	Structure	Therapeutic Effect
Z-ligustilide		Smooth muscle relaxation, vasodilation, and as an insecticidal, antibacterial, antifungal, and anti-inflammatory (Beck and Chou, 2007; Ran et al., 2011), neuroprotection against $A\beta_{25–35}$ and $A\beta_{1-40}$ neurotoxicity (Ho et al., 2009), cytotoxins produced by activated microglia (Or et al., 2011)
Z-butylidienephtalide		Antianginal, antihypertensive, antispasmodic, vasodilatory, and serotonergic activity—as well as selectivity upon the central cholinergic neuronal system in rats (Beck and Chou, 2007; Ran et al., 2011), Anti-hyperglycymic (Brindis et al., 2010)
ferulic acid		Antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis, and anti-cancer (Srinivasan et al., 2007).
senkyunolide I		Anti-migraine (Wang et al., 2011), anti-platlet, anticoagulation, free radical scavaging (Zhu et al., 2010)

Figure 1.5 Main biologically active constituents of the genus *Ligusticum*. Structures were made using Chemdraw software.

Name	Structure	Therapeutic Effect
senkyunolide A		Neuroprotection against cytotoxins produced by activated microglia (Or et al., 2011), anti-inflammatory (Ran et al., 2011)
tetramethylpyrazine		Vasodilation, relaxation, anti-platlet, anti-oxidant, neuroprotective, chondroprotective (Ran et al., 2011)

1.4.4 Bioassay

Although a variety of therapeutic properties have been associated to *Ligusticum* found within Traditional Chinese Medicine, the exact mechanisms which underline these attributes are still unclear (Beck and Chou, 2007). With respect to pharmacology of 'Osha', extracts of *L. porteri* have shown to effectively inhibit the infective capacity of several strains of bacteria (Dentali and Hoffman, 1992). On the other hand, essential oil and CH_2CL_2 extracts did not exhibit significant antimicrobial activity against a norfloxacin-resistant strain of *Staphylococcus aureus*, however sensitivity to norfloxacin was restored (Cegiela-Carlioz et al., 2005). Additionally, ethanol extracts taken from roots of *L. porteri* (0.05g/ml) have shown inhibitory activity against both *Bacilus subtilis* and *Pseudemonae syringae* (Turi, 2009). In various model systems to test pain susceptibility, extracts of *L. porteri* have shown significant antinociceptive activity (Deciga-Campos et al., 2005). Orally administered

extracts of *L. porteri* have shown antihyperglycymic effects in mice, with (*Z*)-6,6',7,3'a-Diligustilide, (*Z*)-ligustilide, and 3-(*Z*)-butylidenephthalide contributing to the observed effects (Brindis et al., 2010). In contrast, models of cancer metabolism did not find significant inhibition of proliferation of tumor cells or cytotoxic effects (Daniels et al., 2006). It is interesting to note that some toxicity has been determined in roots of *L. porteri* (LD_{50} = 1085 mg/kg and CL_{50} = 777.98 µg/ml,(Deciga-Campos et al., 2007) suggesting further enquiry into safe dosage should be undertaken.

1.5 Model Species II: *Artemisia*

The genus *Artemisia* consists of 350-500 species, with the majority of individuals occuring in temperate zones of the Northern hemisphere (Bora and Sharma, 2011; Riggins and Seigler, 2012; Shultz, 2006) (Figure 1.6). Recent estimates suggest there are 50 species of *Artemisia* (native and introduced) found in North America and which are distributed across the following subgenera: *Artemisia* (Miller) Less, *Absinthium* (Miller) Less, *Dracunculus* Besser, and *Tridentatae* (Rydb.) (Shultz, 2006;Garcia et al., 2011b;Shultz, 2009). Among these, the subgenus *Tridentatae* (Sagebrush) is endemic to North America (Garcia et al., 2011b; Shultz, 2009) (Figure 1.7) and has been subject to numerous taxonomic revisions over the years (Garcia et al., 2011b; Garcia et al., 2011a) (Table 1.4). It is believed there are between 10 and 13 species of Sagebrush in North America (Garcia et al., 2011b; Shultz, 2009; Garcia et al., 2011a).

From an ecological perspective, Big Sagebrush provides food and shelter to hundreds of species such as the pygmy rabbit and greater sage-grouse (Woods et al., 2013; Rowland et al., 2006). Unfortunately, native populations of *A. tridentata* have declined and been fragmented by various environmental pressures including fire, human disturbance and competition from invasive species (Davies et al., 2011). Current methods employed to conserve Sagebrush include: (a) planting of seedlings into the wild (Davies et al., 2013), (b) removal of competitive species (Boyd and Svejcar, 2011), and/or (c) prescribed fire (Ellsworth and Kauffman, 2013). While these methods are crucial for maintaining healthy *in situ* populations, the effectiveness of *ex situ* efforts to conserve plant genetic resources is limited (Engelmann, 2011; Reed et al., 2011). Seed viability in some *A. tridentata* populations is low and propagation efforts relying solely on seeds have been only partially successful to date (Wijayratne and Pyke, 2012). Plant cell culture, cryopreservation, and seed

banking have been proposed as methods to ensure the long-term conservation of the genetic resources (Engelmann, 2011; Campbell et al., 2001). The establishment of plant tissue cultures can provide plants for recovery of populations in the wild, as well as resources for commercial plant production (Pence, 2011). There is no existing protocol to grow wild-harvested or cultivated Big Sagebrush *in vitro*, but protocols have been developed for other *Artemisia* species such as *Artemisia annua* L. (Liu et al., 2004) and *Artemisia judaica* L. (Liu et al., 2003).

Phytochemical and pharmacological investigations of *Artemisia* species have led to the discovery of novel biologically active compounds, most notably artemisinin from *Artemisia annua* L. (Asian sweet wormwood) (Bora and Sharma, 2011; Tan et al., 1998; Jose Abad et al., 2012). Traditionally, Native American or First Nations peoples used indigenous *Artemisia* species as an analgesics, anti-inflammatory, anti-septic, gastrointestinal or immunostimulatory aids or to treat afflictions from spiritual or unknown origins (Turner et al., 1980; Moerman, 2009; Turner, 2009; Kelley et al., 1992). A search in Web of Science on December 4, 2012 identified 891 articles referring to members of the subgenus *Tridentatae*. Of these publications, 64% pertained to research areas in environmental sciences and ecology, while less than 5% encompassed research areas in chemistry and pharmacology (Table 1.5).

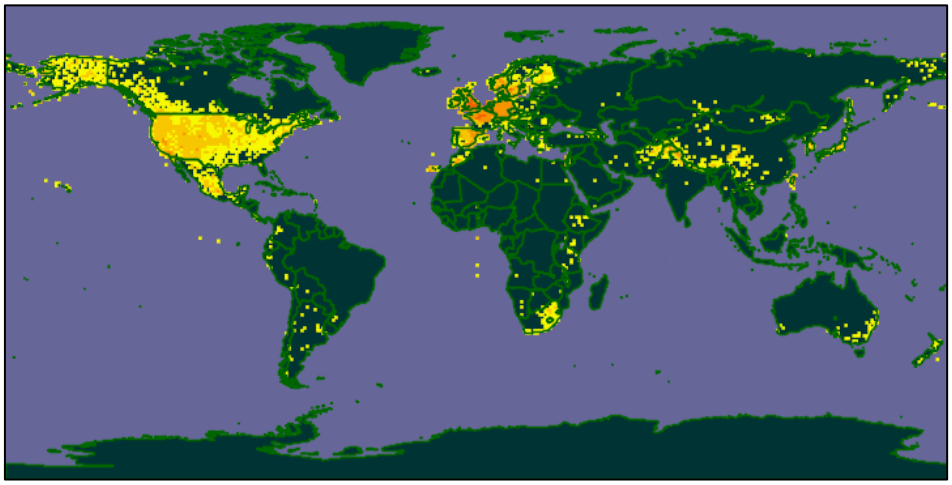


Figure 1.6 Global distribution of *Artemisia* species according to GPS data collected from the Global Biodiversity Information Facility on December 4th, 2012 (GBIF, 2012). GPS data were only available for 308 out of ≈ 500 *Artemisia* species. Picture generated using the GBIF data portal.

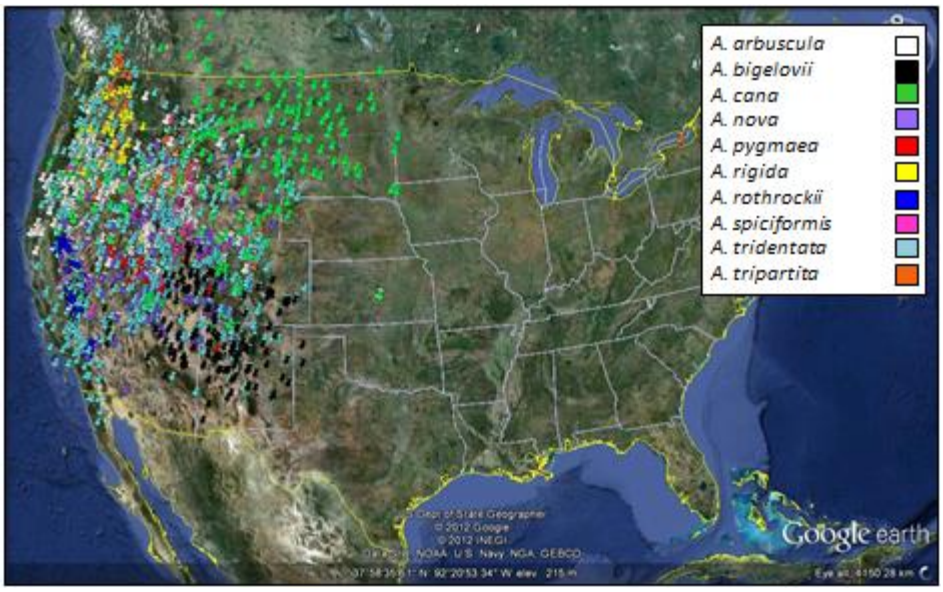


Figure 1.7 Distribution of *Artemisia* species, subgenus *Tridentatae* in North America according to GPS data collected from the Global Biodiversity Information Facility (GBIF, 2012). Picture generated using Google Earth.

Table 1.4 Species and subspecies in the subgenus *Tridentatae* according to the Flora of North America (Shultz, 2006).

Species	Synonyms	Common Names	Subspecies	Synonyms
***Artemisia arbuscula* Nutt.**	*Artemisia tridentata* Nutt. subsp. *arbuscula* (Nutt.) H.M. Hall & Clements; *Artemisia tridentata* var. *arbuscula (*Nutt.) McMinn*; Seriphidium arbusculum* (Nutt.) W.A. Weber	Low Sage, Dark Sagebrush, Scabland Sagebrush	*Artemisia arbuscula* Nutt. subsp. a*rbuscula*	
			Artemisia arbuscula Nutt. subsp. l*ongiloba* (Osterhout) L.M. Shultz	*Artemisia spiciformis* Osterhout var. *longiloba* Osterhout; *Artemisia longiloba* (Osterhout) Beetle; *Seriphidium arbusculum* (Nutt.) W.A. Weber subsp. *longiloburn* (Osterhout) W.A. Weber
			Artemisia arbuscula Nutt. subsp. *thermopola* Beetle	*Seriphidium arbusculum* (Nutt.) W.A. Weber var. *thermopolum* (Beetle) Y.R. Ling
***Artemisia bigelovii* A. Gray**	*Artemisia bigelovii* (A. Gray)	Bigelow Sagebrush		
***Artemisia cana* Pursh**	*Seriphidium canum* (Pursh)	Silver Sagebrush, Sticky Sagebrush, Silver wormwood, Hoary Sagebrush, Dwarf Sagebrush, Silvery Sagebrush	*Artemisia cana* subsp. c*ana*	*Artemisia columbiensis* Nutt.
			Artemisia cana subsp. *bolander*	*Artemisia bolanderi* A. Gray; *Artemisia tridentata* Nutt. subsp. *bolanderi* (A. Gray) H.M. Hall & Clememts; *Seriphidium bolanderi* (A. Gray) Y.R. Ling; *Seriphidium canum* (Pursh) W.A. Weber subsp. b*olanderi (A. Gray).* W.A.Weber
			Artemisia cana subsp. v*iscidula*	*Artemisia cana* var. *viscidula* Osterhout; *Artemisia argillosa* Beetle; *Artemisia viscidula* (Osterhout) Rydberg

Table 1.4 Species and subspecies in the subgenus *Tridentatae* according to the Flora of North America (Shultz, 2006).

Species	Synonyms	Common Names	Subspecies	Synonyms
***Artemisia nova* A. Nelson**	*Artemisia arbuscula* Nutt. subsp. *nova; Artemisia arbuscula* var. *nova* (A. Nelson) Cronquist; *Artemisia tridentata* Nutt. subsp. *nova*(A. Nelson) H.M. Hall & clements; *Seriphidium novum*(A. Nelson) W.A. Weber	Black Sage, Black Sagebrush		
***Artemisia pygmaea* (A. Gray) W.A. Weber**	Seriphidium pygmaeum (A. Gray)	Pygmy Sage		
***Artemisia rigida* (Nutt.) A. Gray**	*Artemisia trifida* Nutt. var. *rigida* Nutt.; *Seriphidium rigidum* (Nutt.) W.A. Weber	Scabland Sagebrush		
***Artemisia rothrockii* A. Gray**	*Artemisia tridentata* Nutt. subsp. *Rothrockii* (A. Gray) H.M. Hall & Clements; Seriphidium rothrockii (A. Gray) W.A. Weber	Rothrock, Sticky Sagebrush		
***Artemisia spiciformis* Nutt.**	*Artemisia tridentata* Nutt. subsp. s*piciformis; Seriphidium spiciformis* (Osterhout) Y.R. Ling	Snowfield Sagebrush		

Table 1.4 Species and subspecies in the subgenus *Tridentatae* according to the Flora of North America (Shultz, 2006).

Species	Synonyms	Common Names	Subspecies	Synonyms
***Artemisia tridentata* Nutt.**	*Seriphidium tridentatum* (Nutt.) W.A. Weber	Basin Sagebrush, Big Sagebrush, Big-Sage, Common Sagebrush, Great Basin Sagebrush, Mojave Sagebrush, Mountain Sagebrush, Sagebrush, Vasey's Sagebrush, Wyoming Sagebrush	*Artemisia tridentata* Nutt. subsp. *tridentata*	*Artemisia angustifolia* (A. Gray) Rydberg; *Artemisia tridentata* subsp. *xericensis* Winward ex R. Rosentreter & R.G. Kelly (NAFLORA)
			Artemisia tridentata Nutt. subsp. p*arishii* (A. Gray) H.M. Hall & Clements	*Artemisia parishii* A. Gray; *Artemisia tridentata* var. *parishii* (A. gray) Jepson; *Seriphidium tridentatum* (Nutt.) W.A. Weber subsp. *parishii* (A. Gray) W.A . Weber
			Artemisia tridentata Nutt. subsp. *vaseyana* (Rydb) Beetle	*Artemisia vaseyana* Rydb; *Artemisia tridentata* var. *pauciflora* Winward & Goodrich; *Artemisia tridentata* var. *vaseyana* (Rydb) B. Boivin; *Seriphidium vaseyanum* (Rydb) W.A. Weber
			Artemisia tridentata Nutt. subsp. w*yomingensis* Beetle & A.M. Young	*Artemisia tridentata* var. *wyomingensis* (Beetle& A.M. Young) S.L. Welsh; *Seriphidium tridentatum* (Nutt.) W.A. Weber subsp. w*yomingense*
***Artemisia tripartita* Rydb**	*Artemisia trifida* Nutt.; *Artemisia tridentata* Nutt. subsp. *trifida*, *Seriphidium tripartitum* (Rydb) W.A. Weber	Three-tipped Sagebrush, Cutleaf Sagebrush	*Artemisia tripartita* Rydb subsp. t*ripartita*	
			Artemisia tripartita Rydb subsp. *rupicola* Beetle	

Table 1.5 Distribution of articles across specific research areas according to Web of Science™ search engine analysis function.

Total Number of Articles Identified Pertaining to Species Found Within the Subgenus *Tridentatae*	**891**	
Research Areas	**Counts**	**Percent**
Agriculture	181	20
Biochemistry Molecular Biology	57	6
Biodiversity Conservation	67	8
Biotechnology Apllied Microbiology	8	1
Chemistry	24	3
Entomology	15	2
Environmental Sciences Ecology	567	64
Evolutionary Biology	30	3
Forestry	77	9
Genetics Heredity	13	1
Geochemistry Geophysics	5	1
Geology	17	2
Marine Freshwater Biology	5	1
Meteorology Atmospheric Sciences	10	1
Pharmacology Pharmacy	7	1
Physical Geography	7	1
Plant Sciences	191	21
Remote Sensing	6	1
Water Resources	15	2
Zoology	48	5

*Only those research areas encompassing at least 1% of publications are shown above

1.5.1 Evolution, Botany and Taxonomy

The exact origin of the subgenus *Tridentatae* is still unknown. Research suggests that Asian relatives belonging to the genus *Artemisia* subgenus *Artemisia* are ancestors to North American species of Sagebrush (Garcia et al., 2011b; Garcia et al., 2011a). It was hypothesized that the subgenus *Tridentatae* emerged in North America during the Pleistocene via herbaceous ancestral members (Garcia et al., 2011b; Shultz, 2009), which would make these species endemic to Western parts of North America (Shultz, 2009). Climatic fluctuations during this time may have facilitated the diversification of the *Tridentatae* by providing new territory for colonization and barriers which limit gene flow (Riggins and Seigler, 2012; Garcia et al., 2011b; Shultz, 2009; Valles et al., 2011).

The evolutionary history of the *Tridentatae* is reticulate, which in turn has led to multiple taxonomic re-arrangements within the *Tridentatae* (Garcia et al., 2008; Stanton et al., 2002) and a plethora of nomenclatural synonyms (Table 1.4). Tremendous efforts have been put forth to effectively group Sagebrush species (Garcia et al., 2011b; Shultz, 2009; Shultz, 2009; Garcia et al., 2011a; Garcia et al., 2008). According to the Flora of North America, the subgenus *Tridentatae* is comprised of the following species: *Artemesia arbuscula* Nutt., *Artemisia bigelovii* A. Gray, *Artemisia cana* Pursh, *Artemisia nova* A. Nelson, *Artemisia pygmaea* (A. Gray) W.A. Weber, *Artemisia rigida* (Nutt.) A. Gray, *Artemisia rothrockii* A. Gray, *Artemisia spiciformis* Nutt., *Artemisia tridentata* Nutt., and *Artemisia tripartita* Rydb (Shultz, 2006) (Table 1.4). It should be noted that recent molecular works suggest further redefinition of this subgenus by splitting the *Tridentatae* into two or more of the following sections: *Tridentatae*, *Nebulosae* and *Filifoliae* in hopes of concentrating the 'true Sagebrushes' within section *Tridentatae* (Garcia et al., 2011b; Shultz, 2009; Valles et al., 2011). Identification of species within the *Tridentatae* is challenging due to overlap of key morphological features (Shultz, 2009). Sub-species can be identified by the unique environments they inhabit (Shultz, 2009). Members of the Asteraceae are often identified by weakly ornamented pollen grains, alternate or sparsely distributed leaves, and by close examination of flower heads or capitula (Valles et al., 2011). All members of the *Tridentatae* are shrubs (Shultz, 2009). The presence of interxylary cork and bisexual flora heads is helpful to identify the subgenus (Shultz, 2006; Shultz, 2009; Valles et al., 2011),

while plant size, shape and size of flowering heads or pollen, leaf lobeing, and shape of lateral shoots are helpful determinants at the species level (Shultz, 2006; Shultz, 2009).

The ability of taxa in the *Tridentatae* to readily adapt to new territories has led to their broad distribution over tens of millions of hectares in North America (Garcia et al., 2011b; Stanton et al., 2002) (Figure 1.7). Sagebrush can thrive in diverse habitats such as canyons, meadows and slopes ranging from steppe to subalpine zones, dry shrublands, foothills, rocky outcrops, scabelands, and valleys (Stanton et al., 2002; Douglas, 1998). *A. tridentata*, *A. arbuscula*, *A. cana*, and *A. nova* are all considered landscape dominant species, while *A. arbuscula*, *A. bigelovii*, *A. pygmaea*, *A. rigida*, *A. rothrockii*, and *A. spiciformis* possess more restricted distributions (Garcia et al., 2008; Stanton et al., 2002) (Figure 1.7). Distribution of subspecies is primarily dictated by moisture-elevation gradients and/or characteristics specific to soils and substrates (Stanton et al., 2002).

1.5.2 Phytochemistry

Research interest for the phytochemical complexity of Sagebrush has been consistent for many decades but declining since the 1970s (Figure 1.8). The reported phytochemistry of the *Tridentatae* spans the major classes of plant specialized metabolism - phenolics (13.2%), monoterpenes (40.5%), sesquiterpenes (39.1%), diterpenes (1.8%) and others (5.5%) (Figure 1.9, Appendix A). This phytochemistry is not a comprehensive representation of the metabolome but rather the result of several decades of targeted phytochemical discovery using primarily GC/FID and GC/MS profiling of the essential oils. Considerable effort has also been made to characterize more complex terpenes, plant phenolics, and other constituents of the metabolome of these species, but the total metabolome has not been described.

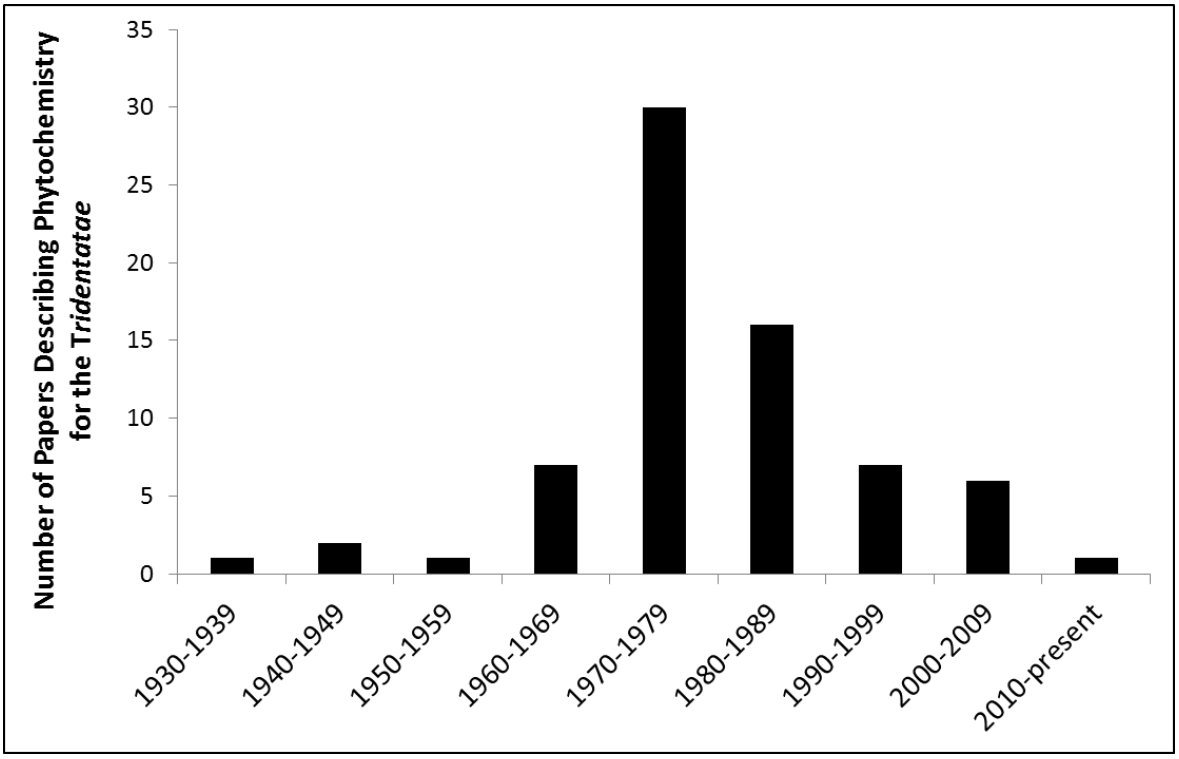


Figure 1.8 Number of publications describing phytochemistry of the *Tridentatae* (1930-Present) according to Web of Science™ search engine results.

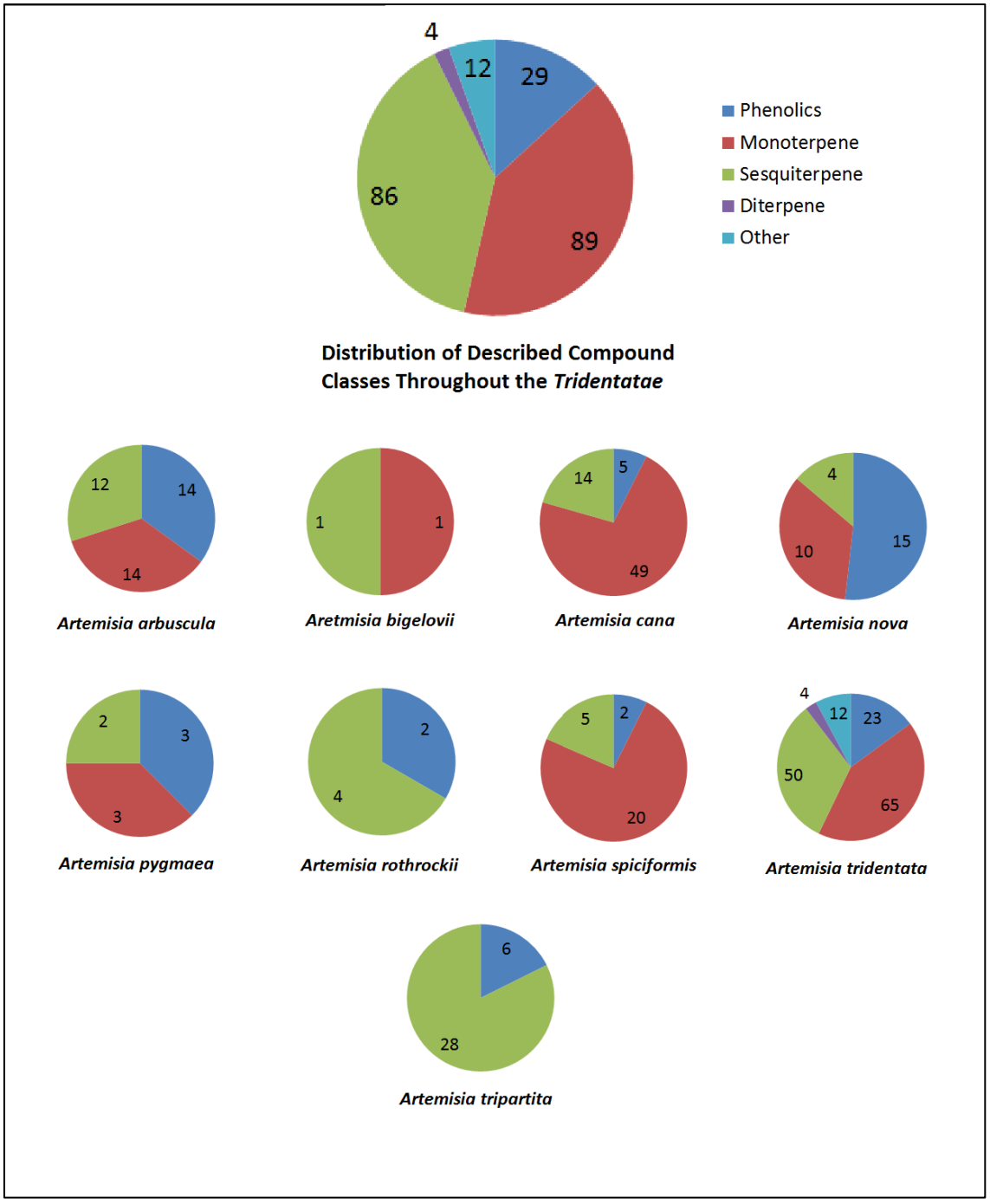


Figure 1.9 Distribution of compound classes in the *Tridentatae* and its members based on 71 publications. Pie charts represent the number of compounds described in the literature for each species and the specific class they pertain too.

Phenolics

A total of 29 plant phenolics were described in the literature for the *Tridentatae* in *A. arbuscula, A. cana, A. nova, A. pygmaea, A. rothrockii, A. spiciformis, A. tridentata,* and *A. tripartita* (Figure 1.9) (Table A.1, Figure A.1-A.3). All are phenylpropanoids, with the majority being flavonoids and glycosylated flavonoids. While the majority of the studies describe the identification of phenolics by HPLC/DAD or HPLC/UV analysis and by comparison with standard compounds, a small number of novel phenylpropanoids were isolated and characterized from the *Tridentatae* including dihydroquercetin 7,3´-dimethyl ether (**1**), artelin (**2**), methylesculin (**3**) and some coumarin sequiterpene ethers that will be described along with the sesquiterpenes. Esculin (**4)**, scopoletin (**5)**, axillarin (**6)**, esculetin (**7)**, isoscopoletin (**8)**, and 6-β-D-glucosyl-7-methoxycoumarin (**9)** were most widely detected (>30% of species) (Figure 1.9 & 1.10).

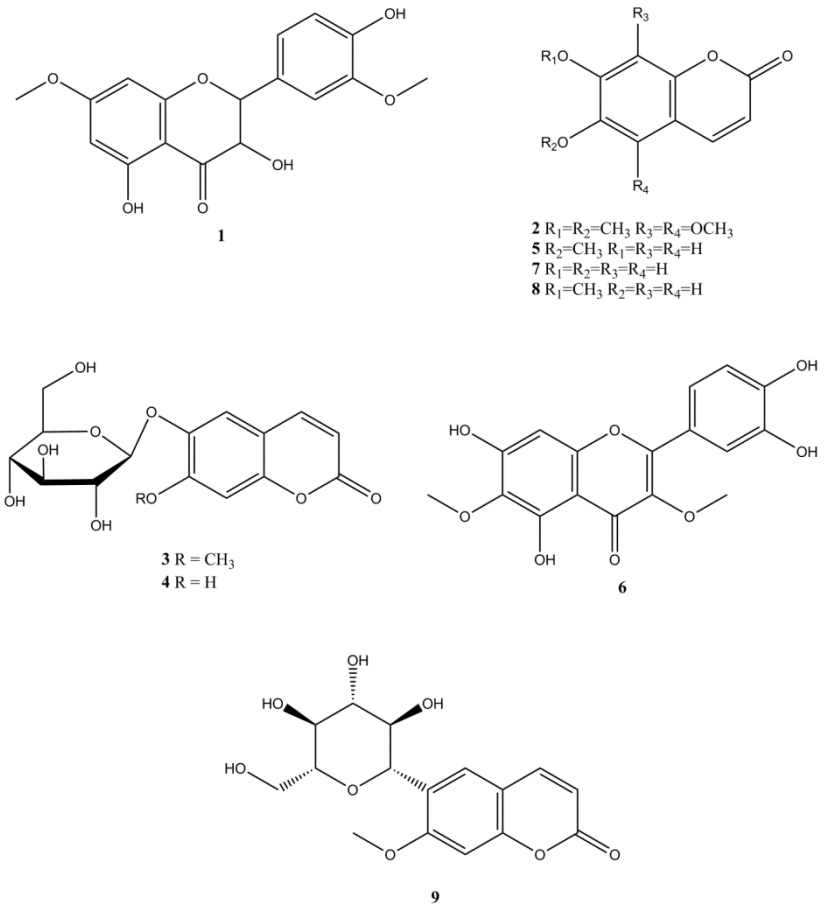


Figure 1.10 Characteristic phenylpropanoids in the *Tridentatae*. Numbers refer to structures found in text. Structures drawn using Chemdraw software.

Monoterpenes

Overall, 89 monoterpenes have been reported in *A. arbuscula, A. bigelovii, A. cana, A. nova, A. pygmaea, A. spiciformis, A. tridentata,* (Figure 1.9) (Table A.1, Figures A.4-A.8). The overwhelming majority of monoterpenes were identified by gas chromatographic (GC) analysis with detection and tentative identification by either EI-MS (electron impact mass spectrometry) followed by database matching of the mass spectra, or by flame ionization detection, where the compounds were either identified by comparison with standards, or tentatively identified by relative retention using the Kovacs retention index.

A number of novel monoterpenes were identified and fully characterized from the *Tridentatae*, of which most are irregular monoterpenes (Figure 1.11). These include chrysanthemol (**10**) and its oxidized derivative chrysanthemal (**11**), methyl santolinate (**12**), santolina triene (**13**), oxido santolina triene (**14**), artemesia triene (**15**), santolinolide A (**16**) and B (**17**), artemiseole (also spelled artemeseole, and artemisol) (**18**), rothrockene (**19**), , neolyratol (**20**), (1,6,6-trimethyl-4-ethenyl-exo-2-oxabicyclo[3.1.0]hexane (**21**), lavandulol (**22**), arbusculone (**23**), 2,2-dimethyl-6-isopropenyl-2H-pyran (**24**), 2,3-dimethyl-6-isopropyl-4H-pyran (**25**), and 2-isopropyl-5-methylhexa-*trans*-3,5-diene-1-ol (**26**) (Figure 1.10). Compounds most commonly described in these species (>40% of *Tridentatae* members) include: α-pinene (**27)**, santolina triene (**13)**, artemiseole (**18)**, camphor (**28)**, methacroleine (**29)** p-cymene (**30)**, camphene (**31**) and eucalyptol (**32)** (Figure 1.10).

Sesquiterpenes

In total, 86 sesquiterpenes and their derivatives were found in *A. arbuscula, A. bigelovii, A. cana, A. nova, A. pygmea, A. rothrockii, A. spiciformis, A. tridentata,* and *A. tridentata* (Figure 1.9) (Table A.1, Figures A.9-A.12). Most of the sesquiterpenes described in the *Tridentatae* were first isolated and structurally characterized from members of the genus *Artemisia*, and represent a remarkable chemical diversity. Sesquiterpenes were identified by a variety of techniques after their original isolation and characterization, including comparisons to standards by TLC, HPLC/DAD, HPLC/UV, and HPLC/MS.

The majority of sesquiterpenes that were first described in the *Tridentatae* are sesquiterpene lactones, with structures based on: the lactone fused to a bicyclo[4.4.0]decane

framework such as arbusculins A (**33**), B (**34**), C (**35**), D (**36**), and E (**37**), colartin (**38**), ridentin B (**39**), rothins A (**40**) and B (**41**), 1-β-hydroxysant-3-en-6,12-olide-C (**42**), 1-β-hydroxysant-4(14)-en-6,12-olide-C (**43**) (Figure 1.12); the lactone fused to the open cyclodecane framework, such as artevasin (**44**), novanin (**45**), badgerin (**46**), deacetyllaurenobiolide (**47**), spiciformin (**48**), and tatricidins A (**49**) and B (**50**) (Figure 1.12); or the lactone fused to the large ring of a bicyclo[5.3.0]decane such as arbiglovin (**51**), artecanin (also referred to in the literature as chrysartemin B) (**52**), canin (**53**), cumambrin A (**54**) and B(**55**), 8-deoxycumambrin B (**56**), cumambrin B oxide (**57**), matricarin (**58**), deacetoxymatricarin (also referred to as leucodin in the literature) (**59**), deacetylmatricarin (**60**), rupicolins A (**61**) and B (**62**), rupins A (**63**) and B (**64**), and viscidulins A (**65**), B (**66**), and C (**67**) (Figure 1.11). Four sesquiterpene coumarin ethers have been isolated and structurally characterized in the *Tridentatae* including tripartol (**68**), secondriol (**69**), secondrial (**70**), and drimAchone (**71**), as well as three other sesquiterpenes, longilobol (**72**), arbusculin E (**37**), and pygmol (**73**) (Figure 1.13A&B). Arbusculins A (**33**), B (**34**), and C (**35**), artevasin (**44**), deacetoxymatricarin (**59**), deacetylmatricarin (**60**), desacetoxymatricarin (**74**), ridentin (**75**), tatridin-A (**49**) and B (**50**) were reported within more than 30% of species in the *Tridentatae* (Figure 1.9 and 1.13A&B).

Diterpenes

Only 4 diterpenes have been characterized in extracts of *A. tridentata* including: methyl isopimarate (**76)**, methyl levopimarate (**77)**, methyl palustrate (**78)**, and methyl trans-communate (**79)** (Figure 1.14)(Table A.1).

Other Phytochemicals

An assortment of polyketides, fatty acids, polysaccharides, tannins, alkaloids, carbohydrates and proteins have also been described in *A. tridentata* and/or *A. tripartita*.

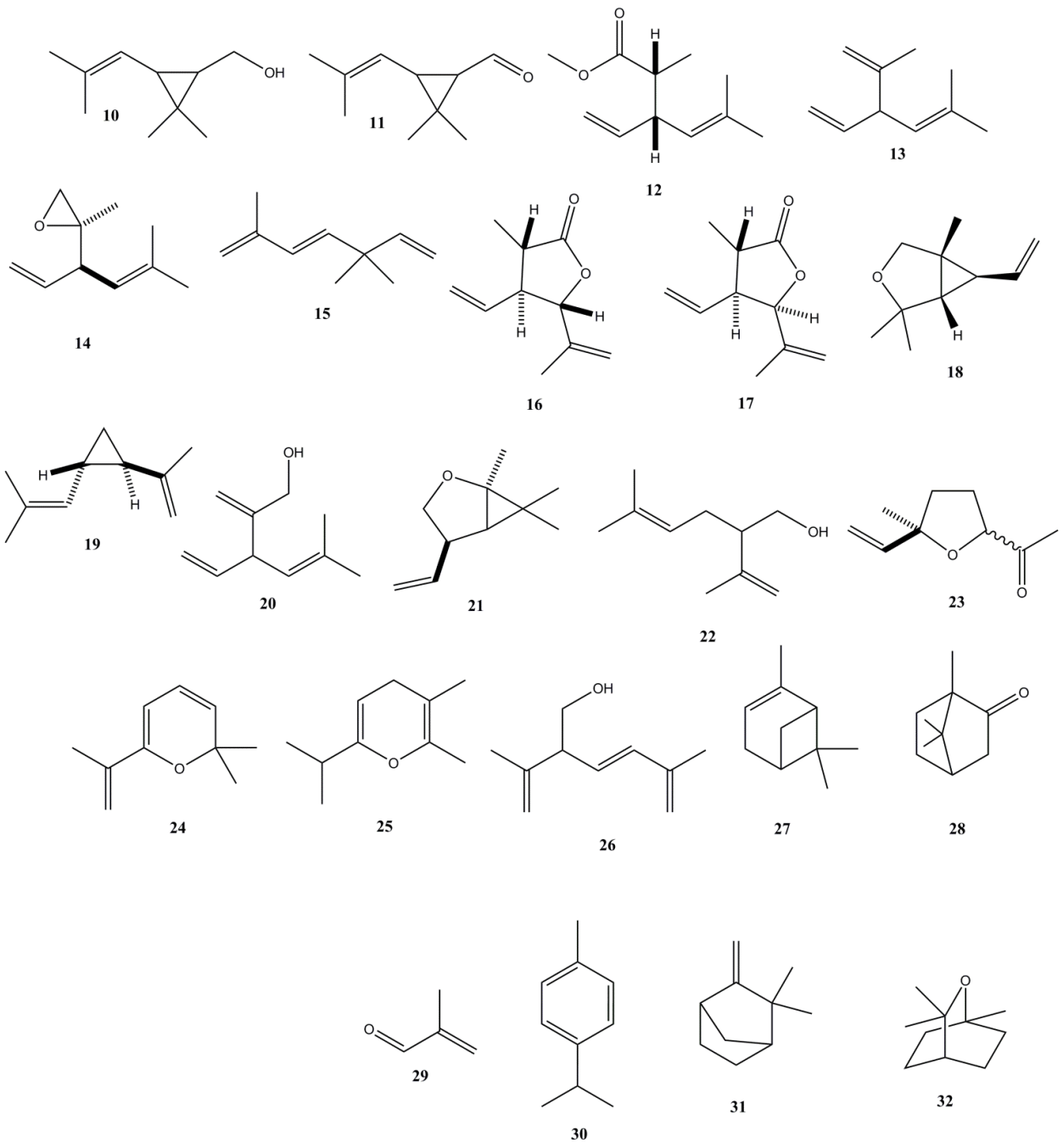


Figure 1.11 Characteristic monoterpenes in the *Tridentatae.* Numbers refer to structures found in text. Structures drawn using Chemdraw software.

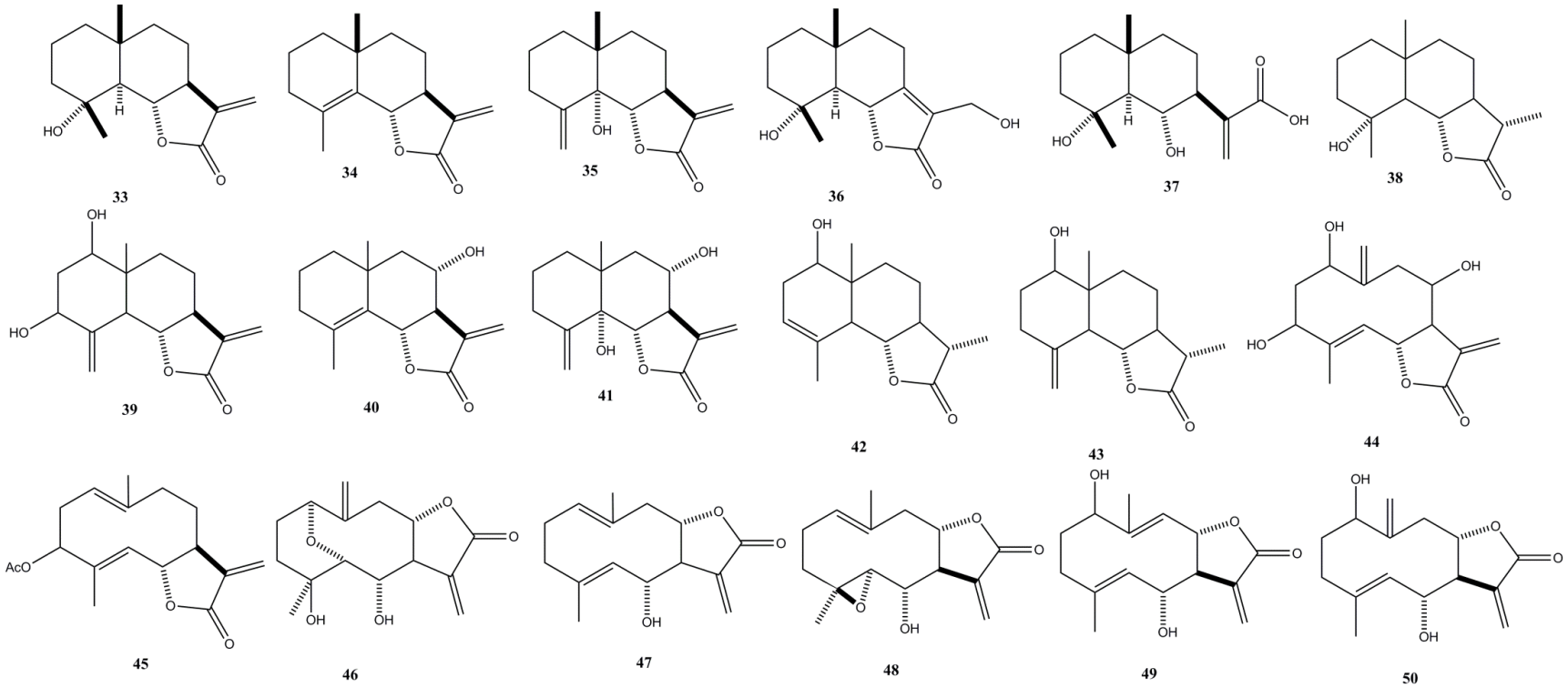


Figure 1.12 Characteristic sesquiterpenes in the *Tridentatae*. Numbers refer to structures found in text. Structures drawn using Chemdraw software.

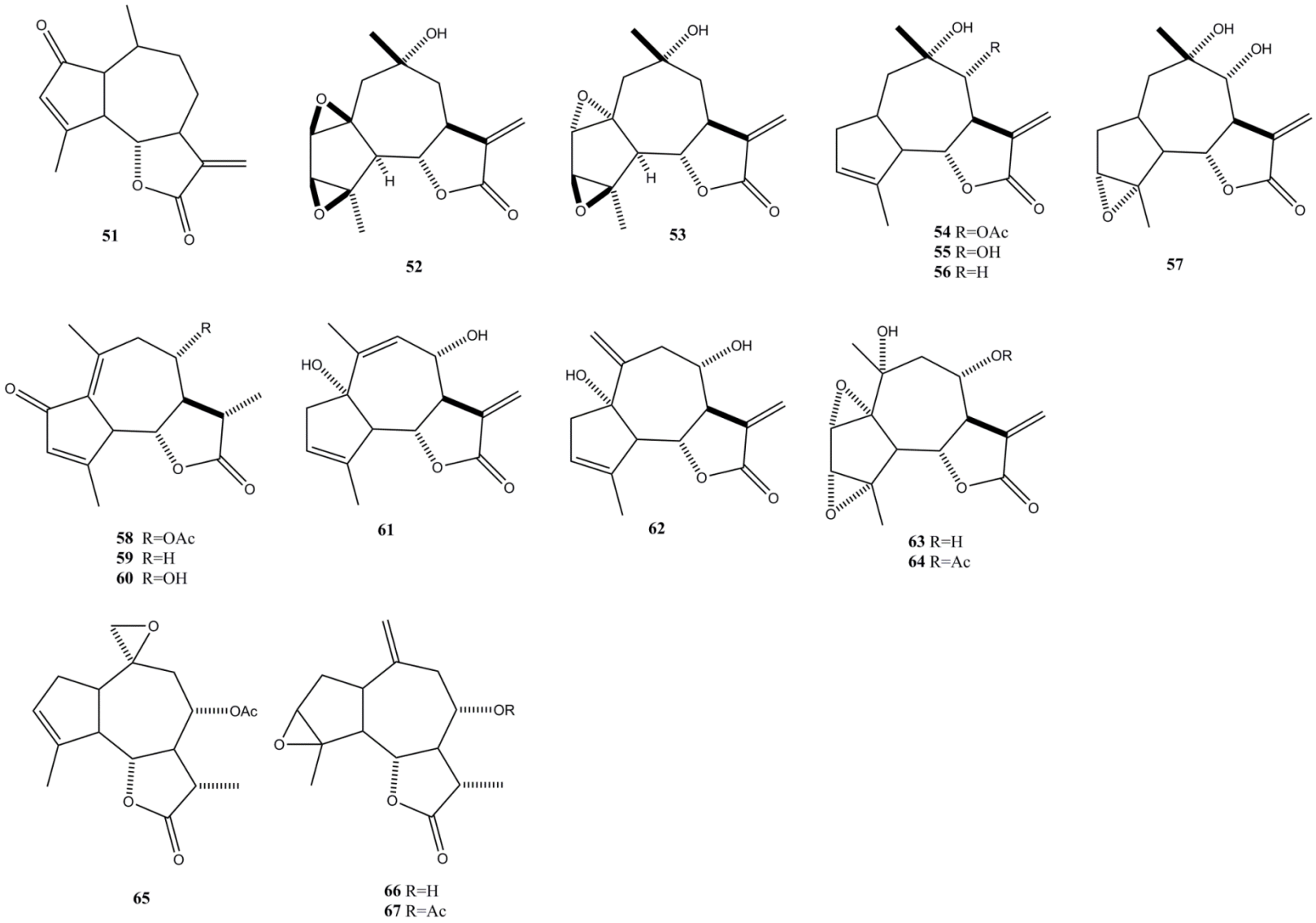


Figure 1.13A Characteristic sesquicoumarins in the *Tridentatae*. Numbers refer to structures found in text. Structures drawn using Chemdraw software.

