An ethnobotanical, pharmacological, and phytochemical analysis of *Achillea millefolium* L. by parts

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ABSTRACT

This thesis investigated the pharmacology and phytochemistry of *Achillea millefolium* L. (yarrow) flowers, roots, stems, and leaves based on ethnobotanical reports in North America, with a focus on applications in a respiratory model. Seasonal changes in the phytochemical profile of yarrow were also assessed.

A comprehensive dataset of medicinal Asteraceae was created after collecting ethnobotanical reports from the Native American Ethnobotany (NAEB) database. Using residual and binomial analyses, 14 tribes of Asteraceae were quantitatively evaluated and ranked within ten therapeutic categories as either over- or under-selected for treatment by North American indigenous peoples. Flora belonging to the Anthemideae tribe were over-utilized as pulmonary aids, particularly species of *Achillea.* Yarrow was selected for further analysis in the subsequent chapters of this thesis.

The respiratory pharmacology of yarrow was examined by testing the immunomodulatory effects of four plant parts in an *in vitro* assay using BEAS-2B human bronchial epithelial cells. Concentrations of the pro-inflammatory cytokines IL-6 and IL-8 were quantified using ELISA kits. Flowers demonstrated significant anti-inflammatory activity at 40 μg/ml in both assays, and also at 20 μg/ml in the IL-8 assay, suggesting a dose-dependent response. Roots displayed significant pro-inflammatory activity at all concentrations. A second mechanism of action via the endocannabinoid system was tested through inhibitory enzyme assays for fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), in which the flowers and roots were most active.

Since extracts of the four plant parts exhibited significantly different bioactivities, active metabolites previously identified in yarrow were quantified in each part through the targeted profiling of phenolics and alkylamides using analytical chromatographic techniques. Phenolic compounds were found at highest concentrations in the flowerheads, while alkylamides were detected predominantly within roots. An accompanying phenological analysis of alkylamide and phenolic levels in all parts was explored.

Collectively, this research provides the first integrated comparison of yarrow ethnobotany, bioactivity, and phytochemistry across different parts of the plant, contributing novel insights into the traditional, contemporary, and future uses of one of North America's most important medicinal plants.

RÉSUMÉ

Cette thèse a porté sur la pharmacologie et la phytochimie des fleurs, racines, tiges et feuilles d'*Achillea millefolium* (yarrow) à partir d'études ethnobotaniques réalisées en Amérique du Nord, en mettant l'accent sur les applications dans un modèle respiratoire. Les variations saisonnières du profil phytochimique de l'achillée ont également été évaluées.

Un ensemble de données sur les Asteraceae médicinales a été créé à partir d'études ethnobotaniques provenant de la base de données Native American Ethnobotany. À l'aide d'analyses statistiques résiduelles et binomiales, 14 tribus d'Asteraceae ont été évaluées quantitativement et classées dans dix catégories thérapeutiques selon qu'elles étaient sur ou sous-utilisées sélectionnées pour traitement par les peuples autochtones d'Amérique du Nord. Les représentants de la tribu Anthemideae étaient fréquemment utilisées pour les troubles pulmonaires, en particulier les espèces du genre *Achillea*. Yarrow a été sélectionné pour une analyse plus approfondie dans les chapitres suivants de cette thèse.

La pharmacologie respiratoire de l'achillée a été examinée en testant les effets anti-inflammatoires de quatre parties de plantes dans un essai in vitro utilisant des cellules épithéliales bronchiques humaines BEAS-2B. Les concentrations des cytokines pro-inflammatoires IL-6 et IL-8 ont été quantifiées à l'aide de kits ELISA. Les fleurs présentaient une activité anti-inflammatoire significative à 40 µg/ml dans les deux essais et également à 20 µg/ml dans le dosage de l'IL-8, suggérant une réponse dose-dépendante. Les racines présentaient une activité pro-inflammatoire significative à toutes les concentrations. Un mécanisme d'action proposé via le système endocannabinoïde a été testé par des dosages enzymatiques inhibiteurs de

la FAAH et de la monoacylglycérol lipase (MAGL), dans lesquels les fleurs et les racines étaient les plus actives.

Étant donné que les extraits des quatre parties de la plante présentaient des bioactivités significativement différentes, les métabolites actifs précédemment identifiés dans yarrow ont été quantifiés dans chaque partie en utilisant un profilage ciblé des composés phénoliques et des alkylamides en utilisant des techniques chromatographiques analytiques. Les composés phénoliques ont été trouvés à des concentrations plus élevées dans les fleurs, tandis que les alkylamides ont été détectés principalement dans les racines. Une analyse phénologique accompagnant les niveaux d'alkylamide et de phénol dans toutes les parties a été explorée.

Collectivement, cette recherche fournit la première comparaison intégrée de l’ethnobotanique achillée, de la bioactivité et de la phytochimie dans différentes parties de la plante, apportant ainsi de nouvelles informations sur les utilisations traditionnelles, contemporaines et futures de l’une des plantes médicinales les plus importantes d’Amérique du Nord.

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Δ9-THC	Δ9-tetrahydrocannabinol
2-AG	2-arachidonoylglycerol
5-LOX	5-lipoxygenase
AMC-AA	7-amino-4-methyl coumarin-arachidonamide
BAL	Bronchoalveolar lavage
CAM	Complementary and alternative medicine
CB	Cannabinoid receptor
chDNA	Chloroplast DNA
DAD	Diode array detector
DMEM/F12	Dulbecco's modified eagle medium: nutrient mixture F-12
DMSO	Dimethylsulfoxide
ECS	Endocannabinoid system
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FAAH	Fatty acid amide hydrolase
HPLC	High-performance liquid chromatography
IC_{50}	Median inhibitory concentration
IL	Interleukin
ITS	Internal transcribed spacer
LC	Liquid chromatography
LPS	Lipopolysaccharide
MAGL	Monoacylglycerol lipase
MRM	Multiple reaction monitoring
MS	Mass spectrometer
m/z	Mass over charge ratio
NAEB	Native American ethnobotany
NMR	Nuclear magnetic resonance
nrDNA	Nuclear ribosomal DNA
OTD	Optimal defense theory
PDA	Photo diode array
QTRAP	Quadrupole ion trap
RCF	Relative centrifugal force
RT	Retention time
SEM	Standard error of the mean
TIC	Total ion count
UPLC	Ultra-performance liquid chromatography
WST-1	Water-soluble tetrazolium salt

CHAPTER 1 – GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The sunflower family (Asteraceae) is the largest family of flowering plants, including over 1500 genera and 23,000 species distributed nearly worldwide (Flora of North America, 2006). Although particularly abundant and diverse in tropical regions including Central America and the Mediterranean, Asteraceae is the largest family in North American flora. Numerous species have been used traditionally in the form of teas, tinctures, or topical agents as medicinal treatments in indigenous healing practices (Moerman, 2003). Traditional knowledge of these herbal remedies remains relevant today, as information has been passed down through previous generations. Within the discipline of ethnobotany, the relationship between plants and humans can be studied to further comprehend how cultures make use of particular species for medicinal purposes, as well as to understand the significance of these flora among indigenous communities. This type of ethnobotanical research continues to thrive as medicinal plants – carefully selected over generations of knowledge transfer – remain popular today, with increasing scientific evidence of their therapeutic potential, including the discovery of major bioactive components and their modes of action.

Further investigation into the ethnobotany, pharmacology, and phytochemistry of a significantly used species of Asteraceae, *Achillea millefolium* L., was conducted within this thesis to facilitate the comparison of different plant organs and the potential discovery of novel marker compounds or pharmacological applications of this traditionally used medicinal plant.

1.2 LITERATURE REVIEW

1.2.1 ETHNOBOTANY OF *ACHILLEA*

The use of plants in healing and medicinal applications is one of the main components of alternative medicine. Complementary and Alternative Medicine (CAM) comprises a group of procedures and therapeutic treatments with roots outside conventional medicine (Eisenberg et al., 1993). These include homeopathy, naturopathy, and indigenous healing practices (e.g. Traditional Chinese Medicine), all of which employ herbal extract remedies to varying degrees. Traditional knowledge of plants and their healing properties has been developed and applied over centuries, even millennia, and passed down as a cultural practice from generation to generation. The study of the relationship between plants and humans is termed ethnobotany, a multidisciplinary field that continues to thrive and evolve today. Not limited to the use of plant species for medicinal purposes, ethnobotany also encompasses the relationships between peoples, cultures, and plants as food, clothing, fuel, and currency, as well as spirits, living beings, and essential components of our natural and designed environments.

Achillea millefolium L. (Asteraceae), more commonly known as yarrow, is one of the most widely used medicinal plants among indigenous peoples of North America. Native to Europe and North America**,** this species possesses active compounds with anxiolytic, gastroprotective, and anti-inflammatory effects (Choudhary et al., 2007; Moerman, 2009). Similar to its global distribution, yarrow's medicinal value has been recognized around the world. The people of ancient Greece made use of this species as a poultice to heal the battle wounds of warriors. Today, research pertaining to yarrow as a herbal product is gaining popularity, as the traditional uses of this species are so widespread and pertinent to a variety of illnesses. Described by Applequist and Moerman (2011) as a "neglected panacea", this ancient

herbal medicine has emerged as the focus of countless scientific studies evaluating and reviewing the plant's ethnobotany, reported phytochemistry, and related bioactivity.

1.2.2 Pharmacology of Asteraceae

Asteraceae species have been used medicinally to treat a variety of illnesses and symptoms including inflammation, cold and flu, and pulmonary and respiratory diseases. Ethnobotanical records suggest that different plant parts are used as treatments for pulmonary illnesses such as asthma, bronchitis, chest constrictions, pneumonia, sinus attacks, colds, and coughs (Moerman, 2003). The rationale behind bioactivity research of plant secondary metabolites is often based upon these ethnobotanical reports. For instance, *Echinacea* species are often used in traditional medicine to treat cold, cough, and flu (Barrett, 2003). Research investigating the anti-inflammatory properties of this genus reported significant inhibitory effects of *Echinacea* extracts on 5-lipoxygenase (5-LOX) activity (Merali et al., 2003). This enzyme is responsible for leukotriene synthesis, resulting in bronchoconstriction and mucous production in various pulmonary conditions. When inhibited, these physiological symptoms would decrease. By examining the pulmonary applications within Asteraceae, this review further investigates the traditional use of North American species as pulmonary and respiratory treatments.

Within this family, the Anthemideae tribe consists of 110 genera distributed predominantly throughout the Mediterranean, southern Africa, and central Asia; however, there is also an abundance of North American species. Over half of the species are classified into five genera, including *Artemisia,* the largest of the group, as well as *Achillea* (Watson et al., 2000). Several *Artemisia* species are widespread across North America, though many are located in the western United States and Canada. *Achillea* and *Artemisia* are two genera mainly used for

treating pulmonary illnesses and their symptoms, such as respiratory infections, colds, coughs, rheumatism, and asthma based on North American ethnobotanical reports. Several *Artemisia* species have been tested for bioactivity and contain phytochemicals responsible for these medicinal properties. The three main classes of compounds within Anthemideae are flavonoids, polyacetylenes, and sesquiterpene lactones (Greger, 1977). Luteolin, a flavonoid found in *Artemisia afra*, contributes to the bronchodilatory effects of this species. Using an isolated perfused lung system to test the effects of *A. afra* and the traditional method of steam inhalation, extracts were found to decrease tidal volume and lung resistance, both involved in the bronchodilatory process (Mjiqiza et al., 2013). Whereas yarrow is referred to as a global panacea and, like *Artemisia* has been widely used to treat pulmonary symptoms, extracts of yarrow have not yet been tested in respiratory models. One objective of this thesis was to reduce this knowledge gap and collect pharmacological data for four separate parts of the yarrow plant. It is important to assess distinct plant organs for variation in bioactivity, not only because ethnobotanical records indicate the selective use of separate parts for medicinal purposes but because different plant tissues and organs generally differ in their phytochemistry.

1.2.3 Phytochemistry of Asteraceae

For centuries, the evolution of cultures and their selective applications of medicinal plants have led to the modern discovery of bioactive compounds and the precise association of different natural products to specific physiological functions. With medicinal plants, such pharmacological activities are generally mediated by secondary metabolites, which vary widely in their structure and distribution among plant taxa. These phytochemicals are often the evolutionary products of plant-animal or plant-microbe interactions (e.g. for plant defense).

Several herbivorous insects have adapted to these defenses and in some cases may sequester them for their own protection. For example, *Longitarsus* beetles sequester and metabolize pyrrolizidine alkaloids found in Asteraceae host plants and use them for their own defense while the same phytochemicals effectively deter herbivory by other insects (Dobler et al., 2000; Macel et al., 2005). The human use of bioactive phytochemicals as medicine (or as pesticides) is rooted in, and in some cases comparable to, such co-evolution of plants and insects, providing another basis of reasoning for investigating the bioactivity of plant secondary metabolites.

The phytochemistry of plants within the Asteraceae has been studied extensively. There are several general phytochemical groups that are characteristic of this family, one being alkylamides. Produced by plants used in several traditional medicinal systems, alkylamides are reported in over 100 species from 26 plant families, with the majority from the Asteraceae (Boonen et al., 2012). The three most common species of *Echinacea* (Heliantheae) include *E. purpurea, E. angustifolia,* and *E. pallida*, all of which contain a wide array of active compounds, including alkylamides (Bauer, Remiger, & Wagner, 1989; Woelkart et al., 2005). Alkylamides are secondary metabolites with anti-inflammatory properties, often acting as agonists to receptors in the endocannabinoid system (ECS). This mechanism is responsible for the significant immunomodulatory and anti-inflammatory properties of medicinal plants such as *Echinacea* (Raduner et al., 2006; Woelkart et al., 2005). Given the close taxonomic relationship of Heliantheae and Anthemideae tribes, it is possible that alkylamides are present in several genera of Anthemideae and contribute to decreased inflammation prevalent in pulmonary diseases. It was hypothesized that higher levels of alkylamides exist in the most used North American Anthemideae species selected for pulmonary treatments. A systematic review of the online Alkamid database (Boonen et al., 2012**)** identified 92 alkylamide compounds isolated within the

Anthemideae. Comparing these data to North American Anthemideae selected for pulmonary aid, a total of 21 distinct alkylamides were found in two of the pulmonary species: *Achillea millefolium*, and *Artemisia dracunculus*. According to this database, the majority of known alkylamides are found within *Achillea* species, while only three have been isolated in *A. dracunculus*. However, the Alkamid database is unlikely an exhaustive list. Further phytochemical research should be completed on Anthemideae to provide updated reports.

1.3 RESEARCH OBJECTIVES AND HYPOTHESES

The main goal of this thesis was to comprehensively assess the medicinal ethnobotany of *Achillea millefolium* by contributing to the pharmacological and phytochemical research pertaining to yarrow, one of North America's most prominent medicinal plants. An overview of the project structure is presented in Figure 1.1. In Chapter 2, data collected from the Native American Ethnobotany (NAEB) database (Moerman, 2003) was used to compile a more simplified database summarizing reported ethnobotanical uses for all Asteraceae species across North America, as well as information on the plant organ used. Though 'indigenous' has now replaced the term 'Native American', for the sake of continuity this thesis will refer to the NAEB database by its original name stated above. Ten medicinal categories were evaluated at familial and tribal levels of phylogeny, with specific interest placed on Asteraceae selected as treatments for pulmonary illness. It was hypothesized that the selection of North American medicinal species is non-random, and that specific flora are actively utilized or avoided depending on the therapeutic category. Using residual statistical methods described by Moerman (1991) as well as a secondary level of analysis employing binomial statistics, 14 tribes of Asteraceae were ranked as either over-selected or under-selected for treatment in each therapeutic category based on the total size of the flora. Species belonging to Anthemideae were found to be over-utilized as pulmonary aids. These quantitative analyses presented *A. millefolium* as one of the most selected species in North America for treating respiratory and pulmonary symptoms, and thus the subsequent chapters of this project focused on evaluating the pharmacology and chemistry of this species with regards to this promising activity.

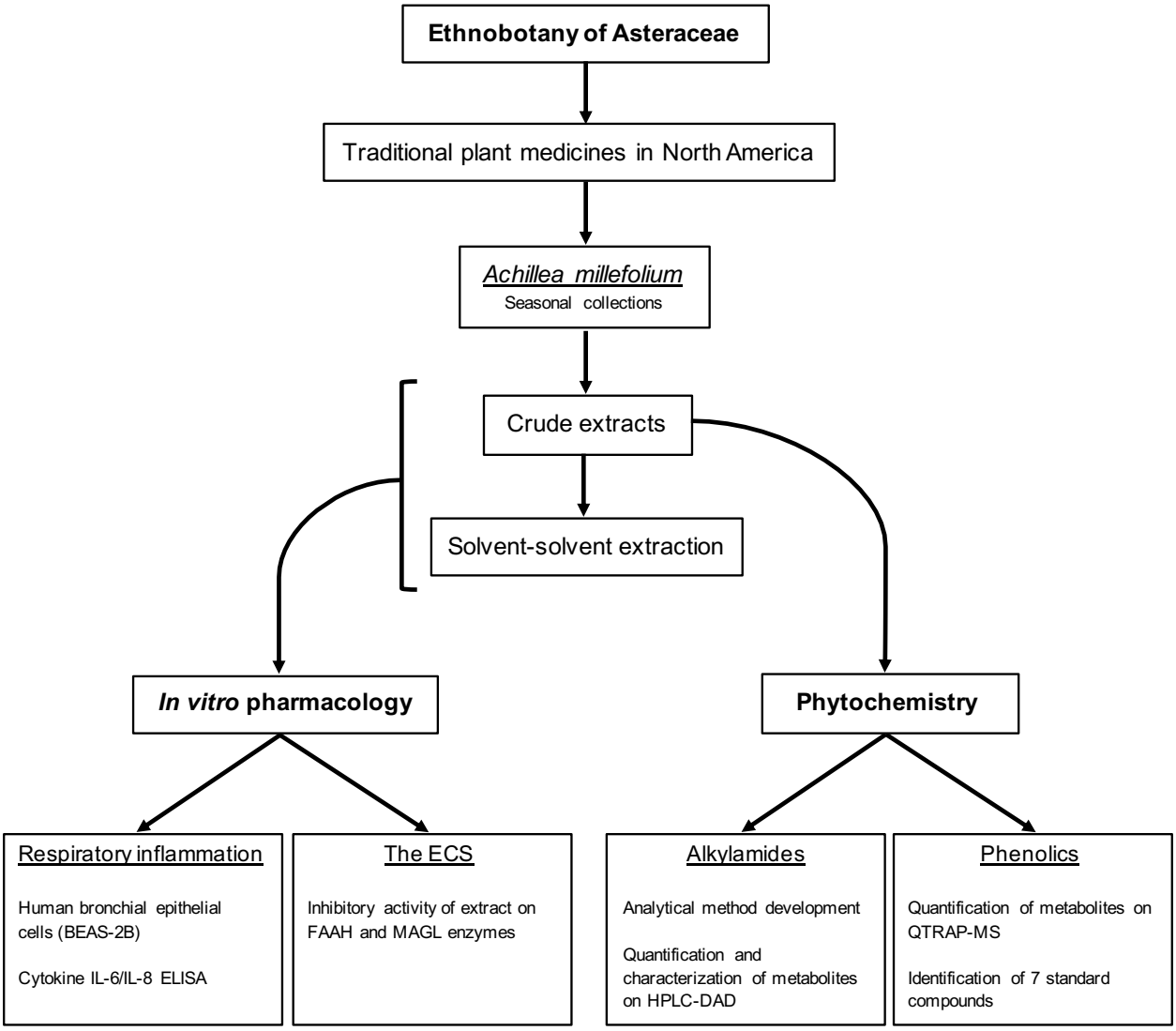


Figure 1.1 – Outline of the research methods for this thesis. Flow chart beginning with an ethnobotanical analysis of Asteraceae then leading to a pharmacological assessment and phytochemical characterization of *Achillea millefolium*.

Next, Chapter 3 provides a pharmacological analysis of the flowers, roots, leaves, and stems of yarrow by exploring two mechanisms of action. Firstly, the immunomodulatory activity of yarrow extracts was assessed in an *in vitro* model using BEAS-2B human bronchial epithelial cells. The concentration of cytokines interleukin 6 (IL-6) and interleukin 8 (IL-8) were measured using an enzyme-linked immunosorbent assay (ELISA), where the activities of four yarrow parts were compared. Second, potential interactions with the ECS were studied. Four parts of yarrow were tested for inhibitory activity of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), two enzymes responsible for the degradation of endogenous cannabinoids. Organic and aqueous extracts of yarrow flowers and roots were also tested in IL-6 ELISA and ECS enzyme assays for a supplementary analysis.

Finally, Chapter 4 summarizes a seasonal analysis of the phytochemistry of flowerheads, roots, leaves, and stems of yarrow grown in and collected from the Carleton University medicinal gardens. An evaluation of the results from Chapter 3 suggested that the differing bioactivities of yarrow parts can likely be attributed to their distinct phytochemical profiles. Based on these findings, as well as phytochemical reports from recent literature, this chapter employed a targeted profiling of alkylamides and phenolic compounds in *A. millefolium* parts collected approximately every two weeks over four months. The developmental trends of yarrow were also assessed through the comparison of early- and late-season collections. It was hypothesized that phenology would have a significant effect on phytochemical profile.

CHAPTER 2 – ASSESSMENT OF NORTH AMERICAN MEDICINAL ASTERACEAE COMPARING RESIDUAL AND BINOMIAL ANALYSES

2.1 INTRODUCTION

The Asteraceae family of flowering plants is the largest family in North America. Across the continent, Asteraceae is also the most medicinally used family among indigenous peoples. Patterns of medicinal plant selection worldwide have been investigated and show significant similarities in both over-selected and under-selected taxa by communities throughout regions across the globe (Moerman et al., 1999). As the first ethnobotanical meta-analysis of its kind, a combination of regression and residual analyses developed by Moerman (1991) was used to evaluate potential relationships between the size of a plant family and the number of species within that family selected for medicinal use. If this relationship is random, it is expected that, as the size of a family increases, the number of medicinal species within that family will increase accordingly. Interestingly, this was not the case. Certain families are used more or less often than expected based on family size. These data were ranked by their corresponding residual values where a large positive residual represents a family favoured for medicinal use. Moerman (1991) compared 14 taxa at the family level and concluded that the Asteraceae family contains the largest number of medicinally used species. More importantly, Asteraceae species are much more highly selected as medicinal aids than expected. This was consistent across cultures from well-separated geographical regions.

Due to the ethnobotanical significance of this plant family, the medicinal applications of North American Asteraceae were evaluated and their prevalence in traditional practices were assessed. Making use of Moerman's regression and residual analytical methods, this review provides a summary and comparative analysis of medicinal Asteraceae species selected by

indigenous peoples of North America. Since this method biases large families, a binomial analysis of plant uses was also executed, which similarly hypothesizes that the proportion of medicinal species in the total flora is the same as the proportion of medicinal species within a family. Statistically significant deviation from this proportion for any given family thereby indicates over- or under-selection for medicinal use. Ranking based on probability rather than residuals, binomial analysis does not bias based on the family size (Bennett & Husby, 2008).

Using the online Native American Ethnobotany (NAEB) database, a list of Asteraceae species with known medicinal uses was compiled and reviewed. An analysis of residual and binomial statistical approaches was compared. Based on significant results from an initial assessment of Asteraceae, the Anthemideae tribe was selected for further evaluation, where the phylogenetic relationships of the most medicinally used species were compared. The objective of this project was to review the NAEB database and identify genera or species that are over-utilized as medicinal aids and thus provide a foundation for future analysis of these flora. It was hypothesized that the selection of North American Asteraceae species, and subsequently the selection of Anthemideae species, by indigenous peoples is non-random and in fact actively favoured or avoided for use as medicinal aids in ten therapeutic categories.

2.2 METHODS

2.2.1 Collection and Organization of Ethnobotanical Data

Reports of ethnobotanical North American Asteraceae species were collated and reviewed using the online Native American Ethnobotany (NAEB) database (Moerman, 2003). This collection of data assembled over several decades summarizes reports of traditional plant uses by 291 indigenous tribes across North America into the following use categories: drug, food, fiber, dye, and other. The term 'tribe' as defined in the NAEB database will be replaced with the proper Canadian terms 'First Nations' or 'indigenous' throughout this thesis. The database contains over 44,000 entries from 243 different plant families. Each database entry provides a concise report of a particular species, its use category, the part(s) of the plant used and how it is administered, as well as the indigenous community referenced to have used the species. All North American Asteraceae taxa were identified based on reports from the Flora of North America (2006), consisting of 2413 species belonging to 418 genera. The database was queried with each genus name and reports were collected and organized by appropriate taxa, creating a complete dataset of ethnobotanical Asteraceae. These entries were grouped into one of three categories: medicine, technology, or food. Tribal analyses within Asteraceae incorporate reports only identified as medicine.

2.2.2 Residual Analysis

All residual analyses were performed in RStudio (Version 1.0.153) based on methods used by Moerman (1991; 1996; et al., 1999). Asteraceae taxa were assessed in terms of their likelihood to be over- or under-used as a medicinal aid. Variables were compared using least squares linear regression analysis using the lm() function and the 'dplyr' R package. Residual

values were calculated in order to accurately compare results across the Asteraceae family, by subtracting the observed value from the expected value attained from the linear regression.

2.2.3 Binomial Analysis

All binomial analyses were performed in Excel based on methods used by Bennett and Husby (2008) and Turi and Murch (2013). For each analysis, the null hypothesis was that the proportion of uses or species selected as ethnobotanical aids was equal to the total number of uses or species. The alternative hypotheses stated that this proportion was either greater or less than the total proportion within the flora. Analyses were carried out using the BINOM.DIST function in Excel.

2.3 RESULTS AND DISCUSSION

2.3.1 Analysis of Medicinal Asteraceae

Initially, a general analysis of reported ethnobotanical uses within the Asteraceae was completed, evaluating the usage of particular taxa for medicine, technology, and food. Residual analyses (Table 2.1) show that Anthemideae was the highest ranked over-selected tribe for medicinal aids and for technological applications, and the highest ranked under-selected tribe for use as food. Similarly, binomial analysis (Table 2.2) ranked Anthemideae first for its significant over-use in technology, but second in medicine closely following Eupatorieae. Both residual and binomial analyses show the significant under-utilization of Heliantheae and Cichorieae in medicine and technology. Astereae ranked first as significantly over-used as food and second, after Anthemideae, in terms of technology-related uses. A least squares linear regression of total medicinal uses relative to total ethnobotanical uses displays Anthemideae as the most over-selected tribe for medicinal use (Figure 2.1).

Further analysis of Asteraceae tribes compared the proportion of reported medicinal species in each tribe to the total number of species in North America (Figure 2.2). Both residual values and binomial statistics rank Anthemideae as the top tribe in which species are significantly over-used and selected more often than predicted as medicinal aids (Table 2.3, 2.4). Eupatorieae and Senecioneae emerge as the two most significantly under-used tribes in both analyses. Comparison of total medicinal uses and total available species across families offers the most pronounced illustration of this selection bias among North American indigenous peoples (Figure 2.3). Arguments for successful binomial analyses require the probability of success on each trial to be between 0 and 1, and that number of successes ≤ number of independent trials. These arguments were not met since use counts (number of successes) were

Table 2.1 – Residual analysis of ethnobotanical uses of Asteraceae.

