SEASONAL AND TISSUE VARIATION OF FALCARINDIOL, AN ANTIMYCOBACTERIAL CONSTITUENT OF THE CANADIAN MEDICINAL PLANT *HERACLEUM MAXIMUM*

by

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ABSTRACT

First Nations peoples of the Canadian Maritime provinces use the roots of *Heracleum maximum* as a treatment for tuberculosis, traditionally collecting the roots in the fall. A recent study confirmed that *H. maximum* roots exhibit significant antimycobacterial activity *in vitro* and identified falcarindiol as the bioactive constituent. Our objective was to determine whether there is a correlation between the amount of falcarindiol and the bioactivity of *H. maximum* crude extracts. Extracts of four *H. maximum* tissues collected over three seasons for three consecutive years were tested for antimycobacterial activity and analyzed for their falcarindiol content. Although there was no difference in bioactivity between seasons, flower extracts exhibited the highest bioactivity whilst the summer root extracts contained the most falcarindiol. There was no relationship between antimycobacterial activity and falcarindiol content of the extracts suggesting the presence of other compounds in the extracts that may be responsible for the observed antimycobacterial activity.

DEDICATION

To my parents, Jim and Michele, for everything you do.

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everything you do for me. The last few years would not have been possible without your endless encouragement.

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LIST OF SYMBOLS, NOMENCLATURE, OR ABBREVIATIONS

^{13}C NMR	carbon nuclear magnetic resonance spectroscopy
1D	one dimensional
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
2D	two-dimensional
ABS	absolute value
ACS	American Chemical Society
bd	broad doublet
bs	broad singlet
bt	broad triplet
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
d	doublet
ddd	double double doublet
dt	double triplet
DMSO	dimethyl sulfoxide
EISMS	electro spray ionization mass spectrometry
FT-IR	Fourier transform infrared spectroscopy
g	gram
H	hydrogen
HPLC	high performance liquid chromatography
HRESIMS	high-resolution electron spray ionization mass spectroscopy
IR	infrared
J	coupling constant
KBr	potassium bromide
LC	liquid chromatography
LTQ	linear trap quadrupole
µ	mean
m	multiplet
MDR	multidrug-resistant
MDR-TB	multidrug-resistant tuberculosis
MHz	megahertz
MRA	microplate resazurin assay
NMR	nuclear magnetic resonance spectroscopy
NPRG	Natural Products Research Group
qNMR	quantitative nuclear magnetic resonance spectroscopy
s	singlet
SEM	standard error of the mean
t	triplet
TB	tuberculosis
td	triple doublet
TS	test statistic
UV	ultraviolet

XDR	extensively drug-resistant
XDR-TB	extensively drug-resistant tuberculosis

Chapter 1: General introduction

1.1 Natural products and ethnopharmacology

Natural products, also known as secondary metabolites, are compounds produced by living organisms for social, physiological or defensive purposes that aren't essential for the survival of the organism (Williams et al., 1989). Social and physiological roles of secondary metabolites might include pheromones, fragrances, flavours, and colours (Bennett and Wallsgrove, 1994; Meinwald and Eisner, 2008; Yang et al., 2012). Secondary metabolites may also be produced for protection against other species; for instance, compounds produced by a plant that are toxic to a competing species (Arnason et al., 1981; Cowan, 1999; Fraenkel, 1959; Meinwald, 2011; O'Brien and Wright, 2011; Yang et al., 2012). Since plants, fungi, and bacteria lack mobility and have no adaptive immune system (Jones and Dangl, 2006), they must employ alternate defenses against competitors, predators, and pathogens (Lattanzio et al., 2006). Plant secondary metabolism and the production of bioactive compounds can be affected by a variety of biotic as well as abiotic factors such as environmental conditions (Lattanzio et al., 2006). Cues for production of plant-produced secondary metabolites include temperature changes, leaf damage and phenological stages (Bennett and Wallsgrove, 1994; Cirak et al., 2013; Zangerl and Berenbaum, 1990). The accumulation of plant-produced secondary metabolites are often specific to the developmental stage of the plant as well as the tissue of the plant (Yang et al., 2012).

The diversity of plants and their ability to synthesize structurally different compounds (Bednarek and Osbourn, 2009; Bennett and Wallsgrove, 1994; Cowan, 1999; Yang et al., 2012) makes them a valuable source of natural products. Despite this, fewer than 10% of the approximately 250,000 known flowering plant species have been studied in depth for their chemical diversity (Meinwald and Eisner, 2003). Plant metabolic pathways are continuously evolving and are thus capable of developing new secondary metabolites (Pichersky and Gang, 2000). Therefore, plant extracts have been, and continue to be, a rich source of lead structures for drug discovery and development (Al-Fatimi et al., 2007; Eisner and Meinwald, 1995; Gertsch, 2009; Heinrich and Gibbons, 2001; Jones et al., 2000) and account for more than 50% of drug candidates on the market (Chin et al., 2006; Newman et al., 2003).

Plant extracts have been used for their medicinal properties for centuries (Rates, 2001; Tringali, 2012; Uprety et al., 2012; Wondimu et al., 2007) and more than fifty thousand plant-derived compounds have been isolated (De Luca and St Pierre, 2000). Due to the adverse side effects, the overuse or misuse of drugs, and the limited availability of pharmaceuticals in many parts of the world, the use of plant extracts for therapeutic purposes has become increasingly popular world-wide (Gu et al., 2014; Rates, 2001)

Ethnopharmacology is the exploration of indigenous plants and investigation of their biological activity as it relates to the plants' use by respective ethnic groups (Etkin, 1988; Fabricant and Farnsworth, 2001; Nanyingi et al., 2008). It is estimated that between 65% and 80% of the world's population use traditional medicines as components of their primary health care (Fabricant and Farnsworth, 2001; Mandal et al., 2012; WHO, 2005). Additionally, more than 60% of approved and preclinical drug candidate applications are natural products or derivatives of natural products (Cragg et al., 1997). In the United States of America, plants and their bioactive compounds comprise approximately 25% of the total prescriptions issued every year (Cox and Balick, 1994; Rates, 2001; Wondimu et al., 2007). In Canada, close to 400 plants have been used as traditional medicines for centuries and many have been studied in depth for their chemical diversity and bioactive properties (Arnason et al., 1981; Jones et al., 2000; Uprety et al., 2012). One of these plants, the cow parsnip *Heracleum maximum* (Moerman, 1998), has gained attention because of its traditional use to treat tuberculosis and has recently been shown to produce antimycobacterial natural products (O'Neill, 2012).

1.2 *Heracleum maximum*

Heracleum maximum Bartr.[synonyms: *Heracleum lanatum* Michx., *Heracleum sphondylium* L. var. *lanatum* (Michx.) Dorn and *Heracleum sphondylium* L. ssp. *montanum* (Schleich. ex Gaudin) Briq.], commonly known as cow parsnip, is an abundant perennial plant native to North America that belongs to the Apiaceae (or Umbelliferae) family that can be found in all Canadian provinces and territories with the exception of Nunavut (Walker, 2008). It has been noted to be the most intensively used green springtime vegetable among indigenous peoples in Canada (Kuhnlein and Turner, 1991). *Heracleum maximum* can grow up to two metres tall with large stems and leaves and has small white flowers that appear in the early summer and form umbrella shaped clusters approximately 20 cm across (Walker, 2008). It has been noted by Walker (2008) from information passed down from First Nations peoples that stalks and stems from *H. maximum* should be harvested in the spring, whereas the roots may be harvested in the spring and the fall.

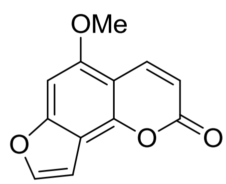
Heracleum maximum was first reported as a Canadian medicinal plant in the Journal of Canadian Pharmacy in 1868, however, its medicinal properties were acknowledged hundreds of years prior to this classification (Erichsen-Brown, 1979). *Heracleum maximum* has been employed by Canadian First Nations to treat many illnesses (Table 1.2.1) including colds, tuberculosis and skin ailments (Moerman, 1998; Webster et al., 2010).

Table 1.2.1 Traditional medicinal uses of *Heracleum maximum* by North American First Nations (Moerman 1998).

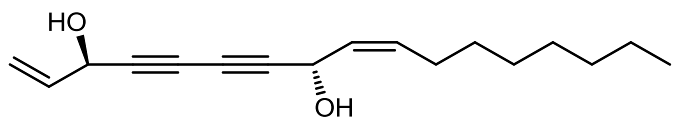
Organ	Use	First Nation
Flower	Dermatological aid	Ojibwa
Stem	Antidiarrheal	Blackfoot
	Dermatological aid	Blackfoot, Meskwaki
Leaf	Cold remedy	Aleut
	Dermatological aid	Aleut
	Orthopedic aid	Aleut, Quinault
	Throat aid	Aleut
	Eye medicine	Makah
	Analgesic	Quinault
Root	Analgesic	Nuxálk, Cree, Meskwaki, Omaha, Sanpoil, Sekani, Woodland Cree
	Antirheumatic	Nuxálk, Woodland Cree, Gitxsan, Paiute, Pomo, Sekani
	Dermatological aid	Nuxálk, Blackfoot, Cree, Woodland Cree, Gitxsan, Haisla, Kwakiutl, Ojibwa, Okanagan, Paiute, Pawnee, Pomo, Salish, Sanpoil, Secwepemc, Sekani
	Orthopedic aid	Nuxálk, Okanagan
	Pulmonary aid	Nuxálk, Paiute, Ojibewa
	Venereal aid	Cree, Nlaka’pamux
	Gastrointestinal aid	Iroquois, Meskwaki, Omaha, Okanahan, Nlaka’pamux
	Panacea, tonic	Nlaka’pamux, Okanagon, Secwepemc
	Anti-inflammatory	Blackfoot, Sekani
	Antiviral	Maliseet, Mi’kmaq
	Miscellaneous	Blackfoot, Cree, Shoshoni, Washo, Kwakiutl, Okanagon, Omaha, Nlaka’pamux, Sanpoil Secwepemc, Gitxsan, Maliseet, Meskwaki

The First Nations of the Maritime Provinces traditionally prepare *H. maximum* for medicinal applications by steeping roots collected in the fall to make a tea or infusion (Moerman, 1998; Uprety et al., 2012; Walker, 2008; Webster et al., 2010). Indeed, crude (aqueous or methanolic) extracts of the root have been shown to exhibit immunostimulant (Webster et al., 2006), antifungal (McCutcheon et al., 1994), antibacterial (McCutcheon et al., 1992), and antimycobacterial (McCutcheon et al., 1997; Webster et al., 2010) activities. McCutcheon *et al*. (1992) found that *H. maximum* methanolic root extracts displayed antibiotic activity against six species of bacteria as well as broad-scale anti-fungal activity against nine fungal species (McCutcheon et al., 1995) and completely inhibited the growth of both *Mycobacterium tuberculosis* and *M. avium* (McCutcheon et al., 1997). Despite these findings, few studies have focused on the bioactive natural products of *H. maximum*.

Plants of the Apiaceae family are well known to contain cytotoxic furanocoumarins (Figure 1.2.1), as class of compounds commonly produced by plants, that were initially assumed to be responsible for the antimycobacterial activity observed in *H. maximum* extracts (McCutcheon et al., 1997). However, the linear polyacetylene, falcarindiol was recently isolated from root extracts of *H. maximum* by the Natural Products Research Group (NPRG) and identified as the primary antimycobacterial constituent of *H. maximum* roots collected in the fall (O'Neill, 2012). Polyacetylenes are also commonly isolated from the Apiaceae family and have a broad spectrum of bioactivity (Christensen, 2010). These bioactivities, including anti-inflammatory, antifungal, and antibacterial activity, occur at levels in the tea that are effective and nontoxic to humans (Christensen, 2010; Christensen and Brandt, 2006; Christensen and Jakobsen, 2008). The triple bonds that are fundamental to the structure of the polyacetylenes may explain the array of bioactivities as they can readily react with proteins and other biomolecules (Christensen, 2011). In fact, it has been proposed that falcarindiol and these falcarindiol-type polyacetylenes have the potential to be integrated into the development of food products and pharmaceuticals because of their beneficial properties (Christensen, 2011).



Isobergapten (**1**)



Falcarindiol (**2**)

Figure 1.2.1 An example of an angular furanocoumarin (**1**) and a polyacetylene (**2**), natural products that are common in the Apiaceae family.

1.3 Objective

O’Neill et al. (2012) determined that falcarindiol was the primary antimycobacterial constituent of *H. maximum*. The objective of the research described in this thesis was to determine whether there is a correlation between the amount of falcarindiol and the observed bioactivity in different tissue types of *H. maximum*, and also to evaluate whether the falcarindiol content and observed bioactivity of *H. maximum* varies seasonally.

Chapter 2 discusses the antimycobacterial activities of *H. maximum* crude extracts to determine if the observed bioactivity varies with season and plant organ and Chapter 3 evaluates the amount of falcarindiol in *H. maximum* crude extracts to determine whether the amount of falcarindiol is correlated with bioactivity.

Chapter 2: Antimycobacterial activity of *Heracleum maximum* crude extracts

2.1 Introduction

Recently, it was estimated by the World Health Organization (WHO) that 9 million people developed tuberculosis (TB) and 1.5 million died from this disease in 2013 (WHO, 2014). Controlling the global TB pandemic has become an immense challenge because of the development of drug resistance by *Mycobacterium tuberculosis*, co-infection with other diseases, and exacerbation by environmental factors such as pollution and alcoholism (Lawn and Zumla, 2011). However, the emergence of drug resistant strains of the pathogen is by far the greatest of these problems (Dover and Coxon, 2011). There are currently five first-line drugs used for the treatment of TB: isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin (WHO, 2010). Misuse of the first-line drugs compounded by poor therapeutic compliance by patients has resulted in the development of TB strains that are both multidrug-resistant (MDR) and extensively drug-resistant (XDR) (CDC, 2012). MDR-TB does not respond to the two most common anti-TB drugs, isoniazid and rifampicin. It was estimated that 480 000 people had developed MDR-TB worldwide with approximately 9% of those cases having been XDR-TB (WHO, 2014). Furthermore, XDR-TB is not only resistant to the first-line drugs but also second-line drugs that are available, specifically any fluoroquinolone and at least one of the injectable aminoglycosides capreomycin, kanamycin, and amikacin (Lawn and Zumla, 2011).

Despite the urgent need for new treatment options to combat both drug-resistant and drug-sensitive TB, the development of new TB therapies has been slow. Current first-line drugs used for TB treatment are over 40 years old and it is only within the past seven years that there has been development of possible new anti-TB drugs (Zumla et al., 2013). Additionally, only a few of the drug-leads that are discovered will make it through the drug development pipeline and be successful in the subsequent clinical trials to eventually be approved as TB drugs (Dover and Coxon, 2011). The importance of discovering new TB therapeutics has never been higher, especially as TB is now found in all areas in the world (WHO, 2014).

Historically, natural products research has provided the foundation for drug discovery and development (Dias et al., 2012) since natural products are a promising source of small molecules with bioactivity including antimycobacterial activity (Copp, 2003; Copp and Pearce, 2007; Garcia et al., 2012). Indeed, natural product-derived drugs are key components of current TB treatment regimens and natural products will continue to provide TB drug leads in the future (Dashti et al., 2014). Medicinal plants have long been a mainstay of natural products-based drug discovery programmes (Balunas and Kinghorn, 2005; Farnsworth et al., 1985) and ethnobotanical and ethnopharmacological information is often used to focus discovery efforts (Harvey et al., 2010; Ngo et al., 2013). It is assumed that plants that have been used to treat specific ailments produce natural products with therapeutically relevant biological activities (Fabricant and Farnsworth, 2001). Plants that have traditionally been used to treat TB should therefore represent a valuable source of antimycobacterial natural products.

The aboriginal peoples of North America have used plants for millennia resulting in many ethnobotanical applications that include therapeutics (Moerman, 1998, 2009). In Canada, the First Nations peoples use plants extensively as medicines to treat injuries and illnesses resulting from both infectious and non-infectious diseases (Gray et al., 2013). *Heracleum maximum*, for example, is a Canadian medicinal plant that is used by various First Nations peoples to treat TB (Moerman, 2009). Traditionally, the roots of *H. maximum* are collected in the fall, dried, and used to make aqueous infusions that are ingested as a tea (Webster et al., 2010). Indeed, Webster et al (2010) found that aqueous extracts of *H. maximum* roots exhibited significant antimycobacterial activity and the recent report of the isolation of the antimycobacterial C17 diyne falcarindiol from the roots of this plant (O'Neill, 2012) prompted further investigation into the traditional use of *H. maximum* as a TB therapy.