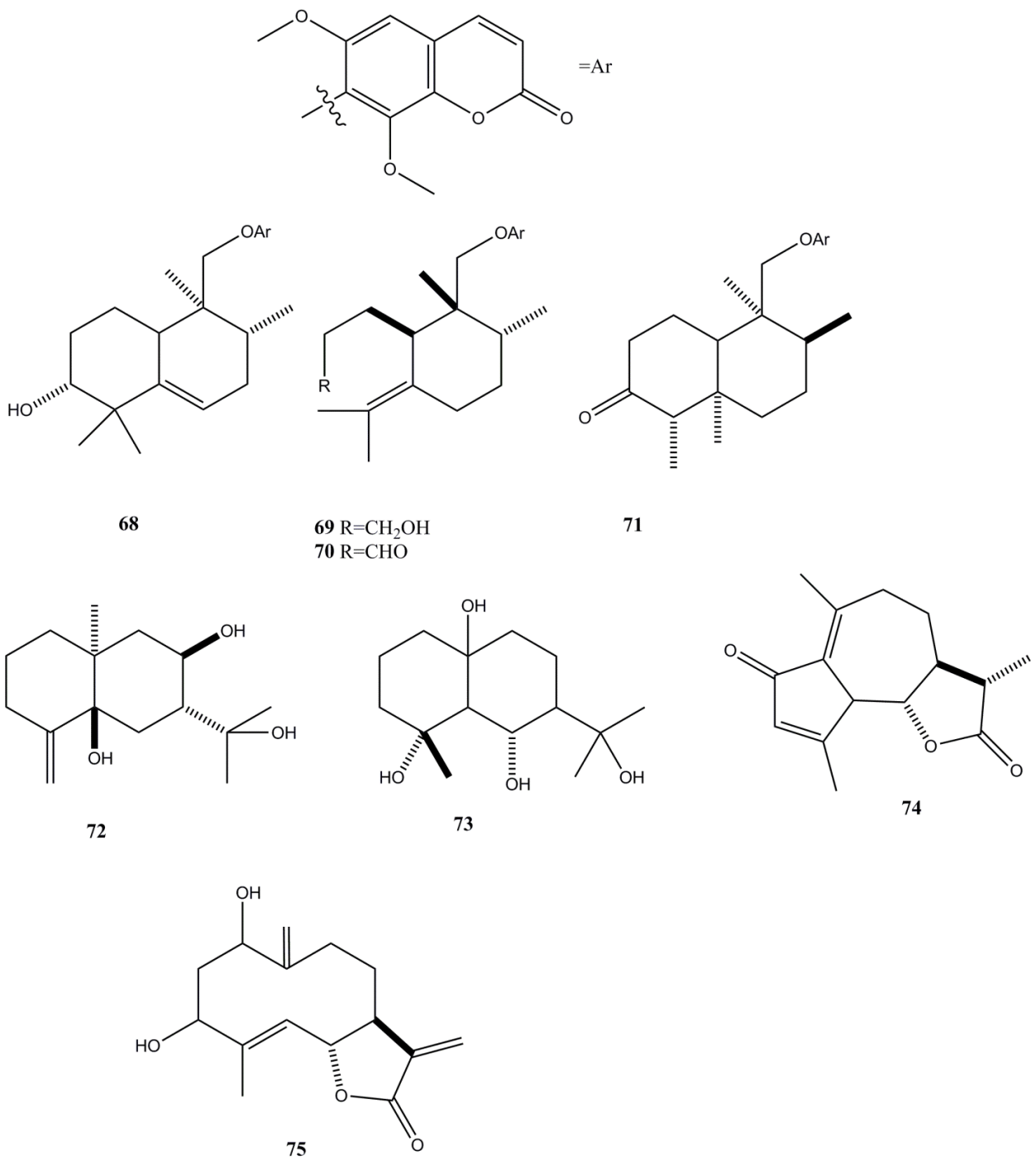


Figure 1.13B Characteristic sesquicoumarins in the *Tridentatae*. Numbers refer to structures found in text. Structures drawn using Chemdraw software.

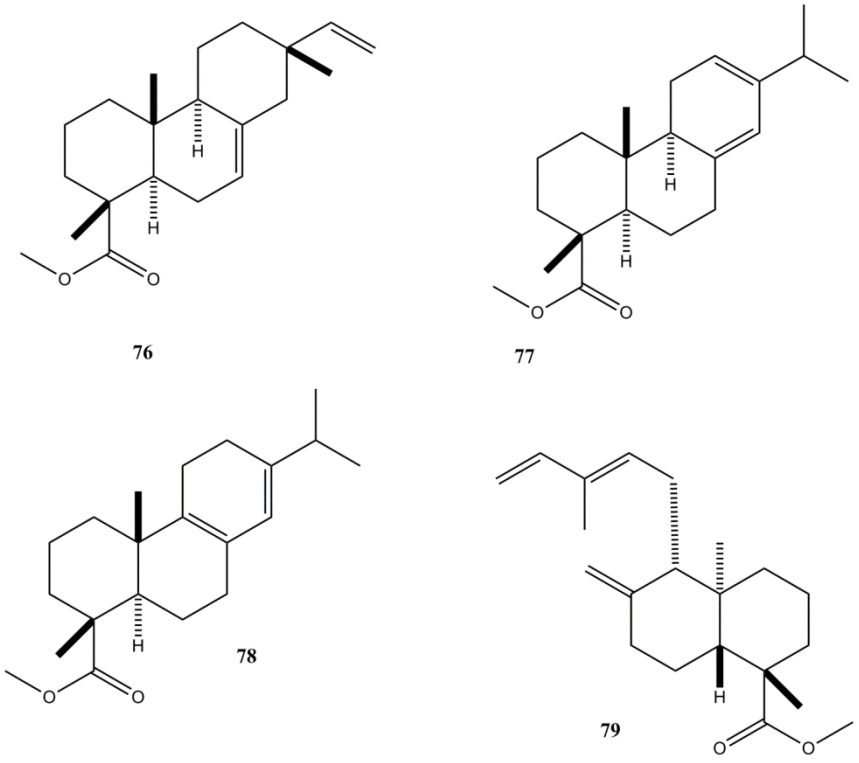


Figure 1.14 Characteristic diterpenes in the *Tridentatae*. Numbers refer to structures found in text. Structures drawn using Chemdraw software.

1.5.3 Bioassay

The majority of bioassay data describing the activity of *Artemisia* species in the *Tridentatae* indicate anti-microbial, antioxidant, antiviral and insecticidal activity.

Anti-microbial

Plant-based antimicrobial compounds that have the potential to combat food spoilage (Tiwari et al. 2009) and multi-drug resistance (Saleem et al., 2010) are under extensive investigation. Amongst members of the *Tridentatae* anti-microbial activity has been observed in oils collected from *A. cana*, *A. nova*, and *A. tridentata* (Lopes-Lutz et al., 2008a; Lopes-Lutz et al., 2008b; Nagy and Tengerdy, 1967) and methanol extracts of *A. arbuscula*, *A. bigelovi*, *A. cana* subsp. *bolanderi*, *A. nova*, *A. rothrockii*, *A. spiciformis*, *A. tridentata* subsp. *parishii*, *A. tridentata* subsp. *tridentata*, *A. tridentata* subsp. *vaseyana*, *A. tridentata* subsp. *wyomingensis*, and *A. tripartita* subsp. *tripartita*, (McCutcheon et al., 1994; McCutcheon, 1996) using *in vitro* bioassays with various strains of bacteria, yeast, and fungi. A common

mechanism or specific mode of action has not been determined and the diversity of extracts makes data interpretation difficult.

Anti-mycobacterium

Methanolic extracts from *A. arbuscula, A. bigelovii, A. cana* subsp. *bolanderi, A. cana* subsp. *cana, A. nova, A. rothrockii, A. spiciformis, A. tridentata* subsp. *tridentata, A. tridentata* subsp. *vaseyana,* and *A. tripartita* ssp. *tripartita* all displayed varying degrees of anti-mycobacterial activity (McCutcheon, 1996). Furthermore, from the above extracts, *A. cana, A. nova, A. tridentata* spp. *tridentata,* and *A. tripartita* show the greatest potential for the discovery of new TB drugs, they were found to exhibit the most activity against *Mycobacterium tuberculosis* and/or *Mycobacterium avium* (McCutcheon, 1996). Further investigation is required to determine the specific mode of activity of the growth inhibition.

Antioxidant Activity

Oils extracted from *A. cana* and *A. tridentata* exhibited weak anti-oxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene/linoleate model (Lopes-Lutz et al., 2008a).

Antiviral Activity

Methanolic extracts from *A. rothrockii* displayed weak anti-viral activity *in vitro* against Coxsackie B6, Polio 1, and Sindbis viruses at concentrations equal to the threshold for non-cytotoxic behaviour (McCutcheon, 1996).

Cytotoxicity

Variability for cytotoxicity has been observed in mono-layer forming "Vero" cells (Green Monkey Kidney) exposed to extracts of *A. arbuscula, A. bigelovii, A. cana, A. rothrockii, A. spiciformis, A. tridentata* subsp. *parishii, A. tridentata* subsp. *tridentata, A. tridentata* subsp. *wyomingensis* (McCutcheon, 1996). Of the species described above, *A. arbuscula, A. cana, A. rothrockii* and *A. spiciformis* were the most toxic (McCutcheon, 1996) and may provide promising pharmacological leads.

Fumigant and Insecticidal Activity

Ethanolic extracts from *A. tridentata* inhibited growth of *Peridroma saucia* (Hbn) (variegated cutworm) (Salloum and Isman, 1989), while volatile compounds released from leaves inhibited oviposition for *Zabrotes subfasciatus* (Boheman) (Mexican bean weevil) (Weaver,D,K, et al., 1995) *A. tridentata* subsp. *vaseyana* demonstrated activity against lesser grain *Rhyzopetha dominica* (F.) (lesser grain borer) and *Plodia interpunctella* (Hubner) (Indian meal moth) (Dunkel and Sears, 1998).

1.6 Metabolomics, Metabonomics, and the Plant Metabolome

Metabolomics is the qualitative and quantitative analysis of all small (less then 1000 Da) metabolites in a biological sample at a specific time (Yuliana et al., 2011). Comparison between metabolomes is referred to as metabonomics (Nicholson, 2002). Subtractive metabonomics is the process of applying logical algorithms in order to subtract metabolites which are common or unique to a specific treatment or sample (Brown et al., 2012a; Brown et al., 2012b). The average plant leaf can contain upwards of 30,000 phytochemicals (Verpoorte et al., 2010), consequently a standard metabolomics dataset will contain vast amounts of data which requires multiple approaches to effectively explore relationships between metabolites or metabolic changes (Goodacre et al., 2004). Two types of approaches are applied to explore the plant metabolome: 1) targeted approaches which are generally hypothesis driven and target well known metabolites in order to answer a specific research question; and 2) untargeted approaches which are hypothesis generating and aim to uncover patterns or relationships within the whole dataset regardless of whether the identity of a specific analyte is known (Lankadurai et al., 2013; Patti et al., 2012; Wishart, 2007).

1.6.1 Targeted vs Untargeted Approaches

Targeted approaches have close resemblance to traditional analytical methods which aim to quantify specific metabolites by optimizing extraction, separation and detection methods for a certain class of compound. On the other hand, untargeted analysis is considered a ‘true metabolomics’ approach and aims to characterize or profile all the ‘unknowns’ within a sample without bias (Hall, 2011; Lankadurai et al., 2013). There are three distinctly different research approaches that are being taken in metabolomics research. Some researchers are using a strictly targeted approach with quantification of known compounds using authenticated standards and spectral libraries sometimes associated with genomic or proteomic data (Davis et al., 2013; Rochfort et al., 2008). Other works are

targeted-untargeted and encompass other 'omics' technologies (i.e. transcriptomics, genomics, proteomics) to find chemical leads by quantifying known compounds that have not previously been described in the species of interest. These data may be mapped in association with the genomic data for compound and pathway discovery but are not truly untargeted analyses (Gao et al., 2014; Wahyuni et al., 2014). The third approach is completely untargeted identification and quantification of the entire metabolome using statistical modelling and mapping to eliminate false discoveries, to explore relationships between unknowns and to target specific characteristic unknowns for fingerprinting, future targeted phytochemical studies, pathway elucidation, chemical association with physiological responses and other applications (Brown et al., 2012b; Jandric et al., 2014; Turi and Murch, 2013).

1.6.2 Untargeted Metabolomics

While great advancements have been made to detect and quantify specific knowns within a biological sample (i.e. targeted-targeted or targeted-untargeted approaches), the use of a truly untargeted approach for the purpose of prioritizing metabolites of interest, prior to structural identification, has not been fully realized (Patti et al., 2012) . Early untargeted works focused on the establishment of a 'metabolic fingerprint' in order to target specific metabolite differences between samples and/or treatments through both multivariate and univariate statistics (Aharoni et al., 2002). As a result, current approaches rely heavily on principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) (Brown and Murch, 2012; Turi and Murch, 2013a). Once specific 'unknowns' have been targeted, metabolites of interest are subjected to putative identification. The identification of metabolites during untargeted analysis is a well-known bottleneck in metabolomics research and consists of two groups: 'known unknowns' and 'unknown unknowns' (Fukishima and Kusano, 2013). Known unknowns are metabolites which have been identified as an already known compound (i.e. MEL), while unknown unknowns are compounds whose structures are considered truly novel (Fukishima and Kusano, 2013; Wishart 2009). Known unknowns can be identified by matching RT/index, m/z, fragmentation mass spectrum to either chemical standards or mass spectral libraries (Dunn et al., 2013; Xiao et al., 2012). The metabolomics standard initiative suggests four levels for metabolite identification: 1) identified, 2)

putatively annotated compounds, 3) putatively characterized compound classes, and 4) unknowns (Summers et al. 2007). Definitive compounds are those which have been identified using authentic standards while under identical analytical conditions. Putatively annotated compounds can be identified by comparing mass: charge (m/z), fragmentation spectra and/or RT to metabolite databases (Dunn et al., 2013; Xiao et al., 2012). A plethora of databases are currently available to assist with compound annotation such as The Dictionary of Natural Products, KNApSAcK (Afendi et al., 2012), Plant Metabolic Network (http://plantcyc.org), PubChem (http://pubchem.ncbi.nlm.nih.gov/), and Chemspider (Hall, 2011). The application of metabolite databases has been comprehensively reviewed for plant metabolomics, thus this topic will not be covered here (see Fiehn et al., 2011; Fukushima and Kusano, 2013; Hall, 2011). Although metabolite databases are a resourceful tool, most are incomplete for a majority of plant species and are not universally compatible with all analytical platforms (Dunn et al., 2013). Furthermore, they are not helpful if metabolites have not previously been described (unknown unknowns). In this case, the use of *in silico* fragmentation software or isotope labeling should then be considered (Dunn et al., 2013; Xiao et al., 2012).

1.7 Synthetic Biotransformation and Significant Ion Generation as a Hypothesis Generating Techniques

1.7.1 Synthetic Biotransformation

The use of statistical tools has been pivotal for conducting untargeted analysis (Brown et al., 2012a; Brown et al., 2012b), however alternative approaches such as synthetic biotransformation and significant ion generation have not been investigated to their fullest (Turi and Murch, 2013a; Murch et al., 2004). As previously mentioned, metabolites are not independent of each other. Instead, many unique and complex structures are produced through enzymatic modifications which cumulatively elucidate biochemical pathways for secondary metabolite biosynthesis (Li et al., 2013; Turi and Murch, 2013a; Murch et al., 2004). Metabolomics provides a unique opportunity to explore enzymatic processes *in vivo*. Synthetic biotransformation involves the numerical manipulation of a known compound's monoisotopic mass in order to simulate enzymatic processes which could be occurring at the cellular level, for example removal and/or addition of amine, carboxyl, hydrogen, hydroxyl, methyl, oxygen or sugars to a specific metabolite's monoisotopic mass (Li et al., 2013; Turi

and Murch, 2013). Newly calculated masses can be mined within a metabolomics dataset to generate hypothesis regarding biochemical pathways and responses to the environment (Kai et al., 2011; Murch et al., 2004) or biological activity of specific extracts (Turi and Murch, 2013a).

1.7.2 Significant Ion Generation

Significant ion generation involves the application of univariate statistics in order to identify specific analytes which possess an ion intensity that is significantly greater in comparison to the remaining dataset. Once prominent ions have been detected putative identification can be applied, by matching monoisotopic masses of each metabolite to secondary metabolite databases such as metabosearch, Chemspider, Metacyc, Dictionary of Natural Products, Chemspider or KNApSAcK (Hall, 2011). Furthermore, average ion counts can be calculated for a specific metabolite and plotted in order to determine increasing or decreasing trends in response to a specific treatment.

1.7.3 Application of Synthetic Biotransformation and Significant Ion Generation

Pathway elucidation

The use of mass spectrometry based methods to characterize biochemical pathways in plants can be challenging due to the lack and/or cost of analytical standards (De Vos et al., 2007). Synthetic biotransformation allows researchers to explore possible enzymatic reactions which are crucial for the synthesis of metabolites. Using FTMS Murch et al. (2004) applied a series of biotransformations to the flavone baicalin in order to develop a putative pathway for synthesis of novel derivatives in *Scutellaria baicalensis* Georgi. Similarly, Kai et al. (2011) applied this approach to characterize previously unknown steps involved in the biosynthesis of indolic glucosinolates found in *Arabidopsis thaliana* using FT-ICR/MS (Kai et al., 2011). Turi and Murch (2013a) applied 182 putative transformations to Z/E-ligustilide a biologically active constituent found in *Ligusticum canbyi*, Coult. & Rose. To date, biosynthesis of ligustilide is still unknown. 82 novel phthalide derivatives possessing ion intensities greater than 1.0 were detected in *L. canbyi* tissue types and suggesting that enzymatic modification of ligustilide is contributing to phthalide diversity in *L. canbyi* (Turi and Murch, 2013a) (Chapter 3).

Plant responses to the environment

In response to their environment, plants produce a wide array of phytochemicals called secondary metabolites (Wink, 2010). In recent years, the application of metabolomics has provided an interesting avenue for understanding how plants respond to specific environmental stressors (Sanchez et al., 2012; Obata and Fernie, 2012), for example environmental cues such as light, temperature, drought, or salt stresses have been examined (Kral'ova et al., 2012), with broader applications towards crop selection (Arbona et al., 2013; Rasmussen et al., 2012). Despite current interest, the majority of these works use multivariate statistics to infer metabolic changes caused by biotic and abiotic elicitors. Synthetic biotransformation can be used to explore relationships between plants and their surrounding environment or how plants produce/store metabolites in response to stress. Kluger et al. (2013) used stable isotope labelling in order to identify novel conjugates of the *Fusarium* mycotoxin deoxynivalenol in Wheat (Kluger et al., 2013). Ntakadzeni et al. (2013) subjected Tobacco cell suspension cultures to isonitrosoacetophenone and monitored metabolomic changes over time by plotting the ion intensity of specific analytes. Analytes of interest were putatively identified using online databases such as Chemspider (Madala et al., 2013). Similarly, Turi and Murch (2014b) compared significant ions between field and culture grown treatments in order to explore how *in vitro* methods can affect the production of secondary metabolites in *A. tridentata* (Chapter 4). In another experiment *A. tridentata* shoot cultures were treated with 0, 5, and 10 µM of galanthamine and plotted ion intensity as a dose response curve to investigate metabolic changes that may be occurring (Chapter 5).

Biological Activity

The use of metabolomics as an alternative approach to bioassay-guided fractionation for the characterization of biologically active constituents in plant extracts has been recently reviewed (see Yuliana et al., 2011; Yuliana et al., 2013). While metabolomics coupled with putative identification is an important tool to characterize observed biological activity within crude extracts (Yuliana et al., 2011) metabolite databases are limited to specific phytochemicals which are already known. Thus, synthetic biotransformation of known biologically active constituents can permit one to explore the contribution of specific metabolite classes or transformations present within a biologically active extract. For example, synthetic biotransformation and putative identification were applied in order to

explore phytochemical changes that are occurring while burning leaf tissues and how observed changes may affect the observed biological activity of smoke (Chapter 6).

1.8 Research Aims and Objectives

The overall objectives of this research were 1) to identify candidate species for studying plant neurochemicals and 2) to develop novel untargeted metabolomic approaches for investigating the phytochemical and biological underpinnings of two model medicinal plants. To achieve this overall objective, the following specific objectives were investigated:

1) To compare and contrast four satistical approaches that are commonly used to evaluate ethnobotanical databases.

2) To select two model species based on the traditional knowledge of use in ceremony or ritual by North American peoples.

3) To establish germplasm collections for the two model species, *L. canbyi* and *A. tridentata*, in order to provide material for chemical studies and bioassay.

4) To determine whether the two model species used in ceremony and ritual contain known phytochemicals that affect human brain metabolism.

5) To develop novel statistical tools and logical algorithms for analysis of metabolomics datasets.

6) To use metabolomics analysis and statistical tools to discover new compounds and biochemical relationships in phthalide metabolism in *L. canbyi*.

7) To use metabolomics analysis and statistical tools to discover new biochemical relationships in response to mediation of cholinergic metabolism in *A. tridentata*.

8) To use metabolomics analysis and statistical tools for investigating the biological activity of *A. tridentata* leaf and smoke extracts.

Chapter 2 Selection of Model Species

2.1 Synopsis of Research

Ethnobotanical research and the study of plants used in rituals, ceremonies or to connect with the spirit world have led to the discovery of many novel psychoactive compounds such as nicotine, caffeine, and cocaine. In North America, spiritual and ceremonial uses of plants are well documented and can be accessed online via the University of Michigan's Native American Ethnobotany Database. The objective of this study was to compare residual, bayesian, binomial and Imprecise Dirichlet Model (IDM) analyses of ritual, ceremonial and spiritual plants in Moerman's ethnobotanical database to identify genera that may be good candidates for identifying novel psychoactive compounds. The database was queried with the following format inserted: "Family Name **AND** Ceremonial **OR** Spiritual" for 263 North American botanical families. Spiritual and ceremonial flora consisted of 86 families with 517 species belonging to 292 genera. Spiritual taxa were then grouped further into ceremonial medicines and items categories as described in Moerman (2009). Residual, bayesian, binomial and IDM analyses were performed to identify over and under-utilized families. The 4 statistical approaches were in good agreement when identifying under-utilized families but large families (>393 species) were underemphasized by binomial, bayesian and IDM approaches for over-utilization. Residual, binomial, and IDM analysis identified similar families as over-utilized in the medium (92-392 species) and small (<92 species) classes. The families Apiaceae, Asteraceae, Ericaceae, Pinaceae and Salicaceae were identified as significantly over-utilized as ceremonial medicines in medium and large sized families. Analysis of genera within the Apiaceae and Asteraceae suggest that the genera *Ligusticum* and *Artemisia* are good candidates for facilitating the discovery of novel psychoactive compounds.

2.2 Objectives and Hypothesis

I hypothesized that the report of use of specific North American plant species in ceremony or spirituality indicates the potential presence of neurologically active constituents in the plant family. The objectives I designed to test this hypothesis were: 1) to analyze ceremonial and spiritual uses of plants by Residual, bayesian, binomial and IDM analyses, 2) to determine the frequency and distribution of spiritual and ceremonial uses within plant

families and 3) to identify genera with which have potential for containing psychoactive compounds.

2.3 Materials and Methods

2.3.1 Acquisition of Ethnobotanical and Flora Data

Moerman's online Native American Ethnobotany Database, was accessed on July 3, 2012 (http://herb.umd.umich.edu). The online database holds a repository of ethnobotanical data for 3618 species and consists of 47,000 items describing uses of North American flora for purposes such as food, drug, dye, and fiber among 291 Native American groups (Moerman, 2012). All ceremonial taxa were identified using Cronquist (1981) via the USDA's PLANTS Database (http://plants.usda.gov/java/). The database was queried with the following format "Family Name **AND** Ceremonial **OR** Spiritual" for 263 North American botanical families (Moerman and Estabrook, 2003). Spiritual/Ceremonial data were exported and sorted into two categories: (1) species identified as 'ceremonial medicines' (incense, smudges, scrubs, smoking tobacco, snuffs, teas, topical rubs, washes) or (2) species identified being used as 'ceremonial items' (ceremonial masks, charms, paints (including ash), prayer sticks, rattles, statues, symbolic items, tools). The total number of species for each family was based on the data collected by Moerman and Estabrook (2003). The metadata on the Apiaceae and Asteraceae were acquired from the USDA's Plants Profile http://plants.usda.gov/java/.

2.3.2 Residual Analysis and Ranking

Residual analysis was performed in Excel using methods outlined in Moerman (1991, 1996). In brief, each family was plotted with dependent values equal to the number of species found within the entire North American family and independent values equal to number of species identified in the database as utilized for spirituality/ceremony, item or medicine. Linear regression analysis was used to identify the number of expected species for each family. Residual values were then calculated by subtracting the observed value from the expected value if the family was of average size. The families were then ranked from largest to smallest residual to determine the 10 most and least represented families, which were then classified as over- and under-utilized respectively.

2.3.3 Binomial Analysis and Ranking

Binomial analyses were conducted using methods described by Bennett and Husby (2008). The null hypothesis was that the proportion of spiritual species in the total flora was equal to the proportion of spiritual species in each family. The alternative hypothesis was that the proportion of species used in spirituality within each family is either 1) greater or 2) less than the total proportion found within the flora. Significance was tested using the Excel BINODIST function and families were than ranked from lowest to highest p-value to determine over- and under-utilization.

2.3.4 Bayesian and IDM Analysis and Ranking

Bayesian and IDM analysis used methods outlined in Bennett and Husby (2008) and Weckerle et al. (2011, 2012) respectively. The null and alternative hypothesis was the same as those tested by binomial analysis. Superior and inferior values were calculated using Excel BETAINV function in order to determine significantly over- and under-utilized species with 95% confidence. Families were then ranked for over- and under-utilization by the highest inferior and lowest superior values respectively.

2.2.5 Division of North American Flora

To apply a challenge to the statistical methods, the analyses were repeated following subdivision of the dataset. The North American flora was divided into small, medium and large sub-groups in accordance with parameters established by Moerman and Estabrook (2003) and reanalyzed using each of the 4 methods.

2.3 Results

2.3.1 Summary of Ethnobotanical Data

Overall, 86 out of the 264 families (32%) were used for spirituality or ceremony and consisted of 517 species belonging to 292 genera. Of these 86 families, 68 (82.5%) were utilized as a ceremonial medicine and included 300 species and 209 genera, while 63 (73%) were utilized as a ceremonial item and consisted of 272 species and 164 genera. Small, medium and large families consisted of less than 92 species, between 92 species and 392 species, and greater then 392 species respectively. In total, 97 indigenous groups were identified using plants for spirituality or ceremony. The Diné (Navajo), Ramah, and Hopi were identified in this database using the most species for spirituality/ceremony (>10% of identified spiritual flora) (Table 1), while the Pinacea, Asteraceae, Rosaceae, Poaceae and

Cupressaceae were the most widely used plant families used for spiritual and ceremonial purposes by Native American/First Nations peoples (>30% of total tribes/nations identified) (Table 2.1).

2.3.2 Over and Under-Utilization of North American Flora

Residual analysis was performed on spiritual/ceremonial, medicinal and ceremonial item categories. R^2 values were 0.8457, 0.8109, and 0.7111 and suggested a linear relationship between family size and species used for spirituality/ceremony (Figures 2.1A-C). The Pinaceae family was ranked the highest-use family in both ceremonial/spiritual and ceremonial item categories, while the Asteraceae ranked 1st in the ceremonial medicine category. The large families Asteraceae, Apiaceae and Ranunculaceae were identified among the 10 most utilized families for spiritual ceremonial and ceremonial medicine uses (Table 2.2). The Rosaceae was not found in the top 10 families in the categories spiritual /ceremonial or ceremonial medicine but ranked 2nd in the ceremonial items category (Table 2.2). The Fabaceae and Poaceae were ranked 1st and 2nd respectively as under-utilized ceremonial medicines and were among the 10 most over-utilized families in the ceremonial items categories (Table 2.2). To determine whether the residual analysis accurately identified families that were oversubscribed, binomial, bayesian and IDM analyses were compared. Like the residual analysis, the binomial analysis ranked the Pinaceae 1st ($p<0.05$) in both spiritual/ceremonial and items categories, but also ranked 1st in the ceremonial medicines category. The Cupressaceae was ranked higher by the binomial analysis than the residual analysis and was ranked 3rd in the ceremonial item category, as compared to a 5th place ranking by the residual analysis. The Apiaceae and Rosaceae were the only large families identified as over-utilized by binomial analysis, ranking 8th and 7th in the ceremonial medicine and ceremonial items categories respectively (Table 2.3). The Boraginaceae was placed in the top 3 for all under-utilized categories, while the Caryophyllaceae placed in the top 4 (Table 2.3). The Hydrophyllaceae placed in the top 4 for under-utilized spiritual/ceremonial and medicinal categories, and then moved to 8th in ceremonial items rankings (Table 2.2). The large families Asteraceae, Apiaceae and Ranunculaceae were identified among the 10 most utilized families for spiritual ceremonial and ceremonial medicine uses (Table 2.2). The Rosaceae was not found in the top 10 families in the categories spiritual/ceremonial or ceremonial medicine but ranked 2nd in the ceremonial

items category (Table 2.2). The Fabaceae and Poaceae were ranked 1st and 2nd respectively as under-utilized ceremonial medicines and were among the 10 most over-utilized ceremonial items (Table 2.2).

Table 2.1 Most commonly cited spiritual and ceremonial plants used by Tribe/Nation and plant family.

Top 10 Nations/Tribes Utilizing Spiritual Plants			
Rank	**Nation/Tribe**	**Number of Ceremonial or Spiritual Species Used**	**Percent of Total Spiritual or Ceremonial Species Used**
1	Diné	229	44.4
2	Ramah	146	28.3
3	Hopi	67	13.0
4	Kayenta	39	7.6
5	Cheyenne	28	5.4
6	Zuni	26	5.0
7	Pomo	24	4.7
8	Iroquois	23	4.5
9	Ojibwa	21	4.1
9	Kashaya	21	4.1
10	Cherokee	19	3.7
10	Blackfoot	19	3.7
Top 10 Plant Families Utilized by First Nation/Native American Peoples			
Rank	**Family**	**Number of Tribes/Nations Using Ceremonial or Spiritual Plants**	**Percent of Total Tribes/Nations Using Ceremonial or Spiritual Plants**
1	Pinaceae	32	33.0
1	Asteraceae	32	33.0
2	Rosaceae	30	30.9
2	Poaceae	30	30.9
3	Cupressaceae	29	29.9
4	Apiaceae	18	18.6
5	Solanaceae	16	16.5
6	Ericaceae	15	15.5
7	Liliaceae	14	14.4
7	Scrophulariaceae	14	14.4
7	Fabaceae	14	14.4
8	Cucurbitaceae	13	13.4
8	Salicaceae	13	13.4
8	Ranunculaceae	13	13.4
9	Typhaceae	12	12.4
10	Agavaceae	11	11.3

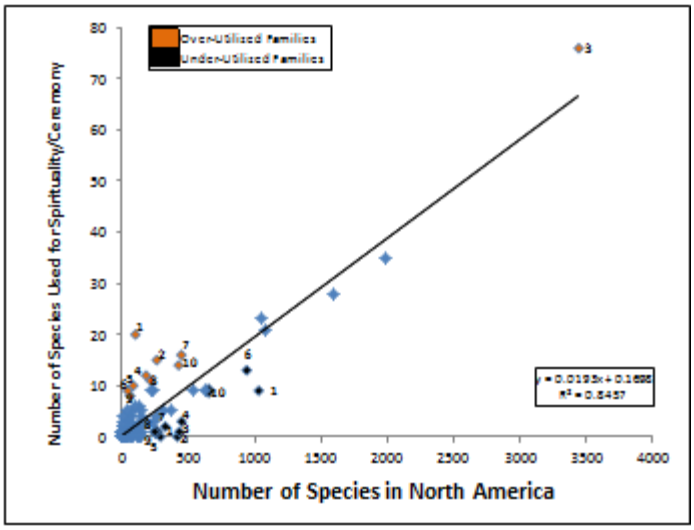


Figure 2.1A Residual analysis of families with reported uses in spirituality or ceremony. Numbers refer to the families identified in Table 2.2.

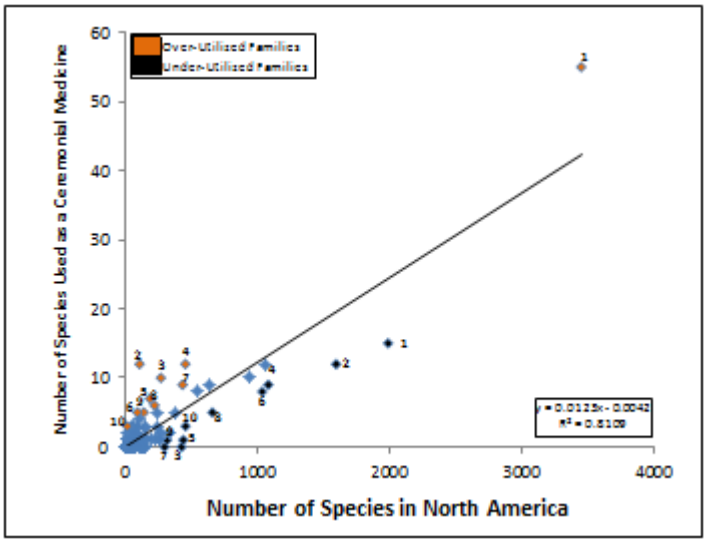


Figure 2.1B Residual analysis of families with reported uses as ceremonial medicines. Numbers refer to the families identified in Table 2.2. Spiritual/Ceremonial data were exported and sorted into two categories: (1) species identified as 'ceremonial medicines' (incense, smudges, scrubs, smoking tobacco, snuffs, teas, topical rubs, washes) or (2) species identified being used as 'ceremonial items' (ceremonial masks, charms, paints (including ash), prayer sticks, rattles, statues, symbolic items, tools).

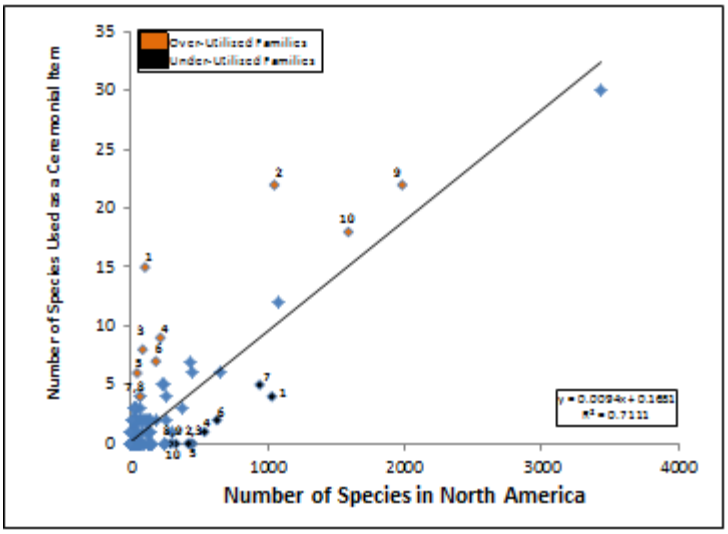


Figure 2.1C Residual analysis of families with reported uses as ceremonial items. Numbers refer to the families identified in Table 2.2. Spiritual/Ceremonial data were exported and sorted into two categories: (1) species identified as 'ceremonial medicines' (incense, smudges, scrubs, smoking tobacco, snuffs, teas, topical rubs, washes) or (2) species identified being used as 'ceremonial items' (ceremonial masks, charms, paints (including ash), prayer sticks, rattles, statues, symbolic items, tools).

To determine whether the residual analysis accurately identified families that were oversubscribed, binomial, bayesian and IDM analyses were compared. Like the residual analysis, the binomial analysis ranked the Pinaceae 1st ($p<0.05$) in both spiritual/ceremonial and ceremonial items categories, but also ranked 1st in the ceremonial medicines category. The Cupressaceae was ranked higher by the binomial analysis than the residual analysis and was ranked 3rd in the ceremonial item category, as compared to a 5th place ranking by the residual analysis. The Apiaceae and Rosaceae were the only large families identified as over-utilized by binomial analysis, ranking 8th and 7th in the ceremonial medicine and ceremonial items categories respectively (Table 2.3). The Boraginaceae placed in the top 3 for all under-utilized categories, while the Caryophyllaceae placed in the top 4 (Table 2.3). The Hydrophyllaceae placed in the top 4 for under-utilized spiritual/ceremonial and medicinal categories, and then dropped to 8th in ceremonial items rankings (Table 2.2).

Table 2.2 Residual analysis of species used in spirituality and ceremony, ceremonial medicine and to make ceremonial items.

Over-Utilized Families											
Ceremony/Spirituality				Ceremonial Medicine				Ceremonial Item			
Rank	Family	Residual	Size	Rank	Family	Residual	Size	Rank	Family	Residual	Size
1	Pinaceae	17.8616	M	1	Asteraceae	12.6676	L	1	Pinaceae	13.8761	M
2	Ericaceae	9.7736	M	2	Pinaceae	10.7496	M	2	Rosaceae	11.9837	L
3	Asteraceae	9.3996	L	3	Ericaceae	6.7816	M	3	Agavaceae	7.0359	S
4	Salicaceae	8.3369	M	4	Apiaceae	6.4938	L	4	Solanaceae	6.8233	M
5	Agavaceae	8.1897	S	5	Salicaceae	4.7779	M	5	Cupressaceae	5.4213	S
6	Cupressaceae	7.9810	S	6	Agavaceae	3.9587	S	6	Salicaceae	5.1335	M
7	Apiaceae	7.1838	L	7	Ranunculaceae	3.7521	L	7	Cucurbitaceae	4.3890	S
8	Solanaceae	6.7000	M	8	Solanaceae	3.3720	M	8	Betulaceae	3.2239	S
9	Cucurbitaceae	6.6336	S	9	Asclepiadaceae	3.3683	M	9	Fabaceae	3.2041	L
10	Ranunculaceae	5.5891	L	10	Elaeagnaceae	2.8935	S	10	Poaceae	2.8983	L
Under-Utilized Families											
Ceremony/ Spirituality				Ceremonial Medicine				Ceremonial Item			
Rank	Family	Residual	Size	Rank	Family	Residual	Size	Rank	Family	Residual	Size
1	Cyperaceae	-11.0488	L	1	Fabaceae	-9.3744	L	1	Cyperaceae	-5.8471	L
2	Boraginaceae	-8.2565	L	2	Poaceae	-7.5405	L	2	Polemoniaceae	-4.3951	L
3	Caryophyllaceae	-7.5074	L	3	Boraginaceae	-5.1495	L	3	Caryophyllaceae	-4.2259	L
4	Polemoniaceae	-5.8548	L	4	Cyperaceae	-4.6648	L	4	Lamiaceae	-4.2129	L
5	Hydrophyllaceae	-5.7668	M	5	Caryophyllaceae	-4.3094	L	5	Boraginaceae	-4.1037	L
6	Brassicaceae	-5.3118	L	6	Scrophulariaceae	-4.2183	L	6	Polygonaceae	-4.0777	L
7	Euphorbiaceae	-4.4809	M	7	Hydrophyllaceae	-3.5628	M	7	Brassicaceae	-4.0011	L
8	Orchidaceae	-4.0913	M	8	Liliaceae	-3.0154	L	8	Euphorbiaceae	-3.2389	M
9	Saxifragaceae	-3.9562	M	9	Cactaceae	-2.7965	M	9	Hydrophyllaceae	-2.8911	M
10	Liliaceae	-3.7534	L	10	Polemoniaceae	-2.5308	L	10	Orchidaceae	-2.5621	M

Table 2.3 Binomial analysis of species used in spirituality and ceremony, ceremonial medicine and to make ceremonial items.

Over-Utilized Families											
Spiritual/Ceremonial				Ceremonial Medicine				Ceremonial Items			
Rank	Family	P-Value	Size	Rank	Family	P-Value	Size	Rank	Family	P-Value	Size
1	Pinaceae	5.08E-14	M	1	Pinaceae	5.7E-09	M	1	Pinaceae	7E-13	M
2	Cupressaceae	3.08E-07	S	2	Elaeagnaceae	0.000147	S	2	Agavaceae	5.3E-06	S
3	Agavaceae	1.32E-05	S	3	Ericaceae	0.001687	M	3	Cupressaceae	9.34E-06	S
4	Elaeagnaceae	2.32E-05	S	4	Agavaceae	0.00406	S	4	Typhaceae	0.000732	S
5	Cucurbitaceae	4.9E-05	S	5	Balsaminaceae	0.006353	S	5	Solanaceae	0.000746	M
6	Salicaceae	0.000531	M	6	Salicaceae	0.007439	M	6	Arecaceae	0.003304	S
7	Ericaceae	0.000548	M	7	Vitaceae	0.009768	S	7	Rosaceae	0.004215	L
8	Vitaceae	0.000925	S	8	Apiaceae	0.010514	L	8	Pedaliaceae	0.004235	S
9	Typhaceae	0.002611	S	9	Lauraceae	0.018139	S	9	Salicaceae	0.004443	M
10	Cornaceae	0.003538	S	10	Anacardiaceae	0.021234	S	10	Cornaceae	0.004473	S
Under-Utilized Families											
Spiritual/Ceremonial Families				Ceremonial Medicine				Ceremonial Items			
Rank	Family	P-Value	Size	Rank	Family	P-Value	Size	Rank	Family	P-Value	Size
1	Boraginaceae	0.000128	L	1	Boraginaceae	0.00565	L	1	Polemoniaceae	0.006491	L
2	Caryophyllaceae	0.001005	L	2	Hydrophyllaceae	0.027806	M	2	Caryophyllaceae	0.00794	L
3	Cyperaceae	0.001571	L	3	Fabaceae	0.029093	L	3	Boraginaceae	0.009184	L
4	Hydrophyllaceae	0.002026	M	4	Caryophyllaceae	0.03065	L	4	Cyperaceae	0.010692	L
5	Polemoniaceae	0.013914	L	5	Poaceae	0.047579	L	5	Lamiaceae	0.017269	L
6	Orchidaceae	0.027887	M					6	Euphorbiaceae	0.025721	M
7	Euphorbiaceae	0.030279	M					7	Polygonaceae	0.028977	L
8	Saxifragaceae	0.031638	M					8	Hydrophyllaceae	0.03892	M
9	Crassulaceae	0.040446	M					9	Brassicaceae	0.050547	L

Bayesian analysis largely identified smaller families as over-utilized and larger families as under-utilized at p-values < 0.05 (Table 2.4). The Elaeagnaceae was ranked 1st in over-utilized spiritual/ceremonial and ceremonial medicine categories and was not identified as a high ranking ceremonial item (Table 2.4). The Fouquieriaceae was ranked 2nd for spiritual/ceremonial uses and 1st for spiritual item uses (Table 2.4). The Pinaceae was the only medium sized family identified as over-utilized, placing in the top 4 for all 3 categories (Table 2.4). IDM analysis varied most from the other analyses. In general, changing the parameters from S1 to S4 changed the outcome of the analysis (Table 2.5). Similar to binomial, IDM analysis identified mostly small and medium sized families (Table 2.5). The Apiaceae was the only large family identified as over-utilized placing 8th and 9th in the ceremonial medicines category (Table 2.5). Similar to bayesian analysis, the Elaegnaceae was identified in the top 2 for over-utilized ceremonial/spiritual and ceremonial medicine categories (Table 2.5). The Pinaceae was also identified in the top 3 for all 3 categories (Table 2.5)

Overall, all 4 methods identified only medium and large families as underutilized. The Boraginaceae, Caryophyllaceae, Hydrophyllaceae, Cyperaceae, and Polemoniaceae were consistently identified in the under-utilized group of families, under the category spirituality and ceremony (Table 2.6). The Boraginaceae, Fabaceae, Poaceae, Caryophyllaceae, Hydrophyllaceae and Cyperaceae were identified as under-utilized ceremonial medicines, while the Boraginaceae, Caryophyllaceae, Cyperaceae and Polemoniaceae were identified as under-utilized for applications in creating ceremonial items (Table 2.6). There was a lack of agreement among all 4 methods with respect to over-utilization when the whole dataset was analyzed but partitioning the dataset into small, medium and large subsets improved the agreement for large and medium sized families (Table 2.7). The Apiaceae, Asteraceae, Ranunculaceae, Pinaceae, Salicaceae, Ericaceae and Solanaceae were consistently identified as over-utilized in the category spirituality and ceremony. The Apiaceae, Asteraceae, Pinaceae, Ericaceae, and Salicaceae were consistently identified as over-utilized in the category ceremonial medicine and the Rosaceae, Pinaceae, Solanaceae, and Salicaceae were identified as over-utilized as ceremonial items (Table 2.7)

Table 2.4 Bayesian analysis of families containing species used in spirituality and ceremony, ceremonial medicine and to make ceremonial items.

Over-Utilized Families														
Spiritual/Ceremonial					Ceremonial Medicine					Ceremonial Item				
Rank	Family	Inf.	Sup.	Size	Rank	Family	Inf.	Sup.	Size	Rank	Family	Inf.	Sup.	Size
1	Elaeagnaceae	0.187086	0.737622	S	1	Elaeagnaceae	0.121552	0.652453	S	1	Fouquieriaceae	0.158114	0.987421	S
2	Fouquieriaceae	0.158114	0.987421	S	2	Staphyleaceae	0.094299	0.905701	S	1	Theophrastaceae	0.158114	0.987421	S
2	Theophrastaceae	0.158114	0.987421	S	3	Pinaceae	0.068948	0.194669	M	2	Typhaceae	0.146633	0.853367	S
3	Typhaceae	0.146633	0.853367	S	4	Balsaminaceae	0.060218	0.517756	S	3	Nelumbonaceae	0.094299	0.905701	S
4	Pinaceae	0.130861	0.283832	M	5	Typhaceae	0.052745	0.716418	S	3	Staphyleaceae	0.094299	0.905701	S
5	Cupressaceae	0.112046	0.345958	S	6	Hamamelidaceae	0.043272	0.641235	S	4	Pinaceae	0.091477	0.228798	S
6	Nelumbonaceae	0.094299	0.905701	S	7	Lauraceae	0.035785	0.34712	S	5	Pedaliaceae	0.06674	0.556095	M
7	Staphyleaceae	0.094299	0.905701	S	8	Araliaceae	0.032071	0.316983	S	6	Cupressaceae	0.064909	0.267925	S
8	Cucurbitaceae	0.067463	0.234966	S	9	Vitaceae	0.030252	0.219096	S	7	Agavaceae	0.048982	0.175089	S
9	Pedaliaceae	0.06674	0.556095	S	10	Agavaceae	0.026032	0.130484	S	8	Arecaceae	0.040336	0.282264	S
10	Agavaceae	0.065616	0.203462	S						9	Cornaceae	0.036302	0.257539	S
										10	Lauraceae	0.035785	0.34712	S
	N.A Population	**0.019429**	**0.02304**			**N.A Population**	**0.010973**	**0.013738**			**N.A Population**	**0.009892**	**0.012527**	
Under-Utilized Families														
Spiritual/Ceremonial					Ceremonial Medicine					Ceremonial Item				
Rank	Family	Inf.	Sup.	Size	Rank	Family	Inf.	Sup.	Size	Rank	Family	Inf.	Sup.	Size
1	Boraginaceae	6.03E-05	0.008745	L	1	Boraginaceae	6.03E-05	0.008745	L	1	Polemoniaceae	5.61E-05	0.008146	L
2	Hydrophyllaceae	8.7E-05	0.012597	M						2	Caryophyllaceae	5.85E-05	0.008483	L
3	Caryophyllaceae	0.00056	0.0128	L						3	Boraginaceae	6.03E-05	0.008745	L
4	Cyperaceae	0.004661	0.016506	L										
5	Polemoniaceae	0.002422	0.019316	L										
	N.A Population	**0.019429**	**0.02304**			**N.A Population**	**0.010973**	**0.013738**			**N.A Population**	**0.009892**	**0.012527**	

Table 2.5 IDM analysis of families containing species used in spirituality and ceremony, ceremonial medicine and to make ceremonial items.

Over-Utilized Ceremonial/Spiritual Species						Over-Utilized Ceremonial Medicine						Over-Utilized Ceremonial Item					
	S1	S2	S3	S4	Size		S1	S2	S3	S4	Size		S1	S2	S3	S4	Size
Agavaceae	1	4	4	4	S	Elaeagnaceae	1	1	2	2	S	Pinaceae	1	1	1	1	M
Elaeagnaceae	2	2	2	2	S	Pinaceae	2	2	1	1	M	Typhaceae	2	2	3	4	S
Pinaceae	3	1	1	1	M	Balsaminaceae	3	3	3	3	S	Cupressaceae	3	3	2	2	S
Cupressaceae	4	3	3	3	S	Agavaceae	4	4	4	4	S	Agavaceae	4	4	4	3	S
Typhaceae	5	6	7	7	S	Ericaceae	5	5	5	5	M	Pedaliaceae	5	5	5	6	S
Cucurbitaceae	6	5	5	5	S	Vitaceae	6	6	6	6	S	Fouquieriaceae	6	17	17	25	S
Vitaceae	7	7	6	6	S	Salicaceae	7	7	7	7	M	Theophrastaceae	6	17	17	25	S
Lauraceae	8	9	10	10	S	Lauraceae	8	9	9	9	S	Arecaceae	7	6	6	5	S
Cornaceae	9	8	8	9	S	Apiaceae	9	8	8	8	L	Cornaceae	8	7	7	7	S
Salicaceae	10	10	9	8	M	Anacardiaceae	10	10	11	10	S	Solanaceae	9	8	8	8	M
												Cucurbitaceae	11	10	10	10	M
												Aceraceae	10	9	9	9	S
Under-Utilized Ceremonial/Spiritual Species						**Under-Utilized Ceremonial Medicine**						**Under-Utilized Ceremonial Item**					
	S1	S2	S3	S4	Size		S1	S2	S3	S4	Size		S1	S2	S3	S4	Size
Boraginaceae	1	1	1	2	L	Boraginaceae	1	2	3	5	L	Polemoniaceae	1	2	4	7	L
Hydrophyllaceae	2	4	4	9	M	Fabaceae	2	1	1	1	L	Caryophyllaceae	2	4	7	8	L
Caryophyllaceae	3	2	3	3	L	Hydrophyllaceae	3	7	11	12	M	Boraginaceae	3	5	8	10	L
Cyperaceae	4	3	2	1	L	Caryophyllaceae	4	5	6	7	L	Cyperaceae	4	1	1	2	L
Polemoniaceae	5	5	6	8	L	Poaceae	5	3	2	2	L	Lamiaceae	5	6	6	6	L
Orchidaceae	6	11	14	16	M	Cyperaceae	6	4	4	3	L	Euphorbiaceae	6	9	12	13	M
Euphorbiaceae	7	9	11	13	M	Scrophulariaceae	7	6	5	4	L	Polygonaceae	7	8	5	4	L
Saxifragaceae	8	13	15	17	M	Liliaceae	8	8	8	10	L	Brassicaceae	8	7	3	3	L
Brassicaceae	9	6	7	5	L	cactaceae	9	13	13	13	M	Asteraceae	9	3	2	1	L
Crassulaceae	10	19	20	22	M	Polemoniaceae	10	12	12	11	L	Hydrophyllaceae	10	11	14	14	M
Fabaceae	11	7	5	4	L	Brassicaceae	11	9	9	9	L	Fabaceae	14	10	9	5	L
Poaceae	12	8	8	6	L	Euphorbiaceae	15	14	14	15	M	Poaceae	15	11	10	9	L
Liliaceae	13	12	10	10	L	Asteraceae	13	10	7	6	L						
Asteraceae	17	10	9	7	L	Rosaceae	12	11	10	8	L						

Not Statistically Significant P > 0.05

Table 2.6 Comparison of residual, bayesian and binomial analysis for under-utilized families.