Over-Selected Tribes								
Medicine			**Technology**			**Food**		
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Anthemideae	72.76	1	Anthemideae	39.29	1	Astereae	528.61
2	Astereae	36.16	2	Astereae	31.18	2	Eupatorieae	134.78
3	Eupatorieae	35.93	3	Plucheeae	14.18			
4	Gnaphalieae	16.28	4	Senecioneae	12.10			
5	Inuleae	9.53	5	Arctotideae	4.89			
6	Mutisieae	1.50	6	Calenduleae	4.89			
7	Vernonieae	0.28	7	Vernonieae	4.83			
			8	Mutisieae	3.80			
Under-Selected Tribes								
Medicine			**Technology**			**Food**		
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Heliantheae	-107.21	1	Cichorieae	-24.54	1	Anthemideae	-324.83
2	Cichorieae	-28.20	2	Eupatorieae	-16.64	2	Gnaphalieae	-58.04
3	Cardueae	-21.85	3	Cardueae	-12.76	3	Senecioneae	-51.76
4	Senecioneae	-7.14	4	Gnaphalieae	-1.86	4	Inuleae	-40.78
5	Plucheeae	-7.02	5	Inuleae	-0.57	5	Vernonieae	-33.37
6	Arctotideae	-0.51				6	Arctotideae	-30.49
7	Calenduleae	-0.51				7	Calenduleae	-30.49
						8	Mutisieae	-23.54
						9	Heliantheae	-21.79
						10	Cardueae	-21.45
						11	Cichorieae	-15.63
						12	Plucheeae	-11.23

Table 2.2 – Binomial analysis of ethnobotanical uses of Asteraceae.

Over-Selected Tribes								
Medicine			**Technology**			**Food**		
Rank	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**
1	**Eupatorieae**	**1.61E-10**	1	**Anthemideae**	**5.79E-06**	1	**Astereae**	**1.47E-222**
2	**Anthemideae**	**2.62E-06**	2	**Astereae**	**0.000048**	2	**Eupatorieae**	**1.01E-88**
3	**Inuleae**	**0.000013**	3	**Plucheeae**	**0.000076**	3	**Plucheeae**	**5.35E-06**
4	**Gnaphalieae**	**0.000186**	4	**Senecioneae**	**0.015397**	4	**Mutisieae**	**0.000031**
5	**Astereae**	**0.002865**	5	Vernonieae	0.559132			
6	Mutisieae	0.104787						
7	Vernonieae	0.459866						
Under-Selected Tribes								
Medicine			**Technology**			**Food**		
Rank	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**
1	**Heliantheae**	**1.16E-12**	1	**Cichorieae**	**4.50E-07**	1	**Anthemideae**	**2.95E-175**
2	**Cichorieae**	**0.000213**	2	**Heliantheae**	**7.32E-06**	2	**Gnaphalieae**	**1.36E-17**
3	**Cardueae**	**0.000691**	3	**Eupatorieae**	**8.61E-06**	3	**Senecioneae**	**4.46E-11**
4	**Plucheeae**	**0.020307**	4	**Cardueae**	**0.000691**	4	**Heliantheae**	**1.83E-10**
5	Senecioneae	0.116404	5	**Inuleae**	**0.005990**	5	**Inuleae**	**3.94E-08**
			6	Gnaphalieae	0.112932	6	**Vernonieae**	**0.014081**
			7	Mutisieae	0.359319	7	Cichorieae	0.054017
						8	Cardueae	0.061272

Bolded values: statistically significant *P*-Value < 0.05

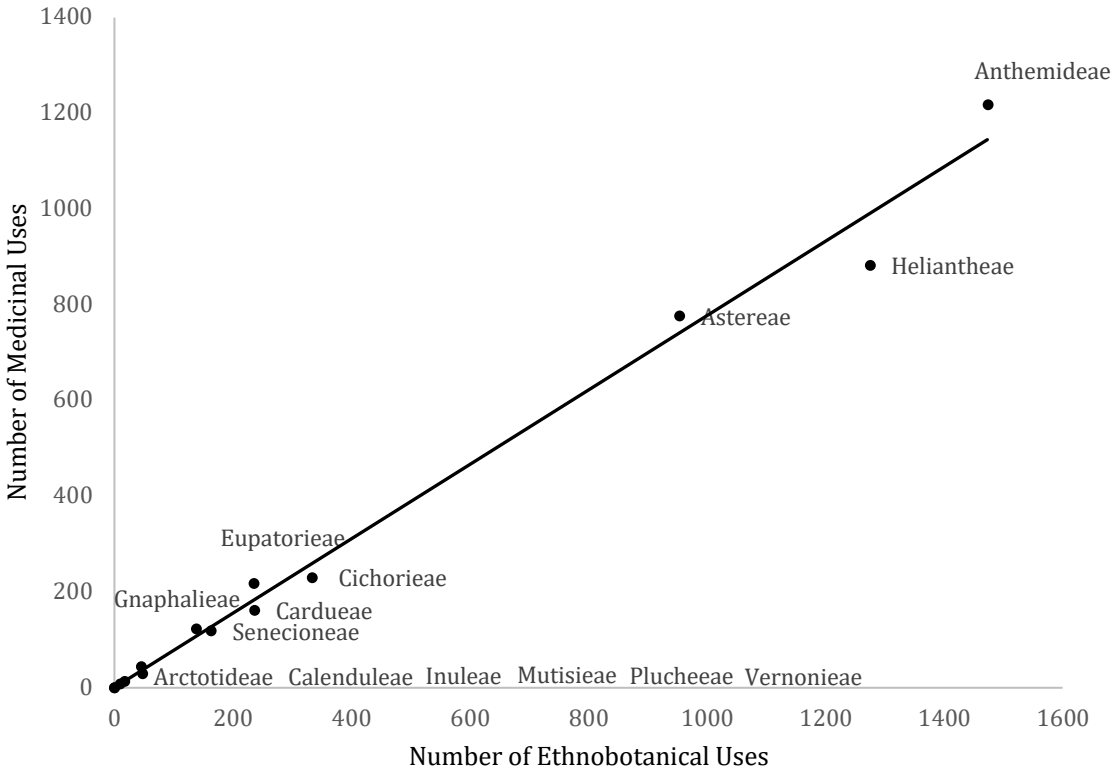


Figure 2.1 – Residual analysis of 14 North American Asteraceae tribes with uses in medicine. Regression follows the equation $y = 0.777x + 0.4738$, $R^2 = 0.98929$.

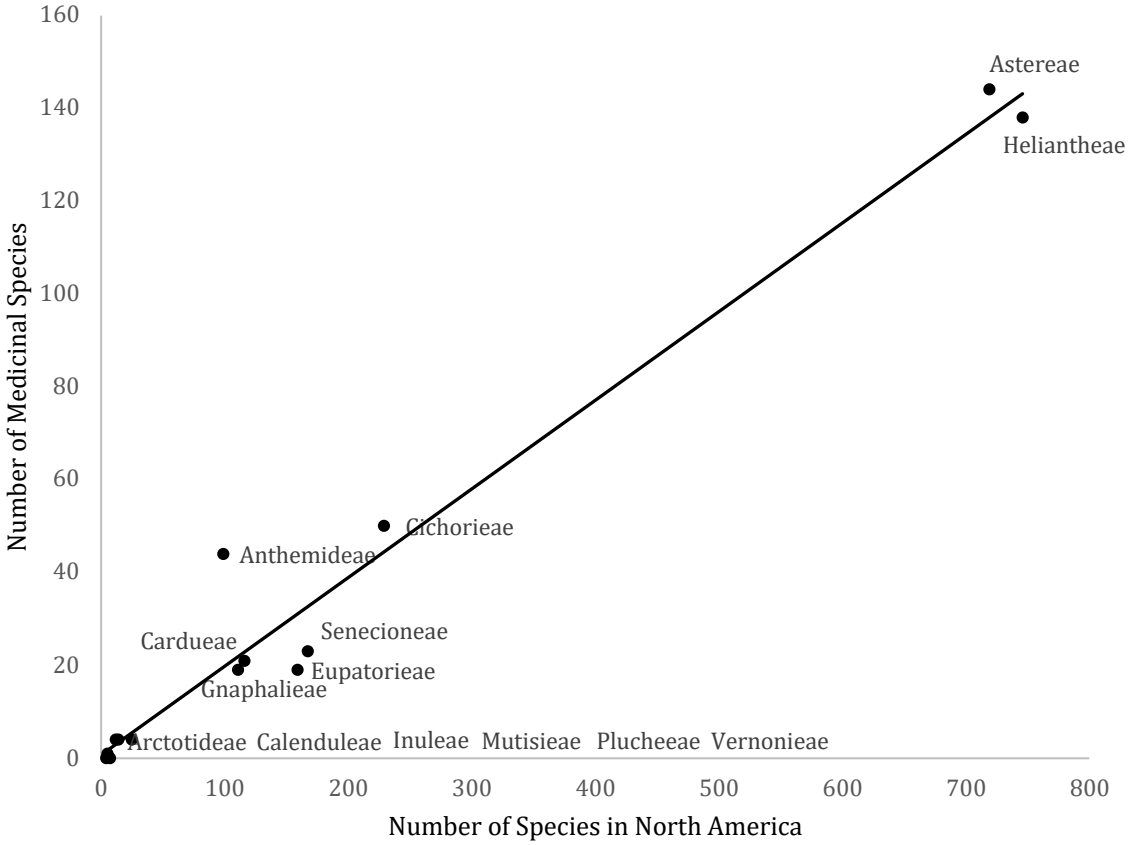


Figure 2.2 - Residual analysis of species found throughout 14 North American Asteraceae tribes with uses in medicine. Regression follows the equation $y = 0.1909x + 0.7314$, $R^2 = 0.9685$.

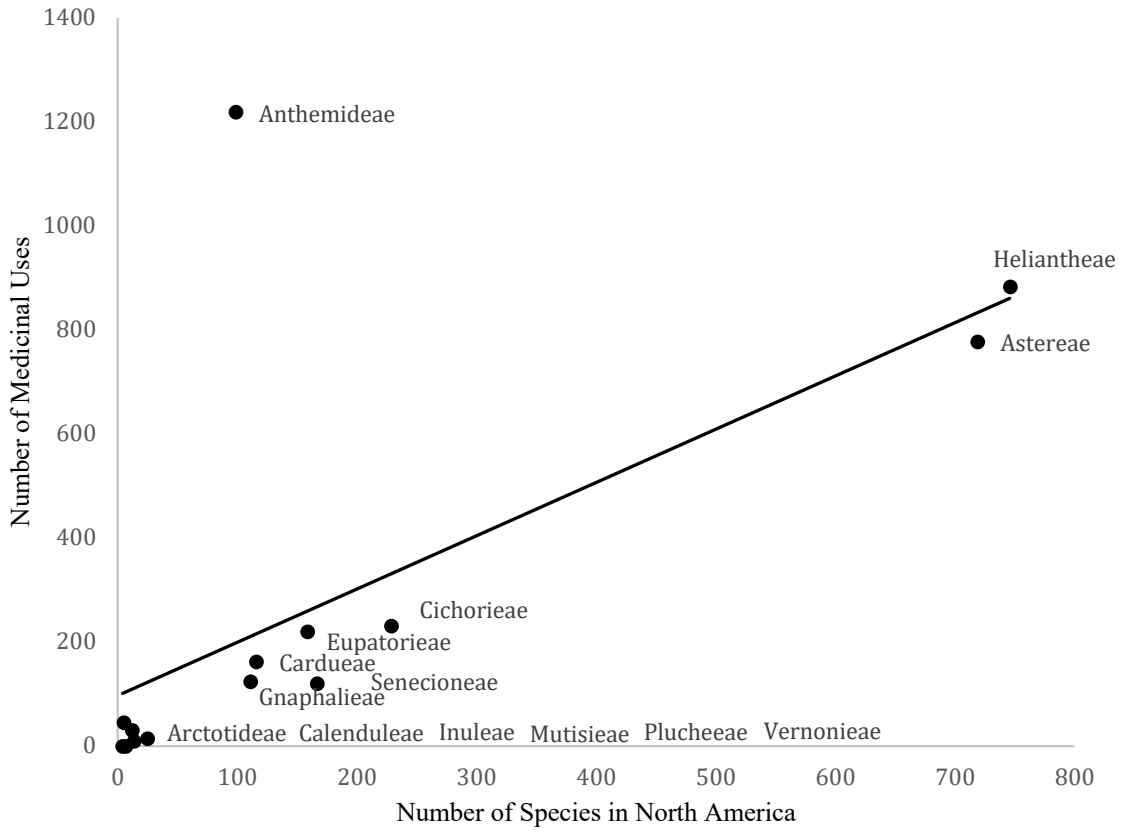


Figure 2.3 - Residual analysis of 14 North American Asteraceae tribes comparing total number of species with number of reported medicinal uses per tribe. Regression follows the equation $y = 1.0238x + 97.253$, $R^2 = 0.42371$.

Table 2.3 - Residual analysis of medicinal species and uses within 14 Asteraceae tribes based on total number of species in North America.

Total spp. vs Medicinal spp.			Total spp. vs Medicinal uses		
Over-Selected Tribes					
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Anthemideae	24.36	1	Anthemideae	1017.55
2	Astereae	5.97	2	Heliantheae	22.02
3	Cichorieae	5.54			
4	Plucheeae	0.97			
5	Mutisieae	0.60			
Under-Selected Tribes					
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Eupatorieae	-12.09	1	Senecioneae	-148.09
2	Senecioneae	-9.62	2	Vernonieae	-108.68
3	Heliantheae	-5.18	3	Calenduleae	-104.25
4	Gnaphalieae	-2.93	4	Mutisieae	-102.41
5	Calenduleae	-2.07	5	Arctotideae	-101.17
6	Cardueae	-1.88	6	Cichorieae	-100.57
7	Vernonieae	-1.51	7	Gnaphalieae	-86.74
8	Arctotideae	-1.50	8	Plucheeae	-79.37
9	Inuleae	-0.69	9	Inuleae	-57.20
			10	Astereae	-56.33
			11	Cardueae	-53.86
			12	Eupatorieae	-40.89

Table 2.4 - Binomial analysis of medicinal species within 14 Asteraceae tribes based on total number of species in North America.

Over-Selected Tribes		
Rank	**Tribe**	***P*-Value**
1	**Anthemideae**	**1.53E-08**
2	Plucheeae	0.192812
3	Cichorieae	0.209952
4	Mutisieae	0.285381
5	Astereae	0.379927
Under-Selected Tribes		
Rank	**Tribe**	***P*-Value**
1	**Eupatorieae**	**0.007810**
2	**Senecioneae**	**0.033867**
3	Calenduleae	0.218697
4	Heliantheae	0.257281
5	Gnaphalieae	0.308384
6	Cardueae	0.403213
7	Arctotideae	0.419534
8	Vernonieae	0.444505

Bolded values: statistically significant *P*-Value < 0.05

always higher than number of species (number of trials), and thus only residual analysis was completed for this data set (Table 2.3).

When comparing North American plant families through regression and residual analyses, Moerman (1991) created ten medicinal therapeutic categories in order to better represent the ethnobotanical reports for each species (Appendix A). Tables 2.5-2.8 summarize the residual and binomial analyses of over- and under-selected tribes of Asteraceae within each therapeutic category. Firstly, the number of species in a tribe selected for use within each of the ten medicinal categories was compared to the total number of medicinal species in a tribe. Across all categories, Anthemideae was the top ranked over-used tribe based on residual data (Table 2.5). A binomial analysis of the same dataset resulted in almost identical results, as Anthemideae was significantly over-used in all medicinal categories except cardiology, in which Vernonieae held the top rank (Table 2.7). Contrastingly, Astereae species were consistently under-utilized by North American indigenous peoples in most therapeutic categories (Table 2.6, 2.8).

Furthermore, comparing the residuals and *P*-Values across ten therapeutic categories resulted in consistent trends. The most significant category in which Anthemideae species were over-utilized was pulmonary. This category held the highest residual value of 15.77 (Table 2.5), and the smallest, most significant *P*-Value of 1.08×10^{-06} (Table 2.7). In both cases, Anthemideae species were the most significantly over-utilized as pulmonary aids.

Not all rankings were consistent between residual and binomial results (Table 2.9). However, Anthemideae was the top ranked significantly over-used tribe in nine out of ten medicinal categories. The binomial analysis of tribes selected for use in the final category (cardiology) placed Vernonieae first, followed by Inuleae and Anthemideae. Categories

Table 2.5 - Residual analysis of Asteraceae species over-used in ten medicinal categories based on total medicinal species in the family.

CAR			DER			GEN			GIM			GYN		
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Anthemideae	2.13	1	Anthemideae	12.85	1	Anthemideae	5.70	1	Anthemideae	7.46	1	Anthemideae	8.14
2	Vernonieae	1.21	2	Heliantheae	4.12	2	Heliantheae	3.48	2	Heliantheae	6.95	2	Heliantheae	2.61
3	Heliantheae	0.90	3	Senecioneae	2.83	3	Eupatorieae	1.63	3	Eupatorieae	3.77	3	Cardueae	0.61
3	Cardueae	0.90	4	Cardueae	2.59	4	Cardueae	1.47	4	Cardueae	1.86	4	Eupatorieae	0.26
4	Cichorieae	0.67				5	Inuleae	0.01	5	Plucheeae	0.55	5	Cichorieae	0.19
5	Inuleae	0.44							5	Vernonieae	0.55	6	Mutisieae	0.13
6	Eupatorieae	0.05										6	Vernonieae	0.13
												7	Inuleae	0.10
NEU			**ORT**			**OTH**			**OTO**			**PUL**		
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Anthemideae	10.34	1	Anthemideae	10.62	1	Anthemideae	8.89	1	Anthemideae	10.65	1	Anthemideae	15.77
2	Plucheeae	2.41	2	Eupatorieae	4.15	2	Senecioneae	1.56	2	Cardueae	1.98	2	Gnaphalieae	2.50
3	Inuleae	0.61	3	Heliantheae	2.58	3	Eupatorieae	0.78	3	Vernonieae	0.67	3	Eupatorieae	1.50
4	Vernonieae	0.41	4	Senecioneae	0.43	4	Cardueae	0.67	4	Astereae	0.09	4	Heliantheae	1.20
5	Eupatorieae	0.38	5	Gnaphalieae	0.15	5	Cichorieae	0.56				5	Senecioneae	0.58
6	Astereae	0.18												
7	Arctotideae	0.01												
7	Calenduleae	0.01												

Table 2.6 - Residual analysis of Asteraceae species under-used in ten medicinal categories based on total medicinal species in the family.

CAR			DER			GEN			GIM			GYN		
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Astereae	-1.56	1	Astereae	-7.16	1	Astereae	-4.98	1	Astereae	-6.77	1	Astereae	-4.34
2	Senecioneae	-1.25	2	Gnaphalieae	-3.65	2	Plucheeae	-1.22	2	Cichorieae	-6.25	2	Gnaphalieae	-3.74
3	Gnaphalieae	-0.95	3	Cichorieae	-2.43	2	Vernonieae	-1.22	3	Gnaphalieae	-3.23	3	Senecioneae	-2.04
4	Mutisieae	-0.79	4	Plucheeae	-1.95	2	Mutisieae	-1.22	4	Senecioneae	-2.04	4	Plucheeae	-0.87
4	Plucheeae	-0.79	4	Vernonieae	-1.95	3	Arctotideae	-0.91	5	Mutisieae	-1.45	5	Arctotideae	-0.57
5	Arctotideae	-0.48	5	Arctotideae	-1.43	3	Calenduleae	-0.91	6	Arctotideae	-0.64	5	Calenduleae	-0.57
5	Calenduleae	-0.48	5	Calenduleae	-1.43	4	Cichorieae	-0.76	6	Calenduleae	-0.64			
			6	Mutisieae	-0.95	5	Senecioneae	-0.68	7	Inuleae	-0.09			
			7	Inuleae	-0.81	6	Gnaphalieae	-0.37						
			8	Eupatorieae	-0.65									
NEU			**ORT**			**OTH**			**OTO**			**PUL**		
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Cichorieae	-6.07	1	Astereae	-4.51	1	Vernonieae	-2.89	1	Senecioneae	-3.57	1	Cichorieae	-8.61
2	Senecioneae	-3.23	2	Cichorieae	-4.47	2	Heliantheae	-2.33	2	Eupatorieae	-3.46	2	Astereae	-3.17
3	Gnaphalieae	-2.62	3	Vernonieae	-2.13	3	Gnaphalieae	-2.22	3	Heliantheae	-2.26	3	Mutisieae	-2.06
4	Mutisieae	-1.59	4	Arctotideae	-1.40	4	Mutisieae	-1.89	4	Mutisieae	-1.33	3	Vernonieae	-2.06
5	Cardueae	-0.42	4	Calenduleae	-1.40	5	Plucheeae	-0.89	5	Cichorieae	-1.01	4	Cardueae	-1.96
6	Heliantheae	-0.41	5	Cardueae	-1.21	6	Astereae	-0.67	6	Inuleae	-0.50	5	Arctotideae	-1.14
			6	Mutisieae	-1.13	6	Arctotideae	-0.67	7	Gnaphalieae	-0.46	5	Calenduleae	-1.14
			6	Plucheeae	-1.13	6	Calenduleae	-0.67	8	Plucheeae	-0.33	6	Plucheeae	-1.06
			7	Inuleae	-0.58	7	Inuleae	-0.22	9	Arctotideae	-0.23	7	Inuleae	-0.37
									9	Calenduleae	-0.23			

Table 2.7 - Binomial analysis of Asteraceae species over-used in ten medicinal categories based on total medicinal species in the family.

CAR			DER			GEN			GIM			GYN		
Rank	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**
1	**Vernonieae**	**0.044130**	1	**Anthemideae**	**0.000145**	1	**Anthemideae**	**0.013346**	1	**Anthemideae**	**0.020425**	1	**Anthemideae**	**0.010224**
2	Inuleae	0.091295	2	Senecioneae	0.120585	2	Inuleae	0.104034	2	Eupatorieae	0.051197	2	Inuleae	0.341826
3	Anthemideae	0.209845	3	Cardueae	0.123351	3	Eupatorieae	0.128216	3	Heliantheae	0.223542	3	Mutisieae	0.422503
4	Cardueae	0.299611	4	Inuleae	0.422505	4	Cardueae	0.168423	4	Cardueae	0.241683	3	Vernonieae	0.422503
5	Cichorieae	0.485218	5	Mutisieae	0.563289	5	Heliantheae	0.469333	5	Plucheeae	0.270784	4	Cardueae	0.431891
6	Eupatorieae	0.528198				6	Gnaphalieae	0.602328	5	Vernonieae	0.270784	5	Heliantheae	0.472776
									6	Inuleae	0.471338	6	Eupatorieae	0.488551
NEU			**ORT**			**OTH**			**OTO**			**PUL**		
Rank	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**
1	**Anthemideae**	**0.001382**	1	**Anthemideae**	**0.000556**	1	**Anthemideae**	**0.005111**	1	**Anthemideae**	**0.000662**	1	**Anthemideae**	**1.084E-06**
2	**Plucheeae**	**0.025928**	2	**Eupatorieae**	**0.013810**	2	Senecioneae	0.299822	2	Cardueae	0.219013	2	Gnaphalieae	0.099994
3	Inuleae	0.401274	3	Inuleae	0.222930	3	Eupatorieae	0.400805	3	Vernonieae	0.317369	3	Eupatorieae	0.212931
4	Eupatorieae	0.516801	4	Senecioneae	0.408474	4	Cardueae	0.431543				4	Inuleae	0.263270
5	Astereae	0.516973	5	Gnaphalieae	0.422271	5	Cichorieae	0.533440				5	Senecioneae	0.402788
6	Vernonieae	0.527000	6	Mutisieae	0.635380	6	Inuleae	0.575372						
			6	Plucheeae	0.635380									

Bolded values: statistically significant *P*-Value < 0.05

Table 2.8 - Binomial analysis of Asteraceae species under-used in ten medicinal categories based on total medicinal species in the family.

CAR			DER			GEN			GIM			GYN		
Rank	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**
1	Astereae	0.226823	1	**Astereae**	**0.026910**	1	**Astereae**	**0.014031**	1	**Cichorieae**	**0.041814**	1	Gnaphalieae	0.068145
2	Senecioneae	0.366144	2	Gnaphalieae	0.118885	2	Cichorieae	0.394686	2	Astereae	0.080749	2	Astereae	0.157285
3	Gnaphalieae	0.471802	3	Cichorieae	0.227300	3	Senecioneae	0.566032	3	Gnaphalieae	0.129078	3	Senecioneae	0.280388
4	Heliantheae	0.504598	4	Plucheeae	0.436711	4	Mutisieae	0.644416	4	Senecioneae	0.289205	4	Cichorieae	0.554589
5	Mutisieae	0.681854	4	Vernonieae	0.436711	4	Plucheeae	0.644416	5	Mutisieae	0.356677	5	Plucheeae	0.577497
5	Plucheeae	0.681854	5	Heliantheae	0.515124	4	Vernonieae	0.644416						
			6	Eupatorieae	0.590989									
NEU			**ORT**			**OTH**			**OTO**			**PUL**		
Rank	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**
1	Cichorieae	0.052000	1	**Astereae**	**0.038755**	1	**Vernonieae**	**0.032511**	1	Eupatorieae	0.063901	1	**Cichorieae**	**0.001119**
2	Senecioneae	0.121449	2	Cichorieae	0.050400	2	Mutisieae	0.208724	2	Senecioneae	0.076181	2	Astereae	0.111005
3	Mutisieae	0.128503	3	Vernonieae	0.364620	3	Heliantheae	0.250144	3	Mutisieae	0.265206	3	Mutisieae	0.294601
4	Gnaphalieae	0.160165	4	Heliantheae	0.404917	4	Gnaphalieae	0.251550	4	Heliantheae	0.324311	3	Vernonieae	0.294601
5	Heliantheae	0.510885	5	Cardueae	0.482034	5	Astereae	0.344517	5	Cichorieae	0.431788	4	Cardueae	0.316523
6	Cardueae	0.518909				6	Plucheeae	0.566875	6	Astereae	0.493388	5	Heliantheae	0.366818
									7	Gnaphalieae	0.542252	6	Plucheeae	0.715702
									8	Plucheeae	0.682631			
									9	Inuleae	0.717622			

Bolded values: statistically significant *P*-Value < 0.05

Table 2.9 - Comparison of top 10 residual and binomial rankings for preferentially selected medicinal species. Analysis of Asteraceae species based on total medicinal species in the family.

