A phytochemical and antibacterial analysis of *Echinacea purpurea* (L.) Moench throughout seasonal development

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ABSTRACT

Echinacea purpurea is consumed as a natural health product around the world. Due to the genus' ethnobotanical relevance, the phytochemistry of *Echinacea* has been extensively studied, revealing a variety of bioactive metabolites including caffeic acid derivatives and alkylamides. Whereas seasonal trends in root chemistry have been established, trends in other plant parts are relatively understudied. Similarly, few studies have evaluated the effects of organic plant growth substances in field trials. With increased demand for organic products, industry is looking for alternative ways to optimize yields and medicinal properties.

For this thesis, my first objective was to quantify the concentrations of *E. purpurea*'s secondary metabolites across organic treatments throughout the plant's first growth year to determine optimal harvesting time and conditions in all parts of the plant. The second objective was to determine how seasonal variations affect its potential bioactivity through inhibition of *Pseudomonas aeruginosa.*

Plants were grown in field plots treated with four different organic treatments: water (control), high cytokinin, low cytokinin, and fish oils; samples were collected biweekly from May-September. Dried plants were separated into major plant parts and were extracted exhaustively in 70% EtOH. Using high-pressure liquid chromatography (HPLC), concentrations of alkylamides and select caffeic acid derivatives were quantified in all samples and compared across plant part, developmental stage, and organic fertilizers. It was determined that while there were no major differences between treatments, phytochemical concentrations changed throughout the season in all plant parts; revealing that aerial parts of the plant also bioactive secondary metabolites and should not be excluded from future studies.

Following this study, an MIC50 assay was used to test these extracts against *P. aeruginosa* PA14. It was noted that seasonality effects of phytochemistry were not consistent with bioactivity and that there were no significant differences between extract and carrier control. While the antibiotic activity of root extracts varied seasonally, the flower extract exhibited the most consistent antibiotic potential. The results presented in this thesis will not only aid in industry practices and yield optimization but, through filling knowledge gaps on seasonality and organic treatments in field trials, will increase the understanding of *E. purpurea*'s chemistry and related bioactivity, with implications on both the medicinal properties and eco-physiology of the species.

RÉSUMÉ

Echinacea purpurea est consommé en tant que produit de santé naturel dans le monde entier. En raison de sa pertinence ethnobotanique, la phytochimie de l'échinacée a été étudiée profondément, révélant une variété de métabolites bioactifs, dont les dérivés de l'acide caféique et les alkylamides. Alors que les tendances saisonnières de la chimie des racines ont déjà été établies, les tendances chimiques dans les autres parties de la plante sont relativement peu étudiées. De même, peu d'études ont évalué les effets des substances organiques de croissance des plantes dans des essais terrain. En raison d'une demande croissante de produits biologiques, l'industrie cherche des alternatifs pour optimiser les propriétés médicinales.

Le premier objectif de cette étude est de quantifier les concentrations des métabolites secondaires d'*E. purpurea* dans des traitements organiques tout au long de la première année de croissance afin de déterminer la durée de croissance et les conditions optimales de récolte à travers les différentes parties de la plante. Le deuxième objectif est de déterminer comment les variations saisonnières affectent la bioactivité potentielle en inhibant *Pseudomonas aeruginosa*.

Les plantes ont été cultivées dans des plantes traitées avec quatre traitements biologiques différents : eau (témoin), concentration élevée de haute cytokinine, faible cytokinine et huile de poisson ; afin d'évaluer les traitements, des échantillons ont été collectés toutes les deux semaines de mai à septembre. Les plantes séchées ont été séparées en composants principales, puis ont été extrait dans une solution d'éthanol de manière exhaustive. En utilisant la CLHP, les concentrations d'alkylamides et de dérivés d'acide caféique sélectionnés ont été quantifiées dans tous les échantillons, permettant de pouvoir comparer les résultats entre les différentes parties de la plante, le stade de développement, et les engrais organiques.

Il a été déterminé qu'il n'y avait pas de différences significatives entre les divers traitements. Cependant, les résultats ont démontré que les concentrations phytochimiques dans les différentes parties de la plante changeaient tout au long de la saison ; révélant que les parties aériennes de la plante contiennent également des taux élevés de métabolites secondaires et devraient être considérées pour des études approfondies.

Suivant cette étude, un test MIC50 a été effectué dans le but de tester les extraits contre *P. aeruginosa* PA14. Il a été noté que les effets saisonniers de la phytochimie n'étaient pas compatibles avec la bioactivité et qu'il n'y avait pas de différences significatives entre les extraits et les contrôles transporteurs. Les résultats présentés dans cette thèse contribueront non seulement à l'optimisation du rendement de l'industrie, mais permettront également de mieux comprendre la chimie et la bioactivité d'*E. purpurea* en comblant les lacunes dans les connaissances sur la saisonnalité et les traitements biologiques.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CAD	Caffeic acid derivatives
CAR	Cardiology
CB	Cannabinoid receptor
CC	Carrier control
CF	Cystic fibrosis
DAD	Diode array detector
DER	Dermatology
GEN	General
GIM	Gastro-Intestinal
GYN	Gynecological-urinary
HPLC	High-performance liquid chromatography
IL-6	interleukin 6
IL-1β	interleukin 1 beta
LB:	Lysogeny broth
MIC	Minimum inhibitory concentration
NAEB	Native American Ethnobotany Database
NEU	Neurological
NHP	Natural health product
ORT	Orthopedic
OTO	Oto-rhino laryngological
PTFE	Polytetrafluoroethylene
PUL	Pulmonary
SEM	Standard error of the mean
SMA	Standard methods agar
TNFα	Tumor necrosis factor alpha
QS	Quorum sensing

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CHAPTER 1: GENERAL INTRODUCTION

1.1 *Introduction*

Herbal remedies for an assortment of illnesses have grown in demand in recent years; however, this form of treatment is undeniably among the oldest forms of medicine (Lindstrom et al., 2014). Throughout history, more than 80,000 plant species have been used for medicinal purposes worldwide (Hossain et al., 2014). With issues such as increasing population, antibiotic resistance, drug interactions and adverse events, standard pharmaceutical drugs are a growing concern for many individuals. For this reason, natural health products (NHPs) are now used by 73% of Canadians on a regular basis (Canada & Ipsos Reid, 2010). One of the leading natural health products in the last 30 years is the *Echinacea* plant, commonly found in dosage forms ranging from tablets to teas.

Echinacea (Asteraceae) is a genus of a perennial flowering plants native to North America and is often referred to as the coneflower (Caruso & Gwaltney, 2005). While native to North America, *Echinacea*'s distribution does not extend to the west coast and remains mostly distributed across central to eastern regions of the continent (Figure 1.1). While there are 9 species in total, the most well-known species within the genus include *Echinacea angustifolia* DC., *Echinacea pallida* (Nutt.) Nutt. and *Echinacea purpurea* (L.) Moench. These species are similar in that they have purple petals, cone shaped flowers and basal leaves. Though similar in appearance, these species differ in morphometrics as well as phytochemistry and genetics (Binns et al., 2002). They also differ in historical uses as *E. angustifolia* has greater abundance of medicinal uses by First Nations communities for a multitude of illnesses (Moerman, 1999). In recent history, the commercialization of *E. purpurea* has surpassed that of the commercialization of *E. angustifolia* as *E. purpurea* does not face the same issues of dormancy and germination,

Figure 1.1. Canadian provinces and US states where *Echinacea* species are reported to be native to – represented by shading. Map created using ArcGis in North America with distribution data from Karetsz, 2015. Scale bar representing 4000km.

germination, making it one of the most popular herbal remedies currently available by NHP companies (Abbasi et al., 2007).

1.2 *Ethnobotany of Medicinal Uses*

Within the Asteraceae family, there is a series of plant tribes and subtribes with a multitude of medicinal uses, but the most commonly used tribes are Anthemideae and Heliantheae (Moerman 2003). In terms of trends in modern science, Heliantheae is a current research focus due to the extensive ethnobotany of *E. angustifolia* and its relatives *E. pallida* and *E. purpurea* which belong to the subtribe Zinniinae. For a visual representation, I have created a phylogenetic tree by referencing Urbastch et al.'s phylogeny from a study in 2009 and reported ethnobotany of medicinal uses found in the Native American Ethnobotany Database (Figure 1.2). Out of the 23 species of Zinniinae in North America, there are seven that have been reported to be used by First Nations traditionally for medicine (Kartesz, 2015; Moerman, 2003). By observation of Figure 1.2 – which highlights the ethnobotany of Zinniinae – the most cited species belong to the *Echinacea* genus with fewer species used throughout the other genera. It is also notable that *Echinacea* diverged later than the other genera as *Heliopsis,* the first to diverge, has the least amount of medicinal uses in comparison to the other genera (Urbatsch et al., 2009). The uses that are exemplified in this figure have been sorted into therapeutic categories and respective indications (Moerman, 1999). Figure 1.3 presents a comparison of the therapeutic categories associated with the four most used species of the Zinniinae, noting that "Oto-rhino-laryngological" (OTO) is the highest use category for *E. angustifolia, Echinacea pallida,* and *Sanvitalia abertii*; and second highest use category for *Zinnia grandiflora*. OTO includes the following sub-categories: ear remedy, eye medicine, nose medicine, oral aid, throat aid and

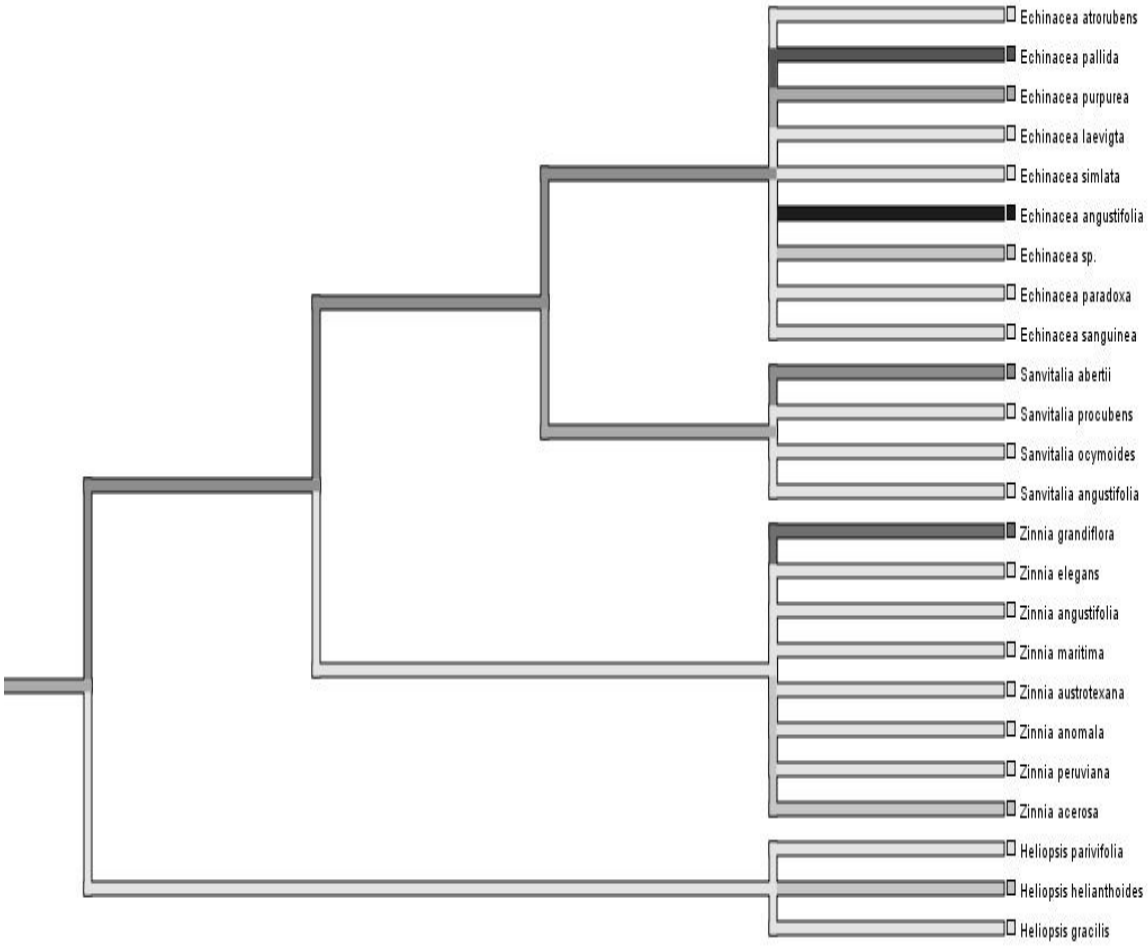

Figure 1.2. Schematic based off Urbatsch et al's (2009) phylogenetic tree showing North American species of subtribe Zinniinae. Shading of branches indicates number of drug uses reported in NAEB Database with increasing darkness representing increasing number of uses. Branch length is not quantified, used for genera grouping.