Under-Utilized Ceremonial/Spiritual				
Family	**Residual Rank**	**Binomial Rank**	**Baysian Rank**	**Average**
Boraginaceae	2	1	1	1.3
Cyperaceae	1	3	4	2.7
Caryophyllaceae	3	2	3	2.7
Hydrophyllaceae	5	4	2	3.7
Polemoniaceae	4	5	5	4.7
Orchidaceae	8	6	6	6.7
Euphorbiaceae	7	7	7	7.0
Saxifragaceae	9	8	8	8.3
Brassicaceae	6	12	9	9.0
Crassulaceae	13	9	10	10.7
Liliaceae	10	14	13	12.3
Gentianaceae	15	10	14	13.0
Under-Utilized Ceremonial Medicine				
Family	**Residual Rank**	**Binomial Rank**	**Baysian Rank**	**Average**
Boraginaceae	3	1	1	1.7
Fabaceae	1	3	2	2.0
Poaceae	2	5	5	4.0
Hydrophyllaceae	7	2	3	4.0
Caryophyllaceae	5	4	4	4.3
Cyperaceae	4	7	6	5.7
Scrophulariaceae	6	8	7	7.0
Cactaceae	9	6	9	8.0
Liliaceae	8	13	8	9.7
Polemoniaceae	10	16	10	12.0
Crassulaceae	14	9	17	13.3
Convolvulaceae	24	10	19	17.7
Under-Utilized Ceremonial Item				
Family	**Residual Rank**	**Binomial Rank**	**Baysian Rank**	**Average**
Polemoniaceae	2	1	1	1.3
Caryophyllaceae	3	2	2	2.3
Cyperaceae	1	4	4	3.0
Boraginaceae	5	3	3	3.7
Lamiaceae	4	5	5	4.7
Euphorbiaceae	8	6	6	6.7
Polygonaceae	6	7	7	6.7
Brassicaceae	7	9	8	8.0
Hydrophyllaceae	9	8	10	9.0
Orchidaceae	10	10	11	10.3
Asteraceae	11	13	9	11.0

Statistically Significant P-Value <0.05

Table 2.7 Comparison of residual, bayesian and binomial analysis for over-utilized families (small, medium, large).

Over-Utilized Spirituality and Ceremony						Over-Utilized Ceremonial Medicine						Over-Utilized Ceremonial Item					
Large						**Large**						**Large**					
Family	**Residual**	**Binomial**	**Baysian**	**IDM-S2**	**Average**	**Family**	**Residual**	**Binomial**	**Baysian**	**IDM-S2**	**Average**	**Family**	**Residual**	**Binomial**	**Baysian**	**IDM-S2**	**Average**
Apiaceae	2	1	1	1	1.3	Apiaceae	2	1	1	1	1.3	Rosaceae	1	1	1	1	1.0
Ranunculaceae	3	2	2	2	2.3	Asteraceae	1	2	2	2	1.8	Fabaceae	2	3	3	2	2.5
Asteraceae	1	3	3	3	2.5	Ranunculaceae	3	3	3	3	3.0	Poaceae	3	4	4	3	3.5
Rosaceae	4	4	4	4	4.0	Lamiaceae	4	4	4	5	4.3	Ranunculaceae	4	2	2	4	3.0
Scrophulariaceae	5	5	5	6	5.3	Polygonaceae	5	5	5	4	4.8	Scrophulariaceae	5	5	5	6	5.3
Fabaceae	9	6	6	5	6.5	Rosaceae	6	6	6	6	6.0	Apiaceae	6	6	6	7	6.3
Poaceae	7	7	7	7	7.0	Brassicaceae	7	7	7	7	7.0	Liliaceae	7	7	6	8	7.0
Lamiaceae	6	8	8	8	7.5	Scrophulariaceae	10	8	9	10	9.3	Asteraceae	8	8	7	5	7.0
Polygonaceae	8	9	10	10	9.3	Liliaceae	9	9	12	12	10.5	Brassicaceae	9	9	9	9	9.0
Brassicaceae	11	11	9	9	10.0	Polemoniaceae	8	10	13	13	11.0	Polygonaceae	10	10	11	11	10.5
Liliaceae	10	10	11	11	10.5	Fabaceae	15	13	8	8	11.0	Cyperaceae	15	11	10	10	11.5
						Poaceae	14	12	10	9	11.3						
Medium						**Medium**						**Medium**					
Family	**Residual**	**Binomial**	**Baysian**	**IDM-S2**	**Average**	**Family**	**Residual**	**Binomial**	**Baysian**	**IDM-S2**	**Average**	**Family**	**Residual**	**Binomial**	**Baysian**	**IDM-S2**	**Average**
Pinaceae	1	1	1	1	1.0	Pinaceae	1	1	1	1	1.0	Pinaceae	1	1	1	1	1.0
Salicaceae	3	2	2	2	2.3	Ericaceae	2	2	2	2	2.0	Solanaceae	2	2	2	2	2.0
Ericaceae	2	3	3	3	2.8	Salicaceae	3	3	3	3	3.0	Salicaceae	3	3	3	3	3.0
Solanaceae	4	4	4	4	4.0	Solanaceae	4	6	6	5	5.3	Fagaceae	4	4	4	4	4.0
Caprifoliaceae	7	5	5	5	5.5	Asclepiadaceae	5	4	4	4	4.3	Chenopodiaceae	5	5	5	5	5.0
Fagaceae	5	6	6	6	5.8	Caprifoliaceae	6	5	5	6	5.5	Ericaceae	6	7	6	6	6.3
Chenopodiaceae	6	8	8	7	7.3	Chenopodiaceae	7	7	7	7	7.0	Caprifoliaceae	7	6	7	7	6.8
Asclepiadaceae	8	7	7	8	7.5	Verbenaceae	8	8	8	8	8.0	Dryopteridaceae	8	8	8	8	8.0
Rhamnaceae	9	9	9	9	9.0	Rhamnaceae	9	9	9	10	9.3	Campanulaceae	9	9	9	9	9.0
Campanulaceae	10	10	10	10	10.0	Onagraceae	10	10	10	9	9.8	Rhamnaceae	10	10	10	10	10.0
Small						**Small**						**Small**					
Family	**Residual**	**Binomial**	**Baysian**	**IDM-S2**	**Average**	**Family**	**Residual**	**Binomial**	**Baysian**	**IDM-S2**	**Average**	**Family**	**Residual**	**Binomial**	**Baysian**	**IDM-S2**	**Average**
Cupressaceae	2	1	4	2	2.3	Elaeagnaceae	2	1	1	1	1.3	Cupressaceae	2	1	5	2	2.5
Elaeagnaceae	5	2	1	1	2.3	Balsaminaceae	7	2	3	2	3.5	Agavaceae	1	2	6	3	3.0
Agavaceae	1	3	8	3	3.8	Agavaceae	1	3	9	3	4.0	Typhaceae	10	3	2	1	4.0
Cucurbitaceae	3	4	6	4	4.3	Vitaceae	3	4	8	4	4.8	Arecaceae	5	5	7	5	5.5
Vitaceae	4	6	10	6	6.5	Lauraceae	8	5	6	5	6.0	Pedaliaceae	11	4	4	4	5.8
Typhaceae	17	5	3	5	7.5	Anacardiaceae	4	8	11	6	7.3	Cornaceae	6	7	8	6	6.8
Cornaceae	6	7	12	7	8.0	Araliaceae	9	7	7	7	7.5	Aceraceae	7	8	10	7	8.0
Lauraceae	10	8	9	8	8.8	Staphyleaceae	17	6	2	10	8.8	Cucurbitaceae	3	10	14	8	8.8
Pedaliaceae	18	10	7	9	11.0	Apocynaceae	5	9	14	8	9.0	Vitaceae	8	9	12	9	9.5
Anacardiaceae	8	16	15	10	12.3	Araceae	10	11	12	9	10.5	Fouquieriaceae	20	6	1	12	9.8
Fouquieriaceae	27	9	2	20	14.5	Typhaceae	18	10	4	16	12.0	Theophrastaceae	20	6	1	12	9.8
Theophrastaceae	27	9	2	20	14.5	Hamamelidaceae	19	12	5	17	13.3	Betulaceae	4	12	16	10	10.5
Amaranthaceae	7	23	25	14	17.3	Amaranthaceae	6	18	21		15.0	Nelumbonaceae	21	11	3	15	12.5
Betulaceae	9	21	24	17	17.8	Myricaceae	20	17	10	20	16.8	Staphyleaceae	21	11	3	15	12.5
Nelumbonaceae	28	13	5	26	18.0							Oleaceae	9	17	19	14	14.8
Staphyleaceae	28	13	5	26	18.0												

Statistically Significant P-Value <0.05

2.3.3 Genera Analysis for the Asteraceae and Apiaceae

Large statistically significant families containing many species used as ceremonial medicines were selected for further assessment at the genus level. The USDA's PLANTS database indicates that the Asteraceae and Apiaceae consist of 482 and 92 genera and 3004 and 428 species respectively. For our analysis of the Asteraceae, the flora was separated into large and small genera using methods similar to Moerman and Estabrook (2003). The mean genus size + 2 standard deviations (1.5 + 2*9.0 = 20) was used to partition genera into small (< 20 species) or large (>20 species) groups. Of these, 55 species from the Asteraceae and 12 species from the Apiaceae were used medicinally in ceremony. Proportion of utilized genera in the Asteraceae was inversely proportional to genus size. Ranking of all 4 methods revealed that all identified *Artemisia* L. as the most significant over-utilized large genus within this family. Furthermore, bayesian and binomial methods agreed that smaller genera such as *Anaphalis* DC, *Petradoria* Greene, *Pericome* A. Gray, *Pyrrhopappus* DC, *Lactuca* L., *Tragopogon L*, and *Xanthium L.* were significantly over-utilized (Table 2.8). In the Apiaceae, the genus *Ligusticum* was observed to be the most significantly over-utilized genus as a ceremonial medicine by the residual and binomial analysis, and ranked 4th with bayesian analysis (Table 2.8). IDM did not identify statistically significant genera in the Apiaceae or among smaller genera within the Asteraceae (Table 2.8).

2.4 Discussion and Selection of Candidate Species

The application of residual, binomial, bayesian and IDM methods to determine under and over-utilization of flora has recently been debated (Moerman, 2012). This research was designed to compare the 4 different approaches and to identify families, genera and species that have a greater probability of discovery of neurologically active phyto-pharmacology. The results indicate that the methods both agreed and disagreed in significant ways and identified some common and several different families depending on the method of applying the data. Dividing the dataset into small, medium and large families significantly altered the outcome. Applying different significance factors also changed which families were identified by the IDM analysis. Similar to previous conclusions, it appeared that different methods resulted in different conclusions and multiple approaches may be required to effectively determine the rates of significance in ethnobotanical data (Leonti et al., 2012; Heinrich and Verpoorte, 2012).

Table 2.8 Summary of significantly over-utilized genera within the Asteraceae and Apiaceae (using ranks).

Over-Utilized Genera as Ceremonial Medicines					
Genera	**Residual**	**Bayesian**	**Binomial**	**IDM-S2**	**Average**
Asteraceae - Large					
Artemisia L.	*1*	*1*	*1*	*1*	*1.0*
Ambrosia L.	2	2	2	2	2.0
Ericameria Nutt.	3	3	3	3	3.0
Brickellia Elliot	4	4	4	5	4.0
Asteraceae - Small					
Anaphalis DC.	3	1	2	3	2.0
Pericome A. Gray	3	1	2	3	2.0
Petradoria Greene	3	1	2	3	2.0
Tragopogon L.	1	3	1	1	2.0
Psathyrotes A. Gray	4	2	4	4	3.0
Pyrrhopappus DC.	4	2	4	4	3.0
Xanthium L.	4	2	4	4	3.0
Lactuca L.	2	5	3	2	3.5
Apiaceae					
Pseudocymopterus J.M. Coult & Rose	2	1	2	2	1.5
Ligusticum L.	1	4	1	1	2.5
Heracleum L.	3	2	3	4	2.5
Cicuta L.	4	3	4	5	3.5
Oreoxis Raf.	4	3	4	5	3.5

Statistically Significant P-Value <0.05

Ethnopharmacological analysis identified the Apiaceae, Asteraceae, Ericaceae, Pinaceae, and Salicaceae as potential families for future phytochemical investigations for the discovery of plant neurotransmitters and human neuroregulators. In the Apiaceae, *Ligusticum* L. was identified as the most over-utilized genus. There are 12 species of *Ligusticum* in North America and ceremonial uses of the plant have been reviewed previously (Turi and Murch, 2011). Some examples of the uses of *Ligusticum* as a ceremonial medicine include: (a) use of infusions of *L. porteri* J.M. Coult. & Rose during ritual curing ceremonies to protect individuals against witches and rattlesnakes, (b) healers chewing on *Ligusticum* roots during healing rituals (Camazine and Bye, 1980), (c) roots burned to console individuals

during mourning (Jordan, 2008), and (d) roots smoked to release individuals from trance or possession (Turner et al., 1980). Recently, we reported the presence of 5HT and MEL in roots and shoots of *L. canbyi* and *L. porteri* (Turi and Murch, 2013a). In the Asteraceae, 55 species and 38 genera were identified as having greater than expected use for ceremonial medicines. The genus *Artemisia* and its members *Artemisia campestris* L., *Artemisia douglasiana* (Besser) W.J. Hooker, *Artemisia frigida* Willd., *Artemisia ludoviciana* Nutt., *A. tridentata*, *A. tripartita* and *Artemisia vulgaris L.* were identified as having greater than expected rates of use in ceremonies and rituals as medicines. There are 50 species (native and introduced) in the genus *Artemisia* in North America (Shultz, 2006). Some examples of the use of *Artemisia* as ceremonial medicines include: (a) the use of *A. douglasiana* or *A. vulgaris* by the Miwok to bring mental clarity during times of mourning, (b) chewing leaves of *A. frigida* during ceremony, (c) the use of *A. tridentata* by Paiutes dancers to become spiritually clean during curing ceremonies and (d) rubbing charcoal made from *A. tripartita* to blacken legs and forearms of patients during the Mountain Chant Ceremony (Moerman, 2009).

One of the challenges of analysis of ethnobotanical datasets is the diversity of responses and the overlap of uses reported in various observations. Medicinal species and species used in rituals or ceremonies have been observed previously within many cultures around the world (de Albuquerque et al., 2007; Kawa, 2012; Morris, 1986; Viladrich, 2006). Native American ceremonial practice is an important component of the maintenance of balanced connections between mind, body and spirit (Portman and Garrett, 2006; Garrett et al., 2011). It is possible that perceived therapeutic effects which render a plant "powerful" or "protective" are purposely sought after in order to maintain this balance. Turner (2009) noted widespread use of fragrant species for spirituality and ceremony by First Nations peoples living within Northwest regions of North America. It is difficult to fully separate the ritual, spiritual or ceremonial use of plants from their sensory or medicinal character and these may overlap (Turner, 2009). Further research is required to determine whether the selection of aromatic plants and the perceived effects attributed to plant reflect phytochemical composition or may be attributed to other influences such as placebo effects (Moerman and Jonas, 2002).

Chapter 3 Metabolomics as a Hypothesis Generating Technique for Phthalide Biosynthesis in the Model Species *Ligusticum canbyi* Coult. & Rose

3.1 Synopsis

Ligusticum canbyi (J.M. Coult. & Rose) is a medicinal understory forest species used in traditional rituals and ceremonies for spiritual enlightenment and to improve mental health. In North America, several endemic species of *Ligusticum* including *L. canbyi* are collectively called Osha and sold in the natural health products industry to boost the immune system or as a respiratory aid. To date, very little is known about the phytochemical complexity of *L. canbyi*. Much of the existing literature suggests that the medicinal activity of *Ligusticum* species stems from phthalide derivatives such as Z-ligustilide and butylidenphthalide and simple phenolics such as ferulic acid. Phthalides are phenolic compounds which are most commonly found in members belonging to the Apiaceae (carrot Family). Although the exact origin of their biosynthesis is yet to be determined, it is believed that phthalide diversity stems from enzymatic modification to Z/E-ligustilide. The current study was undertaken to determine whether *Ligusticum* tissues and extracts contain specifically targeted biologically active phytochemicals such as: MEL, 5HT, Z-ligustilide, E-butylidenephthalide and ferulic acid and to investigate diversity of phthalides using an untargeted approach. The results of these studies identified MEL and 5HT in roots and shoots of *L. canbyi* and *L. porteri*. Z-ligustilide, E-butylidenephthalide and ferulic acid were quantified in roots and shoots of *L. canbyi*. The relative contribution of the known metabolites and the unknown markers to the antioxidant potential of root and shoot tissues were compared and it was determined that the majority of the antioxidant capacity could be attributed to ferulic acid in the tissues. Metabolomic analysis detected approximately 34,000 compounds in each *L. canbyi* extract and predictive analysis suggests the presence of more than 70 putative phthalide metabolites.

3.2 Research Objectives and Hypothesis

I hypothesized that phytochemical diversity and antioxidant potential significantly differs between tissue types, and that synthetic biotranformation can be used as a hypothesis generating technique to investigate phthalide diversity in *L. canbyi*. The current study was designed to address the following objectives; (1) to determine whether *Ligusticum* species

contain the known neurologically active metabolites such as MEL and 5HT; (2) to identify and quantify known *Ligusticum* metabolites ferulic acid, Z-ligustilide and E/Z-butylidenephthalide; (3) to investigate whether phthalides or indoleamines contribute to the reduction of physiological stresses caused by free radicals and (4) to use metabolomics as a hypothesis generating technique for putative identification of novel phthalide derivatives in *L. canbyi.*

3.4 Materials and Methods

3.4.1 Acquisition of Plant Materials

Mature plants of *L. canbyi* were collected from wild populations in southeastern British Columbia, Canada (N49 12'20 W117 53'36, N49 12'09, W117 53'28) in September 2010 and 2011. Voucher specimens were collected, deposited and identified at 2 herbaria (Beaty Biodiversity Centre, Vancouver BC; Accession V235342 and the Biodiversity Resource Center, National Tropical Botanical Garden, Kauai, Hawaii; in process). Intact plants were transplanted into ProMix (Premier Horticulture Ltd, QC) in 6 - 10 inch plastic pots in the greenhouse under ambient light and temperature. A subset of the wild harvested plants were incubated under the following conditions: (1) a controlled environment growth chamber (Conviron, Winnipeg MB) under full spectrum light (35 µmol/m^2/s; T5 Cool Daylight Agrobulb, 6400K, SunBlaster Lighting, Future Harvest Developments, Kelowna BC) with a 16 h light period at 30^0C. (2) Greenhouse conditions under halogen lights (333 lux, 400 watts) for a 10 h light period with automatic water drips set to 5 min/day. Wild-harvested root samples were washed on-site in order to remove all visible soil, transported on ice and stored at -10^0C until analysis.

A flat of seedling plugs (72 x 3 inch plantlets) of *L. porteri* plants (Horizon Herbs Inc., Williams OR) was imported into Canada as bare-root plantlets under appropriate permits and phytosanitary certification. Once inspected and cleared, plants were transplanted into 6 inch plastic pots containing ProMix commercial greenhouse soil and grown under identical conditions. Multiple attempts were made to establish *Ligusticum* collections in tissue culture but results were poor and induction of growth required high concentrations of the growth regulator 2,4 dichloroacetic acid (2-4D), which could alter endogenous phytochemistry (data not shown). Therefore, studies of *Ligusticum* were limited by access to plant material.

3.4.2 Quantification of MEL and 5HT

MEL and 5HT were identified and quantified in *Ligusticum* roots and shoots using previously reported methods (Murch et al., 2010). All samples were prepared in cool darkness in less than 7 min to prevent light and temperature degradation of targeted compounds. In brief, whole root and leaf samples (200 mg) were excised from greenhouse plants or frozen field collections, accurately weighed by difference in a darkened room with a single red light as the only light source and homogenized in 100 µl of methanol: water: formic acid (80:20:1 v/v). for 30 s (Kontes Pellet Pestle disposable tissue grinder, Fisher Scientific, Mississauga, ON). Samples were centrifuged (16,000 x g) for 3 min to settle particulate matter and the supernatant was filtered (0.2 µm, Ultrafree-MC filtered centrifuge tubes; Millipore, MS, USA) prior to chromatography.

MEL and 5HT were separated by ultra-performance liquid chromatography (Acquity UPLC, Waters Corp, Mississauga, ON) on a reverse phase chromatography column (Waters BEH C_{18} UPLC column, 2.1 X 150 mm, 1.7 l µm; Waters, MA, USA) and eluted with a gradient of 1% aqueous formic acid : acetonitrile (0.0 - 4.0 min, 95:5 - 5:95 v/v, 4.0-4.5 min, 95:5—95:5 v/v, 4.5-5.0 min, 95:5 v/v) at 0.25 ml/min and 30^0C over 5 min with a 2 min cleaning and re-equilibration period. Separated plant extracts were introduced into the Time of Flight mass spectrometer (ToF-MS; LCT Premier Micromass MS, Waters) by electrospray ionization (ESI) in the positive mode with m/z determination using the "W" configuration ion flight path using previously described voltages (Murch et al., 2010). MEL and 5HT were quantified by comparison to authenticated standards with purities greater than or equal to and 98% (Sigma) for retention time and mass accuracy. Methods were validated by 5 repeated injections of 10 standards as described in detail in the supplemental methods. LOD and LOQ were determined according to United States Pharmacopoeia standards. The linear range of the detection of MEL was 0.5 µg/ml to 50 µg/ml with a lower limit of quantification (lLOQ) of 0.7 µg/ml and an upper limit of quantification (uLOQ) of 50 µg/ml. Limits of quantification for 5HT were 1800 ng/mL. The lower limits of detection (LOD) were 44 ng/ml (MEL) and 475 ng/ml (5HT) as determined by repeated injections of standards in the sample matrix. Recovery was determined by repeated analysis of spiked samples at 3 concentrations (50 µg/ml, 6.25 µg/ml and 0.07 µg/ml). For all analysis, recovery of MEL was > 76.7% with an average recovery in the linear range of at least 86.9% for the analysis.

Similarly, repeated recovery measures found 5HT at > 69% with an average recovery of 91.2% overall.

3.4.3 Quantification of ferulic acid, Z-ligustilide, and E-butylidenephthalide

Ferulic acid, Z-ligustilide, and E-butylidenephthalide were extracted from plant tissues using previously published protocols with minor modifications (Yi et al., 2006). In brief, frozen root tissues were separated into 3 distinct layers: pith, cortex, and epidermis, while shoot material was separated into stem and leaf tissues (n = 5 replicates). Tissues were ground in liquid nitrogen, accurately weighed by difference, and an internal standard (α-naphthoflavone 40 µg/ml) was added. Samples were macerated (0.05g/ml in 70% EtOH) and extracted for 2 days at room temperature in the dark. Preliminary analysis was performed on each extract in order to determine the appropriate number of extractions per tissue type. For stem tissues, extracts were centrifuged, supernatants were decanted and filtered (UltraFree MC, Millipore, USA), for leaf and root tissues the precipitated pellet was extracted once and twice more respectively using the same procedure. Extracts were pooled and stored at -35^{0}C until chromatographic analysis.

A chromatographic method was optimized for separation of ferulic acid, Z-ligustilide (Chromadex), 3-butylidenephthalide (11.3% E-butylidenephthalide) and α-napthoflavone (Sigma) standards using a Nova-Pak C18 column (300 mm x 3.9 mm, 4 µm Waters, Mississauga, ON) with a flow of 1 ml/min and eluted with 0.1% formic acid and acetonitrile (A:B) delivered by a standardized HPLC system (Alliance 2695, Waters). The gradient was: 0 min, 85A/15B; 10 min, 80A/20B; 20min, 47A/53B; 30min, 32A/68B, 40min, 8A/92B; 50 min, 85A/15B; 55 min, 85A/15B. Compounds were detected by UV absorbance at 260 and 280 nm (Waters 2998 photodiode array detector). Linearity and range were 1-250 µg/ml for ferulic acid (R^2 = 0.98); 1-250 µg for Z-ligustilide (R^2 = 0.98), and 0.2 - 57 µg/ml for E-butylidenephthalide (R^2 = 0.98). Limits of quantification [LOQ] for the analysis were: ferulic acid [LOQ = 1 µg/mL]; Z-ligustilide [LOQ = 1 µg/mL]; E-butylidenephthalide [LOQ = 0.2 µg/mL].

3.4.4 Antioxidant Bioassay

Antioxidant potential of specific tissue types for *L. canbyi* wasdetermined using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) bioassay (Takahata et al., 2001). Specific tissue types

were removed from plant materials which previously underwent targeted phytochemistry (n=5). Tissues were extracted in methanol and diluted to construct a concentration curve. Extracts were randomly placed into a 96 well microplate alongside assay and extract blanks (n=3), and an assay control consisting of 1, 8, 16, 24, 32, 40, and 50 µM dilutions of Trolox ((±)-6-Hydroxy-2,5,7,8 tetramethylchromane-2-carboxylic acid; Sigma, purity ≥ 97%) in triplicate. Ferulic acid, MEL, Z-ligustilide and Z/E-butylidenephthalide standards were dissolved in 100% MeOH. In all instances, 100 µL of 0.1 mM DPPH was added to 100 µL of extract, standard, or blanks and slowly shaken for 2 seconds prior to acquiring absorbance at 520 nm every 60s at 25°C for 30 mins. For each tissue type and standard the DPPH radical-scavenging activity was calculated at T=30 mins and expressed as required to reduce free radicals by 50%.

3.4.5 Untargeted Metabolomics Analysis

Samples of epidermis, cortex and pith tissues were analyzed for the total phytochemical contents as previously described (Brown et al., 2012a; Brown et al., 2012b). Tissues were excised from roots (n=3, 50 mg ± 2.9), homogenized in MeOH: 0.1% Formic (80:20 v:v) and filtered by centrifuged (UltraFree MC, Millipore). Extracts were separated on a Waters BEH Acquity C_{18} (2.1 X 150 mm, 1.7 µm) column with the following gradient: 0.1% aqueous formic acid:acetonitrile (0.0-25 min, 95:5-5:95 v/v, 25.1-30.0 min, 95:5 v/v). The flow rate was set to 0.25 mL/min for 30 mins at 30 °C (Waters Acquity UPLC). A steady flow of leucine enkephalin (Waters 1525 HPLC binary solvent manager, 2 ng/mL) was used as the internal standard for calibration of the Micromass LCT Premier series ToF-MS (Waters Inc.). Time of flight mass spectrometry used previously published optimized conditions including: electrospray ionization and positive and negative ion detection in W mode, mass range of 100-1000 amu and a scan time of 0.1 sec. Details of voltages are included as supplemental information. Data were collected with MassLynx V4.1 and exported via MarkerLynx. Data were processed using Excel (Microsoft) to align retention times and remove multiply charged ions as described previously (Brown et al., 2012a; Brown et al., 2012b).

3.4.6 Exploratory Data Analysis

After processing the above dataset in Excel, PCA was performed using Solo (Eigenvector Research Inc.) in order to illustrate the overall variability between metabolomes

of both tissue types and root ecotypes through clustering. Data was preprocessed using auto scaling prior to applying the PCA algorithms. PLS-DA was also applied (data not shown).

3.5 Results

3.5.1 Detection of Indoleamine Neurotransmitters in *Ligusticum*

A preliminary experiment was undertaken to determine whether 5HT and MEL are present in *Ligusticum* species. In root materials, 92% of *L. porteri* plants (11/12) and 86% of *L. canbyi* plants (13/15) had measurable concentrations of 5HT, $1.01 \times 10^3 \pm 2.59 \times 10^2$ and $9.05 \times 10^2 \pm 2.32 \times 10^2$ ng/g tissue respectively (Figure 3.1A). Similarly, in shoot materials 58% of *L. porteri* plants (7/12) and 53% of *L. canbyi* plants (8/15) were found to contain quantifiable concentrations of 5HT, $1.07 \times 10^4 \pm 3.41 \times 10^3$ and $9.27 \times 10^3 \pm 2.66 \times 10^3$ ng/g of tissue respectively (Figure 3.1B). Concentration of 5HT in both root and shoot materials did not significantly differ between the species (Figure 3.1A & B). Instead, concentration of 5HT was significantly greater in leaves than in roots of *Ligusticum* species (Figure 3.1A & B). In root tissues, 58% of *L. porteri* (7/12) and 80% of *L. canbyi* root samples (12/15), had quantifiable concentrations of MEL (Figure 3.1C). Significantly more MEL was found in root tissues of *L. porteri* than *L. canbyi*, $1.48 \times 10^3 \pm 5.86 \times 10^2$ and $2.20 \times 10^2 \pm 60.7$ ng/g tissue respectively (Figure 3.1C). MEL was found in 50% of *L. porteri* (6/12) and 80% of *L. canbyi* (12/15) shoots (Figure 3.1D). There was no significant difference in the MEL concentration of shoots between *L. porteri* and *L. canbyi* $6.96 \times 10^2 \pm 2.02 \times 10^2$ and $4.76 \times 10^2 \pm 1.18 \times 10^2$ ng/g tissue respectively) (Figure 3.1D). Subsequent experiments focused on *L. canbyi* for detailed phytochemical characterizations.

L. canbyi roots have a complex morphology with distinct tissues and substantially different cell types (Figure 3.2A). Variability in the concentration of MEL and 5HT in root tissues of *Ligusticum* prompted further study to determine whether different cell types within the roots had different indoleamine contents. In roots of *L. canbyi*, 5HT was detected in 80% of epidermis, cortex and pith samples (4/5) (Figure 3.2B). The concentration of 5HT was not significantly different between epidermis, cortex and pith tissues $2.13 \times 10^2 \pm 48.7$, $3.11 \times 10^2 \pm 92.3$, and $1.91 \times 10^2 \pm 33.9$ ng/ g tissue respectively (Figure 3.2B). However, there was significantly more 5HT in the leaf tissues than any of the excised roots (Figure 3.2B). MEL was detected in 100% of the pith, cortex and epidermis samples (5/5) and there was no significant difference between epidermis, cortex and pith tissues $2.01 \times 10^2 \pm 60.7$, 1.28×10^2

± 32.4, 1.06 x 10^2 ± 48.6 ng/g of tissue (Figure 3.2C). There was significantly more MEL in shoot tissues of *L. canbyi* than the roots (Figure 3.2C).

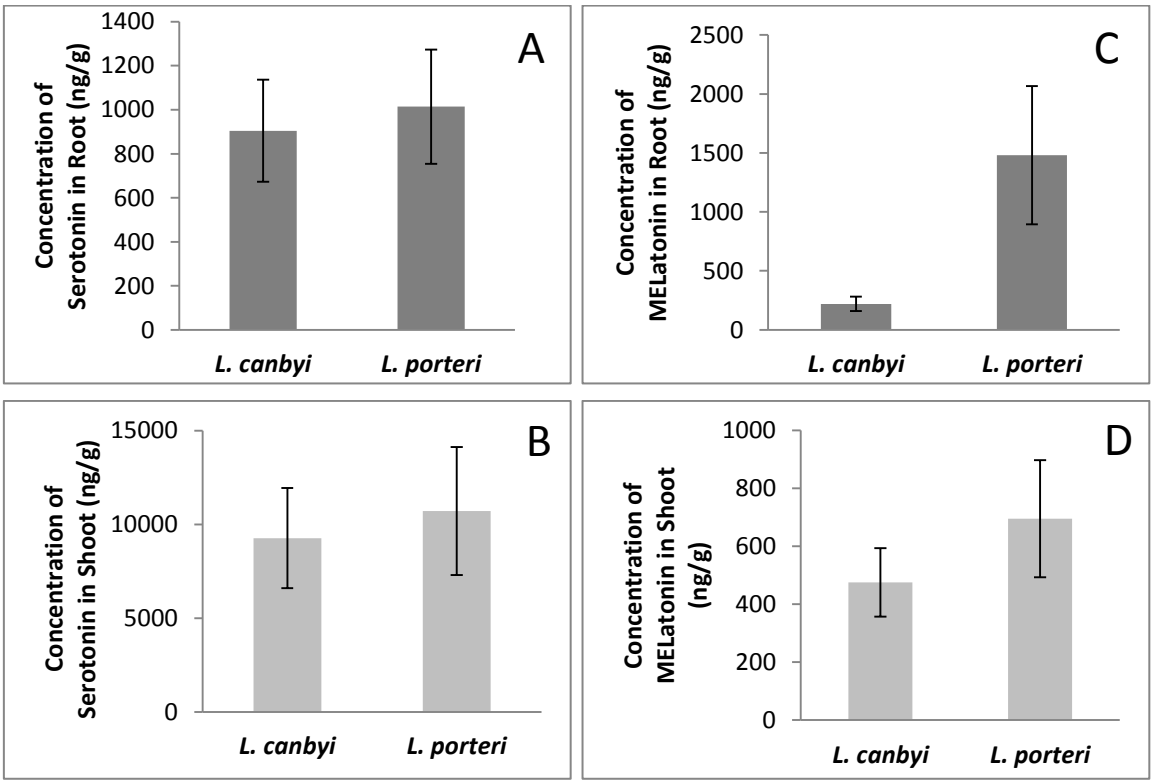


Figure 3.1 Detection and quantification of indoleamines in *L. porteri* and *L. canbyi*. (**A**) 5HT in roots; (**B**) 5HT in shoots; (**C**) MEL in roots; (**D**) MEL in shoots. Error bars represent the standard error of the mean.

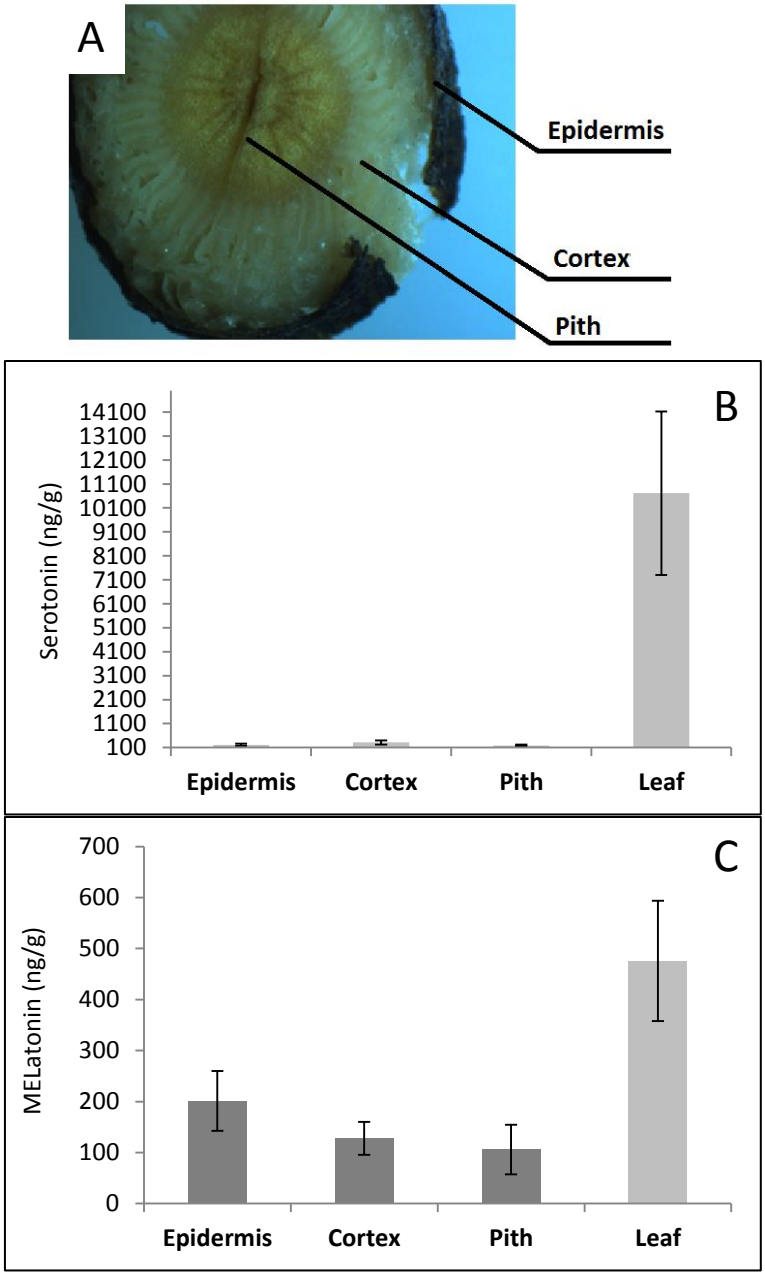


Figure 3.2 Detection and quantification of indoleamines in excised tissues of *L. canbyi*. (**A**) Picture showing root tissue dissection into epidermis, cortex, and pith; (**B**) concentration of 5HT in tissue types; (**C**) concentration of MEL in tissue types. Error bars represent standard error of the mean (n = 5 replicates). Picture taken by Christina E. Turi.

3.5.2 Detection of Phthalides and Ferulic Acid in *L. canbyi*

A variety of phthalides have previously been described in *Ligusticum* species and related plants but a detailed study has not been completed for *L. canbyi*. This data shows that

the concentration of ferulic acid was significantly different between root and shoot tissues (Figure 3.3A). Epidermis, cortex and pith tissues contained $1.14 \times 10^3 \pm 2.22 \times 10^2$, $1.82 \times 10^3 \pm 3.55 \times 10^2$; and $2.39 \times 10^3 \pm 4.71 \times 10^2$ µg/g of ferulic acid, while stem and leaf tissues contained $2.05 \times 10^2 \pm 7.79$ and $2.14 \times 10^2 \pm 10.4$ µg/g of tissue (n=5) (Figure 3.3A). The Z-ligustilide concentration was significantly different between root and shoot material, with epidermis, cortex, and pith tissues containing $5.06 \times 10^3 \pm 1.27 \times 10^3$, $8.20 \times 10^3 \pm 2.77 \times 10^3$, and $1.23 \times 10^4 \pm 3.17 \times 10^3$µg/g of tissue respectively (Figure 3.3B). Interestingly, Z-ligustilide was not detected in shoot material (Figure 3.3B). Z-butylidenephthalide was also not detected in either root or shoot tissues. There was no significant difference in the concentration of E-butylidenephthalide between epidermis, cortex, pith and stem tissues $3.68 \times 10^2 \pm 95.0$, 396.0 ± 87.2, $3.97 \times 10^2 \pm 85.1$, and $3.51 \times 10^2 \pm 1.01 \times 10^2$µg/g of tissue (Figure 3C). The concentration of E-butylidenephthalide was significantly higher in leaf tissues $1.41 \times 10^3 \pm 2.08 \times 10^2$µg/g of tissue (Figure 3.3C).

3.5.3 Determination of Antioxidant Potential in Tissue Types

Both ferulic acid and MEL are thought to be powerful antioxidants but the relative contribution of each of these phytochemicals to the antioxidant potential of the tissues has never been investigated. Antioxidant potential was determined as the amount of tissue required to reduce the free radical generation by DPPH by 50% (Brown et al., 2012b). In *L. canbyi* the antioxidant potential was significantly lower in the stems than any of the other tissues (Figure 3.4A). The amount of tissue required for 50% reduction was $4.46 \times 10^2 \pm 2.32 \times 10^2$; $1.64 \times 10^2 \pm 9.94$; $1.78 \times 10^2 \pm 25.6$; $8.76 \times 10^3 \pm 2.46 \times 10^3$; $1.03 \times 10^3 \pm 8.17 \times 10^2$ µg for epidermis, cortex, pith, stem and leaf tissues respectively (Figure 3.4A). To estimate the relative contribution of the metabolites to the antioxidant capacity of the tissues, I determined the antioxidant potential of each of the pure standards in the DPPH assay and calculated a theoretical contribution to the overall effect (Figure 3.4B). Interestingly, the ferulic acid present in epidermis, cortex, pith, stem, and leaf tissue could theoretically explain 54, 40, 54, 137, and 24% of the observed DPPH reduction respectively (Figure 3.4B).

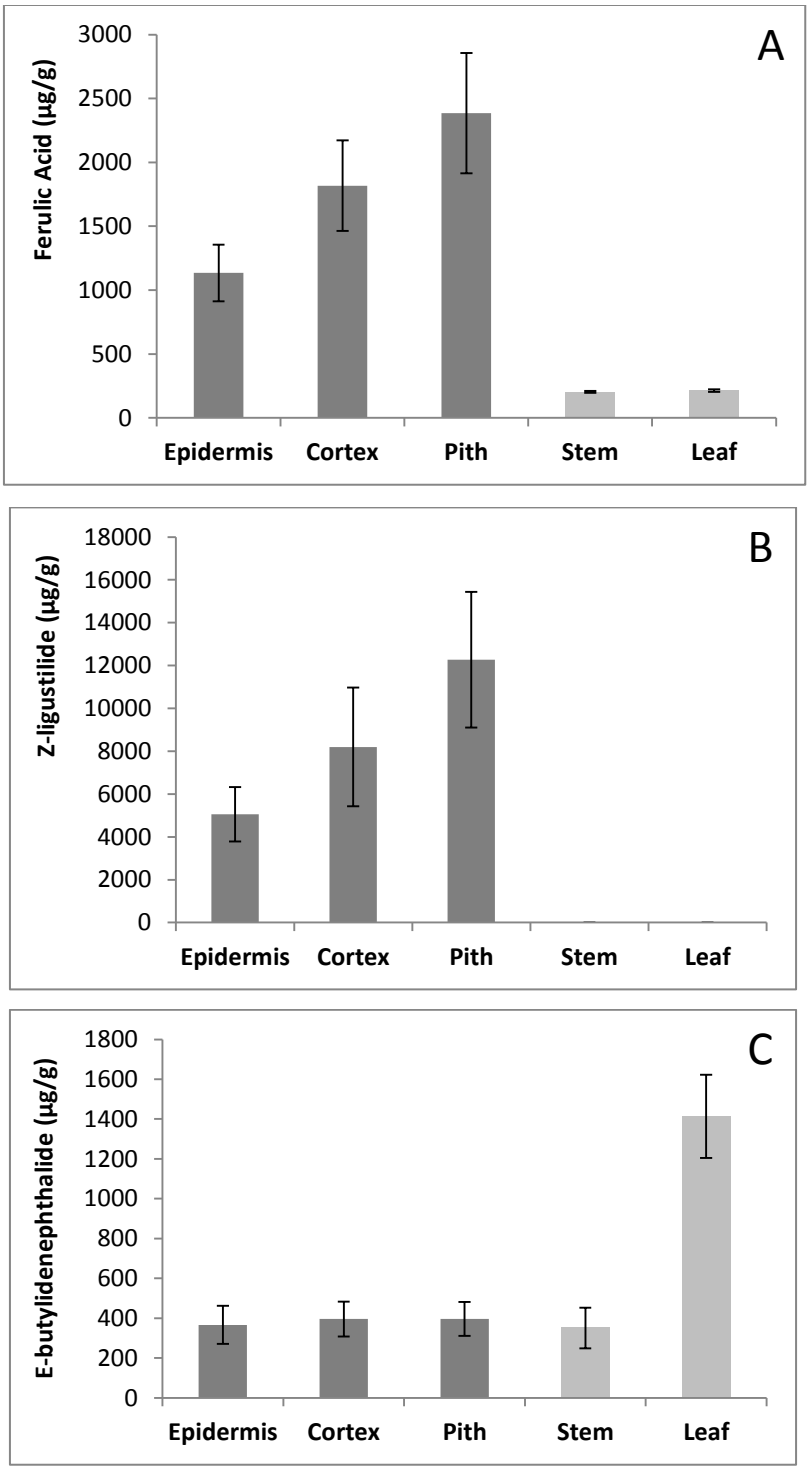


Figure 3.3 Detection and quantification of phenolic compounds in excised tissues of *L. canbyi.* (**A**) ferulic acid; (**B**) Z-ligustilide; (**C**) E-butylidenephthalide. Root tissues were excised as shown in Figure 3.2A. Error bars represent the standard error of the mean (n = 5 replicates).

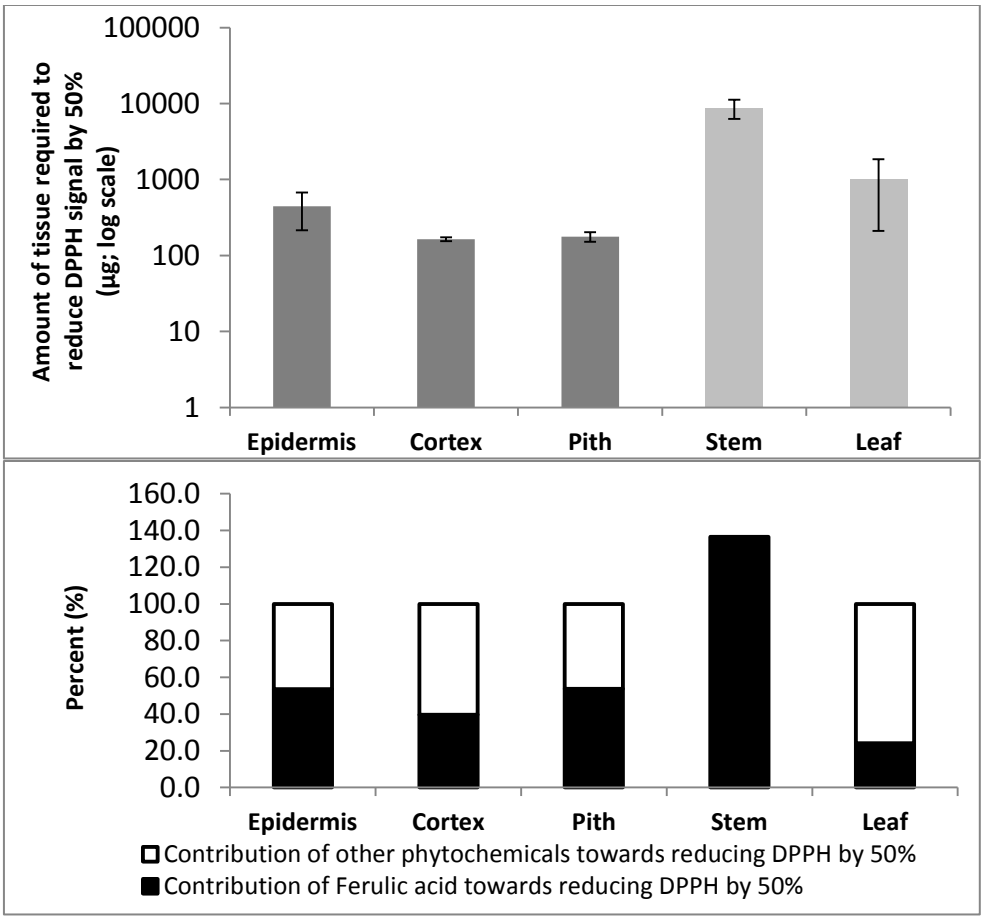


Figure 3.4 Determination of the relative contribution of known targeted metabolites to the antioxidant potential of excised tissues of *L. canbyi.* (**A**) The amount of tissue required to reduce the oxygen free radical generating capacity of DPPH by 50%; (**B**) The theoretical contribution of individual quantified compounds to the overall potential of the excised tissues to detoxify oxygen free radicals. Error bars represent the standard error of the mean (n = 5 replicates).

3.5.5 Metabolomics

An untargeted metabolomics approach was used to investigate the phytochemical complexity of *L. canbyi* root tissues. Mass spectrometry-based metabolomics detected 34,241-35,104 unique compounds in epidermis, cortex and pith tissues (Table 3.1). The highest number of unique compounds was detected within epidermal tissue, with lower chemical diversity in the cortex (Table 3.1). Nearly 70% of the ion signals were common to extracts of all 3 of the tissues (Table 3.1). PCA did not effectively identify distinguishing characteristics between the tissues and showed weak clustering with an overlap of 95% confidence boundaries between pith, cortex and epidermal tissues from different ecotypes for PC1 (21.1%) and PC2 (16.5%) (Figure 3.5A). Interestingly, PC1 (21.1%) and PC3 (15.8%)

indicate that greater commonality is shared amongst epidermis, cortex and pith tissue from the same root (Figure 3.5B).