CAR			DER			GEN			GIM			GYN		
Tribe	**Residual**	**Binomial**	**Tribe**	**Residual**	**Binomial**	**Tribe**	**Residual**	**Binomial**	**Tribe**	**Residual**	**Binomial**	**Tribe**	**Residual**	**Binomial**
Anthemideae	1	3	Anthemideae	1	1	Anthemideae	1	1	Anthemideae	1	1	Anthemideae	1	1
Vernonieae	2	1	Heliantheae	2	7*	Heliantheae	2	5	Heliantheae	2	3	Heliantheae	2	6
Heliantheae	3	9*	Senecioneae	3	2	Eupatorieae	3	3	Eupatorieae	3	2	Cardueae	3	5
Cardueae	4	4	Cardueae	4	3	Cardueae	4	4	Cardueae	4	4	Eupatorieae	4	7
Cichorieae	5	5	Eupatorieae	5*	6*	Inuleae	5	2	Vernonieae	5	6	Cichorieae	5	9*
Inuleae	6	2	Inuleae	6*	4	Gnaphalieae	6*	6	Plucheeae	6	5	Vernonieae	6	4
Eupatorieae	7	6	Mutisieae	7*	5	Senecioneae	7*	10*	Inuleae	7*	7	Mutisieae	7	3
Arctotideae	8*		Arctotideae	8*		Cichorieae	8*	11*	Arctotideae	8*		Inuleae	8	2
Calenduleae	9*		Calenduleae	9*		Arctotideae	9*		Calenduleae	9*		Arctotideae	9*	
Mutisieae	10*	8*	Vernonieae	10*	8*	Calenduleae	10*		Mutisieae	10*	8*	Calenduleae	10*	
NEU			**ORT**			**OTH**			**OTO**			**PUL**		
Tribe	**Residual**	**Binomial**	**Tribe**	**Residual**	**Binomial**	**Tribe**	**Residual**	**Binomial**	**Tribe**	**Residual**	**Binomial**	**Tribe**	**Residual**	**Binomial**
Anthemideae	1	1	Anthemideae	1	1	Anthemideae	1	1	Anthemideae	1	1	Anthemideae	1	1
Plucheeae	2	2	Eupatorieae	2	2	Senecioneae	2	2	Cardueae	2	2	Gnaphalieae	2	2
Inuleae	3	3	Heliantheae	3	9*	Eupatorieae	3	3	Vernonieae	3	3	Eupatorieae	3	3
Vernonieae	4	6	Senecioneae	4	4	Cardueae	4	4	Astereae	4	7*	Heliantheae	4	7*
Eupatorieae	5	4	Gnaphalieae	5	5	Cichorieae	5	5	Arctotideae	5*		Senecioneae	5	5
Astereae	6	5	Inuleae	6*	3	Inuleae	6*	6	Calenduleae	6*		Inuleae	6*	4
Arctotideae	7		Plucheeae	7*	7	Arctotideae	7*		Plucheeae	7*	5*	Plucheeae	7*	6*
Calenduleae	8		Mutisieae	8*	6	Calenduleae	8*		Gnaphalieae	8*	6*	Arctotideae	8*	
Heliantheae	9*	8*	Cardueae	9*	8*	Astereae	9*	8*	Inuleae	9*	4*	Calenduleae	9*	
Cardueae	10*	7*	Arctotideae	10*		Plucheeae	10*	7*	Cichorieae	10*	8*	Cardueae	10*	8*

* Indicates under-selected tribes.

in which the top three residual and binomial rankings were consistent include: neurological, oto-rhino-laryngological, pulmonary, and other.

To differentiate between taxa that are simply over-selected as a medicine, in general, from those that are actively selected for more specific therapeutic purposes, we conducted a subsequent analysis specifying a second level of selection; the number of use counts within each of the ten medicinal categories were statistically evaluated against the total number of medicinal uses in Asteraceae tribes (Appendix A). In contrast to comparing medicinal uses to total species, results relative to total medicinal uses identified the Anthemideae as over-selected for use as pulmonary and orthopedic aids but *under-selected* for use related to cardiology and gynecological symptoms. Relative to the consistent positive bias seen through our initial standard analyses, these findings suggest that Anthemideae species are highly selected for use across all treatment categories (relative to other taxa) but specifically over-selected for pulmonary and orthopedic applications (relative to other medicinal uses).

The significance of Anthemideae as an ethnobotanically over-utilized tribe in conjunction with the high over-selection of species for pulmonary aid – regardless of statistical approach, suggests the presence of inherent bioactivity and phytochemistry characteristic of this tribe.

2.3.2 Anthemideae as a Pulmonary Aid

Following the general analyses of medicinal Asteraceae, the significance of Anthemideae species as promising treatments for pulmonary aid was selected for further assessment. The number of recorded pulmonary uses in Asteraceae tribes is summarized in Table 2.10. When comparing trends across Asteraceae flora, the ratio of uses across categories remains consistent. A closer look at the 1218 total medicinal uses recorded with Anthemideae (Figure 2.4) supports

Table 2.10 - Use counts of Asteraceae tribes selected for 5 pulmonary therapeutic categories. Number of uses collected from NAEB Database (Moerman, 1991; 2003; 2009).

Tribe	Use Category				
	Cold Remedy	Cough Medicine	Expectorant	Pulmonary Aid	Respiratory Aid
Anthemideae	98	32	1	15	17
Astereae	33	26	4	10	11
Cardueae	1	1	1	3	1
Cichorieae	2	1	0	2	0
Eupatorieae	10	2	1	1	1
Gnaphalieae	14	7	0	2	5
Heliantheae	21	11	0	9	7
Inuleae	4	2	0	4	3
Plucheeae	1	0	0	1	0
Senecioneae	6	7	0	3	4

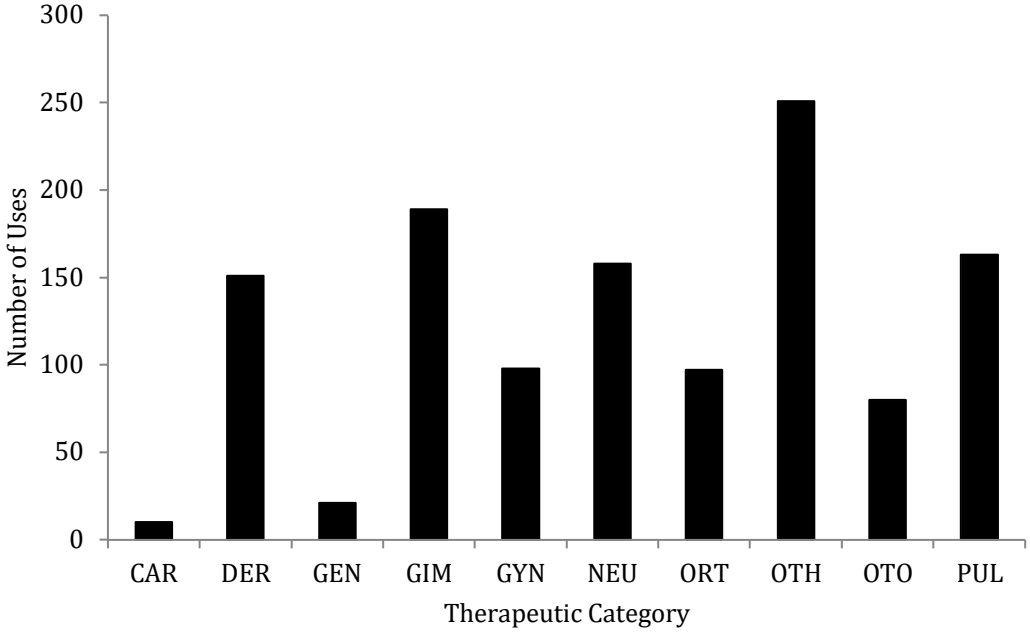


Figure 2.4 - Number of medicinal ethnobotanical uses within Anthemideae. Ten medicinal therapeutic categories are listed as in Moerman (1991) and defined in Appendix A.

the hypothesis that the selection of ethnobotanical species is non-random, and the number of medicinal species in a taxon is not always proportionate to the total number of species in a geographical area. From the distribution of uses across all therapeutic categories, species of Anthemideae are seemingly used equally as dermatological, neurological, and pulmonary aids, and most often for use within 'other' and gastro-intestinal categories. However, residual and binomial statistical analyses suggest otherwise. A least squares linear regression of medicinal species used as pulmonary aids confirmed the over-selection of Anthemideae species by First Nations people of North America (Figure 2.5). Within the pulmonary category, Anthemideae was the only significantly over-used tribe recorded (Table 2.7). Likewise, a least squares linear regression of pulmonary use counts from each tribe presented a similar trend (Figure 2.6). Residual analyses confirmed Anthemideae as the most over-used tribe for pulmonary treatments. Binomial analysis of use counts ranked Anthemideae in second place, closely following Gnaphalieae, though the over-use of both tribes was seen to be statistically significant (Appendix A). North American Anthemideae provide far more species selected for indigenous pulmonary medicines than any other Asteraceae tribe. Although the majority of uses are attributed to two widely distributed genera (*Artemisia* and *Achillea*), Anthemideae species are used almost as often as Heliantheae and Astereae, which are approximately seven times larger in size in terms of number of species.

Regression analysis for each medicinal use category presented a notable trend. When comparing the total number of medicinal uses and number of specific uses for each of the ten categories, Anthemideae species were shown to be selected for certain treatments while preferentially avoided for others (Appendix A). It is possible that these categories may share the

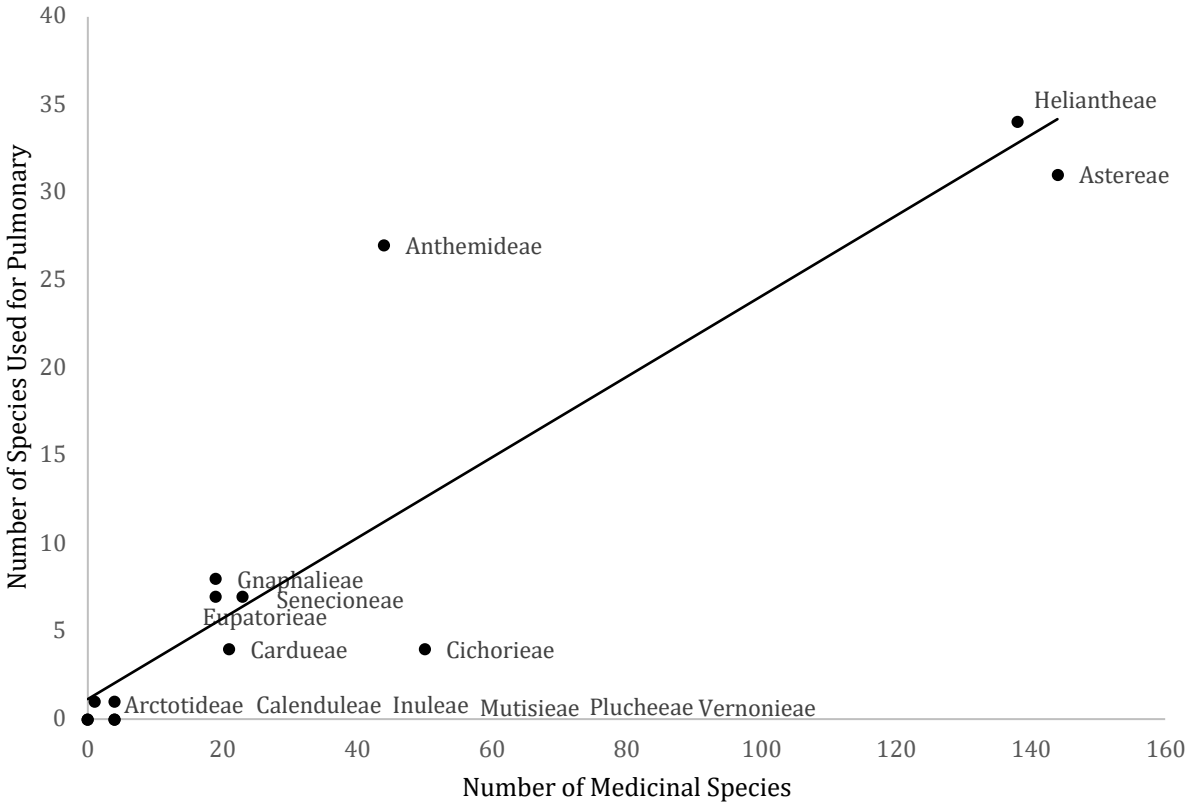


Figure 2.5 - Linear regression plot for 14 North American Asteraceae tribes comparing total number of medicinal species with number of pulmonary medicinal species per tribe. Regression follows the equation $y = 0.2294x + 1.1397$, $R^2 = 0.8152$.

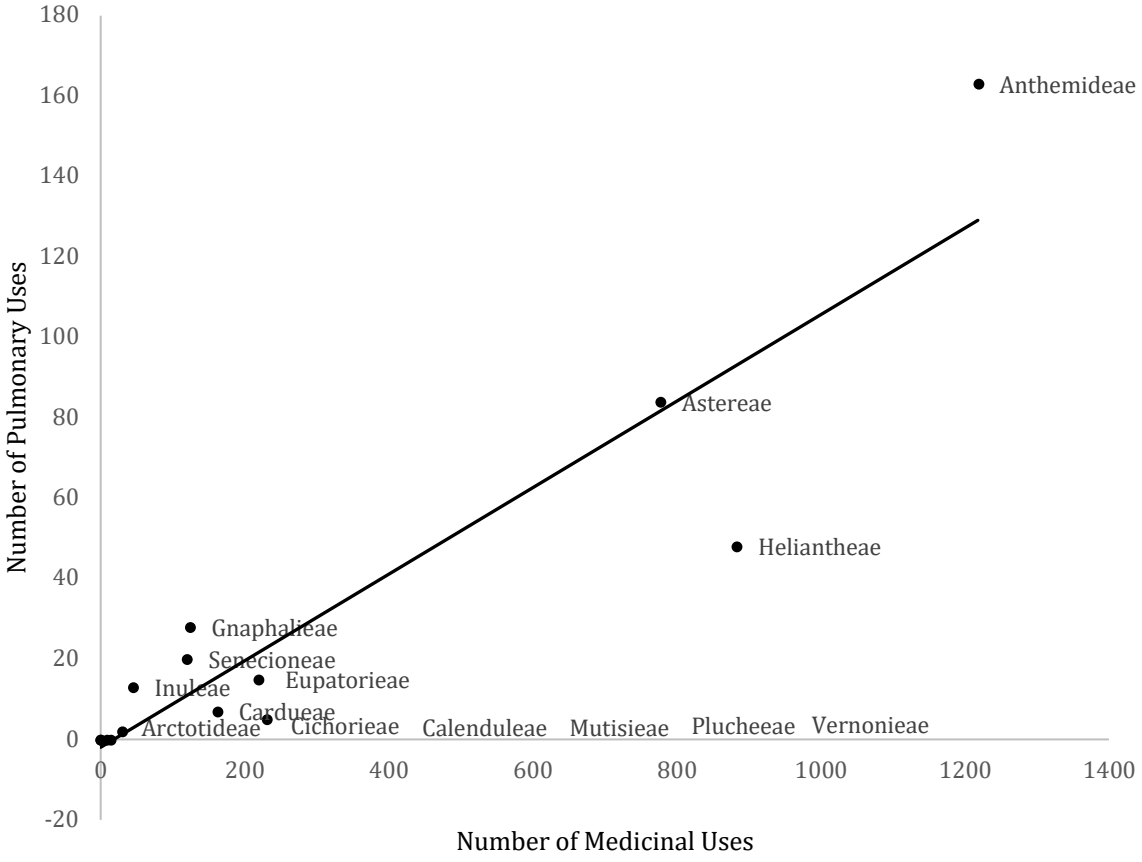


Figure 2.6 – Linear regression plot for 14 North American Asteraceae tribes comparing total number of medicinal uses with number of pulmonary uses per tribe. Regression follows the equation $y = 0.1076x - 1.9514$, $R^2 = 0.8485$.

presence or absence of a common disease symptom, thus resulting in the selection of a certain taxon for medicinal treatment.

2.3.3 Taxonomical Analysis of Anthemideae

Subsequent to tribe-specific statistical analyses, the taxonomic relationships of Anthemideae species selected for pulmonary aid were investigated. Fourteen subtribes are recognized globally, and this phylogeny is presented in Figure 2.7 (Oberprieler, Himmelreich, & Vogt, 2007). It is clear that the entirety of the collected pulmonary medicinal species data falls neatly within three closely related subtribes: Anthemidinae, Matricariinae, and Artemisiinae. Accordingly, these are the only three subtribes found throughout North America. The phylogenetic relationships of 25 species used as pulmonary aids are analyzed in relation to the North American subtribes (Figure 2.8). *Artemisia tridentata* and *Achillea millefolium* L. show the highest use counts, twelve and eight times higher respectively, when compared to the average number of uses for the remaining 23 species.

Residual and binomial analyses of North American Asteraceae tribes suggest that Anthemideae species are highly selected as medicinal treatments for pulmonary illness and related symptoms. This trend may very well be attributed to the similar bioactivity of phytochemicals within Anthemideae. However, if a species is particularly widespread and popular among First Nations, others are more likely to learn about this species and begin using it as well. This development would ultimately result in increased database entries, and thus a higher residual value. This may also be the case with the high medicinal use count for Asteraceae, more generally. Although it is likely that phylogenetically similar species contain

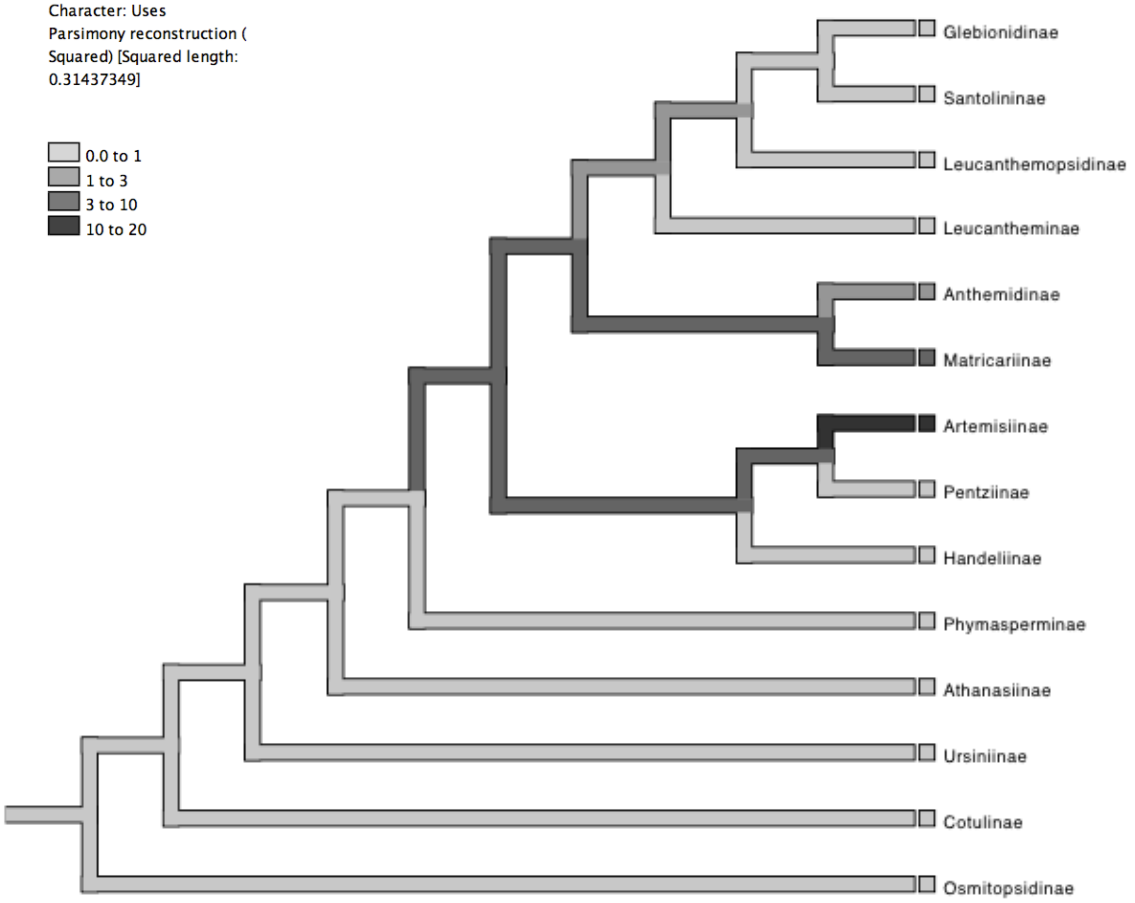


Figure 2.7 - Phylogenetic tree of 14 Anthemideae subtribes based on a maximum-likelihood analysis of nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) sequence variation data. Created by Oberprieler, Himmelreich, & Vogt (2007), adapted and drawn using Mesquite. Branch shading represents number of North American pulmonary medicinal species per subtribe. (Maddison & Maddison, Mesquite, 2017)

Figure 2.8 - Phylogenetic relationship of three Anthemideae subtribes including 25 North American species selected for pulmonary aid. Tree construction based on tribe phylogeny using chloroplast DNA (chDNA), subtribal classification data (nrDNA), and Artemisia genus data (nrDNA) (Oberprieler, Himmelreich, & Vogt, 2007; Torrell et al., 1999; Watson, Evans, & Boluarte, 2000; Watson et al., 2002). Phylogeny adapted and drawn using Mesquite. Branch shading corresponds with number of pulmonary uses per species. (Maddison & Maddison, Mesquite, 2017)

similar classes of phytochemicals, using this information to support taxonomic relationships can be problematic. The subtribal classification of Anthemideae has caused much controversy in the past (Oberprieler, Himmelreich, & Vogt, 2007). Two genera, *Artemisia* and *Absinthium,* were initially placed in the same subtribe due to the isolation of identical sesquiterpene lactones in both taxa (Seaman, 1982). However, it is sometimes difficult to use this information in taxonomy. Oftentimes, phytochemical data is collected unsystematically, and studies that report the identification of specific compounds throughout a taxon usually do not report groups of compounds that are certainly not found in this taxon. Doing so would be nearly impossible without unlimited time and resources.

2.3.4 Geographical Species Selection

Each entry within the NAEB database contains reports of plant part, use description, and association with a First Nations group. Entries for all four of the most used Anthemideae species report similar trends in the selection of plant parts for pulmonary medicinal aids. Leaves are most used across all species, followed by branches and roots. This distribution is comparable to the overall trend of the Anthemideae tribe. After the 40% of uses from the 'unspecified' category, 32% of Anthemideae uses are attributed to leaves, followed by roots (8%), flowers (4%), branches (4%) and stems (3%). Although the 'unspecified' classification seemed to hold the most uses over all, this category was not considered since it is difficult to interpret the reports. If a database entry vaguely stated that a plant was 'used medicinally', this was recorded as 'unspecified'. The original source did not specify a particular plant part for medicinal use, thus it cannot be assumed that this refers to the use of the whole plant rather than a certain organ. Use descriptions for pulmonary medicinal Anthemideae were also collected. The consumption of a

decoction or infusion of plant parts is the most common preparation method for pulmonary medicine, in which the plant is either boiled or simply placed in water without heating.

Each database entry and medicinal use of a species is associated with an indigenous group. Pulmonary Anthemideae species are used by 53 First Nations groups across North America (Moerman, 2003). The top 12 are ranked by use counts in Table 2.11. Pulmonary medicinal uses within Shoshoni, Paiute, and Thompson, all located in western North America, account for 30% of the total pulmonary uses for all species. All three make use of the top Anthemideae species (*Artemisia tridentata, Achillea millefolium,* and *Artemisia ludoviciana ssp. ludoviciana*). *Achillea millefolium* and *Artemisia ludoviciana ssp. ludoviciana* are distributed across the continent, while *Artemisia tridentata* is found predominantly in the western United States. It would be expected that all species are selected equally as pulmonary medicinal aids among these peoples. However, after comparing this data with the geographical distributions of the top three indigenous groups, this was not the case. Shoshoni are selective of *Artemisia ludoviciana ssp. ludoviciana* rather than *A. tridentata* or *A. millefolium*. When comparing distributions of Shoshoni and Paiute with top ranking pulmonary species *A. tridentata* and *A. ludoviciana ssp. ludoviciana*, it is notable that the selection of these medicinal species is not random and does not solely depend on geographical distribution and accessibility of the species.

Both *Artemisia* and *Achillea* species are quite abundant across North America. If there are several polymorphic species in one geographical area, they might be used for a similar purpose. The presence and abundance of phytochemicals in medicinally relevant species could also aid in identification. For example, many *Artemisia* species contain high levels of volatile sesquiterpene lactones with aromatic properties (Turi, Shipley, & Murch, 2014). The scent

Table 2.11 - Top 12 First Nations communities of North America ranked by number of pulmonary uses within Anthemideae. Geographical distribution is included. Data collected from NAEB Database (Moerman, 2003).

First Nations	Geographical Distribution	Number of Uses
Shoshoni	Western US	20
Paiute	Western US	17
Thompson*	South West BC, WA	11
Cheyenne	Montana, Oklahoma	8
Okanagan-Colville*	BC, North East WA	8
Paiute, Northern	Western Nevada	7
Tanana, Upper	Alaska	7
Blackfoot*	MT, AB, SK	5
Flathead	Western MT, Idaho	5
Costanoan	Central CA Coast	4
Sanpoil	North East WA	4
Shuswap*	Southern BC	4

BC: British Columbia; WA: Washington; MT: Montana; AB: Alberta; SK: Saskatchewan

*The Canadian First Nations groups Thompson, Okanagan, Blackfoot, and Shuswap now refer to themselves as Nlaka'pamux, Syilx, Siksika, and Secwepemc respectively.

released by these compounds is likely distinguishable from that of other species, allowing for the accurate selection of these particular species.

Comparing the distribution of highly ranked pulmonary species with the top indigenous groups demonstrates that these relationships are not always congruent. For example, Shoshoni more often select *Artemisia tridentata*, widely distributed throughout North America, over *Artemisia* species distributed only within the geographical location of Shoshoni. This could indicate that traditional knowledge of the selected species as a pulmonary aid is more established and thus these are selected over other species. It is also possible that the abundance or accessibility of individual plants might account for this discrepancy. If there are more *Artemisia tridentata* plants growing in a geographical area, this makes them more accessible and the people in this location might be more likely to select this species for medicinal treatment. Future analysis comparing the distribution of First Nations and Anthemideae species across North America would provide valuable insight into this issue. If a species is distributed equally across the continent, one could hypothesize that it would be equally selected for among indigenous communities. However, this might not be the case if indigenous peoples across North America have differing traditional knowledge of medicinal plant species, as is often the case. A least squares regression analysis similar to those reported in this review might investigate and compare the use data of widely distributed species, as well as if all First Nations equally use all widespread species.

2.4 LIMITATIONS AND CONCLUSIONS

2.4.1 NAEB DATABASE

The analyses and comparisons made throughout this review can be described as secondary ethnobotany. Written documents and reports of firsthand ethnobotany have been carefully studied and are presented here in various forms. This indirect method of investigation can oftentimes present problems in the accuracy and consistency of ethnobotanical reports. Primary ethnobotany studies human relationships with plants through verbal accounts of customary use from community members or traditional healers. The 'passing on' of verbal information may sometimes be unclear, and its written translation has potential to result in miscommunications or ambiguity. Additionally, given the vast number of indigenous communities as well as the number of native and introduced species throughout North America, it is possible that records of traditionally used medicinal species have been missed or disproportionally studied, and thus have been passively excluded from the extensive NAEB database. Following the establishment of the NAEB database, a secondary search of North American medicinal plants was conducted, resulting in only a marginal increase in the number of described species (Moerman, 1991). Consequently, we can assume that any supplementary primary ethnobotanical reports would add only a negligible increase in the database of reported medicinal species. This validates the consistency by which traditional knowledge of medicinal plants is passed down through generations. However, it may also reflect that the same Nations in the same regions are being studied, or that certain indigenous communities or regions that were neglected at this time may not have been added since. Furthermore, novel medicinal plants unknown to indigenous communities are nearly never discovered; however, new uses of already known medicinal species are often found. Particularly for Asteraceae species, it is noteworthy to

mention that various cultures, communities, and indigenous groups across the northern hemisphere are known to use certain species for similar medicinal applications (Moerman et al., 1999). The independent derivation of the uses of these species within geographically distinct groups of people implies that similar knowledge of medicinal plants has somehow been generated in these separate groups. It has also been demonstrated that traditional knowledge can be transmitted vertically and horizontally through familial generations and peers, and also between Nations or cultures (Lozada, Ladio, & Weigandt, 2006). Empirical analysis of an indigenous Amazonian community determined that the most significant mode of transmission of ethnobotanical knowledge was through oblique measures, in which one person transmits information to a group of people, or when one person receives information from a group of people (e.g. a group of elders) (Reyes-García et al., 2009).