Cognisant that the antitubercular application of *H. maximum* involved a single plant organ harvested in a specific season, we were particularly interested in evaluating whether extracts from other parts of the plant exhibited similar or higher antimycobacterial activity than the roots and whether the activity of the root extracts differed among seasons. The objective of this chapter, therefore, was to determine whether the antimycobacterial activity of *H. maximum* extracts varied among tissues and/or seasons.

2.2 Materials and Methods

2.2.1 *Heracleum maximum* collection and extraction

Individual *H. maximum* plants (10 plants per season and tissue) were collected from the Kingston Peninsula, New Brunswick, Canada (within 0.5 km^2 of 45° 30 N; 65° 53 W) in spring, summer, and fall over a three-year period (2009 – 2011). Root tissues could not be collected in the winter as the ground was frozen. All plants were collected at least 10 m from each other in an attempt to ensure they were not clonal. Plants collected in the summer were separated into four tissue types (root, stem, leaf, and flower) after collection; only the roots were used for the spring and fall collections. Plant material was separately freeze-dried, ground into a powder using a household blender and approximately 5 g of each sample was extracted with methanol (200 mL) for 8 hours using a Soxhlet apparatus. The resulting extracts were concentrated *in vacuo* to give 178 distinct crude extracts (two plants did not have enough flower material) that were tested for antimycobacterial activity as described in section 2.2.2 and analyzed for their falcarindiol content as described in chapter 3.

2.2.2 Microplate resazurin assay

All crude extracts were tested against *M. tuberculosis* H37Ra using the microplate resazurin assay (MRA) as described by O'Neill et al. (2014). Samples were prepared in concentrations of 50 µg/mL and 100 µg/mL in 2% aqueous dimethyl sulfoxide (DMSO) and were tested in triplicate. Inhibition of mycobacterial growth was evaluated fluorometrically and is reported as percent inhibition of growth relative to untreated

M. tuberculosis grown in the respective vehicle control (O'Neill et al., 2014). The antimycobacterial data used for analyses in this thesis was that tested at 100 µg/mL as this is the standard used for this assay based on the results obtained with the optimization of the microplate resazurin assay (O'Neill et al., 2014).

2.3 Statistical Analyses

2.3.1 Randomization

A randomization-based method was employed to analyze bioactivity because the data did not meet the assumptions of traditional parametric tests due to non- normality and heterogeneity of variances (Shapiro-Wilk and Bartlett's tests respectively), and equivalent non-parametric tests (e.g., Freidman's test) could not be used as they cannot analyze data with different sample sizes. Randomization was performed by using the formulae given in section 2.3.2 in Microsoft Excel to reveal differences in root extract bioactivity between seasons and differences in bioactivity between tissue type extracts.

2.3.2 Calculations for seasonal and tissue type differences in bioactivity

To test for a difference among seasons the following test statistic was used:

$$TS_T = \sum_{i=1}^{3} ABS(\mu_T - \mu_i)$$

where TS_T = test statistic, ABS = absolute value, μ_T=the mean percent inhibition for all samples, and μ_i= the mean percent inhibition for season i. The test statistics were calculated for the observed data and these were compared to test statistics calculated for 1000 randomized datasets (i.e., where observed inhibitory activity are assigned randomly to each of the treatment groups). To test for pairwise differences in bioactivity between seasons the following test statistics were used:

$$TS_{spring:summer} = ABS(\mu_{spring-}\mu_{summer})$$

$$TS_{spring:fall} = ABS(\mu_{spring-}\mu_{fall})$$

$$TS_{summer:fall} = ABS(\mu_{summer-}\mu_{fall})$$

A similar randomization approach was used to test for differences among tissues:

$$TS_T = \sum_{j=1}^{4} ABS(\mu_T - \mu_j)$$

where TS_T = test statistic, ABS = absolute value, μ_T=the mean percentage inhibition for all samples, and μ_i= the mean percentage inhibition for tissue j. To test for pairwise difference between tissue types in percentage inhibition we used the following test statistics:

$$TS_{root:stem} = ABS(\mu_{root-}\mu_{stem})$$

$$TS_{root:leaf} = ABS(\mu_{root-}\mu_{leaf})$$

$$TS_{root:flower} = ABS(\mu_{root-}\mu_{flower})$$

$$TS_{stem:leaf} = ABS(\mu_{stem-}\mu_{leaf})$$

$$TS_{stem:flower} = ABS(\mu_{stem-}\mu_{flower})$$

$$TS_{leaf:flower} = ABS(\mu_{leaf-}\mu_{flower})$$

P-values were then calculated by comparing the observed value to the distribution of 1000 randomized values. Data were considered significantly different at an α of 0.05.

2.4 Results

2.4.1 Seasonal differences in the bioactivity of crude extracts from *H. maximum* roots

Across seasons, the inhibitory activity of *H. maximum* root extracts ranged from −2 to 100%; although little variation was observed, the highest mean activity was exhibited by extracts derived from roots collected in the fall. When tested for antimycobacterial activity at 100 μg/mL, no statistical difference in the antimycobacterial activity among the root extracts from different seasons (P=0.866) was found (Figure 2.4.1).

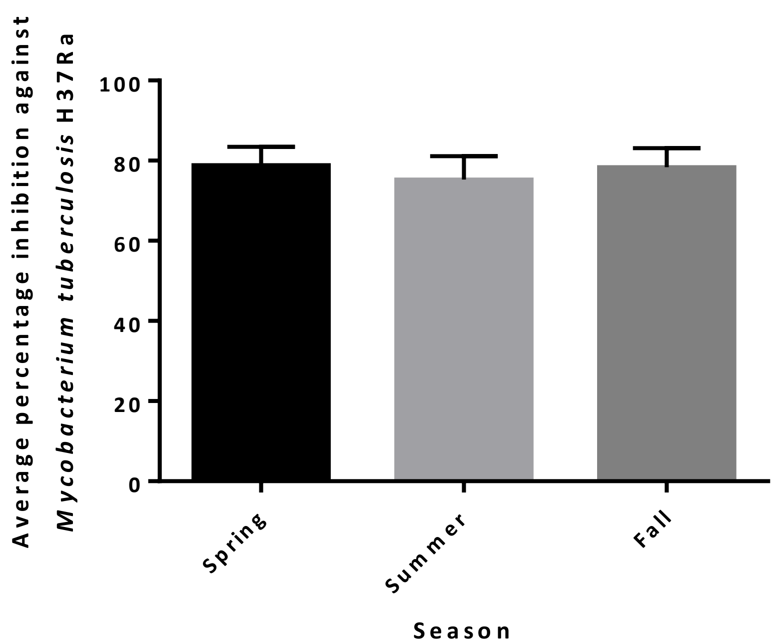


Figure 2.4.1 Percentage inhibition of *Heracleum maximum* root extracts from different seasons (spring, summer and fall), collected over three years. Extracts were tested against *Mycobacterium tuberculosis* H37Ra at 100 μg/mL (error bars indicate standard error; no significant differences were observed between groups).

2.4.2 Differences in bioactivity of *H. maximum* tissue extracts collected in the summer

The inhibitory activity of summer extracts from different tissues ranged from −14 to 100%. Overall, there were differences seen in the antimycobacterial activity of the *H. maximum* tissue extracts collected in the summer (P<0.001). Flower extracts inhibited the growth of *M. tuberculosis* the most, and this activity was statistically different from that of the roots, stems and leaves, whereas extracts from the stems exhibited the lowest inhibitory activity [post-hoc pairwise comparison (P=0.002, P<0.000, P=0.001 respectively) (Figure 2.4.2)].

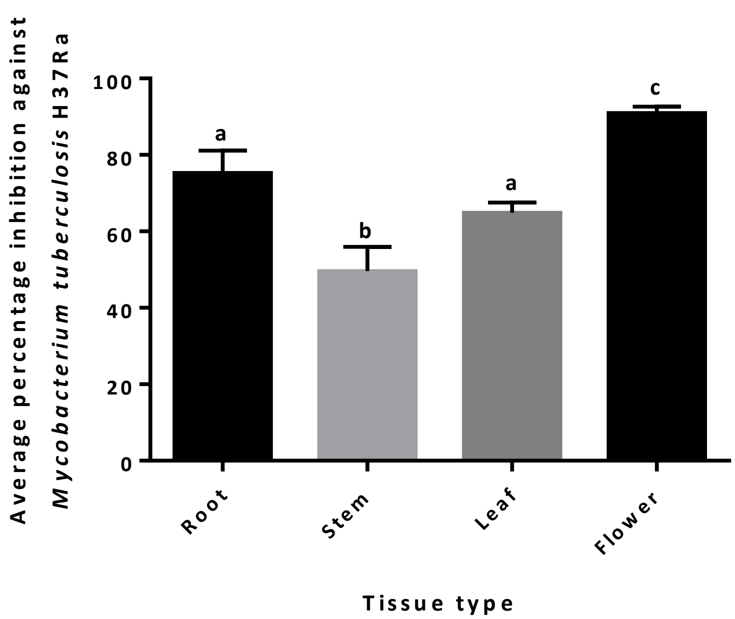


Figure 2.4.2 Percentage inhibition of *Heracleum maximum* summer extracts in different tissues (root, stem, leaf, and flower) over three years. Extracts were tested against *Mycobacterium tuberculosis* H37Ra at 100 µg/mL (error bars indicate SEM; homogenous groups are denoted by lowercase letters).

2.5 Discussion and conclusion

Crude extracts from *H. maximum* inhibited the growth of *M. tuberculosis* by varying amounts depending on the tissue and season from which the extracts were derived. From the results found in this study, it can then be concluded that the season in which roots of *H. maximum* are collected does not make a difference to the antimycobacterial activity of extracts prepared from the tissue. Traditionally, in the Maritime Provinces *H. maximum* roots are collected by First Nations in the fall, which is consistent with what was found in our study as the fall root extracts did have high bioactivity. Regarding differences in tissue type, it is interesting to note that the flower extracts had the greatest inhibitory effect on the growth of mycobacteria, which may be expected as plants are known to produce more defense chemicals in their flowers to protect the tissues that are their means of reproduction (Prior et al., 2007).

A study by Pira et al. (1989) analyzed furanocoumarins in the steams, leaves, and fruit from *Heracleum mantegazzianum*, a closely related species to *H. maximum*. It was found that the furanocoumarin content was highest in the fruit and lowest in the stem. These results are in accordance with our observations of antimycobacterial activity in which the highest activity was observed in extracts from the flowers, followed by the roots, leaves, and the lowest bioactivity in the extracts from the stems. Although flowers show the highest inhibition against *M. tuberculosis*, First Nations may not have collected flowers because of the short flowering period and therefore availability of flowers. The roots, which were traditionally collected by First Nations, are available throughout the year and had the second highest bioactivity and was significantly higher than the observed bioactivity of the stem and leaf extracts.

To date, there are no known studies on seasonal or tissue type variation in bioactivity with regards to *H. maximum*. However, there are a few recent studies on other plants that have looked at variation in bioactivity based on different seasons as well as different plant tissues (Brenes-Arguedas et al., 2008; El-Readi et al., 2013; Kellogg et al., 2010; Manilal et al., 2009; Mediani et al., 2012; Ncube et al., 2015; Pacifico et al., 2015; Scognamiglio et al., 2014; Vuorela et al., 2005). For example, Pacifico et al. (2015) found that the bioactivity of *Calamintha nepta* (L.), a medicinal herb, varied with season tested where the highest antioxidant bioactivity was found in extracts from the summer and the highest anti-inflammatory bioactivity was found in extracts from the winter. Together, these studies suggest that the bioactivity of plant extracts from different tissues and different seasons can be dependent on environmental stresses as well as growth stage of the plant in question (Ramakrishna and Ravishankar, 2011). Environmental variables such as precipitation levels, temperature, and sunlight availability have been suggested to have an impact on the quality and quantity of bioactive compounds from plants such as different species of Alaskan berries (Kellogg et al., 2010). However, prolonged studies over several years would help solidify this evidence (Kellogg et al., 2010; Pacifico et al., 2015). There can also be variation in bioactivity due to the compounds and types of plants under investigation and the sought bioactivity.

Randomization offers an alternative approach to classical parametric statistical methods when analyzing complex data with significant sources of variation (MacKenzie and Manly, 2001). One of the major premises behind randomization is that it is a valuable tool for analysis of variance in situations where data are non-normal (Gonzalez and Manly, 1998). As the antimycobacterial data obtained for the *H. maximum* extracts were not suitable for analysis by multivariate repeated measures ANOVA and in the absence of a satisfactory non-parametric alternative, the data were analyzed using a randomization approach to determine if there were significant differences among the three seasons and four tissues. The randomization approach was adapted from Manly (1997). For data sets that were applicable, parametric and non-parametric tests were run to assess the accuracy of the randomization method and the results from these tests were in accordance with the results obtained from the randomization approach.

The next step in this research would be to determine whether the antimycobacterial activity seen in these crude extracts has similar variation patterns to antimycobacterial compounds from *H. maximum*. This will help determine if the variation in activity is caused by a sole compound. The first compound to be assessed for its variation and correlation with antimycobacterial activity is falcarindiol (Chapter 3) as it showed the highest antimycobacterial activity when tested against *M. tuberculosis* in the MRA (O'Neill, 2012).

Chapter 3: Falcarindiol quantification and correlation with antimycobacterial activity

3.1 Introduction

Members of the Apiaceae family, such as *Heracleum maximum* are known to contain furanocoumarins and polyacetylenes (Crowden et al., 1969) that have been shown to exhibit various bioactivities including antimycobacterial activity (McCutcheon et al., 1997; O'Neill, 2012; Webster et al., 2010). The fact that First Nations traditionally collected *H. maximum* roots in the fall led us to wonder what the chemical connection was between the presence of a specific compound and the observed bioactivity. It was these furanocoumarins and polyacetylenes that were thought to exhibit the anti-TB activity that were observed for the crude extracts of *H. maximum* when they were subjected to the MRA assay (Chapter 2). More specifically, the polyacetylene, falcarindiol, which was isolated from the roots of *H. maximum,* had the highest anti-TB activity (O'Neill, 2012) and led to this research.

There have been many studies performed on the bioactivity of falcarindiol isolated from different sources, and these are summarized in Table 3.1.1. However, only three studies have been performed on falcarindiol specifically isolated from *H. maximum* (Degen et al., 1999; O'Neill, 2012; Webster et al., 2010) and there are no reports of the quantification of falcarindiol from *H. maximum*. Thus, this traditionally used Canadian medicinal plant has not been studied in depth with regards to this important natural product. Our research is the first to link falcarindiol from *H. maximum* to the plants' antimycobacterial activity following an ethnopharmacological approach and the traditional use of the plant.

Table 3.1.1 Bioactivity of falcarindiol isolated from various plant sources.

Bioactivity	Activity against	Plant species	Author
Antibacterial	*Micrococcus luteus, Bacillus cereus*	*Crithmum maritimum* L.	Meot-Duras et al. (2010)
Anticancer	leukemia, lymphoma, myeloma	*Anethum graveolens*	Zidorn et al. (2005)
Antifungal	*Mycocentrospora acerina*	*Daucus carota* L.	Garrod et al. (1979)
	Alternaria brassicicola, Septoria nodorum	*Aegopodium podagraria*	Kemp (1978)
	Microsporum gypseum, Microsporum nanum, Trichophyton mentagrophytes, Tricohphyton rubrum, Epidermophyton floccosum	*Schefflera digitata*	Muir (1982)
Anti-inflammatory	Cyclooxygenase, 5-lipoxygenase	*Angelica pubescens* Maxim *f. biserrata*	Liu et al. (1998)
Antimutagenic	Furylfuramide, Trp-P-1	*Peucedanum praeruptorum*	Miyazawa et al. (1996)

Table 3.1.1 continued.