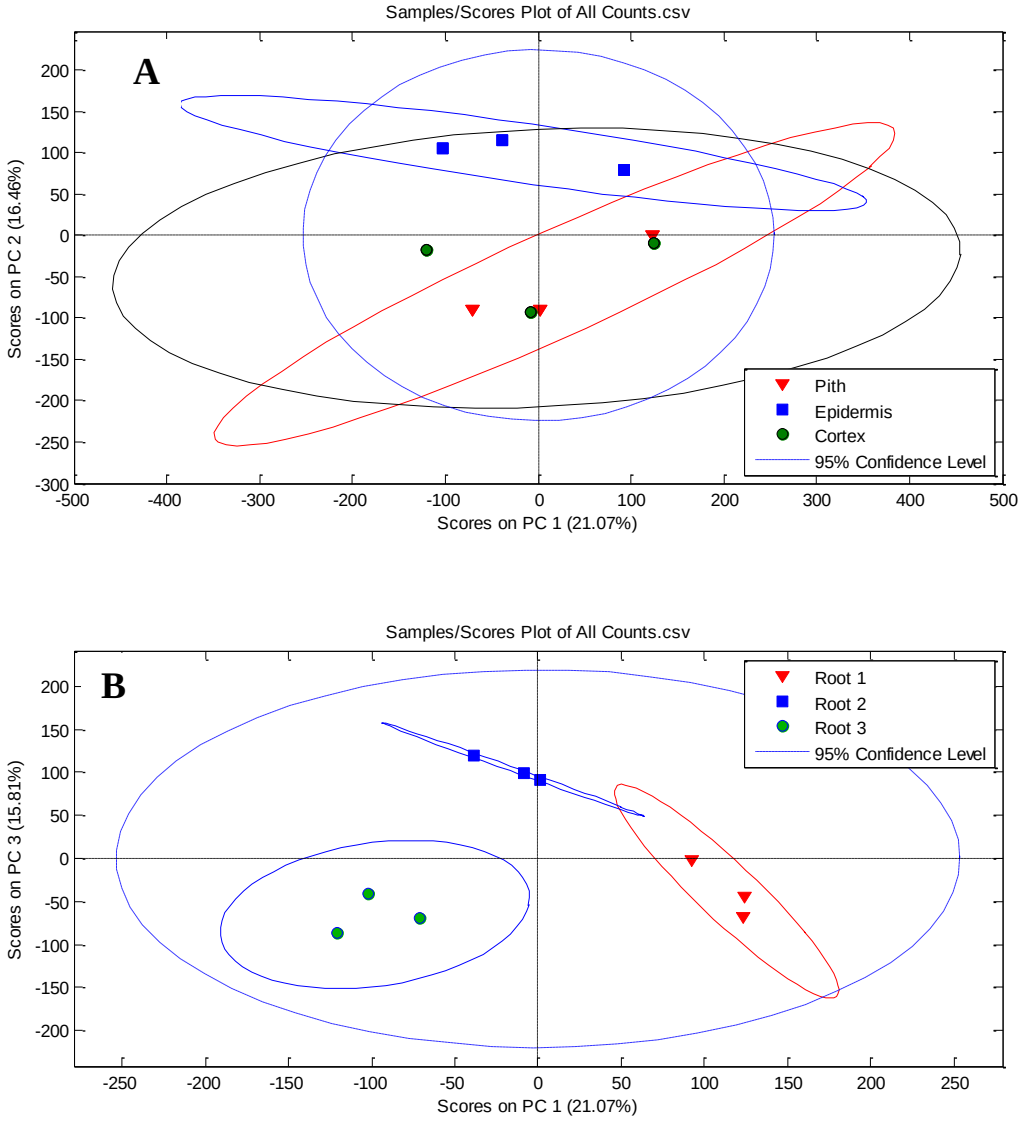


Figure 3.5 PCA illustrating illustrating observed phytochemical variability for *L. canbyi* roots. (**A**) weak clustering between specific tissue types collected from 3 different ecotypes for *L. canbyi;* (**B**) phytochemical commonality between epidermis, cortex and pith tissue from the same ecotype.

Table 3.1 Summary of metabolomic data.

	Epidermis	Cortex	Pith
Total number of compounds	3.51×10^4 (88.7)	3.51×10^4 (88.6)	3.42×10^4 (86.5)
Average compounds	2.27×10^4 (64.4)	2.22×10^4 (63.3)	2.20×10^4 (64.4)
Compounds in all replicates	1.15×10^4 (33.0)	1.10×10^4 (31.3)	1.15×10^4 (33.5)
Compounds in 2/3 replicates	1.21×10^4 (34.5)	1.19×10^4 (34.0)	1.12×10^4 (32.8)
Compounds in 1/3 replicates	1.14×10^4 (32.6)	1.22×10^4 (34.8)	1.15×10^4 (33.7)
Common Ions			
Compounds common to all tissues	2.72×10^4 (68.9)		
Common to Epidermis & Cortex	1.30×10^3 (3.3)		
Common to Cortex & Pith		1,317 (3.3)	
Common to Pith & Epidermis	1.72×10^3 (4.4)		
Unique Ions			
Compounds unique to each tissue (at least 1 replicate)	3.25×10^3 (9.26)	2.22×10^3 (6.3)	2.75×10^3 (8.0)
Compounds unique to each tissue (present in all replicates)	65.0 (0.2)	2.79 (0.8)	65.0 (0.2)
Significant Ions			
	Time	**m/z**	**Relative Intensity**
Unique to Epidermis	12.4214	632.4037	6.6541
Unique to Cortex	26.5311	339.2885	7.1331
	2.9015	183.0113	2.5933
	6.3023	572.3803	1.8043
	21.4506	398.3039	1.2334
	16.3299	214.1277	1.0892

*Numbers in () are values expressed as percent

Using methods similar to Murch et al. (2004), the monoisotopic mass for Z/E-ligustilide and its isomer 3-butylphthalide was calculated (191.1072) and chemically transformed using algorithms in Excel which simulated the following enzymatic reactions: $+H_2$, $-H_2$, $-CH_3$, $+ CH_3$, $+C_6H_{12}O_6$, +OH, +OH(x2), +COOH, $+NH_2$, $+NH_3$, +Angelic Acid ($C_5H_8O_2$), + Ligustilide, + 3-Butylidenephthalide (See Appendix 2 for further details). Chemical transformations were applied again to produce 182 putative transformations (Figure 3.6 & 3.7). The calculated mass for each transformation was matched with accurate mass data (±0.02). The putative presence of each transformation within samples was then determined by selecting those compounds which possessed counts > 1.0 within at least 6 of 9 samples. Chemical transformations of the monoisotopic mass for ligustilide/3-butylphthalide detected 82 potential phthalides within 6 of 9 samples with counts >1.0, (Figure 3.6 & 3.7, Table 3.2). The highest ion counts (>15.0) were identified for compounds **5-7,17-19**, and **49** (Table 3.2). Compound **5** with m/z 191.1072 can putatively be identified as Z/E-butylidenephthalide, compounds **6 -7** with m/z of 191.1072 is consistent with ligustilide, 3-butylphthalide, or (Z)-iso-butylidene-3a,4-dihydrophthalide, compound **17** with m/z 193.1228 is consistent with either senkyunolide A or (Z)-6,7-dihydro-ligustilide), compounds **18-19** with m/z 205.1228 are consistent with (Z)-iso-validene-3a,4-dihydrophthalide, (Z)-iso-validene-3a,4-dihydrophthalide, while compound **49** with mz/223.0970 is consistent with senkyunolide D (Figure 3.6 & 3.7). With the exception of dimers, previously known derivatives for the dehydrogenated transformations of Z/E-ligustilide/3-butylphthalide (compound **5**) were not found at significant concentrations in at least 6/9 samples (i.e. senkyunolide B, senkyunolide C, 3-butylidene-7-hydroxyphthalide, Z/E-senkyunolide E, m/z = 205.0864). Compounds **18-21** could potentially be novel amino derivatives of **5,** however their m/z of 205.1103 also corresponds to previously known methylated derivatives of Z/E-ligustilide/3-butylphthalide (Compounds **18-21**, m/z = 205.1228)(Figure 3.**7**).

Low concentrations of compounds consistent with well-known derivatives of hydrogenated Z/E-ligustilide/3-butylphthalide transformations (compounds **16 -17)** were also observed. For example, m/z consistent with neocnidilide, isocnidilide and cnidilide (average counts = 0.277±0.072, n=17, m/z = 195.1385) were putatively identified. Only 1 compound **(51),** consistent with a hydroxylated (x2) derivative of **16 & 17** (m/z =225.1126) could indicate several derivatives (i.e. senkyunolide I, senkyunolide H, E-3-butylidene-6,7-

dihydroxy-4,5,6,7-tetrahydrophthalide, ligustilidiol, trans-E-3-butylidene-6,7-dihydroxy-4,5,6,6-tetrahydrophthalide, Cis-6,7-dihydroxy-ligustilide) (see supplementary data). Hydrogenated derivatives (m/z =227.1283) of **51** also possessed average counts < 1.0 (i.e. senkyunolide N, senkyunolide J). Interestingly, adequate counts were observed for compounds **52** and **74-76,** which are consistent with methylated (i.e. (Z)-6-hydroxy-7-methoxydihydro-ligustilide) and dimer transformations of **51** (not yet known) (Table 3.2).

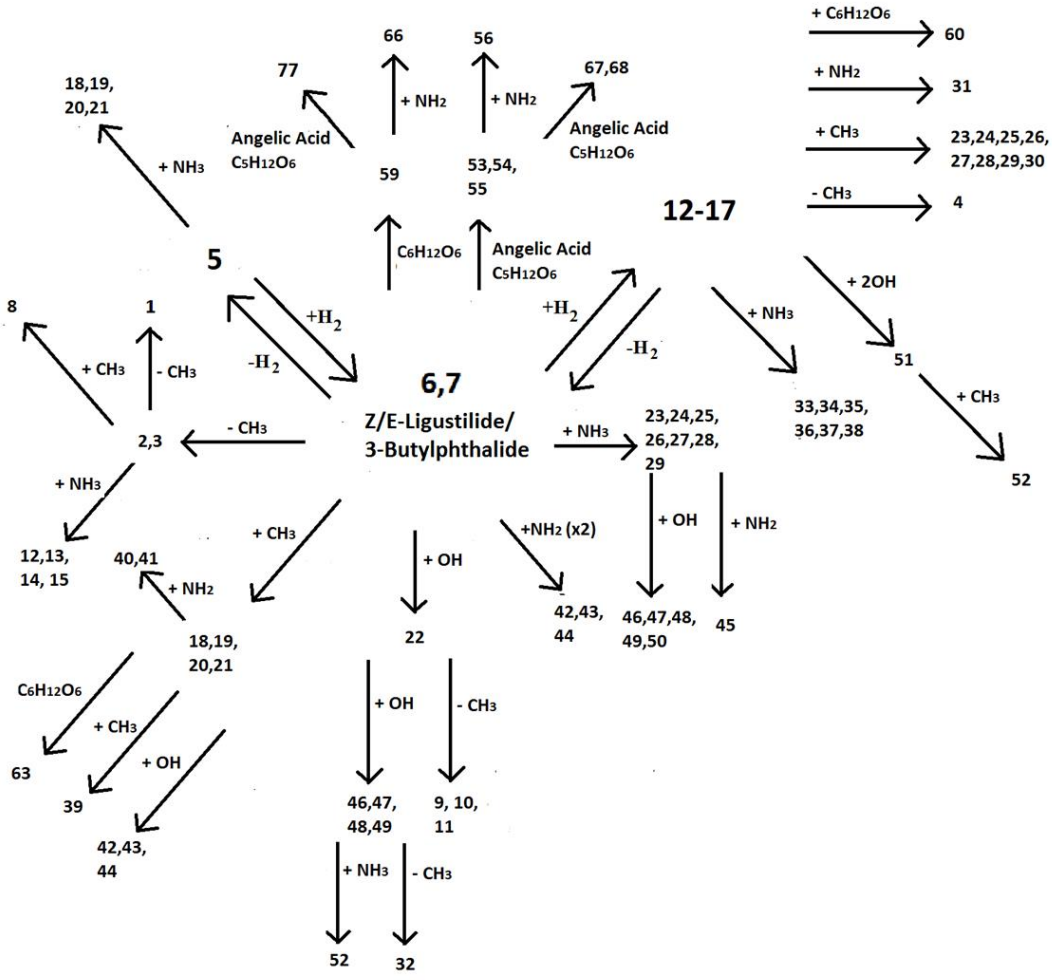


Figure 3.6 Predicted biotransformations of phthalide precursors in *L. canbyi.* Numbers refer to specific biotransformations found in Table 5.2 (see Appendix 2 for calculations).

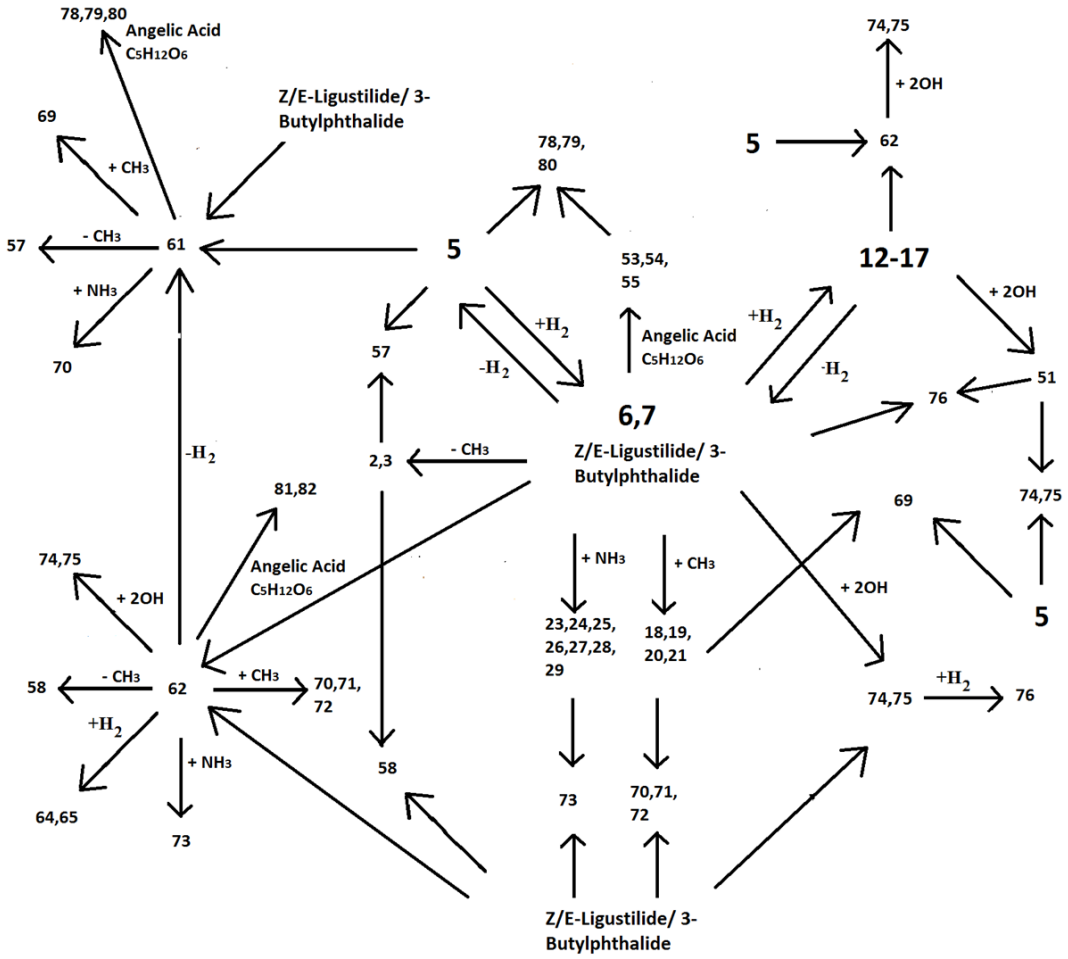


Figure 3.7 Predicted biotransformations of phthalide dimer precursors in *L. canbyi*. Numbers refer to specific biotransformations found in Table 5.2 (see Appendix 2 for calculations).

Table 3.2 Predicted products of ligustilide / butylphthlaide biotransformations.

ID	Transformation	Chemical Formula	Expected Mass (Monoisotopic Mass + H)	Observed m/z	Average Counts	Unknown trans-formation (Y/N)
1	Lig/Butyl -CH_3 (x2)	$C_{10}H_9O_2$	163.0759	163.0682	1.76	**Y**
2	Lig/Butyl -CH_3	$C_{11}H_{12}O_2$	177.0915	177.0828	8.31	**Y**
3				177.0881	4.60	**Y**
4	Lig/Butyl +H_2 -CH_3	$C_{11}H_{14}O_2$	179.1072	179.1061	1.44	**Y**
5	Lig/Butyl - H_2	$C_{12}H_{12}O_2$	189.0915	189.1161	15.64	**N**
6	Lig/Butyl	$C_{12}H_{14}O_2$	191.1072	191.1225	16.33	**N**
7				191.1227	15.00	**N**
8	Lig/Butyl -CH_3 + CH_3	$C_{12}H_{14}O_2$	191.1072	191.1252	8.08	**N**
9	Lig/Butyl +OH - CH_3	$C_{11}H_{12}O_3$	193.0865	193.0848	2.24	**Y**
10				193.089	2.14	**Y**
11				193.0904	1.46	**Y**
12	Lig/Butyl -CH_3 + NH_3 OR Lig/Butyl + H_2	$C_{11}H_{11}O_2N$/ $C_{12}H_{16}O_2$	193.1103/ 193.1228	193.1168	12.08	**Y / N**
13				193.1185	1.73	**Y / N**
14				193.12	6.95	**Y / N**
15				193.1226	1.21	**Y / N**
16	Lig/Butyl + H_2	$C_{12}H_{16}O_2$	193.1228	193.1393	11.87	**N**
17				193.1422	16.93	**N**
18	Lig/Butyl - H_2 + NH_3 OR Lig/Butyl + CH_3	$C_{12}H_{15}O_2N$/ $C_{13}H_{16}O_2$	205.1103/ 205.1228	205.112	16.19	**Y / N**
19				205.1166	17.06	**Y / N**
20				205.1238	2.12	**Y / N**
21				205.1245	1.81	**Y / N**
22	Lig/Butyl + OH	$C_{12}H_{14}O_3$	207.1021	207.1042	2.05	**N**
23	Lig/Butyl +H_2 + CH_3 OR Lig/Butyl + NH_3	$C_{13}H_{18}O_2$/ $C_{12}H_{16}O_2N$	207.1385/ 207.1259	207.1391	1.54	**N / Y**
24				207.1421	2.63	**N / Y**
25				207.1423	2.23	**N / Y**
26				207.1425	2.24	**N / Y**
27				207.1434	1.40	**N / Y**
28				207.1435	1.69	**N / Y**
29				207.1451	2.66	**N / Y**
30	Lig/Butyl +H_2 + CH_3	$C_{13}H_{18}O_2$	207.1385	207.1489	1.52	**Y**
31	Lig/Butyl +H_2 +NH_2	$C_{12}H_{17}O_2N$	208.1337	208.1439	3.34	**Y**
32	Lig/Butyl + 2OH - CH_3	$C_{11}H_{12}O_2$	209.0814	209.0815	2.26	**Y**

Table 3.2 Predicted products of ligustilide / butylphthlaide biotransformations.

ID	Transformation	Chemical Formula	Expected Mass (Monoisotopic Mass + H)	Observed m/z	Average Counts	Unknown trans-formation (Y/N)
33	Lig/Butyl + H_2 + NH_3	$C_{12}H_{18}O_2N$	209.1416	209.1542	5.40	**Y**
34				209.1563	1.38	**Y**
35				209.157	3.77	**Y**
36				209.1582	1.39	**Y**
37				209.1586	2.11	**Y**
38				209.1592	2.69	**Y**
39	Lig/Butyl +2CH_3	$C_{14}H_{18}O_2$	219.1385	219.1435	4.94	**Y**
40	Lig/Butyl +CH_3 + NH_2	$C_{13}H_{17}O_2N$	220.1337	220.1539	9.62	**Y**
41				220.1539	9.62	**Y**
42	Lig/Butyl + NH_2 (x2) OR Lig/Butyl + CH_3 + OH	$C_{12}H_{17}O_2N_2$/ $C_{13}H_{16}O_3$	221.1290/ 221.1178	221.1237	1.35	**Y / Y**
43				221.1251	1.18	**Y / Y**
44				221.1302	2.12	**Y / Y**
45	Lig/Butyl + NH_3 + NH_2	$C_{12}H_{17}O_2N_2$	222.1130	222.1297	4.04	**Y**
46	Lig/Butyl + 2(OH) OR Lig/Butyl + NH_3 + OH	$C_{12}H_{16}O_4$/ $C_{12}H_{16}O_3N$	223.0970/ 223.1208	223.1016	1.58	**N**
47				223.1025	2.63	**N**
48				223.1182	12.23	**N / Y**
49				223.119	15.28	**N / Y**
50	Lig/Butyl + NH_3 + OH	$C_{12}H_{16}O_3N$	223.1208	223.1391	6.09	**Y**
51	Lig/Butyl +H_2 +OH(x2)	$C_{12}H_{16}O_4$	225.1127	225.1169	4.38	**N**
52	Lig/Butyl + 2OH + NH_3 OR Lig/Butyl + H_2 +2OH + CH_3	$C_{12}H_{16}O_4N$/ $C_{13}H_{16}O_4$	239.1158/ 239.1283	239.1349	7.96	**Y / Y**
53	Lig/Butyl + Angelic Acid	$C_{17}H_{20}O_4$	289.1440	289.1468	2.34	**N**
54				289.1508	1.79	**N**
55				289.1514	4.24	**N**
56	Lig/Butyl + Angelic Acid + NH_2	$C_{17}H_{20}O_4$	304.1549	304.1718	13.45	**Y**
57	Lig/Butyl + (Lig/Butyl-H_2) - CH_3 OR Lig/Butyl - CH_3 + (Lig-H_2)	$C_{23}H_{24}O_4$	365.1753	365.1694	1.38	**Y**
58	Lig/Butyl + Lig/Butyl - CH_3 OR Lig/Butyl -CH_3 + (Lig/Butyl)	$C_{23}H_{26}O_4$	367.1909	367.2109	5.43	**Y**

Table 3.2 Predicted products of ligustilide / butylphthlaide biotransformations.

ID	Transformation	Chemical Formula	Expected Mass (Monoisotopic Mass + H)	Observed m/z	Average Counts	Unknown trans-formation (Y/N)
59	Lig/Butyl + glucose	C18H24O8	369.1549	369.1554	1.17	N
60	Lig/Butyl + H_2 + glucose	$C_{18}H_{26}O_8$	371.1706	371.1636	1.26	**Y**
61	Lig/Butyl + Lig -H_2	$C_{24}H_{26}O_4$	379.1909	379.2072	2.06	**N**
62	Lig/Butyl + Lig/Butyl OR Lig/Butyl -H_2 + (Lig + H_2)	$C_{24}H_{28}O_4$	381.2066	381.1886	1.64	**N**
63	Lig/Butyl + CH_3 + glucose	$C_{19}H_{26}O_8$	383.1706	383.1903	1.61	**Y**
64	Lig/Butyl + H_2 + (Lig/Butyl)	$C_{24}H_{30}O_4$	383.2222	383.2065	11.11	**N**
65				383.2101	1.41	**N**
66	Lig/Butyl + $C_6H_{12}O_6$ + NH_2	$C_{18}H_{26}O8N$	384.1658	384.1848	3.61	**Y**
67	Lig/Butyl + Angelic Acid (X2)	$C_{22}H_{26}O_6$	387.1808	387.1849	4.89	**Y**
68				387.2048	1.37	**Y**
69	Lig/Butyl + (Lig/Butyl-H2) +CH_3 OR Lig/Butyl +CH_3 + (Lig/Butyl-H_2)	$C_{25}H_{28}O_4$	393.2066	393.2007	3.36	**Y**
70	Lig/Butyl + (Lig/Butyl) + CH_3 OR Lig/Butyl + (Lig/Butyl-H_2) + NH_3	$C_{25}H_{30}O_4$/ $C_{24}H_{28}O_4N$	395.2222/ 395.2096	395.2165	3.53	**Y / Y**
71	Lig/Butyl + Lig/Butyl + CH_3	$C_{25}H_{30}O_4$	395.2222	395.2253	2.13	**Y**
72	Lig/Butyl + Lig/Butyl + CH_3	$C_{25}H_{30}O_4$	395.2222	395.2259	1.11	**Y**
73	Lig/Butyl + Lig/Butyl + NH_3	$C_{24}H_{30}O_4N$	397.2253	397.2352	9.24	**Y**
74	Lig/Butyl + Lig/Butyl + 2OH OR Lig/Butyl -H2 + (Lig + H_2) +2OH	$C_{24}H_{28}O_6$	413.1964	413.2159	1.72	**Y**
75				413.216	8.44	**Y**
76	Lig/Butyl + Lig/Butyl + 2OH + H_2	$C_{24}H_{28}O_6$	415.2121	415.2091	1.09	**Y**

Table 3.2 Predicted products of ligustilide / butylphthlaide biotransformations.

ID	Transformation	Chemical Formula	Expected Mass (Monoisotopic Mass + H)	Observed m/z	Average Counts	Unknown trans-formation (Y/N)
77	Lig/Butyl + Angelic Acid + Glucose	$C_{23}H_{30}O_{10}$	467.1917	467.1998	1.36	**Y**
78	Lig/Butyl + (Lig/Butyl -H2) + Angelic Acid	$C_{29}H_{32}O_6$	477.2277	477.233	1.56	**Y**
79				477.2343	7.77	**y**
80				477.2351	2.70	**Y**
81	Lig/Butyl + Lig/Butyl + Angelic Acid	$C_{29}H_{34}O_6$	479.2434	479.2461	1.98	**Y**
82				479.2508	2.18	**Y**

*Lig = Z/E-ligustilide, Butyl = 3-butylphthalide *

3.6 Discussion

Ligusticum species are sold as natural health products under the collective name Osha (The Scientific Authority of the United States of America, 2000; Turi and Murch, 2011; UMP, 2011) and while there has been some phytochemical and pharmacological investigation of *L. porteri,* very little is known about the related and often mistaken species *L. canbyi* . The current work has identified 2 neurologically active indoleamines, MEL and 5HT, quantified ferulic acid and 2 phthalides previously associated with other species of *Ligusticum*, characterized the role of these known compounds in the capacity of the plant tissues to detoxify free radicals and provided the first metabolomics analysis of *L. canbyi* roots.

Production of antioxidants is crucial for plant survival (Blokhina et al., 2003) and has been used to promote specific plants for the purpose of maintaining good health (Gulcin, 2012; Iriti et al., 2010). The determination of antioxidant potential in medicinal plants has been associated with treatment or prevention of multiple diseases and conditions which are attributed to free radical generation (Gulcin, 2012; Chanda and Dave, 2009; Krishnaiah et al., 2011). As a result, specialized metabolites such as MEL (Rosales-Corral et al., 2012), resveratrol (Cuzzola et al., 2011), and curcumin (Darvesh et al., 2012) are now considered promising leads for the treatment of neurodegenerative or cardiovascular diseases. Previous works have suggested the potential role of ferulic acid, indoleamines and phthalides in the protection of plant and human tissues against oxidative stress (Kikuzaki et al., 2002; Ho et

al., 2009). For example, in the presence of excess UV radiation or drought, ferulic acid has shown to be up-regulated in Barley leaves potentially countering stresses caused by photoinhibition (Hura et al., 2008, 2009, 2010; Liu et al., 1995).

MEL and 5HT have previously been quantified in medicinal plants which are known to have effects on the brain such as St. John's wort (*Hypericum perforatum* L.), feverfew (*Tanacetum parthenium* (L.) Sch. Bip) and skullcap (*Scutellaria baicalensis* Georgi), and devil's trumpet (*Datura metel* L.) (Murch et al., 2009; Murch et al., 1997). Furthermore, MEL has shown a potential role for providing protection to plant tissues from environmental stresses such as UV irradiation (Posmyk and Janas, 2009), metal contamination (Tan et al., 2007) and cold (Zhao et al., 2011). It has also been hypothesized that high levels of MEL in reproductive tissues such as seeds, fruit and flowers could indicate a role for indoleamines as antioxidants to protect the genetic resources of gametes (Murch et al., 2009; Murch et al., 2010; Manchester et al., 2000; Murch and Saxena, 2002).

Methods of comparing the relative importance of the different phytochemicals in antioxidant activity are inherently flawed since the activity of the compounds in the tissue extracts may be different from the activity of isolated compounds. However, the experiment was undertaken to determine whether it was possible to assess the relative contribution of different compounds. Data indicates that the ferulic acid content accounted for the majority of the antioxidative activity of *Ligusticum* tissues. It is likely that MEL contributed greater antioxidant activity than could be identified by this method. It has previously been reported that oxidized derivatives of MEL (cyclic 3-hydroxyMEL (C3-OHM), N^1-acetyl-N^2-formyl-5 methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK)) collectively contribute to the reducing potential in living organisms (Galano et al., 2011; Reiter et al., 2007). With the exception of stem tissues, the targeted metabolites could not account for all the observed antioxidant activity at 50% DPPH, which suggests that other phytochemicals contribute to reduction of reactive oxygen species in the plants.

Metabolomics is the qualitative and quantitative analysis of all small metabolites in a biological sample (Yuliana et al., 2011; Verpoorte et al., 2010). For medicinal plant researchers it has served as an invaluable tool by enabling the investigation of synergies for complex phytochemical mixtures (Urich-Merzenich et al., 2009), the authentication of

medicinal crops through fingerprinting (Tianniam et al., 2009; Lee et al., 2012) and the discovery of novel therapeutic compounds (Murch et al., 2004). Methods generally employed to analyze large metabolomic data sets include multivariate statistics such as PCA, PLS-DA, or OPLS analysis (Yuliana et al., 2011). Better approaches to interpret and effectively model complex data sets are needed since simply determining that one extract is different from another has limited utility (Brown and Murch, 2012; Verpoorte et al., 2010). To date, a full biochemical pathway describing phthalide biosynthesis, catabolism and the production of derivatives has not been fully elucidated. Early works using radiolabelled acetate in cultivated *Levisticum officinale* determined that biogenesis of Z/E-ligustilide occurs through head to tail addition of acetate units (Beck and Chou, 2007; Kobayashi and Mitsuhashi, 1987; Mitsuhashi and Nomura, 1966). It has been hypothesized that 3-butylidenephthalide, Z/E-ligustilide, 3-butylphthalide and senkyunolide A may be the main precursors for phthalide biosynthesis (Beck and Chou, 2007). Metabolomics data suggests that predictive transformation of Z/E-ligustilide/3-butylphthalide and putative identification of their derivatives is a useful approach towards gaining preliminary understanding of potential phthalide branches and metabolites within *L. canbyi* root tissues, which in turn has led us to hypothesize the presence of novel methylated, demethylated, angeloyl ester, amine, and dimer derivatives in *L. canbyi*. Methylated (Gold and Wilson, 1963; Gijbels et al., 1982; Deng et al., 2006), and de-methylated (Gold and Wilson, 1963; Gijbels et al., 1982) derivatives of Z/E-ligustilide/3-butylphthalide have previously been detected in other members of the Apiaceae (Beck and Chou, 2007). Angeloyl ester phthalides have previously been identified within members of the genus *Angelica* (Deng et al., 2006; Tsuchida et al., 1987). Previous reports of amine derivatives were not found in my search of the literature. The identification of potentially novel phthalide dimers may be an artifact of the ionization and mass spectrometry analysis or may provide new avenues for investigation of medicinal activity.

L. canbyi is an important traditional ceremonial plant in North America and is gaining in popularity in the natural product marketplace. Mechanisms of activity, evidence of efficacy and phytochemical composition are all limited for this species and further enquiry into the therapeutic potential of this crop is warranted. Targeted data provides the first evidence of the biologically active neuroindoleamines 5HT and MEL in *L. canbyi* as well as

the identification and quantification of other medicinal metabolites in the plants. The metabolomics data indicate a wealth of potential for medicinally active phytochemicals in these plants that traditionally bring a sense of comfort and well-being. Further research may lead to the discovery of novel treatments for neurological ailments and better understandings of the use of plants in traditional rituals and ceremonies.

Chapter 4 Metabolomics as a Hypothesis Generating Tool for Plant Tissue Culture

4.1 Synopsis

The classic hypothesis of the regulation of plant growth and regeneration attributes morphological changes to changes in the relative ratio of auxins and cytokinins (Skoog and Miller, 1957). Interpretations and applications of this approach are the foundation of modern plant tissue culture and biotechnology. However, not all plants respond in the same way as model systems like tobacco and other plant growth regulators have been discovered that mediate, accelerate or enhance fundamental plant regeneration mechanisms, especially in medicinal plants (Murch and Saxena, 2005). In particular, it has been hypothesized that human neurosignaling compounds may regulate plant metabolism, growth, and development (Murch and Saxena, 2002; Murch and Saxena, 2004; Cao et al., 2006). *A. tridentata* and related species in the subgenus *Tridentatae* are ecologically important plants with a rich history of ceremonial and medicinal use by the indigenous peoples of North America. With the exception of antimicrobial and insecticidal bioassays, there is limited data to support the traditional uses of *A. tridentata*. Additionally, wild *A. tridentata* populations are declining and conservation of genetic resources is warranted. A collection of *in vitro* grown germplasm lines was established from wild-harvested seeds of *A. tridentata*. Neurotransmitters Ach, GABA, MEL and 5HT were identified and quantified in the plant tissues. Crude extracts of *A. tridentata* inhibited AchE in a bioassay. A metabolomics analysis with chemometric statistics quantified changes in the phytochemical profiles of wild-harvested plants and plantlets in axenic culture. A total of 1,543 phytochemicals were found in all samples of *A. tridentata* including 52 significant ions putatively identified as monoterpene, phenolic or sesquiterpene compounds.

4.2 Research Objectives and Hypothesis

I hypothesized that sterile *A. tridentata* cultures contain neurotranmitters Ach, GABA, MEL and 5HT and that metabolomics can be used as a hypothesis generating technique for plant tissue culture. The current study was designed with the following objectives: 1) to develop a protocol for establishment of a living germplasm collection of North American Sagebrush species using *A. tridentata* as a model species; 2) to investigate the phytochemistry underlying the traditional medicinal and ceremonial use of *A. tridentata*

by identification and quantification of neurotransmitters Ach, GABA, MEL, and 5HT; 3) to determine whether *Artemisia* leaf tissues exhibit anti-Achstetrase activity that might support the traditional knowledge of use; and 4) to develop a metabolomics toolkit as an investigative technique for understanding plant secondary metabolism under aseptic conditions.

4.3 Materials and Methods

4.3.1 Wild Harvest of *A. tridentata* Leaf Tissue and Seed

Seed and leaf tissue for *A. tridentata* was collected in the Fall of 2011 from 2 sites located near the Osoyoos Desert Centre in Osoyoos, British Columbia site 1(N 49 03'15 W 119 31'14W) and site 2(N 49 03'14, W 119 31'16). Seeds were stratified at 4 $^{\circ}$C for 6 months in a dry environment, while leaf material was stored at -80 C for analysis.

4.3.2 Seed Germination

Stratified seeds were surface sterilized in a solution of 10% bleach containing a 10% commercial solution of zinc pyrithione for ten minutes, and rinsed twice with sterile deionized water. Approximately twenty seeds were cultured onto sterile media plates containing a basal media consisting of ½ MS salts (Murashige and Skoog 1962), ½ Gamborg B5 vitamins (Gamborg et al. 1968) and solidified with phytagel ^{TM} (Sigma) with pH adjusted to 5.7. After two months, plants were large enough to excise multiple shoot clusters for subculture onto a fresh MSO medium containing full strength MS salts and B5 vitamins with 30 g/l sucrose, pH 5.7, solidified with phytagel.

4.3.3 Establishment of Germplasm Lines

Individual intact seedlings were excised from individual germinated seeds and placed in numbered isolated cultures so that seed origin could be tracked throughout the experiments. Clonal propagation of individual seedling explants established a collection of perpetual germplasm lines maintained by serial subculture onto basal media MSO at 2 week intervals. Germplasm lines have been maintained for more than 18 months and subcultured every 4-6 weeks.

4.3.4 Leaf Surface Imaging

Leaf surface structures on freshly excised tissues of *in vitro* grown and wild-harvested *A. tridentata* were compared using the Tescan Mira3 XMU Field Emission Scanning Electron Microscope in the UBC Okanagan Facility for Scanning-Electron Microscopy.

4.3.5 Quantification of GABA, MEL and SER

Four *A. tridentata* germplasm lines derived from individual seeds were selected to represent the 2 sites (site 1, N49 03'15, W119 31'14W = GPL-9A & GPL-13A; site 2, N49 03'14, W119 31'16W = GPL-3B & GPL-7B). Extracts were performed in triplicate using previously described methods from Murch et al. (2010) with minor modifications (see Chapter 3).

4.3.6 Quantification of Ach

Tissues were sampled in triplicate, flash frozen, weighed and stored at -80 ˚C. Using an Amplex© Red Ach / Acetylcholinesterase Assay Kit (Molecular Probes Inc. Eugene, Oregon), Ach was quantified in tissues using a modification of the spectrophotometric method originally described previously (Ellman et al., 1961). In brief, 100µL of Ach standard (n=3) (0, 1, 5, 10, 20, 40, 60, 80, and 100µM, positive control (n=4), 0.5 g/mL *A. tridentata* were added to a 96 well plate. All samples were prepared in 1X buffer. 100 µL of working solution (200 µM Amplex red reagent, 1 U/mL Horseradish peroxidase, 0.1 U/mL choline oxidase, 0.5 U/mL AchE, Buffer) was added to each well, lightly shaken and placed into a microplate reader (Synergy Multi-detection Microplate Reader, Biotech), with excitation and emission wavelengths set to 530-560 nm and 590 nm respectively. Fluorescence was measured every 5 min until the reaction was complete (1 hour). A standard curve for Ach was generated by subtracting standards by a negative control (0 µM Ach). Ach was then quantified for each sample by subtracting the observed fluorescence values by the negative control and sample blank and comparing to standard curve data.

4.3.7 AchE Inhibition Activity

Using a modified version of the same methods for quantification of Ach, MeOH extracts from sterile and field collected tissue (n=3) were assayed for AchE activity. In brief, 50 µL of extract or buffer (control) were lightly mixed with 100 mU of AchE for 30 seconds and left to incubate in the dark at room temperature. After 30 minutes, 100 µL of working solution (400 µM Amplex red reagent, 2 U/mL horseradish peroxidase, 0.2 U/mL choline oxidase, 100 µM Ach) was added to each well, lightly shaken and placed into the same microplate reader, with excitation and emission wavelengths set to between 530-560 nm and 590 nm respectively. Fluorescence was measured every 5 min until the reaction was complete (1 hour). Assay and a positive control were also used, and consisted of either

buffer or 10 µM H_2O_2 solution respectively. AchE activity was determined using Michaelis-Menten enzyme kinetics, and enzyme inhibition was determined by the amount of extract required to reduce V_{max} by 50% of the assay control. Galanthamine hydrobromide (Chromadex) was included as a negative control for all experiments.

4.3.8 Untargeted Metabolomic Analysis

A. tridentata leaf tissue from both field and culture were sampled in triplicate and subjected to untargeted phytochemical analysis using previously published protocols from (Brown et al., 2012 a & b) (see Chapter 3).

4.3.9 Analysis of Metabolomic Data

Multivariate analysis compared metabolomes of *in vitro* and field grown *A. tridentata* tissues and used PCA to model data with SOLO software (Eigenvector Research Inc.). Average ion intensity was calculated for each compound in order to select for metabolites which are significant throughout all treatments and replicates. Putative identifications (±0.02 Daltons) was performed using m/z data previously collected from an extensive literature of all known phytochemistry for North American *Artemisia* found in the subgenus *Tridentatae* (Turi et al., 2014a).

4.4 Results

4.4.1 *In Vitro* Culture of *A. tridentata*

Following stratification, seeds germinated at varying rates in sterile cultures (Figure 4.1A). No difference was observed between seeds germinated on the basal or TDZ-supplemented media. Germination rates were found to be site specific and varied between 10-25%. Individual seedlings were selected for establishment of germplasm lines (Figure 4.1B) and subcultured onto the same media. Tissue browning and discoloured purple leaves were removed during subculture and healthy tissues were subcultured onto fresh sterile media at 2 week intervals (Figure 4.1C). After two months, individual axenic plants had established sufficiently for regeneration. Clusters of shoots were excised and subcultured onto new media where *de novo* root formation was observed within a month (Figure 4.1D). Attempts to regenerate *A. tridentata* from single shoot explants were unsuccessful and supplementation of the media with either benzylaminopurine or thidiazuron did not significantly improve regeneration (data not shown).

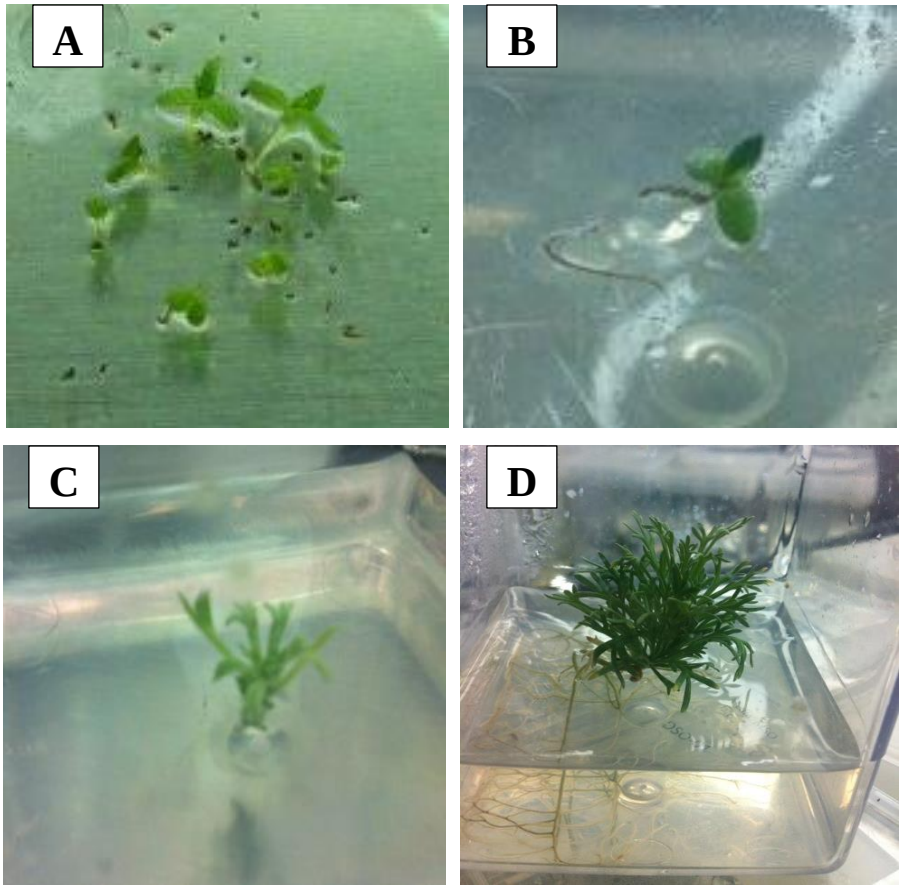


Figure 4.1 Establishment of *A. tridentata* in axenic culture. (**A**) *A. tridentata* seed germination; (**B**) *A. tridentata* plantlet growth; (**C**) *de novo* shoot regeneration on an individual seedling; (**D**) larger multiple shoot clusters were the ideal explants for establishment of perpetual germplasm lines *in vitro.* Pictures taken by Katie Axwik.

4.4.2 Comparison of Leaf Surface of Field and Culture Collected Tissues

Given sesquiterpene and monoterpene compounds are believed to be contained on the surface of leaves in structures called glandular trichomes, leaf tissues were examined using scanning electron microscopy. Leaves excised from *in vitro* grown plantlets and wild-harvested plants were covered in abundant hairs (Figure 4.2A, B & D). Glandular trichomes were observed on both types of leaf explants (Figure 4.2B, C, E & F). The wild-harvested leaves had additional structures that appeared similar to pollen trapped in the hairs but could also be modified cells (Figure 4.2C). Further work is required to positively determine the identity of these structures.

The known plant neurotransmitter metabolites Ach, GABA, MEL and 5HT were identified and quantified in shoot tissues of *A. tridentata*. The concentration of GABA varied significantly between germplasm lines collected from different sites. Since quantification of Ach is dependent on its hydrolysis by AchE, preliminary experiments were conducted to optimize the time for the enzymatic reaction and quantification measurements were made at 60 minutes (Figure 4.3) when the rate of reaction was greatest. The concentration of Ach was not significantly different between GPL-9A (7.08 ± 3.55 ng/g of tissue) and GPL-13A (12.1 ± 7.07 ng/g of tissue) (Figure 4.3). GABA concentrations were significantly greater in extracts of GPL-9A & GPL-13A than in extracts of GPL-3B & GPL-7B (Figure 4.4). Across all of the germplasm lines, the concentration of GABA spanned the range from 290 μg / g to 650 μg / g. The concentration of MEL did not significantly differ between germplasm lines collected from different sites (Figure 4.5). The average MEL concentration of all germplasm lines was about 264 ng / g. The concentration of 5HT was significantly different between germplasm lines collected from different sites (Figure 4.6). The 5HT concentration was significantly greater in GPL-9A & 13A while GPL-3B & 7B had only trace amounts (Figure 4.6). The concentration of 5HT spanned the range from not detected to almost 1.5 μg/g (Figure 4.6).

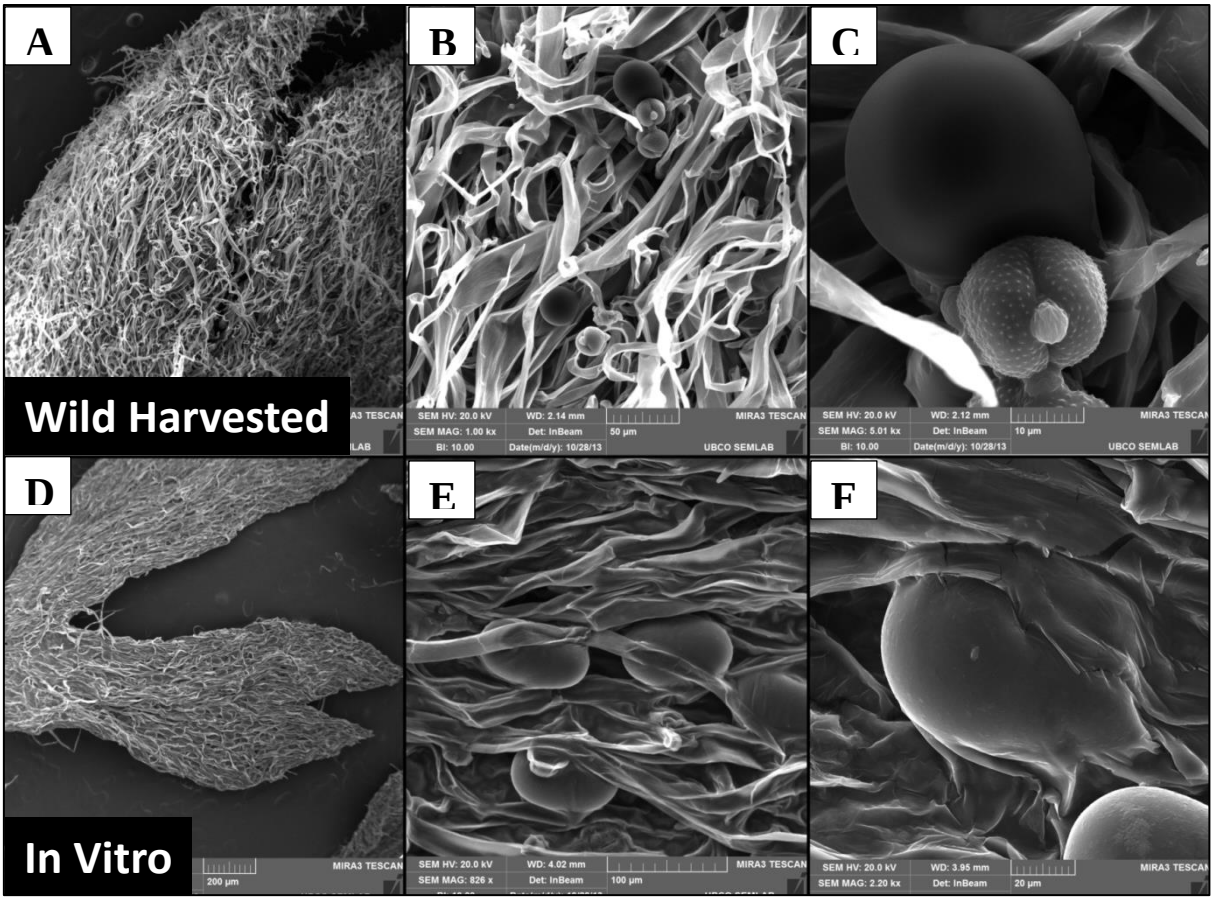


Figure 4.2 Scanning electron microscopy of wild-harvested and *in vitro* grown leaf tissues of *A. tridentata*. (**A**) Whole leaf of wild-harvested *A. tridentata* (178 x); (**B**) leaf hairs, glandular trichomes and unidentified structure on the surface of wild-harvested *A. tridentata* leaves (1,000 x); (**C**) individual trichome and unidentified structure on wild-harvested leaf (5,000 x); (**D**) whole leaf of *in vitro*-grown *A. tridentata* (180 x); (**E**) leaf hairs and glandular trichomes on the surface of *in vitro* grown *A. tridentata* (826 x); (**F**) glandular trichome on the leaf of an *in vitro* grown *A. tridentata* plantlet (2,200 x)

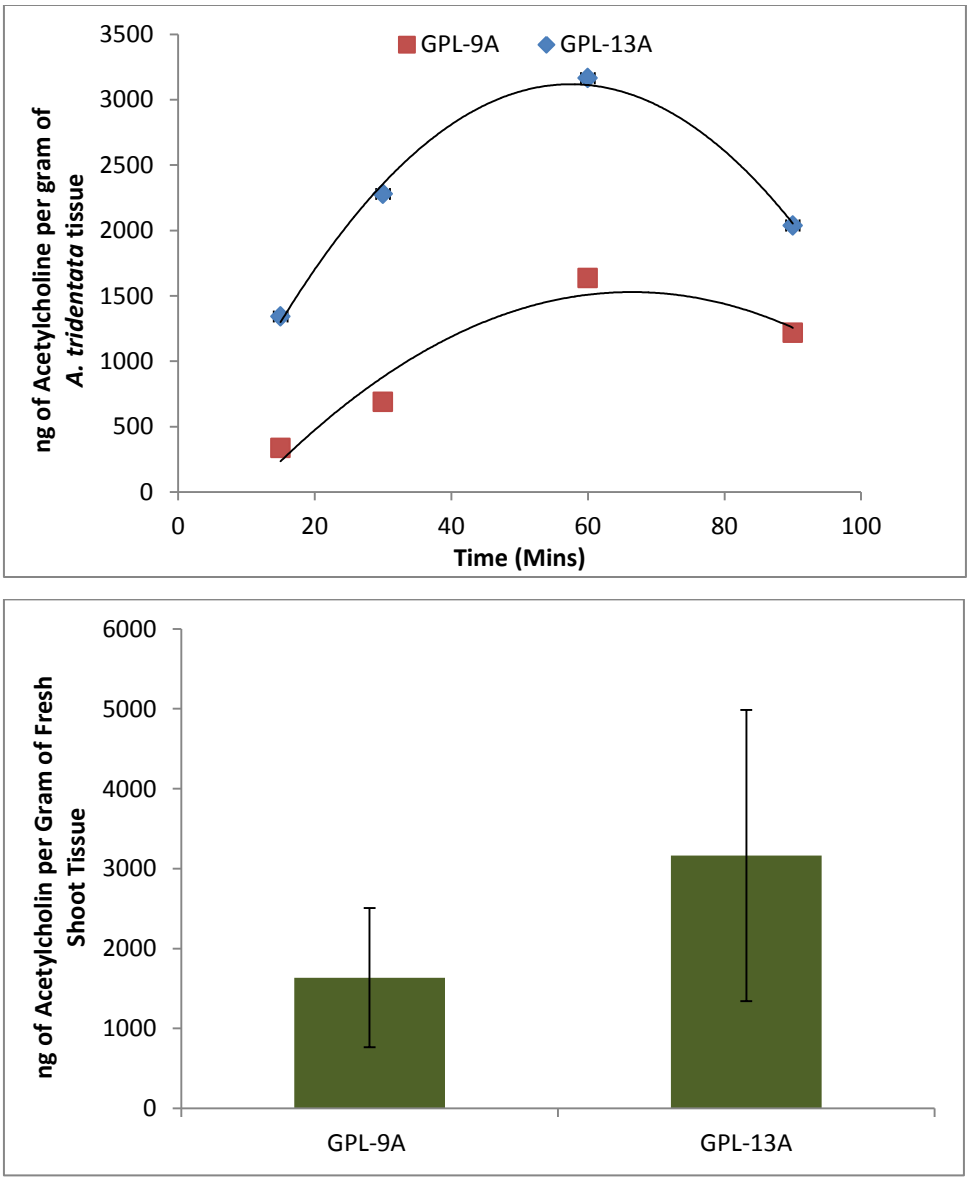


Figure 4.3 Detection and quantification of Ach in axenic shoot tissues of *A. tridentata* germplasm lines.