The NAEB database itself presents several inconsistencies. Firstly, the potential overlap of certain drug use descriptions may result in skewed data collections. Pulmonary use descriptions for each entry may specifically describe an illness the plant is used to treat (e.g. asthma, bronchitis), or refer to related symptoms (e.g. shortness of breath, headache). In some cases, the use description is rather vague, simply stating the plant is 'used for medicine'. It is possible that entries associated with the same indigenous community specifying an illness are the same use records as those stating a symptom, but from two different primary sources. The interpretation of the primary ethnobotanical reports could also provide a source of human error. Primary sources often include a description of symptoms which are then subjectively diagnosed by the researcher and categorized for specific biomedical applications. Additionally, use category terms might hinder the possibility of a comprehensive search. Within the database, categories 'other' and 'unspecified' were not mined for data. The 'unspecified' category contains

567 reports including inexplicit descriptions of medicinal plant use. The use category termed 'tuberculosis remedy' was also omitted from analysis. These entries are used only when the original source mentions tuberculosis, consumption, or scrofula exclusively (Moerman, 2009). Lastly, specific reference to the use of a plant part poses another spot for uncertainty. As formerly mentioned, use descriptions for several species referred to the 'plant' used as a pulmonary medicinal treatment. These data were compiled into one 'unspecified' plant part category which accounted for one of the highest part use numbers overall. Issues also arise as primary sources may often use subjective descriptions of plant parts. For instance, when describing 'branches' and 'leaves' it is unknown whether the branches collected and used medicinally still have leaves attached.

The physical identification of plants by First Nations might also cause discrepancies within the database. Although the entry of reports into the database made use of an electronic system that aimed to resolve overlap with species names, there are still a few issues with species organization. Occasionally, only a genus will be included in an entry and written as follows: *Achillea* sp. In this case, the source may have referred to one or several unknown species within the *Achillea* genus and consolidated these together in this particular use description. Several North American yarrow species are polymorphic, thus arises a potential for misidentification. If multiple species have similar appearances, it is questioned how individuals select the stated species for particular medicinal uses or how researchers confirmed botanical identity.

2.4.2 Statistical Limitations

Moerman's regression and residual approach is beneficial as the analysis of proportional values may highlight smaller flora (Moerman, 1991). Nevertheless, this method provides no

means by which to compare statistical hypotheses, and the significance of over- or under-utilized taxa can be biased depending on the dataset in question. These regression techniques are now outdated and not entirely statistically sound but were applied in this thesis for consistency. Employing binomial methodology resolves these issues as certain arguments must be met in order to provide distinct values of significance (Bennett & Husby, 2008; Turi & Murch, 2013). Still, constraints arise when comparing two sets of data from different sources (e.g. species vs uses) which may be abated with the use of supplementary statistical methods such as Bayesian tests.

2.4.3 Conclusions

Using the NAEB database, the medicinal significance of Asteraceae tribes used by indigenous peoples in North America were assessed. Two methods, residual analysis and binomial statistics, were investigated and presented generally parallel results. Anthemideae were significantly over-selected in most therapeutic categories, but particularly for use as pulmonary aids. Across all classifications, pulmonary was the top ranked category in which First Nations over-utilized plants for healing. In some cases, residual and binomial analyses did not agree, thus indicating that the use of several different statistical approaches might be essential to evaluate significance in the use of ethnobotanical reports. This review and evaluation of the NAEB database highlights the selection and use of distinct plant parts for specific medicinal aids. As the top ranked genera for pulmonary use, a comprehensive assessment of the pharmacology and phytochemistry of *Achillea* and *Artemisia* parts would provide useful tools to strengthen these findings.

CHAPTER 3 – EVALUATING THE IMMUNOMODULATORY EFFECTS OF *ACHILLEA MILLEFOLIUM* EXTRACTS

3.1 INTRODUCTION

Achillea millefolium L. (Asteraceae) is used globally by First Nations and numerous cultures to treat a wide variety of symptoms and conditions. Whereas the widespread and varied uses of this species suggest a role as a panacea remedy, the ethnobotanical meta-analysis completed in the previous chapter of this thesis framed the active selection of *Achillea* for certain medicinal ailments but not for others. Notably, *A. millefolium* is commonly used for skin, gastrointestinal, and neurological conditions, as well as pulmonary and orthopedic aid.

More commonly known as yarrow, *A. millefolium* has recently been the subject of many *in vitro* and *in vivo* studies to determine its potential physiological effects. Indeed, several reported bioactivities reflect yarrow's known ethnobotanical applications. For example, established antibiotic activities (Maz, Mirdeilami, & Pessarakli, 2013; Sarker, Nahar, & Kumarasamy, 2007) may help protect against infections of the skin, lungs or gut. *In vivo* studies of the aqueous extract of yarrow flowers have displayed anti-inflammatory and anxiolytic activity (Goldberg et al., 1969; Molina-Hernandez et al., 2004), which are relevant to the plant's use as a respiratory/orthopedic aid and mild sedative, respectively. Analyses of aerial parts resulted in significant hepatoprotective and choleretic activity, and an investigation of the combination of leaves, stems, and stalks displayed gastroprotective activity (Benedek et al., 2006; Cavalcanti et al., 2006; Yaeesh et al., 2006;). Just as different parts of the yarrow plant have different ethnobotanical applications, bioactivity studies testing separate parts have shown diverse pharmacological activities. However, the majority of yarrow bioactivity studies have reported testing only flower heads or aerial parts. The goal of this project was to study more

deeply the differences in the parts of yarrow and if these display distinct characteristics in the inflammatory response in an *in vitro* respiratory model.

Many common symptoms resulting from general respiratory illnesses may result from activity within the same endogenous system. Symptoms such as inflammation, anxiety, and pain can all be regulated by the endocannabinoid system (ECS). The ECS provides a mechanism of action for these symptoms that are common within orthopedic and pulmonary diseases, for which *Achillea* is generally selected. One emerging direction of considerable interest involves the ECS as a target to treat major symptoms in respiratory and pulmonary patients. Notably, *Achillea spp*. are known to produce alkylamides, some of which are structurally similar to the endogenous cannabinoid anandamide and may interact with endocannabinoid receptors or metabolic enzymes (Chicca et al., 2009).

The cannabinoid receptors, CB1 and CB2, are classified as seven-transmembrane G-protein-coupled receptors (GPCRs) and are involved in various physiological mechanisms in both central and peripheral nervous systems. Traditionally, CB1 receptors were assumed to be located primarily in the central nervous system, while CB2 receptors were predominantly found in periphery organs and on immune cells (Galiègue et al., 1995). However, recent investigations suggest that the individual receptors are not limited to these precise locations and are distributed more widely than initially reported. CB1 receptors also exist at lower concentrations in peripheral organs including the lungs (Galiègue et al., 1995). Unpublished research from the Harris lab provides evidence for the existence of CB1 receptors on bronchial epithelial cells (Jason Kwan, Honours 2016). Fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) are the primary degradative enzymes for anandamide and 2-arachidonoylglycerol (2-

AG) respectively, though often interchangeable (Cravatt et al., 1996). Molecular inhibition of these enzymes proves a useful approach to stimulate and study the endocannabinoid system.

The previous chapter of this research project highlighted the particular ethnobotanical uses of yarrow and the active selection of this species as an orthopedic and pulmonary aid. Using these data as well as results from published works discussing various bioactivities of yarrow and their applications within an endogenous system, this chapter compared the immunomodulatory activity of four yarrow parts in a respiratory model. This research will examine how the pharmacological analysis of plant parts compares to the ethnobotany and traditional uses of *A. millefolium* as a medicinal aid by exploring two possible mechanisms of action: a general immunomodulatory pathway via the production of pro-inflammatory cytokines IL-6 and IL-8 in an *in vitro* respiratory model using BEAS-2B human bronchial epithelial cells, and an ECS-specific pathway via an *in vitro* model of endocannabinoid degradation.

3.2 METHODS

3.2.1 Extraction of Plant Material

3.2.1.1 *Crude extracts*

Dried flowers were purchased (Premier Herbal, Toronto), while all leaves, stems, and roots were grown and collected from the medicinal gardens at Carleton University (Voucher #UO-H-AK-001). Collected plant material was cleaned to remove soil and other materials then dried using a food dehydrator at 37°C. Each part was milled in a Wiley Mill using mesh size 20 (1 mm) then transferred to a large Erlenmeyer flask with 80% ethanol, 10:1 per gram of material. Flasks were sealed with parafilm, covered with aluminum foil, and then placed on a shaker overnight (200-250 rpm). Using a large Buchner funnel, extracts were filtered to remove any particulate material. Samples were rotoevaporated then lyophilized to produce a crude extract free of any remaining water.

3.2.1.2 *Organic and aqueous extracts*

A liquid-liquid extraction was used to prepare organic and aqueous extracts of both yarrow root and flower. Crude extracts were transferred to flasks with Milli-Q H_2O, 100:1 per gram, then washed in a separatory funnel with 50 mL dichloromethane. Organic solvent wash was repeated twice more. Organic samples were rotoevaporated, then both organic and aqueous fractions were lyophilized.

3.2.2 Cell Culture

3.2.2.1 *Cytotoxicity*

Prior to anti-inflammatory testing, the toxicity of yarrow extracts was determined using Cell Proliferation Reagent WST-1 (Sigma-Aldrich), which assesses cell viability using the

activity of NAD(P)H-dependent cellular oxidoreductase enzymes as an indicator of cell density. Each extract was administered to BEAS-2B cells at various concentrations (1-200 ug/mL) to determine the highest non-toxic concentrations (<15% reduction in cell viability) over the 96h experimental period. The highest non-toxic concentration for each extract was used as the maximum concentration tested in the anti-inflammatory assays.

3.2.2.2 *BEAS-2B and immunomodulatory assay*

The respiratory immunomodulatory activity of yarrow extracts was tested by assessing the concentration of cytokines IL-6 and IL-8 within a BEAS-2B bronchial epithelial cell line stimulated with LPS (adapted from Sharma et al., 2006). Cells were cultured in DMEM/F12 1:1 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), and maintained in a humidified incubator at 37°C with 5% CO_2. Cells were plated at 1.5 x 10^3 cells/well in complete media in a 96-well plate and incubated overnight (16-24 h). Following incubation, complete media was aspirated and replaced with serum-free media containing 200 ng/ml lipopolysaccharide (LPS, *Escherichia coli,* Sigma-Aldrich, St. Louis, MO, USA) and various plant extract dissolved in 50% DMSO. Wells contained a total volume of 200 µl with 0.25% DMSO. Extracts were assayed between 2 and 200 µg/ml, dependent on cytotoxicity. Parthenolide (Thermo Fisher Scientific, Waltham, MA, USA) was used as a positive control (1.25 µg/ml) and 0.25% DMSO was used as a vehicle control. Cells were incubated for 96 h, after which supernatants were collected and analyzed for levels of IL-6 and IL-8 using ELISA kits (BD OptEIA, San Jose, CA, USA) according to manufacturer's protocol. All extracts and controls were tested in triplicate.

3.2.3 Fatty Acid Amide Hydrolase (FAAH) Assay

Four yarrow extracts (stem, leaf, root, and flower) were solubilized in MeOH and incubated with 10 µg/ml FAAH (Cayman, Ann Arbor, Michigan, USA) and 20 µM 7-amino-4-methyl Coumarin-Arachidonamide (AMC-AA) in 125 mM Tris-HCl buffer with 1 mM EDTA at 37°C for 60 min, following manufacturer's instructions. A Cytation-3 image reader was used to monitor the reaction fluorescence, with excitation at 355 nm and emission at 460 nm. For crude extract analysis, each sample was run at 6 different serially diluted concentrations. Wells contained a final volume of 200 µl. JZL 195 was used as a positive control at 5 µg/ml, and 1% (in well) MeOH as a vehicle control. Crude samples were run in triplicate. Organic and aqueous samples were run in duplicate.

3.2.4 Monoacylglycerol Lipase (MAGL) Assay

Yarrow extracts (stem, leaf, root, and flower) were solubilized in MeOH and incubated with MAGL and 4-nitrophenylacetate in Tris-HCl buffer at room temperature for 10 minutes. A Cytation-3 image reader was used to monitor the reaction fluorescence at 405-415 nm. Each sample was run at 3 serially diluted concentrations. The most concentrated stock solutions provided in-well concentrations of 111 µg/ml. Wells contained a final volume of 180 µl. JZL 195 was used as a positive control and MeOH as a vehicle control. All samples were run in triplicate.

3.2.5 Ultra Performance Liquid Chromatography (UPLC)

Extracts were analyzed on a Shimadzu UPLC-PDA system (Mandel scientific company Inc, Guelph, Ontario) which contains LC30AD pumps, a CTO20A column oven, a SIL-30AC autosampler, and a SPD-M20A (PDA – photo diode array) UV detector. Briefly, Acquity CSH

C18 column (100 x 2.1mm, 1.7 μm particle size, Waters, Mississauga, Ontario) with an Acquity CSH C18 VanGuard Pre-column (5x2.1 mm) were used for separation. Mobile phase was H_2O (A) and acetonitrile (B) with 0.1% formic acid in both. The gradient elution method initiated with 5% B then increased to 70% B in 20 min. The column was then washed with 100% B for 3 minutes and changed back to the initial condition in 1 minute. The system was equilibrated for 5 minutes before the next injection. The flow rate was set at 0.5 ml/min with the column temperature at 50˚C. PDA detector was set to monitor for 220 to 600 nm wavelength.

3.2.6 STATISTICS

All statistical analyses were performed with GraphPad InStat software (Version 3.0, San Diego CA USA, 2003). Data were compared using repeated measures ANOVA. Levels of cytokines in response to extract were analyzed with subsequent Dunnett *post-hoc* tests, and enzyme inhibition data were compared with subsequent Tukey *post-hoc* tests. All data are reported as mean ± SEM.

3.3 RESULTS

3.3.1 INFLAMMATORY RESPONSE IN BEAS-2B

Cytotoxicity testing revealed that the stem extract was nontoxic across the tested range of concentrations while the root and leaf extracts were mildly toxic at high concentrations (200 μg/ml) and the flower extract significantly reduced cell viability at 50 μg/ml. Of the four *A. millefolium* parts tested for levels of IL-6, flower extracts displayed dose-dependent anti-inflammatory activity in BEAS-2B stimulated with LPS (Figure 3.1). Cytokine production decreased with increasingly higher concentrations of flower extract. At 40 μg/ml, yarrow flowers significantly inhibited the release of IL-6 in comparison to the stimulated control (Media + LPS + DMSO). Leaf extracts appeared to exhibit a dose-dependent response, though not significant. Stem extracts at all concentrations had no significant effect of IL-6 release in BEAS-2B. In contrast, root extract exhibited a significant pro-inflammatory response at all tested concentrations, approximately doubling levels of IL-6 (% control) seen within the stimulated control.

Similar trends are presented for the release of cytokine IL-8 in LPS-stimulated BEAS-2B (Figure 3.2). Flower extracts displayed a significant dose-dependent anti-inflammatory response in comparison to the stimulated control. Leaf, stem, and root extracts showed no significant effect, though IL-8 levels in root extracts exceed those found in all other parts, suggesting a pro-inflammatory response.

Two separate fractions of both yarrow flower and root extracts were tested for their effects on the release of IL-6 within LPS-stimulated BEAS-2B (Figure 3.3). Organic extracts were tested at n=1, thus statistical analysis was only completed with aqueous extracts for both parts. At 150 μg/ml, aqueous root extracts were significantly pro-inflammatory compared to the

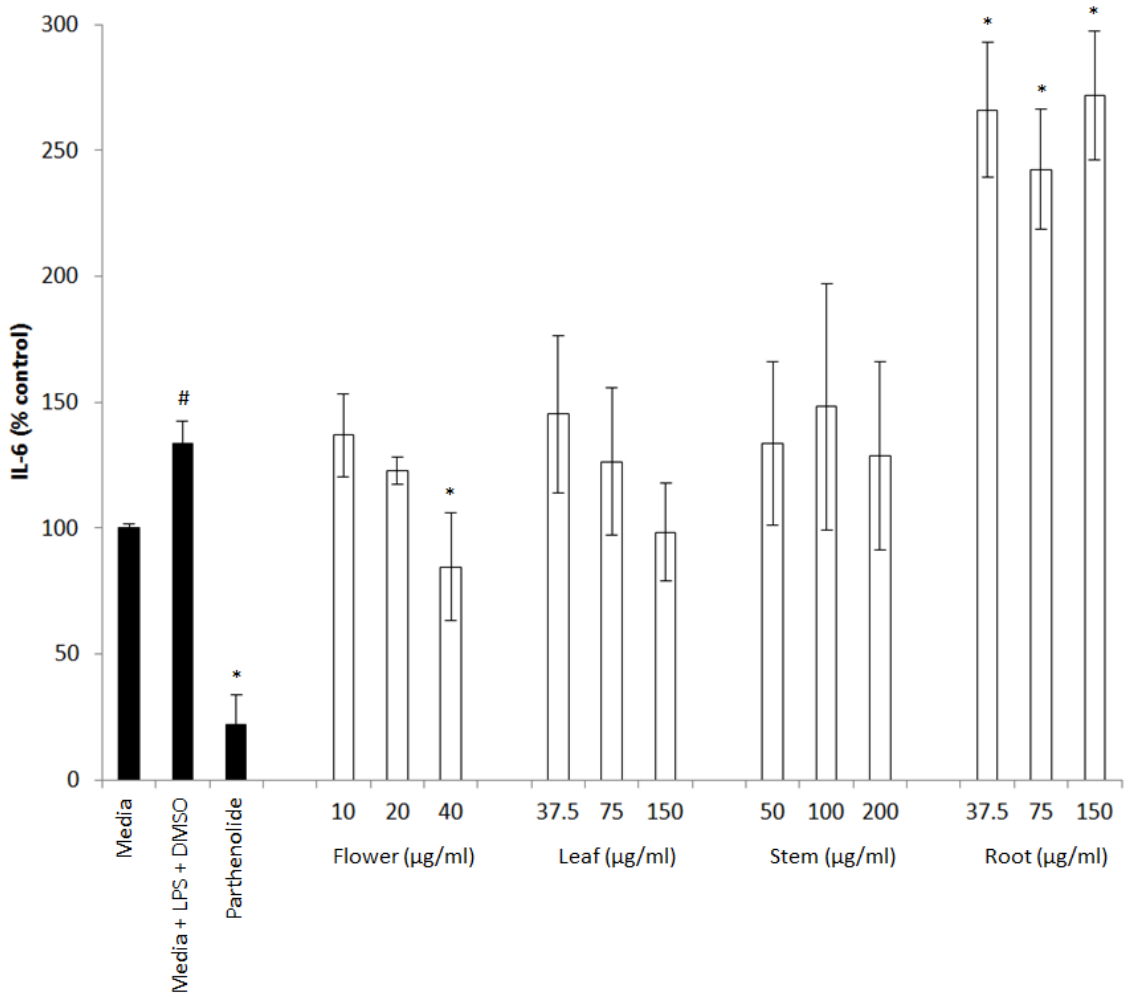


Figure 3.1 - Immunomodulatory activity of *Achillea millefolium* extracts via release of IL-6 from LPS-stimulated BEAS-2B presented as % media control. DMSO 0.25% was used as a vehicle control and parthenolide (1.25 µg/ml) was used as a positive control. Means ± SEM are presented for three replicates ($p < 0.05$). # Denotes significant difference from Media control. * Denotes significant difference from Media + LPS + DMSO control.

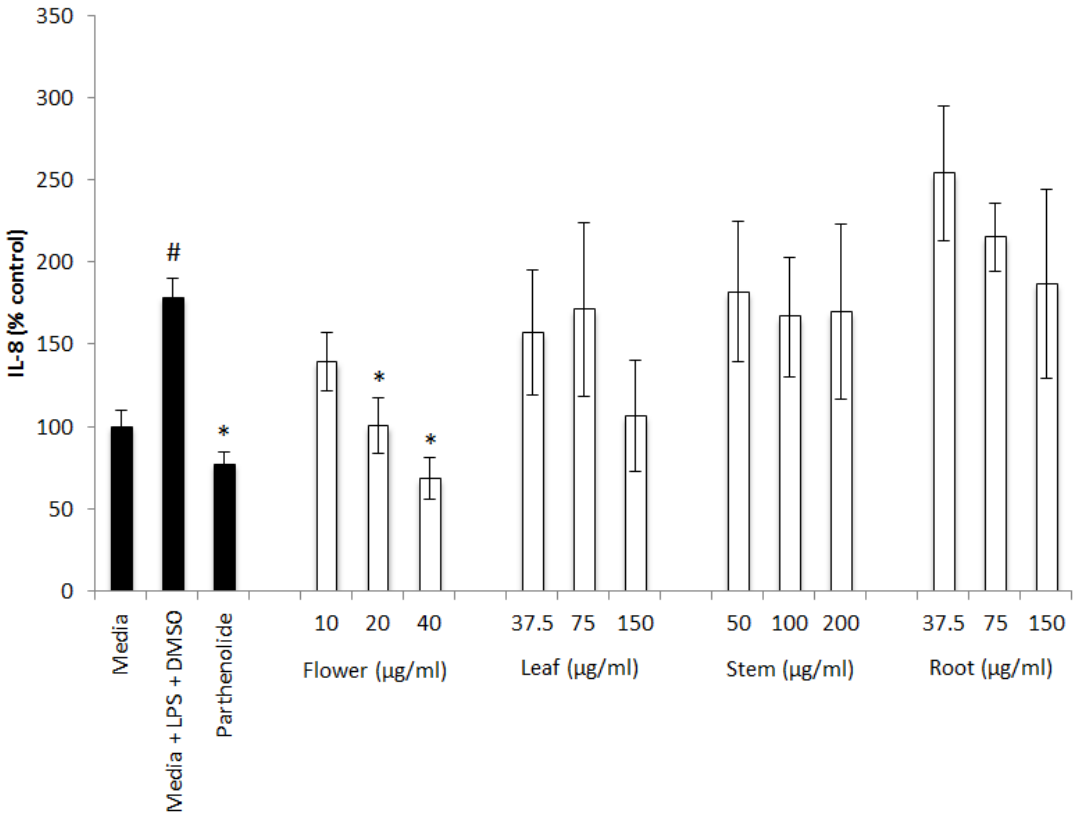


Figure 3.2 - Immunomodulatory activity of *Achillea millefolium* extracts via release of IL-8 from LPS-stimulated BEAS-2B presented as % media control. DMSO 0.25% was used as a vehicle control and parthenolide (1.25 µg/ml) was used as a positive control. Means ± SEM are presented for three replicates ($p < 0.05$). # Denotes significant difference from Media control. * Denotes significant difference from Media + LPS + DMSO control.

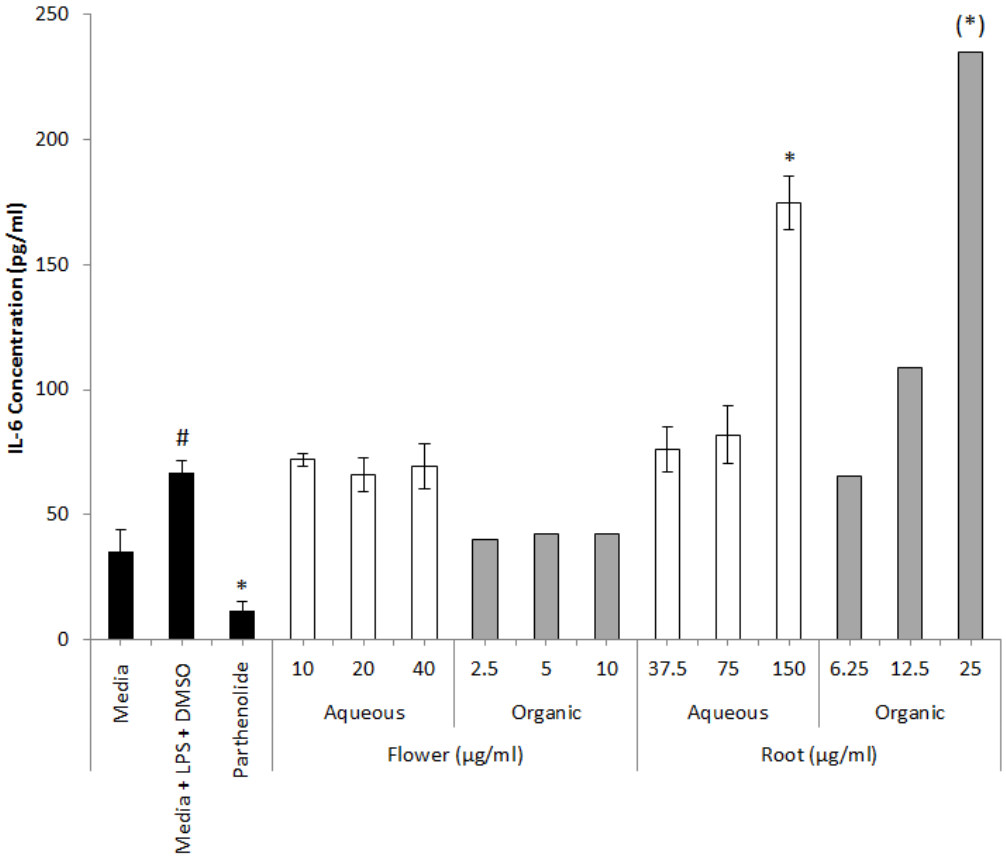


Figure 3.3 - Immunomodulatory activity of *Achillea millefolium* root and flower fractions via release of IL-6 from LPS-stimulated BEAS-2B. DMSO 0.25% was used as a vehicle control and parthenolide (1.25 µg/ml) was used as a positive control. Means ± SEM are presented for three replicates ($p < 0.05$). Analysis of organic extracts using n=1. # Denotes significant difference from Media control. * Denotes significant difference from Media + LPS + DMSO control.

stimulated control. Similarly, organic extracts of yarrow root seemingly displayed dose-dependent pro-inflammatory effects, and at 25 μg/ml this effect would likely be significant. Aqueous flower extracts showed no significant difference from the stimulated control, though organic extracts appear to display potential anti-inflammatory effects.

3.3.2 Enzyme Assays

Yarrow extracts of flower, leaf, stem, and root displayed similar inhibitory activities of FAAH and MAGL enzymes. Testing the highest concentrations of each part within the dynamic range of FAAH inhibition (Figure 3.4) resulted in root extracts having the most activity (92.1%), followed by flower (86.5%), leaf (83.6%), then stem (66.0%). Similarly, results from the MAGL assay (Figure 3.5) revealed yarrow flowers as most active (70.3%), followed closely by root (69.9%), leaf (67.1%), then stem (34.8%). Inhibitory activities of yarrow extracts on FAAH and MAGL enzymes are presented as IC_{50} values and compared in Table 3.1. The most potent inhibitor of FAAH activity was yarrow flower, followed by root, leaf, and stem. No significant difference was found between the four parts. In the MAGL assay, roots and flowers displayed the most potent inhibition, followed closely by leaf extract, then stem. Yarrow root, flower, and leaf extracts were all significantly more inhibitory than stem extracts on MAGL activity.