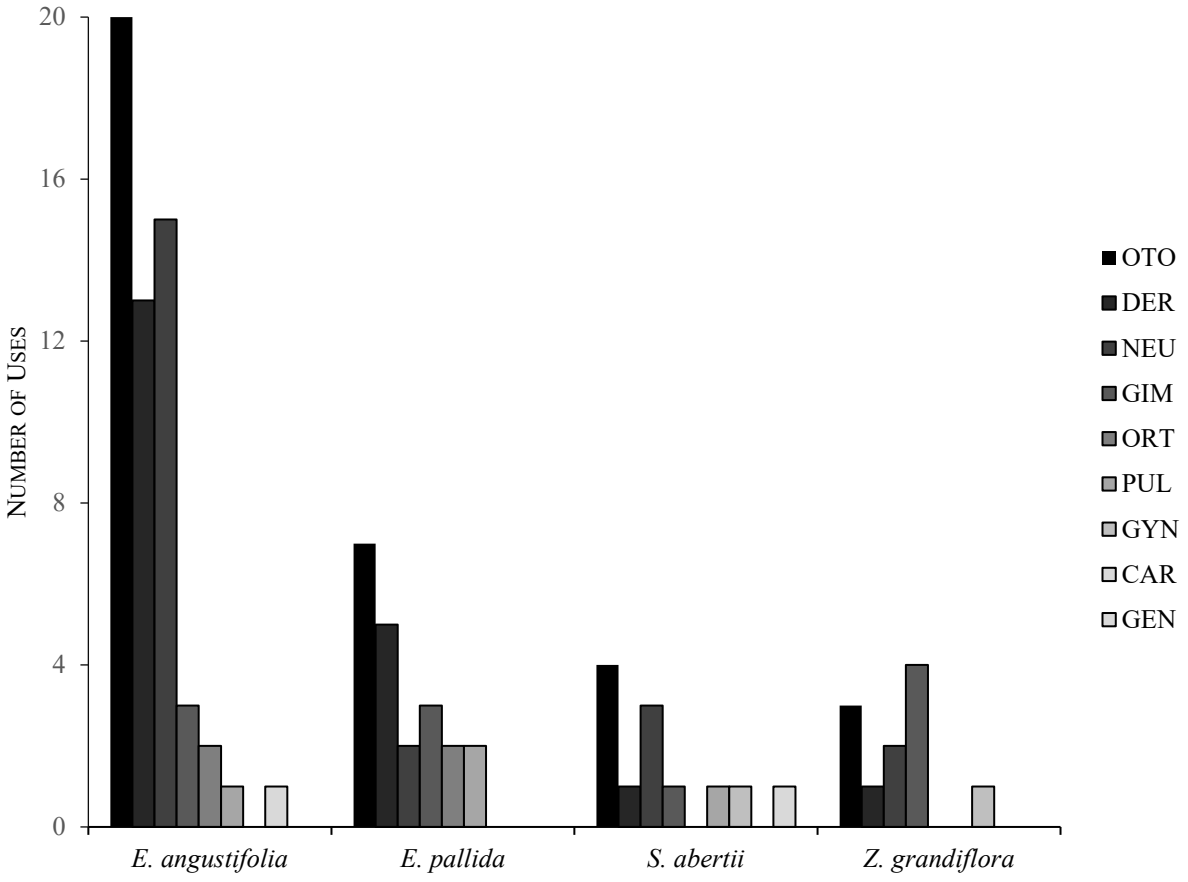

Figure 1.3. Number of medicinal use reports among commonly cited species of subtribe Zinniinae in North America by First Nations people. Uses separated by category based on characterizations in Moerman's paper in 1991. (OTO: Oto-rhino laryngological, DER: Dermatology, NEU: Neurological, GIM: Gastro-Intestinal, ORT: Orthopedic, PUL: Pulmonary, GYN: Gynecological-urinary, CAR: Cardiology, GEN: General).

toothache remedy. Zinniinae species appear to have been used disproportionately for these purposes across many different Indigenous peoples.

For example, as seen in Table 1.1, *E. angustifolia*'s roots have been used as a toothache remedy, oral aid, throat aid and an eye medicine by Blackfoot, Cheyenne, Dakota, Lakota, and at least six more First Nation communities in North America (Blankinship, 1905; Densmore, 1918; Gilmore, 1919; Johnston, 1987; Kraft, 1990; Rogers, 1980; Vestal and Schultes, 1939). The fact that several First Nation groups all select roots for similar treatments can be used not only as reason for further investigation as a remedy but can also be evidence of its effectiveness.

1.3 *Phytochemistry and bioactivity of Echinacea*

The chemistry of *E. purpurea* is complex and demonstrates a variation between major organs. Many constituents of the plant – most of which belong to the three large classes alkylamides, caffeic acid derivatives (CADs) and polysaccharides – have shown bioactivity against both plant and human pathogens (Barnes et al., 2005; Bergeron et al., 2002). These compounds are found throughout the plant but in different concentrations depending on plant part as well as variation within parts. The chemical composition of *Echinacea* may be complex, but not all compounds are found in high concentration. Not only do alkylamides and CADs represent the species' dominant phytochemistry they are also suspected to be the prominent factors in the plant's medicinal properties (Wills and Stuart 1999).

Phytochemical partition throughout the plant are often complemented by its bioactivity. In many cases, antimicrobial inhibition is differentiated between roots, leaves, flowers and stems with roots often showing greater inhibition. Chiellini et al. (2017) demonstrated that when tested

against pathogens that affect Cystic Fibrosis patients, roots showed the highest inhibition. However, a product containing only 5% roots and 95% above ground parts inhibited growth of *Streptococcus pyogenes, Haemophilus influenzae*, and *Legionella pneumophila* in another study (Sharma et al., 2010). This demonstrates that, although roots may be the most active, aerial parts are also important antimicrobials. *Echinacea* as a remedy for the common cold has possibly generated the most controversy. Many studies have shown contradicting results; however, some have shown significant reduction in the symptoms of rhinoviruses. Using juice pressed from *E. purpurea* leaves, Schoeneberger et al. (1992) examined its effects on cold symptoms in 108 participants. In children, there was a 50% decrease in common cold symptoms for those taking the *E. purpurea* juice over the participants who received a placebo. A meta-analysis conducted by Shah et al. (2007), observed 14 studies demonstrating evidence of the benefit of *Echinacea* in decreasing the development and longevity of the common cold by an average of 58%. While rhinoviruses may be the most well-known association with *Echinacea*, the plant has been reported to have viricidal effects against other viruses as well as inhibiting microbial growth (Table 1.2).

1.3.1 ALKYLAMIDES

Alkylamides or N-alkylamides are bioactive compounds found within over 100 plant species. Of the 178 identified alkylamides found in the Asteraceae family, at least 90 have been identified in species belonging to the tribe Heliantheae (Boonen et al., 2012). Their most studied role in natural health products is their effectiveness with immunomodulation through the interaction with the Endocannabinoid System (ECS) (Clifford at al., 2002; Woelkart & Bauer

Table 1.1. Reported Oto-rhino-laryngological uses of Zinniinae species in Native American

Plant name	Parts used	Traditional drug use	First Nation Community
Echinacea angustifolia DC.	Roots, whole plant	Toothache remedy, oral aid, throat aid, eye medicine	Blackfoot, Cheyenne, Dakota, Lakota, Montana, Omaha, Pawnee, Ponca, Sioux, Teton, Winnebago
Echinacea pallida (Nutt.) Nutt.	Roots	Throat aid, oral aid, toothache remedy, eye medicine	Cheyenne, Crow, Sioux
Echinacea sp.*	Roots	Throat aid, toothache remedy	Comanche
Sanvitalia abertii Gray	Whole plant	Oral aid, throat aid, toothache remedy	Navajo, Ramah
Zinnia grandiflora Nutt.	Whole plant	Throat aid, Eye medicine, Nose medicine	Navajo

Ethnobotany Database (NAEB) (Moerman, 2003)

* Species not recorded, could belong to *E. angustifolia*, *E. pallida* or another species not listed (Blankship 1905; Carlson & Volney 1940; Densmore 1918; Elmore 1944; Gilmore 1919; Grinnell 1972; Hart 1981; Hart 1992; Johnston 1987; Kraft 1990; Rogers 1980; Vestal & Schultes 1939; Vestal 1952).

2007; Woelkart et al., 2005). Select alkylamides can bind to the two receptors that are responsible for regulating pain and inflammation, among other physiological functions, mimicking the well-studied endocannabinoid ligand anandamide (Di Marzo et al., 2004; Urbatsch et al., 2000; Gertsch et al., 2010; Raduner et al., 2006). Since cannabinoids are defined as "any plant-derived natural product capable of either directly interacting with cannabinoid receptors or sharing chemical similarity with cannabinoids or both" (Gertsch et al., 2010), alkylamides can be considered cannabinoids. Plants containing alkylamides have been used in many traditional medicine systems for various illnesses including coughing, toothaches, joint pain, rhinitis, bronchitis, epilepsy, headaches and more; many of these symptoms belonging to the OTO system (Table 1.1) (Zhang et al., 2005; Sharma et al., 2011; Khare, 2008; Wilson et al., 2007; Leporatti & Ghedira, 2009; Di Stasi et al., 2002).

1.3.2 Caffeic acid derivatives

Caffeic acid derivatives are a group of phenolics often associated with their antioxidant activity (Silva et al., 2000). They are found across many families in the plant kingdom and are more widespread than alkylamides (Silva et al., 2014; Razzaghi-Asl et al., 2013; Boonen et al., 2012). Some of *E. purpurea*'s most abundant CADs include caftaric acid and cichoric acid. The majority of the reported bioactivities are often associated with cichoric acid, and include effects such as antiviral activity, inhibiting HIV-1 integrase, antihyaluoranidase activity, and the protection of collagen from free radicals (Pellati et al., 2004; Charvat et al., 2006; Liu et al., 2006; Healy et al., 2009; Cheminat et al., 1988). While caftaric acid is often overlooked in comparison, it is responsible for the inhibition of free radical production and lipid peroxidation which are often associated with the development of inflammation (Stanisavljević et al., 2009;

Speroni et al., 2002). While these are important medicinal effects often used as a marker of *Echinacea*`s effectiveness; it has been reported that CADs are not bioavailable through oral administration, which makes their effects in humans questionable (Matthias et al 2004; Zolgharnein et al., 2010), or at least limited to the gut.

1.4 *Rationale and research objectives*

The goal of this study was to evaluate the phytochemical variability of *E. purpurea* through analyzing concentrations of bioactive compounds across plant parts, growing season and field treatments. This variability was also studied in terms of antimicrobial potential throughout growth to determine if phytochemistry concentrations correspond to the level of bacterial growth inhibition. Due the extensive ethnobotanical uses of *Echinacea*, its reported bioactivity, and its value in the natural health product market, it is useful to further study its chemical and antimicrobial properties in relation to seasonal variation and agricultural practice. Investigation into these variables may aid in mitigating the inconsistencies found amongst natural health products as well as provide further insight into the potential applications of the plant.

Table 1.2. Antiviral, antibacterial and antifungal reports of *E. purpurea* (+: inhibitory activity; ++: high inhibitory activity)

Species/Virus	Type	Activity of *E. purpurea* (+ or ++)
Clostridium difficile	Bacterium	++
Hemophilus influenzae	Bacterium	++
Klebsiella pneumoniae	Bacterium	+
Legionella pneumophila	Bacterium	++
Mycobacterium smegmatis	Bacterium	+
Propionibacterium acnes	Bacterium	++
Staphylococcus aureus	Bacterium	+
Streptococcus pyogenes	Bacterium	++
Candida albicans	Fungi	+
Calicivirus	Virus	+
Coronavirus	Virus	+
Herpes viruses	Virus	++
Influenza viruses	Virus	++
Rhinoviruses	Virus	+

Data from Sharma et al. 2008, Sharma et al. 2010, Vimalanathan et al. 2005 and Pleschka et al. 2009.

CHAPTER 2: A QUANTITATIVE PROFILE OF SEASONAL VARIATIONS OF PHYTOCHEMICALS IN ORGANICALLY TREATED *ECHINACEA PURPUREA* (L.) MOENCH

2.1 INTRODUCTION

The phytochemistry of *Echinacea purpurea* (L.) Moench is complex and varies between organs and developmental stage. Many chemical constituents throughout the plant have shown medicinal effects but these chemicals vary in concentrations (Barnes et al., 2005; Bergeron et al., 2002). Not only do these secondary metabolite levels vary between species, there is also a variation in levels between different plant parts of the same individual (Perry et al., 1997; Hudson et al., 2005), as well as within the same plant part during different times in the growing season (Thomsen et al., 2012). For this reason, it was of interest to determine the concentrations of these compounds across both plant organs and developmental stages.

The main bioactive chemicals include alkylamides, caffeic acid derivatives, polysaccharides, polyacetylenes, alkenes and glycoproteins (Barnes et al., 2005; Manček and Kreft 2005). While polysaccharides and glycoproteins are often associated with the immune-stimulant bioactivity of the plant, research suggests that alkylamides and caffeic acid derivatives (CADs) are the prominent factors in the plant's antimicrobial properties (Tsai et al., 2012).