Error bars represent the standard error of the mean (n = 3 replicates).

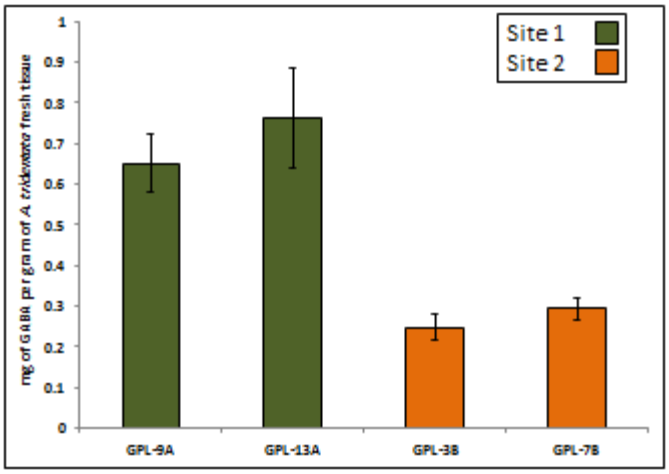


Figure 4.4 Detection and quantification of GABA in axenic shoot tissues of *A. tridentata* germplasm lines.

Error bars represent the standard error of the mean (n = 3 replicates).

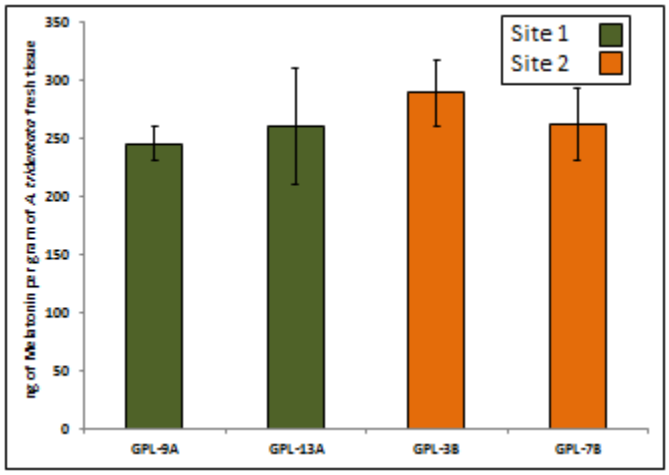


Figure 4.5 Detection and quantification of MEL in axenic shoot tissues of *A. tridentata* germplasm lines.

Error bars represent the standard error of the mean (n = 3 replicates).

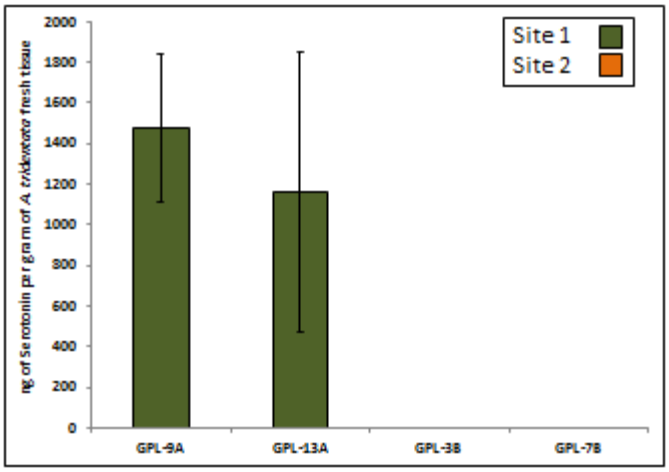


Figure 4.6 Detection and quantification of 5HT in axenic shoot tissues of *A. tridentata* germplasm lines. Error bars represent the standard error of the mean (n = 3 replicates).

4.4.3 Anti-AchE Activity

Inhibition of AchE was observed in both culture and field collected *A. tridentata* MeOH extracts. Field collected leaf material for *A. tridentata* (0.5 mg +/- 0.05 mg) was significantly more potent then culture grown (2.8 mg +/- 1.1 mg) with respect to inhibiting V_{max} by 50% of the control AchE activity (Figure 4.7).

4.4.4 Metabolomic Analysis

PCA using PC1 and PC2 captured 64.3% of existing metabolic variability, and revealed that metabolomes significantly differed between field and culture grown *A. tridentata* tissues (Figure 4.8). The average number of compounds detected in *in vitro* grown tissues (17, 930 +/- 1072) was significantly less then wild-harvested *A. tridentata* tissue (41, 120 +/- 741) (Table 4.1). The field collected tissue contained more than eight times the number of unique compounds (11, 087) compared to *in vitro* grown tissues (1, 377) (Table 4.1). Further analysis revealed the presence of 52 significant ions present in all samples (Table 4.1), of which 7 were putatively identified as monoterpene (n=2), phenolic (n=3), or sesquiterpene (n=2) compounds (Table 4.2). A greater number of significant ions was detected in wild-harvested tissues (n=90) than culture grown (n=27) (Table 4.1). Of these, 1 and 16 compounds were putatively identified in *in vitro* grown and wild-harvested tissues respectively (Table 4.2). 69% and 100% of significant ions putatively identified in culture or field collected tissues were sesquiterpene (Table 4.2).

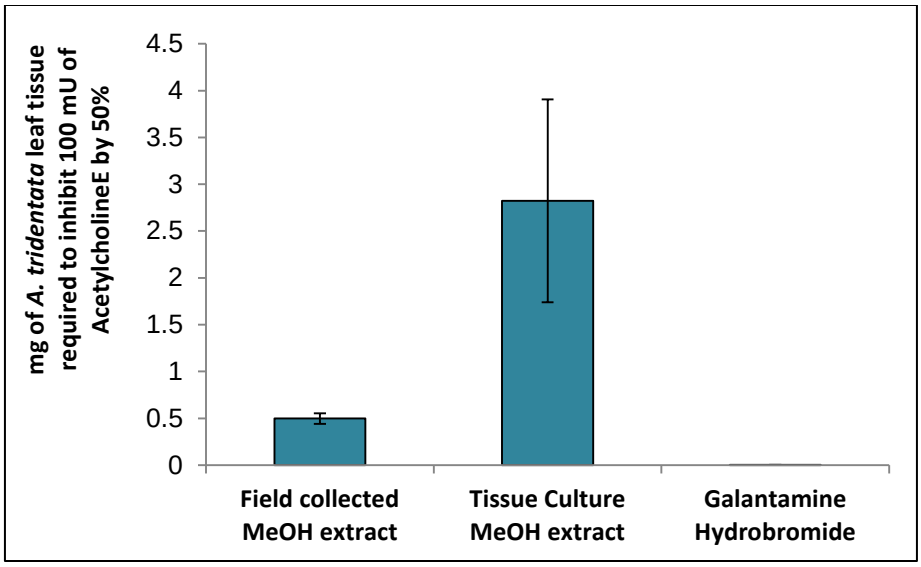


Figure 4.7 Determination of inhibition of AchE activity of methanol extracts of field-grown and axenic cultures of *A. tridentata*. Error bars represent the standard error of the mean (n = 3 replicates).

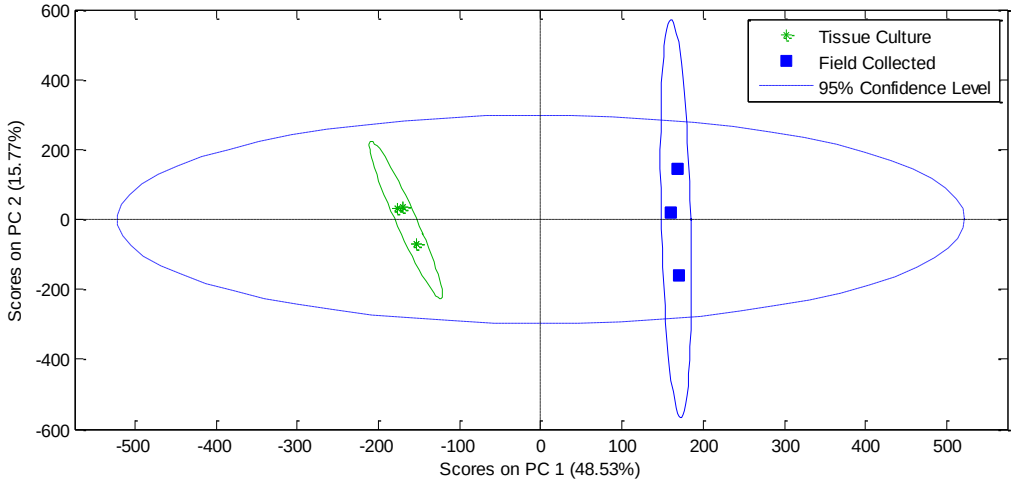


Figure 4.8 PCA of biologically active *A. tridentata* extracts.

Table 4.1 Summary of metabolomic data.

Summary of Metabolomic Data		
	Tissue Culture	**Field Collected**
Total Number of compounds Observed	33,752	55,955
Average Number of Compounds per Sample	17,930 +/- 1072 (%RSD = 10.35)	41,120 +/- 741 (%RSD = 1.80)
Compounds Common to All Samples	1,543	
Number of Compounds Only Found in Culture or Field Collected Tissues	1,377	11,087
Number of Significant Ions Common to All Samples	52	
Number of Significant Ions Only Found In Culture or Field Collected Tissues	27	90

Table 4.2 Putative identification of significant ions present in *A. tridentata* field and culture collected leaf tissues.

Putative Identification of Significant Ions Found in All Samples			
Ret. Time	Mass	Average Ion Count	Putative Identification
11.7757	249.1472	8.9	[S] 8-deoxycumambrin B, arbusculin-C, deacetyllaurenobiolide, rothin-A
7.0888	433.1339	9.7	[P] apigenin 7-0-glucoside
14.9044	233.1523	13.5	[S] arbusculin-B
5.2042	355.1117	31.9	[P] esculin 7-methylether (methylesculin)
5.0115	193.1116	12.7	[M] lyratyl acetate, sabinyl acetate
12.7954	361.0961	25.0	[P] quercetagetin-3,6,7-trimethyl ether
8.0415	169.1452	8.5	[M] santolinic acid, trans-3-(1-oxo-2-methyl-2-propenyl)-2,2-dimethylcyclopropylmethanol
Putative Identification of Significant Ions Unique to Field Tissue			
Ret. Time	Mass	Average	Putative Identification
8.7088	153.1419	3.1	[M] 2,3-dimethyl-6-isopropyl-4*H*-pyran, 2-Isopropenyl-5-methylhexa-trans-3,5-dien-1-ol , α-Thujone, artemiseole, artemeseole, arthole,artemisia ketone, β-Thujone, fenchone, isolyratol, lyratol,myrtenol,neral,oxidosantolinatriene,sabinol, santolina epoxide, trans-pinocarveol
14.272	167.1082	4.2	[M] santolinolide-A, santolinolide-B, santolinolide-B', satonlinolide-C
19.224	183.1355	11.6	[M] methyl santolinate, santolinyl ester
21.4477	233.152	6.8	[S] arbusculin-B
15.4297	249.1367	12.4	[S] 8-deoxycumambrin B, arbusculin-C, deacetyllaurenobiolide, rothin-A
17.207	249.1388	4.8	[S] 8-deoxycumambrin B, arbusculin-C, deacetyllaurenobiolide, rothin-A
15.724	249.1406	4.0	[S] 8-deoxycumambrin B, arbusculin-C, deacetyllaurenobiolide, rothin-A
16.8333	249.1407	15.4	[S] 8-deoxycumambrin B, arbusculin-C, deacetyllaurenobiolide, rothin-A
15.0636	249.1596	4.2	[S] 8-deoxycumambrin B, arbusculin-C, deacetyllaurenobiolide, rothin-A
14.4797	251.1566	6.8	[S] 1-β-hydroxysant-3-en-6,12-olide-C, 1-β-hydroxysant-4(14)-en-6,12-olide-C, arbusculin-A
10.2153	265.1217	20.5	[S] ridentin
15.1179	267.1475	3.7	[S] arbusculin D
13.8551	267.1562	8.7	[S] arbusculin D
13.0774	279.1389	17.6	[S] artecanin, canin
12.0512	331.0614	18.2	[P] quercetin 7,3'-dimethyl ether (rhamnazine)
13.0122	361.0748	12.4	[P] quercetagetin-3,6,7-trimethyl ether
Putative Identification of Significant Ions Unique to Tissue Culture			
Ret. Time	Mass	Average Ion Count	Putative Identification
6.5911	305.1591	9.3	[S] matricarin

[M] Monoterpene, [P] Phenolic, [S] Sesquiterpene,

4.5 Discussion

Historically, Big Sagebrush occupied 62 million hectares of land in North America and in recent years it has been suggested that only 56% of its original distribution remains (Davies et al., 2011). The establishment of *in vitro* propagation methods for *A. tridentata* provided a ready source of plant material grown in a sterile controlled environment for chemical analysis and bioassays, as well as a source of shoot material for long term potential conservation of the genetic resources in cryopreservation and a source of plants for ecological restoration projects. Plants that commonly grow in desert climates can be difficult to maintain in the moist *in vitro* environment (Liu et al., 2004) but the optimized nutrient media determined in these studies effectively mediates the water balance and hygroscopic damage was not observed.

This study provides the first evidence of the presence of Ach, GABA, MEL, and 5HT in the genus *Artemisia*, and indicates that significant differences between selected populations. MEL was first described in growing plants in 1997 in a study of plants used in the treatment of neurological disorders (Murch et al., 1997). Although work in 2000 reported the biosynthesis of MEL and 5HT in axenic cultures of St. John's wort (Murch et al., 2000), it is only recently that the genetic basis and regulation of the indoleamine pathway has begun to be characterized (Park et al., 2013; Okazaki et al., 2010). There have been several hypothesized physiological roles for MEL and 5HT in plants. Alterations of the ratios of MEL and 5HT in *in vitro* cultured explants were found to significantly alter root and shoot development (Murch et al., 2001; Jones et al., 2007; Murch and Saxena, 2002; Murch and Saxena, 2004). MEL has been shown to protect plant tissues from damage induced by reactive oxygen species in photosynthesis (Lazár et al., 2013) as well as other antioxidant responses (Galano et al., 2011; Park et al., 2013; Paredes et al., 2009). MEL levels were found to accumulate during specific phases of flower and seed development indicating a potential role in plant reproduction (Murch et al., 2009; Murch et al., 2010; Murch and Saxena, 2002).

The related indoleamine 5HT has been found in many different plant species and tissues (Roshchina, 2001). In particular, 5HT has been associated with tendrils, shoots and pulvini associated with plant movements (Applewhite, 1973). There are several proposed roles for 5HT in plant tissues including detoxification of by-products of metabolism (Grobe,

1982) and regulation of *de novo* regeneration, root and shoot development (Murch et al., 2001; Murch and Saxena, 2004). It is interesting that this analysis quantified 5HT in only one of the populations of *A.tridentata* indicating that detectible levels of 5HT may not be required for plant growth. Similar to ecological studies (Karban et al., 2010), these data may be indicative of the potential physiological and biochemical diversity in the wild populations or the effects of differing microclimates.

GABA has been found in many plant tissues and the metabolism is well established (Bown and Shelp, 1997; Bown and Shelp, 1989). In plants, glutamate is decarboxylated in order to produce GABA which then undergoes other modifications in order to enter the tricarboxylic acid cycle as succinate (Seifi et al., 2013). Genetic regulation of the GABA shunt has been investigated extensively in plants due to its importance in facilitating healthy plant growth by maintaining adequate C:N ratios through nitrogen storage (Seifi et al., 2013; Fait et al., 2008; Shelp et al., 2012). Thus, it is not surprising that higher levels of GABA have been found in angiosperm plants compared to more primitive groups such as gymnosperms, algae and lichens (Seher et al., 2013). The mechanisms of GABA and the GABA shunt in plants seem almost to mimic the neurological activity of GABA in humans, where the GABA shunt can deplete glutamate under conditions of oxidative stress (MaMELak, 2012). For example, production of GABA has been found to occur in response to many environmental signals (Wallace et al., 1984) including insect feeding (Shelp et al., 2003). This analysis quantified GABA concentrations that were significantly higher in plantlets collected from one of the two locations. Interestingly, higher levels were detected in the same plants containing detectible levels of 5HT indicating the potential for cross-talk or shared responses between these metabolites.

Ach has been described in plants in the early 1970s (Hartmann and Kilbinger, 1974) and has been found in almost all plant tissues (Tretyn and Kendrick, 1991; Roshchina, 2001). In roots and dark adapted tissues, higher concentrations of Ach have been observed (Roshchina, 2001; Hartmann and Kilbinger, 1974; Tretyn et al., 1997; Wisniewska and Tretyn, 1999) leading to the hypothesis that Ach may be involved in phytochrome-linked signalling (Jaffe, 1970), phytochrome-modulated calcium signaling (Mehta et al., 1993), induction of primary and secondary rooting (BaMEL et al., 2007), regulation of phloem

transport (Yang et al., 2007) and regulation of pollen tube development (Tezuka et al., 2007). Targeted data showing Ach in *in vitro* grown leaf tissues of *A. tridentata* provides the foundation for further studies of the role of this potential plant growth regulator in *Artemisia* development.

Similar to other studies with other *Artemisia* species, bioassay data for *A. tridentata* indicates the presence of phytochemicals that inhibit AchE (Liu et al., 2012; Orhan et al., 2010). Natural inhibitors of AchE are attractive drug targets for treatment of Alzheimer's disease and other neurological ailments (Mukherjee et al., 2007). While many plants contain compounds that inhibit the enzyme, the role of these regulators in the physiology and biochemistry of the plants has not been established. It is interesting to note that the AchE capacity of wild-harvested plants was significantly greater than the inhibitory capacity of *in vitro* grown plants. Generally, *in vitro* grown plants are exposed to less stress and accumulate less specialized metabolites than plants that face the daily challenges of a native landscape such as fall temperatures, cold nights, insect or animal feeding, soil microbiota, plant maturity, communications with neighbouring plants (Karban et al., 2010) and a host of other factors.

Metabolomics has been defined as the qualitative and quantitative analysis of all existing metabolites found within a biological sample at a specific time (Yuliana et al., 2011) and can be used as a tool for discovery of novel biologically active compounds (Brown and Murch, 2012). Recently, we proposed mining of untargeted metabolomics datasets to discover metabolic families, environmental responses and specialized metabolites (Turi and Murch, 2013a). In the current study, we compared wild-harvested tissues with *in vitro* cultures generated from seeds of the same plants. There was significantly greater phytochemical complexity in wild-harvested *A. tridentata* leaf tissues than *in vitro* grown leaves. This increase in phytochemical complexity corresponds to the observed AchE activity. Putative metabolite identifications indicated more monoterpenes and sesquiterpenes in field collected tissues than *in vitro* cultures. These data are consistent with previous studies that demonstrated lower concentrations of plant secondary metabolites in *in vitro* grown medicinal plants and suggested that elicitors provided by other species in the environment contribute to the chemical diversity (Jones et al., 2007; Jones et al., 2009; Jones

et al., 2009). In *Artemisia*, monoterpene and sesquiterpene compounds are produced in the glandular trichomes present on leaf surfaces (Ruhland et al., 2013; Polichuk et al., 2010). SEM images indicate that there were glandular trichomes on both *in vitro* grown and wild harvested leaves, however I did not determine the contents of the structures or whether physiological differences existed such as stage of maturity or development. Plant age, microorganisms, environmental stresses, nutrient availability and other factors can affect the production of both glandular trichomes and the associated specialized metabolites (Ruhland et al., 2013; Biswas et al., 2009; Lommen et al., 2006; Chaudhary et al., 2008). Thus, the current protocol is adequate for the conservation of plant tissues but may need to be optimized through modification of growth hormones, vitamins, or sucrose levels (Nguyen et al., 2013) or elicitors (Jones et al., 2009) for production of specific medicinal phytochemistry.

The phytochemistry of *A. tridentata* provides a rich and diverse resource for discovery of biologically active compounds. Previous research identified a wide-spectrum of monoterpenes and sesquiterpenes in *A, tridentata* and close relatives, however the phytochemical underpinnings of reported biological activity are not fully understood (reviewed in Turi et al. 2014a). Given the long record of traditional use for *A. tridentata* by First Nations and Native American peoples (Moerman, 2009), the observation that MeOH extracts inhibited AchE helps to support its medicinal use in North America. We have established an effective protocol for growth of *A tridentata* plants *in vitro* to provide a continuous source of tissue for phytochemical analysis, determinations of medicinal efficacy, commercial production, and/or preservation of wild germplasm. Although further work is required to understand how the phytochemistry and biological activity of *A. tridentata* varies within and between *ex situ* and *in vitro* grown populations, mining of the metabolomics datasets provides a hypothesis-generating technique for future studies to optimize tissue culture protocols so that model systems can better reflect the phytochemical diversity *ex situ*.

Chapter 5 Metabolomics as a Hypothesis Generating Technique to Understand Plant Signaling and Behavior

5.1 Synopsis

Previous work has shown that many synthetic and natural drugs that affect human neurotransmitter metabolism can also mediate plant regeneration (Murch et al., 2001; Jones et al., 2007; Murch and Saxena, 2004). The current study was designed to extend upon pervious findings that serotonergic drugs mediate root and shoot development in plants in order to investigate whether manipulation of cholinergic signaling will affect auxin and indoleamine responses in plants. Furthermore, this research aims to investigate whether metabolomics can be used as a hypothesis generating technique for understanding plant signalling and behavior. Galanthamine is a naturally occurring AchE inhibitor that has been well established as a drug for treatment of mild to moderate Alzheimer's disease but the role of the compound in plant metabolism is not known. Exposure of *A. tridentata* to 10 µM galanthamine in axenic cultures significantly altered plant growth in a manner reminiscent of auxin toxicity, significantly decreased the concentration of endogenous Ach, auxin and MEL, and significantly inhibited the activity of AchE. While plants exposed to galanthamine had lower levels of auxin, galanthamine itself demonstrated auxin activity in a classical auxin bioassay. Statistical analysis identified about 2200 significant differences in the phytochemical profiles of *A. tridentata* plantlets exposed to 5 or 10 µM galanthamine indicating significant metabolic mediation. Metabolomic analysis putatively identified coumarins scopoletin/isoscopoletin, and scopolin in *A. tridentata* leaf extracts and these metabolites linearly increased in response to galanthamine treatments.

5.2 Research Objectives and Hypothesis

In order to investigate the potential role of the cholinergic system in plant growth and development, I hypothesized the following: 1) that the AchE inhibiting natural product galanthamine redirects morphogenesis in *A. tridentata* cultures by altering the concentration of endogenous plant neurochemicals Ach, MEL, 5HT and the plant hormone auxin and 2) that metabolomics can be used as a hypothesis generating technique to understand plant signalling and behavior. To investigate this hypothesis, I designed the following specific objectives: 1) to quantify plant neurochemicals and auxin in *in vitro* cultures of *A. tridentata;* 2) to determine whether inhibition of plant cholinergic signalling affects MEL, 5HT and/or

auxin in *A. tridentata;* and 3) to develop and analyze a metabolomics dataset as a hypothesis generating tool for abiotic stresses.

5.3 Materials and Methods

5.3.1 Establishment of Germplasm Lines

A collection of axenic stock plants of *A. tridentata* plants was established from individual seeds *in vitro* with previously established protocols (see Chapter 4) and maintained on a sterile medium hereafter referred to as "MSO" and containing 4.4g/L Murashige and Skoog salts (Murashige and Skoog, 1962) with B5 vitamins (Gamborg et al., 1968) and 30 g/l sucrose at pH 5.7. 3g/L phytagel was added to all media and were solidified with phytagel prior to autoclaving for 20 minutes at 121^0C at 1.4 x 10^4 kg/m/s^2. Germplasm lines were established by regeneration of shoot cultures from individual seedlings as described previously (Chapter 4), maintained at standard temperature (28^0C) with a 16 hour photoperiod (35-50 µmol/m^2/s) and sub-cultured on MSO medium into GA7 boxes (Magenta Corp.) at 2 week intervals.

5.3.2 Galanthamine Treatments

Shoot tissue from the *A. tridentata* germplasm line DCO-1-09 (described in Turi et al., 2013) was sub-cultured onto MSO medium in Petri dishes (n=`12). For each treatment (n=4), a solution of galanthamine hydrobromide (1.0 mL of 0, 5, or 10 µM; Chromadex, Vancouver) was filter sterilized (Millipore PVDF, 0.22 µm, 25 mm sterile syringe filters; Fisher Scientific). An aliquot of 1.0 mL was layered onto the sterile media twice weekly for a period of 1 month to ensure a continual dose. At the end of the experiment, tissues were excised, flash frozen and stored at -80 ˚C until further testing.

5.3.3 Preparation of Extracts

A method was developed for analysis of neurotransmitters, auxin and antioxidant potential in the same extract to ensure valid data comparison. In brief, an explant of *A. tridentata* leaf tissue was extracted 1:2 (v:v) in MeOH in complete darkness and an aliquot of 100 µL of each sample was dried under nitrogen gas and resuspended in 18 mΩ water (Millipore). Remaining MeOH extract was stored at -80 ˚C for further analysis.

5.5.4 Quantification of Ach and AchE

The cholinergenic system was quantified with a Amplex© Red Ach / Aceytlcholinesterase Assay Kit (Molecular Probes Inc. Eugene, Oregon). Ach was

quantified in tissues using a modification of the spectrophotometric method originally described in 1961 (Ellman et al., 1961) (see Chapter 4).

5.5.5 Analysis of MEL, 5HT, and Auxin

Auxin and indoleamines were separated from a 1 µL aliquot of the extract on a reverse phase column (150 x 2.1 mm, 1.7 µm C18 BEH, Waters) using a Waters I-Class UPLC. A gradient of 0.1% formic (Eluent A) and acetonitrile (Eluent B) [(A%:B%): 0.0-0.5 min, 99:1; 0.5-2.5 min, 70:30; 2.5-2.6 min, 5:95; 2.6-3.0 min, 5:95; 3-3.5 min, 5:95; 3.5-3.6 min, 99:1; 3.6-5.0 min, 99:1] separated MEL, 5HT and auxin with a flow rate of 0.5 ml/min. Analytes were detected and quantified with a tandem mass spectrometer (Xevo TQ-S triple quadrupole mass spectrometer, Waters, Mississauga) using detection parameters optimized for signal intensity and specificity (Table 5.1). The capillary voltage was 3500, the desolvation gas was 800 L/hr, the cone gas was 150 L/hr, the desolvation temperature was 400^0C and the source temperature was 150^0C for all analyses. Authentic standards were injected over a wide range to establish the limits of detection (LOD) and limits of quantification (LOQ). The linear ranges were 1-100 ng/mL for MEL and 1-500 ng/mL for 5HT and Auxin. In preliminary experiments it was determined that a gradient of auxin and indoleamine was present across the plant tissues. Therefore, to determine the response to galanthamine, plantlets were subsampled by sectioning into 3-5 pieces (259 ± 10 mg) prior to analysis and each subsample was analyzed in triplicate.

5.5.6 Auxin and Indoleamine Data Analysis

Indoleamines and auxin were quantified by comparison to the linear range of standard curves established with authenticated standards (Sigma). Data for each plantlet was determined by a proportioned approach. In brief, the fresh weight of each excised section was divided by the total weight of the plantlet it originated from in order to scale the proportionate MEL, 5HT and auxin concentrations in the entire tissues.

Table 5.1 Optimized detection of indoleamines and auxin by tandem mass spectrometry (Waters Xevo TQ-S).

	Cone voltage (V)	Collision voltage (V)	MRM Transition
Auxin	30	25	176 > 103
Auxin	30	13	176 > 130
5HT	45	27	177 > 115
5HT	45	10	177 > 160
MEL	30	23	233 > 159
MEL	30	15	233 > 174

5.5.7 Antioxidant Potential

Antioxidant potential was determined in the same extracts with a DPPH bioassay as described previously in Chapter 3 (Turi and Murch, 2013a).

5.5.8 Auxin Bioassay

Auxin activity was determined using an oat (*Avena sativa*) coleoptile elongation bioassay as previously described (Sirois, 1966). In brief, the coleoptile tips (about 0.3 mm) were removed and 10 mm sections were incubated in 10 ml of sterile distilled water supplemented with various concentrations of galanthamine (0, 0.05, 0.1, 1. or 10 µM), 1-naphthaleneacetic acid (5 µM), or auxin (5 µM) in 15 mm petri plates. After 24 hours, the coleoptiles were measured again to determine the degree of elongation. Each plate contained ten sections and each treatment was replicated thrice for a total of 30 sections per treatment. The plates were arranged in a completely randomized design and the experiment was conducted twice.

5.5.9 Metabolomic Analysis

A. tridentata shoot cultures treated with 0, 5, and 10 µM of galanthamine were sampled in triplicate and subjected to untargeted phytochemical analysis using previously published protocols (Turi and Murch, 2013a; Brown et al., 2012a; Brown et al., 2012b) (see Chapter 3).

5.5.10 Analysis of Metabolomic Data

Average ion intensity was calculated for each compound in order to select for metabolites which are most prominent throughout all treatments and replicates. Once ion averages were calculated they were divided by the average total ion count in order to identify compounds which possess average ion intensities greater than 5.0 (0.09% of the average total

ion intensity). Using excel, average ion intensity for each prominent ion (dependent) was plotted against 0, 5, and 10 µM galanthamine treatments (independent), in order to determine whether a linear relationship exists. Compounds exhibiting exponential or linear behaviour ($R^2 > 0.75$) in response to galanthamine treatments were then subjected to putative identification using Metabosearch http://omics.georgetown.edu/MetaboSearch.html or the Plant Metabolic Pathway Database http://www.plantcyc.org/. Putative identifications (±0.02 daltons) were performed using previously published methods (Turi and Murch, 2013a).

5.6 Results

5.6.1 *In vitro* Regeneration

A. tridentata shoot proliferation was sustained on basal MSO medium and altered morphologies were observed in response to galanthamine (Figure 5.1A-C). Treatment with 5 µM galanthamine appeared to increase the leaf length and reduce the turgor. Cultures treated with 10 µM galanthamine appeared to have increased yellowing, leaf curling, and vitrification compared to the control or 5 µM treatments (Figure 5.1A-C).

5.6.2 Ach and Anti-AchE Activity

Ach concentration in *A. tridentata* cultures was significantly reduced by treatment with 10 µM galanthamine (45.9 ± 3.01 µg/g) as compared to the control (92.8 ± 12.6 µg/g) or 5 µM galanthamine treatment (98.4 ± 3.01 µg/ g) (Figure 5.2A). Exposure of plant tissues to 10 µM galanthamine also significantly decreased the activity of AchE in the tissues (454.1 ± 19.8 mU/g) as compared to the control (1004.6 ± 178.4 mU/ g) and 5 µM galanthamine treatments (1129.1 ± 207.3 mU/ g) (Figure 5.2B).

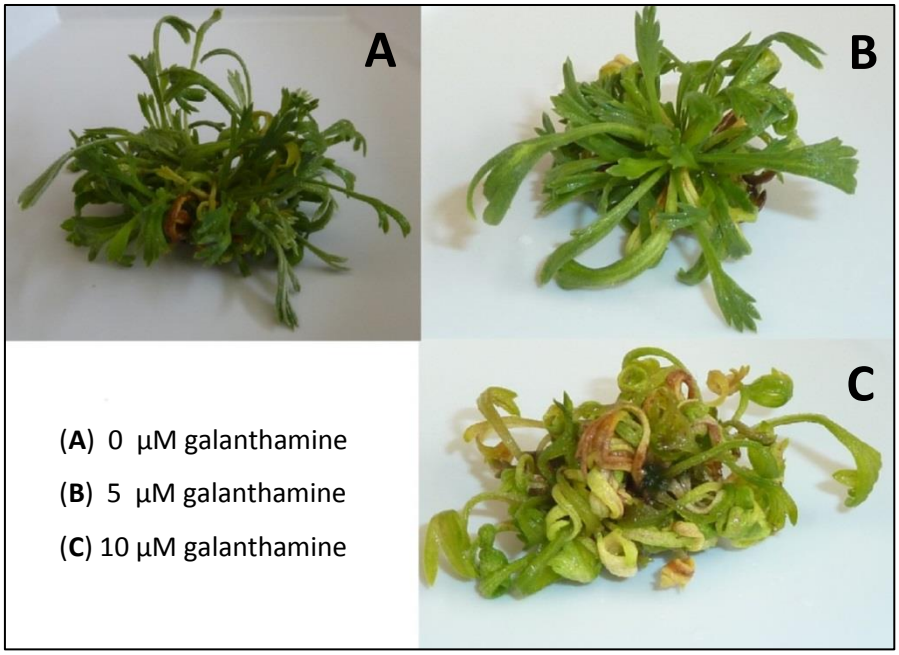


Figure 5.1 Effect of galanthamine on the growth and development of *A. tridentata* cultures. (**A**) 0 µM galanthamine; (**B**) 5 µM galanthamine; (**C**) 10 µM galanthamine. Pictures taken by Christina E. Turi.

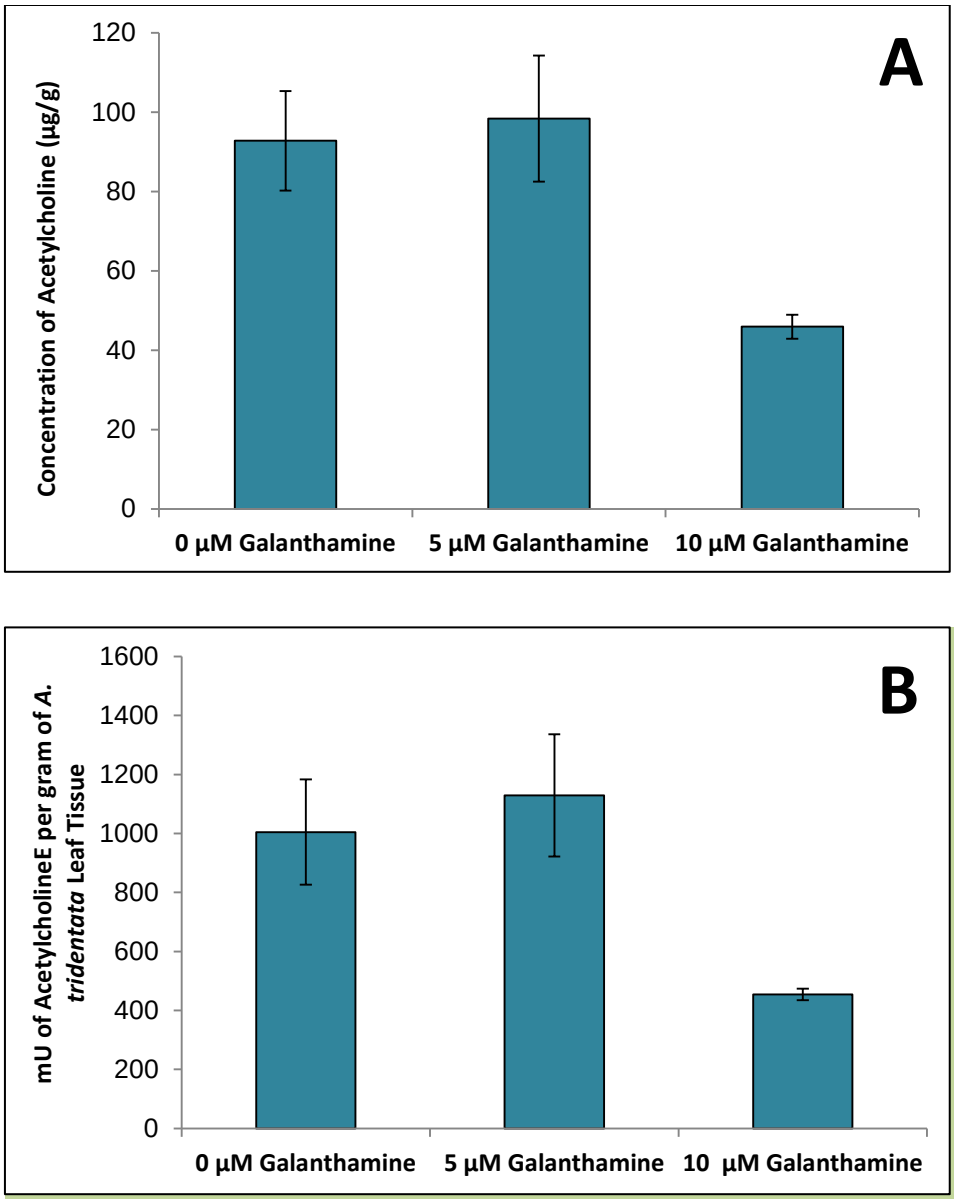


Figure 5.2 Effect of galanthamine on Ach concentration and AchE activity in *A. tridentata* cultures. (A) Ach concentration; (**B**) Inhibition of AchE. Error bars represent the standard error of the mean (n = 4 replicates).

5.6.3 Effects of Galanthamine on Auxin and Indoleamines

The detected concentration of MEL was linearly decreased in response to galanthamine treatment (R^2=0.991), and significantly differed between control (9.6 ± 3.1 ng/g) and 10 µM treatments (2.3 ± 0.9 ng/g) (Figure 5.3A). Interestingly, 5HT levels were significantly decreased in response to 5 µM galanthamine (10.7 ± 3.6 ng/g) as compared to control (37.1 ± 16.5 ng/g) and 10 µM treatments (35.4 1 ± 15.3 ng/g) (Figure 5.3B). Auxin

levels were also found to decrease in a linear response to galanthamine treatment (R^2=0.9989), but the overall differences between treatments were not significant (Figure 5.3C).

5.6.4 Analysis of Oxidative Stress in Galanthamine Treated Cultures

Quantification of the antioxidant potential of control and galanthamine-treated tissues by DPPH bioassay indicated that significantly less tissue was required to inhibit DPPH by 50% in 5 µM (473 ± 42 µg) and 10 µM (529 ± 87 µg) treatments as compared to the control (930 ± 83 µg) (Figure 5.4).

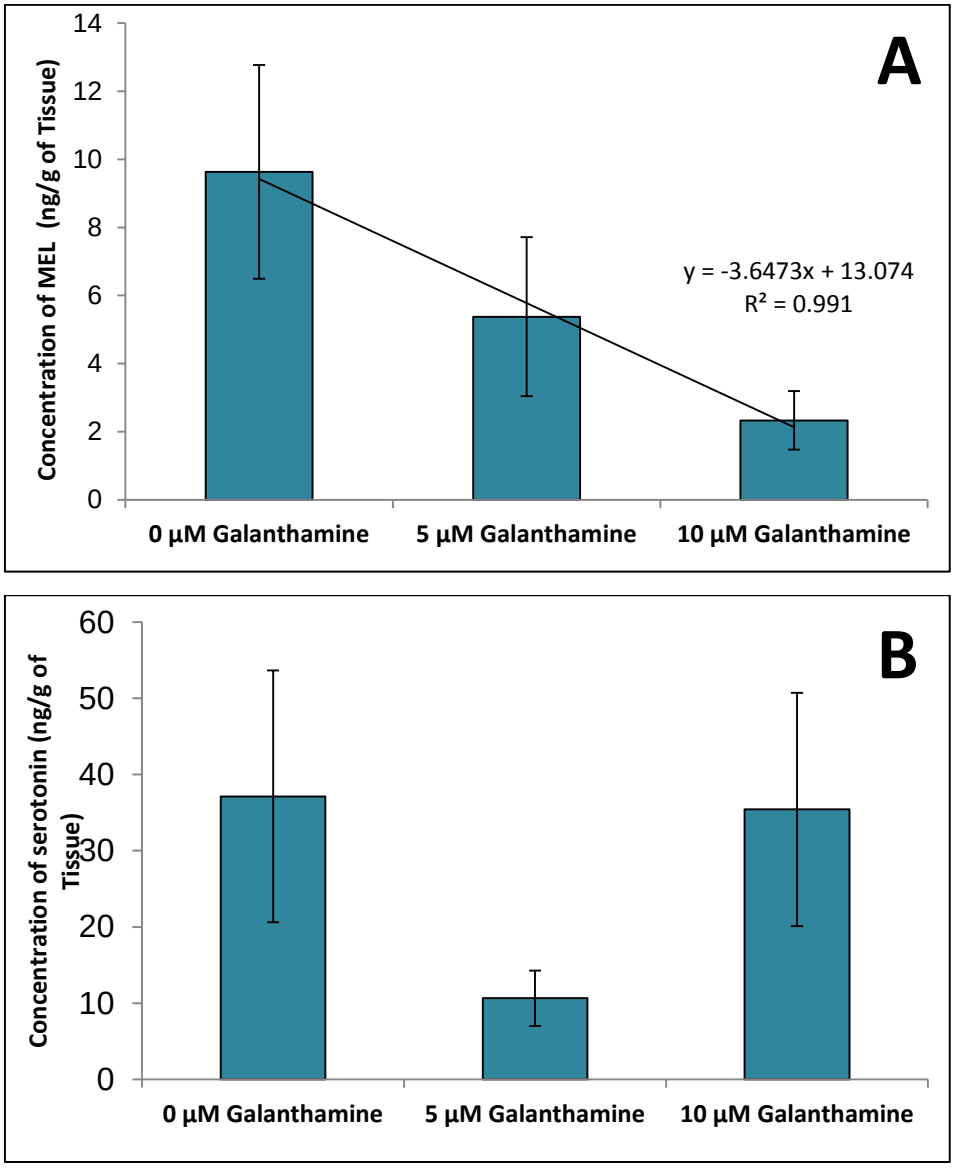


Figure 5.3 Effect of galanthamine on indoleamine and auxin in *A. tridentata* cultures. (**A**) Effects on MEL; (**B**) Effects on 5HT; (**C**) Effects on auxin. Error bars represent the standard error of the mean (n =4 replicates).

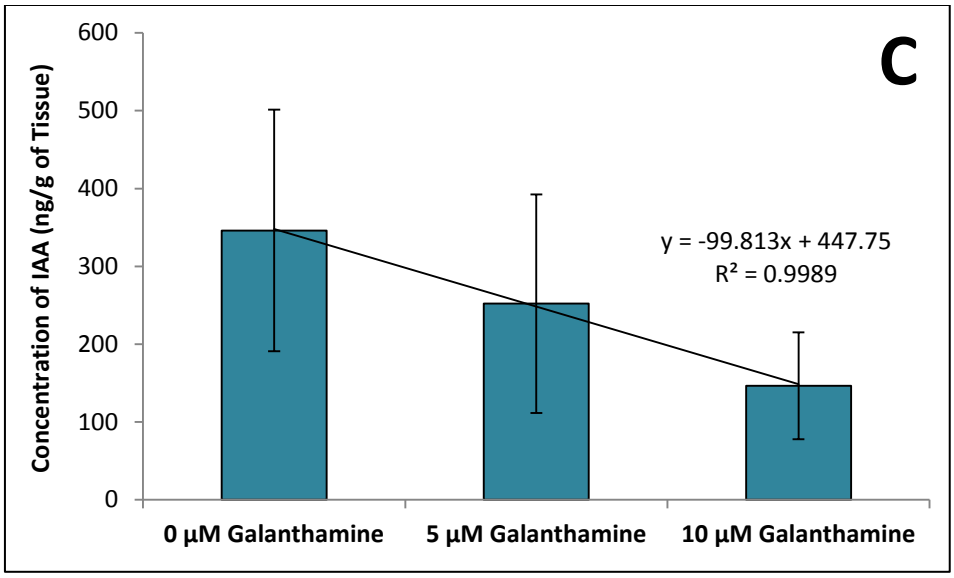


Figure 5.3 Effect of galanthamine on indoleamine and auxin in *A. tridentata* cultures. (**A**) Effects on MEL; (**B**) Effects on 5HT; (**C**) Effects on auxin. Error bars represent the standard error of the mean (n =4 replicates).

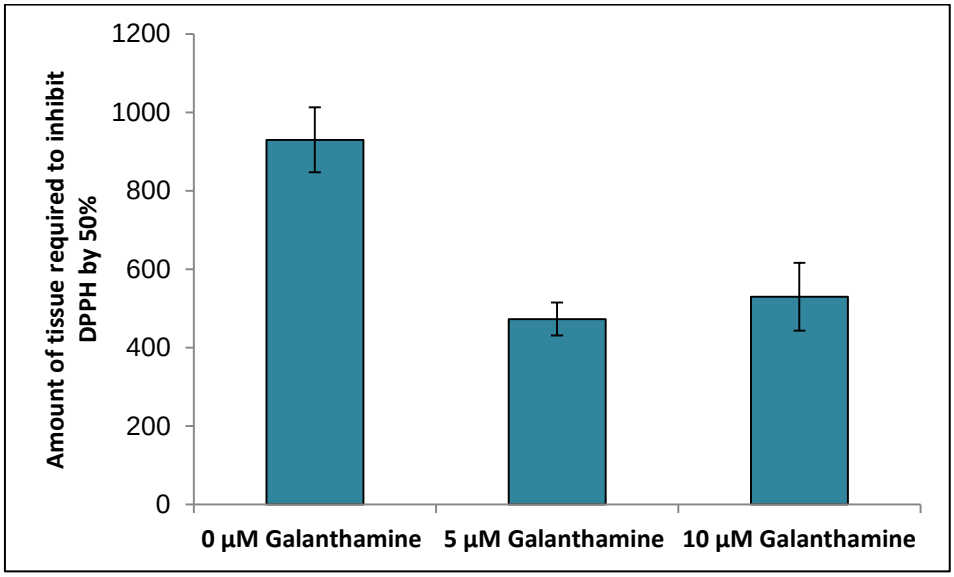


Figure 5.4 Effect of galanthamine on the antioxidant capacity of *A. tridentata* tissues grown *in vitro*. Error bars represent the standard error of the mean (n = 4 replicates).

5.6.5 Activity of Galanthamine in an Auxin Bioassay

Inclusion of galanthamine in the solution significantly increased the elongation of oat coleoptiles in a manner similar to the classic auxin response (Figure 5.5). Exposure of the coleoptiles to 20 µM galanthamine significantly increased the growth as compared to

controls and was not significantly different from the auxin response observed with either 5 µM auxin or 5 µM 1-naphthaleneacetic acid.