Further analysis of organic and aqueous extracts of the most active yarrow parts, flower and root, resulted in similar inhibitory profiles for each part against FAAH activity (Figure 3.6). Organic extracts showed higher inhibition compared to aqueous. Organic root extracts displayed increased inhibition compared to organic flower, and aqueous root displayed lower inhibition compared to aqueous flower. Similar trends occurred within a range of concentrations of organic and aqueous root extracts tested in the MAGL assay (Figure 3.7). Comparable to root inhibition

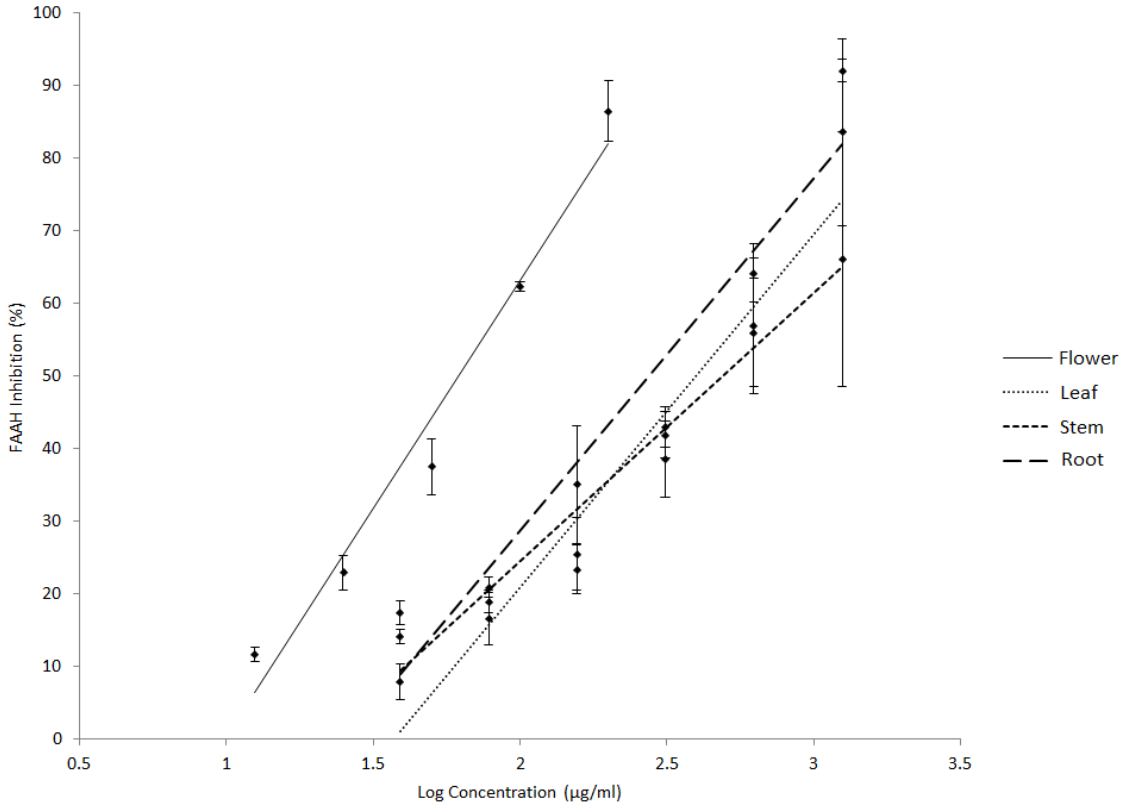


Figure 3.4 - Inhibition (%) of *Achillea millefolium* flower, leaf, stem, and root of FAAH enzyme activity presented as concentration response curves. Data are log transformed. Using initial stock concentrations of 20 mg/ml (flower) or 50 mg/ml (leaf, stem, and root), serial dilutions and subsequent dilutions with Milli-Q water of factors 5 (flower) or 2 (leaf, stem, and root) were performed. Mean totals (± SEM) of 3 counts are presented. A linear regression is fitted using the *Trendline* function in Microsoft Excel.

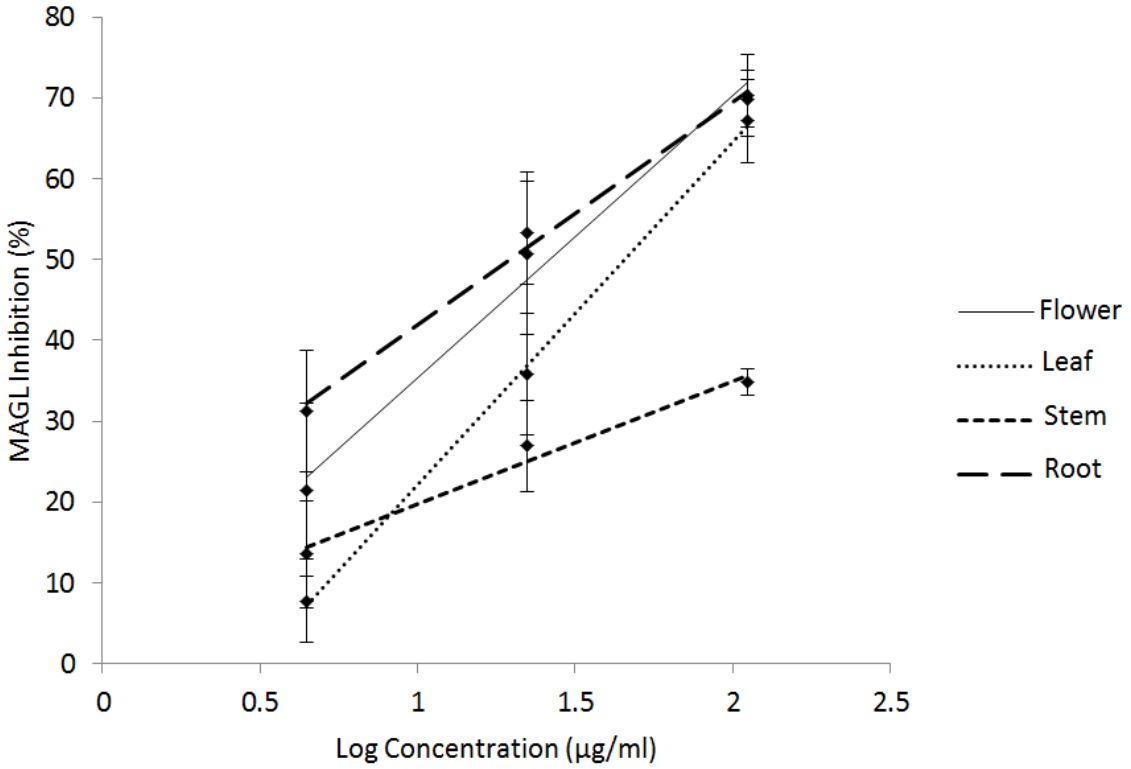


Figure 3.5 - Inhibition (%) of *Achillea millefolium* flower, leaf, stem, and root of MAGL enzyme activity presented as concentration response curves. Data are log transformed. Highest concentration for all parts was 111 µg/ml, followed by two serial dilutions of factor 5. Mean totals (± SEM) of 3 counts are presented. A linear regression is fitted using the *Trendline* function in Microsoft Excel.

Table 3.1 – Mean IC_{50} values ± SEM calculated from individual samples (n=3) for FAAH and MAGL inhibition. Significant differences for MAGL ($p < 0.05$), as determined by one-way analysis of variance with Tukey *post-hoc* test, are designated by letters (a, b).

Yarrow Part	IC_{50} (μg/ml) ± SEM	
	FAAH	MAGL
Flower	59.5 ± 1.83	31.4 ± 16.4[a]
Leaf	510.8 ± 199	49.4 ± 13.2[a]
Stem	2650 ± 2340	1090 ± 214[b]
Root	280 ± 31.78	22.2 ± 9.96[a]

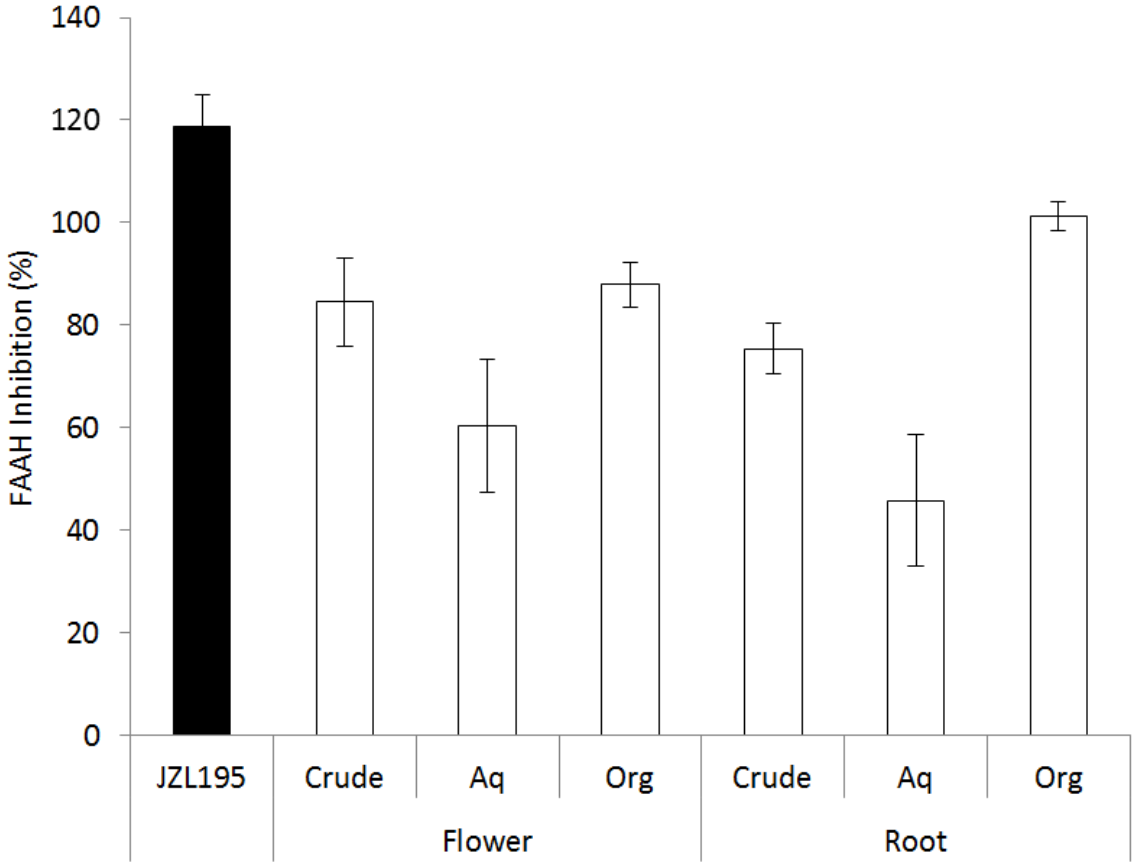


Figure 3.6 - Inhibition (%) of *Achillea millefolium* flower and root of FAAH enzyme activity. All extracts tested at 10 µg/ml. JZL 195 was used as a positive control at 5 µg/ml. Mean totals (± SEM) of 2 counts are presented.

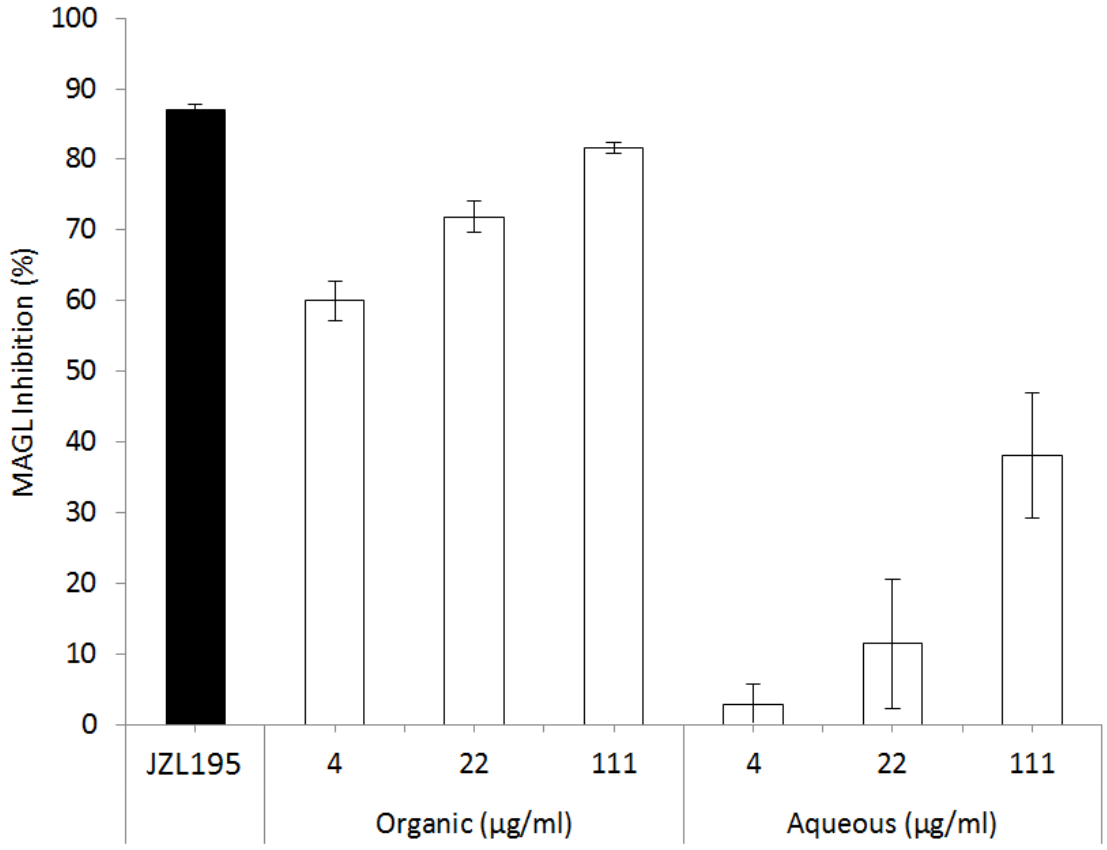


Figure 3.7 - Inhibition (%) of *Achillea millefolium* root of MAGL enzyme activity. JZL 195 was used as a positive control at 5 µg/ml. Mean totals (± SEM) of 3 counts are presented.

of FAAH, organic root extracts show increased inhibition of MAGL compared to aqueous extracts. Both organic and aqueous extracts seem to exhibit a dose-dependent inhibition of MAGL.

3.3.3 UPLC-PDA

Following the pharmacological assays, subsequent chemical screening of the four parts of yarrow was analyzed by ultra-performance liquid chromatography and photo diode array (UPLC-PDA). Each part presented a distinct phytochemical profile (Figure 3.8). Based on the elution profiles, alkylamide-like compounds were found to be more abundant in root extract, while phenolic compounds were identified mainly within yarrow flowers.

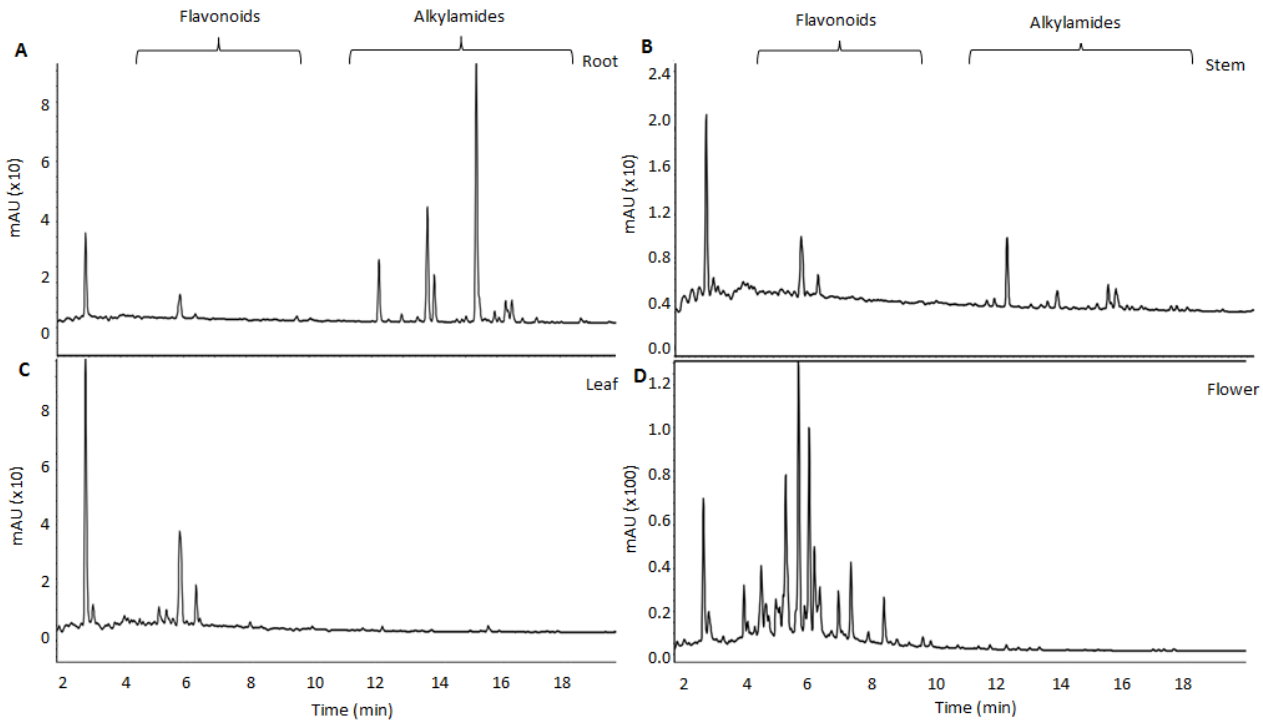


Figure 3.8 - UPLC-PDA chromatograms of yarrow root (A), stem (B), leaf (C), and flower (D). Tentative identification of chemical classes indicated by brackets.

3.4 DISCUSSION

Achillea is highly over-used in disease categories that could encompass general symptoms involved in respiratory conditions. General symptoms such as inflammation often involved in the orthopedic category are also similar to symptoms seen in many pulmonary and respiratory diseases. In most orthopedic disorders we see inflammation and pain in the joints or muscles, and within the pulmonary scope there is often inflammation isolated within the bronchi or the lungs (Gompertz et al., 2001; Kung et al., 1995).

Like yarrow, *Echinacea* is used traditionally as treatment for symptoms that result from many respiratory and pulmonary diseases. Similar to reports in which *Achillea* is medicinally applicable to a variety of diseases based on the generality of the symptoms it treats (e.g. inflammation), *Echinacea* is pharmaceutically marketed towards treating respiratory symptoms involved in cough, cold, and flu, and its traditional uses reflect this established bioactivity (Lindenmuth & Lindenmuth, 2000). When tested *in vitro*, *Echinacea purpurea* exhibited anti-inflammatory properties in rhinovirus infected BEAS-2B human bronchial epithelial cells. The application of both aerial and root extracts reversed the stimulation of pro-inflammatory cytokines IL-6 and IL-8 (Sharma et al., 2006). Ethanol extracts of *Salvia plebeia* (Lamiaceae) aerial parts and roots also improved the induced inflammatory response in BEAS-2B by inhibiting the production of the same cytokines. The aerial parts exhibited a more effective inhibitory response than the root extracts, reflecting the results of yarrow flower and root extracts presented in this research chapter. Furthermore, an *in vivo* assessment of an ovalbumin-induced asthma mouse model displayed the aerial extract of *S. plebeia* as a promising treatment for asthmatic symptoms after resulting in a significant reduction in the inflammatory response of Th2 cytokines (Jang et al., 2016). Although recent studies such as these are now beginning to

investigate distinct plant organs more closely, research is still lacking in the study of separate bioactivities of plant parts.

The observed differences in bioactivity reflected the clearly distinct phytochemical profiles observed by UPLC-PDA. Reported phytochemical localization in closely related Asteraceae such as species of *Echinacea* reflects the variation of bioactivity within yarrow. Alkylamides have been isolated in higher concentrations in the roots of the plant compared to aerial parts (Binns et al., 2002; Kabganian et al., 2003; Qu et al., 2005). On a more finite level, fractions of the fruit of *Echinacea* were analyzed and alkylamides were most concentrated within the perianth, and phenolic compounds were isolated at higher concentrations in the seed (Parsons et al., 2018). Within each part exists an individual chemical profile that may parallel and likely contribute to the selective bioactivity of yarrow parts presented here.

Alkylamides mimic anandamide by acting as agonists mostly with CB2, although sometimes with CB1, explaining the strong immuno-modulatory and anti-inflammatory properties of medicinal plants such as *Echinacea* (Raduner et al., 2006; Woelkart et al., 2005). A number of alkylamides in yarrow have been reported, though research in this area is lacking. There likely exist many other undiscovered alkylamides in the *Achillea* genus. As both *Echinacea* and *Achillea* genera belong within the Asteraceae, it is possible that alkylamides within yarrow are responsible for the similar medicinal properties of these species. Moreover, given the structural similarity of alkylamides to the endogenous anandamide, alkylamides may also interact with endocannabinoid metabolic enzymes and transporters to elicit medicinal effects. Although studies suggest mixed findings, exposure to allergens in respiratory and asthmatic patients activates the ECS as anandamide is positively correlated with inflammatory markers of the airway. Following the hyper-responsiveness of the bronchi and an inflammatory

response to an asthmatic trigger, anandamide activates CB1 receptors in the airways and inhibits the release of excitatory neurotransmitters to elicit a response. However, application of anandamide may elicit opposite effects in some cases depending on the state of the bronchi (Calignano et al., 2000). This directional regulation of the inflammatory response parallels the distinct effects seen on the inhibition of IL-6/IL-8 production by yarrow flowers or roots (Figure 3.1, 3.2). Perhaps the *in vitro* cultured BEAS-2B manipulated in these experiments exist consistently in a state in which the distinct chemical properties of yarrow roots will elicit effects opposite to those seen in other parts.

The ECS and associated ligands have promising anti-inflammatory properties and are involved in the regulation of various physiological functions including appetite, pain, and anxiety. Once activated, the ECS mainly inhibits the production of cAMP and subsequently the activity of PKA, while also participating in the attenuation of TNF-α induced inflammation (Melck et al., 1999). CB1 receptors have been found on the axon terminals of airway nerves in rodents, providing considerable evidence that endocannabinoids may regulate bronchial smooth muscle (Calignano et al., 2000). Th2 type cytokines are the predominant immune cells involved in triggering an asthma attack as a response to an allergen, and high levels of mostly eosinophils and lymphocytes subsequently accumulate in the bronchoalveolar lavage (BAL) and lung tissue (Bradley et al., 1991; Robinson et al., 1993). Studies have demonstrated that levels of eosinophils are reduced following treatment with Δ9-tetrahydrocannabinol (Δ9-THC); thus, quantitative tests examining levels of these immune cells may provide substantial support for novel medications (Braun et al., 2011). Ongoing research on the ECS and plant based cannabinomimetic compounds has resulted in significant insight into the interactions of this system within an asthmatic model. Various compounds that mimic the structure and function of

endocannabinoids stimulate the ECS via CB1 or CB2 receptor activation. Indeed, the ECS was essentially identified in studies testing the activity of Δ9-THC, the primary active component in *Cannabis sativa*. The administration of Δ9-THC and cannabinol in an ovalbumin sensitized murine model lead to the reduction of serum IgE, IL-2, IL-4, IL-5, and IL-13 mRNA expression in addition to a decrease in mucus production (Jan et al., 2003).

Together, these results demonstrate that *A. millefolium* exhibits localized bioactivity across plant parts on the inflammatory response of respiratory cells *in vitro*. Data from flowers and roots in particular warrant for further investigation. The promising activity of these two parts coincides with their favourable interactions with the ECS, and consequently supports this mechanism as a reasonable mode of action.

CHAPTER 4 – PROFILING THE DISTRIBUTION AND SEASONAL VARIABILITY OF SECONDARY METABOLITES IN *ACHILLEA MILLEFOLIUM*

4.1 INTRODUCTION

Although portrayed as a universal cure, ethnobotanical reports of *Achillea millefolium* L. (Asteraceae) indicate its popularity as a treatment for skin conditions, bleeding wounds, gastrointestinal illnesses, and respiratory infections (Applequist and Moerman, 2011). When exploring at a broader scale and relative to other members of the Asteraceae family, analyses at the tribal level frame Anthemideae species as significantly over-selected medicinal aids in almost all categories, and specifically for pulmonary and orthopedic maladies (see Chapter 2). A closer look at the active selection of particular *Achillea* plant parts by North American indigenous peoples showed that there are differences in part selection based on the therapeutic category they are used for. The selection of plant parts is likely attributed, at least in part, to varying levels and types of phytochemicals found within different parts of the plant. The distinct chemical profiles of separate plant parts of *A. millefolium* likely reflect distinct bioactivities that underlie their value in the treatment of these particular illnesses.

The reported biological activities of *A. millefolium* (yarrow) reflect the diversity of its ethnobotanical applications, and more generally its use as an anti-inflammatory agent. *In vivo* analyses of aerial parts have revealed significant anti-inflammatory (Goldberg et al., 1969), anxiolytic (Molina-Hernandez et al., 2004), and hepatoprotective (Yaeesh et al., 2006) activities. Similarly, *in vitro* testing of the aerial parts of *A. millefolium* displayed anti-inflammatory activity (Zaidi et al., 2012), consistent with results from Chapter 3. When studying the levels of respiratory cytokines in response to the extracts of four *A. millefolium* parts, flowers exhibited a concentration dependent anti-inflammatory effect. This pattern is not consistent throughout each

plant part. Roots in particular are pro-inflammatory, supporting the notion that different plant parts appear to elicit distinct bioactivity profiles. Furthermore, when the organic and aqueous extracts of *A. millefolium* roots and flowers were tested, both elicited differing immunomodulatory responses. The organic fraction of flower extract showed anti-inflammatory activity, while the organic fraction of root resulted in a pro-inflammatory response in pulmonary epithelial cell cultures, seemingly more potent than that of crude extract. These results demonstrate the different bioactivities of yarrow part extracts with separate chemical profiles and provide support for the rationale behind the diverse traditional uses of the species.

Alkylamides are a defining chemical class among species of Anthemideae. Over half of the identified alkylamides in the Asteraceae family are found in the Anthemideae (Boonen et al., 2012). Reports of alkylamides within *A. millefolium* (Greger, 1984; Greger and Hofer, 1989; Greger and Werner, 1990) suggest that these compounds contribute to and justify the therapeutic anti-inflammatory effects observed with the ethnobotanical use of this species. Recent analyses on the identification of alkylamides in *A. millefolium* have characterized 15 structures through HPLC-ESI-MS (Veryser et al., 2017). Phenolics constitute another noteworthy class of phytochemicals isolated within *Achillea*. Reported phenolics include rutin, luteolin, apigenin, chlorogenic acid, and caffeic acid, all found primarily within aerial parts (Jesionek et al., 2015a,b; Pires et al., 2009; Vitalini et al., 2011). The 7-O-glucosides of both apigenin and luteolin have demonstrated significant bioactivity as antiplasmodial, antioxidant, and anti-inflammatory agents (Vitalini et al., 2011; Yassa et al., 2007). Based on these findings (as well as my own results from Chapter 3), the current study employed a targeted profiling of alkylamides and phenolic compounds in *A. millefolium* flowerheads, roots, leaves, and stems.