2.1.1 *Alkylamides*

Alkylamides are lipophilic compounds that have been of interest for decades due to their numerous biological activities, most notably their activity as anti-inflammatory phytoceuticals

(Boonen et al., 2012; Clifford at al., 2002). In *Echinacea,* they are mainly made up of a poly-unsaturated fatty acid with acetylenic bonds and an isobutylamide moeity (Boonen et al., 2015; Romero et al., 2009). These compounds are commonly recognized as being the core of *Echinacea*'s phytochemistry (Barnes et al., 2005). Even though nearly 30 alkylamides can be found in *E. purpurea,* the E and Z isomers of alkylamide dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide (Figure 2.1) account for nearly one-third of the total alkylamides within the plant (Stuart and Wills, 2000). These isomers are difficult to separate through high-performance liquid chromatography (HPLC) and thus they are often referred to together as alkylamides 8/9 (Cech et al., 2006). Alkylamide variation throughout *Echinacea*'s major organs is notable as roots contain ~70% of the plants total alkylamides with flowers, stems and leaves containing 20%, 10% and 1%, respectively (Stuart and Wills, 2000).

2.1.2 *Caffeic Acid Derivatives*

Another group of phytochemicals that contribute to *Echinacea*'s pharmacological activity are caffeic acid derivatives (CADs). Unlike alkylamides, which are more restricted in their distribution across taxa, phenolic metabolites are ubiquitous among plants. While the collection of CADs found in *Echinacea* is distinctive, these compounds are found in many plant families. Two of *E. purpurea*'s most well-studied CADs are caftaric acid and cichoric acid (Figure 2.1) as they are the most prominent polyphenols present in this species (Ortiz-Castro et al., 2010). Along with their roles in plant defenses such as anti-herbivory and interspecies defence, CADs are also associated with the immunostimulant and antioxidant effects of *Echinacea* (Bergeron et al., 2002; Thygesen et al., 2007; Dias et al., 2012); however, tests in oral administration show that they lack bioavailability thus making the extent of their activity in humans debateable (Matthias

et al., 2004). Cichoric acid is a highly abundant CAD in *E. purpurea* with up to 20% of total concentrations in the roots, with flowers, stems and leaves containing 35%, 10% and 35%, respectively (Stuart and Wills, 2000). In terms of natural health products, the CAD allocation in parts would suggest that products should include flowers and leaves as those are the most highly concentrated which is true for certain NHPs, whereas other companies choose to focus on alkylamides which are mainly localized in the roots with lesser concentrations in aerial parts.

2.1.3 *Phytochemical variations*

In terms of the seasonal fluctuation of *E. purpurea*'s phytochemicals, alkylamides and CADs follow different trends. A study on these variations in roots and stems showed that both caftaric acid and cichoric acid are at their highest concentrations in late spring, just prior to flowering; whereas the highest concentrations of alkylamides in roots and stems are reached is during early spring (Thomsen et al., 2012). In contrast to *E. purpurea*, one study looked at the roots of *E. angustifolia* during its first year of growth. The roots were shown to have the greatest levels of alkylamides at the end of vegetative growth and prior to the reproductive stage (Berti at al., 2002). Although there are many studies on roots of both species, fluctuations in both alkylamides and CADs throughout seasonal growth in all plant parts have yet to be reported.

2.1.4 *Organic fertilizers and foliar sprays*

Several other environmental variables contribute to changes in phytochemical composition in *Echinacea*, including biotic factors such as herbivory or microbial interactions

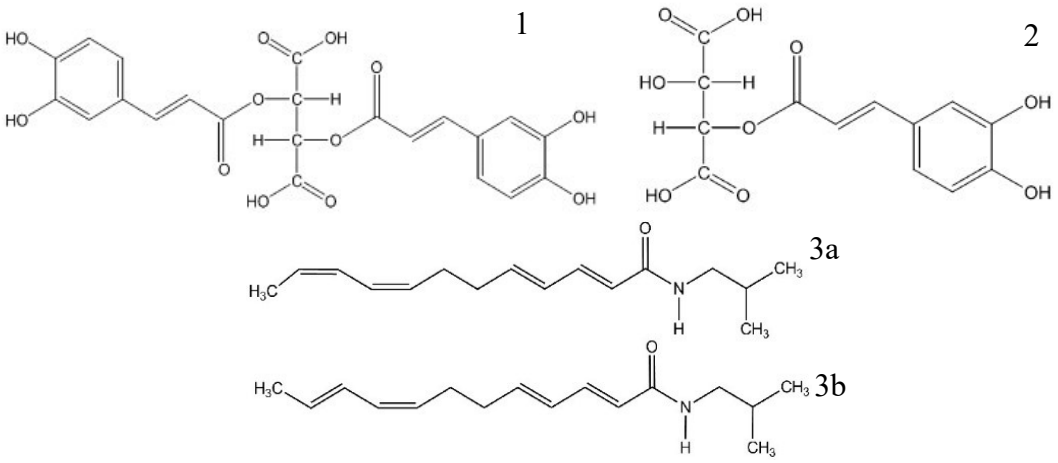

Figure 2.1. Chemical structures of the isolated active compounds under investigation from *Echinacea purpurea* extracts. (1) Cichoric acid: 2,3-O-dicaffeoyl tartaric acid, (2) Caftaric acid: 2-O-caffeoyl tartaric acid, (3) Alkylamides 8,9: (3a) Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide; (3b) Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide.

and abiotic factors such as soil health and fertilizer use (Binns et al., 2001; Maggini et al., 2017). The success of a plant in terms of health and biomass is often associated with the use of fertilizers. However, both non-organic and organic fertilizers also influence plant chemistry and energy allocation. In terms of non-organic fertilizers, the presence of nitrogen and potassium results in an increase in percent alkylamide content while aiding in plant growth of *Echinacea* (El-Sayed et al., 2012). Due to the demand for organically farmed products (both food and natural health products), industry is moving away from inorganic fertilizers (Greene & Dimitri, 2003). Moreover, consumers will also pay more for a product labelled as organic due to their association with health and environmental friendliness (Bauer et al. 2013). Accordingly, organic farming practices are continuously evolving to maintain competitiveness.

Organic fertilizers, such as those rich in plant hormones auxin and cytokinin, can increase plant biomass by promoting cell division (Metting et al., 1990). The presence of cytokinin in an organic fertilizer may interact with the production of alkylamides in *E. purpurea.* A study by Lopez-Bucio et al. (2007) showed that alkylamides found in *Arabidopsis* activates cytokinin signaling which results in cell division and shoot formation. Other marine based fertilizers such as fish oils naturally contain nitrogen and potassium which could result in increased percent alkylamide (Illera-Vives et al., 2015). While fish oils on their own have not produced significant increase in yields, multiple studies have combined fish oils with kelp-based fertilizers resulting in increased biomass in fruits of other plants (Illera-Vives et al., 2015; Wiens & Reynolds 2008). By testing organic fertilizers, such as those mentioned above, not only can organic farms expand on techniques, but also optimize yields of the chemical constituents within their products. In collaboration with an organic farm, we have tested the phytochemical composition of *E. purpurea* organically treated with marine based fertilizers.

2.1.5 *Rationale and research objectives*

The objectives of this study were to determine the effect of organic fertilizers on plant biomass and phytochemical concentrations, as well as determine how phytochemicals of interest fluctuate in concentrations throughout the growing season across plant parts.

2.2 METHODS

2.2.1 *Sample collection*

Echinacea purpurea seeds were obtained from Franklin Johnson at Trout Lake Farm germplasm and planted in an experimental plot at Trout Lake Farm-East Site (Ephrata, WA) on April 16th, 2016 in a 533.3m^2 (1600ft^2) area at a planting density of 30.5 cm (1ft.) spacing both in-row and between-rows. Irrigation was applied soon after planting, and subsequently every three days while germinating, then weekly once plants were established. Each of the four treatment blocks contained 50 plants and were treated with one of the foliar treatments. There were four replicates per block, except for the non-treated control group which only had three replicates. Solutions were made according to the product label for use as a foliar spray. The plants were sprayed heavily, or drenched, and both the upper and lower sides of the leaves were treated. Whole plant sample collections and treatments started at the 2-4 leaf stage, on May 17th, 2016 (Table 2.1) and occurred thereafter on a bi-weekly basis. Whole plants of average size and development for that growth stage were harvested until full flowering on July 26th, 2016, with additional collections monthly at the end of August (maturity), September, and October. Loose soil was removed manually from roots, then plant materials were washed with water, air dried at 18°C at maximum fan speed in a Harvest Saver Tray Dryer (Commercial Dehydrator Systems, Inc., Eugene, OR, USA), before obtaining a fresh weight. Then, whole plants were completely dried at 57°C at maximum fan speed until a moisture content of ≤10% was achieved, and the dry weight was measured prior to packaging for shipment to the University of Ottawa.

Table 2.1. Description of developmental stages of *E. purpurea* plants with corresponding sample collection dates through first year growth in 2016.

Collection Date (2016)	Developmental Stage
May 17th	2-4 leaf stage; early development
May 30th	Developed leaves
June 27th	Developed roots
July 11th	Pre-flowering; mature leaves
July 26th	Full flowering
August 26th	Maturity; fallen petals

2.2.2 *Treatments*

Approximately 800 plants underwent varying organic growth treatments while growing at Trout Lake Farm over a ten-week period. Nitrozyme (Agri-Growth International Inc., Alberta, Canada), Microplus (AgPro Systems Inc., Spokane, WA, USA), Fish Agra (Northeast Organics, Manchester, MA, USA), or irrigation water (non-treated control group) were the treatments that were applied (Table 2.2). Nitrozyme is a kelp fertilizer containing 300-400ppm cytokinin; Microplus is made up of 30% kelp and enzyme complexes such that it contained 150 ppm cytokinin; Fish-Agra is a fertilizer extract of whole Atlantic Ocean wild fish (fish oil); and the negative control group was treated with water. The field experiment carried out in this study was in an area with a starting base nitrogen content of 110 pounds per acre at the start of the season.

2.2.3 *Sample Extraction*

Dried samples of whole *E. purpurea* plants, stored in sealed plastic bags, were provided by Trout Lake Farm to the University of Ottawa in the Fall of 2016. Fresh and dried weights were provided by Trout Lake Farm for each whole plant sample, which were subsequently sorted by treatment and harvest date and separated into root, leaf, stem and flower subsamples. Each subsample was weighed and ground using a Wiley mill with a 1 mm mesh size. 1.0 g of the ground samples were extracted with 15 mL of 70% ethanol. The samples were sonicated for 30 minutes, shaken for three hours and centrifuged at 3200 xg for ten minutes. After centrifugation, the supernatant was separated, and the extraction process was repeated twice more. The pooled supernatant (~45 mL) was transferred to a 50 mL volumetric flask to which 70% ethanol was added to reach a final volume of 50 mL.

Table 2.2. Described treatments sprayed onto samples in the respective field plots with used shorthand codes found in figures.

Name	Description	Treatment Code
Irrigation water	Control group; only sprayed with water	CTL
Nitrozyme	High cytokinin; kelp-based fertilizer	400 ppm
Fish-Agra	Fish oil; extract of whole wild fish	Fish Oil
Microplus	Low cytokinin; kelp-based fertilizer	150 ppm

2.2.4. *Chemical Analysis: HPLC-DAD*

Prior to chemical analysis, 1 mL of the 50 mL samples was filtered by a 0.2 μm PTFE filter into a high-performance liquid chromatography (HPLC) vial. HPLC was used to separate and quantify chemicals and compounds in the extracts – more specifically, levels of separate alkylamides (quantified as alkylamide 8/9 equivalents), caftaric acid and cichoric acid based on reported methods (Cech et al., 2006). Equipment used was Agilent Technologies 1100 series (Montreal, Canada). Hardware components included a solvent degasser, autosampler, quaternary pump, column oven and a diode array detector (DAD). Analysis was performed using a Phenomenex Luna C18 column (100X2.1 mm 5 um particle size) (Phenomenex, Mississauga, Canada). Samples were injected at a volume of 2 μL. The column oven temperate was set at 50°C with a flow rate of 0.4 mL per minute. Mobile phases A and B were 0.01% trifluoroacetic acid in Milli-Q H_2O and 0.01% tifluoroacetic acid in acetonitrile, respectively. The detection wavelengths were 268 nm for alkylamides and 330 nm for CADs. The gradient elution was set as follows: initial 1 minute 10% in B; 19 minutes linear gradient from 10-100% in B; hold 100% in B for 5 minutes. The post-running time was 5 minutes.

2.2.5 *Identification and Quantification of Phytochemicals*

Seven alkylamides were identified based on established phytochemistry and quantified relative to the standard curve of alkylamide 8/9 (Parsons et al., 2018; Binns et al., 2001). A cichoric acid standard was individually injected to generate a standard curve for CADs. The total alkylamides value was calculated by summing the total amounts of each quantified alkylamide peak.

2.2.6 *Statistical Analysis*

Statistical analysis was conducted in Microsoft Excel with the RealStatistics extension. Treatments were compared throughout growth in each plant part for each observed phytochemical using a two-way ANOVA. This was followed by *post hoc* Tukey test to determine where significance occurred. P-values ≤ 0.05 were considered statistically significant. Analysis of biomass was tested using a one-way ANOVA for each plant part during one time point; this was also followed by *post hoc* Tukey test to determine where significance occurred.