Bioactvity	Activity against	Plant species	Author
Antimycobacterial	*Mycobacterium tuberculosis*	*Opolopanax horridus*	Kobaisy et al. (1997)
		Angelica sinensis	Deng et al. (2008)
	Mycobacterium avium	*Opolopanax horridus*	Kobaisy et al. (1997)
		Anethum graveolens	Stavri & Gibbons (2005)
	Mycobacterium fortuitum	*Anethum graveolens*	Stavri & Gibbons (2005)
	Mycobacterium smegmatis	*Anethum graveolens*	Stavri & Gibbons (2005)
	Mycobacterium abscessus	*Anethum graveolens*	Stavri & Gibbons (2005)
Antitumor	colon cancer cells	*Opolopanax horridus* Miq.	Jin et al. (2012)
Apoptosis	colon cancer cells	*Opolopanax horridus* Miq.	Jin et al. (2012)
Cytotoxic	human myeloma, lympocytic leukemia	*Crithmum maritimum* L.	Meot-Duras et al. (2010)

There are several different techniques that can be employed for quantification of natural products in biological extracts such as electrospray ionization mass spectrometry (ESIMS) coupled with liquid chromatography, high performance liquid chromatography (HPLC) with diode-array UV spectroscopy, and quantitative nuclear magnetic resonance (qNMR), to name a few (Liang et al., 2009). Electrospray ionization mass spectrometry has been a successful method for characterizing polyacetylenes including falcarindiol (Rai et al., 2011). Falcarindiol has also been quantified from various different plant sources using HPLC (Ishizuka et al., 1998; Prior et al., 2007; Wittstock et al., 1995; Zschocke et al., 1997) and, additionally, some of these investigations analyzed falcarindiol from different plant tissues and seasons.

High performance liquid chromatography (HPLC) is a technique that separates compounds in a mixture based on absorption, partition and ion exchange. Coupled with diode array detection, HPLC can be used to quantify the amount of a specific molecule in a mixture. Quantitative HPLC can be performed by injecting a standard of the pure compound in question and determining the retention time of this compound. This can then be coupled with a series of known concentrations of the pure compound to create a standard curve (Skoog et al., 2007). This is generally achieved by finding the area under a UV absorbance curve and creating a linear regression from which the regression equation can be used to correlate how much of the analyte is present in each sample.

The amount of falcarindiol has been assessed in various other plant sources (Aguilo-Aguayo et al., 2014; Christensen and Kreutzmann, 2007; Czepa and Hofmann, 2003; Huang et al., 2011; Huang et al., 2010; Ishizuka et al., 1998; Kjellenberg et al., 2010; Kolodziej et al., 2013; Kramer et al., 2011; Prior et al., 2007; Schulz-Witte et al., 2010; Soltoft et al., 2010; Wittstock et al., 1995; Zidorn et al., 2005; Zschocke et al., 1997), however, we believe this is the first instance where the amount of falcarindiol present in *H. maximum* extracts has been determined.

Quantitative nuclear magnetic resonance (qNMR) is an analytical technique used to determine the concentration of a compound through the use of NMR (Bernstein et al., 2013). This can be performed using a standard curve of the known compound under analysis or through the use of an internal standard. With an internal standard, a known amount of a chemical is added to each extract being analyzed. The ratio of integrations of the analyte can then be related to those of the standard (Liang et al., 2009), allowing the amount of the analyte present in the extract to be calculated.

There were three overall objectives for this chapter. The first objective was to isolate falcarindiol from *H. maximum* using various fractionation techniques in order to use falcarindiol as a standard to analyse its concentration in crude methanolic extracts from different seasons and plant organs. The second objective of this chapter was to quantify the amount of falcarindiol present in *H. maximum* methanolic crude extracts. The final objective of this chapter was to determine whether there is a correlation between the amount of falcarindiol present in the crude extracts and the level of bioactivity against *Mycobacterium tuberculosis* H37Ra.

3.2 Material and methods

3.2.1 General experimental procedures

All solvents used were ACS or high performance liquid chromatography grade (Sigma Aldrich®). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity 400 instrument at 100 MHz for ^{13}C and 400 MHz for ^{1}H. Chemical shifts are reported in ppm and referenced to residual protonated solvent using MestReNova software (Mestrelab Research®, 2010). Optical rotations were determined using an AA-10 polarimeter (Optical Activity Ltd., Terochem Scientific®) and measured at 589 nm. Flash chromatography was performed on a Biotage® Flash+™ system using KP-Sil 25+S silica cartridges (40-63 µm, 60Å; Biotage®). Semi-preparative high performance liquid chromatography (HPLC) was performed using a Waters® 510 isocratic pump and R401 refractive index detector combined with a silica column (Phenomenex® Luna®; 10 µm, 100 Å, 250 × 10mm) at a flow rate of 4 mL/min. Infrared spectra were recorded on a Nicolet iS10 Fourier transform infrared (FT-IR) spectrometer on NaCl discs. High-resolution electron spray ionization mass spectrometric (HRESIMS) data were recorded on a linear trap quadrupole (LTQ) Exactive Orbitrap liquid chromatography-mass spectrometer (Thermo Scientific®). Electrospray ionization mass spectrometric (EISMS) data were recorded using Agilent 1100 LC system coupled to an AB Sciex® API 2000 triple quadrupole mass spectrometer. Statistical analyses were performed using randomization as described in section 2.3. Linear regression analyses were performed using SPSS (IBM® SPSS® Statistics 21.0, 2012). Bioactivity of *H. maximum* crude extracts was determined as per Chapter 2.

3.2.2 *Heracleum maximum* collection and extraction for the isolation of falcarindiol

Heracleum maximum roots (526 g) were collected from the Kingston Peninsula, New Brunswick, Canada (N 45° 30.778 W 65° 53.949) in November of 2011, washed with deionized water and stored at -40°C. The roots were freeze-dried (-28°C, dry weight: 114 g) and ground using a domestic blender. The ground roots (114 g) were exhaustively extracted with methanol (600 mL) using a Soxhlet apparatus (80°C, 8 hours) to give, after being concentrated *in vacuo*, a crude methanolic extract (15 g).

3.2.3 Fractionation, isolation and identification

The crude extract (15.0 g) was separated by polarity using a modified Kupchan liquid-liquid partition (Kupchan et al., 1973) as follows: the extract was initially dissolved in 9:1 methanol/water (1000 mL), extracted with hexanes (3×300 mL), diluted with water (500 mL) and extracted with dichloromethane (3×300 mL). The aqueous fraction was concentrated using a TurboVap® 500 (Zymark®), dissolved in water (400 mL), and extracted sequentially with ethyl acetate (3×200 mL) and *n*-butanol (3×200mL). All fractions were concentrated *in vacuo* to give the following five fractions: hexanes (960 mg), dichloromethane (2.5 g), ethyl acetate (191 mg), *n*-butanol (953 mg), and aqueous (7.8 g) (Figure 3.2.1).

Flash chromatography columns were run for the hexanes and dichloromethane fractions. The hexanes fraction (945 mg) was adsorbed onto silica and eluted through a Biotage + Flash column using a stepwise gradient of 100% hexanes to 100% ethyl acetate with 10% increments (150 mL per elution) with a final elution of 1:1

hexanes/methanol. Each fraction was collected in 250 mL round bottom flasks, transferred to vials and evaporated. The dichloromethane fraction was treated in the same manner. Each fraction was collected in 500 mL round bottom flasks, transferred to vials and concentrated *in vacuo*.

The ethyl acetate fraction was subjected to flash column chromatography on silica (5.04 g). The extract (158 mg) was adsorbed onto silica from a mixture of hexanes, dichloromethane, and ethyl acetate, and the silica was added to the column. A stepwise gradient of 100% hexanes to 100% ethyl acetate was used with 10% increments (50 mL per elution). Each fraction was collected in 100mL round bottom flasks, transferred to vials and concentrated *in vacuo.*

Fraction 2 (66 mg) from the flash column of the dichloromethane fraction was further purified using HPLC in 4:1 hexanes/ethyl acetate and the major fraction obtained was determined to be falcarinol (**1**: 29 mg). Fraction 3 (39 mg) from the flash column of the ethyl acetate fraction was further purified using HPLC in 4:1 hexanes/ethyl acetate to give falcarindiol (**2**: 8 mg) (Figure 3.2.2).

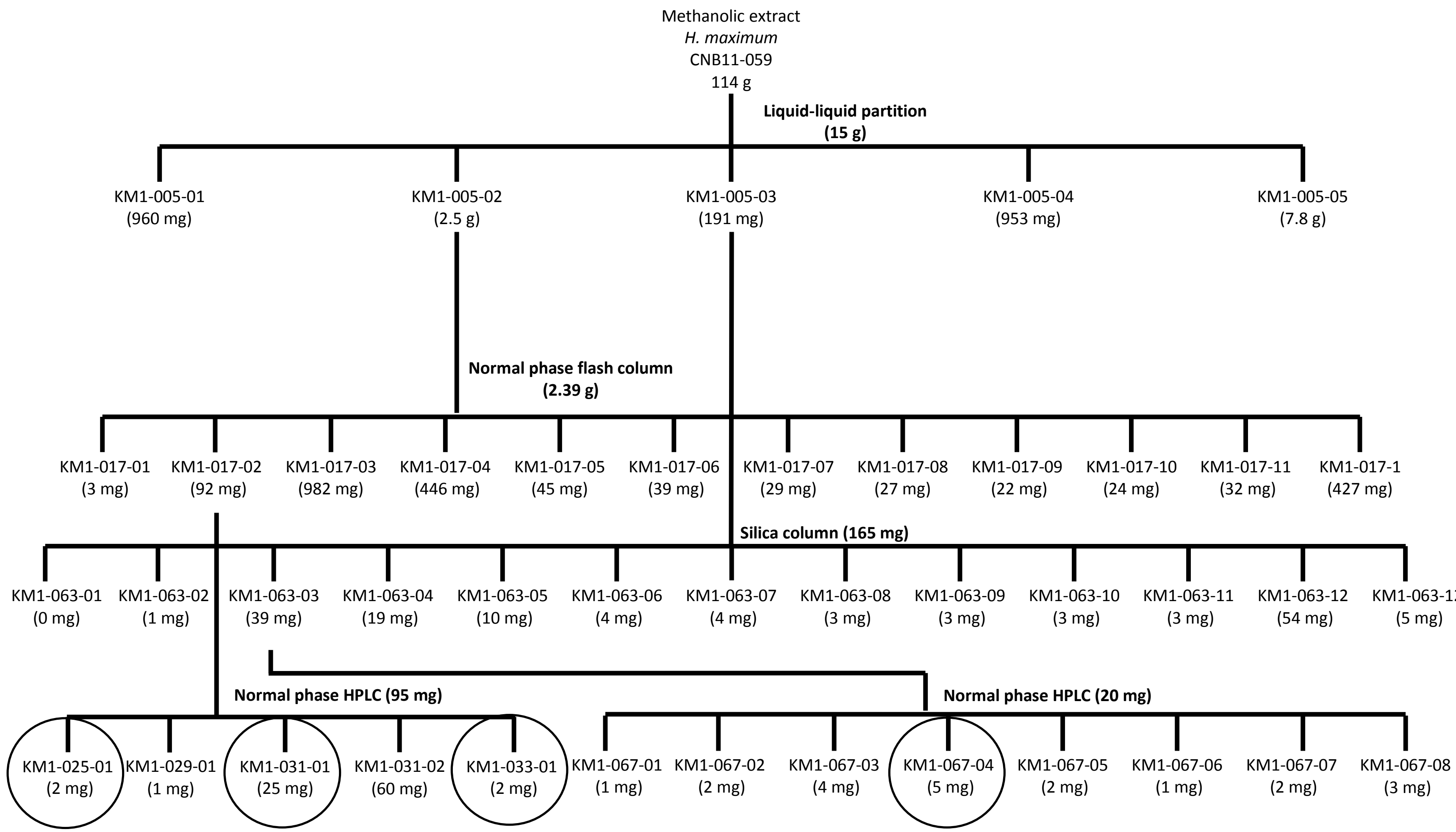


Figure 3.2.1 Flowchart displaying the isolation of compounds **1** (falcarinol) and **2** (falcarindiol) from *Heracleum maximum.*

3.2.4 Reflux of falcarindiol

Due to the instability of falcarindiol, a pure sample of the compound was refluxed in order to determine whether the compound degrades on prolonged heating in methanol, i.e., during the extraction process. A sample of pure falcarindiol (0.5 g) was dissolved in methanol (5 mL), heated (80°C) for approximately eight hours and concentrated *in vacuo*. Spectroscopic data were recorded to determine if the compound degraded through the extraction process.

3.2.5 Spectroscopic data

Falcarinol (**1**). Colorless oil; $[\alpha]^{25}_{D}$ = -19 (*c* 0.026, $CHCl_3$); IR (thin film) υ_{max}; 3405, 2921, 2851, 2209, 1702, 1384, 1099 cm^{-1}; ^{1}H NMR (benzene-d_6, 400 MHz) δ 5.68 (1H, ddd, *J*= 17.0, 10.2, 5.2 Hz, H-2), 5.34 (1H, m, H-10), 5.32 (1H, m, H-9), 5.24 (1H, dt, *J*= 17.1, 1.3 Hz, H-1a), 4.88 (1H, dt, *J*= 10.2, 1.3 Hz, H-1b), 4.53 (1H, bd, *J*= 5.2 Hz, H-3), 2.73 (2H, m, H_2-8), 1.81 (2H, m, H_2-11), 1.32 (1H, m, 3-OH), 1.27 (2H, m, H_2-16), 1.23 (2H, m, H_2-15), 1.19 (6H, m, H_2-12, H_2-13, H_2-14), 0.91 (3H, t, *J*= 6.8 Hz, H_3-17); ^{13}C NMR (DMSO, 100 MHz) δ 137.6 (d, C-2), 132.6 (d, C-10), 122.3 (d, C-9), 115.3 (t , C-1), 80.0 (s, C-7), 77.0 (s, C-4), 69.1 (s, C-5), 64.0 (s, C-6), 61.6 (d, C-3), 31.1 (t, C-16), 28.7 (t, C-12), 28.5 (t, C-13), 28.5 (t, C-14), 26.5 (t. C-11), 22.0 (t, C-16), 16.9 (t, C-8), 13.9 (q, C-17)

Falcarindiol (**2**). Colorless oil; $[\alpha]^{25}_{D}$ = +308 (*c* 0.0097, $CHCl_3$); IR (thin film) υ_{max}; 3398, 2956, 2926, 2855, 2252, 2151, 1644, 1016 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.94 (1H, ddd, *J*= 17.0, 10.2, 5.4 Hz, H-2), 5.62 (1H, m, H-10), 5.53 (1H, m, H-9), 5.47 (1H, m, H-1_b), 5.26 (1H, dt, *J*= 10.2, 1.2 Hz, H-1_a), 5.21 (1H, m, H-8), 4.94 (1H, bt, *J*= 5.7 Hz, H-3), 2.11 (2H, m, H_2-11), 1.94 (1H, bd, *J*= 6.7 Hz, 3-OH), 1.86 (1H, bd, *J*= 5.0 Hz, 8-OH), 1.38 (2H, m, H_2-12), 1.27 (8H, m, H_2-13, H_2-14, H_2-15, H_2-16), 0.88 (3H, t, *J*= 7.0 Hz, H_3-17); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 135.9 (d, C-2), 134.9 (d, C-10), 127.8 (d, C-9), 117.5 (t, C-1), 80.0 (s, C-7), 78.4 (s, C-4), 70.5 (s, C-5), 68.9 (s, C-6), 63.7 (d, C-3), 58.8 (d, C-8), 31.9 (t, C-15), 29.4 (t, C-11), 29.3 (t, C-12), 29.3 (t, C-13), 27.9 (t, C-14), 22.3 (t, C-16), 14.3 (q, C-17); ESIMS *m/z* (rel. int.); 299.3 (100), 282.4 (17), 278.3 (16); HRESIMS *m/z* [M-H^+] 259.1711 (calculated for $C_{17}H_{23}O_2$, 259.1704)