While the compounds are believed to contribute to yarrow's medicinal properties, their distribution across the plant remains poorly defined.

Ecological implications must also be considered when investigating the phytochemistry and ethnobotanical applications of yarrow. Yarrow is a herbaceous perennial species that begins to flower from late May to June. However, as a rhizomatous species able to produce many separate stems from its horizontal rootstock (Hurteau, 2018), younger stems may continue to flower throughout the summer. It is possible that, within a growing season, biosynthesis, storage, and transport of phytochemicals may vary across plant organs and tissues. Accordingly, this study also characterized and compared the chemical profiles of *A. millefolium* plant parts throughout the growing season. It was hypothesized that the presence and abundance of certain compounds would differ between parts, and that phenology would have a significant effect on phytochemical profile.

4.2 METHODS

4.2.1 Collection and Extraction of Plant Material

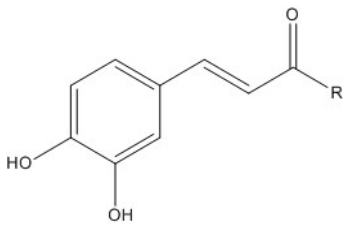
Achillea millefolium was grown within the medicinal gardens at Carleton University (Voucher #UO-H-AK-001), where multiple collections were made across a growing season (May - September 2016). Approximately every two weeks, whole plant samples were collected, cleaned, separated and pooled into four main plant organs (flowerhead, root, leaf, and stem), and dried using a food dehydrator. Dried plant parts were milled in a Wiley Mill using mesh size 20 (1 mm). For each sample, 15 ml of 70% ethanol was added to 1 gram of ground material, followed by vortexing, sonication for 10 minutes, then shaking for 2 h (200-250 rpm). Extracts were centrifuged at 3200 rcf for 10 minutes, after which supernatants were collected. This process was repeated twice, and supernatants were pooled and adjusted to 50 ml.

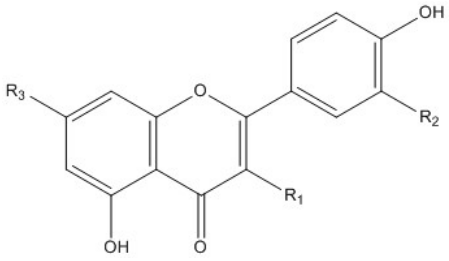
4.2.2 QTRAP-MS and MRM Conditions

Identification and quantification of selected marker compounds were performed on a 4000 QTRAP MS system (AB SCIEX, Concord, ON, Canada) connected with a Shimadzu LC20 series high performance liquid chromatograph. The separations of seven selected marker compounds (Figure 4.1) was performed on a Phenomenex Luna® omega polar C18 Column (100 mm x 2.1 mm I.D., particle size 2.1 mm; Phenomenex, Torrance, CA, USA). The column thermostat was maintained at 50°C. The flow rate was maintained at 0.3 ml/min with an initial mobile phase composition of 90% A (water with 0.1% formic acid) and 10% B (acetonitrile with 0.1% formic acid), linearly changing to 60% B in 8 min, followed by a hold at 100% B for 5 minutes. Instrument was then re-equilibrated with the initial conditions for an additional 5 minutes. Analyst software (version 1.6.3) was used for data acquisition.

For Multiple Reaction Monitoring (MRM) method development, each marker compound was dissolved at 10 ng/ml in 50:50 water and acetonitrile (both LC/MS grade) with 0.1% formic acid and infused at 10 µl/min into a Turbo V™ ion source in negative electrospray ionization mode to determine optimal compound ionization conditions. Two major product ions were monitored for each marker compound and devolved into MRM transitions by Analyst (version 1.6.3) software compound optimization wizard. The highest intensity MRM transition of each compound was used for quantitation and the MRM transition with less intensity was used for compound identity conformation (Table 4.1). The optimized source temperature and voltage were 350°C and -4500 V respectively. Source gas 1, 2 and Curtain gas was set at 40, 50 and 20 psi respectively.

Quantitation was performed with Analyst software (Version 1.6.3) quantification wizard based on area under the peak using external calibration curves generated by reference standards injected at multiple concentrations. The calibration range for all compounds was from 25 pg to 5 ng on column.





Compound	R
1	Quinic acid
2	OH

Compound	R_1	R_2	R_3
3	Rutinose	OH	OH
4	H	Glucose	OH
5	H	H	Glucose
6	H	OH	OH
7	H	H	OH

Figure 4.1 - Chemical structures of phenolic compounds in *Achillea millefolium*. 1: Chlorogenic acid; 2: Caffeic acid; 3: Rutin; 4: Luteolin Glucoside; 5: Apigenin Glucoside; 6: Luteolin; 7: Apigenin.

Table 4.1 - Optimized retention time (RT) and multiple reaction monitoring (MRM) transitions for the detection and quantification of marker phenolic compounds.
Quantification was performed on a QTRAP MS system and separation of marker compounds was performed on a Phenomenex Luna omega polar C18 Column.

Peak #	Compound	RT (min)	Q1	Q3	DP	EP	CE	CXP
1	Chlorogenic acid	2.76	353.066	191.000[a]	-65	-10	-26	-11
				84.900[b]	-65	-10	-60	-3
2	Caffeic acid	2.89	179.024	135.100[a]	-70	-10	-22	-7
				133.900[b]	-70	-10	-36	-9
3	Rutin	4.21	609.148	299.800[a]	-130	-10	-52	-19
				270.900[b]	-130	-10	-78	-17
4	Luteolin Glucoside	4.41	447.055	284.800[a]	-140	-10	-36	-7
				283.900[b]	-140	-10	-50	-7
5	Apigenin Glucoside	4.87	430.990	267.900[a]	-155	-10	-48	-17
				268.800[b]	-155	-10	-36	-15
6	Luteolin	5.81	284.993	132.900[a]	-120	-10	-48	-9
				64.900[b]	-120	-10	-72	-1
7	Apigenin	6.45	140.900	150.900[a]	-105	-10	-34	-9
				116.800[b]	-105	-10	-48	-7

[a]: MRM transition used for quantification; [b]: MRM transition used for confirmation.
Q1: m/z parent; Q3: m/z major fragment; DP: declustering potential; EP: entrance potential; CEP: collision cell entrance potential; CE: collision energy; CXP: collision cell exit potential.

4.2.3 HPLC-DAD CONDITIONS

Chromatographic separations were performed on an Agilent 1100 series analytical HPLC-DAD system (Agilent Technologies, Montreal, QC, Canada) equipped with a Phenomenex Luna® C-18 column (150 mm x 2 mm, 3 micron, Phenomenex, Torrance, CA, USA). To quantify potential alkylamide compounds, methods were optimized by applying a linear gradient over 40 minutes at 50°C starting at 90% A (0.1% TFA in water) and 10% B (0.1% TFA in acetonitrile), and ending at 100% B, with a flow rate of 4 ml/min and an injection volume of 5 µl. A five minute post-run equilibration was completed at 90% A. Mean absorbance was calculated as the area under the peak of each of the 15 alkylamides as measured by DAD at 268 nm.

4.2.4 UPLC-MS CONDITIONS

Samples were then run on a UPLC-MS system (Mandel Inc., Guelph, Ontario) containing a CTO20A column oven, SIL-30AC autosampler, and a LCMS-2020 mass spectrometer. A CSH-C18 column was used (100 x 2.1 mm, 1.7 µm particle size, Acquity, Waters, Mississauga, Ontario). A linear gradient starting at 90% A (0.1% TFA in water), 10 % B (0.1% TFA in acetonitrile) and increasing to 100% B after 8 minutes, with a flow rate of 0.6 ml/min at 50°C. The mass spectrometer was operating in positive and negative ion modes. The nebulizing gas flow was set to 1.5 L/min and drying gas flow at 10 L/min. The dissolution line temperature and heat block temperature were set at 300°C and 450°C respectively. The m/z range was from 150 to 600 with 938 u/sec scan speeds.

4.2.5 STATISTICS

Developmental comparisons of *Achillea millefolium* parts were analyzed in GraphPad Instat using one-way ANOVA tests followed by Tukey *post-hoc* tests.

4.3 RESULTS

4.3.1 PHENOLIC COMPOUNDS

Phenolic compounds were detected at varying concentrations across the collection time points using a QTRAP MS system (Figure 4.2). Of all phenolic compounds analyzed, chlorogenic acid is the only phytochemical found within the whole plant at all points throughout the growth period (Figure 4.3). Seasonal trends of chlorogenic acid remained consistent throughout the whole plant. Levels within the flowerhead peak June 1, while the stem, leaf, and root peak about a week later around June 8. Chlorogenic acid in all extracts then drops down to initial concentrations and remains relatively low for the remainder of the growth period. Initially, stem extracts contain the highest concentration of chlorogenic acid on the first collection date (May 26) then result in a decrease in concentration by over 95% over the course of one week.

The concentrations of the remaining phenolic compounds within flowerheads, stems, and leaves were analyzed and compared (Figures 4.4-4.6). These figures clearly show that stem extracts are qualitatively similar to leaf, while flowerhead has a distinct profile and seasonal trend. Flowerheads contained the highest levels of most phenolics. Flavonoids and their glucosides shared similar trends, and glucosides were found in lower concentrations at all points throughout the season. Caffeic acid, chlorogenic acid, and apigenin glucoside concentrations were omitted from particular figures as their levels were very low, and thus deemed negligible, relative to the other detected compounds. Rutin profiles within stem and leaf followed similar trends to one another. Concentrations start high and end high, with low relative abundance mid-season. Abundance peaked early season during the beginning of June, decreased in both parts at the end of this month, then increased to their highest concentrations within the month of August. Levels of rutin within the flowerhead remained relatively low and consistent. When monitoring

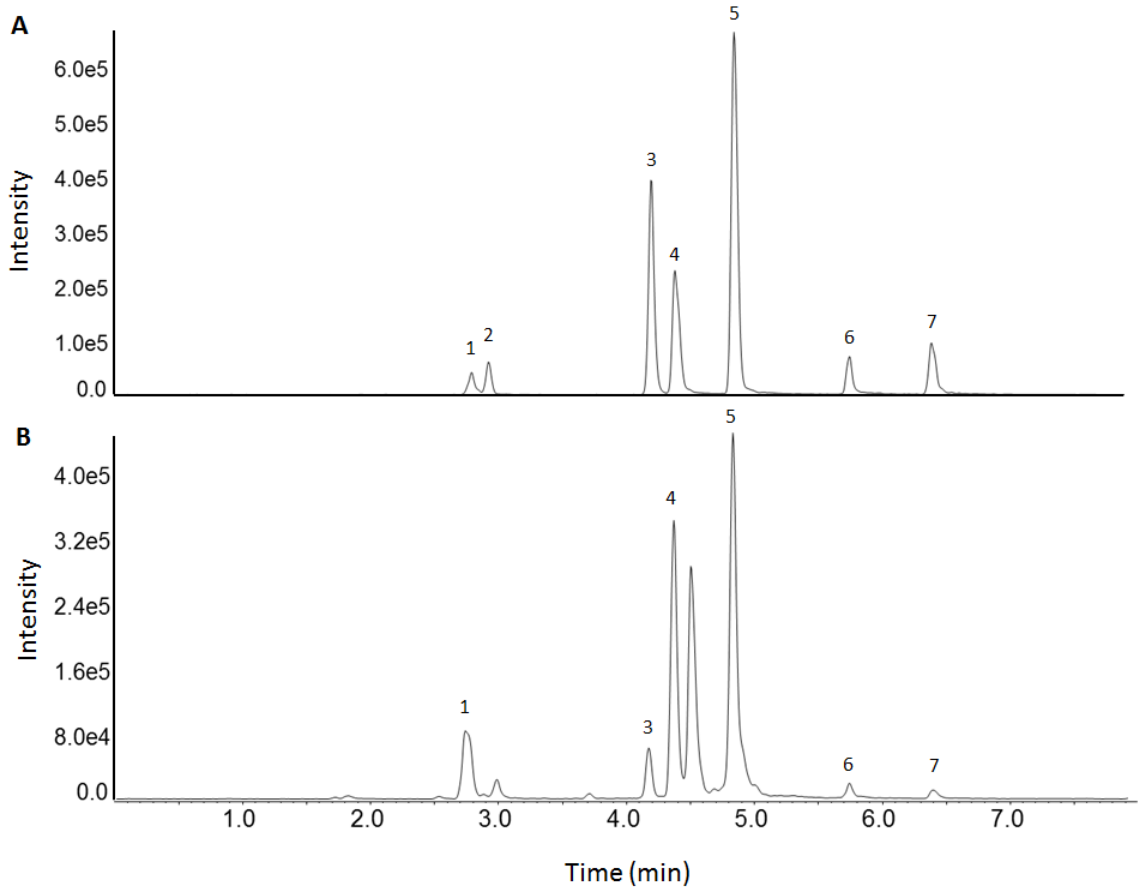


Figure 4.2 - QTRAP total ion count (TIC) chromatograms of standards (A) and yarrow flowerhead extract (B) generated using LC-MS/MS in negative ionization mode. Yarrow flowerhead extract from 1/6/16. 1: Chlorogenic acid; 2: Caffeic acid; 3:Rutin; 4: Luteolin Glucoside; 5: Apigenin Glucoside; 6: Luteolin; 7: Apigenin

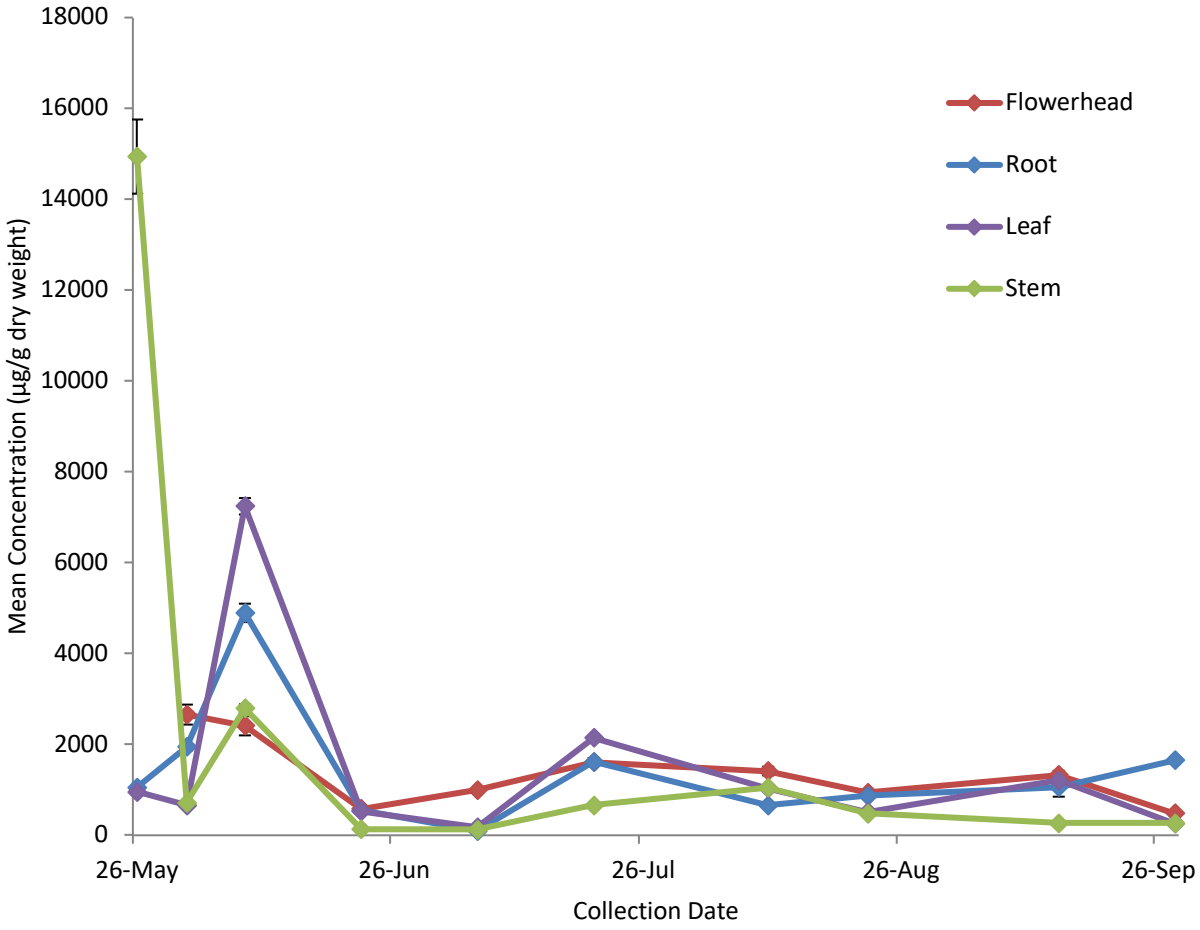


Figure 4.3 - Seasonal profiles of chlorogenic acid quantified within four *A. millefolium* parts (n=3, ± SEM). Samples quantified using QTRAP-MS and MRM.

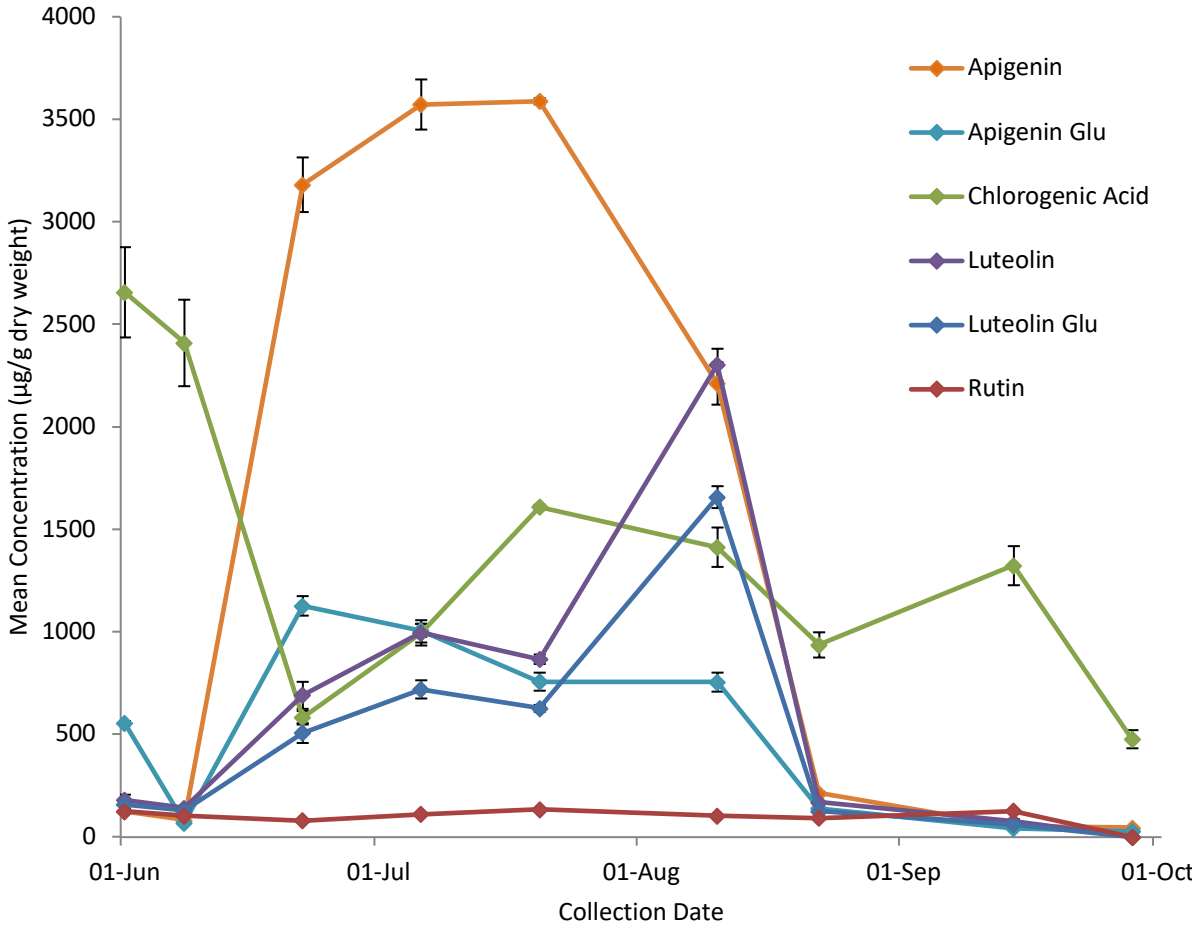


Figure 4.4 - Seasonal phenolic flowerhead profile (n=3, ± SEM). Samples quantified using QTRAP-MS and MRM.

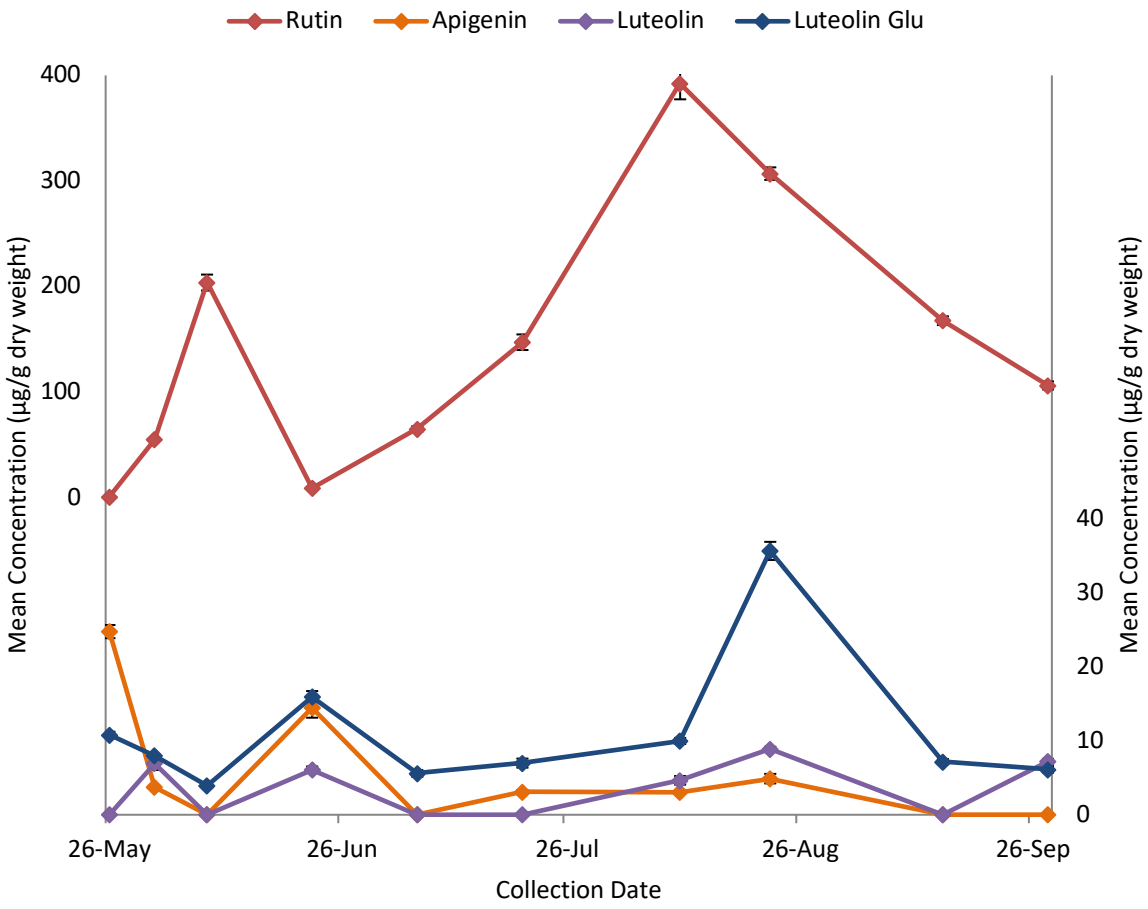


Figure 4.5 - Seasonal phenolic stem profile (n=3, ± SEM). Samples quantified using QTRAP-MS and MRM.

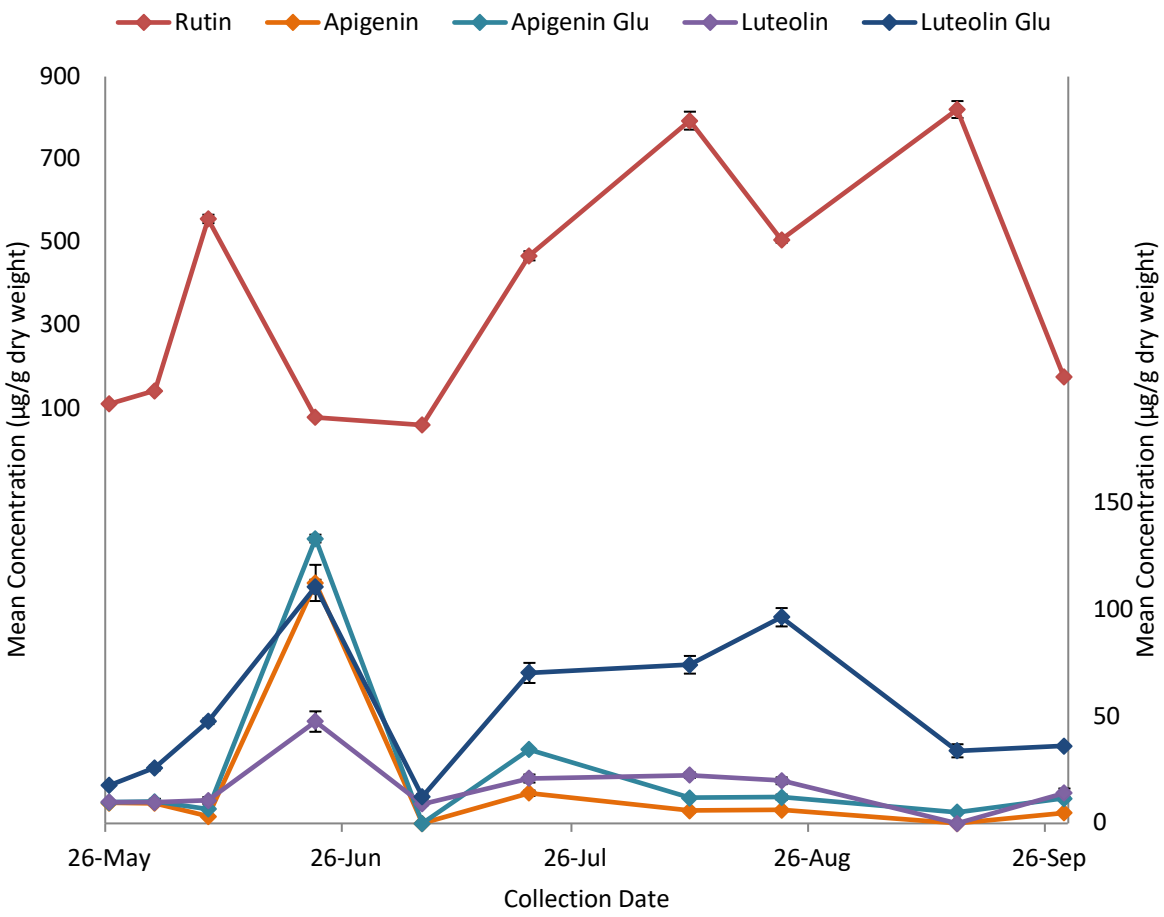


Figure 4.6 - Seasonal phenolic leaf profile (n=3, ± SEM). Samples quantified using QTRAP-MS and MRM.

trends of apigenin and luteolin concentration as well as their glucosides (apigenin-7-O-glucoside, luteolin-7-O-glucoside), concentrations within the stem and leaf were observed at relatively low levels when compared to flowerhead extracts. Apigenin was present at low concentrations at both the start and end of the growing period. During mid-season, concentration increased over 60-fold. Similarly, luteolin levels within the flowerhead peaked during August, though following a more gradual increase (Figure 4.4).