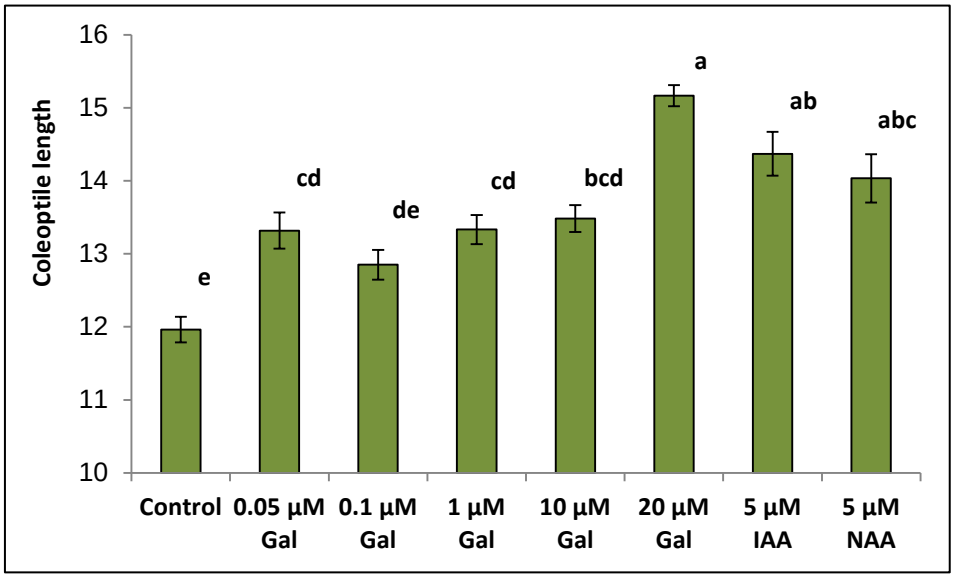


Figure 5.5 Galanthamine has auxin-like activity in an oat coleoptile bioassay. Gal = galanthamine; IAA = auxin, and NAA = 1-naphthaleneacetic acid. Error bars represent the standard error of the mean (n = 30 sections per treatment). Significance between treatments was determined using the Tukey means separation test (α=0.05).

5.6.6 Untargeted Phytochemical Analysis on Galanthamine Treated Cultures

The metabolomes of *A. tridentata* shoot cultures contained more than 20,000 distinct compounds (Table 5.2) with about 27,000 detected in control and 5 µM galanthamine treatments. Fewer compounds were detected in the 10 µM galanthamine-treated tissues than the control or 5 µM galanthamine treatments. Scores plots for PC1 (23.12%) and PC2 (19.28%) showed significant clustering for 10 µM treated cultures in contrast to the significant overlap observed between control and 5 µM cultures (Figure 5.6). Of the 20,921-27,891 compounds found, 161 had average ion intensities greater than 5.0 (>0.09% of average total ion count). Linear or exponential responses to galanthamine treatments were observed for 32 (decreasing) and 50 (increasing) compounds (Table 5.3). Putative identifications were performed for all prominent compounds (data not shown). The most significant findings were the putative identification of coumarins (scopoletin/isoscopoletin and scopolin) and their precursor phenylalanine as compounds significantly impacted by the exposure to galanthamine (Table 5.3). The average ion intensity of phenylalanine (m/z =

165.0790) significantly decreased (R^2 =0.9986), while the ion intensities of scopoletin/isoscopoletin (m/z = 192.0423, R^2 = 0.8044) and its glucocylated derivative scopolin (m/z = 354.0951, R^2 >0.95) increased in response to galanthamine treatments (Figure 5.7).

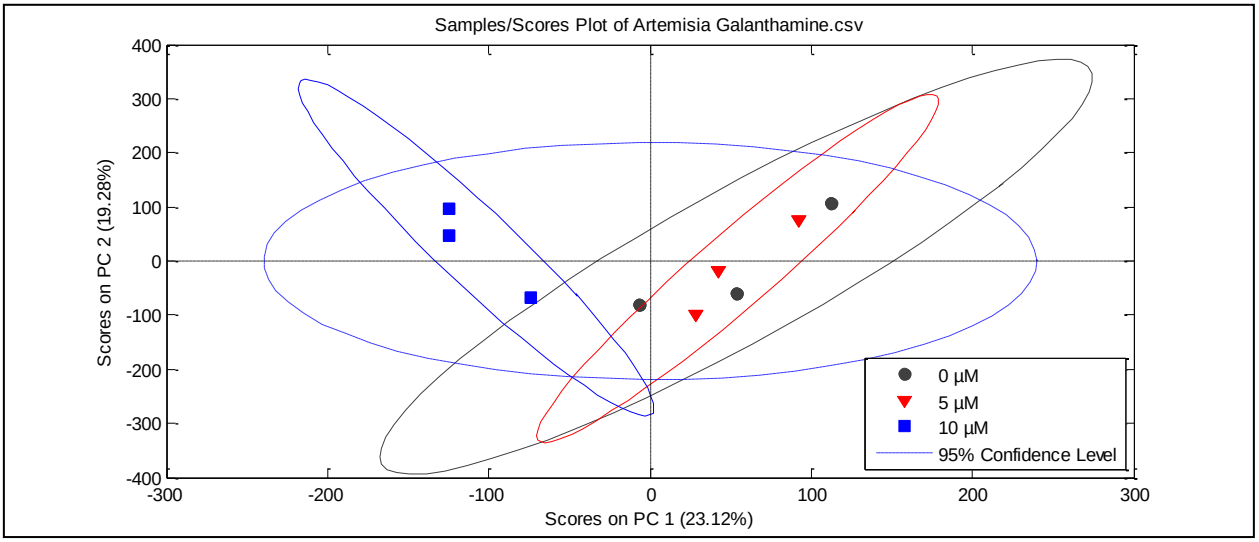


Figure 5.6 PCA identifying the characteristic responses of axenic cultures of *A. tridentata* to galanthamine treatment.

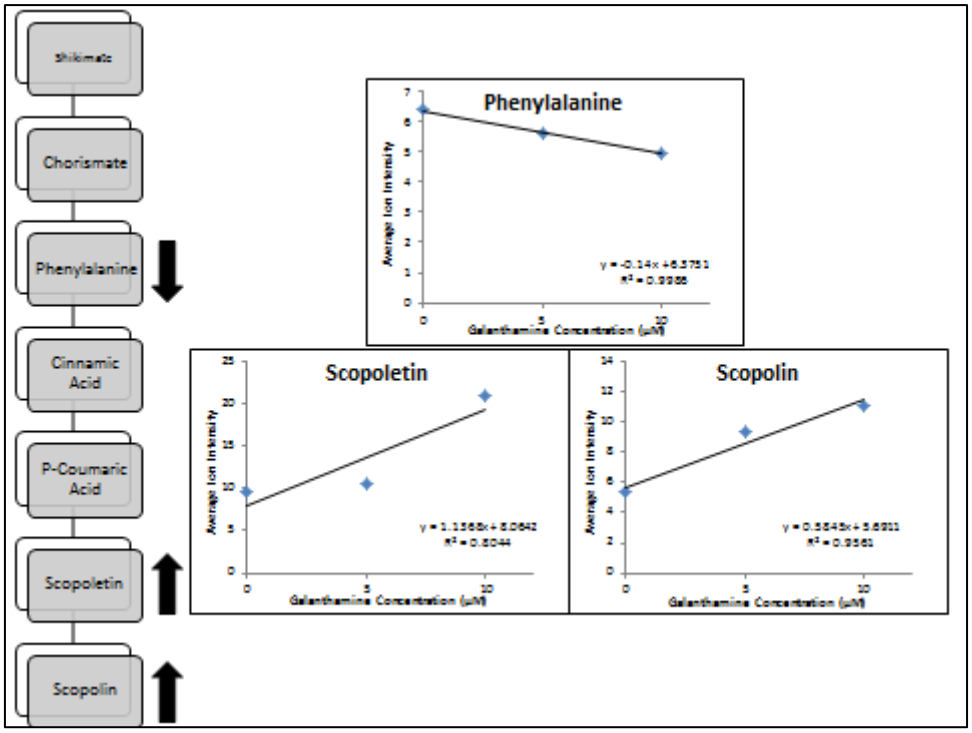


Figure 5.7 Putative identification of prominent ions with linear responses to galanthamine treatment.

Table 5.2 Summary of metabolomic data of galanthamine treated cultures.

	Control	5 µM Galanthamine	10 µM Galanthamine
Total Number of compounds	27,891	27,851	20,921
Average Number of Compounds	16,663 +/- 241	17,083 +/- 491	13,786 +/- 192
Compounds Common to All	6,219	6,799	4,798
Compounds Unique Between Treatments			
	Control	**5 µM Galanthamine**	**10 µM Galanthamine**
10 µM Galanthamine	2,291	2,213	0
5 µM Galanthamine	9137	0	2,213

Table 5.3 Most prominent ions (average ion counts > 5.0 (0.09%)) showing linear or exponential trends in *A. tridentata* plantlets treated with galanthamine.

Decreasing with Treatment					
ID	**Ret. Time**	**Mass**	**Line of Best fit**	**Equation**	**R^2**
1	6.9558	340.0927	Linear	y = -0.2425x + 29.744	0.9929
2	6.0175	241.0592	Linear	y = -0.2425x + 29.744	0.9929
3	13.5308	334.1383	Linear	y = -2.1247x + 28.83	0.8788
4	1.7363	213.0418	Linear	y = -1.0884x + 21.545	0.9578
5	8.9819	434.1102	Exponential	y = 22.657e-0.087x	0.9545
6	6.7	233.113	Exponential	y = 16.501e-0.016x	0.9827
7	1.8035	258.0591	Linear	y = -2.3942x + 26.101	0.9964
8	13.5301	233.1084	Linear	y = -1.4829x + 21.035	0.8013
9	6.0152	276.0961	Linear	y = -0.4833x + 14.438	0.9926
10	6.2874	227.0927	Linear	y = -0.7726x + 15.239	0.9506
11	6.0038	517.1004	Linear	y = -1.4169x + 18.422	0.9510
12	15.1925	505.1853	Linear	y = -1.4143x + 15.892	0.8489
13	1.6532	349.1665	Exponential	y = 16.153e-0.169x	0.9935
14	7.4304	480.1565	Linear	y = -0.6014x + 11.605	0.9843
15	9.9656	434.1394	Exponential	y = 9.5567e-0.04x	0.9144
16	15.2634	375.0408	Linear	y = -0.6859x + 11.272	0.9619
17	8.5777	399.0983	Exponential	y = 9.3338e-0.046x	0.9243
18	6.6231	352.0954	Linear	y = -0.2273x + 8.1602	0.9322
19	9.8383	345.0929	Linear	y = -0.3421x + 8.5447	0.9997
20	12.416	284.1271	Exponential	y = 18.081e-0.391x	0.9544
21	5.658	406.0925	Linear	y = -0.8878x + 10.342	0.9593

Table 5.3 Most prominent ions (average ion counts > 5.0 (0.09%)) showing linear or exponential trends in *A. tridentata* plantlets treated with galanthamine.

ID	Ret. Time	Mass	Line of Best fit	Equation	R^2
22	6.0164	205.0516	Linear	y = -0.2074x + 6.8009	0.9392
23	2.0929	284.0493	Linear	y = -0.5524x + 8.4621	0.9663
24	14.6013	284.1245	Linear	y = -0.6884x + 9.1244	0.9908
25 [A]	3.9644	166.0617	Linear	y = -0.14x + 6.3751	0.9986
26	8.6167	210.11	Exponential	y = 7.8604e-0.079x	0.9960
27	8.9827	399.0978	Exponential	y = 8.0114e-0.086x	0.9980
28	1.7025	387.1295	Linear	y = -0.7391x + 9.0713	0.9968
29	8.4876	169.1004	Linear	y = -0.6547x + 8.467	0.7922
30	22.367	235.1366	Linear	y = -0.7584x + 8.9401	0.9744
31	10.7236	582.172	Linear	y = -0.7747x + 8.91	0.9526
32	9.8427	327.0888	Linear	y = -0.2612x + 6.339	0.9882
Increasing with Treatment					
ID	**Ret. Time**	**Mass**	**Line of Best fit**	**Equation**	**R^2**
21	9.5537	199.0965	Linear	y=1.1095x + 34.389	0.9410
22	1.8738	380.991	Linear	y = 1.3206x + 33.245	0.9778
23	13.9895	215.116	Linear	y = 1.1765x + 31.268	0.9722
24	6.3407	245.0937	Exponential	y = 28.216e0.0427x	0.7988
25	5.61	355.004	Linear	y = 1.8953x + 14.458	0.9560
26	5.1247	205.0568	Exponential	y = 5.5215e0.1735x	0.8681
27	8.4323	354.1065	Linear	y = 0.2937x + 17.805	0.8137
28	8.5697	516.9717	Linear	y = 3.1768x - 0.6695	0.9845
29 [B]	5.3907	193.0583	Exponential	y = 8.7431e0.0777x	0.8284

Table 5.3 Most prominent ions (average ion counts > 5.0 (0.09%)) showing linear or exponential trends in *A. tridentata* plantlets treated with galanthamine.

ID	Ret. Time	Mass	Line of Best fit	Equation	R^2
30	7.9198	399.0968	Exponential	y = 8.8366e0.078x	0.9997
31	5.6303	355.0657	Linear	y = 0.7487x + 7.7082	0.9981
32	5.3821	390.1043	Exponential	y = 7.8235e0.0536x	0.8414
33	5.1447	341.0321	Exponential	y = 5.4765e0.1026x	0.8517
34	9.8245	523.021	Exponential	y = 2.0228e0.2333x	1
35	1.742	290.7999	Linear	y = 0.9645x + 4.58	0.9966
36 C	5.5567	355.0838	Linear	y = 0.5845x + 5.6911	0.9561
37	11.4011	453.0584	Exponential	y = -0.6014x + 11.605	0.9843
38	7.8306	534.1533	Linear	y = 0.4806x + 5.0447	0.7993
39	5.1249	188.0433	Exponential	y = 1.9949e0.1862x	0.8455
40	1.8441	218.9918	Exponential	y = 3.5563e0.1186x	0.9998
41	1.5434	184.9998	Linear	y = 0.3741x + 5.2801	0.9420
42	5.131	246.0826	Exponential	y = 2.4741e0.1565x	0.7967
43	8.0024	194.0502	Exponential	y = 4.3066e0.0751x	0.7676
44	22.1758	593.2024	Linear	y = 1.3187x - 0.1007	0.9993
45	12.5212	469.1415	Exponential	y = 1.108e0.2394x	0.9468
46	11.8184	418.1451	Exponential	y = 4.0479e0.06x	0.8826
47	12.2741	416.1586	Exponential	y = 0.3947e0.3309x	0.9005
48	6.3831	422.0663	Exponential	y = 2.3529e0.1352x	0.9879
49	1.5636	122.5363	Linear	y = 0.1846x + 4.1701	0.9528
50	9.9414	659.1121	Exponential	y = 1.3175e0.1987x	0.9541

Putative Identifications: A = Phenylalanine, B=Scopoletin/Isoscopoletin, C=Scopolin

5.7 Discussion

The classic hypothesis of the regulation of plant growth and regeneration attributes morphological changes to changes in the relative ratio of auxins and cytokinins (Skoog and Miller, 1957). Interpretations and applications of this approach are the foundation of modern plant tissue culture and biotechnology. However, not all plants respond in the same way as model systems like tobacco and other plant growth regulators have been discovered that mediate, accelerate or enhance fundamental plant regeneration mechanisms (Murch and Saxena, 2005). In particular, it has been hypothesized that plant neurotransmitters may help regulate plant metabolism, growth, and development (Murch and Saxena, 2002; Murch and Saxena, 2004; Cao et al., 2006). With respect to plant tissue cultures, the capacity for plant neurochemicals to enhance cell survival and regeneration warrants further study.

Ach was first described in plants in the early 1970s using bioassays and detected in plants by gas chromatography in 1974 (Hartmann and Gupta, 1989). Ach occurs in all parts of plants, but higher concentrations of Ach have been quantified in roots and dark adapted tissues than in shoots or light exposed cells (Hartmann and Gupta, 1989; Tretyn and Kendrick, 1991; Roshchina, 2001; Hartmann and Kilbinger, 1974). The hypothesized roles of Ach in plants include almost all aspects of growth and development (Roshchina, 2001) including phytochrome-linked signalling (Jaffe, 1970) phytochrome-modulated calcium signaling (Mehta et al., 1993), induction of primary and secondary rooting (BaMEL et al., 2007), regulation of phloem transport (Yang et al., 2007) and regulation of pollen tube development (Tezuka et al., 2007).

Galanthamine is a natural inhibitor of AchE first isolated from snowdrop (*Galanthus* species) and commonly used in the treatment of Alzheimer's disease (Heinrich and Teoh, 2004). In humans, galanthamine increases Ach levels in the brain by reversibly inhibiting AchE, while also increasing sensitivity of neuronal nicotinic receptors to surrounding Ach (Ago et al., 2011). The initial observation of Ach in *Artemisia* tissues (Turi et al. 2014b) prompted studies to investigate the role of Ach in plant development. The most significant findings of the current investigations were the diversity of responses of *A. tridentata* tissues to galanthamine exposure. There was an overall inhibition of the cholinergic system with decreases in both Ach and AchE in response to galanthamine. In addition, there was a decrease in auxin and MEL in the galanthamine treated *A. tridentata* but the effects on plant

5HT were less clear. Interestingly, galanthamine induced a clear auxin-like growth response in the classic oat coleoptile bioassay and the galanthamine treated tissues appeared elongated and vitrified. The metabolomics analysis identified the increase of polyphenols as the most significant phytochemical response to galanthamine. Together these data indicate the induction of a cascade of responses that includes growth regulation, stress, and the induction of defence mechanisms by the natural product.

It has been proposed that Ach regulates basic cellular functions that are required for survival in vertebrate, invertebrates, plants, fungi, and mosses (Horiuchi et al., 2003; Wessler et al., 1999; Grando et al., 2012). Ach has been reported in many plant species but specific physiological roles remain under debate (Roshchina, 2001). Targeted data indicated that an overall decrease in cholinergic metabolism in *A. tridentata* was manifested mainly as stress in the tissues. BaMEL et al. (2007) found that the application of exogenous Ach or inhibitors of its breakdown induced rhizogenesis in tomato cultures (*Lycopersicon esculentum* Miller) which led them to propose that Ach may interact with other plant regulators such as auxin or MEL (BaMEL et al., 2007). Bioassay and targeted data support this observation, indicating an interaction of galanthamine with auxin. The observation that oat coleoptiles elongated in the presence of galanthamine could indicate that the mechanism of action may involve cross talk between auxin and Ach pathways.

Regulation of shoot and root development by 5HT and MEL was previously investigated with a variety of pharmaceuticals used to regulate neuronal 5HT metabolism in humans (Murch et al., 2001; Murch and Saxena, 2002; Murch and Saxena, 2004) and served as a model for the current study. Several researchers have proposed roles for MEL and 5HT in mediation of stress including protection of plant organelles and structures from oxidative damage caused by free radicals (Lazár et al., 2013; Galano et al., 2011; Paredes et al., 2009; Park, 2011) and the elimination of extraneous metabolic products through detoxification (Grobe, 1982). The current data is the first suggestion of synergy between cholinergic metabolism, neuroindoleamines (5HT, MEL) and auxin signalling in plants. In human systems, the interaction between cholinergic and serotonergic systems during cognitive processes has been well documented (Matsukawa et al., 1997) and 5HT receptor subtypes are

under investigation as potential drug targets for diseases such as Alzheimer's (Terry et al., 2008).

Metabolomics is the qualitative and quantitative analysis of all small metabolites in a biological sample (Yuliana et al., 2011; Verpoorte et al., 2010). In recent years, the application of metabolomics has provided an interesting avenue for understanding how plants respond to specific environmental stressors (Sanchez et al., 2012; Obata and Fernie, 2012). Understanding how the plant metabolome responds to neurologically active phytochemicals has not been investigated but environmental cues such as light, temperature, drought, or salt stresses have been examined (Kral'ova et al., 2012), with broader applications towards crop selection (Arbona et al., 2013; Rasmussen et al., 2012). In the current work, changes to the whole plant metabolome, in response to galanthamine treatment was investigated. Untargeted analysis identified phenylpropanoid metabolism as a primary response for further study. Coumarins are a class of secondary metabolites derived from the shikamate pathway via phenylalanine and contribute to plant defense by exhibiting anti-fungal and anti-microbial properties (Gnonlonfin et al., 2012). Previous research identified coumarins including scopoletin, isoscopoletin, esculin, esculetin, methylesculin and umbelliferone in leaf extracts of *A. tridentata* (Wilt et al., 1992; Wilt and Miller, 1992). Exploratory analysis found increasing amounts of scopoletin and its monoglucoside scopolin in response to galanthamine exposure. Increased levels of scopoletin and scopolin have been shown to occur in response to stresses such as microbial attack, water stress, and wounding (Gnonlonfin et al., 2012). Furthermore, manipulation of cultures with growth regulators such as 2-4D, kinetin, salicylic acid and methyl jasmonate was previously found to affect levels of both these compounds (Gnonlonfin et al., 2012), most notably in tobacco cultures (Sharan et al., 1998; Petit-Paly et al., 1999; Taguchi et al., 2001). Although far more enquiry is still needed, this untargeted exploratory analysis has allowed me to formulate the hypothesis that cultures treated with galanthamine increase production of phenolic compounds.

Galanthamine is a very interesting natural drug that is found in a wide range of species including snowdrops, Narcissis and other common bulbs. The utility of galanthamine for modulation of human neurochemistry is well established but less is known about the role of galanthamine in plant metabolism. This study indicates a possible role for

galanthamine as an allelopathic phytochemical released by one species into the environment and inducing a wide spectrum of responses in the phytochemistry of other plants. In order to survive abiotic and biotic environmental stresses, plants produce many phytochemicals including plant neurotransmitters or compounds which can mediate human brain function. Previous researchers have hypothesized that these neuro-mimicking plant responses indicate plant communication, plant neurosignaling or plant avoidant behaviours necessary for survival (Darwin, 1880; Baluska and Mancuso, 2009). The systems approach to generating novel phytochemical hypotheses through metabonomic analysis provides new avenues for future studies of the responses of plant tissues to environmental stimuli, allelochemicals and natural products.

Chapter 6 Metabolomics as a Hypothesis Generating Technique for Investigating Biological Activity of Plant Derived Smoke

6.1 Synopsis

The traditional use of plant derived smoke within many cultures is well documented as air purifiers, febrifuges, disinfectants, ear and eye remedies, hallucinogens or as gastrointestinal, genito-urinary, dermatological, neurological, and oral aids (Mohagheghzadeh et al., 2006). Since then, isolated compounds derived from smoke produced from species such as *Cannabis sativa* have been under extensive investigation as alternative treatments for Parkinsons and Huntingtons diseases. Traditional use of plant derived smoke by First Nations and Native American people is well documented as fumigants, smudges, and inhalants in North America. This research aims to identify plant families and genera which are sought after as ceremonial smokes and to use metabolomics as a hypothesis generating techniques for investigating anti-cholinesterase activity of smoke. A majority of the relevant ethnobotanical literature pertaining to the ritualistic use of plant derived smoke has been curated in Daniel Moerman's Ethnobotanical database via the University of Michigan http://herb.umd.umich.edu/. This database was queried with the following format "Family Name **AND** Ceremonial **AND** Burned **OR** Smoke **OR** Smoked **OR** Smoking **OR** Incense **OR** smudge **OR** fumigant" for 263 North American botanical families. Of the 263 families searched, 23 contained species that were used as ceremonial smokes and encompassed 60 species and 43 genera total. The Cupressaceae, Pinaceae, Asteraceae, Apiaceae and Solanaceae were the five most sought after families. Further analysis of the Asteraceae revealed that members from the genus *Artemisia* L. are good candidates for investigating biological activity of smoke. MeOH and smoke extracts collected from *A. tridentata* were screened using an anti-AchE bioassay and revealed that approximately 0.5 ± 0.06 and 1.9 ± 0.24 mg of leaf tissue were required to inhibit 100 mU of AchE at room temperature respectively. Metabolomic analysis of smoke revealed that significantly more compounds were observed in leaf tissues (41, 120 +/- 741) than in smoke (10, 851 +/- 44). Furthermore, putative identification of significant ions present in smoke showed that monoterpene and sesquiterpene compounds are potentially influencing the observed anti-AchE activity of smoke.

6.2 Research Objectives and Hypothesis

I hypothesized the following: 1) that specific plant families and genera are sougth after by North American indigenous peoples aceremonial smokes; 2) plants burned during ceremony contain biologically active constituents in their smoke; and 3) metabolomics can be used as a hypothesis generating technique to investigate biological activity in smoke: The objectives of this research were 1) to use residual and binomial analysis in order to identify specific genera which are over-utilized as smokes during ritual or ceremony; 2) to determine whether smoke and MeOH extracts collected from *A. tridentata* Nutt. possess phytochemicals which are inhibitory to AchE; and 3) to characterize biological activity in smoke using metabolomics as a hypothesis generating technique.

6.3 Materials and Methods

6.3.1 Acquisition and Analysis of Ethnobotanical and Flora Data

Moerman's online Native American Ethnobotany Database was accessed on March 24, 2013 (http://herb.umd.umich.edu/). The online database holds a repository of ethnobotanical data for 3618 species and consists of 47,000 items describing uses of North American flora for purposes such as food, drug, dye, and fiber among 291 Native American and Canadian First Nations groups (Moerman, 2012). All ceremonial taxa were identified using Cronquist (1981) via the USDA's PLANTS Database (http://plants.usda.gov/java/). The database was queried with the following format: "Family Name **AND** Ceremonial **AND** Burned **OR** Smoke **OR** Smoked **OR** Smoking **OR** Incense **OR** smudge **OR** fumigant" for 263 North American botanical families (Moerman and Estabrook, 2003). Residual and binomial analysis was performed using methods previously described in Turi and Murch (2013) (Turi and Murch, 2013b). The Asteraceae was assessed further at the genus level using binomial analysis in order to identify genera which are significantly over-utilized as ceremonial smokes.

6.3.2 Collection of Plant Material

Leaf material for *A. tridentata* subsp. *tridentata* were collected near the Osoyoos Desert Centre, British Columbia (N49 03'15 W119 31'14W, N49 03'14, W119 31'16W, N49 03'12 W119 31'18) during September 2011, and stored at -80 °C until further analysis. Voucher specimens were collected and deposited at the herbaria of the Beaty Biodiversity Centre, Vancouver BC (Accession in progress).

6.3.3 Preparation of Leaf Extracts

Stock solutions (0.5g/mL) made from fresh leaf material for *A. tridentata* were prepared by homogenizing and subsequently vortexing material in MeOH for 30 seconds (Kontes Pellet Pestle disposable tissue grinder, Fisher Scientific, Mississauga, ON). Extracts were then centrifuged (16,000 x g, Eppendorf Microfuge) for 1 min with the resulting supernatant being filtered (0.2 µm, Ultrafree-MC filtered centrifuge tubes; Millipore, MS, USA) and stored at -20 ˚C.

6.3.4 Acquisition of Smoke Extracts

Leaf tissue from *A. tridentata* was individually plucked from branches and dried at room temperature in perforated bags for 1 week. Approximately 5 g of leaf material was weighed prior to burning (n=3). Plant materials were ignited in a sealed vessel in the presence of a continuous stream of oxygen and slowly burned for approximately 1 hour, until mostly ash remained. Outwards airflow from the vessel passed through a filter gas diffuser which was contained between water (500 mL) and pentane (300 mL) layers within a 2 litre separatory funnel. A secondary pentane fraction was collected by rinsing the gas diffuser with approximately 20 mL of pentane after every burn (smoke capture). Pentane fractions were collected under vacuum and heat, while smoke captures were dried down using nitrogen. Both fractions were then re-dissolved in MeOH and stored at -80 ◦C until further testing.

6.3.5 AchE Inhibition Bioassay

Galantamine hydrobromide (Chromadex), smoke, and smoke capture extracts (n=3) were dried down under nitrogen and diluted (n=8) in buffer or a 10% MeOH buffer solution. AchE inhibition was measured using a modification of the spectrophotomertric method originally described by Ellman et al. (1961) using an Amplex© Red Ach / Acetylcholinesterase Assay Kit (Molecular Probes Inc. Eugene, Oregon) (see Chapter 4).

6.3.6 Metabolomics Analysis

MeOH and smoke capture extracts were dried down in the presence of nitrogen and diluted in 70% EtOH (0.05 g/mL) and analyzed using a previously published protocol (see Brown et al. 2012 a & b) (see Chapter 4).

6.3.7 Putative Identification and Biotransformations

Using previous protocols (Brown et al., 2012b) logical algorithms were applied using Excel in order to identify compounds which are solely present in *A. tridentata* leaf tissue, only present in smoke, or common to both smoke and leaf extracts. Monoisotopic masses were collected from an extensive literature search for all *Artemisia* species belonging to the *Tridentatae* subgenus (see Turi et al., 2014a) and used to putatively identify compounds in both smoke and tissue extracts (Table 3). Using methods outlined in Turi and Murch (2013a), compounds putatively identified within all 3 replicates of *A. tridentata* tissue were biosynthetically transformed in excel in order to simulate the following bio-transformations: -H2, +H2, -CH3, -CH3, +OH, +OH(x2), +COOH, +NH2, +NH3, +O. Masses for each transformation were then searched within the metabolomics dataset in order to identify transformations in leaf and smoke extracts.

6.4 Results

6.4.1 Selection of Biologically Active Smokes

Of the 263 families found within North America, 23 contained species that were used as ceremonial smokes and encompassed 60 species and 43 genera total. Due to the small number of species being employed within the total population as ceremonial smokes, the observed linear regression value for the total population was low with an R^2 value equal to 0.3555 (Figure 6.1). Within the total population, residual analysis identified the Cupressaceae, Pinaceae, Asteraceae, Apiaceae and Solanaceae as the 5 most sought after families with residual values equal to 6.9, 5.8, 4.1, 3.1, and 2.5 respectively (Table 6.1). With the exception of the Asteraceae, binomial analysis on the whole population suggests that selection of the above families is not random (p-value < 0.05) (Table 6.1). Given binomial analysis has been found to favour small and medium sized families in some circumstances (Turi and Murch, 2013b), the population was divided into small, medium and large subsets and subjected to binomial analysis once more. Using this method, all the above families including the Asteraceae were found to be over-utilized (p-value < 0.05) (Table 6.1). Analysis at the genus level for the Asteraceae identified the genus *Artemisia* as significantly over-utilized (p-value = 0.0001) (Table 6.2).

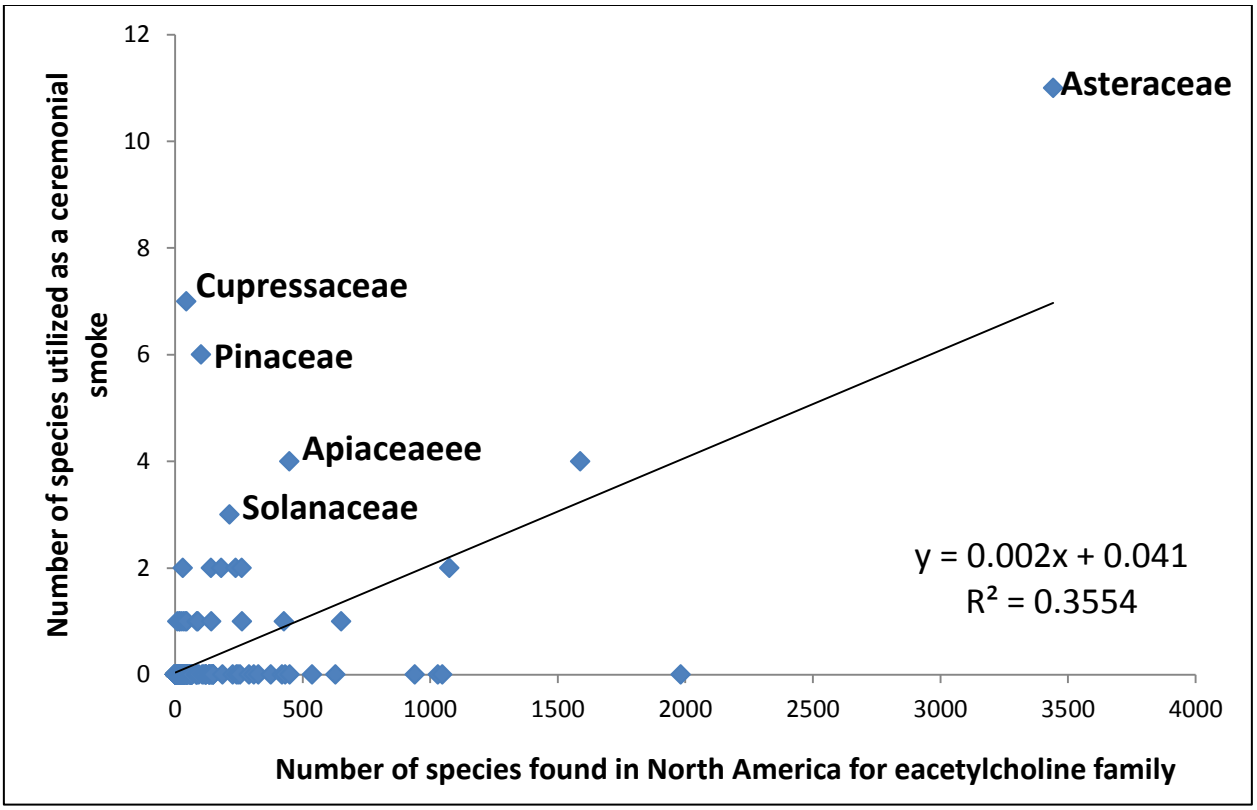


Figure 6.1 Residual analysis of ethnobotanical data showing over-use of North American plant families as smokes during ceremony.

Table 6.1 Most over-utilized families as ceremonial smokes identified by Residual and corresponding p-values.

Size	Family	Number of species in North America	Number of species used as ceremonial smokes	Residual	Binomial Analysis p-value (Whole Population)	Binomial Analysis p-value (Small, Medium, and Large Family Subsets)
Small	Cupressaceae	44	7	6.871	1.90 x 10^-11	2.95 x 10^-9
Medium	Pinaceae	102	6	5.755	2.41 x 10^-7	1.26 x 10^-6
Large	Asteraceae	3442	11	4.075	2.31 x 10^-1	1.86 x 10^-2
Large	Apiaceae	448	4	3.063	2.56 x 10^-2	5.22 x 10^-3
Medium	Solanaceae	214	3	2.531	1.62 x 10^-2	3.39 x 10^-2

Table 6.2 Binomial analysis on genera belonging to the Asteraceae.

Rank	Genus	Number of species in North America	Number of species used as ceremonial smokes	Binomial Analysis p-value (Whole Population)
1	*Artemisia* L.	68	4	1.0 x 10^-4
2	*Anaphalis* DC.	1	1	3.7 x 10^-3
3	*Gutierrezia* Lag.	10	1	3.6 x 10^-2
4	*Madia* Molina	11	1	4.0 x 10^-2
5	*Balsamorhiza* Nutt..	14	1	5.0 x 10^-2

6.4.2 Anti-AchE Activity

Anti-AchE activity was observed for both extracts. Approximately 0.5 ± 0.06 and 1.9 mg ± 0.24 of leaf tissue were required to inhibit 100 mU of AchE at room temperature by MeOH and smoke capture extracts respectively (Figure 6.2). The pentane smoke fraction for *A. tridentata* showed weak activity and is not shown below.

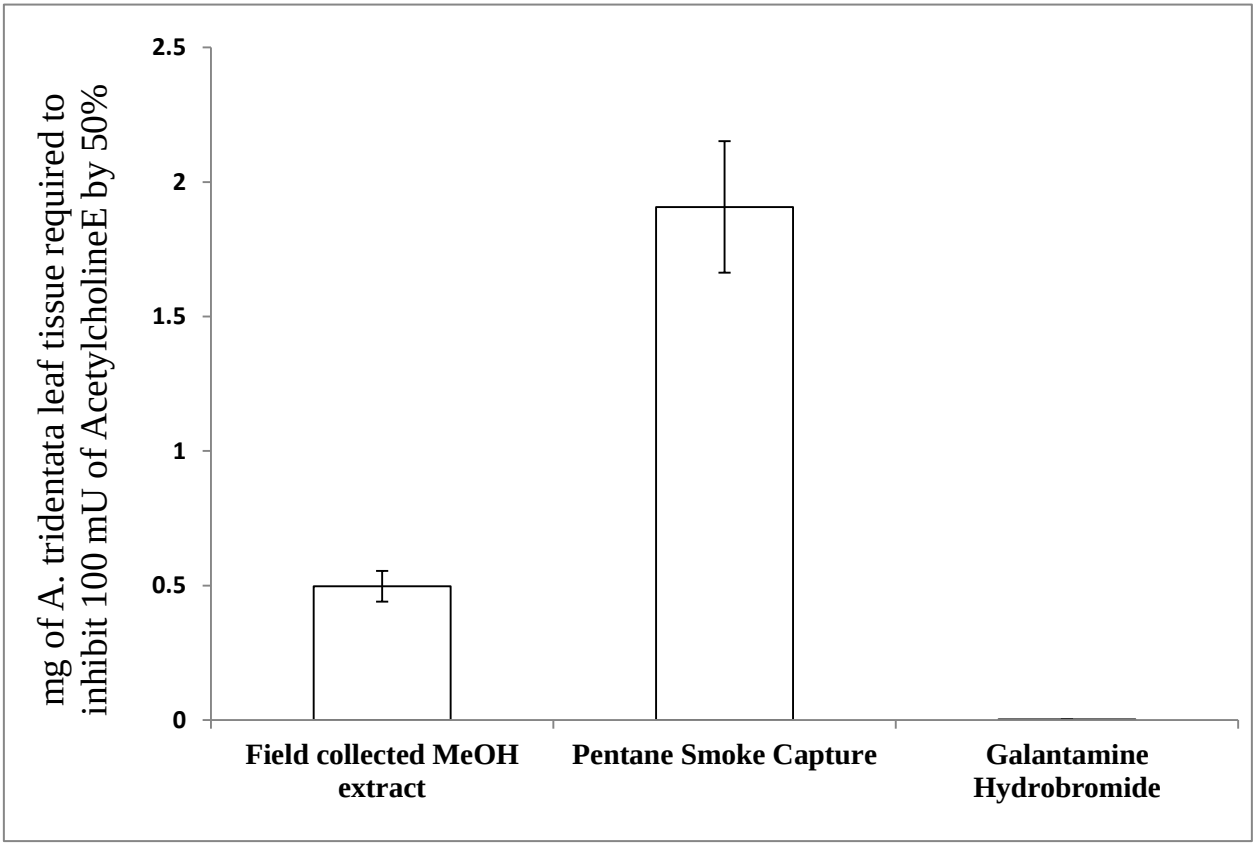


Figure 6.2 Anti-AchE activity for MeOH and smoke capture *A. tridentata* extracts. Error bars represent the standard error of the mean (n =3 replicates).

6.4.3 Metabolomic Analysis of Leaf and Smoke

Significantly more compounds were observed in leaf tissues (41,120 +/- 741) than in smoke (10, 851 +/- 44) (Table 6.3). Of these 13, 895 and 521 compounds were unique to all 3 replicates for leaf and smoke extracts respectively (Table 6.3). Interestingly, 756 compounds were shared between leaf tissue and smoke extracts (Table 6.3).

Table 6.3 Summary of metabolomic analysis of leaf and smoke extracts for *A. tridentata*.

	Smoke	Leaf
Total Number of compounds Observed	23,601	55,955
Average Number of Compounds per Sample	10,851 +/- 44 (%RSD = 0.40)	41,120 +/- 741 (%RSD = 1.80)
Number of unique metabolites identified in all 3 replicates	521 (29)	13, 895 (141)
Number of metabolites common to both extracts	756 (35)	

*Numbers in () represent the number of compounds putatively identified (see table 6.4)

6.4.4 Putative Identification of Compound Classes in Tissue and Smoke

Monoisotopic masses for diterpene (1 mass, 4 compounds), monoterpene (14 masses, 78 compounds), phenolic (25 masses, 29 compounds) and sesquiterpene (30 masses, 83 compounds) compounds were mined in order to putatively identify metabolites which are either unique to leaf tissue, unique to smoke or common between both extracts (Table 6.4). Overall, 205 compounds were identified in leaf and smoke extracts, with 141 residing solely in plant tissues, 29 solely in smoke, and 35 common between both extracts (Table 6.3 & 6.4). Of the 141 putatively identified compounds solely present in plant tissue, 0.7%, 15%, 53%, and 31% were diterpenes, monoterpenes, sesquiterpene, and phenolic compounds (Figure 6.3). Furthermore, of the 35 putatively identified compounds present in both plant tissue and smoke, 0%, 37%, 57% and 6% were diterpene, monoterpene, sesquiterpene and phenolic compounds (Figure 6.3).

Table 6.4 Putative identification in leaf and smoke extracts.

Chemical Formula	*M/z*	Example	Only Leaf	Smoke and Leaf	Only Smoke
C_4H_60	71.048	methacroleine	0	0	0
$C_{10}H_{16}$	137.138	rothrockene,6 -3-carene,α-phellandrene,α-pinene,α-terpinene,α-thujene, artemisia triene, β-pinene, camphene, γ-terpinene, limonene, myrcene, sabinene, santolina triene, terpinolene, tricyclene	1	2	2
$C_{10}H_{14}O$	151.108	2,2-dimethyl-6-isopropenyl-2H-pyran, carvacrol, carvone, chrysanthenone, filifolone, lyratal, pinocarvone,	5	0	0
$C_{10}H_{16}O$	153.128	2,3-dimethyl-6-isopropyl-4*H*-pyran, 2-Isopropenyl-5-methylhexa-trans-3,5-dien-1-ol , α-thujone, artemiseole, artemeseole, arthole, artemisia ketone, β-thujone, fenchone, isolyratol, lyratol, myrtenol,neral,oxidosantolinatriene,sabinol, santolina epoxide, trans-pinocarveol	3	3	0
$C_9H_{14}O_2$	155.108	arbusculone, nor-chrysanthemic acid methyl ester	0	0	2
$C_{10}H_{18}O$	155.148	α-santolina alcohol, α-terpineol, artemisia alcohol, borneol, chrysanthemol, cis-sabinene hydrate, eucalyptol, 1,8-cineole, cineole, fenchyl alcohol (fenchol), fraganol, isopulegol, lavandulol, terpinen-4-ol, terpineol, thujyl alcohol, trans-carveol, trans-isocarveol, trans-para-menth-2-en-1-ol, trans-piperitol, yomogi alcohol	0	0	0
$C_9H_6O_3$	163.038	umbelliferone	0	0	0
$C_{10}H_{14}O_2$	167.108	santolinolide-A, santolinolide-B, santolinolide-B', satonlinolide-C	0	3	0
$C_{10}H_{16}O_2$	169.128	santolinic acid, trans-3-(1-oxo-2-methyl-2-propenyl)-2,2-dimethylcyclopropylmethanol	4	0	1
$C_9H_6O_4$	179.038	esculetin	1	0	0
$C_{11}H_{18}O_2$	183.138	methyl santolinate, santolinyl ester	2	3	0
$C_{10}H_8O_4$	193.048	isoscopoletin, scopoletin (7-hydroxy-6-methoxycoumarin)	0	0	0
$C_{13}H_{20}O$	193.158	α-ionone	1	2	0
$C_{12}H_{18}O_2$	195.138	lyratyl acetate, sabinyl acetate	0	0	0
$C_{12}H_{20}O_2$	196.158	artemisyl acetate	2	0	1
$C_{12}H_{20}O_2$	197.158	artemisia acetate, bornyl acetate, chrysanthemyl acetate, fraganyl acetate, geranyl acetate	2	2	1
$C_{15}H_{22}$	203.178	γ-curcumene	6	1	1

Table 6.4 Putative identification in leaf and smoke extracts.

Chemical Formula	*M/z*	Example	Only Leaf	Smoke and Leaf	Only Smoke
$C_{15}H_{24}$	205.198	α-bulnesene, α-copaene, α-humulene, α-longipinene, β-caryophyllene, β-chamigrene, β-elemene, β-gurjunene, β-selinene, bicyclogermacrene, cyclosativene, delta-cadiene, γ-himAchalene, germacrene-D, longicyclene, longifolene, silphinene	1	1	2
$C_{11}H_{10}O_4$	207.068	scoparone	0	0	0
$C_{16}H_{26}$	219.208	α-himAchalene, β-himAchalene	0	0	0
$C_{15}H_{24}O$	221.188	β-copaen-4-α-ol, spathulenol	1	2	1
$C_{11}H_{10}O_5$	223.058	isofraxidin (7-hydroxy-6,8-dimethoxycoumarin)	0	0	1
$C_{15}H_{26}O$	223.208	nerolidol	0	0	1
$C_{14}H_{24}O_2$	225.188	geranyl isobutanoate	0	0	0
$C_{15}H_{20}O_2$	233.158	arbusculin-B	9	5	0
$C_{15}H_{26}O_2$	239.198	neryl isovalerate	2	0	1
$C_{14}H_{26}O_3$	243.198	cryptomeridiol	1	1	2
$C_{15}H_{16}O_3$	245.118	dehydroleucodin	4	0	0
$C_{15}H_{18}O_3$	247.138	Achillin, arbiglovin, deacetoxymatricarin, deacetylmatricarin, desacetoxymatricarin,parishin-B, santonin	2	0	0
$C_{15}H_{20}O_3$	249.148	8-deoxycumambrin B, arbusculin-C, deacetyllaurenobiolide, rothin-A	4	4	1
$C_{15}H_{22}O_3$	251.168	1-β-hydroxysant-3-en-6,12-olide-C, 1-β-hydroxysant-4(14)-en-6,12-olide-C, arbusculin-A	2	1	1
$C_{15}H_{24}O_3$	253.178	11,13-dihydroarbusculin, colartin	6	1	1
$C_{15}H_{26}O_3$	255.198	longilobol	0	0	0
$C_{14}H_{26}O_4$	259.188	pygmol	2	0	2
$C_{15}H_{18}O_4$	263.128	artemisin, parishin-A, parishin-C, rupicolin A, rupicolin B, tatridin-C	0	0	1
$C_{15}H_{20}O_4$	265.128	ridentin	4	0	0
$C_{15}H_{20}O_4$	265.148	artecalin, cumambrin B, dentatin-A, dentatin-B, isophotosantonic lactone, ridentin B, rothin-B, spiciformin, tatridin-A, tatridin-B, viscidulin-C	4	0	0
$C_{13}H_{14}O_6$	267.088	artelin	0	0	0
$C_{15}H_{22}O_4$	267.158	arbusculin D	3	1	0
$C_{15}H_{24}O_4$	269.278	arbusculin-E	0	0	0
$C_{15}H_{10}O_5$	271.058	apigenin	1	0	0

Table 6.4 Putative identification in leaf and smoke extracts.

Chemical Formula	*M/z*	Example	Only Leaf	Smoke and Leaf	Only Smoke
$C_{15}H_{18}O_5$	279.128	artecanin, canin	1	0	0
$C_{15}H_{20}O_5$	281.138	artevasin, badgerin, cumambrin B oxide	1	0	0
$C_{16}H_{12}O_5$	285.078	apigenin 4'-methyl ether (acacetin)	2	0	0
$C_{15}H_{10}O_6$	287.058	kaempferol, luteolin	1	0	0
$C_{17}H_{22}O_4$	291.158	novanin	0	0	0
$C_{15}H_{18}O_6$	295.118	rupin A	2	0	0
$C_{15}H_{10}O_7$	303.048	quercetin	4	0	1
$C_{17}H_{18}O_5$	303.128	dehydromatricarin	2	0	0
$C_{17}H_{20}O_5$	305.138	matricarin	3	0	0
$C_{17}H_{22}O_5$	307.158	cumambrin A, viscidulin-A, viscidulin-B	1	0	1
$C_{16}H_{12}O_7$	317.068	eupafolin, kaempferol 6-methyl ether	0	1	0
$C_{21}H_{32}O_2$	317.248	methyl isopimarate, methyl levopimarate, methyl palustrate, methyl trans-communate	1	0	0
$C_{15}H_{10}O_8$	319.048	quercetagetin	1	0	0
$C_{15}H_{16}O_8$	325.088	skimmin	1	0	0
$C_{17}H_{14}O_7$	331.078	quercetin 7,3'-dimethyl ether (rhamnazine)	2	0	0
$C_{17}H_{16}O_7$	333.098	dihydroquercetin 7,3'-dimethyl ether	3	0	0
$C_{17}H_{20}O_7$	337.128	rupin B	2	0	3
$C_{16}H_{18}O_8$	339.108	6-β-D-glucosyl-7-methoxycoumarin	1	0	0
$C_{15}H_{16}O_9$	341.088	cichoriin (esculetin 7-O-glucoside), esculin	0	0	0
$C_{18}H_{16}O_7$	345.098	penduletin	1	0	0
$C_{17}H_{14}O_8$	347.078	axillarin	4	0	0
$C_{16}H_{18}O_9$	355.108	esculin 7-methylether (methylesculin)	3	0	0
$C_{18}H_{16}O_8$	361.088	quercetagetin-3,6,7-trimethyl ether	3	0	0
$C_{19}H_{18}O_8$	375.108	5,4'-dihydroxy-3,6,7,3'-tetramethoxyflavone	2	1	1
$C_{26}H_{34}O_5$	427.248	farnochrol	8	1	0
$C_{21}H_{20}O_{10}$	433.118	apigenin 7-0-glucoside	4	0	0
$C_{26}H_{34}O_6$	443.248	drimartol B, epoxyfarnochrol, tripartol	5	0	1
$C_{21}H_{20}O_{11}$	449.108	luteolin-7-O-glucoside	2	0	0
$C_{30}H_{38}O_{14}$	623.238	magnoloside	8	0	0
Total			**141**	**35**	**29**

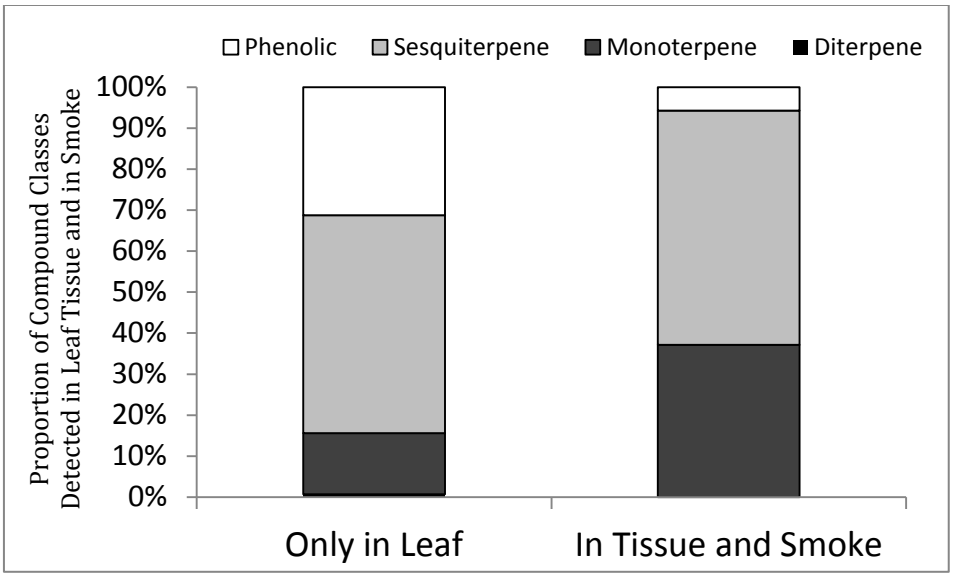


Figure 6.3 Comparison between putatively identified metabolite classes in leaf and smoke extracts.