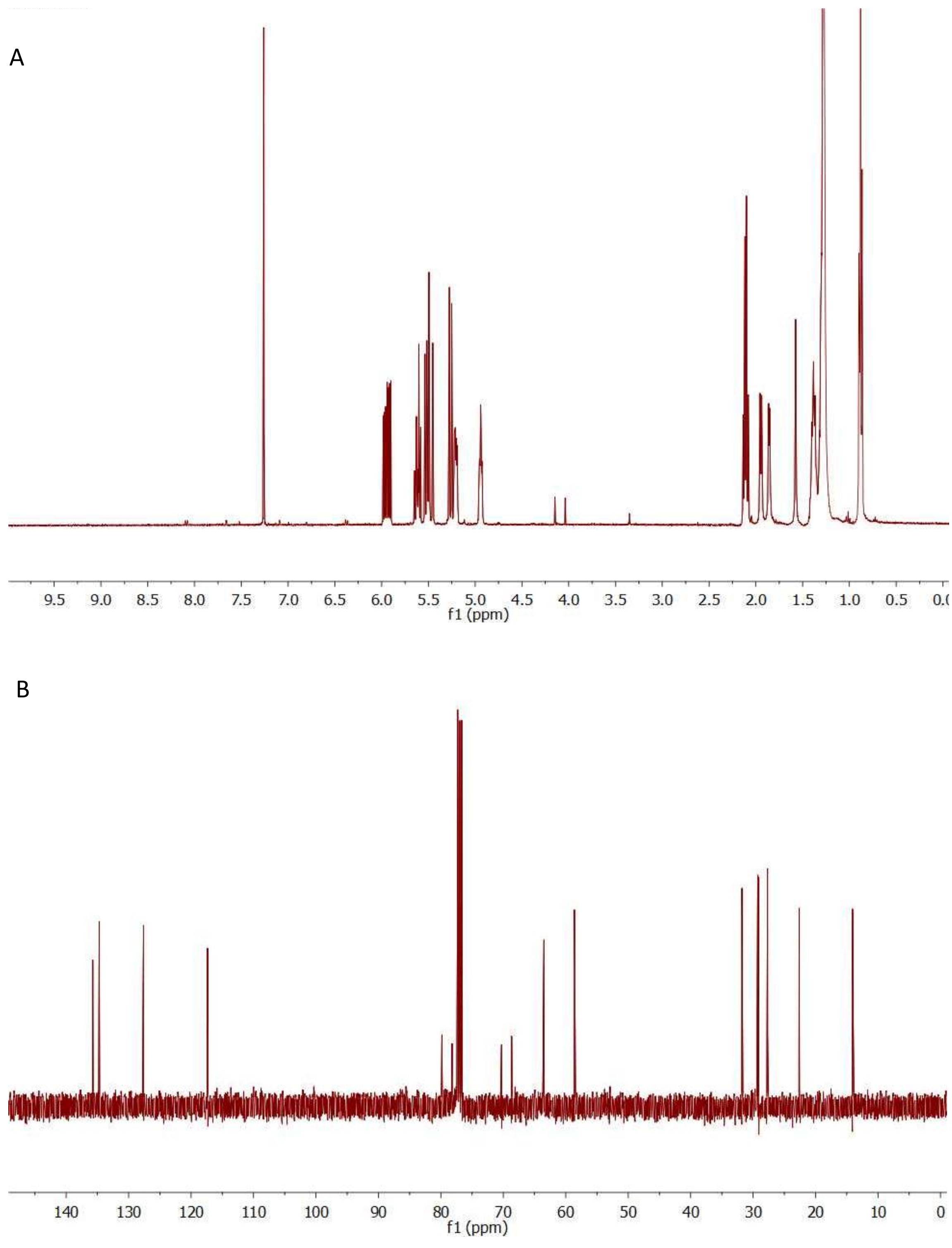


Figure 3.2.2 A) ^{1}H (400 MHz, $CDCl_3$) and B) ^{13}C (100 MHz, $CDCl_3$) NMR spectra of falcarindiol (**2**) isolated from the roots of *Heracleum maximum* collected from the Kingston Peninsula, New Brunswick, Canada.

3.2.6 Attempted quantification of falcarindiol using ESIMS

Electrospray ionization mass spectrometric (EISMS) data were recorded using Agilent 1100 LC system coupled to an AB Sciex® API 2000 triple quadrupole mass spectrometer. This technique was employed in both positive and negative ion mode. Voltage, temperature, curtain gas, declustering potential, entrance potential and focusing potential parameters were varied in an attempt to optimize the conditions for the ionization of falcarindiol. Various solvent systems employing acetonitrile and methanol both unmodified and with modifiers (formic acid and ammonium acetate) were used for the analyses. None of the methods used were found to be suitable for the analysis of compound **2**.

3.2.7 Quantification of falcarindiol using HPLC

Analytical HPLC was performed using a Kinetex™ C-18 analytical column (50 × 4.6mm, 2.6µm, 100Å, Phenomenex, Torrance, CA, USA) and the following parameters: 100µL injection at a flow rate of 500µL/min with the oven temperature set to 20°C, and UV detector set to 190-210 nm. The column was eluted with a solvent gradient as shown in Table 3.2.1 and Figure 3.2.3.

Table 3.2.1 Flow gradient used for high performance liquid chromatography to quantify falcarindiol in *Heracleum maximum* crude extracts.

Total time (min)	Flow Rate (µL/min)	Solvent A: water (%)	Solvent B: methanol (%)
0.00	500	92.0	8.0
2.00	500	92.0	8.0
15.00	500	8.0	92.0
18.00	500	9.0	92.0
18.50	500	92.0	8.0
20.00	500	92.0	8.0

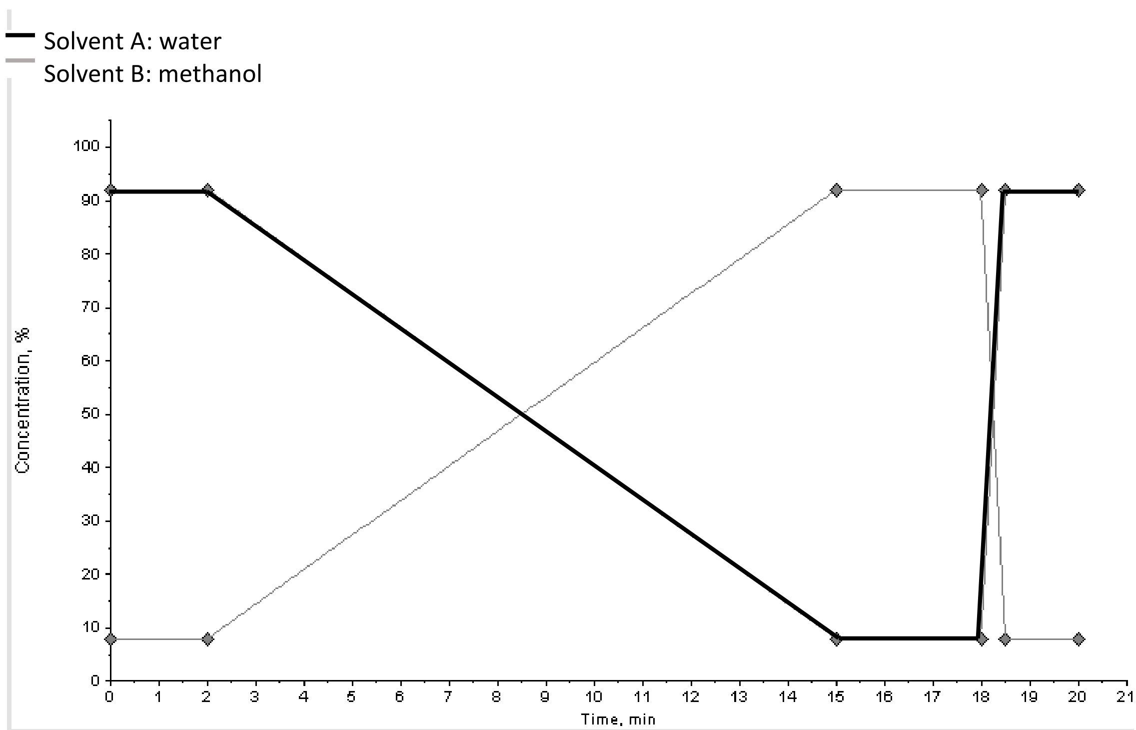


Figure 3.2.3 Graphical representation of the elution gradient used for the analytical high performance liquid chromatography of *Heracleum maximum* crude extracts.

Using this HPLC method, pure falcarindiol samples of known concentrations were analyzed to create a standard curve. For each of the known concentrations, the HPLC trace (Figure 3.2.4) was analyzed and the area under the absorbance curve was calculated (using the quantitation wizard on AB Sciex Analyst® 1.4). The absorbance was plotted on the y-axis and the concentration on the x-axis and a regression was run to create the analytical curve (Figure 3.2.5).

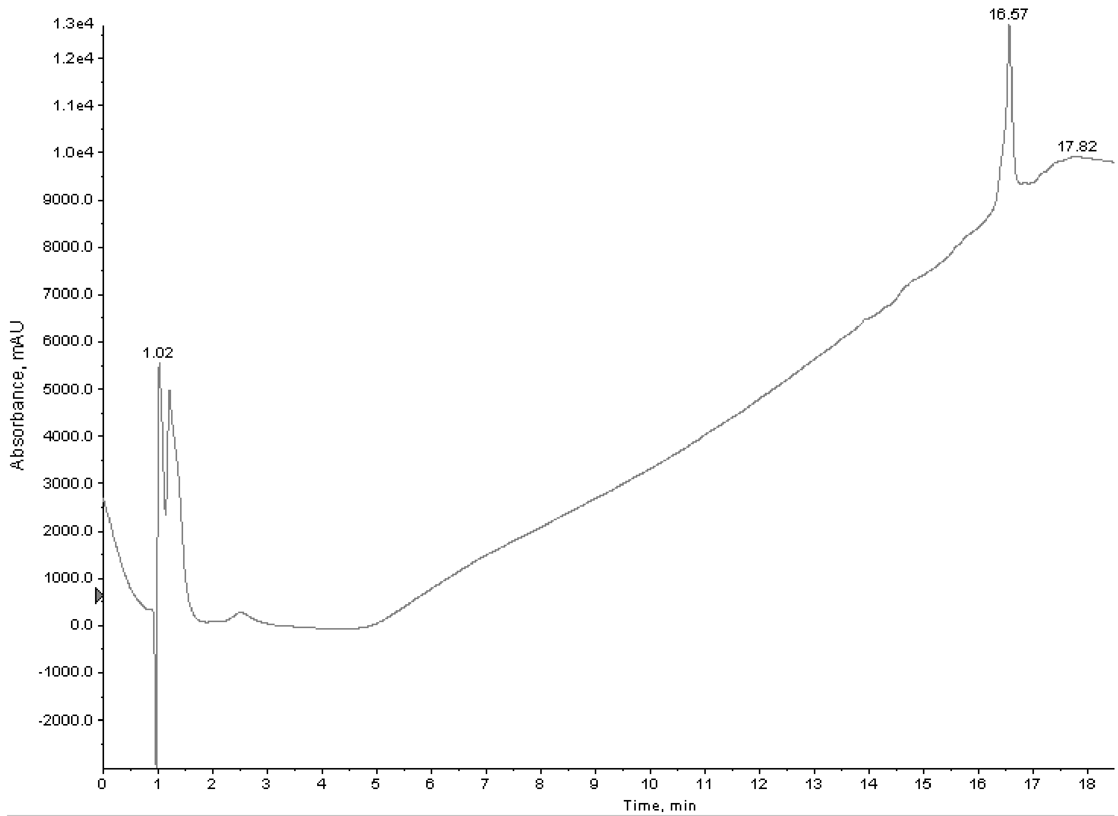


Figure 3.2.4 An example of a high performance liquid chromatogram of falcarindiol (**2**) demonstrating a retention time of 16.57 min for the pure compound.

This standard curve was used to determine the amount of falcarindiol present in each crude extract by using the following formula,

$$concentration\ of\ falcarindiol\ (mg/mL) = \frac{area\ under\ the\ curve}{1798.9}$$

Each chromatogram was analyzed three times and the average area under the falcarindiol absorbance peak was used to calculate the concentration of falcarindiol. The concentrations of falcarindiol were converted into percentages of the extract to account for differences in amount of crude extract tested.

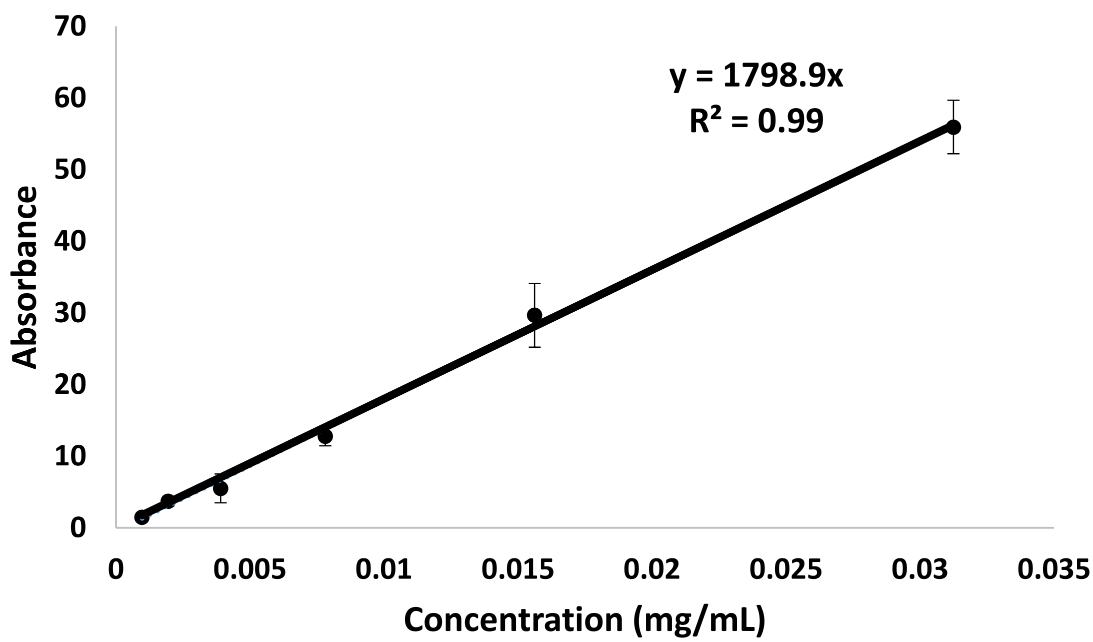


Figure 3.2.5 Standard curve obtained for the analytical HPLC of falcarindiol.

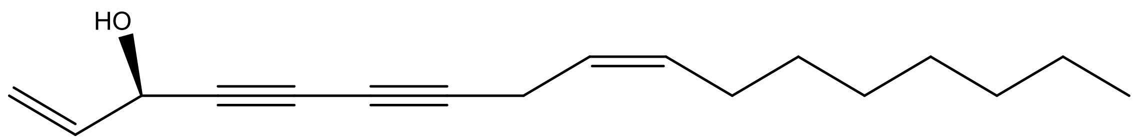
3.2.8 Quantification of falcarindiol using qNMR

All qNMR samples were prepared by dissolving a known amount of extract (20-30 mg) in 0.7 mL of dimethyl sulfoxide-d6. Benzoic acid (2.0 mg per sample) was added to each sample as an internal standard. All spectra were analyzed using MestreNova. The number of moles of benzoic acid was calculated for each sample and determined to be 1.61×10^{-5}. The benzoic acid resonance at 7.90 ppm represents two protons thus the number of moles of protons represented by the integral of that resonance was calculated to be 3.23×10^{-5}. The resonance that was chosen to be characteristic of falcarindiol has a chemical shift of 5.94 ppm and represents one proton thus the integral of that resonance was calculated to be 1.61×10^{-5} moles of protons. Relating the area under the analyte peak at 5.85 ppm to that of the standard at 7.90 ppm therefore allows the number of moles of falcarindiol present in the sample to be calculated. The molecular weight of falcarindiol, 260.371 g/mol can then be used to calculate the mass of the compound present in the sample and hence in the extract. All spectra were analyzed three separate times to allow for an average percentage falcarindiol.

3.3 Results

3.3.1 Falcarindiol isolation

Through the use of liquid-liquid partitioning, silica flash column and normal phase high-performance liquid chromatography, falcarinol (**1**) and falcarindiol (**2**) were isolated and their structures were elucidated using nuclear magnetic resonance and high and low resolution mass spectrometry (Figure 3.3.1). It is important to note that falcarinol was not isolated from the roots of *H. maximum* by O'Neill (2012), and this is the first known instance of falcarinol being isolated from this plant species.