2.3. RESULTS

Prior to quantification, the chromatograms were inspected to provide information on which plant parts and treatments contained the greatest level of alkylamides and CADs by observing relative peak heights. During flowering, it was observed that roots have the highest absorbance of alkylamides whereas leaves had the highest absorbance of cichoric acid (Figure 2.2; Figure 2.3).

2.3.1 *Root Phytochemistry*

The roots of *E. purpurea* contain the highest concentration of alkylamides in comparison to the other tested plant parts. Alkylamides varied by >30 % over the season and 2-fold across treatments with the control and high cytokinin treatments resulting in greater concentrations than fish oil and low cytokinin treatments (Figure 2.4). However, the total concentration of alkylamides significantly decreased after early development (Figure 2.5). In terms of impact of treatments, no treatment consistently resulted in greater alkylamide concentrations throughout growth. It was notable that high cytokinin and the control were significantly greater in alkylamide concentration in comparison to fish oil and low cytokinin during the first half of the growing season (Figure 2.5). With regards to root CADs, both caftaric and cichoric acids were significantly higher in concentration during flowering and after flowering than the earlier developmental stage – a contrasting trend to alkylamides (Figure 2.4; Figure 2.5). There was no treatment that consistently produced higher CAD concentrations relative to the other treatments throughout development.

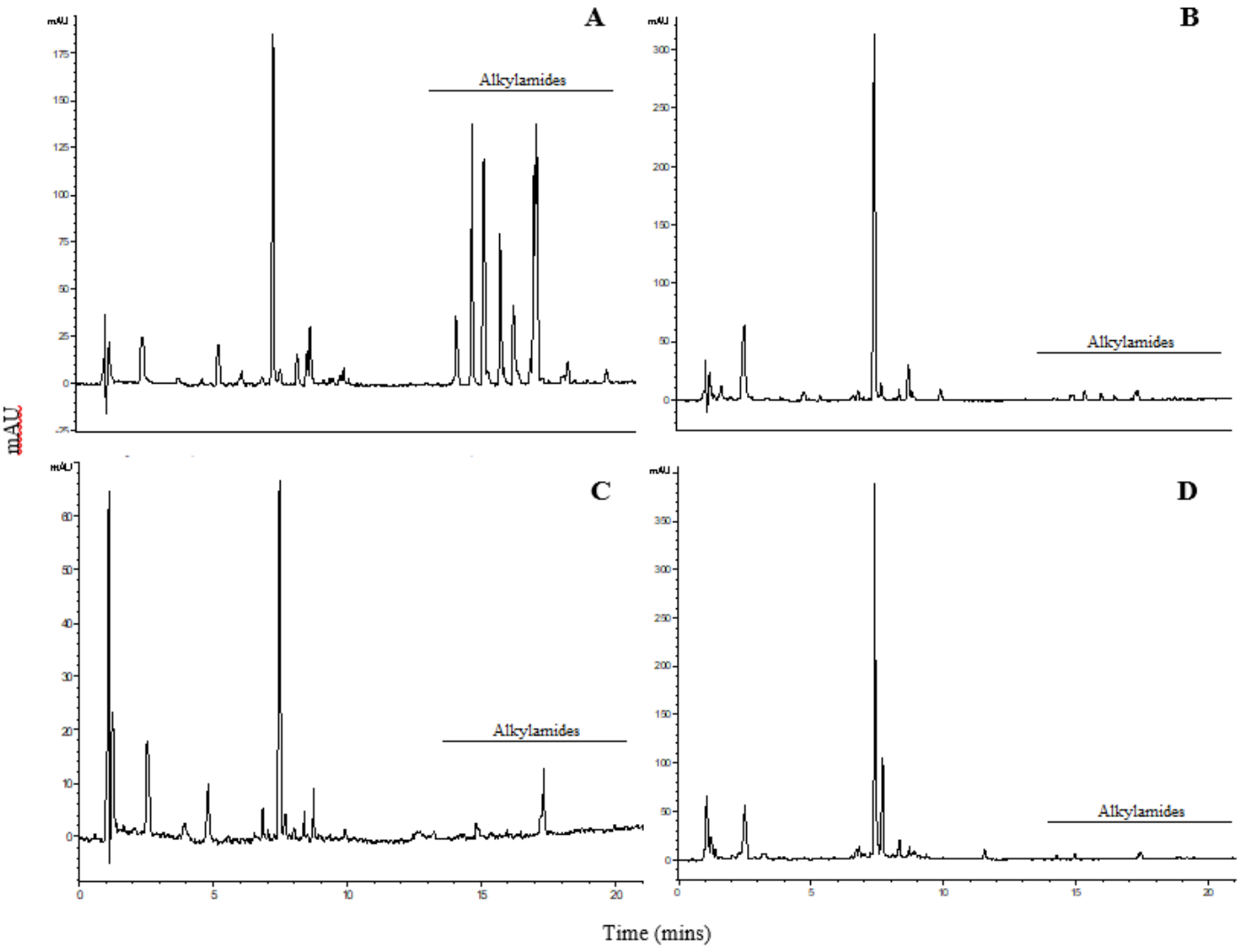

Figure 2.2. HPLC-DAD chromatograms for control samples at July 26th, 2016 of A) roots B) leaves C) stems and D) flowers. Showing figures at 268 nm for alkylamide visualization (alkylamides eluted at 14-20 mins).

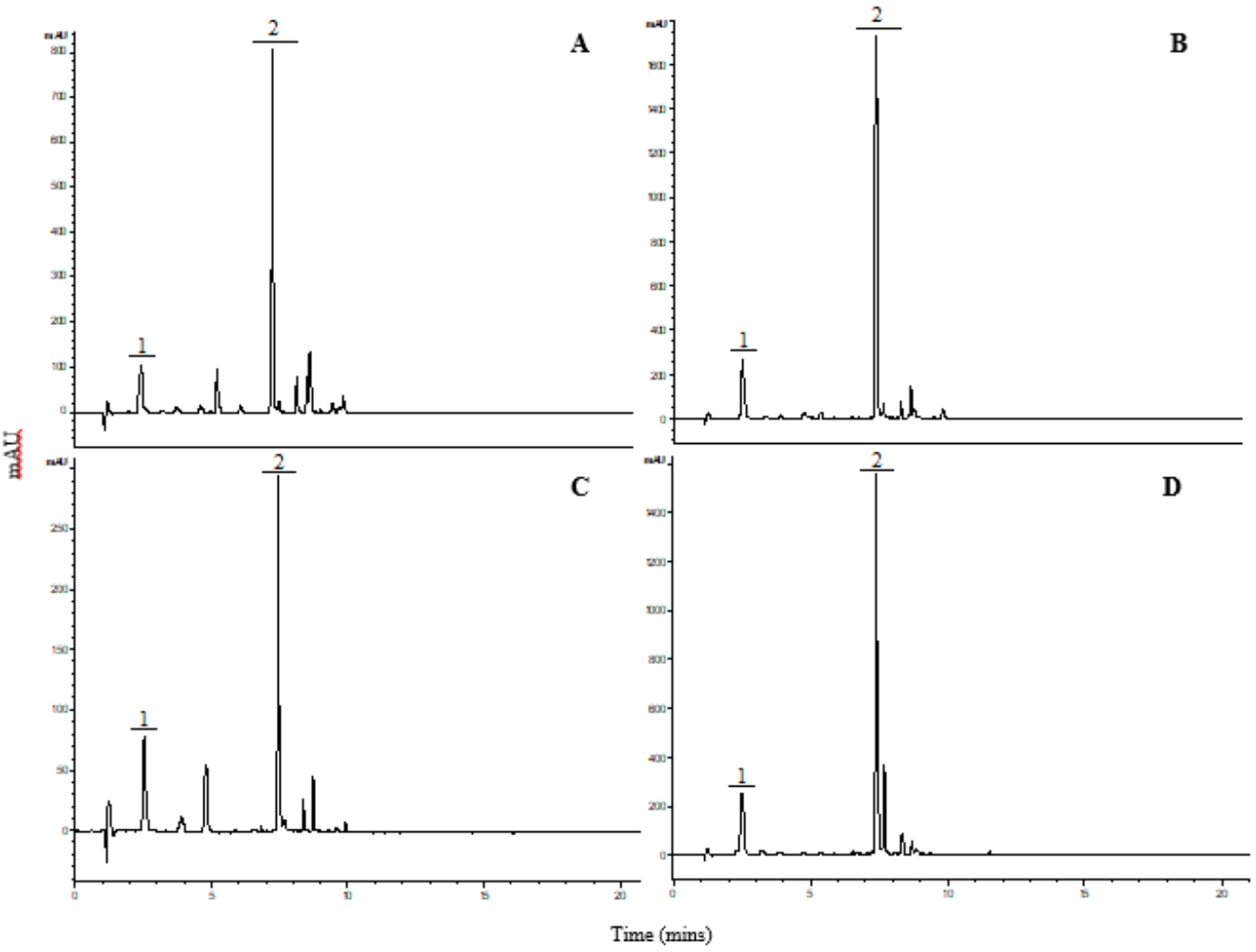

Figure 2.3. HPLC-DAD chromatograms for control samples at July 26th, 2016 of A) roots B) leaves C) stems and D) flowers. Showing figures at 330 nm for CAD visualization 1: caftaric acid. 2: cichoric acid (CADs eluted at 2-9 mins).

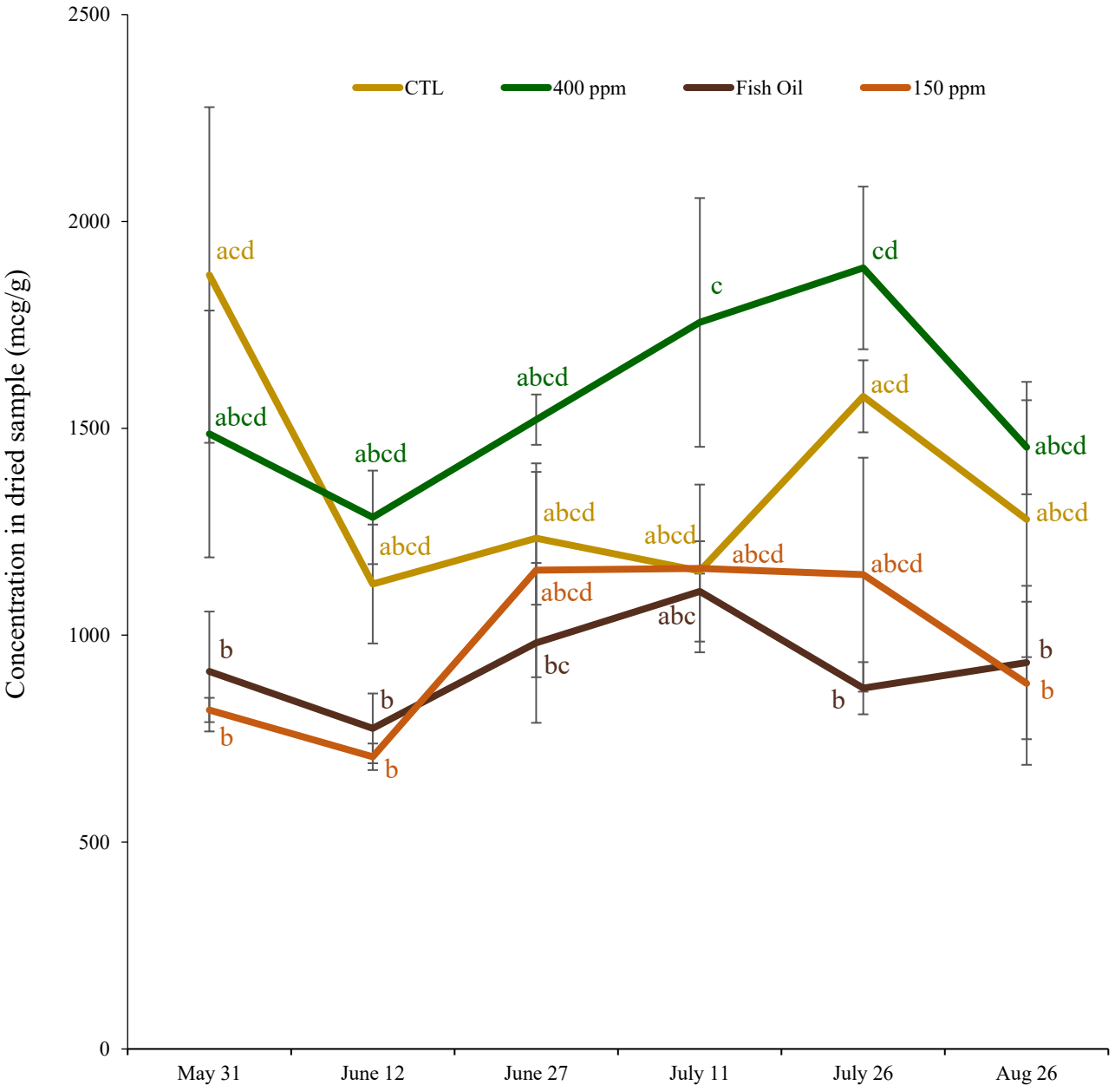

Figure 2.4. Quantitative comparisons of alkylamide 8/9 concentrations throughout growth (May 31st to August 26th, 2016) of *Echinacea purpurea* root extracts across organic treatments. Means and SEM for three replicates. Letters used to indicate significant ($p \leq 0.05$) differences between samples as determined by two-way ANOVA with Tukey post hoc test.