4.3.2 ALKYLAMIDES

Based on the described identification criteria using HPLC-DAD, fifteen potential alkylamides were identified in four parts of *A. millefolium* and quantified across one growing season. Quantification in roots is presented in Appendix B. Elution of alkylamides extended between 18 to 30 minutes and compounds were mainly isolated in the roots (Figure 4.7). Isolation and characterization through UPLC-MS of the top five alkylamides is presented in Table 4.2. Analysis of the five most prominent root compounds in extracts of root and flowerhead showed variation in qualitative and quantitative measures (Figure 4.8). The top five alkylamides found in roots were all isolated above a threshold level of 200 absorbance units (area under the curve per gram dry weight). Abundance of all alkylamides in flowerhead, stem, and leaf never exceeded 250, with the exception of late season flowerhead extracts at one collection date. Within the root, compounds 1, 4, 11, and 13 are found in relatively similar concentrations at the start and end of the season, with significant variations throughout. Compound 8 seemed to decrease until early August, where it quickly increased above initial levels by the end of the season. Flowerhead and stem extracts showed similar quantity and trends

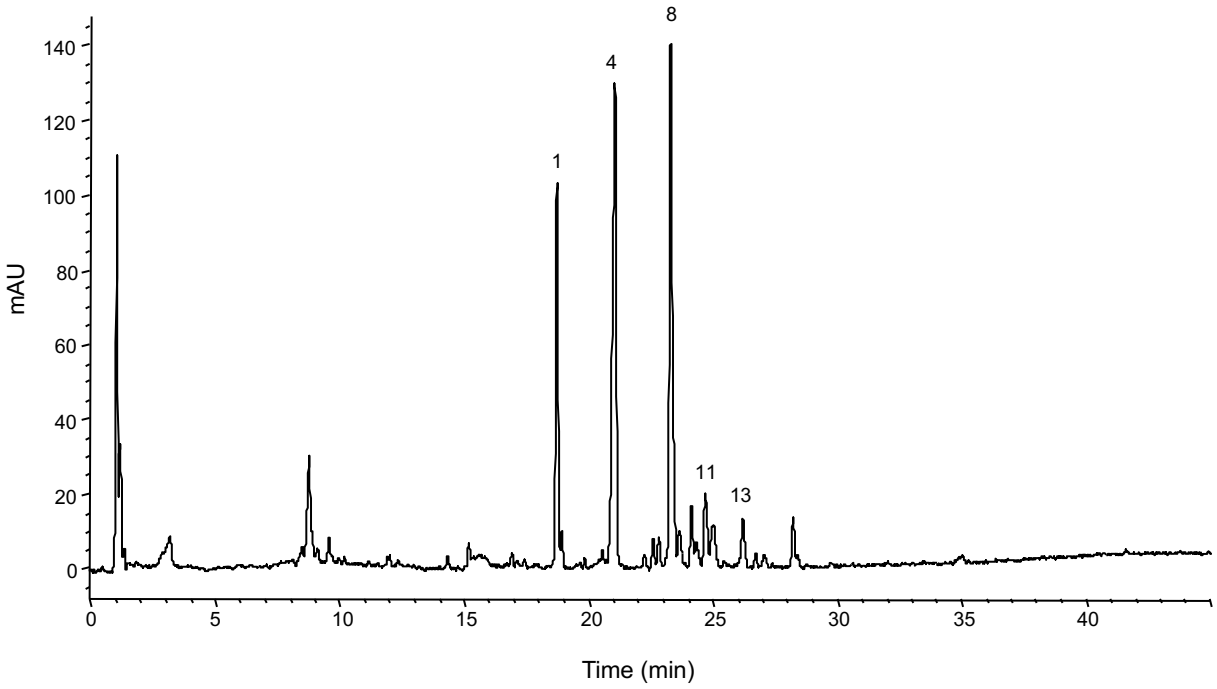


Figure 4.7 – HPLC-DAD chromatogram of yarrow root at 268 nm. Sample collected on 1/6/16. Labelled peaks correspond to the top 5 most abundant alkylamide compounds.

Table 4.2 – Detection and quantification of top 5 alkylamide compounds. Quantification was performed using an optimized linear gradient on a UPLC-MS system.

Compound	RT (min)	m/z [parent]/[fragment]
1	18.78	[271+] / [230+]
4	21.11	[329+] / [288+]
8	23.37	[265+] / [224+]
11	24.75	[313+] / [272+]
13	26.23	[277+] / [236+]

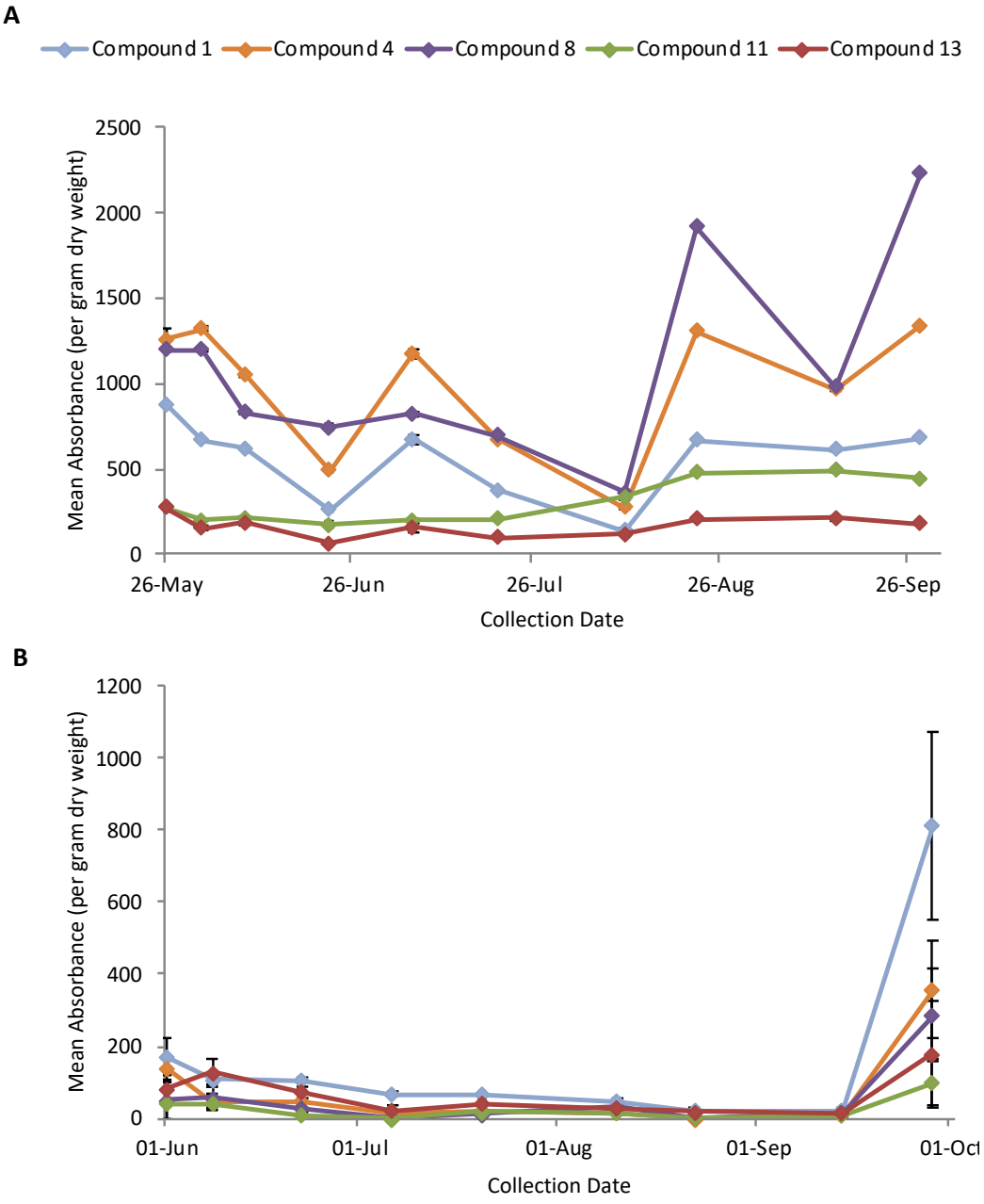


Figure 4.8 - The five most abundant alkylamides in root (A) and flowerhead (B), as determined by peak area of DAD chromatogram at 268 nm. Quantified as relative absorbance per gram dry plant material.

of alkylamide concentrations from early- to mid-season. Stem compounds displayed a more rapid decline in abundance when compared to the apparent gradual decrease in flowerhead, but both trends showed an overall drop in alkylamides over the growing season. However, a significant difference between these two parts is clear from collections made in September. Concentrations of alkylamides in stem do collectively begin to increase, albeit at significantly lower rates as compounds in the flowerhead. The concentration of alkylamides seen within flowerheads collected in September increased to levels comparable within the root (Figure 4.9). Contrastingly, leaf extracts displayed the lowest concentrations of all four parts at this date. There are no significant changes in concentration as these phytochemicals vary throughout the season (Appendix B).

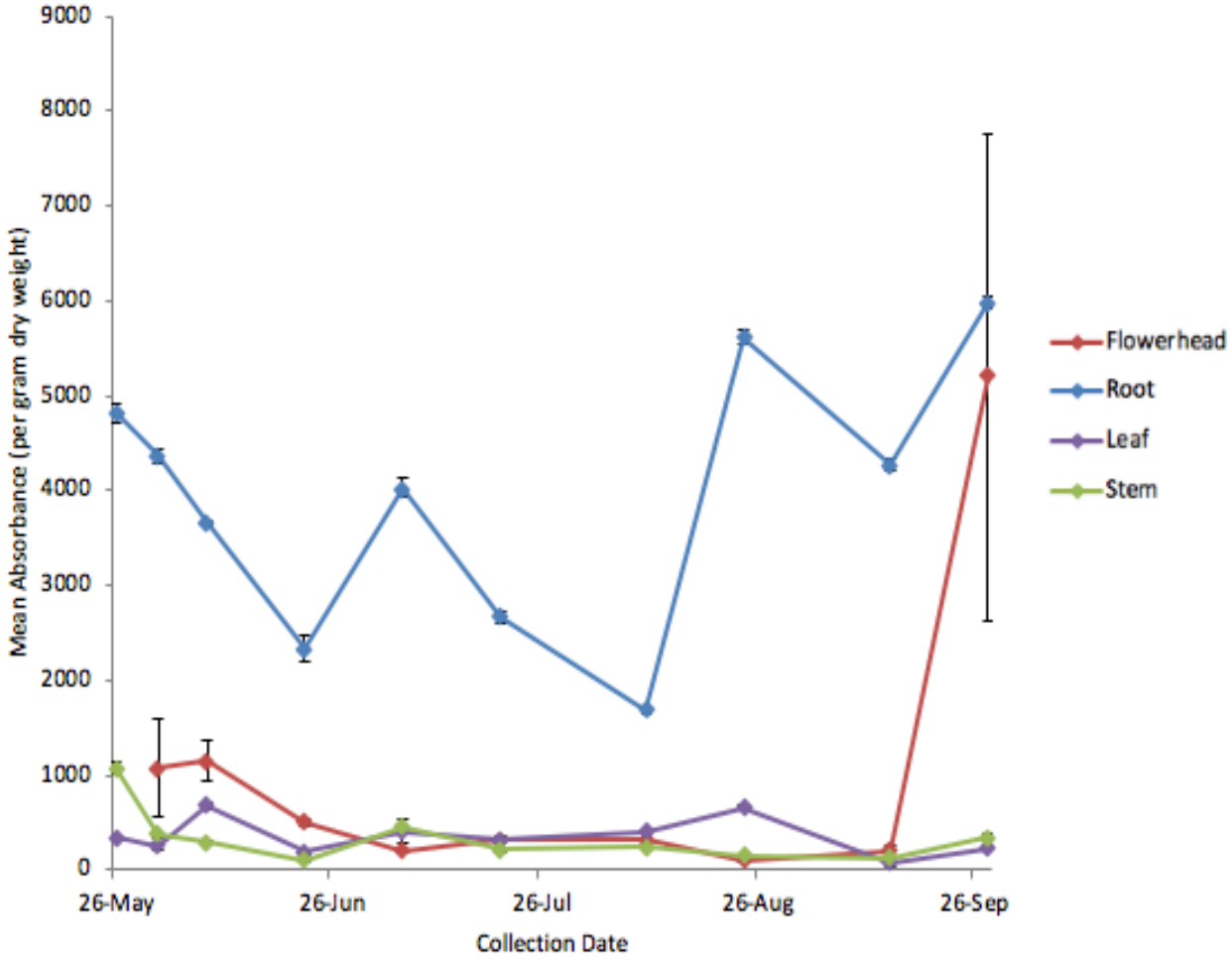


Figure 4.9 – Alkylamide profiles in four yarrow parts as determined by peak area of DAD chromatogram at 268 nm.

4.3.3 Seasonal Analysis

Phytochemical composition was then analyzed based on developmental trends. We compared the chemistry of root, stem, and leaf samples collected on the same date from individual plants at distinct developmental stages to the chemistry of previously collected samples. A phenological analysis of three flavonoids is compared in Table 4.3. New growth collections are significantly lower in phenolic compounds in all parts. However, this variation in concentration does not follow phenological trends as new collections are almost always significantly different from older growth. Different trends in alkylamide content were observed in all parts (Figure 4.10). Within the root, there are fairly equal concentrations of alkylamides for both new and old growth, with no significant difference from early season. Stem and leaf extracts from all three collections contained significantly different concentrations of alkylamides. Stems showed little to no alkylamides in the old growth compared to new. The leaf extracts shared the same general trend as in stem, where only low levels of alkylamide compounds were seen in old growth compared to new. However, late-season new growth was significantly higher in alkylamides in comparison to both early season, and old growth from the same time point.

Table 4.3 - Phenological comparison of three flavonoids. Means of three counts presented (± SEM). Significance determined by Tukey *post-hoc* test, or unpaired t-test (Luteolin in stem).

Plant part	Date	Mean Concentration (µg/g dry weight)		
		Chlorogenic acid	**Rutin**	**Luteolin**
Leaf	June 1	656 ± 34.1^{a}	142 ± 0.735^{a}	9.92 ± 1.61
	Sept 28 New	181 ± 13.4^{b}	44.9 ± 0.746^{b}	14.1 ± 0.162
	Sept 28 Old	253 ± 35.3^{b}	175 ± 2.92^{c}	14.2 ± 2.11
Stem	June 1	716 ± 6.90^{a}	55.1 ± 0.920^{a}	6.95 ± 0.896
	Sept 28 New	130 ± 4.53^{b}	11.5 ± 0.192^{b}	TRACE
	Sept 28 Old	263 ± 9.15^{c}	106 ± 3.77^{c}	7.21 ± 0.348
Root	June 1	1950 ± 75.5^{a}	6.14 ± 0.443	ND
	Sept 28 New	796 ± 60.2^{b}	ND	ND
	Sept 28 Old	1650 ± 6.77^{a}	ND	ND

TRACE: Detected but not quantifiable
ND: Not detected
Different letters indicate significant difference ($p < 0.001$).

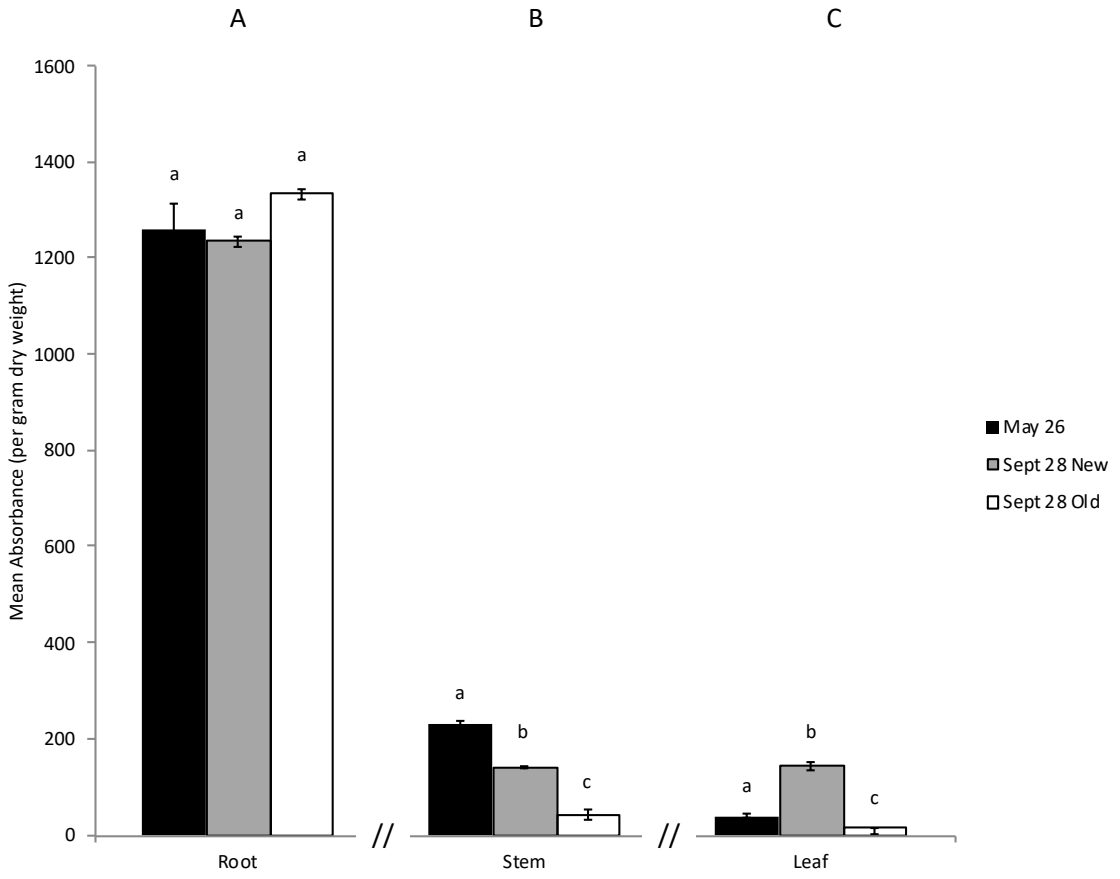


Figure 4.10 - Developmental comparison of alkylamide Compound 4 within *A. millefolium* root (A), stem (B), and leaf (C) (n=3, ± SEM). Samples quantified using HPLC-DAD. Bars within the same plant part which do not share a common letter represent significant differences in concentrations as determined by Tukey *post-hoc* test ($p < 0.05$).

4.4 DISCUSSION

Two major phytochemical classes identified here in *A. millefolium* appear to be distributed across the plant but concentrated mostly within two distinct organs. Alkylamides have been detected mainly within the roots, consistent with results published by Veryser et al. (2017), while phenolics remain highly concentrated in the flowerheads. These chemical markers are consistent across populations throughout recent published findings. Previous reports of *Achillea* chemistry demonstrate the variability of compound profiles in different parts of the plant (Jesionek et al., 2015a,b; Pires et al., 2009; Vitalini et al., 2011). In North America, *A. millefolium* exists as a composite of both native and introduced varieties from Europe (TWC, 2017). The observed and expected chemical variation across populations is related to the global distribution and co-existence of native and introduced varieties that collectively form a complex. The seasonal variations observed in this study – and the different time points at which the plant was collected in other studies – likely also contribute to inconsistent reports in the literature.

The seasonal variation of phytochemicals in separate organs provides a rationale for the variable use of certain parts in specific medicinal applications. It is equally important to consider why the plant synthesizes and transports these compounds at distinct growth stages. Interesting trends appear within the phenolic profiles of the four parts of yarrow. Chlorogenic acid and its chemical derivatives may be largely involved in the initial growth stages of the plant (Figure 4.3), possibly providing chemical defense from herbivory when photosynthetic tissues are most limited. In *Vaccinium angustifolium* leaves and stems, concentrations of chlorogenic acid similarly peak during early growth then drop throughout flowering (McIntyre et al., 2009).

Phytochemicals may also be translocated and redistributed throughout the plant or upregulated at a specific time in response to upstream regulation. The profile of apigenin in

yarrow flowerheads is seemingly opposite to the trend of rutin concentrations seen in both leaves and stems. The selective transportation of flavonoids between plant organs has been established and thus provides an explanation for these opposing quantitative trends (Buer et al., 2008; Petrussa et al., 2013). Flavonoids are involved in growth and development as well as the stress response of a plant. At times around mid-season, when the reproductive structures are reaching the peak of their growth, they require increased resources to do so. The detected phenolic compounds may be redistributed from the leaves and stems to the inflorescences during the peak of the reproductive stage. In yarrow, the expression of different enzymes involved in flavonoid biosynthesis may be organ-specific.

The alkylamide profiles for each of the four organs also display variation in both type and concentration of phytochemicals. The most abundant alkylamides for each part are not the same compounds, again supporting variability between plant organs and also differences phenologically. Similar research published on work with *Echinacea* reflects these findings regarding phytochemical localization. Alkylamides isolated in *Echinacea* species were found at higher concentrations in the roots than in aerial parts and seed heads (Binns et al., 2002; Kabganian et al., 2003; Qu et al., 2005). Although alkylamide concentrations in yarrow flowerheads remain relatively low throughout the season, the spike in late September collections is particularly noteworthy and perhaps indicates that alkylamides are localized within yarrow seeds. This inference conclusively parallels unpublished findings in *Echinacea*, reflecting the increased concentration of alkylamides in late-season flowers (Elizabeth Daley, Harris lab). Both *Echinacea* and *Achillea* are known to produce a diversity of alkylamides, mostly within the roots (Bauer et al., 1989; Boonen et al., 2012; Woelkart et al., 2005). The presence of this phytochemical class is associated with the induction of immunity for the individual by initiating

the biosynthesis of signaling molecules and the subsequent release of volatile compounds. Several unsaturated alkylamides are known to accumulate in the presence of methyl jasmonate (Binns et al., 2001). Derived from linoleic acid, both methyl jasmonate and jasmonic acid function as signal molecules to regulate and induce plant defense strategies. Interestingly, this accumulation of alkylamides as a stress response is mimicked in the mammalian system in various physiological conditions. Recent studies on *Arabidopsis thaliana* have identified the presence of a gene for fatty acid amide hydrolase (AtFAAH), based on its similarly to the mammalian FAAH gene (Shrestha, Dixon, and Chapman, 2003). Fatty acid amide hydrolase is the primary degradative enzyme for endocannabinoids and endocannabinoid-like compounds in mammals. Alkylamides mimic the structure and function of endocannabinoids and are recognized by the same FAAH enzymes as targets for degradation. Overexpression of AtFAAH in *Arabidopsis thaliana* resulted in decreased immunity and suppressed defense mechanisms (Kang et al., 2008). Individuals were significantly more susceptible to bacterial pathogens *Pseudomonas syringae* pv. *tomato* and *P. syringae* pv. *maculicola*. The plant also expressed lower levels of defense signaling molecules, jasmonic acid and salicylic acid, as well as abscisic acid, a phytochemical involved in physical defense (Kang et al., 2008). It is possible that Asteraceae species contain similar genes to AtFAAH, and this could provide reasoning for their interactions with signaling molecules and defense mechanisms, although further research should be conducted. Since there are relatively consistent concentrations of alkylamides in the roots at all growth stages, this may indicate the existence of a constitutively expressed defense mechanism in this organ.

An assessment of the distinct developmental stages of yarrow provided insight into the effects of phenology versus development on the phytochemical profiles of each part. If chemical

variations are affected by development, then the compounds and trends seen in late-season 'new' growth should be similar to trends seen early in the season during the young developmental stages (May/June). Alternatively, if phenological factors have a stronger impact, the chemistry of 'new' growth would be more similar to that of older samples collected at the same time. In the case of phenolics, concentrations of chlorogenic acid and rutin in all parts were almost always significantly different in comparisons across three collections. It is possible that since the plant has already reached maturity, it has no need to input energy into biosynthesizing these phytochemicals for defense. These observations support the optimal defense theory (ODT), which predicts that the within-plant quantitative allocation of defenses is not random and that chemical defenses will be allocated within a plant as a function of tissue value (e.g. more concentrated in young leaves compared to older leaves). Since flowers contribute more to overall fitness than leaves, the higher concentrations of phenolics in flowerheads compared to leaves (at any time point) is also predicted by ODT (McCall & Fordyce, 2010). Similarly, the reduced phenolic defenses expressed by new vegetative growth produced late in the season follows ODT predictions; reproductive structures from these new shoots may not develop in time to produce viable seed. Besides chlorogenic acid, however, concentrations of other individual phenolic metabolites do not peak during early growth, suggesting more complex regulation involving developmental or environmental cues beyond defense from organ loss.

Concentrations of alkylamides remained relatively consistent across the season and extracts from 'new' and 'old' growth from late season reflected this trend. As a rhizomatous, clonally spreading species, the roots of yarrow are always developing (Hurteau, 2018). As yarrow spreads, the rhizome and roots develop more extensively below the new shoot, so early stage root growth would be expected throughout the season. Thus, it is likely they require

relatively constant concentrations of the same phytochemicals. Perhaps there is no need for significant seasonal changes, and visible fluctuations, though not significant, are likely explained by the age of the root rather than above-ground developmental stage.

The phytochemical profiles in certain organs may reflect the ethnobotanical applications of yarrow as a medicinal treatment. Through trial and error of selecting specific plant parts, traditional healers may have learned to choose certain parts so that they may be more confident in a successful treatment. For example, if at one point they gathered mainly flowers to treat an illness and the illness persisted, individuals may learn from this and in the future, avoid collecting flowers. Therefore, it is a possibility that these healers are not actively selecting the leaves, but instead actively avoiding other parts. Because the majority of ethnobotanical reports of yarrow's medicinal use do not describe collection practices like time of harvest, any seasonal or developmental factors that may have guided collection or medicinal application by different healers was not captured by meta-analysis (Chapter 2). However, given that alkylamide levels are low throughout the season in leaf and stem, and that levels in flowerheads only rise during seed set, phenolics likely predominate in most natural products derived from yarrow's aerial parts and would serve as more appropriate markers for quality control. From the results discussed in Chapter 2 of this report, yarrow roots were determined to be over-used as oto-rhino-laryngological and pulmonary aids. *Echinacea* roots have been tested in similar models and contain compounds that show significant bioactivity against symptoms encompassed by these categories. It is well established that *Echinacea* roots are also abundant in alkylamides, so the incidence of a similar mechanism of action is probable. The selection of yarrow roots may reflect a similar use to *Echinacea* for treating respiratory symptoms.