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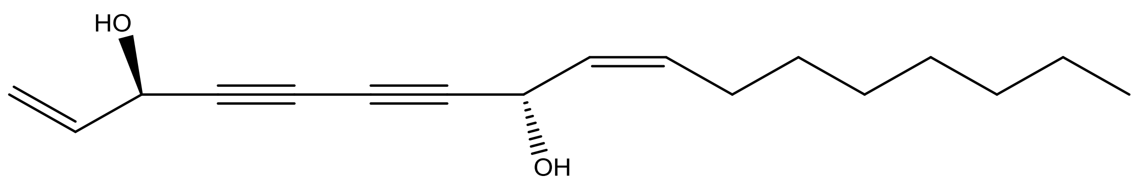

2

Figure 3.3.1 The two diynes isolated from *Heracleum maximum*: falcarinol (**1**) and falcarindiol (**2**).

3.3.2 Quantification of falcarindiol using qNMR

The results from the quantification of falcarindiol through the use of qNMR indicate that the *H. maximum* root extracts showed significant differences between seasons (P = 0.004). The extracts from the summer contained significantly more falcarindiol than the extracts from the spring and fall (post-hoc pairwise comparison, P = 0.022 and P < 0.001 respectively) (Figure 3.3.2). Falcarindiol content ranged from 0 to 20% in the extracts from the three different seasons. These results also show that overall, there was a difference in the amount of falcarindiol present in extracts from different tissues of *H. maximum* (P < 0.001). The extracts obtained from the roots have significantly more falcarindiol than extracts from stem, leaf or flower (post-hoc pairwise comparison, P < 0.001 for all significant comparisons) (Figure 3.3.3). Ranges for falcarindiol content from different tissues were 0 to 17%.

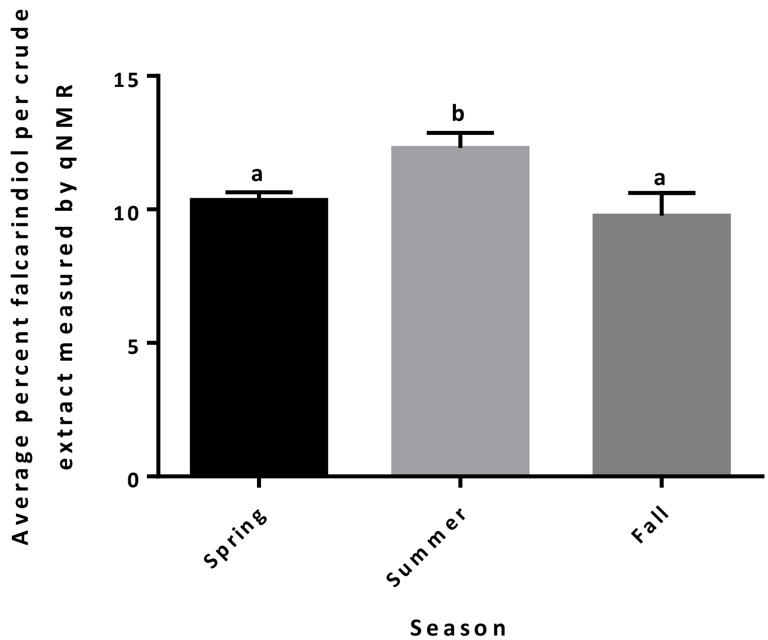

Figure 3.3.2 Average percentage of falcarindiol in *Heracleum maximum* root extracts in different seasons (spring, summer, and fall), collected over three years, quantified by qNMR (error bars indicate standard error; homogenous groups are denoted by lowercase letters).

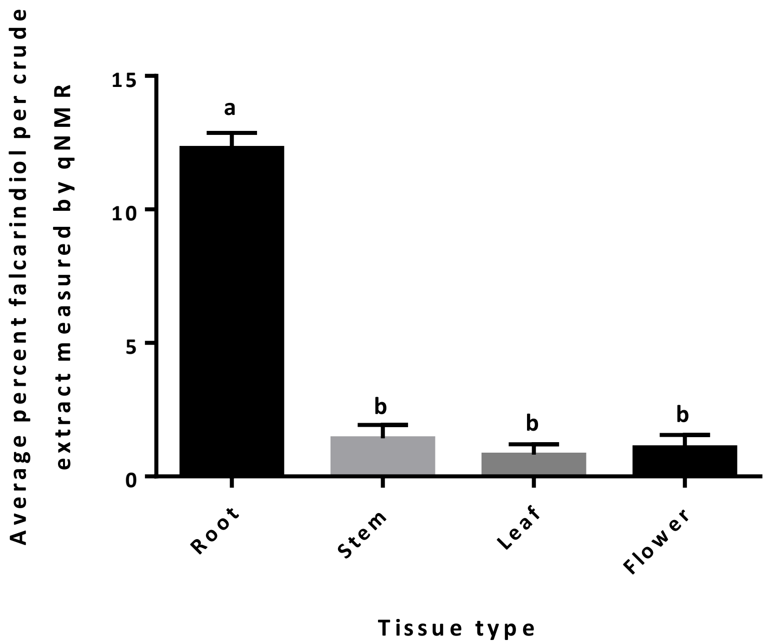

Figure 3.3.3 Average percentage of falcarindiol in *Heracleum maximum* summer extracts in different tissues (root, stem, leaf, and flower), collected over three years, quantified by qNMR (error bars indicate standard error; homogenous groups are denoted by lowercase letters).

3.3.3 Comparison of qNMR and HPLC methods for quantification of falcarindiol

Analysis of the relationship between falcarindiol content measured by qNMR and the falcarindiol content measured by HPLC reveals that there is a weak correlation between the two methods (R^2=0.2946) (Figure 3.3.4). On average the amount of falcarindiol was estimated to be 81% more in the crude extracts when analyzed by qNMR quantification methods compared to HPLC.

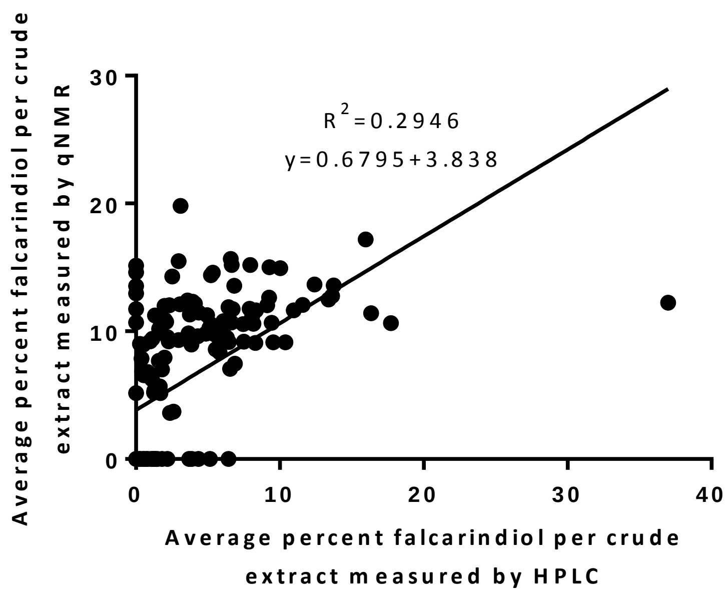

Figure 3.3.4 Comparing methods of quantification of average percentage of falcarindiol in *Heracleum maximum* extracts, collected over three years, measured by HPLC plotted against percentage of falcarindiol in *Heracleum maximum* extracts measured by qNMR.

3.3.4 Correlations between antimycobacterial activity and falcarindiol quantification using qNMR

A regression analysis was completed to determine if there was a correlation between the amount of bioactivity displayed by the crude extracts and the amount of falcarindiol present in these extracts. This analysis revealed a very weak correlation (R^2=0.0435) (Figure 3.3.5). When analyzed by qNMR, the only correlation that was not very weak between falcarindiol content and bioactivity was with the *H. maximum* root extracts from the spring (Figure 3.3.6) and there were no significant correlations found with any of the extracts from different tissues (Figure 3.3.7). It is important to note that the data for the root extracts and the data for the summer extracts are identical as the roots were the only tissue collected for the seasonal study due to availability of plant tissues in different seasons.

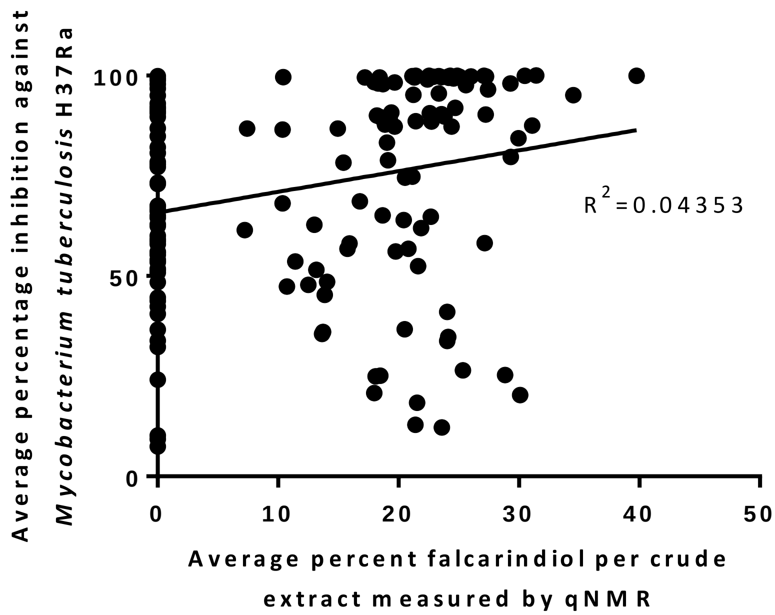

Figure 3.3.5 Average percentage inhibition of *Heracleum maximum* extracts, collected over three years, tested against *Mycobacterium tuberculosis* H37Ra measured at 100 µg/mL versus average percentage falcarindiol per crude extract measured by qNMR.

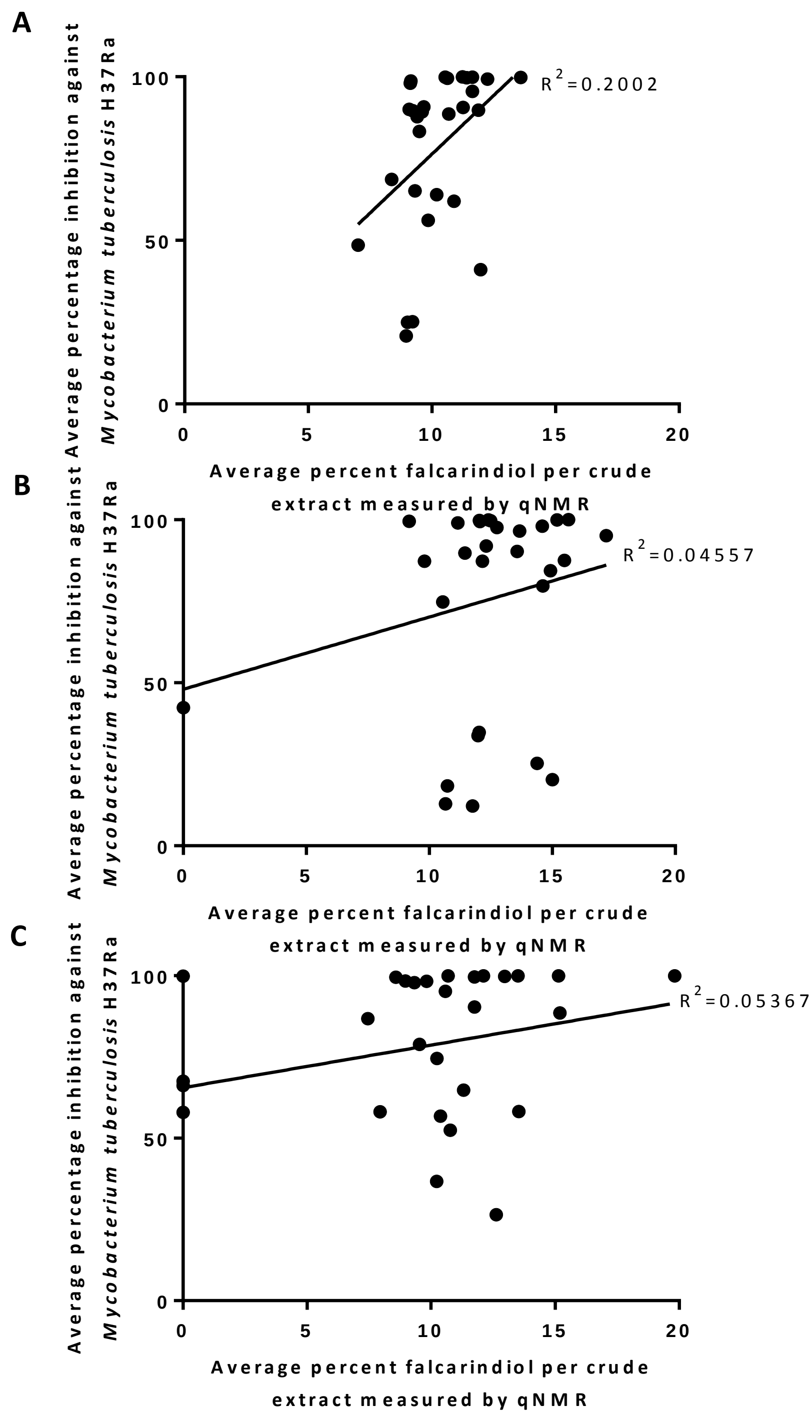

Figure 3.3.6 Average percentage inhibition of *Heracleum maximum* root extracts, collected over three years, tested against *Mycobacterium tuberculosis* H37Ra measured at 100 µg/mL versus average percentage falcarindiol per crude extract measured by qNMR for different seasons: A) spring, B) summer*, C) fall. *These data are identical to that presented in Figure 3.3.7 panel A as the seasonal comparison uses only root data and the tissue comparison uses only summer data.

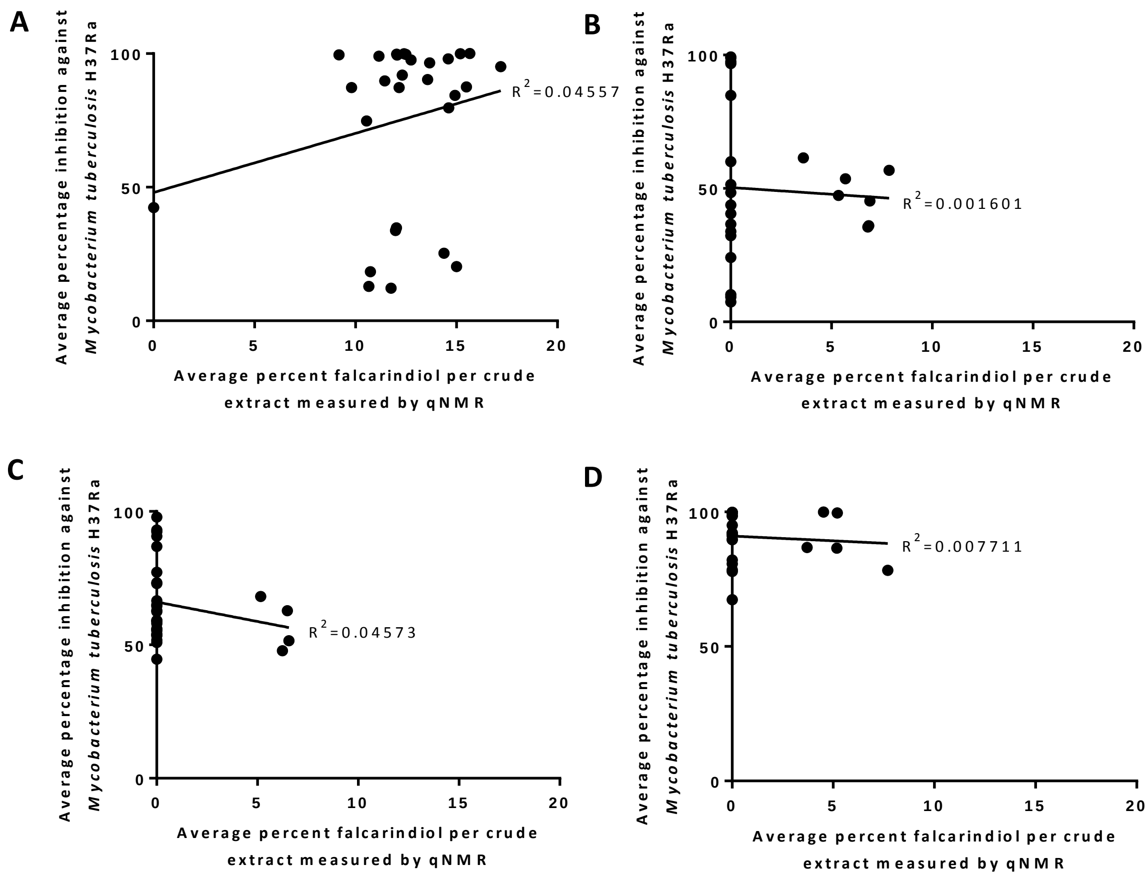

Figure 3.3.7 Average percentage inhibition of *Heracleum maximum* summer extracts, collected over three years, tested against *Mycobacterium tuberculosis* H37Ra measured at 100 µg/mL versus average percentage falcarindiol per crude extract measured by qNMR for different tissues: A) root*, B) stem, C) leaf, D) flower. *These data are identical to that presented in Figure 3.3.6 panel B as the seasonal comparison uses only root data and the tissue comparison uses only summer data.