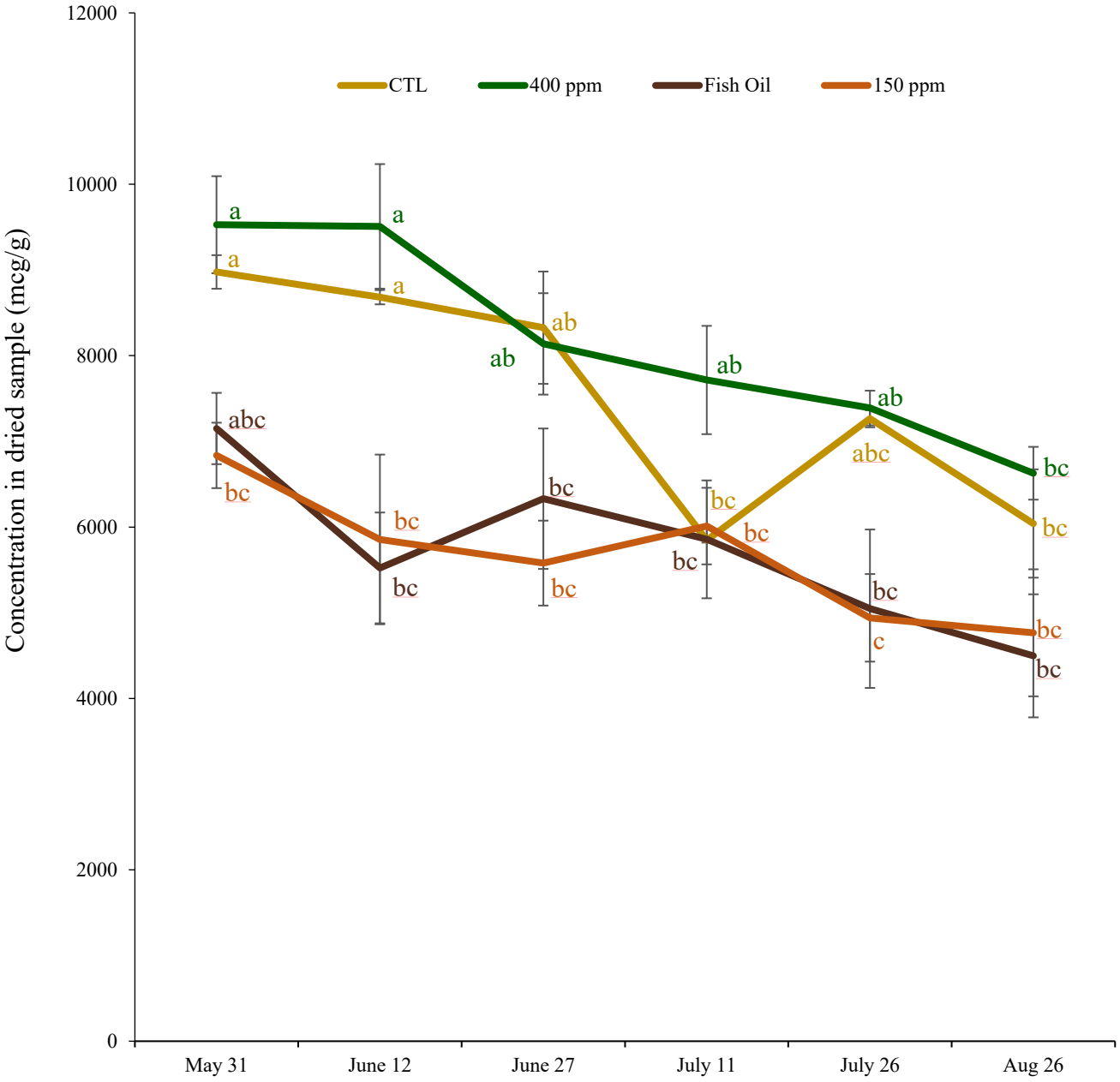

Figure 2.5. Quantitative comparisons of total alkylamide concentrations throughout growth (May 31st to August 26th, 2016) of *Echinacea purpurea* root extracts across organic treatments. Means and SEM for three replicates. Letters used to indicate significant ($p \leq 0.05$) differences between samples as determined by two-way ANOVA with Tukey post hoc test.

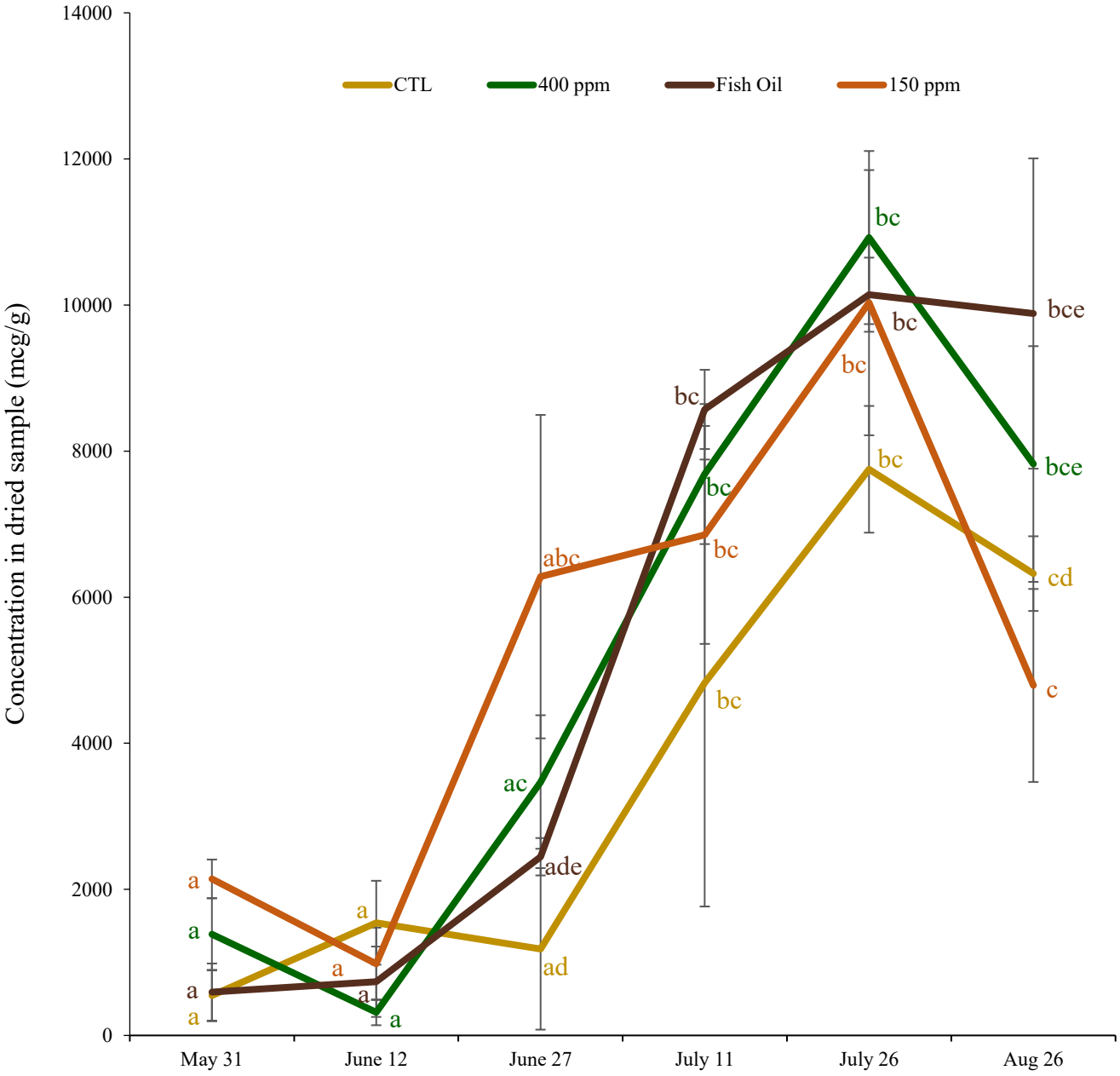

Figure 2.6. Quantitative comparisons of cichoric acid concentrations throughout growth (May 31st to August 26th, 2016) of *Echinacea purpurea* root extracts across organic treatments. Means and SEM for three replicates. Letters used to indicate significant ($p \leq 0.05$) differences between samples as determined by two-way ANOVA with Tukey post hoc test.

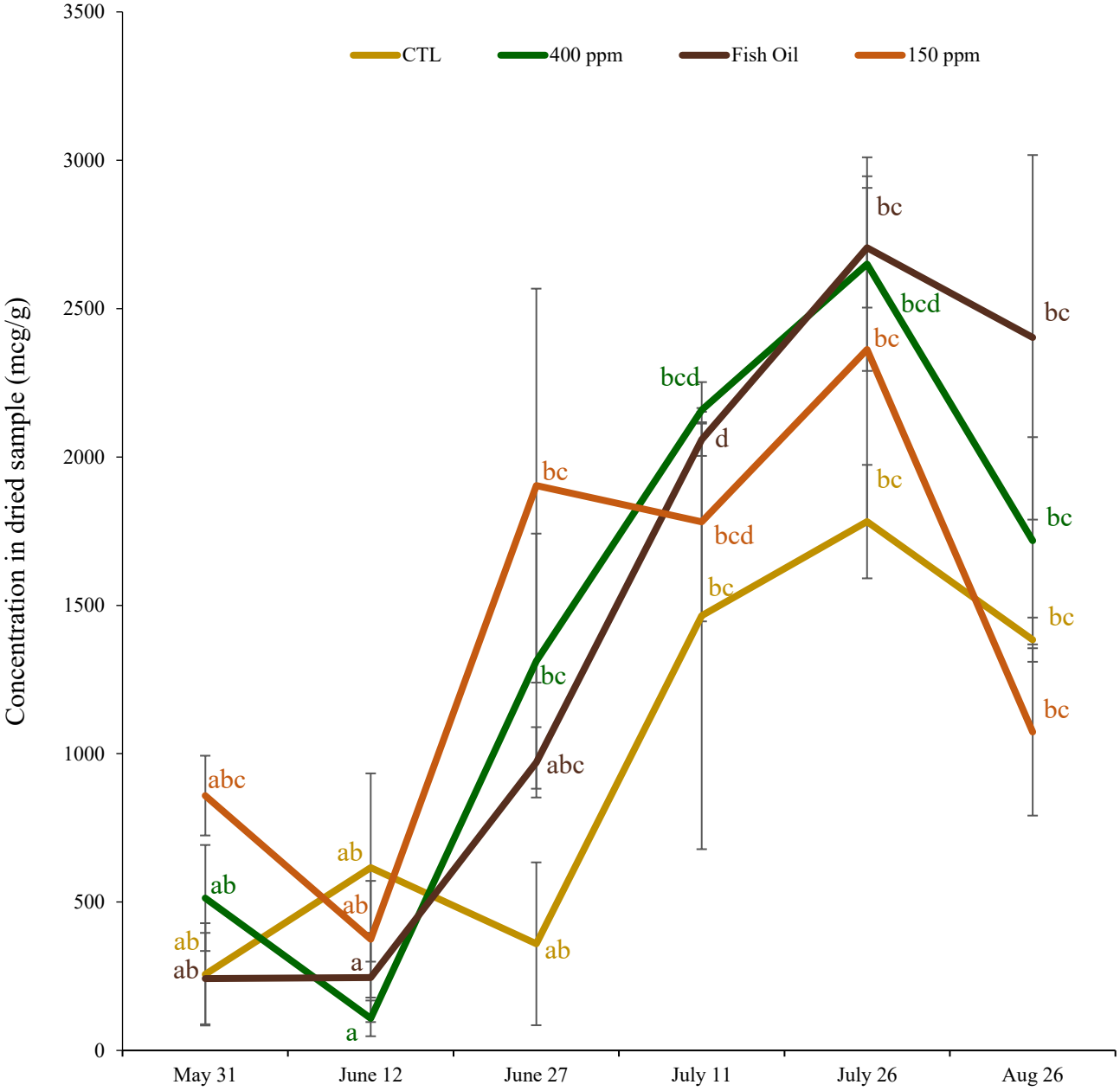

Figure 2.7. Quantitative comparisons of caftaric acid concentrations throughout growth (May 31st to August 26th, 2016) of *Echinacea purpurea* root extracts across organic treatments. Means and SEM for three replicates. Letters used to indicate significant ($p \leq 0.05$) differences between samples as determined by two-way ANOVA with Tukey post hoc test.