6.4.5 Synthetic Biotransformation

In order to investigate whether specific compound classes are more likely to undergo modification during burning, synthetic biotransformation was applied to all the monoisotopic masses described in table 6.4. For each metabolite class, a ratio was derived by dividing the total number of biotransformations found in only smoke (originating from burning) by the total number of biotransformations found in both smoke and leaf tissue (originating from plant tissue) (Figure 6.4). Biotransformed diterpene, phenolic and sesquiterpenes detected in smoke, all possessed a ratio greater than 1 (biotransformations originating from burning: biotransformations originating from plant metabolism) which may suggest that these classes are more susceptible to modification during burning (Figure 6). Interestingly, the number of monoterpene biotransformations detected in both plant tissue and smoke (n=122 biotransformations) was less than the number of biotransformations detected solely in smoke (n=70 biotransformations) (Figure 6.4).

6.4.7 Putative Identification of Significant Ions in Smoke

Standard deviations of average ion counts for compounds found in smoke and tissue were calculated. Compounds present in smoke possessing ion counts greater than twice the standard deviation (3.4 + (2 x 7.0) = 17.4) were identified as significant (Table 6.5). Putative

identification of significant ions present in smoke revealed the presence of both monoterpene and sesquiterpenes compounds arbusculin B, α-ionone, artemisia acetate, bornyl acetate, chrysanthemyl acetate, fraganyl acetate, geranyl acetate, 8-deoxycumambrin B**,** arbusculin C, deacetyllaurenobiolide, and rothin-A, (Table 6.5). Sesquiterpene and monoterpene derivatives were also found (Table 6.5).

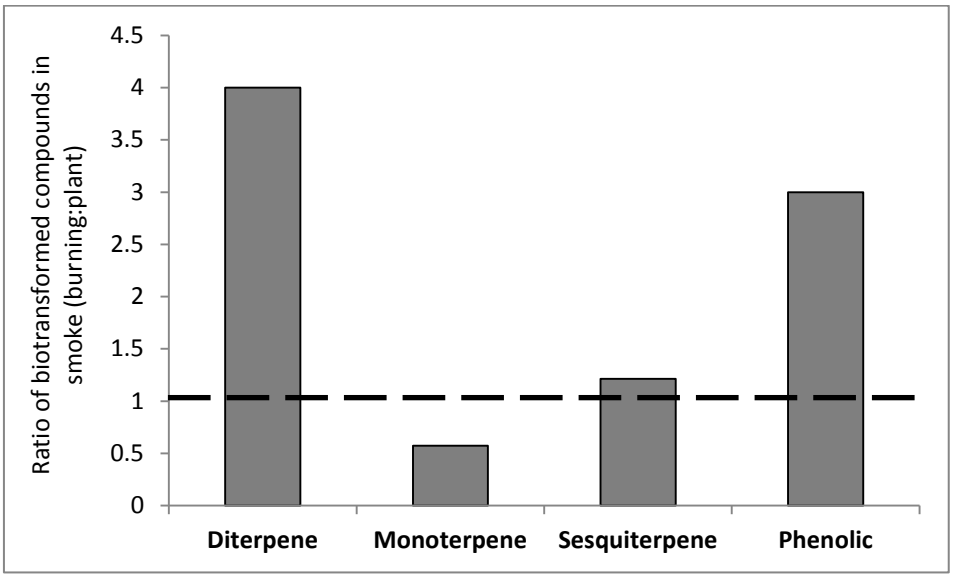


Figure 6.4 Analysis of biotransformations detected in smoke.

A burning:plant ratio was calculated by dividing the number of biotransformations detected in solely smoke by the number of biotransformations detected in both plant and smoke extracts. A burning:plant ratio > 1 suggests that a specific class is more likely to undergo modification during burning.

Table 6.5 Putative identification of significant ions present in both tissue and smoke.

Ret. Time	Mass	Average Field Counts	Average Smoke Counts	Putative Identifation
21.6252	235.1661	20.9	92.6	**ADD H_2** arbusculin B**, ADD OH (x2)** γ-curcumene
17.1632	233.1536	20.7	80.9	arbusculin B
21.0172	235.1943	3.2	72.9	**ADD H_2** arbusculin B,
21.0128	276.2213	2.2	66.9	

Table 6.5 Putative identification of significant ions present in both tissue and smoke.

Ret. Time	Mass	Average Field Counts	Average Smoke Counts	Putative Identifation
14.6045	161.1414	17.3	57.0	
24.7491	279.257	4.1	43.3	
14.6284	217.1795	7.8	42.0	**ADD CH_3** γ-curcumene, **Minus H_2** α-himAchalene, β-himAchalene
11.806	193.1508	6.6	35.5	α-ionone
12.843	284.1962	20.0	35.0	
13.1441	197.1421	14.7	34.0	artemisia acetate, bornyl acetate,chrysanthemyl acetate,fraganyl acetate, geranyl acetate **ADD CH_3** methyl santolinate, santolinyl ester, **ADD H_2** lyratyl acetate, sabinyl acetate
14.8386	249.1627	4.1	28.3	8-deoxycumambrin B**,** arbusculin C, deacetyllaurenobiolide, rothin-A, **ADD H_2** Achillin, arbiglovin, deacetoxymatricarin, deacetylmatricarin, desacetoxymatricarin, parishin-B, santonin **Minus H_2** 1-β-hydroxysant-3-en-6,12-olide-C **,** 1-β-hydroxysant-4(14)-en-6,12-olide-C, arbusculin A **ADD NH_3/OH** arbusculin B **ADD COOH** α-bulnesene, α-copaene**,** α-humulene, α-longipinene, β-caryophyllene, β-chamigrene, β-elemene, β-gurjunene, β-selinene, bicyclogermacrene, cyclosativene, delta-cadiene, γ-himAchalene, germacrene-D, longicyclene, longifolene, silphinene, β-selinene
14.9044	233.1523	18.2	24.6	arbusculin B
12.8723	267.1853	4.0	23.9	**ADD H_2** artecalin, cumambrin B, dentatin-A, dentatin-B, isophotosantoniclactone, ridentin-B, rothin-B, spiciformin, tatridin-A, tatridin B, viscidulin C **ADD CH_3** 11,13-dihydroarbusculin**,** colartin **ADD COOH** nerolidol, **ADD OH/NH_3** 1-β-hydroxysant-3-en-6,12-olide-C, 1-β-hydroxysant-4(14)-en-6,12-olide-C, arbusculin-A
11.7923	284.2049	19.9	22.8	
11.7402	165.1055	14.4	21.5	**ADD CH_3** carvacrol, carvone, chrysanthenone, filifolone, lyratal, pinocarvone **OR MINUS H_2** santolinolide-A, santolinolide-B, santolinolide-B', santolinolide-C

Table 6.5 Putative identification of significant ions present in both tissue and smoke.

Ret. Time	Mass	Average Field Counts	Average Smoke Counts	Putative Identifation
12.9751	334.2038	13.0	19.9	
14.2392	135.1186	6.5	18.8	**MINUS H_2 -** rothrockene, 6-3 carene, α-phllandrene, α-terpinene, α thujene, artemisia triene, β-pinene, camphene, γ-terpinene, limonene, myrcene, sabinene, santolina triene, terpinolene, tricyclene
14.6464	294.2469	1.7	18.4	

6.5 Discussion

North America is home to a vast repository of traditional ecological knowledge, originating largely from First Nations and Native American traditions (Turner et al., 1980; Moerman, 2009). Although previous works have investigated the therapeutic potential of medicinal species found within North America, the phytochemical underpinnings of smoke have been severely overlooked. The current work has applied residual and binomial analysis in order to identify specific plant families and genera thay are over-used during ceremony as smokes, and used untargeted metabolomic analysis to generate hypothesis for future investigations of AchE inhibition activity in smoke captured from *A. tridentata* leaves. The most significant findings were that: 1) members from the Asteraceae such as the genus *Artemisia* are sought after significantly as ceremonial smokes; and 2) that smoke from *A. tridentata* exhibited anti-AchE activity. Although we did not isolate specific phytochemicals responsible for the observed anti-cholinesterase activity, metabolomic analysis suggests that monoterpene and sesquiterpene compounds present in smoke are involved in the observed biological activity.

A compendium by Pennacchio et al. (2010) on plant derived smokes suggests that approximately 400 species are burned for magical, religious or protective purposes (Pennacchio et al., 2010). According to my analysis of Moerman's database, plant families such as the Asteraceae, Apiaceae, Cupressaceae, Pinaceae, and Solanaceae are over-utilized

as ceremonial smokes by First Nations and Native American peoples. Previous work by Turner (2009) discusses the wide spread use of aromatic plants during ceremony by First Nations peoples living within Northwest regions of North America (Turner, 2009). Similar to this observation, the majority of families identified as ceremonial smokes are aromatic in nature, which supports further that organoleptic properties are a contributing factor towards the selection of ceremonial plants. However, it is still possible that a placebo-like effect is also occurring while spiritual plants are being burned (Moerman and Jonas, 2002).

The traditional use of smoke derived from the genus *Artemisia* L. is widespread. World-wide the genus *Artemisia* consists of between 350-500 members which are distributed mostly in temperate regions of the northern hemisphere (Turi et al., 2014a). In parts of China, species of *Artemisia* are burned or used to ignite incense made from *Juniperus squamata* Buch. and *Cupressus funebris* (Staub et al., 2011). Similarly, in parts of India and Nepal, *Artemisia* species are burned as incense (Ahuja et al., 2011; Shah and Joshi, 1971;BhattAcharyya, 1991) or are used ritualistically as offerings to local deities (Shah, 2013), while in Pakistan, *Artemisia scoparioa* Waldst & Kit and *Artemisa herba-alba* Asso are burned to treat burns and muscle AchEs respectively (Hayat et al., 2009). North America is home to approximately 50 species of *Artemisia* (Shultz, 2006; Shultz, 2009) many of which have reported uses as smudges, incense, or fumigants including *A. tridentata*, *A. dracunculus*, *Artemisia frigida*, *A. ludovicina*, and *A. tripartita* (Turner et al., 1980; Moerman, 2009;Turner, 2009; Turner et al. 1990). Similar to previous works for this genus (Liu et al., 2012; Orhan et al., 2010), *A. tridentata* tissue and smoke inhibited AchE *in vitro*. Although the therapeutic effects of *Artemisia* species have been reviewed extensively (see Bora and Sharma, 2011; Tan et al., 1998; Jose Abad et al., 2012; Turi et al., 2014a), phytochemical understanding towards the therapeutic potential of smoke derived from *Artemisia* species is limited. Xu et al. (2013) subjected mice to smoke derived from *Artemisia vulgaris* daily for 15 or 30 minutes and observed an increase in levels of cerebral 5HT, dopamine and norepinephrine. When exposed to smoke derived from *Artemisia princeps*, human breast cancer cells underwent apoptosis, possibly due to the presence of specific phytochemicals which cause the mitochondrial membrane to depolarize and down-

regulate the expression of the anti-apoptitic protein BCL-2 (Sarath et al., 2007). Antimicrobial activity has also been observed against *Bacillus cereus*, *Klebsiella pneumonia* and *Cryptococcus neoformans* from smoke and tissue extracts collected from *Artemisia afra* Jacq. Ex Willd. (Braithwaite et al., 2008). Based on the above, research investigating the medicinal chemistry of smoke derived from the genus *Artemisia* is warranted.

Physical and chemical changes which occur while burning a plant sample are not well understood. Similar to previous works, we were able to putatively identify many monoterpene and sesquiterpene compounds in smoke. Furthermore, synthetic biotransformation revealed that terpenes, particularly sesquiterpenes are likely to undergo modification during burning in order to produce interesting structures which could affect biological activity of smoke. Traditional approaches to drug discovery involve screening of plant extracts, fractionating and re-screening until a single active constituent is present (Brown and Murch, 2012; Wang and Chen, 2013). While this is a valid approach to drug discovery, bioassay guided fractionation does not favor the isolation of bioactive compounds which are unstable or in low abundance, or consider synergism between compounds (Brown and Murch, 2012; Wang and Chen, 2013). Given the broad metabolic overview metabolomics provides for a given biological sample, multivariate and univariate statistics have been applied to datasets in order to investigate bioactivity of complex mixtures (Brown and Murch, 2012; Yuliana et al., 2011;Yuliana et al., 2013; Wang and Chen, 2013). These methods have limited utility for investigating relationships between specific analytes or metabolite classes present within a complex mixture, thus novel approaches to visualize and interpret biological activity are still needed. Although we did not isolate specific phytochemicals, untargeted analysis suggests that monoterpenes and sesquiterpene compounds may have facilitated the observed anti-AchE activity. A review of the literature revealed that several of the compound putatively identified in smoke such as α-ionone (Miyazawa et al., 2004), bornyl acetate, pinene and 1,8-cineole (Savelev et al., 2003) have all been shown to exhibit anti-AchE activity. Interestingly, significant ion generation identified many unique sesquiterpenes structures such as arbusculin A & C, deacetyllaurenobiolide, deoxycumambrin B and rothin-A present in smoke. Though they are widespread in the

subgenus *Tridentatae* (Turi et al., 2014a), little information pertaining to the pharmacology of these compounds exists and has led us to hypothesize that the above compounds are facilitating the observed biological activity *in vitro*.

The traditional use of plant derived smokes for healing is well described across many cultures of the world. Despite widespread use of these species, little attention has been paid to the biological activity of their smoke. This in part is largely due to challenges associated with standardization and reproducibility of smoke extracts and scarcity of works or analytical tools which investigate how burning plant material may effect the phytochemical composition of smoke derived from plants. Thus, metabolomics provides a unique opportunity to examine the phytochemical complexity of smoke.

Chapter 7 Conclusion

This research was designed to explore the application of untargeted metabolomics analysis for investigating the phytochemical and biological underpinnings of two model medicinal plants *L. canbyi* and *A. tridentata*. To achieve this, I selected model species, established plant collections, analyzed known phytochemicals and bioassay systems and developed statistical approaches for analysis of large metabolomics datasets.

7.1 Selection of Medicinal Species

I assessed the frequency of use of North American plants for ritual, ceremony and spirituality using 4 different methods of statistical analysis. Comparison between methods revealed: 1) that selection of plant species for ceremony or ritual by Native American and First Nations peoples is not random; 2) that all 4 approaches showed variability towards the selection of over-used flora, but agreed with respect to under-use; and 3) partitioning of data into small, medium and large subsets in conjunction with residual, bayesian, binomial or IDM approaches permitted complete statistical characterization. Together, these approaches were able to identify species found within the families Apiaceae, Asteraceae, Ericaceae, Pinaceae and Salicaceae as over-utilized ceremonial medicines. In other words, their use is not random and significantly over-represented within the North American flora (p-value < 0.05). After further analysis at the genus level for the Apiaceae and Asteraceae, I identified the genus *Artemisia* and *Ligusticum* as excellent candidates for studies of plant neurochemistry.

Once I established an adequate statistical approach for analyzing Moerman's database, the traditional use of plant derived smokes by First Nations and Native American people during ceremony was also investigated. Similar to the above, I found that ceremonial use of plant derived smokes from the Asteraceae, Apiaceae, Cupressaceae, Pinaceae, and Solanaceae is not random (p-value<0.05). After further analysis of the Asteraceae at the genus level, I identified members of the genus *Artemisia* as good candidates for investigating biological activity of smoke (p-value < 0.05).

7.2 Establishment of Plant Collections

During the course of my studies, I established *in vitro* regeneration protocols for *A. tridentata* and various other Sagebrush species. Unfortunately, I had limited success establishing reliable and sterile cultures for *L. canbyi* and *L.* porteri. This outcome can be attributed to the following: low germination rates of seeds, slow regeneration of meristematic tissue in culture, contamination, and complex growth requirements i.e. a cool, moist, alpine understory. To date, there is no adequate protocol to conserve North American *Ligusticum* species, despite growing concern that wild populations are at risk from over-harvesting and habitat loss. Thus, further efforts are still needed to conserve *Ligusticum* species *in vitro*.

7.2 Targeted Analysis of Known Phytochemicals

Bioactive compounds Z-ligustilide, E-butylidenephthalide, ferulic acid, MEL, and 5HT were quantified in epidermis, cortex, pith, stem and leaf tissues. Significantly higher levels of ferulic acid and Z-ligustilide were detected in root tissues, while E-butylidenephthalide, MEL and 5HT were significantly higher in above ground tissues. Analysis of antioxidant potential revealed that root tissues were able to scavenge free radicals more effectively then shoot material, and that a majority of the observed activity could be explained by the presence of ferulic acid. Together the above data support the traditional medicinal use of *L. canbyi* root.

MEL, 5HT, GABA and Ach are human neurotransmitters which have all previously been found in plants. Despite their widespread occurrence, the importance of these neurochemicals towards plant growth and development is not fully understood. I quantified MEL, 5HT, GABA and Ach in four germplasm lines which were collected from two distinct sites at the Osoyoos Desert Centre. Interestingly, both 5HT and GABA showed significant variability between germplasm lines collected from different sites, and reinforces further the idea that large scale collection and screening for elite lines is needed to successfully curate germplasm collections. In order to investigate further the role of these neurochemicals in mediating plant growth and development, I manipulated cholinergic signalling of *A. tridentata* cultures using the Alzheimer's drug galanthamine, a well-known partial inhibitor of AchE. Using this approach I was able to show that changes in cholinergic signalling will

also affect levels of Auxin, MEL and 5HT in *A. tridenta* cultures and produces a distinctly different phenotype at 10 µM levels of galanthamine. Using the pea coleoptile bioassay, it was determined that leaf curling and yellowing are likely due to an Auxin response which was elicited by galanthamine. Together the above data quantified neurotransmitters MEL, 5HT, GABA and Ach for in the genus *Artemisia*, and demonstrated cross-talk between cholinergic and neuroindoleamine pathways for the first time in plants.

7.3 Untargeted Analysis

7.3.1 Metabolomics as a Hypothesis Generating Tool for Putative Pathway Construction

I used an untargeted approach to compare metabolomes between three root tissue types belonging to *L. canbyi*. Overall, the number of metabolites detected in pith, cortex, and epidermis did not significantly differ. Furthermore, PCA revealed that greater metabolite variability existed between root types then between tissues for the same root. Given, *L. canbyi* is not commercially grown, it is possible that the medicinal efficacy of wild harvested roots will significantly vary in the environment. In order to explore the medicinal chemistry of *L. canbyi* further, I applied synthetic biotransformation to Z-ligustilide which is believed to be the precursor to all phthalides in plants. Using this approach, I detected more than 70 putative phthalide derivatives in *L. canbyi* root tissue, which includes many novel derivatives such as methylated, de-methylated, angeloyl ester, amine, and dimer derivatives. Thus, it is likely that phthalide diversity and the subsequent medicinal chemistry of *Ligusticum* species is far more complex than once thought. Phthalide compounds are incredibly challenging to isolate, thus the above approach provides an opportunity to explore the diversity of phthalides in order to generate hypothesis regarding their biosynthesis and diversity.

7.3.2 Metabolomics as a Hypothesis Generating Tool for Plant Tissue Culture

A collection of *in vitro*-grown germplasm lines was established from wild-harvested seeds of *A. tridentata*. Using PCA, I analyzed the metabolome of wild-harvested and *in vitro* collected tissues, and deduced significant difference between treatments using PCA. In order to generate hypothesis regarding this observation, significant ion generation and putative identification was performed using a database which I constructed from an extensive review on the phytochemistry of *A. tridentata* and close relatives in the subgenus *Tridentata*e. Using

this untargeted approach I detected a greater number of monoterpene and sesquiterpene compounds in wild-harvested tissues and generated the hypothesis that morphological structures responsible for their biosynthesis differ. This notion was supported by performing scanning electron microscopy on glandular trichomes of *A. tridentata* and observing morphological differences between culture grown and wild harvested tissues. Given the importance of establishing *in vitro* model systems for phytochemical studies, mining of the metabolomics datasets provides a hypothesis-generating technique for future studies to optimize tissue culture protocols, so that model systems can better reflect the phytochemical diversity *ex situ*.

7.3.3 Metabolomics as a Hypothesis Generating Tool for Plant Signalling and Behavior

I treated *A. tridentata* cultures with a known AchE inhibitor galanthamine and significant morphological and phytochemical difference were observed in response to treatment. Untargeted analysis revealed that 0 and 5 µM treatments differed significantly from 10 µM. In order to investigate specific metabolite changes, I applied significant ion generation and putative identification in order to explore other unknown metabolic changes and detected increasing levels of scopolin and scopoletin. Using this approach, I was able to generate the hypothesis that galanthamine treatment is affecting production of phenylpropanoids in *A. tridentata*. Generally, experiments investigating the effects of abiotic stress on a model system aim to detect changes in specific genes, proteins, or metabolites, however this approach does not allow one to tackle unknown biochemical changes which may be pertinent to the model system under investigation. Thus, my approach may be useful to researchers by facilitating the generation of novel phytochemical hypotheses regarding plant responses to abiotic stress.

7.3.4 Metabolomics as a Hypothesis Generating Tool for Understanding Biological Activity of Smoke

I have shown that smoke from *A. tridentata* is inhibitory to AchE *in vitro*, which in turn supports the traditional use of smoke derived from *A. tridentata* during ceremony. Untargeted analysis revealed significant differences between smoke and leaf metabolomes. In order to investigate the observed variability further, I applied logical algorithms,

significant ion generation and putative identification to accurate mass data and observed that monoterpene compounds are less likely to undergo modification during burning of leaf tissue, while diterpene, phenolic and sesquiterpene classes were more likely to be modified. Significant ion generation and putative identification revealed that anti-AchE activity may be attributed to the presence of both monoterpene and sesquiterpenes compounds such as arbusculin B, α-ionone, artemisia acetate, bornyl acetate,chrysanthemyl acetate,fraganyl acetate, geranyl acetate, 8-deoxycumambrin B**,** arbusculin C, deacetyllaurenobiolide, and rothin-A in smoke. Previous works have shown that several of the above compounds exhibit anti-AchE activity including: α-ionone, bornyl acetate, pinene and 1,8-cineole. Thus, it is likely that these compounds in conjunction with lesser known ones are mediating the observed anti-AchE effect. Despite widespread use of plant derived smokes, little attention has been paid to the biological activity of their smoke. This in part is largely due to challenges associated with standardization and reproducibility of smoke extracts and scarcity of works or analytical tools which investigate how burning plant material might affect the presence or absence of metabolite classes in plant derived smokes. Using the above, I have shown how untargeted metabolomics can provide a unique opportunity to examine the chemical complexity of smoke.

7.4 Limitations and Applications of this Thesis

7.4.1 Ethnobotanical Analysis

I have developed a more robust approach for analyzing Moerman's ethnobotanical database, which in turn can be used to identify good botanical while conducting natural products research. Limitations to this analysis include: the chance that the selection or spiritual perception of a specific plant family or genus was not dependent on chemistry, but rather due to a placebo like effect. It is also possible that important ceremonial species were absent from Moerman's database which in turn could have increased or decreased p-values for specific taxa. Lastly, the application of other statistics may provide a different perspective.

7.4.2 Tissue Culture

The establishment of *in vitro* propagation methods for *A. tridentata* provided a ready source of plant material grown in a sterile controlled environment for chemical analysis and bioassays, as well as a source of shoot material for long term potential conservation of the genetic resources in cryopreservation and a source of plants for ecological restoration projects. Differences in phytochemistry between *in vitro* and field plants remain unestablished and wild-harvested plants may have more active secondary metabolism induced by environmental interaction.

7.4.2 Targeted Phytochemistry

Although *L. canbyi* is currently being sold within the natural health products industry as a panacea or respiratory aid, this is the first time that the medicinal chemistry of this species has been evaluated. The method I used for comparing the relative importance of the different phytochemicals found in *L. canbyi* tissue in order to describe antioxidant activity is inherently flawed since the activity of the compounds in the tissue extracts may be different from the activity of isolated compounds. Although the data indicates that the ferulic acid content accounted for the majority of the antioxidative activity of *Ligusticum* tissues, it is possible that MEL contributed greater antioxidant activity *in vivo* than could be determined by this method.

7.4.3 Untargeted Phytochemistry

According to Hall (2011) "Metabolomics is strongly data driven. Nevertheless, it is essential that the biologist maintains the lead in order to gurantee the development and proper implementation of those technologies specifically required to tackle unsolved, fundamental biological questions. Howerver, with metabolomics being a multi-disciplinary technology, giving biologists the lead entails that these researchers need to have a broad understanding of a variety of scientific disciplines…This requires a new generation of biologists who are confident in multi-variate statistical approaches for complex, large-scale data management and manipulation. Such broad-minded and broadly disciplined individuals are not easy to find". Previous research in Dr. Murch's lab established statistical methods for eliminating false discoveries in metabolomics datasets. My work built on these earlier efforts

and established logical algorithms for detection of metabolite relationships. I applied these statistical tools to compare tissues, to understand new aspects of phthalide biosynthesis, plant regeneration, plant cell communication, environmental responses, and to investigate the phytochemical underpinnings of smoke derived by burning *A. tridentata* leaf tissue.

Metabolomics datasets as a whole are multi-faceted, requiring several distinct approaches to effectively visualize and interpret data. Given this notion, many interesting patterns and relationships likely went undetected during the course of my analysis, and the application of other approaches in the future may provide further insight. Currently, there is significant disagreement in the literature about experimental design, standards, method validation, accuracy, precision, and data interpretation. As instrumentation, statistical tools and databases continue to be developed, one can expect for greater reconciliation between metabolomics approaches, and ultimately a complete characterization of plant metabolic responses and biological activity. As a field of study, metabolomics is still in its infancy. The next 10 years are likely to bring exciting advancements and opportunities for plant biologists to explore the depths of the 'unknowns'.

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Appendices

Appendix A: Phytochemistry of the subgenus *Tridenatae*

Table A.1 List of all metabolites previously identified in the subgenus *Tridentatae*. Distribution of metabolites between members found in the subgenus *Tridentatae* has been described in Turi et al. (2014a). ID numbers 1-79 pertain to structures described in Chapter 1, while S0-S128 can be found in Appendix 1, Figures 1-5.

ID	Name	Chemical Formula	M/Z	Molecular weight	Compound Class
1	dihydroquercetin 7,3'-dimethyl ether	$C_{17}H_{16}O_7$	332.09	332.3	Phenolic
2	artelin	$C_{13}H_{14}O_6$	266.08	266.25	Phenolic
3	esculin 7-methylether (methylesculin)	$C_{16}H_{18}O_9$	354.10	354.31	Phenolic
4	esculin	$C_{15}H_{16}O_9$	340.08	340.28	Phenolic
5	scopoletin (7-hydroxy-6-methoxycoumarin)	$C_{10}H_8O_4$	192.04	192.17	Phenolic
6	axillarin	$C_{17}H_{14}O_8$	346.07	346.29	Phenolic
7	esculetin	$C_9H_6O_4$	178.03	178.14	Phenolic
8	isoscopoletin	$C_{10}H_8O_4$	192.04	192.17	Phenolic
9	6-β-D-glucosyl-7-methoxycoumarin	$C_{16}H_{18}O_8$	338.10	338.31	Phenolic
S-0	5,4'dihydroxy-3,6,7,3'-tetramethoxyflavone	$C_{19}H_{18}O_8$	374.10	374.34	Phenolic
S-1	apigenin	$C_{15}H_{10}O_5$	270.05	270.24	Phenolic
S-2	apigenin 4'-methyl ether (acacetin)	$C_{16}H_{12}O_5$	284.07	284.26	Phenolic
S-3	apigenin 7-O-glucoside	$C_{21}H_{20}O_{10}$	432.11	432.38	Phenolic
S-4	cichoriin (esculetin 7-O-glucoside)	$C_{15}H_{16}O_9$	340.08	340.28	Phenolic
S-5	eupafolin	$C_{16}H_{12}O_7$	316.06	316.26	Phenolic
S-6	isofraxidin (7-hydroxy-6,8-dimethoxycoumarin)	$C_{11}H_{10}O_5$	222.05	222.19	Phenolic
S-7	kaempferol	$C_{15}H_{10}O_6$	286.05	286.24	Phenolic
S-8	kaempferol 6-methyl ether	$C_{16}H_{12}O_7$	316.06	316.26	Phenolic
S-9	luteolin	$C_{15}H_{10}O_6$	286.05	286.24	Phenolic
S-10	luteolin-7-O-glucoside	$C_{21}H_{20}O_{11}$	448.10	448.38	Phenolic
S-11	magnoloside	$C_{30}H_{38}O_{14}$	622.23	622.61	Phenolic

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ID	Name	Chemical Formula	M/Z	Molecular weight	Compound Class
S-12	penduletin	$C_{18}H_{16}O_7$	344.09	344.32	Phenolic
S-13	quercetagetin	$C_{15}H_{10}O_8$	318.04	318.24	Phenolic
S-14	quercetagetin-3,6,7-trimethyl ether	$C_{18}H_{16}O_8$	360.08	360.31	Phenolic
S-15	quercetin	$C_{15}H_{10}O_7$	302.04	302.24	Phenolic
S-16	quercetin 7,3'-dimethyl ether (Rhamnazine)	$C_{17}H_{14}O_7$	330.07	330.29	Phenolic
S-17	scoparone	$C_{11}H_{10}O_4$	206.06	206.19	Phenolic
S-18	skimmin	$C_{15}H_{16}O_8$	324.08	324.28	Phenolic
S-19	umbelliferone	$C_9H_6O_3$	162.03	162.14	Phenolic
10	chrysanthemol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
11	trans-chrysanthemal	$C_{10}H_{16}O$	152.12	152.24	Monoterpene
12	methyl santolinate	$C_{11}H_{18}O_2$	182.13	182.26	Monoterpene
13	santolina triene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
14	oxidosantolinatriene	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
15	artemisia triene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
16	santolinolide-A	$C_{10}H_{14}O_2$	166.1	166.22	Monoterpene
17	santolinolide-B	$C_{10}H_{14}O_2$	166.1	166.22	Monoterpene
18	artemiseole, artemeseole, arthole	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
19	rothrockene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
20	neolyratol	$C_{10}H_{16}O$	152.12	152.24	Monoterpene
21	1,6,6-trimethyl-4-ethenyl-exo-2-oxabicyclo[3.1.0]hexane	$C_{10}H_{16}O$	152.12	152.24	Monoterpene
22	lavandulol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
23	arbusculone	$C_9H_{14}O_2$	154.1	154.21	Monoterpene
24	2,2-dimethyl-6-isopropenyl-2H-pyran	$C_{10}H_{14}O$	150.1	150.22	Monoterpene
25	2,3-dimethyl-6-isopropyl-4*H*-pyran	$C_{10}H_{16}O$	152.1	152.23	Monoterpene
26	2-Isopropenyl-5-methylhexa-trans-3,5-dien-1-ol	$C_{10}H_{16}O$	152.12	152.23	Monoterpene

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ID	Name	Chemical Formula	M/Z	Molecular weight	Compound Class
27	α-pinene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
28	camphor	$C_{10}H_{16}O$	152.12	152.24	Monoterpene
29	methacroleine	C_4H_60	70.04	70.09	Monoterpene
30	p-cymene	$C_{10}H_{16}O$	152.12	152.24	Monoterpene
31	camphene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
32	eucalyptol, 1,8-cineole, cineole	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-20	2,2,-dimethyl-6acetyl-2H-pyran	$C_9H_{12}O_2$	152.08	152.19	Monoterpene
S-21	6 -3-carene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
S-22	α-phellandrene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
S-23	α-santolina alcohol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-24	α-terpinene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
S-25	α-terpineol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-26	α-thujene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
S-27	α-thujone	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
S-28	artemisia acetate	$C_{12}H_{20}O_2$	196.15	196.29	Monoterpene
S-29	artemisia alcohol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-30	artemisia ketone	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
S-31	artemisyl acetate	$C_{12}H_{20}O_2$	195.15	196.29	Monoterpene
S-32	β-ocimene	$C_{10}H_{16}$	136.13	136.24	Monoterpene
S-33	β-pinene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
S-34	β-thujone	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
S-35	borneol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-36	bornyl acetate	$C_{12}H_{20}O_2$	196.15	196.29	Monoterpene
S-37	carvacrol	$C_{10}H_{14}O$	150.1	150.22	Monoterpene
S-38	carvone	$C_{10}H_{14}O$	150.1	150.22	Monoterpene
S-39	chrysanthemyl acetate	$C_{12}H_{20}O_2$	196.15	196.29	Monoterpene

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ID	Name	Chemical Formula	M/Z	Molecular weight	Compound Class
S-40	chrysanthenone	$C_{10}H_{14}O$	150.1	150.22	Monoterpene
S-41	cis-sabinene hydrate	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-42	fenchone	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
S-43	fenchyl alcohol (fenchol)	$C_{10}H_{18}O$	154.14	154.14	Monoterpene
S-44	filifolone	$C_{10}H_{14}O$	150.1	150.22	Monoterpene
S-45	fraganol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-46	fraganyl acetate	$C_{12}H_{20}O_2$	196.15	196.29	Monoterpene
S-47	γ-terpinene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
S-48	geranyl acetate	$C_{12}H_{20}O_1$	196.15	196.29	Monoterpene
S-49	geranyl isobutanoate	$C_{14}H_{24}O_2$	224.18	224.34	Monoterpene
S-50	isolyratol	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
S-51	isopulegol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-52	limonene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
S-53	lyratal	$C_{10}H_{14}O$	150.1	150.22	Monoterpene
S-54	lyratol	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
S-55	lyratyl acetate	$C_{12}H_{18}O_2$	194.13	194.27	Monoterpene
S-56	myrcene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
S-57	myrtenol	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
S-58	neral	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
S-59	neryl isovalerate	$C_{15}H_{26}O_2$	238.19	238.37	Monoterpene
S-60	nor-chrysanthemic acid methyl ester	$C_9H_{14}O_2$	154.1	154.21	Monoterpene
S-61	p-cymene acetate	N/A	N/A	N/A	Monoterpene
S-62	pinene	$C_{10}H_{14}O$	134.11	134.22	Monoterpene
S-63	pinocarvone	$C_{10}H_{14}O$	150.1	150.22	Monoterpene
S-64	sabinene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
S-65	sabinol	$C_{10}H_{16}O$	152.12	152.23	Monoterpene

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ID	Name	Chemical Formula	M/Z	Molecular weight	Compound Class
S-66	sabinyl acetate	$C_{12}H_{18}O_2$	194.13	194.27	Monoterpene
S-67	santolina epoxide	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
S-68	santolinic acid	$C_{10}H_{16}O_2$	168.12	168.23	Monoterpene
S-69	santolinolide-B'	$C_{10}H_{14}O_2$	166.1	166.22	Monoterpene
S-70	santolinyl ester	$C_{11}H_{18}O_2$	182.13	182.26	Monoterpene
S-71	satonlinolide-C	$C_{10}H_{14}O_2$	166.1	166.22	Monoterpene
S-72	terpinen-4-ol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-73	terpineol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-74	terpinolene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
S-75	thuja-2,4(10)-diene	$C_{10}H_{14}$	134.11	134.22	Monoterpene
S-76	thujone	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
S-77	thujyl alcohol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-78	trans-3-(1-oxo-2-methyl-2-propenyl)-2,2-dimethylcyclopropylmethanol	$C_{10}H_{16}O_2$	168.12	168.13	Monoterpene
S-79	trans-carveol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-80	trans-isocarveol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-81	trans-para-menth-2-en-1-ol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-82	trans-pinocarveol	$C_{10}H_{16}O$	152.12	152.23	Monoterpene
S-83	trans-piperitol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
S-84	tricyclene	$C_{10}H_{16}$	136.13	136.23	Monoterpene
S-85	yomogi alcohol	$C_{10}H_{18}O$	154.14	154.25	Monoterpene
33	arbusculin-A	$C_{15}H_{22}O_3$	250.16	250.33	Sesquiterpene
34	arbusculin-B	$C_{15}H_{20}O_2$	232.15	232.32	Sesquiterpene
35	arbusculin-C	$C_{15}H_{20}O_3$	248.14	248.32	Sesquiterpene
36	arbusculin D	$C_{15}H_{22}O_4$	266.15	266.33	Sesquiterpene
37	arbusculin-E	$C_{15}H_{24}O_4$	268.27	268.35	Sesquiterpene
38	colartin	$C_{15}H_{24}O_3$	252.17	252.35	Sesquiterpene
39	ridentin B	$C_{15}H_{20}O_4$	264.14	264.32	Sesquiterpene
40	rothin-A	$C_{15}H_{20}O_3$	248.14	248.32	Sesquiterpene
41	rothin-B	$C_{15}H_{20}O_4$	264.14	264.32	Sesquiterpene

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ID	Name	Chemical Formula	M/Z	Molecular weight	Compound Class
42	1-β-hydroxysant-3-en-6,12-olide-C(W-A)	$C_{15}H_{22}O_3$	250.16	250.33	Sesquiterpene
43	1-β-hydroxysant-4(14)-en-6,12-olide-C(W-B)	$C_{15}H_{22}O_3$	250.16	250.33	Sesquiterpene
44	artevasin	$C_{15}H_{20}O_5$	280.13	280.32	Sesquiterpene
45	novanin	$C_{17}H_{22}O_4$	290.15	290.35	Sesquiterpene
46	badgerin	$C_{15}H_{20}O_5$	280.13	280.32	Sesquiterpene
47	deacetyllaurenobiolide	$C_{15}H_{20}O_3$	248.14	248.32	Sesquiterpene
48	spiciformin	$C_{15}H_{20}O_4$	264.14	264.32	Sesquiterpene
49	tatridin-A	$C_{15}H_{20}O_4$	264.14	264.32	Sesquiterpene
50	tatridin-B	$C_{15}H_{20}O_4$	264.14	264.32	Sesquiterpene
51	arbiglovin	$C_{15}H_{18}O_3$	246.13	246.3	Sesquiterpene
52	artecanin	$C_{15}H_{18}O_5$	278.12	278.3	Sesquiterpene
53	canin	$C_{15}H_{18}O_5$	278.12	278.3	Sesquiterpene
54	cumambrin A	$C_{17}H_{22}O_5$	306.15	306.35	Sesquiterpene
55	cumambrin B	$C_{15}H_{20}O_4$	264.14	264.32	Sesquiterpene
56	8-deoxycumambrin B	$C_{15}H_{20}O_3$	248.14	248.32	Sesquiterpene
57	cumambrin B oxide	$C_{15}H_{20}O_5$	280.13	280.32	Sesquiterpene
58	matricarin	$C_{17}H_{20}O_5$	304.13	304.34	Sesquiterpene
59	deacetoxymatricarin	$C_{15}H_{18}O_3$	246.13	246.3	Sesquiterpene
60	deacetylmatricarin	$C_{15}H_{18}O_3$	246.13	246.3	Sesquiterpene
61	rupicolin A	$C_{15}H_{18}O_4$	262.12	262.3	Sesquiterpene
62	rupicolin B	$C_{15}H_{18}O_4$	262.12	262.3	Sesquiterpene
63	rupin A	$C_{15}H_{18}O_6$	294.11	294.3	Sesquiterpene
64	rupin B	$C_{17}H_{20}O_7$	336.12	336.34	Sesquiterpene
65	viscidulin-A	$C_{17}H_{22}O_5$	306.15	306.35	Sesquiterpene
66	viscidulin-B	$C_{17}H_{22}O_5$	306.15	306.35	Sesquiterpene
67	viscidulin-C	$C_{15}H_{20}O_4$	264.14	264.32	Sesquiterpene
68	tripartol	$C_{26}H_{34}O_6$	442.24	442.54	Sesquiterpene
69	secondriol	$C_{26}H_{36}O_6$	444.25	444.57	Sesquiterpene
70	secondrial	$C_{26}H_{34}O_6$	442.24	442.54	Sesquiterpene
71	drimAchone	$C_{26}H_{34}O_6$	442.24	442.54	Sesquiterpene

Table A.1 List of all metabolites previously identified in the subgenus *Tridentatae*. Distribution of metabolites between members found in the subgenus *Tridentatae* has been described in Turi et al. (2014a). ID numbers 1-79 pertain to structures described in Chapter 1, while S0-S128 can be found in Appendix 1, Figures 1-5.

ID	Name	Chemical Formula	M/Z	Molecular weight	Compound Class
72	longilobol	$C_{15}H_{26}O_3$	254.19	254.37	Sesquiterpene
73	pygmol	$C_{14}H_{26}O_4$	258.18	258.35	Sesquiterpene
74	desacetoxymatricarin	$C_{15}H_{18}O_3$	246.13	246.3	Sesquiterpene
75	ridentin	$C_{15}H_{20}O_4$	264.12	264.32	Sesquiterpene
S-86	11,13-dihydroarbusculin	$C_{15}H_{24}O_3$	252.17	252.35	Sesquiterpene
S-87	Achillin	$C_{15}H_{18}O_3$	246.13	246.3	Sesquiterpene
S-88	α-bulnesene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-89	α-copaene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-90	α-himAchalene	$C_{16}H_{26}$	218.2	218.38	Sesquiterpene
S-91	α-humulene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-92	α-ionone	$C_{13}H_{20}O$	192.15	192.3	Sesquiterpene
S-93	α-longipinene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-94	artecalin	$C_{15}H_{20}O_4$	264.14	264.32	Sesquiterpene
S-95	artemisin	$C_{15}H_{18}O_4$	262.12	262.3	Sesquiterpene
S-96	β-caryophyllene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-97	β-chamigrene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-98	β-copaen-4-α-ol	$C_{15}H_{24}O$	220.18	220.35	Sesquiterpene
S-99	β-elemene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-100	β-gurjunene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-101	β-himAchalene	$C_{16}H_{26}$	218.2	218.38	Sesquiterpene
S-102	β-selinene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-103	bicyclogermacrene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-104	cryptomeridiol	$C_{14}H_{26}O_3$	242.19	242.35	Sesquiterpene
S-105	cyclosativene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-106	dehydroleucodin	$C_{15}H_{16}O_3$	244.11	244.29	Sesquiterpene
S-107	dehydromatricarin	$C_{17}H_{18}O_5$	302.12	302.32	Sesquiterpene
S-108	delta-cadiene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-109	dentatin-A	$C_{15}H_{20}O_4$	264.14	264.32	Sesquiterpene
S-110	dentatin-B	$C_{15}H_{20}O_4$	264.14	264.32	Sesquiterpene
S-111	drimartol B	$C_{26}H_{34}O_6$	442.24	442.54	Sesquiterpene
S-112	epoxyfarnochrol	$C_{26}H_{34}O_6$	442.24	442.54	Sesquiterpene
S-113	farnochrol	$C_{26}H_{34}O_5$	426.24	426.55	Sesquiterpene

Table A.1 List of all metabolites previously identified in the subgenus *Tridentatae*. Distribution of metabolites between members found in the subgenus *Tridentatae* has been described in Turi et al. (2014a). ID numbers 1-79 pertain to structures described in Chapter 1, while S0-S128 can be found in Appendix 1, Figures 1-5.

ID	Name	Chemical Formula	M/Z	Molecular weight	Compound Class
S-114	γ-Curcumene	$C_{15}H_{22}$	202.17	202.34	Sesquiterpene
S-115	γ-HimAchalene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-116	germacrene-D	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-117	isophotosantonic lactone	$C_{15}H_{20}O_4$	264.14	264.32	Sesquiterpene
S-118	longicyclene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-119	longifolene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-120	nerolidol	$C_{15}H_{26}O$	222.2	222.37	Sesquiterpene
S-121	parishin-A	$C_{15}H_{18}O_4$	262.12	262.3	Sesquiterpene
S-122	parishin-B	$C_{15}H_{18}O_3$	246.13	246.3	Sesquiterpene
S-123	parishin-C	$C_{15}H_{18}O_4$	262.12	262.3	Sesquiterpene
S-124	santonin	$C_{15}H_{18}O_3$	246.13	246.3	Sesquiterpene
S-125	silphinene	$C_{15}H_{24}$	204.19	204.35	Sesquiterpene
S-126	spathulenol	$C_{15}H_{24}O$	220.18	220.35	Sesquiterpene
S-127	tatridin-C	$C_{15}H_{18}O_4$	262.12	262.3	Sesquiterpene
S-128	terelactone	C_6H_8O	112.05	112.13	Sesquiterpene
76	methyl isopimarate	$C_{21}H_{32}O_2$	316.24	316.48	Diterpene
77	methyl levopimarate	$C_{21}H_{32}O_2$	316.24	316.48	Diterpene
78	methyl palustrate	$C_{21}H_{32}O_2$	316.24	316.48	Diterpene
79	methyl trans-communate	$C_{21}H_{32}O_2$	316.24	316.48	Diterpene

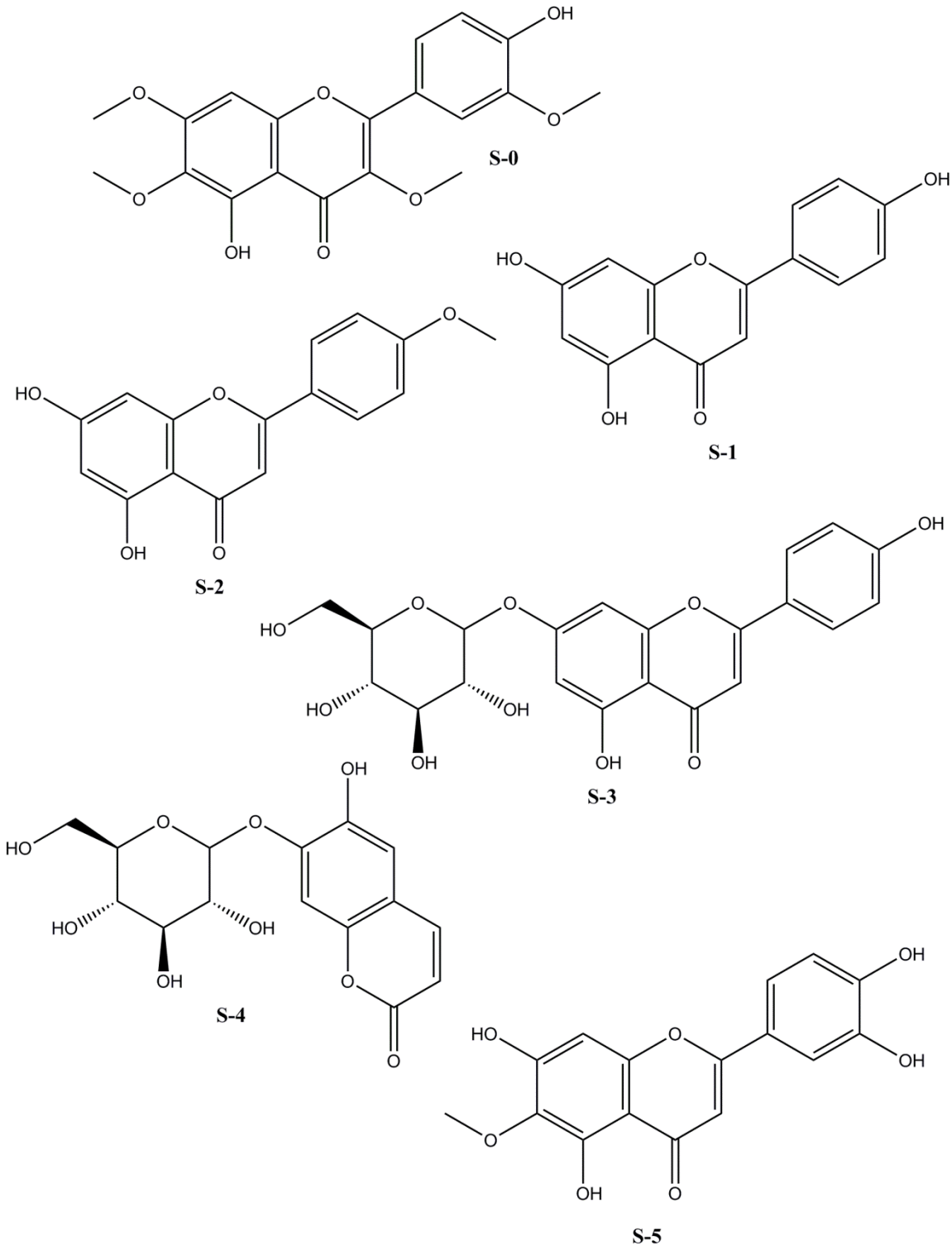

Figure A.1 Phenolic compounds identified within the subgenus *Tridentatae*.