3.4 Discussion and conclusion

Heracleum maximum root extracts contained the most falcarindiol when quantified with the use of qNMR. This many explain why this was the tissue that First Nations traditionally collected for the medicinal uses of this plant. The root extracts also showed high antimycobacterial activity (Chapter 2), further supporting the traditional uses of the plant. Although summer root extracts had the highest amount of falcarindiol present, there was still a significant amount of falcarindiol present in *H. maximum* extracts from other seasons. Again, considering the traditional use of *H. maximum*, although the summer extracts had the highest amount of falcarindiol present, it would still be present in extracts from roots collected in the fall.

Although there were no strong correlations in content and antimycobacterial activity, in almost all cases root extracts contained falcarindiol and exhibited antimycobacterial activity. Analysis of the extracts obtained from different tissue types collected in the summer indicated that only roots tissues consistently contained falcarindiol. Although the flower extracts had higher antimycobacterial activity than the extracts from the other tissues tested, many of the flower extracts (as well as many of the stem and leaf extracts) did not contain detectable amounts of falcarindiol. Relating this back to First Nations traditional uses of *H. maximum* tissues, the roots would be the tissues that would provide the most reliable source of falcarindiol whilst maintaining high levels of bioactivity.

Originally, electrospray ionization mass spectrometry (ESIMS) was going to be used to analyze the amount of falcarindiol present in the *H. maximum* extracts; however, despite numerous attempts, a method could not be developed that allowed the ionization and detection of falcarindiol. Electrospray ionization mass spectrometry coupled with liquid chromatography was attempted in both positive and negative mode, along with several different solvent systems (both with and without buffers) to try to observe the compound. As there are few reports of the use of ESIMS coupled with LC for the characterization of polyacetylenes (Rai et al., 2011) our inability to use LC-MS is not surprising and alternative approaches, i.e., HPLC and qNMR had to be employed to analyze the falcarindiol content in the *H. maximum* extracts.

Through both the analysis of HPLC and qNMR quantitation methods, there were a significant number of extracts that were found to contain no falcarindiol by one of the analytical techniques (Figure 3.3.5). Data that lie on the Y-axis represent extracts that were found to contain no falcarindiol when analyzed by qNMR but were found to contain falcarindiol when analyzed by HPLC. Conversely, data that lie on the X-axis represent extracts that were found to contain no falcarindiol when analyzed by HPLC but were found to contain falcarindiol when analyzed by qNMR. In the first scenario, this would be indicative of a co-eluting peak in the HPLC chromatogram that was misidentified as falcarindiol. A co-eluting peak could be due to the presence of one or more compounds with polarity similar to falcarindiol that would therefore have the same retention time. In a complex biological extract it is very possible that many compounds will have the same retention time which reduces our confidence in this method of analysis. In the second scenario, this would be indicative of a false positive. With NMR, it is possible for two compounds to show a proton resonance at the same chemical shift, however, in this case, it is more likely that the compounds are similar in structure than is the case with co-eluting peaks that occur with HPLC.

Therefore if one method were to be used for the quantification of falcarindiol, qNMR would be chosen as a co-elution in HPLC could be any type of compound that has the same retention time as falcarindiol; however, a false-positive measured with qNMR would most likely be due to compounds with similar structures to falcarindiol. Thus out of the two methods used in this study, qNMR would be a more reliable method for the quantification of falcarindiol as other falcarindiol-like compounds have been shown to exhibit antimycobacterial activity (Li et al., 2012). Therefore, we chose to only focus on the results from the qNMR data these data were deemed more reliable, however, data from the HPLC analysis are available in Appendix 4.

Although this research focuses on the results obtained through the use of qNMR rather than HPLC, as demonstrated above, there has been success with the quantification of falcarindiol with ESI-MS and HPLC. Due to the fact that there was not a strong correlation between the two quantification methods used in this study, it would suggest that one of these methods may not be reliable in determining the concentration of falcarindiol in *H. maximum* crude extracts. Further research should focus on assessing the quantification of this natural product with differing quantification techniques such as different mass spectrometry methods such as those used by Rai et al. (2011).

Prior et al. (2007) tested the seasonal and tissue variations of falcarindiol from *Aeogpodium podagraria* roots, leaves, stems, and flower. Similar to our qNMR results, this member of the Apiaceae family showed varying levels of falcarindiol in the different tissues when quantified using HPLC. Contrary to my qNMR results, they found that the flower extracts in the spring yielded the highest amounts of falcarindiol and suggest that the highest levels of falcarindiol are found before flowering season as it is a critical time in the development of the plant (Prior et al., 2007). Prior et al. (2007) also found that the leaves generally had more falcarindiol content than the roots, stems, and flowers.

A study by Kolodziej et al. (2013) used HPLC to quantify the amount of falcarinol, a polyacetylene closely related to falcarindiol. They found that falcarinol content in the roots of ginseng (*Panax quinquefolius*) comprised 14% of hexane extracts and 1% of ethanolic extracts. They also found that the leaves of the plant did not contain any polyacetylenes (Kolodziej et al., 2013). Although this was a different plant and a different compound was quantified, these results are comparable to our data when quantifying falcarindiol from *H. maximum* using qNMR, where it can be seen that the root extracts had more falcarindiol than the leaves. It can also be noted that the percentage of analyte found in extracts by Kolodziej et al. (2013) were similar to those found in our study.

Zobel and Brown (1990) found that the concentrations of three furanocoumarins in the leaves of *Heracleum lanatum* varied over a period from April to November. Another study by Pira et al. (1989) found that furanocoumarin content of *H. mantegazzianum* was highest at the end of June which would be the vegetation season for the plant. They also found that in August and November there were low levels of furanocoumarins which was attributed to the flower season of the plant (August) and the beginning of dormancy (November). This data is in agreement with ours as the highest amounts of falcarindiol were found in the *H. maximu*m extracts from the summer (collected in June).

Falcarindiol was quantified in the roots of *Saposhnikovia divaricata,* a plant in the Umbelliferae family by Ishizuka et al. (1998) and it was found that the amount of falcarindiol decreased during the flowering season of the plant. It was also noted that the falcarindiol concentration increased after the flowering period (Ishizuka et al., 1998). In a study by Wittstock et al. (1995), they determined that all of the polyacetylenes they were quantifying, including falcarindiol were present in the rhizomes, roots, leaves, fruits and young shoots of *Cicuta virosa*, a member of the Apiaceae family. In this plant species, falcarindiol was one of three major components of the leaves and fruits and one of four major components of roots and young shoots (Wittstock et al., 1995). An additional study by Zschocke et al. (1997) found that the amount of falcarindiol found in *Notopterygium incisum,* an east Asian medicinal plant from the Umbelliferae family exhibited large concentration variations in their samples (0.08% to 3.2%) (Zschocke et al., 1997).

Correlations between antimycobacterial activity and falcarindiol content were made between extracts tested against *M. tuberculosis* at 100 µg/mL in the MRA. This concentration was chosen for comparison because as previously mentioned, it is the standard used for this assay from the results obtained with the optimization of the microplate resazurin assay (O'Neill et al., 2014). There was no correlation between falcarindiol content and antimycobacterial activity and this could be the result of a number of scenarios. One such explanation could be that our research demonstrated poor estimates of falcarindiol content or poor estimates of percentage inhibition. These poor estimates, particularly with regards to a poor estimate of falcarindiol content, could be due to the sensitivity of the spectroscopic technique. Additionally, this lack of correlation demonstrates that falcarindiol may not be the only antimycobacterial compound present in these *H. maximum* crude extracts. In fact, these results would indicate that there are compounds present in the extracts that would either demonstrate higher antimycobacterial activity than falcarindiol or may possibly have a synergistic effect with this compound and possibly others. Indeed, in this study we isolated a significant amount of falcarinol, a compound that is known to possess antimycobacterial activity (Christensen, 2011; Kobaisy et al., 1997; Li et al., 2012; Schinkovitz et al., 2008), from *H. maximum* roots.

Chapter 4: Summary of thesis research

The objective of this research was to determine whether a relationship exists between the antimycobacterial activity observed in *Heracleum maximum* crude extracts and the amount of falcarindiol the crude extracts contain. The crude extracts from different tissues (root, stem, leaf, flower) and different seasons (spring, summer, fall) over three years (2009-2011) showed varying bioactivity and varying amounts of falcarindiol.

The antimycobacterial activity of *H. maximum* crude extracts differed when tested at different concentrations, which was expected. These crude extracts also differed in bioactivity when testing different tissue collected in the summer and when testing roots from different seasons. Bioactivity of crude extracts did not differ significantly between seasons and, in the summer, only flower extracts were significantly more bioactive than extracts of the others tissues.

Falcarindiol concentrations assessed using qNMR showed that summer root extracts contained more falcarindiol than extracts from other seasons and flower extracts had significantly more falcarindiol present than extracts from any other tissue.

There was no relationship observed between the antimycobacterial activity of extracts and the amount of falcarindiol in the extracts when quantified by qNMR. This lack of a correlation could be indicative of other very potent antimycobacterial compounds that are displaying the observed bioactivity against *Mycobacterium tuberculosis* H37Ra.

Future studies should employ more sensitive methods for the quantification of this compound to determine which of the two methods used in this study would be considered the most reliable for detecting falcarindiol concentrations in *H. maximum* crude extracts.

Overall, it can be concluded that the ethnopharmacological background knowledge of *H. maximum* collection should be followed. If *H. maximum* was collected for its medicinal uses, it would be practical to follow the traditional First Nations approach and collect the roots of the plant. *Heracleum maximum* roots, especially those collected in the fall, are not always the tissues from which the highest amount of falcarindiol is present, but there will always be some of this natural product present, which cannot be said for all other tissues and seasons. It can also be concluded that further fractionation of *H. maximum* crude extracts has the potential to lead to additional antimycobacterial compounds with high bioactivity.

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APPENDICES

Appendix 1 Extraction efficiency of *Heracleum maximum* methanolic crude extracts.

Table A1.1 *Heracleum maximum* root crude extracts from three seasons and their extraction efficiency.

Season	Extract ID	Extraction efficiency
Spring	TO2-043-01	9.1
	TO2-047-01	17.3
	TO2-051-01	14.7
	TO2-058-01	11.2
	TO2-059-01	18.8
	TO2-063-01	3.5
	TO2-067-01	9.8
	TO2-071-01	18.0
	TO2-075-01	6.6
	TO2-079-01	16.6
	TO4-003-01	25.5
	TO4-003-02	18.3
	TO4-003-03	24.0
	TO4-003-04	22.8
	TO4-003-05	21.8
	TO4-003-06	25.5
	TO4-003-07	19.8
	TO4-003-08	22.0
	TO4-003-09	22.5
	TO4-003-10	21.3
	TO6-002-01	24.6
	TO6-002-02	25.1
	TO6-002-03	25.2
	TO6-002-04	27.7
	TO6-002-05	30.4
	TO6-002-06	28.4
	TO6-002-07	26.2
	TO6-002-08	26.4
	TO6-002-09	27.6
	TO6-002-10	32.1
	Mean (± S. E.)	**20.8 (± 1.3)**

Table A1.1 continued

Season	Extract ID	Extraction efficiency
Summer	TO2-083-01	8.5
	TO2-087-01	8.2
	TO2-091-01	9.0
	TO2-095-01	6.7
	TO2-099-01	5.9
	TO2-103-01	7.3
	TO2-107-01	12.0
	TO2-115-01	10.7
	TO2-119-01	5.5
	TO4-043-01	19.7
	TO4-043-02	14.2
	TO4-043-03	16.2
	TO4-043-04	16.2
	TO4-043-05	24.4
	TO4-043-06	17.1
	TO4-043-07	14.9
	TO4-043-08	15.2
	TO4-043-09	21.6
	TO4-043-10	13.8
	TO6-104-01	16.5
	TO6-104-02	14.9
	TO6-104-03	19.3
	TO6-104-04	13.0
	TO6-104-05	13.2
	TO6-104-06	15.4
	TO6-104-07	15.7
	TO6-104-08	15.5
	TO6-104-09	14.0
	TO6-104-10	11.4
	Mean (± S. E.)	**13.7 (± 0.9)**

Table A1.1 continued

Season	Extract ID	Extraction efficiency
Fall	TO3-005-01	16.6
	TO3-007-01	17.7
	TO3-009-01	17.6
	TO3-011-01	18.3
	TO3-013-01	16.7
	TO3-015-01	15.4
	TO3-017-01	17.3
	TO3-019-01	21.1
	TO3-021-01	18.6
	TO3-023-01	17.3
	TO4-167-01	19.4
	TO4-167-02	1.6
	TO4-167-03	20.3
	TO4-167-04	18.9
	TO4-167-05	20.6
	TO4-167-06	20.8
	TO4-167-07	17.5
	TO4-167-08	21.0
	TO4-167-09	17.5
	TO4-167-10	22.5
	TO7-020-01	20.6
	TO7-020-02	19.7
	TO7-020-03	21.8
	TO7-020-04	23.3
	TO7-020-05	19.9
	TO7-020-06	21.6
	TO7-020-07	19.7
	TO7-020-08	20.8
	TO7-020-09	26.9
	TO7-020-10	22.5
	Mean (± S. E.)	**19.1 (± 0.7)**

Table A1.2 *Heracleum maximum* summer crude extracts from four tissues and their extraction efficiency.

Tissue	Extract ID	Extraction efficiency
Root	TO2-083-01	8.5
	TO2-087-01	8.2
	TO2-091-01	9.0
	TO2-095-01	6.7
	TO2-099-01	5.9
	TO2-103-01	7.3
	TO2-107-01	12.0
	TO2-111-01	9.3
	TO2-115-01	10.7
	TO2-119-01	5.5
	TO4-043-01	19.7
	TO4-043-02	14.2
	TO4-043-03	16.2
	TO4-043-04	16.2
	TO4-043-05	24.4
	TO4-043-06	17.1
	TO4-043-07	14.9
	TO4-043-08	15.2
	TO4-043-09	21.6
	TO4-043-10	13.8
	TO6-104-01	16.5
	TO6-104-02	14.9
	TO6-104-03	19.3
	TO6-104-04	13.0
	TO6-104-05	13.2
	TO6-104-06	15.4
	TO6-104-07	15.7
	TO6-104-08	15.5
	TO6-104-09	14.0
	TO6-104-10	11.4
	Mean (± S. E.)	**13.5 (± 0.8)**

Table A1.2 continued

Tissue	Extract ID	Extraction efficiency
Stem	TO2-129-01	12.8
	TO2-131-01	16.2
	TO2-133-01	22.4
	TO2-135-01	24.3
	TO2-137-01	18.1
	TO2-139-01	16.6
	TO2-141-01	9.0
	TO2-143-01	15.8
	TO2-145-01	14.5
	TO2-147-01	17.0
	TO4-047-01	19.0
	TO4-047-02	27.7
	TO4-047-03	12.1
	TO4-047-04	11.8
	TO4-047-05	16.8
	TO4-047-06	11.2
	TO4-047-07	24.8
	TO4-047-08	17.8
	TO4-047-09	15.2
	TO4-047-10	12.8
	TO6-106-01	17.9
	TO6-106-02	9.1
	TO6-106-03	9.5
	TO6-106-04	13.2
	TO6-106-05	12.8
	TO6-106-06	11.7
	TO6-106-07	11.2
	TO6-106-08	11.9
	TO6-106-09	18.7
	TO6-106-10	10.6
	Mean (± S. E.)	**15.4 (± 0.9)**

Table A1.2 continued

Tissue	Extract ID	Extraction efficiency
Leaf	TO4-051-01	26.9
	TO4-051-02	25.5
	TO4-051-03	2.6
	TO4-051-04	8.3
	TO4-051-05	32.1
	TO4-051-06	28.7
	TO4-051-07	27.4
	TO4-051-08	22.6
	TO4-051-09	24.1
	TO4-051-10	9.8
	TO2-149-01	23.9
	TO2-151-01	15.8
	TO2-153-01	18.6
	TO2-155-01	13.6
	TO2-157-01	19.7
	TO2-159-01	26.3
	TO2-161-01	19.7
	TO2-163-01	18.6
	TO2-165-01	20.2
	TO2-167-01	19.4
	TO6-107-01	17.9
	TO6-107-02	14.7
	TO6-107-03	13.6
	TO6-107-04	15.3
	TO6-107-05	15.9
	TO6-107-06	16.0
	TO6-107-07	9.9
	TO6-107-08	14.5
	TO6-107-09	13.9
	TO6-107-10	19.9
	Mean (± S. E.)	**18.5 (± 1.2)**

Table A1.2 continued

Tissue	Extract ID	Extraction efficiency
Flower	TO2-169-01	13.3
	TO2-171-01	-0.7
	TO2-173-01	11.0
	TO2-175-01	-1.6
	TO2-177-01	8.8
	TO2-179-01	14.6
	TO2-181-01	0.2
	TO2-183-01	0.2
	TO4-055-01	8.6
	TO4-055-02	6.1
	TO4-055-03	6.9
	TO4-055-04	6.7
	TO4-055-05	7.1
	TO4-055-06	9.2
	TO4-055-07	8.3
	TO4-055-08	5.9
	TO4-055-09	10.0
	TO4-055-10	8.8
	TO6-105-01	6.4
	TO6-105-02	4.8
	TO6-105-03	5.5
	TO6-105-04	7.3
	TO6-105-05	6.6
	TO6-105-06	6.9
	TO6-105-07	6.5
	TO6-105-08	8.2
	TO6-105-09	5.4
	TO6-105-10	5.9
	Mean (± S. E.)	**6.7 (± 0.7)**

Appendix 2 Antimycobacterial activity of *Heracleum maximum* crude extract against *Mycobacterium tuberculosis* H37Ra.