2.3.2 *Leaf Phytochemistry*

Total alkylamide concentrations and the 8/9 subset were significantly greater at early development (May 31st), with other stages stabilizing at a lower concentration (Figure 2.8; Figure 2.9). Like root alkylamides, there were no significant differences between treatments throughout the season in leaf alkylamides. Differences became more convoluted when examining CADs. Looking at cichoric acid, plants treated with low cytokinin and fish oil treatments were significantly richer than plants grown with the other treatments, but only at the flowering and maturity stages, respectively (Figure 2.10). Cichoric acid concentrations were not higher in one growth stage for all four treatments as treatments remain at statistically similar concentrations across development. Caftaric acid levels were significantly higher at flowering than the other developmental stages for the fish oil and low cytokinin treatments; however, throughout growth, all treatments were similar in concentration and thus none were significantly different from each other (Figure 2.11).

2.3.3 *Flower Phytochemistry*

Because flowers were produced later in first year growth, flowers and stems were only compared between their most distinct stages: flowering and maturity (fallen petals). In most cases there were no significant differences between treatments and thus only differences in developmental stages were presented. The alkylamides in the flowerheads were significantly higher at maturity than during flowering (Figure 2.12; Figure 2.13). The CADs demonstrate a different pattern with greater concentrations occurring at flowering rather than maturity in both cichoric and caftaric acid (Figure 2.13A-B).

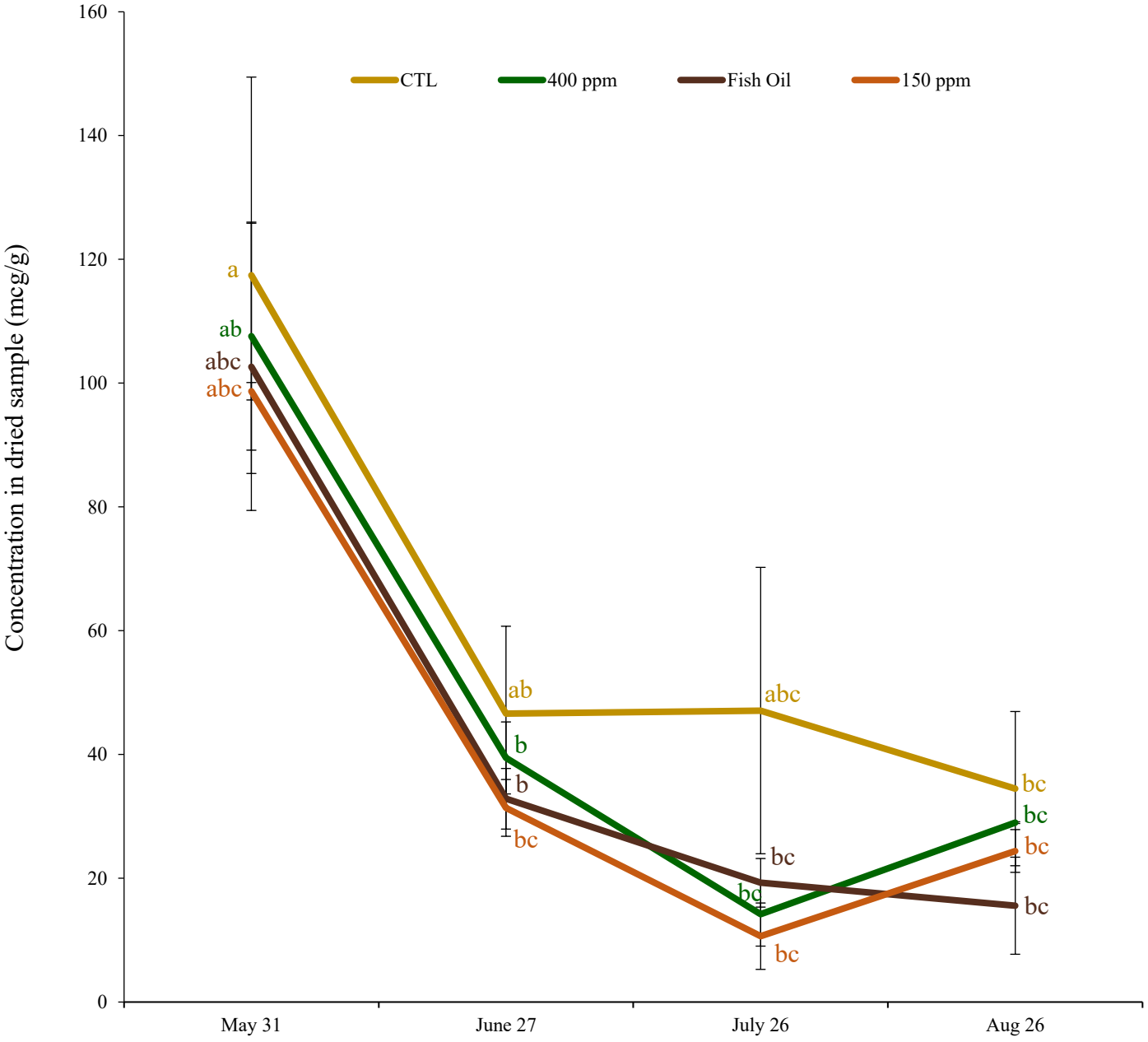

Figure 2.8. Quantitative comparisons of alkylamide 8/9 concentrations throughout growth (May 31st to August 26th, 2016) of *Echinacea purpurea* leaf extracts across organic treatments. Means and SEM for three replicates. Letters used to indicate significant ($p \leq 0.05$) differences between samples as determined by two-way ANOVA with Tukey post hoc test.

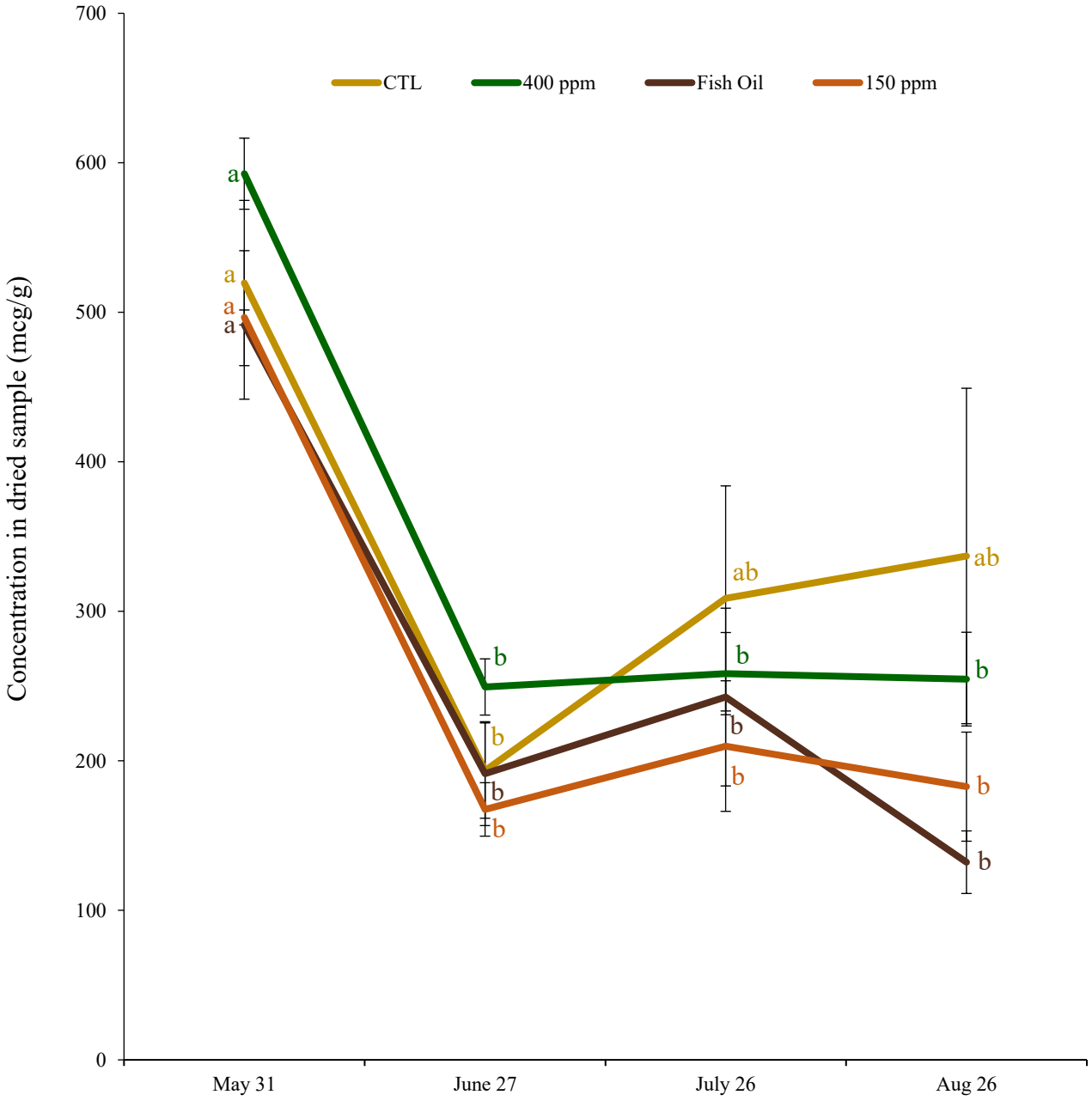

Figure 2.9. Quantitative comparisons of total alkylamide concentrations throughout growth (May 31st to August 26th, 2016) of *Echinacea purpurea* leaf extracts across organic treatments. Means and SEM for three replicates. Letters used to indicate significant ($p \leq 0.05$) differences between samples as determined by two-way ANOVA with Tukey post hoc test.

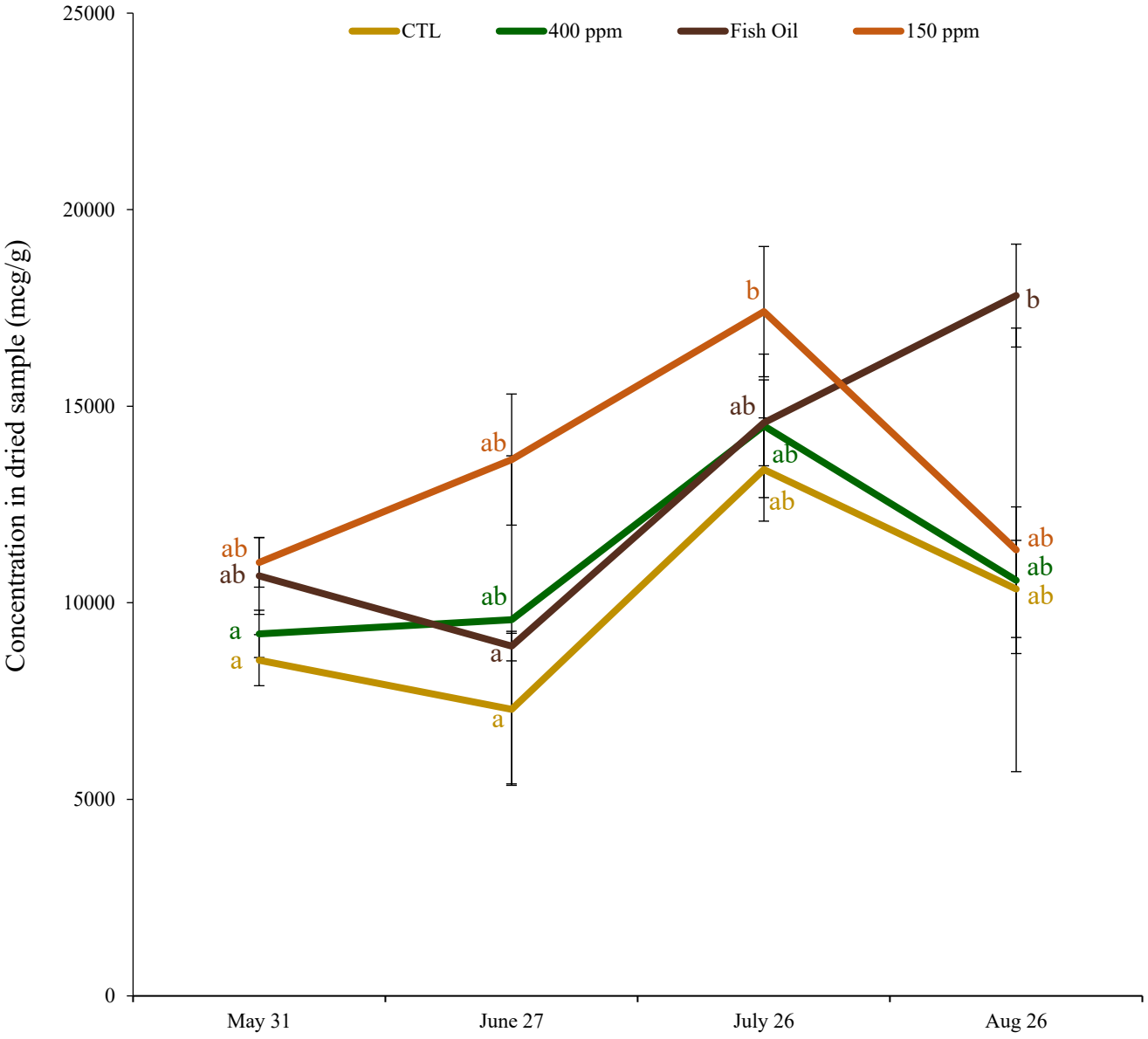

Figure 2.10. Quantitative comparisons of cichoric acid concentrations throughout growth (May 31st to August 26th, 2016) of *Echinacea purpurea* leaf extracts across organic treatments. Means and SEM for three replicates. Letters used to indicate significant ($p \leq 0.05$) differences between samples as determined by two-way ANOVA with Tukey post hoc test.