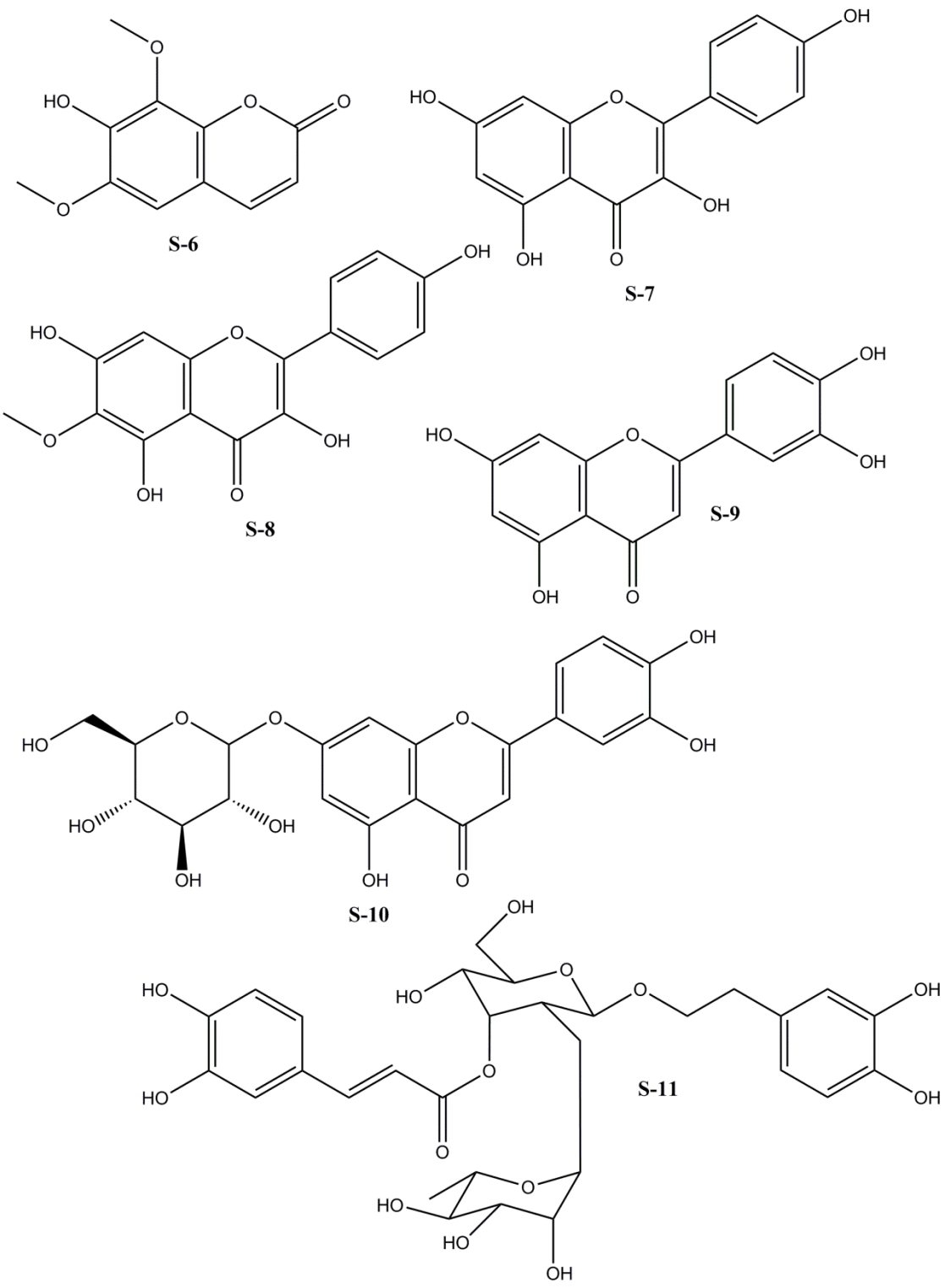

Figure A.2 Phenolic compounds identified within the subgenus *Tridentatae*.

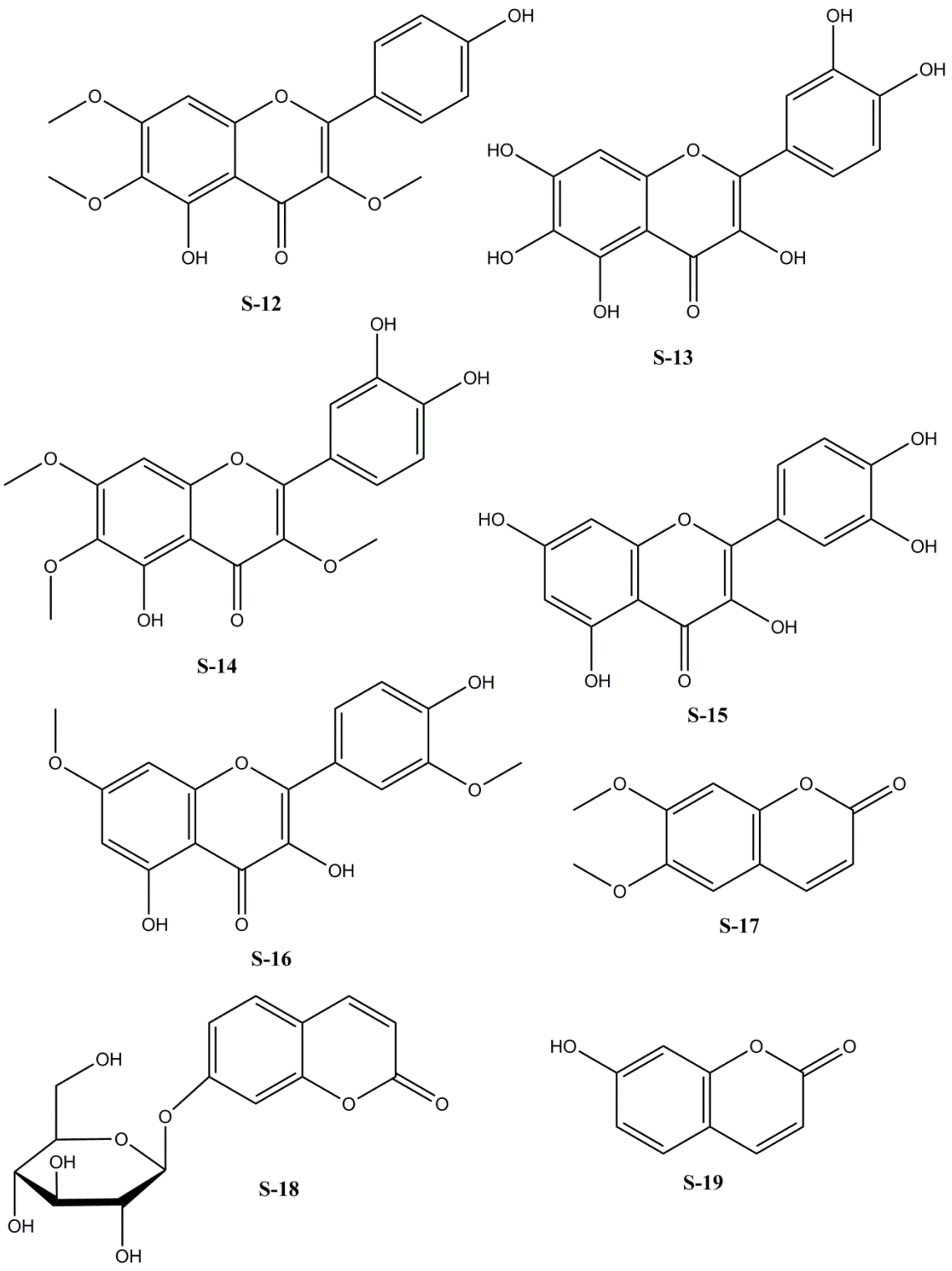

Figure A.3 Phenolic compounds identified within the subgenus *Tridentatae*.

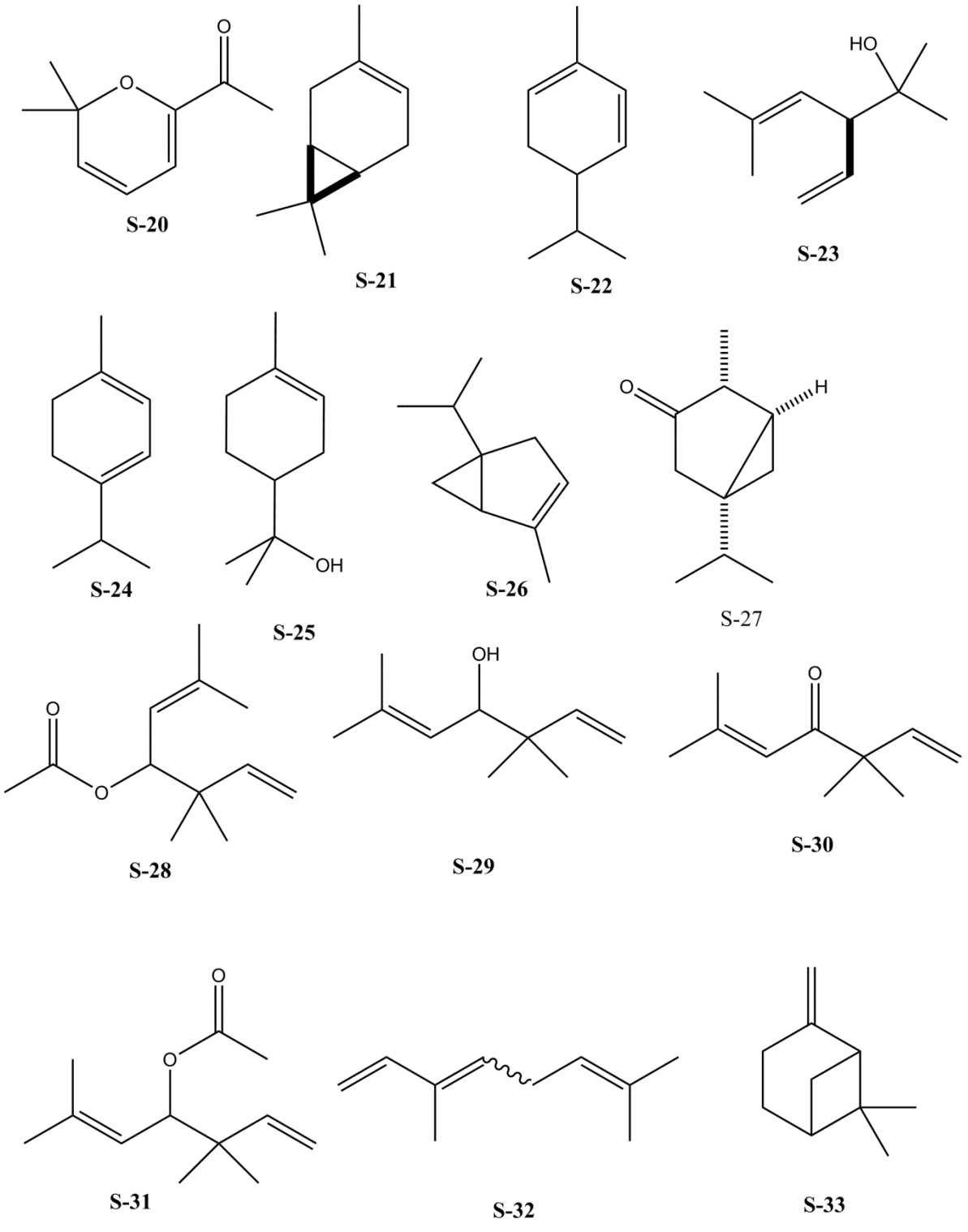

Figure A.4 Monoterpene compounds identified within the subgenus *Tridentatae*.

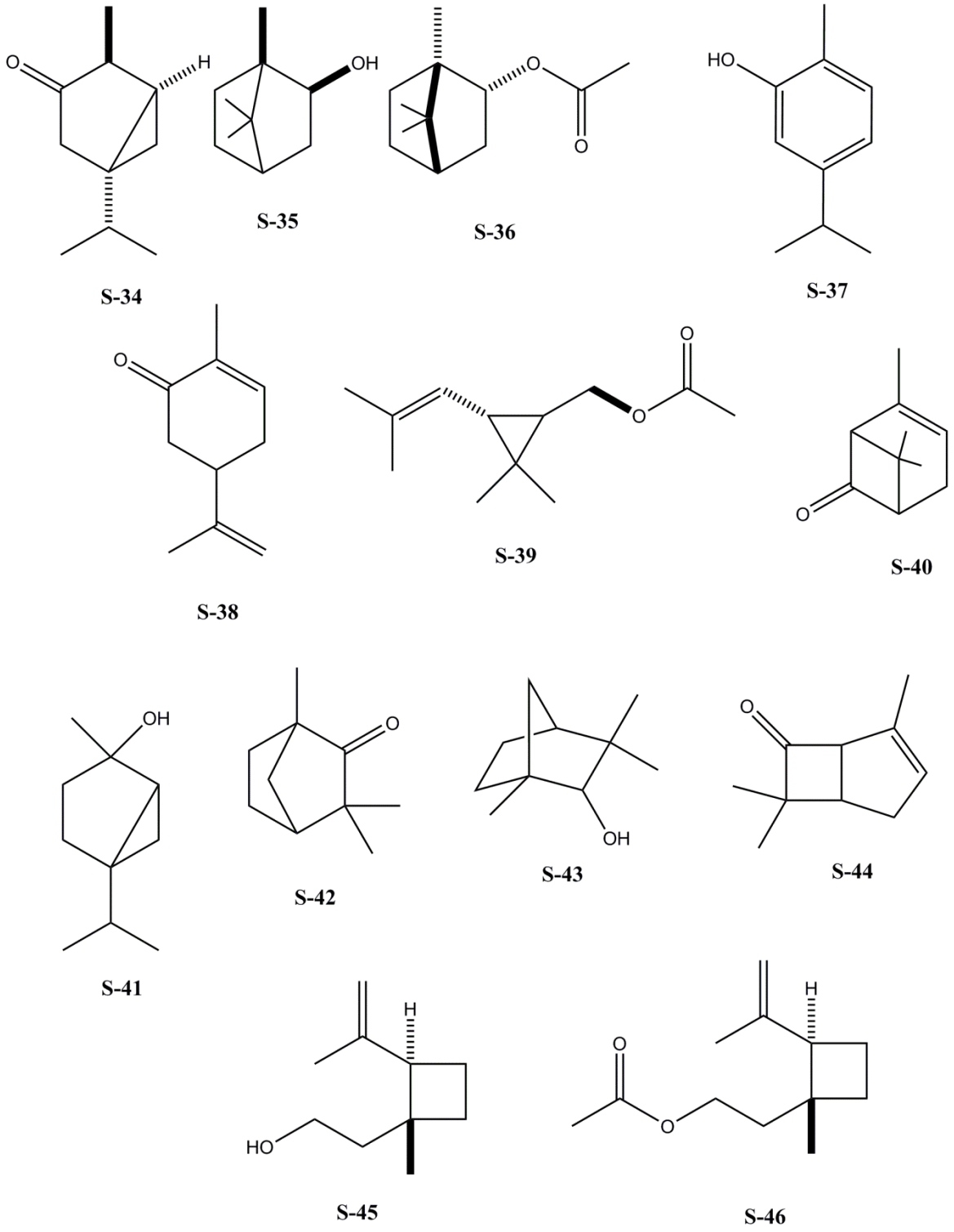

Figure A.5 Monoterpene compounds identified within the subgenus *Tridentatae*.

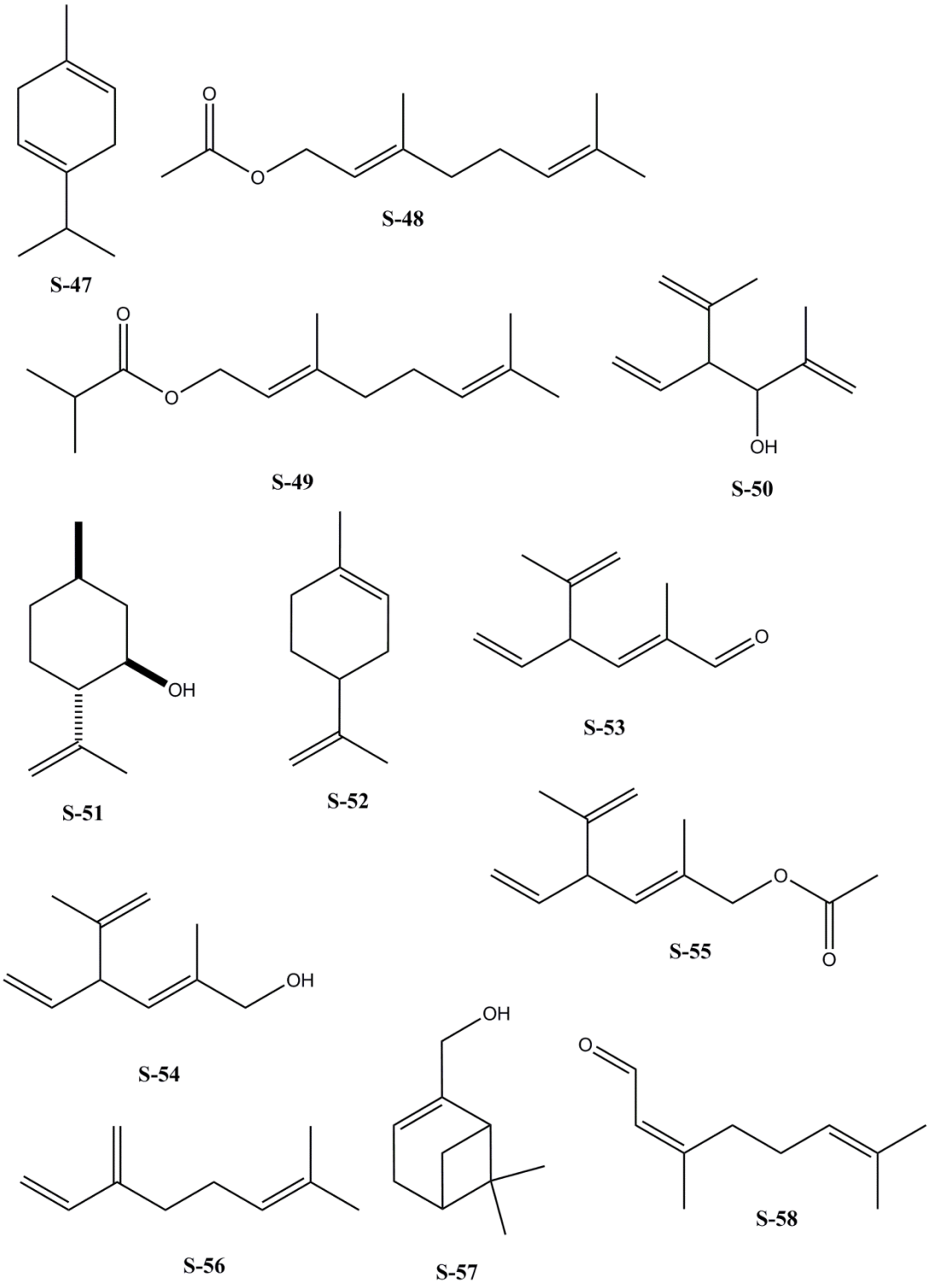

Figure A.6 Monoterpene compounds identified within the subgenus *Tridentatae*.

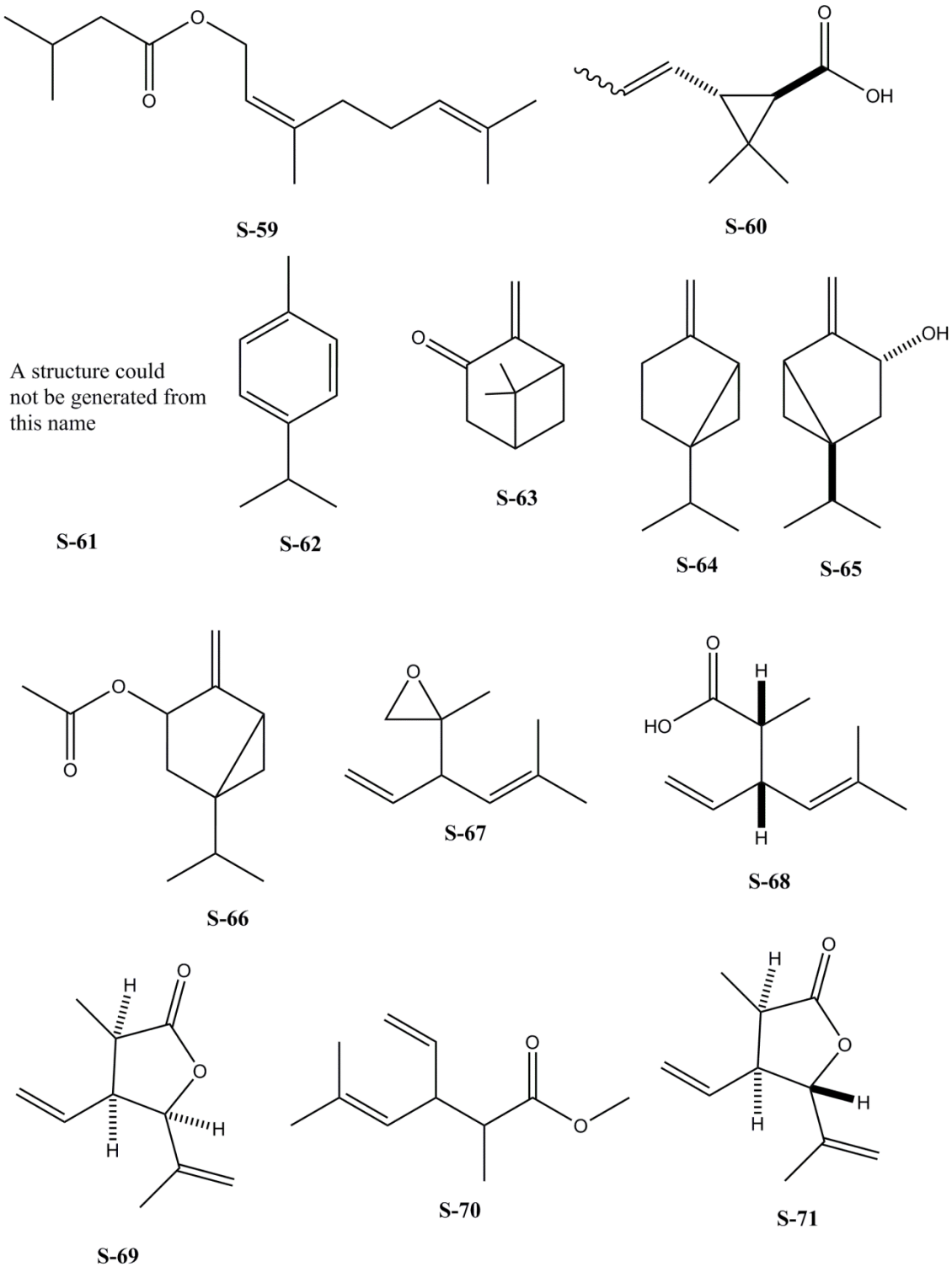

Figure A.7 Monoterpene compounds identified within the subgenus *Tridentatae*.

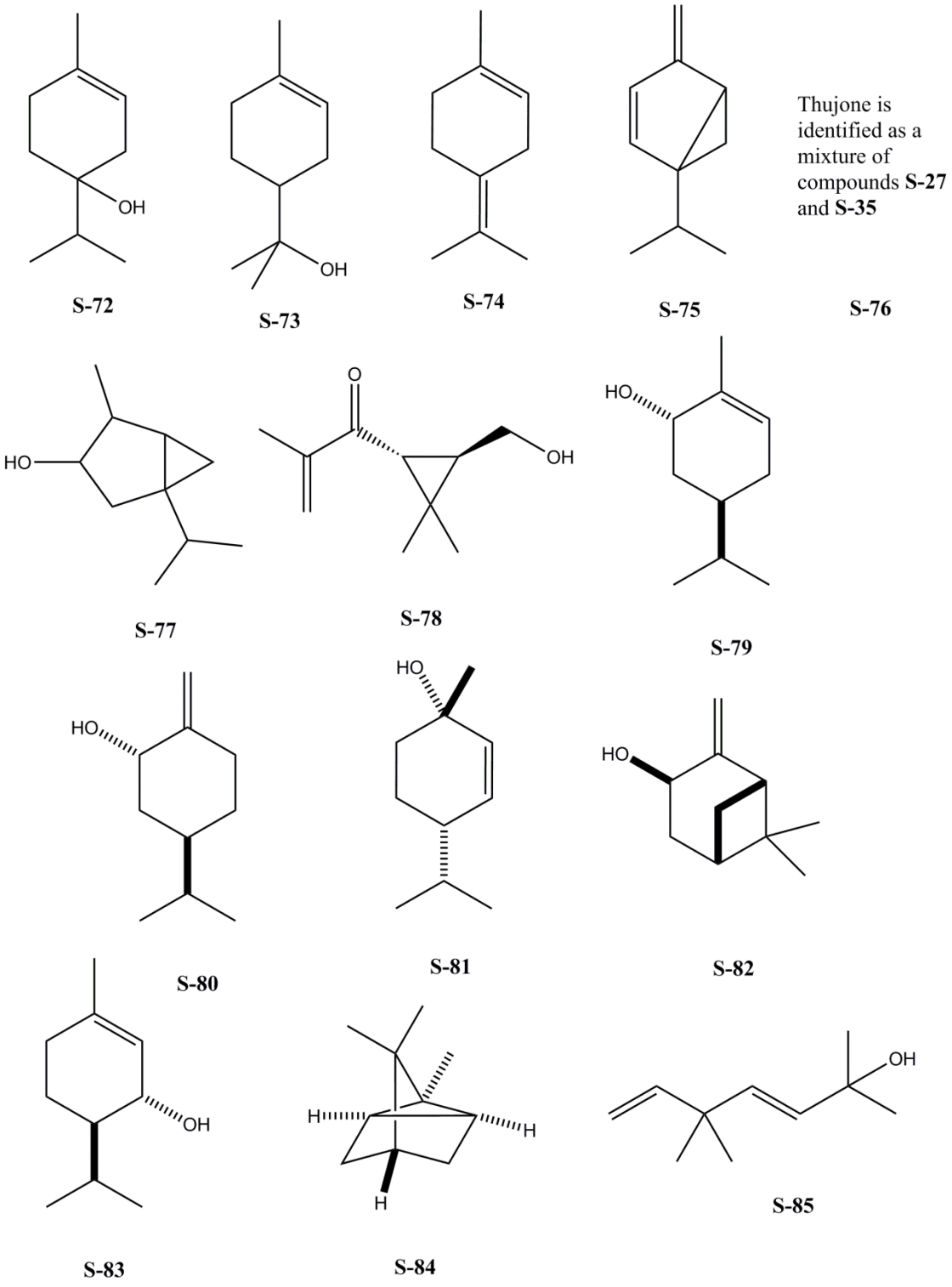

Figure A.8 Monoterpene compounds identified within the subgenus *Tridentatae*.

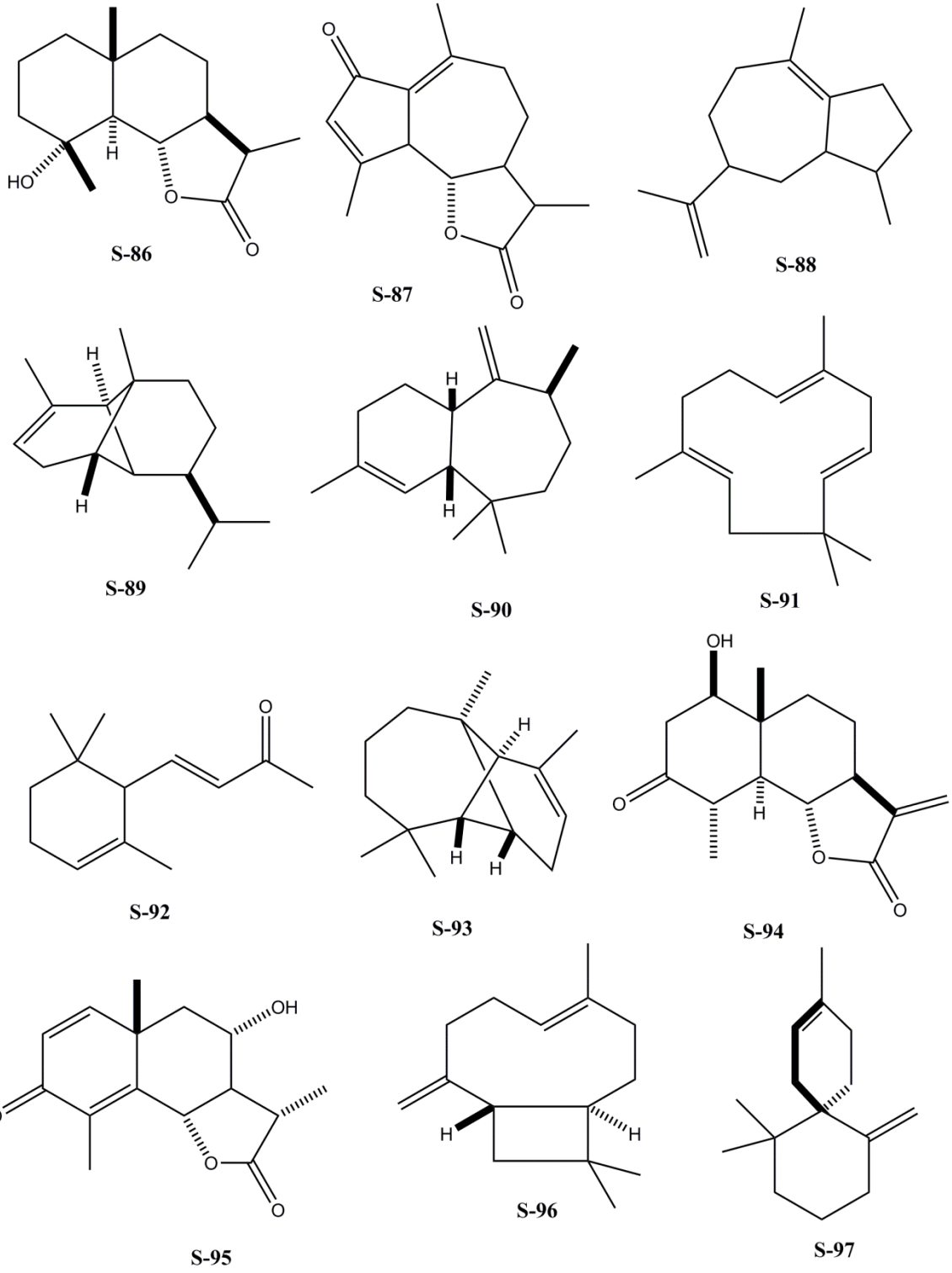

Figure A.9 Sesquiterpene compounds identified within the subgenus *Tridentatae*.

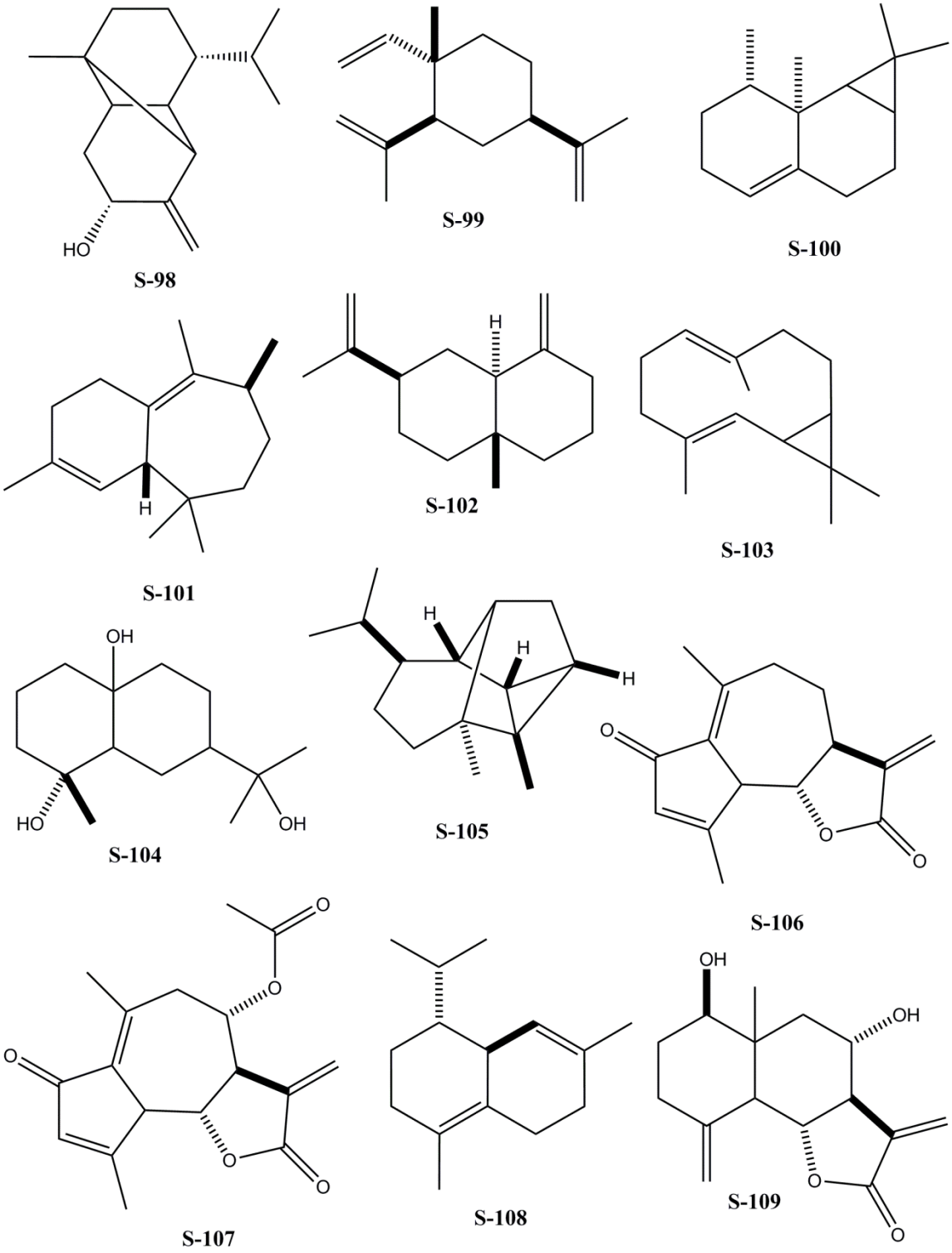

Figure A.10 Sesquiterpene compounds identified within the subgenus *Tridentatae*.

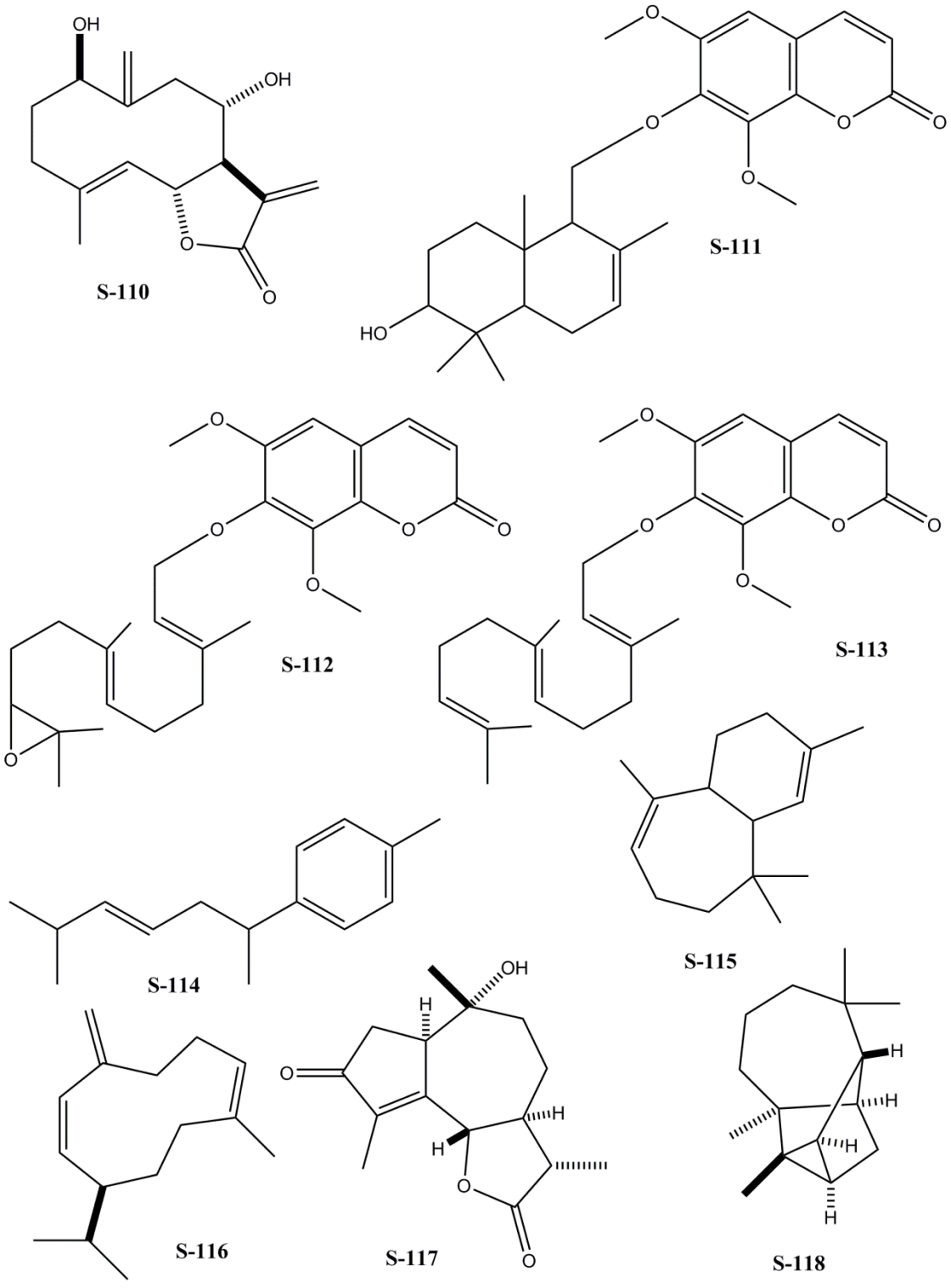

Figure A.11 Sesquiterpene compounds identified within the subgenus *Tridentatae*.

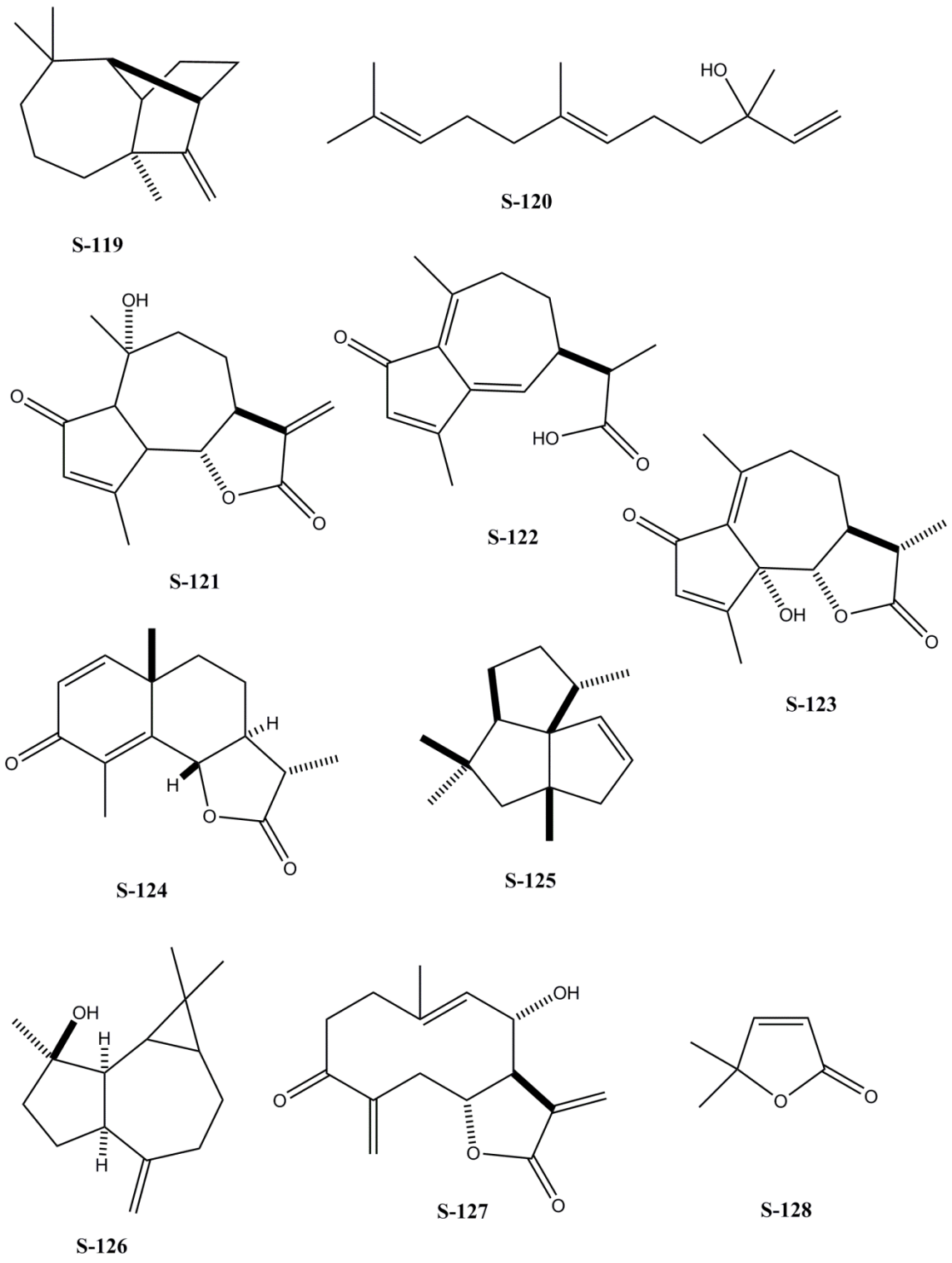

Figure A.12 Sesquiterpene compounds identified within the subgenus *Tridentatae*.

Appendix B: Sample Calculations for Synthetic Biotransformation

Using methods similar to Murch et al. (2004), the monoisotopic mass of Z/E-ligustilide and its isomer 3-butylphthalide was calculated (190.0994 Da) and transformed using algorithms in excel in order to simulate common enzymatic modifications. See example below for further clarification (Table B.1 and Figure B.1).

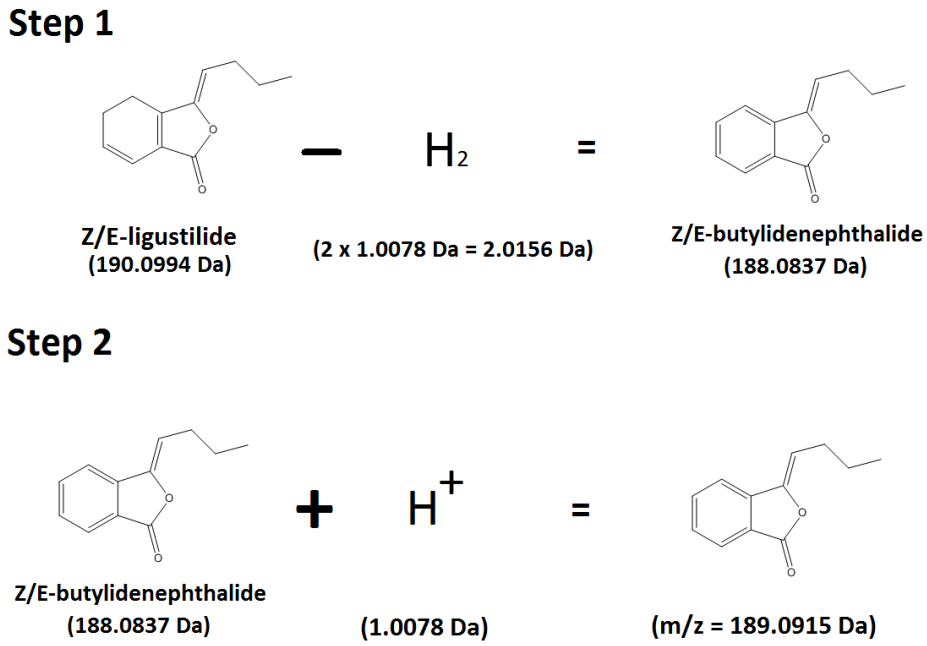

Figure B.1 Sample calculation and diagram showing a synthetic biotransformations to Z/E-ligustilide. Step 1: calculate the monoisotopic mass for the compound of interest (i.e. ligustilide) and add/subtract the monoisotopic mass of a specific biotransformation (i.e. dehydrogenase = 2.0156 Da). Step 2: Add another hydrogen atom synthetically in order to calculate the m/z for the structure. Please note that step 2 is not always required and depends on the specific structure/biotransformation under investigation. Structures were drawn using Chemdraw software.

Table B.1 Summary of all numerical transformations made to Z/E ligustilide/3-butylphthalide in Excel. Each number below represents the calculated m/z values for each transformation. Calculated m/z were then mined in the metabolomics raw dataset (±0.02 Da).

			Step 2												
Z-Ligustilide/3-butylphthalide/(Z)-iso-butylidene-3a,4-dihydrophthalide Monoisotopic mass + Hydrogen 191.107199			Minus H2	H2	Minus CH3	CH3	C6H12O6	OH	OH (x2)	COOH	NH2	NH3	Angelic Acid	Ligustilide	Butyldiene phthatlide
Step 1	Minus H2	189.0915	187.0759	191.1072	175.0759	203.1072	367.1393	205.0865	221.0814	233.0814	204.1024	205.1103	287.1283	379.1909	377.1753
	H2	193.1228	191.1072	195.1385	179.1072	207.1385	371.1706	209.1178	225.1127	237.1127	208.1337	209.1416	291.1596	383.2222	381.2066
	Minus CH3	177.0915	175.0759	179.1072	163.0759	191.1072	355.1393	193.0865	209.0814	221.0814	192.1024	193.1103	275.1283	367.1909	365.1753
	CH3	205.1228	203.1072	207.1385	191.1072	219.1385	383.1706	221.1178	237.1127	249.1127	220.1337	221.1416	303.1596	395.2222	393.2066
	C6H12O6	369.1549	367.1393	371.1706	355.1393	383.1706	547.2027	385.1499	401.1448	413.1448	384.1658	385.1737	467.1917	559.2543	557.2387
	OH	207.1021	205.0865	209.1178	193.0865	221.1178	385.1499	223.0970	239.0919	251.0919	222.1130	223.1208	305.1389	397.2015	395.1858
	OH (x2)	223.0970	221.0814	225.1127	209.0814	237.1127	401.1448	239.0919	255.0869	267.0869	238.1079	239.1158	321.1338	413.1964	411.1808
	COOH	235.0970	233.0814	237.1127	221.0814	249.1127	413.1448	251.0919	267.0869	279.0869	250.1079	251.1158	333.1338	425.1964	423.1808
	NH2	206.1181	204.1024	208.1337	192.1024	220.1337	384.1658	222.1130	238.1079	250.1079	221.1290	222.1368	304.1549	396.2175	394.2018
	NH3	207.1259	205.1103	209.1416	193.1103	221.1416	385.1737	223.1208	239.1158	251.1158	222.1368	223.1446	305.1627	397.2253	395.2096
	Angelic Acid	289.1440	287.1283	291.1596	275.1283	303.1596	467.1917	305.1389	321.1338	333.1338	304.1549	305.1627	387.1808	479.2434	477.2277
	Ligustilide	381.2066	379.1909	383.2222	367.1909	395.2222	559.2543	397.2015	413.1964	425.1964	396.2175	397.2253	479.2434	571.3059	569.2903
	Butyldienephthatlide	379.1909	377.1753	381.2066	365.1753	393.2066	557.2387	395.1858	411.1808	423.1808	394.2018	395.2096	477.2277	569.2903	567.2746
	H2 + OH(x2)	225.1127	223.0970	227.1283	211.0970	239.1283	403.1604	241.1076	257.1025	269.1025	240.1236	241.1314	323.1495	415.2121	413.1964