Table A2.1 *Heracleum maximum* root crude extracts from three seasons in 2009 and their respective antimycobacterial activity (percentage inhibition) against *Mycobacterium tuberculosis* H37Ra at 50 µg/mL and 100 µg/mL.

Season	Extract ID	Percentage inhibition at 50 µg/mL	Percentage inhibition at 100 µg/mL
Spring	TO2-043-01	81.9	99.9
	TO2-047-01	21.9	68.6
	TO2-051-01	85.0	99.8
	TO2-055-01	69.9	98.7
	TO2-059-01	73.4	99.7
	TO2-063-01	69.2	99.3
	TO2-067-01	71.4	99.5
	TO2-071-01	58.3	98.0
	TO2-075-01	39.9	89.3
	TO2-079-01	17.2	56.1
	TO4-003-01	39.4	90.6
	TO4-003-02	31.8	90.0
	TO4-003-03	33.9	87.8
	TO4-003-04	16.2	65.2
	TO4-003-05	57.7	95.5
	TO4-003-06	46.5	89.8
	TO4-003-07	8.8	48.5
	TO4-003-08	26.0	63.9
	TO4-003-09	44.6	90.8
	TO4-003-10	43.3	88.7
	TO6-002-01	90.5	100.1
	TO6-002-02	-2.5	41.1
	TO6-002-03	91.8	100.0
	TO6-002-04	44.1	87.6
	TO6-002-05	-15.7	20.8
	TO6-002-06	-15.0	25.1
	TO6-002-07	84.8	99.8
	TO6-002-08	-19.4	24.9
	TO6-002-09	19.9	83.3
	TO6-002-10	14.1	61.9
	Mean (± S. E.)	**41.0 (± 5.9)**	**78.8 (± 4.6)**

Table A2.1 continued

Season	Extract ID	Percentage inhibition at 50 µg/mL	Percentage inhibition at 100 µg/mL
Summer	TO2-083-01	33.0	90.3
	TO2-087-01	59.5	96.6
	TO2-091-01	71.7	99.4
	TO2-095-01	74.1	99.7
	TO2-099-01	60.1	97.6
	TO2-103-01	29.3	84.4
	TO2-107-01	73.2	99.9
	TO2-111-01	81.3	100.2
	TO2-115-01	64.8	99.6
	TO2-119-01	50.6	99.1
	TO4-043-01	55.2	95.1
	TO4-043-02	90.9	99.8
	TO4-043-03	46.1	89.8
	TO4-043-04	43.8	87.6
	TO4-043-05	44.8	87.3
	TO4-043-06	100.2	100.0
	TO4-043-07	42.3	91.9
	TO4-043-08	36.2	87.3
	TO4-043-09	67.6	98.0
	TO4-043-10	-1.7	66.2
	TO6-104-01	24.8	79.7
	TO6-104-02	-16.3	25.3
	TO6-104-03	-15.0	34.8
	TO6-104-04	-23.2	18.4
	TO6-104-05	88.6	100.0
	TO6-104-06	-4.5	33.8
	TO6-104-07	-38.1	12.2
	TO6-104-08	-32.5	20.3
	TO6-104-09	9.7	74.8
	TO6-104-10	-44.6	99.9
	Mean (± S. E.)	**38.5 (± 7.5)**	**78.0 (± 5.3)**

Table A2.1 continued

Season	Extract ID	Percentage inhibition at 50 µg/mL	Percentage inhibition at 100 µg/mL
Fall	TO3-005-01	36.0	90.4
	TO3-007-01	30.3	86.8
	TO3-009-01	13.1	64.8
	TO3-011-01	-29.5	26.5
	TO3-013-01	3.0	36.7
	TO3-015-01	4.8	52.5
	TO3-017-01	-41.2	-2.3
	TO3-019-01	56.2	97.9
	TO3-021-01	12.5	74.6
	TO3-023-01	36.1	88.5
	TO4-167-01	24.5	99.9
	TO4-167-02	85.4	58.0
	TO4-167-03	26.0	98.3
	TO4-167-04	59.6	99.5
	TO4-167-05	74.5	56.8
	TO4-167-06	28.2	67.5
	TO4-167-07	37.2	58.2
	TO4-167-08	26.4	78.8
	TO4-167-09	34.2	98.4
	TO4-167-10	64.5	88.5
	TO7-020-01	84.4	100.1
	TO7-020-02	100.3	58.2
	TO7-020-03	-31.0	100.0
	TO7-020-04	99.4	100.0
	TO7-020-05	100.1	95.2
	TO7-020-06	31.9	99.8
	TO7-020-07	100.2	100.0
	TO7-020-08	100.1	99.6
	TO7-020-09	99.9	99.9
	TO7-020-10	100.1	88.5
	Mean (± S. E.).	**45.6 (± 7.8)**	**78.7 (± 4.8)**

Table A2.2 *Heracleum maximum* summer crude extracts and their respective antimycobacterial activity (percent inhibition) against *Mycobacterium tuberculosis* at 50 µg/mL and 100 µg/mL.

Tissue type	Extract ID	Percentage inhibition at 50 µg/mL	Percentage inhibition at 100 µg/mL
Root	TO2-083-01	33.0	90.3
	TO2-087-01	59.5	96.6
	TO2-091-01	71.7	99.4
	TO2-095-01	74.1	99.7
	TO2-099-01	60.1	97.6
	TO2-103-01	29.3	84.4
	TO2-107-01	73.2	99.9
	TO2-111-01	81.3	100.2
	TO2-115-01	64.8	99.6
	TO2-119-01	50.6	99.1
	TO4-043-01	55.2	95.1
	TO4-043-02	90.9	99.8
	TO4-043-03	46.1	89.8
	TO4-043-04	43.8	87.6
	TO4-043-05	44.7	87.3
	TO4-043-06	100.2	100.0
	TO4-043-07	42.3	91.9
	TO4-043-08	36.1	87.3
	TO4-043-09	67.6	98.0
	TO4-043-10	-1.7	42.4
	TO6-104-01	24.8	79.7
	TO6-104-02	-16.3	25.3
	TO6-104-03	-15.0	34.8
	TO6-104-04	-23.2	18.4
	TO6-104-05	88.6	100.0
	TO6-104-06	-4.5	33.8
	TO6-104-07	-38.1	12.2
	TO6-104-08	-32.5	20.3
	TO6-104-09	9.7	74.8
	TO6-104-10	-44.6	12.8
	Mean (± S. E.)	**35.7 (± 7.5)**	**75.3 (± 5.8)**

Table A2.2 continued

Tissue type	Extract ID	Percentage inhibition at 50 µg/mL	Percentage inhibition at 100 µg/mL
Stem	TO2-129-01	5.5	40.5
	TO2-131-01	49.5	96.8
	TO2-133-01	-0.9	36.6
	TO2-135-01	2.2	36.0
	TO2-137-01	2.6	48.4
	TO2-139-01	8.8	45.3
	TO2-141-01	14.7	61.5
	TO2-143-01	14.7	60.0
	TO2-145-01	8.5	53.6
	TO2-147-01	5.3	47.4
	TO4-047-01	2.0	51.4
	TO4-047-02	-6.2	9.3
	TO4-047-03	6.0	35.6
	TO4-047-04	-15.0	7.5
	TO4-047-05	-16.9	10.2
	TO4-047-06	-2.6	24.2
	TO4-047-07	-22.9	-14.8
	TO4-047-08	-21.9	-0.5
	TO4-047-09	-7.9	43.8
	TO4-047-10	-12.3	-4.6
	TO6-106-01	81.0	98.9
	TO6-106-02	53.3	97.5
	TO6-106-03	-3.9	33.9
	TO6-106-04	-7.5	32.3
	TO6-106-05	65.4	99.3
	TO6-106-06	24.7	84.9
	TO6-106-07	83.2	99.4
	TO6-106-08	49.5	99.00
	TO6-106-09	53.6	98.9
	TO6-106-10	2.8	56.8
	Mean (± S. E.)	**13.8 (± 5.4)**	**49.6 (± 6.3)**

Table A2.2 continued

Tissue type	Extract ID	Percentage inhibition at 50 µg/mL	Percentage inhibition at 100 µg/mL
Leaf	TO2-149-01	30.5	50.9
	TO2-151-01	32.7	62.8
	TO2-153-01	31.4	58.0
	TO2-155-01	31.7	62.9
	TO2-157-01	28.2	59.1
	TO2-159-01	37.0	55.3
	TO2-161-01	42.4	62.4
	TO2-163-01	37.1	59.1
	TO2-165-01	35.7	64.5
	TO2-167-01	38.6	66.6
	TO4-051-01	33.9	58.7
	TO4-051-02	32.4	54.0
	TO4-051-03	27.4	51.8
	TO4-051-04	-11.7	65.0
	TO4-051-05	2.7	47.8
	TO4-051-06	28.8	53.5
	TO4-051-07	29.5	51.6
	TO4-051-08	27.6	53.7
	TO4-051-09	2.6	44.7
	TO4-051-10	-7.1	97.9
	TO6-107-01	40.4	77.2
	TO6-107-02	26.7	68.1
	TO6-107-03	27.1	55.8
	TO6-107-04	48.6	93.1
	TO6-107-05	52.6	92.4
	TO6-107-06	46.3	86.9
	TO6-107-07	42.3	90.7
	TO6-107-08	27.4	56.0
	TO6-107-09	36.5	73.3
	TO6-107-10	30.8	73.0
	Mean (± S. E.)	**29.7 (± 2.7)**	**64.9 (± 2.7)**

Table A2.2 continued

Tissue type	Extract ID	Percentage inhibition at 50 µg/mL	Percentage inhibition at 100 µg/mL
Flower	TO2-169-01	45.3	78.4
	TO2-171-01	58.0	92.4
	TO2-173-01	40.8	78.3
	TO2-175-01	61.5	94.5
	TO2-177-01	54.9	86.5
	TO2-179-01	55.6	86.8
	TO2-181-01	58.6	84.2
	TO2-183-01	65.1	92.1
	TO4-055-01	59.1	91.8
	TO4-055-02	28.1	77.9
	TO4-055-03	62.9	92.2
	TO4-055-04	43.1	91.1
	TO4-055-05	30.2	67.4
	TO4-055-06	38.1	80.7
	TO4-055-07	-3.31	95.1
	TO4-055-08	33.2	89.7
	TO4-055-09	38.1	82.1
	TO4-055-10	48.4	89.7
	TO6-105-01	82.5	99.8
	TO6-105-02	99.6	100.1
	TO6-105-03	81.6	99.7
	TO6-105-04	82.9	99.8
	TO6-105-05	86.2	99.8
	TO6-105-06	90.8	99.9
	TO6-105-07	94.5	99.8
	TO6-105-08	85.8	98.5
	TO6-105-09	75.2	98.5
	TO6-105-10	98.5	99.9
	Mean (± S. E.)	**60.5 (± 4.7)**	**91.0 (± 1.7)**

Appendix 3 Average percent falcarindiol in *Heracleum maximum* crude extracts.

Table A3.1 *Heracleum maximum* root crude extracts from three seasons and their respective average percentage falcarindiol content as measured by HPLC and qNMR respectively.

Season	Extract ID	Average % falcarindiol measured by HPLC	Average % falcarindiol measured by qNMR
Spring	TO2-043-01	5.2	10.5
	TO2-047-01	5.8	8.4
	TO2-051-01	13.7	13.6
	TO2-055-01	9.5	9.1
	TO2-059-01	16.3	11.4
	TO2-063-01	37.0	12.2
	TO2-067-01	17.7	10.6
	TO2-071-01	10.4	9.1
	TO2-075-01	4.3	9.6
	TO2-079-01	3.7	9.8
	TO4-003-01	4.9	11.3
	TO4-003-02	8.3	9.1
	TO4-003-03	1.1	9.4
	TO4-003-04	3.0	9.3
	TO4-003-05	8.3	11.6
	TO4-003-06	6.4	11.9
	TO4-003-07	1.8	7.0
	TO4-003-08	1.6	10.2
	TO4-003-09	5.6	9.7
	TO4-003-10	6.6	10.7
	TO6-002-01	2.5	14.3
	TO6-002-02	2.0	12.0
	TO6-002-03	1.3	11.2
	TO6-002-04	2.3	9.2
	TO6-002-05	0.5	9.0
	TO6-002-06	1.0	9.2
	TO6-002-07	11.0	11.6
	TO6-002-08	0.3	9.0
	TO6-002-09	6.3	9.5
	TO6-002-10	2.0	10.9
	Mean (± S. E.)	**6.7 (± 1.3)**	**10.3 (± 0.3)**

Table A3.1 continued

Season	Extract ID	Average % falcarindiol measured by HPLC	Average % falcarindiol measured by qNMR
Summer	TO2-083-01	6.8	13.6
	TO2-087-01	12.4	13.7
	TO2-091-01	11.6	12.1
	TO2-095-01	13.4	12.5
	TO2-099-01	13.6	12.8
	TO2-103-01	10.0	14.9
	TO2-107-01	8.0	15.2
	TO2-111-01	6.5	9.2
	TO2-115-01	7.5	9.2
	TO2-119-01	8.1	11.2
	TO4-043-01	16.0	17.2
	TO4-043-02	9.1	12.0
	TO4-043-03	4.4	11.5
	TO4-043-04	3.0	15.5
	TO4-043-05	4.9	9.8
	TO4-043-06	6.6	15.7
	TO4-043-07	3.9	12.3
	TO4-043-08	4.1	12.2
	TO4-043-09	5.3	14.6
	TO4-043-10	0.5	0.0
	TO6-104-01	0.0	14.6
	TO6-104-02	5.2	14.4
	TO6-104-03	2.3	12.0
	TO6-104-04	2.1	10.7
	TO6-104-05	3.6	12.4
	TO6-104-06	1.9	12.0
	TO6-104-07	4.1	11.8
	TO6-104-08	9.3	15.0
	TO6-104-09	7.5	10.6
	TO6-104-10	9.4	10.7
	Mean (± S. E.)	**6.7 (± 0.7)**	**12.3 (± 0.6)**

Table A3.1 continued

Season	Extract ID	Average % falcarindiol measured by HPLC	Average % falcarindiol measured by qNMR
Fall	TO3-005-01	6.7	11.7
	TO3-007-01	6.9	7.5
	TO3-009-01	3.8	11.3
	TO3-011-01	9.2	12.6
	TO3-013-01	6.0	10.2
	TO3-015-01	6.1	10.8
	TO3-017-01	6.5	7.1
	TO3-019-01	5.9	9.3
	TO3-021-01	5.1	10.2
	TO3-023-01	6.6	15.2
	TO4-167-01	4.9	0.0
	TO4-167-02	8.3	0.0
	TO4-167-03	1.1	0.0
	TO4-167-04	3.0	9.8
	TO4-167-05	8.3	8.6
	TO4-167-06	6.4	10.4
	TO4-167-07	1.8	0.0
	TO4-167-08	1.6	7.9
	TO4-167-09	5.6	9.5
	TO4-167-10	6.6	9.0
	TO7-020-01	3.1	19.8
	TO7-020-02	0.0	11.7
	TO7-020-03	0.0	13.5
	TO7-020-04	0.0	10.7
	TO7-020-05	0.0	15.1
	TO7-020-06	8.2	10.6
	TO7-020-07	0.0	13.0
	TO7-020-08	0.0	13.5
	TO7-020-09	7.9	11.8
	TO7-020-10	3.1	12.1
	Mean (± S. E.)	**4.4 (± 0.6)**	**9.8 (± 0.8)**

Table A3.2 *Heracleum maximum* summer crude extracts from four tissues and their respective average percentage falcarindiol content as measured by HPLC and qNMR respectively.