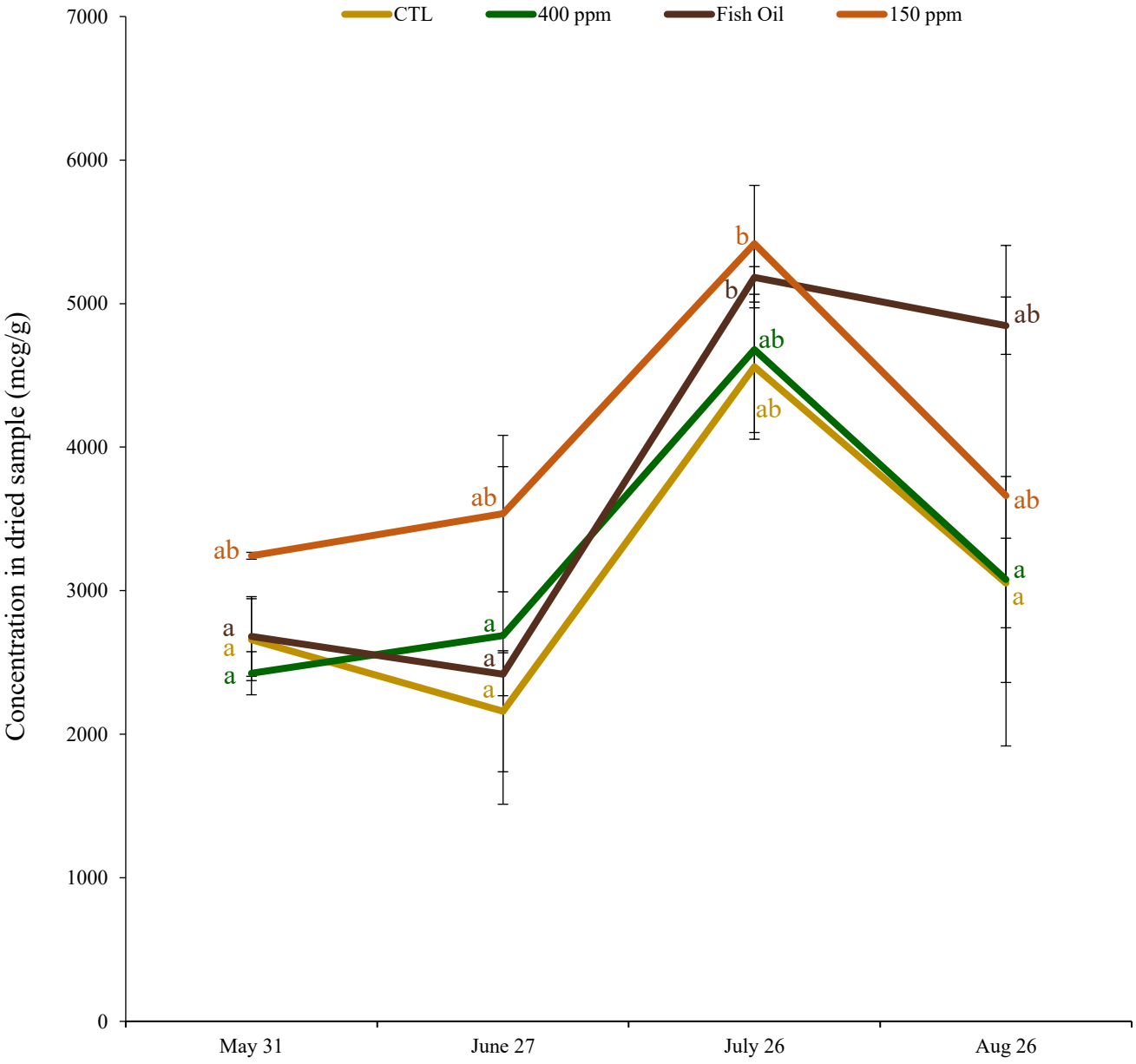

Figure 2.11. Quantitative comparisons of caftaric acid concentrations throughout growth (May 31st to August 26th, 2016) of *Echinacea purpurea* leaf extracts across organic treatments. Means and SEM for three replicates. Letters used to indicate significant ($p \leq 0.05$) differences between samples as determined by two-way ANOVA with Tukey post hoc test.

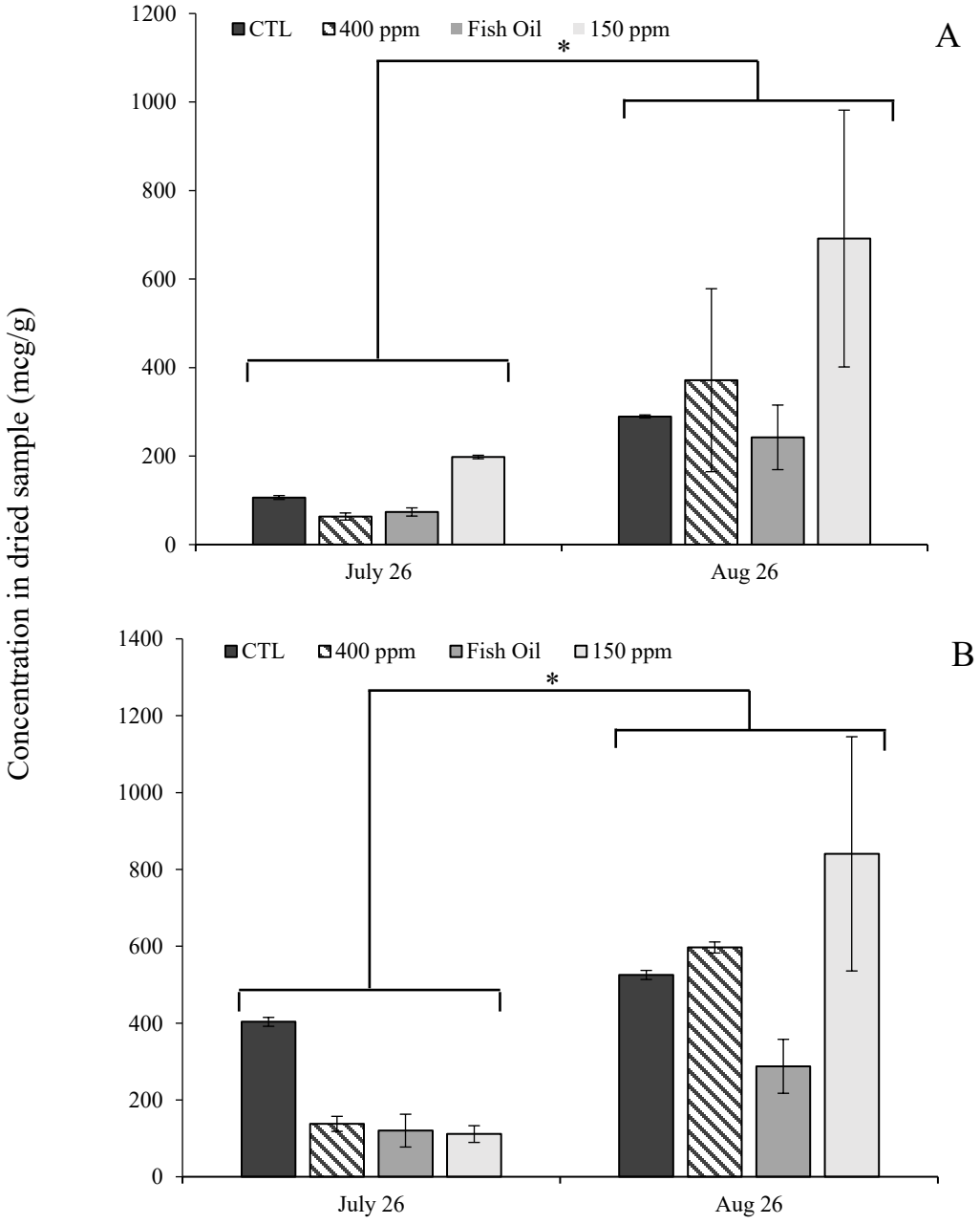

Figure 2.12. Quantitative comparisons of A) Alkylamide 8/9 and B) Total alkylamide content at flowering and maturity (July 26th and August 26th, 2016) in *Echinacea purpurea* flower extracts across organic treatments. Means and SEM for three replicates. * used to indicate significant ($p \leq 0.05$) differences between collection dates as determined by two-way ANOVA with Tukey post hoc test.

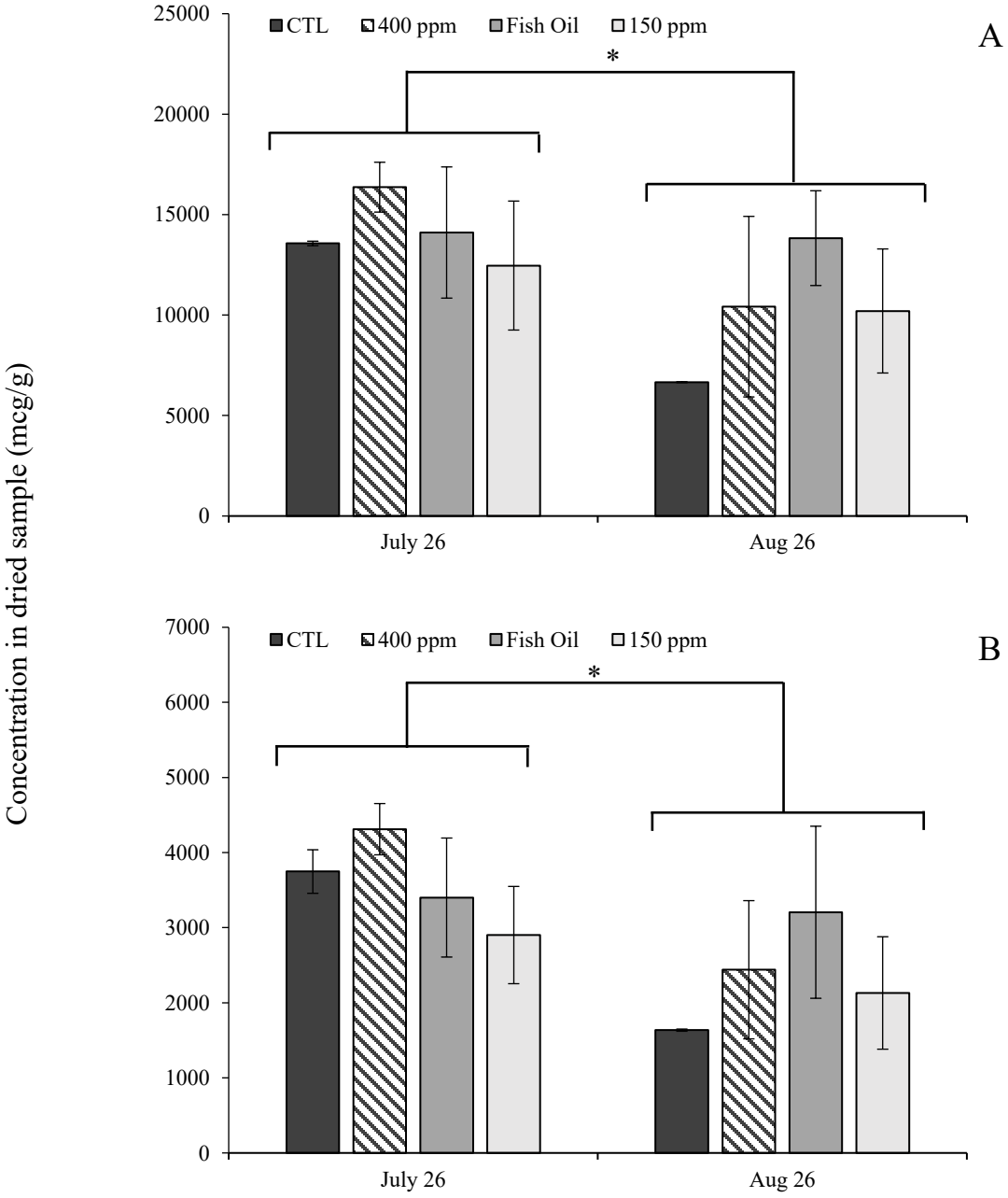

Figure 2.13. Quantitative comparisons of A) Cichoric acid and B) Caftaric acid content at flowering and maturity (July 26th and August 26th, 2016) in *Echinacea purpurea* flower extracts across organic treatments. Means and SEM for three replicates. * used to indicate significant ($p \leq 0.05$) differences between collection dates as determined by two-way ANOVA with Tukey post hoc test.