In summary, this chapter highlights the phytochemical variations of *A. millefolium* throughout development. Alkylamides are mainly localized in roots, while phenolic compounds are abundant within flowerheads. In all parts, concentration of phenolics is dependent upon growth stage as well as seasonal time point, although not necessarily governed by these factors exclusively. To further this research, analyses involving the MS characterization of phytochemicals will be employed in a non-targeted approach, as well as potential structure elucidation using NMR.

CHAPTER 5 – GENERAL DISCUSSION

5.1 SUMMARY OF ORIGINAL CONTRIBUTIONS

This thesis presented a systematic review of medicinal Asteraceae used traditionally by indigenous peoples of North America. Together with a targeted pharmacological analysis and a seasonal assessment of phytochemical profiles, the results presented here will augment the knowledge and communication in the field of natural health products in the scientific community.

As the first ethnobotanical meta-analysis of its kind, Chapter 2 offered a novel set of enquiries addressed by way of residual and binomial statistics. Results from both analyses supported the general hypothesis that the selection of North American Asteraceae species for ethnobotanical use is in fact non-random and is not always dependent on the size of the flora. Anthemideae were found to be over-selected for medicinal use in most therapeutic categories defined by Moerman (1991), and most significantly as a pulmonary aid. Taxonomic analysis of 25 Anthemideae species used as pulmonary aids showed that most uses come from *Artemisia* and *Achillea*. Phylogenetic trends were presented. The significance of Anthemideae as an ethnobotanically over-utilized tribe in conjunction with the high over-selection of species for pulmonary aid suggests the presence of inherent bioactivity and phytochemistry characteristic of this tribe.

Steps were then taken to broadly confirm the efficacy of these ethnobotanical reports and indigenous traditions across North America through pharmacological assays. First, *Achillea millefolium* L. (Asteraceae) flowers, roots, stems, and leaves were tested for activity in the respiratory immunomodulatory response of BEAS-2B bronchial epithelial cells. Flower extracts

were significantly anti-inflammatory and displayed dose-dependent activity in most cases. Leaf extracts followed a similar trend, although not significant. In contrast, root extracts exhibited a significant pro-inflammatory response in stimulated BEAS-2B cells. Organic and aqueous extracts of the most active parts, flowers and roots, were then tested within the same assays. Just as separate organs displayed distinct bioactivity, the products of a solvent-solvent extraction of crude flowers and roots showed differing activities. Furthermore, the potential for *A. millefolium* (yarrow) to interact with the endocannabinoid system (ECS) was assessed for the first time, quantified through inhibitory assays on fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) activity. Flowers and roots were the most potent inhibitors of ECS enzymes. Analysis of organic and aqueous extracts resulted in higher inhibitory activity from organic extracts, though both fractions seemed to exhibit a dose-dependent inhibition of MAGL. These data confirmed the localized bioactivity across yarrow plant parts and suggested that variation in phytochemical composition is responsible for this apparent effect.

Finally, a seasonal targeted profiling of two major phytochemical classes was achieved, providing novel insight into the distribution and variability of established bioactives. Alkylamides and phenolic compounds were quantified and identified across four parts of yarrow at different timepoints throughout a growing season. Alkylamides were mainly distributed in roots, while phenolics were found in high concentrations in flowerheads. Phytochemical profiles varied between parts and also across developmental stages. A phenological analysis of flavonoids showed that phenolic compounds were significantly lower in September new growth collections of all parts, while seasonal alkylamide levels fluctuated. Results suggest that this seasonal relationship is complex and should be further studied to deduce significant conclusions.

5.2 PHARMACOLOGY OF YARROW

The cannabinomimetic properties of alkylamides allow for interactions with the ECS and thus elicit physiological effects regulated by this endogenous system. Chapter 3 concluded that yarrow flowers and roots were the most active parts in inhibiting FAAH and MAGL activities *in vitro*. Due to yarrow's close phylogenetic relation to *Echinacea*, it was hypothesized that these activities were attributed to an abundance of alkylamide compounds. However, phytochemical profiling in Chapter 4 suggested that this may not in fact be the case. Though roots were highly concentrated in alkylamides, flowerheads contained much lower concentrations at all points throughout the season, except the final September collection date when levels were comparable to root. Consequently, it is unlikely that this group of phytochemicals is solely responsible for the promising inhibitory results seen within the FAAH and MAGL assays. Unpublished results from the Harris lab suggest certain phenolic metabolites inhibit FAAH and that inhibitory activity may result of phytochemicals acting by means of synergy (Rui Liu, PhD; Etienne Rheaume, Honours, 2017). Future work should focus on isolating single compounds and evaluating their bioactivities in comparison to crude extracts of yarrow.

Though not necessarily apparent in this study, the ECS has been exercised as a means by which to treat symptoms of asthma in the past. Cannabis was prescribed as a bronchodilator in the mid-19th century, however with the use of synthetic drugs on the rise by the start of the 1900s its significance and popularity began to decline (Ziment & Tashkin, 2000). Studies from the late 20th century presented data supporting a dose-dependent bronchodilation response in male subjects treated with cannabis. When compared to the administration of a β2-agonist, cannabis provided a greater functional response in reducing antigen-induced bronchoconstriction (Tashkin, Shapiro, & Frank, 1973; Vachon et al., 1973). Lancelot medicinal cigarettes contained

cannabis and stramonium for immediate relief and relaxation of the bronchi. Additional brands of asthma cigarettes contained atropine from Deadly Nightshade, arsenic, or other toxic compounds to aid in bronchodilation, presumably via cholinergic pathways (Chu & Drazen, 2005; Elliott & Reid, 1980).

More recent explorations into the bioactivity of traditionally used Asteraceae species support the medicinal use of yarrow as a respiratory aid. Acetylcholine induced contractions of ileum smooth muscle in rats are decreased in the presence of yarrow extract (Moradi et al., 2013). Similarly, luteolin-rich *Artemisia afra* extracts have been found to possess bronchodilatory properties (Mjiqiza et al., 2013). Results summarized in this thesis showed that yarrow flowers exhibited the most significant anti-inflammatory activity and contained the highest concentration of luteolin across parts. It is probable that similar principles are responsible for bronchiolar contractions occurring in chronic respiratory diseases and future studies should focus on testing the effects of yarrow on muscle constriction. Another species of Asteraceae, *Eclipta prostrata*, has been studied as a pulmonary aid. A methanol extract showed a significant reduction in bronchial hyperresponsiveness, a characteristic of chronic pulmonary diseases. Levels of pro-inflammatory cytokines including interleukin 4 (IL-4), IL-5, and IL-13 were also significantly reduced (de Freitas Morel et al., 2017). Furthermore, *Echinacea* is most commonly used to treat cold and flu symptoms, and extracts have the potential to stimulate anti-inflammatory, analgesic, and anxiolytic activity (Haller, Hohmann, & Freund, 2010; Raso et al., 2002; Yu et al., 2013). Although limited research exists on the use of *Echinacea* as a supplementary treatment for asthma, recent studies support this possibility as extracts proved to be effective bronchodilators and anti-inflammatory agents both *in vivo* and *in vitro* (Šutovská et al., 2015). However, it should be noted that some patients with allergy or asthma experienced

bouts of asthmatic symptoms immediately following the ingestion of *Echinacea* tea or tablets, suggesting possible cross-interactions (Mullins & Heddle, 2002).

5.3 PHYTOCHEMISTRY OF YARROW

Phytochemical variation across a growing season is often large, and thus it must be considered why the plant is making these changes for its own fitness. Phenolics are mostly found in the flowerheads, and a speculative conclusion proposes that these phytochemicals are produced to aid in or defend the reproduction of the plant. In most chemical profiles, we do see changes over time. However, the alkylamides in the roots tend to remain consistent. Yarrow clones and spreads its roots, in contrast to *Echinacea*, which clones yet remains as individual plants. In cases where there are minimal chemical changes across a growth period, the difficulty in determining whether a root sample was part of new growth may have been increased. When collecting samples, all the roots were connected underground through rhizomes and older roots were indistinguishable from new.

The chemical differences between parts suggests that these compounds may be produced or stored at different levels over the course of plant development. For example, phytochemicals can be transported throughout the plant or synthesized in response to attack. The qualitative and quantitative profiles of isobutylamides and cichoric acid within *Echinacea* have been shown to vary throughout its developmental stages (Binns et al., 2001; Letchamo et al., 1999). The role of certain phytochemicals of the Asteraceae in chemical ecology is now well described, as several of these compounds (e.g. polyacetylenes) are involved in defense against a pathogen or herbivory by eliciting toxic effects on the attacking organism (Zid & Orihara, 2005). However, the ecophysiological functions of others (e.g. alkylamides) remain unclear. Future work should incorporate phytochemical profiling results from Chapter 4 and compare these to additional analyses of ecological function.

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APPENDIX A - THERAPEUTIC CATEGORIES AND SUPPLEMENTARY STATISTICS

Table A.1 - Medicinal abbreviations and therapeutic categories. (Moerman, 1991)

Medicinal category	Terms
Cardiology (CAR)	Blood medicine
	Heart medicine
	Hypotensive
Dermatology (DER)	Burn dressing
	Dermatological aid
	Hemostat
Gastro-Intestinal (GIM)	Anthelmintic
	Antidiarrheal
	Antiemetic
	Antihemorrhagic
	Carminative
	Diaphoretic
	Dietary aid
	Emetic
	Gastro-Intestinal aid
	Hemorrhoid remedy
	Laxative
	Liver aid
General (GEN)	Adjuvant
	Alterative
	Panacea
Gynecological-urinary (GYN)	Abortifacient
	Breast treatment
	Diuretic
	Gynecological aid
	Kidney aid
	Reproductive aid
	Urinary aid
	Venereal aid
Neurological (NEU)	Analgesic
	Anesthetic
	Anticonvulsive
	Febrifuge
	Hallucinogen
	Narcotic
	Sedative
	Stimulant

Orthopedic (ORT)	Antirheumatic (External) Antirheumatic (Internal) Orthopedic aid
Other (OTH)	Antidote Cancer treatment Ceremonial medicine Disinfectant Herbal steam Hunting medicine Insecticide Love medicine Misc. disease remedy Other Pediatric aid Poison Psychological aid Snake bite remedy Strengthener Tonic Tuberculosis remedy Unspecified Veterinary aid Witchcraft medicine
Oto-rhino-laryngological (OTO)	Ear medicine Eye medicine Nose medicine Oral aid Throat aid Toothache remedy
Pulmonary (PUL)	Cold remedy Cough medicine Expectorant Pulmonary aid Respiratory aid

Table A.2 - Residual analysis of Asteraceae tribes over-selected for medicinal use. Analysis compared specific use counts to total medicinal uses.

CAR			DER			GEN			GIM			GYN		
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Cichorieae	4.75	1	Heliantheae	9.21	1	Cardueae	4.11	1	Eupatorieae	12.08	1	Eupatorieae	17.41
2	Heliantheae	4.69	2	Senecioneae	7.50	2	Heliantheae	1.79	2	Heliantheae	7.89	2	Cichorieae	12.38
3	Cardueae	4.49	3	Cardueae	5.09	3	Anthemideae	1.56	3	Plucheeae	4.31	3	Astereae	8.38
4	Inuleae	1.76	4	Mutisieae	4.79	4	Eupatorieae	1.21	4	Cichorieae	1.17	4	Cardueae	1.32
5	Astereae	0.83	5	Arctotideae	1.95	5	Cichorieae	0.03	5	Inuleae	0.91	5	Heliantheae	1.26
6	Vernonieae	0.10	5	Calenduleae	1.95				6	Vernonieae	0.87			
			6	Vernonieae	1.15				7	Arctotideae	0.11			
									7	Calenduleae	0.11			
NEU			**ORT**			**OTH**			**OTO**			**PUL**		
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Heliantheae	5.54	1	Anthemideae	16.59	1	Heliantheae	12.78	1	Heliantheae	11.22	1	Anthemideae	34.03
2	Plucheeae	2.26	2	Cardueae	3.71	2	Cichorieae	10.85	2	Astereae	5.14	2	Gnaphalieae	16.60
3	Eupatorieae	2.06	3	Senecioneae	2.50	3	Gnaphalieae	10.01	3	Cichorieae	3.97	3	Inuleae	10.11
4	Gnaphalieae	1.73	4	Gnaphalieae	2.24	4	Astereae	4.72	4	Vernonieae	2.19	4	Senecioneae	9.03
5	Astereae	1.67	5	Mutisieae	1.89	5	Senecioneae	2.87	5	Plucheeae	2.00	5	Astereae	2.30
6	Vernonieae	1.39	6	Arctotideae	1.48	6	Eupatorieae	0.45	6	Arctotideae	1.24	6	Arctotideae	1.95
7	Arctotideae	1.26	6	Calenduleae	1.48				6	Calenduleae	1.24	6	Calenduleae	1.95
7	Calenduleae	1.26	7	Eupatorieae	0.92				7	Cardueae	1.13	7	Mutisieae	0.99
8	Cardueae	0.66	8	Vernonieae	0.55				8	Mutisieae	0.57	8	Plucheeae	0.72
9	Mutisieae	0.06	9	Plucheeae	0.49							9	Vernonieae	0.45

Table A.3 - Residual analysis of Asteraceae tribes under-selected for medicinal use. Analysis compared specific use counts to total medicinal uses.

CAR			DER			GEN			GIM			GYN		
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Anthemideae	-4.92	1	Eupatorieae	-14.26	1	Astereae	-5.55	1	Gnaphalieae	-11.72	1	Anthemideae	-10.41
2	Gnaphalieae	-2.09	2	Gnaphalieae	-7.02	2	Plucheeae	-0.82	2	Senecioneae	-6.08	2	Gnaphalieae	-9.41
3	Plucheeae	-2.08	3	Anthemideae	-3.68	3	Vernonieae	-0.57	3	Anthemideae	-5.37	3	Inuleae	-4.61
4	Mutisieae	-1.85	4	Inuleae	-2.84	4	Mutisieae	-0.49	4	Cardueae	-2.80	4	Arctotideae	-3.74
5	Arctotideae	-1.75	5	Cichorieae	-1.80	5	Arctotideae	-0.35	5	Astereae	-1.16	4	Calenduleae	-3.74
5	Calenduleae	-1.75	6	Astereae	-1.13	5	Calenduleae	-0.35	6	Mutisieae	-0.33	5	Senecioneae	-3.07
6	Eupatorieae	-1.12	7	Plucheeae	-0.91	6	Gnaphalieae	-0.29				6	Mutisieae	-2.51
7	Senecioneae	-1.05				7	Senecioneae	-0.23				7	Plucheeae	-2.32
						8	Inuleae	-0.05				8	Vernonieae	-0.94
NEU			**ORT**			**OTH**			**OTO**			**PUL**		
Rank	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**	**Rank**	**Tribe**	**Residual**
1	Senecioneae	-6.74	1	Astereae	-15.20	1	Anthemideae	-14.27	1	Eupatorieae	-12.13	1	Heliantheae	-45.12
2	Cichorieae	-6.54	2	Heliantheae	-9.25	2	Cardueae	-9.22	2	Anthemideae	-9.68	2	Cichorieae	-17.92
3	Anthemideae	-3.86	3	Cichorieae	-6.88	3	Vernonieae	-5.19	3	Senecioneae	-4.73	3	Cardueae	-8.49
4	Inuleae	-0.74	4	Inuleae	-0.51	4	Plucheeae	-3.66	4	Inuleae	-2.12	4	Eupatorieae	-6.62
						5	Mutisieae	-3.11	5	Gnaphalieae	-0.03			
						6	Arctotideae	-2.16						
						6	Calenduleae	-2.16						
						7	Inuleae	-1.90						

Table A.4 - Binomial analysis of Asteraceae tribes over-selected for medicinal use. Analysis compared specific use counts to total medicinal uses.

CAR			DER			GEN			GIM			GYN		
Rank	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**
1	**Cardueae**	**0.007419**	1	**Mutisieae**	**0.016630**	1	**Cardueae**	**0.021350**	1	**Eupatorieae**	**0.019221**	1	**Eupatorieae**	**0.000132**
2	**Inuleae**	**0.007488**	2	Senecioneae	0.054974	2	Eupatorieae	0.315533	2	**Plucheeae**	**0.039619**	2	**Cichorieae**	**0.004896**
3	**Cichorieae**	**0.019784**	3	Heliantheae	0.090897	3	Heliantheae	0.430424	3	Heliantheae	0.239790	3	**Vernonieae**	**0.043769**
4	**Vernonieae**	**0.023551**	4	Cardueae	0.179638	4	Anthemideae	0.497794	4	Vernonieae	0.391286	4	Mutisieae	0.224260
5	Heliantheae	0.454235	5	Anthemideae	0.407492	5	Inuleae	0.537108	5	Inuleae	0.429964	5	Cardueae	0.262415
			6	Astereae	0.409657	6	Cichorieae	0.552389	6	Cichorieae	0.445658	6	Plucheeae	0.350712
												7	Astereae	0.445993
NEU			**ORT**			**OTH**			**OTO**			**PUL**		
Rank	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**
1	Heliantheae	0.214015	1	**Anthemideae**	**0.007050**	1	**Gnaphalieae**	**0.012840**	1	**Heliantheae**	**0.041005**	1	**Gnaphalieae**	**3.46E-05**
2	Plucheeae	0.341906	2	Cardueae	0.192596	2	**Cichorieae**	**0.048194**	2	Astereae	0.164994	2	**Anthemideae**	**0.000113**
3	Astereae	0.350275	3	Senecioneae	0.312102	3	Senecioneae	0.214001	3	Cichorieae	0.196284	3	**Inuleae**	**0.000350**
4	Eupatorieae	0.386873	4	Gnaphalieae	0.346570	4	Heliantheae	0.272093	4	Vernonieae	0.257701	4	**Senecioneae**	**0.016601**
5	Gnaphalieae	0.429773	5	Mutisieae	0.432993	5	Eupatorieae	0.469405	5	Plucheeae	0.353230	5	Astereae	0.256922
6	Anthemideae	0.496598	6	Eupatorieae	0.469969	6	Astereae	0.488857	6	Cardueae	0.467382	6		
7	Cardueae	0.521381												
8	Vernonieae	0.554236												

Bolded values: statistically significant *P*-Value < 0.05

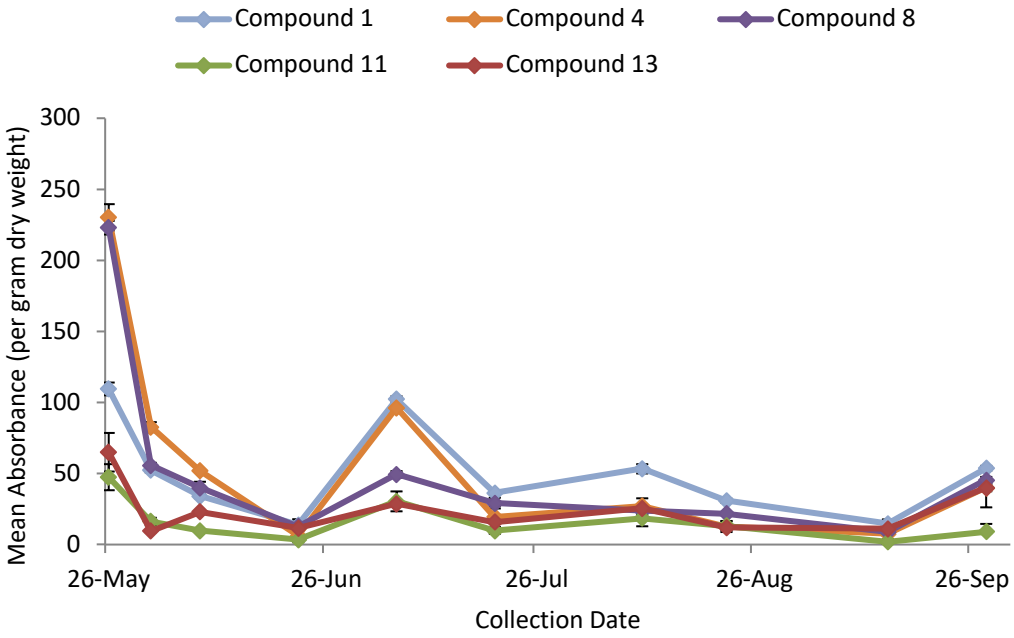
Table A.5 - Binomial analysis of Asteraceae tribes under-selected for medicinal use. Analysis compared specific use counts to total medicinal uses.

CAR			DER			GEN			GIM			GYN		
Rank	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**
1	**Anthemideae**	**0.006079**	1	**Eupatorieae**	**0.000706**	1	**Astereae**	**0.047642**	1	**Gnaphalieae**	**0.001225**	1	**Gnaphalieae**	**0.012522**
2	Astereae	0.312876	2	**Gnaphalieae**	**0.012499**	2	Plucheeae	0.598393	2	Senecioneae	0.074796	2	**Anthemideae**	**0.012768**
3	Gnaphalieae	0.367748	3	**Inuleae**	**0.021083**	3	Gnaphalieae	0.648197	3	Cardueae	0.314099	3	Heliantheae	0.232399
4	Eupatorieae	0.477457	4	Plucheeae	0.105199	4	Senecioneae	0.666705	4	Anthemideae	0.365541	4	Inuleae	0.331007
5	Plucheeae	0.593643	5	Cichorieae	0.382045	5	Vernonieae	0.786913	5	Astereae	0.486661	5	Senecioneae	0.459308
6	Senecioneae	0.658155	6	Vernonieae	0.478028	6	Mutisieae	0.857227	6	Mutisieae	0.566755			
7	Mutisieae	0.855180												
NEU			**ORT**			**OTH**			**OTO**			**PUL**		
Rank	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**	**Rank**	**Tribe**	***P*-Value**
1	**Senecioneae**	**0.022305**	1	**Cichorieae**	**0.026181**	1	**Vernonieae**	**0.028566**	1	**Eupatorieae**	**0.000106**	1	**Heliantheae**	**5.51E-07**
2	Cichorieae	0.107591	2	**Astereae**	**0.032205**	2	Cardueae	0.066918	2	**Senecioneae**	**0.027541**	2	**Cichorieae**	**2.65E-06**
3	Mutisieae	0.289346	3	Heliantheae	0.224495	3	Anthemideae	0.071201	3	**Inuleae**	**0.037739**	3	**Cardueae**	**0.006199**
4	Inuleae	0.296085	4	Inuleae	0.230219	4	Plucheeae	0.306215	4	Anthemideae	0.294864	4	Eupatorieae	0.066083
			5	Vernonieae	0.413707	5	Mutisieae	0.366351	5	Gnaphalieae	0.491043	5	Vernonieae	0.226917
			6	Plucheeae	0.445419	6	Inuleae	0.570542	6	Mutisieae	0.519229	6	Mutisieae	0.385402
												7	Plucheeae	0.407662

Bolded values: statistically significant *P*-Value < 0.05

APPENDIX B - SEASONAL PROFILING OF ALKYLAMIDES

A



B

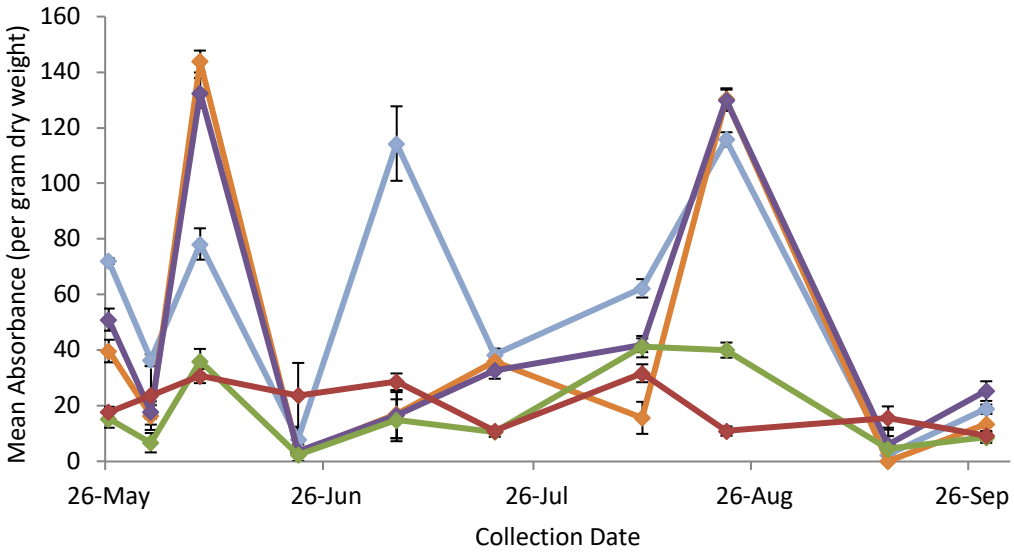


Figure B.1 – Seasonal profiles of the top 5 alkylamides in yarrow stem (A) and leaf (B).

Table B.1 – Mean absorbance of 15 alkylamides in yarrow roots across a season identified by HPLC-DAD. RT: Retention time.

Compound	RT	Mean Absorbance (per gram dry weight)											
		26-May	**01-Jun**	**08-Jun**	**22-Jun**	**06-Jul**	**20-Jul**	**10-Aug**	**22-Aug**	**14-Sep Old**	**14-Sep New**	**28-Sep Old**	**28-Sep New**
1	18.78	880.56	668.05	622.54	261.26	671.13	372.32	138.74	666.38	610.56	601.39	682.87	729.02
2	18.97	87.92	72.85	103.66	64.59	101.94	46.25	18.40	33.20	62.54	43.20	47.32	72.97
3	19.72	36.68	17.69	11.20	37.11	100.09	74.22	41.20	162.20	83.44	140.28	100.59	124.70
4	21.11	1260.37	1321.37	1050.79	490.38	1177.50	674.53	276.52	1303.17	968.84	949.27	1336.12	1234.70
5	22.32	72.13	41.02	30.38	52.99	103.15	51.80	33.99	145.77	107.81	119.29	86.03	143.78
6	22.65	59.47	63.90	83.04	98.10	56.99	56.49	25.17	54.98	106.57	51.13	86.99	108.61
7	22.88	66.53	75.78	55.30	56.27	57.91	46.30	26.06	92.60	62.89	41.26	94.50	67.10
8	23.37	1196.54	1194.64	827.05	741.68	824.80	693.32	361.43	1912.68	977.46	1002.36	2232.78	1091.36
9	23.73	172.93	122.20	140.17	74.00	120.60	80.66	42.25	146.59	185.95	195.23	136.64	217.07
10	24.19	136.67	125.32	109.05	67.84	98.35	89.06	84.89	83.67	104.76	175.39	113.65	99.78
11	24.75	272.64	200.47	215.98	170.93	202.34	207.07	332.34	475.92	487.67	316.51	445.28	530.44
12	25.02	154.29	170.47	147.90	71.95	147.74	111.49	88.07	199.14	185.27	71.72	279.20	192.55
13	26.23	272.55	152.29	183.69	65.53	158.39	96.24	120.39	206.83	210.29	244.60	176.11	264.23
14	28.24	120.52	119.15	45.26	48.92	174.77	48.21	82.69	83.46	85.23	102.99	97.39	109.78
15	29.78	30.28	18.55	38.57	34.23	29.67	24.30	16.04	46.49	36.08	15.88	66.24	47.74