Tissue type	Extract ID	Average % falcarindiol measured by HPLC	Average % falcarindiol measured by qNMR
Root	TO2-083-01	6.8	13.6
	TO2-087-01	12.4	13.7
	TO2-091-01	11.6	12.1
	TO2-095-01	13.4	12.5
	TO2-099-01	13.6	12.8
	TO2-103-01	10.0	14.9
	TO2-107-01	7.9	15.2
	TO2-111-01	6.5	9.2
	TO2-115-01	7.5	9.2
	TO2-119-01	8.1	11.2
	TO4-043-01	16.0	17.2
	TO4-043-02	9.1	12.0
	TO4-043-03	4.4	11.5
	TO4-043-04	3.0	15.5
	TO4-043-05	4.8	9.8
	TO4-043-06	6.6	15.7
	TO4-043-07	3.9	12.3
	TO4-043-08	4.1	12.2
	TO4-043-09	5.3	14.6
	TO4-043-10	0.5	0.0
	TO6-104-01	0.0	14.6
	TO6-104-02	5.2	14.4
	TO6-104-03	2.3	12.0
	TO6-104-04	2.1	10.7
	TO6-104-05	3.6	12.4
	TO6-104-06	1.9	12.0
	TO6-104-07	4.1	11.8
	TO6-104-08	9.3	15.0
	TO6-104-09	7.5	10.6
	TO6-104-10	9.4	10.7
	Mean (± S. E.)	**6.7 (± 0.7)**	**12.3 (± 0.6)**

Table A3.2 continued

Tissue type	Extract ID	Average % falcarindiol measured by HPLC	Average % falcarindiol measured by qNMR
Stem	TO2-129-01	0.8	0.0
	TO2-131-01	6.4	0.0
	TO2-133-01	1.2	0.0
	TO2-135-01	0.4	6.9
	TO2-137-01	0.5	0.0
	TO2-139-01	1.8	6.9
	TO2-141-01	2.4	3.6
	TO2-143-01	1.3	0.0
	TO2-145-01	1.7	5.7
	TO2-147-01	1.3	5.4
	TO4-047-01	1.2	0.0
	TO4-047-02	0.4	0.0
	TO4-047-03	0.8	6.8
	TO4-047-04	0.0	0.0
	TO4-047-05	0.0	0.0
	TO4-047-06	13	0.0
	TO4-047-07	0.2	0.0
	TO4-047-08	0.0	0.0
	TO4-047-09	1.2	0.0
	TO4-047-10	0.0	0.0
	TO6-106-01	0.3	0.0
	TO6-106-02	0.0	0.0
	TO6-106-03	0.0	0.0
	TO6-106-04	0.0	0.0
	TO6-106-05	0.0	0.0
	TO6-106-06	0.0	0.0
	TO6-106-07	0.0	0.0
	TO6-106-08	0.0	0.0
	TO6-106-09	0.2	0.0
	TO6-106-10	0.4	7.9
	Mean (± S. E.)	**1.2 (± 0.5)**	**1.4 (± 0.5)**

Table A3.2 continued

Tissue type	Extract ID	Average % falcarindiol measured by HPLC	Average % falcarindiol measured by qNMR
Leaf	TO2-149-01	0.5	0.0
	TO2-151-01	0.9	6.5
	TO2-153-01	0.5	0.0
	TO2-155-01	0.7	0.0
	TO2-157-01	1.2	0.0
	TO2-159-01	0.7	0.0
	TO2-161-01	0.9	0.0
	TO2-163-01	0.5	0.0
	TO2-165-01	0.6	0.0
	TO2-167-01	1.2	0.0
	TO4-051-01	0.5	0.0
	TO4-051-02	0.3	0.0
	TO4-051-03	0.2	0.0
	TO4-051-04	0.0	0.0
	TO4-051-05	1.1	6.2
	TO4-051-06	0.3	0.0
	TO4-051-07	0.5	6.6
	TO4-051-08	0.4	0.0
	TO4-051-09	0.4	0.0
	TO4-051-10	0.0	0.0
	TO6-107-01	0.0	0.0
	TO6-107-02	0.0	5.2
	TO6-107-03	0.0	0.0
	TO6-107-04	0.0	0.0
	TO6-107-05	0.0	0.0
	TO6-107-06	0.0	0.0
	TO6-107-07	0.0	0.0
	TO6-107-08	0.0	0.0
	TO6-107-09	0.0	0.0
	TO6-107-10	0.0	0.0
	Mean (± S. E.)	**0.4 (± 0.07)**	**0.8 (± 0.4)**

Table A3.2 continued

Tissue type	Extract ID	Average % falcarindiol measured by HPLC	Average % falcarindiol measured by qNMR
Flower	TO2-169-01	2.2	0.0
	TO2-173-01	1.6	7.7
	TO2-177-01	1.7	5.2
	TO2-179-01	2.6	3.7
	TO2-183-01	0	0.0
	TO4-055-01	1.1	0.0
	TO4-055-02	0.0	0.0
	TO4-055-03	1.4	0.0
	TO4-055-04	0.8	0.0
	TO4-055-05	0.8	0.0
	TO4-055-06	0.9	0.0
	TO4-055-07	0.2	0.0
	TO4-055-08	0.3	0.0
	TO4-055-09	0.0	0.0
	TO4-055-10	1.1	0.0
	TO6-105-02	1.8	0.0
	TO6-105-03	1.2	5.2
	TO6-105-04	4.3	0.0
	TO6-105-05	0.5	0.0
	TO6-105-06	3.7	0.0
	TO6-105-07	1.1	0.0
	TO6-105-08	0.9	0.0
	TO6-105-09	0.5	0.0
	TO6-105-10	N/A	4.5
	Mean (± S. E.)	**1.2 (± 0.23)**	**1.1 (± 0.5)**

Appendix 4 Falcarindiol content in *Heracleum maximum* crude extracts measured by HPLC.

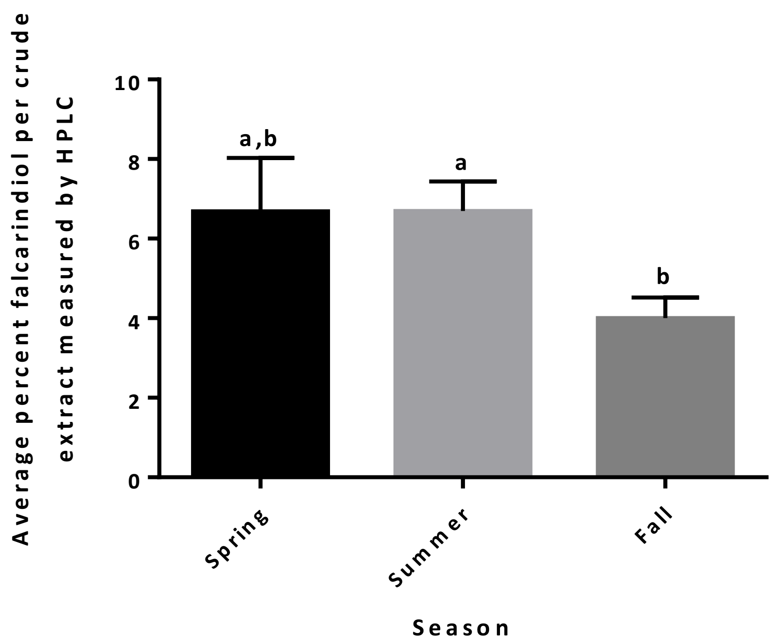

Figure A4.1 Average percentage of falcarindiol in *Heracleum maximum* root extracts in different seasons (spring, summer, and fall) measured by HPLC (bars indicate standard error; separate homogenous groups are denoted by lowercase letters).

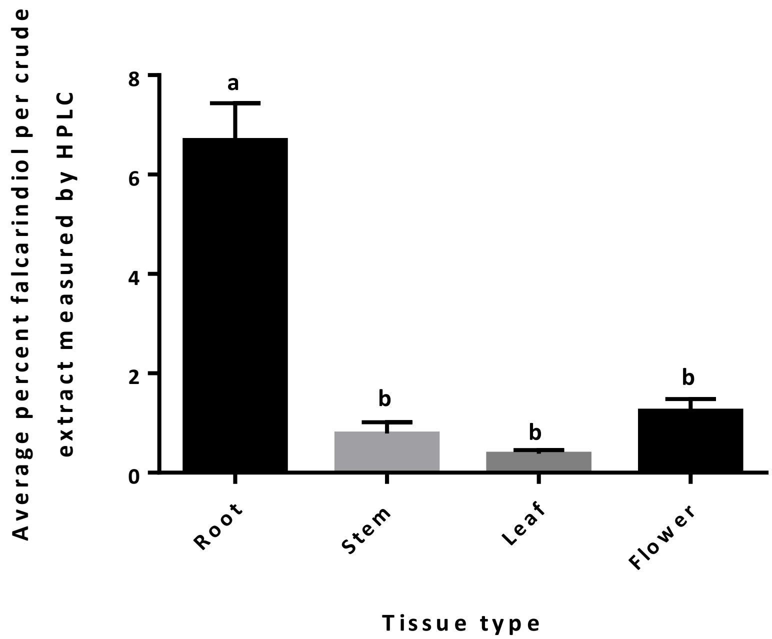

Figure A4.2 Average percentage of falcarindiol in *Heracleum maximum* summer extracts in different tissues (root, stem, leaf, and flower) measured by HPLC (bars indicate standard error; separate homogenous groups are denoted by lowercase letters).

Appendix 5 P values for statistical analyses

Table A5.1 P values for seasonal differences in percent inhibition of *Mycobacterium tuberculosis* at 50 µg/mL against *Heracleum maximum* root extracts.

Difference	P value
Overall	0.666
Spring vs. summer	0.583
Spring vs. fall	0.657
Summer vs. fall	0.308

Table A5.2 P values for seasonal differences in percent inhibition of *Mycobacterium tuberculosis* at 100 µg/mL against *Heracleum maximum* root extracts.

Difference	P value
Overall	0.866
Spring vs. summer	0.621
Spring vs. fall	0.947
Summer vs. fall	0.677

Table A5.3 P values for tissue differences in percent inhibition of *Mycobacterium tuberculosis* at 50 µg/mL against *Heracleum maximum* summer extracts.

Difference	P value
Overall	0.000
Root vs. stem	0.015
Root vs. leaf	0.513
Root vs. flower	0.002
Stem vs. leaf	0.095
Stem vs. flower	0.000
Leaf vs. flower	0.001

Table A5.4 P values for tissue differences in percent inhibition of *Mycobacterium tuberculosis* at 100 µg/mL against *Heracleum maximum* summer extracts.

Difference	P value
Overall	0.000
Root vs. stem	0.003
Root vs. leaf	0.189
Root vs. flower	0.041
Stem vs. leaf	0.048
Stem vs. flower	0.000
Leaf vs. flower	0.002

Table A5.5 P values for seasonal differences in falcarindiol content in *Heracleum maximum* extracts measured by HPLC.

Difference	P value
Overall	0.053
Spring vs. summer	0.994
Spring vs. fall	0.051
Summer vs. fall	0.051

Table A5.6 P values for tissue differences in falcarindiol content in *Heracleum maximum* extracts measured by HPLC.

Difference	P value
Overall	0.000
Root vs. stem	0.000
Root vs. leaf	0.000
Root vs. flower	0.000
Stem vs. leaf	0.664
Stem vs. flower	0.433
Leaf vs. flower	0.380

Table A5.7 P values for seasonal differences in falcarindiol content in *Heracleum maximum* extracts measured by qNMR.

Difference	P value
Overall	0.004
Spring vs. summer	0.022
Spring vs. fall	0.409
Summer vs. fall	0.001

Table A5.8 P values for tissue differences in falcarindiol content in *Heracleum maximum* extracts measured by qNMR.

Difference	P value
Overall	0.000
Root vs. stem	0.000
Root vs. leaf	0.000
Root vs. flower	0.000
Stem vs. leaf	0.691
Stem vs. flower	0.989
Leaf vs. flower	0.844

Appendix 6 R square and P values for correlations

Table A6.1 R square and P values for the correlation between falcarindiol content measured by qNMR and antimycobacterial activity in *Heracleum maximum* seasonal extracts.

Difference	R^2 value	P value
Spring	0.200	0.013
Summer	0.046	0.257
Fall	0.054	0.218

Table A6.2 R square and P values for the correlation between falcarindiol content measured by qNMR and antimycobacterial activity in *Heracleum maximum* tissue extracts.

Difference	R^2 value	P value
Root	0.046	0.257
Stem	0.002	0.834
Leaf	0.046	0.256
Flower	0.008	0.683

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2015 Science Atlantic Chemistry Conference, Fredericton, New Brunswick (May 2015)
Poster Presentation: "Variation of the amount of falcarindiol, an antimycobacterial natural product, within the Canadian medicinal plant *Heracleum maximum*." Melvin, K., O'Neill, T. E., Webster, D., Houlahan, J. E., Johnson, J. A., Gray, C. A.

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2015 Interprofessional Health Research Day, Saint John Regional Hospital, Saint John, New Brunswick (March 2015)
Poster Presentation: "Variation of the amount of falcarindiol, an antimycobacterial natural product, within the Canadian medicinal plant *Heracleum maximum*." Melvin, K., O'Neill, T. E., Webster, D., Houlahan, J. E., Johnson, J. A., Gray, C. A.

2014 New Brunswick Health Research Foundation 6th Annual Health Research Conference, Moncton, New Brunswick (November 2014)
Poster Presentation: "Variation of the amount of falcarindiol, an antimycobacterial natural product, within the Canadian medicinal plant *Heracleum maximum*." Melvin, K., O'Neill, T. E., Webster, D., Houlahan, J. E., Johnson, J. A., Gray, C. A.

2014 Maritime Natural Products Conference, University of Prince Edward Island, Charlottetown, Prince Edward Island (August 2014)
Oral Presentation: "Seasonal and tissue variation of falcarindiol, an antimycobacterial constituent of *Heracleum maximum*." Melvin, K., O'Neill, T. E., Webster, D., Houlahan, J. E., Johnson, J. A., Gray, C. A.

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2014 Interprofessional Health Research Day, Saint John Regional Hospital, Saint John, New Brunswick (March 2014)
Poster Presentation: "Seasonal and tissue variation of falcarindiol, the primary antimycobacterial constituent of the Canadian medicinal plant *Heracleum maximum.*" Melvin, K., O'Neill, T. E., Webster, D., Johnson, J. A., Gray, C. A.

2013 New Brunswick Health Research Foundation 5th Annual Health Research Conference, Saint John, New Brunswick (November 2013)
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2013 Maritime Natural Products Conference, St. Francis Xavier University, Antigonish, Nova Scotia (August 2013)
Oral Presentation: "Seasonal and tissue variation of falcarindiol, an antimycobacterial constituent of *Heracleum maximum*." Melvin, K., O'Neill, T. E., Webster, D., Johnson, J. A., Gray, C. A.

2013 Interprofessional Health Research Day, Saint John Regional Hospital, Saint John, New Brunswick (March 2013)
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