2.3.4 *Stem Phytochemistry*

In the stems, the highest concentrations of alkylamides were found earlier as they were highest in flowering and decrease during maturity (Figure 2.14A-B). In particular, the fish oil treatment showed significantly greater concentrations during flowering than any of the other treatments (Figure 2.14A-B). CADs of stems were similar to flower trends as concentrations of cichoric and caftaric acid was significantly greater during flowering (Figure 2.15A-B).

2.3.5 *Biomass Across Parts*

The dry plant material biomass of each plant part demonstrated differing effects based on the treatment applied (Figure 6). Since biomass is naturally different between plant parts, they were analyzed separately from each other. Root mass was unaffected by all organic treatments as they were all statistically similar. The leaf control group has a significantly greater biomass than the other three treatments. While there appears to be variation in flower biomass between treatment groups, there were no significant differences. Lastly, the stems differ in treatments with control and high cytokinin treatments resulted in a lower yield than the fish oil and low cytokinin treatments.

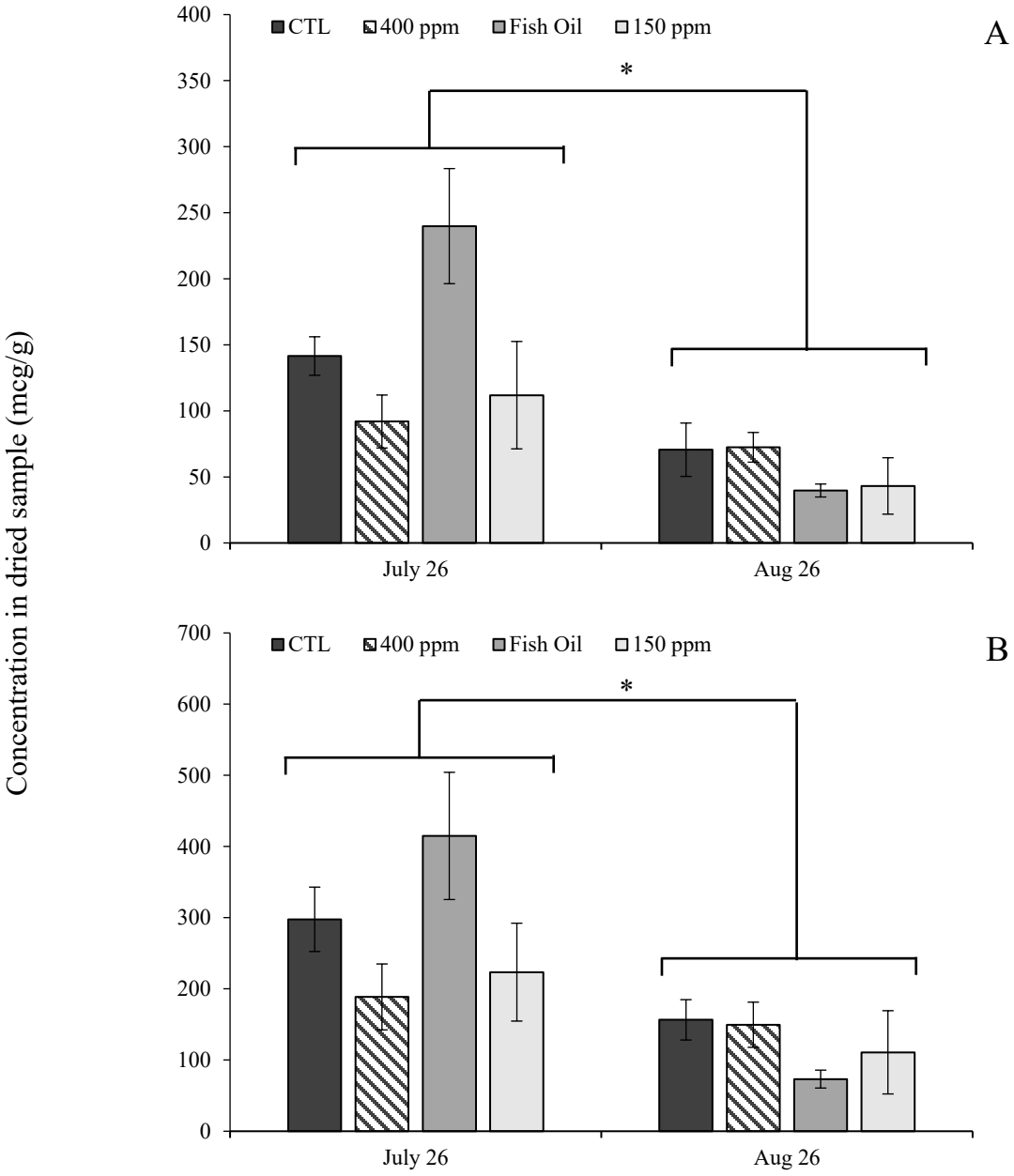

Figure 2.14. Quantitative comparisons of A) Alkylamide 8/9 and B) Total alkylamide content at flowering and maturity (July 26th and August 26th, 2016) in *Echinacea purpurea* stem extracts across organic treatments. Means and SEM for three replicates. * used to indicate significant ($p \leq 0.05$) differences between collection dates as determined by two-way ANOVA with Tukey post hoc test.

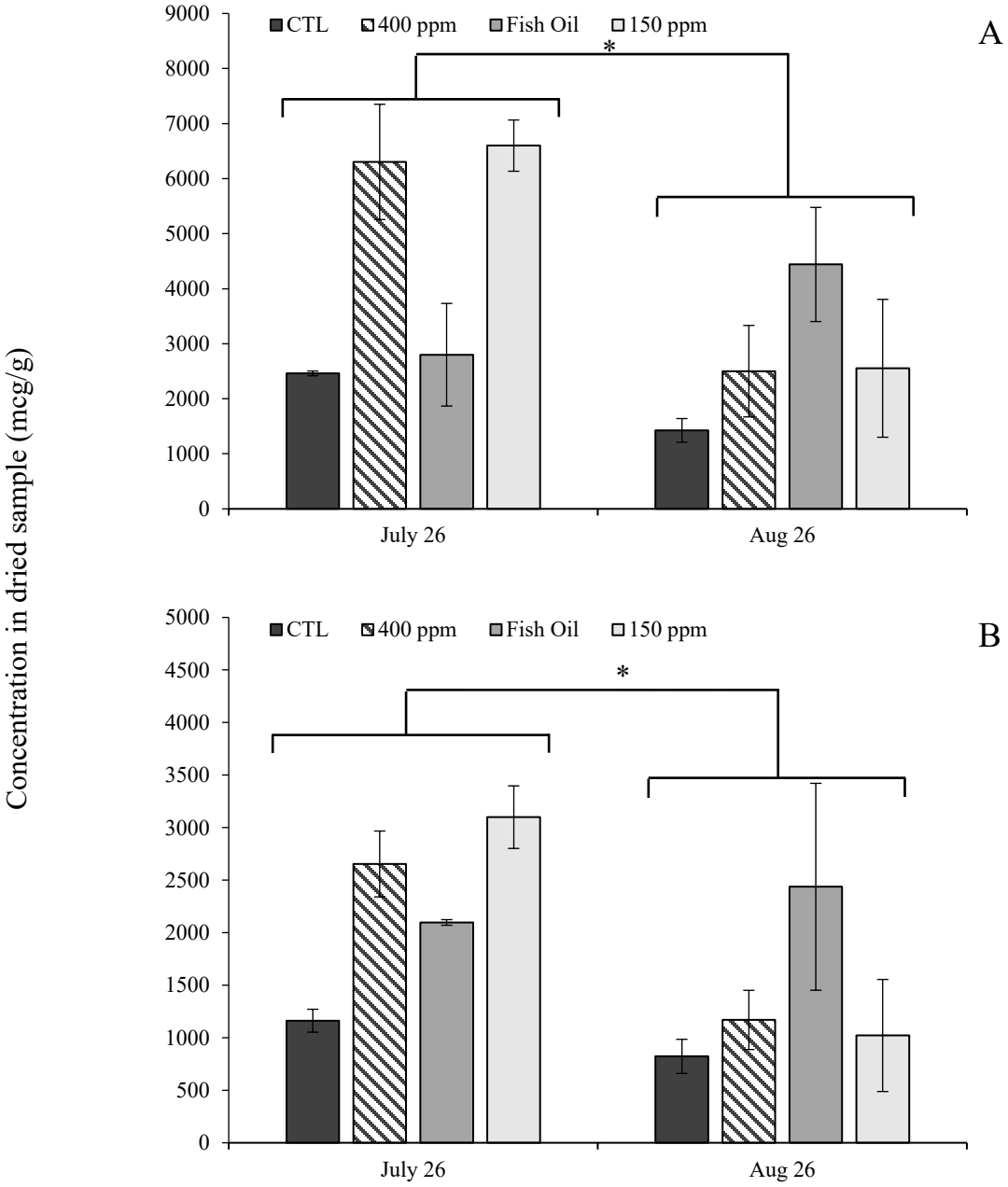

Figure 2.15. Quantitative comparisons of A) Cichoric acid and B) Caftaric acid content at flowering and maturity (July 26th and August 26th, 2016) in *Echinacea purpurea* stem extracts across organic treatments. Means and SEM for three replicates. * used to indicate significant ($p \leq 0.05$) differences between collection dates as determined by two-way ANOVA with Tukey post hoc test.

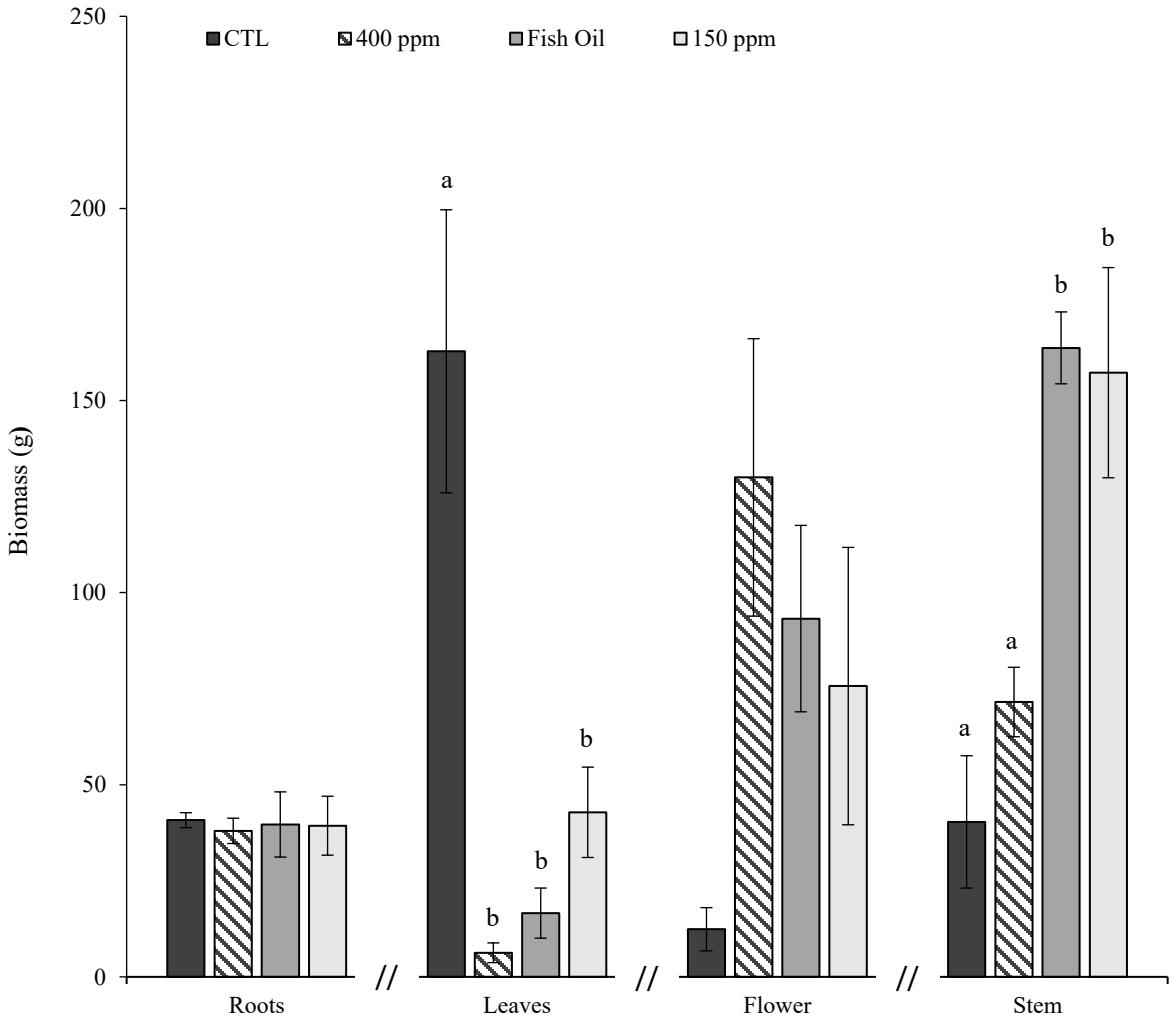

Figure 2.16. Biomass yields in grams across organic treatments of *Echinacea purpurea* in roots, leaves, flowers and stems at maturity (August 26th, 2016). Roots and flowers showed no significant differences between treatments; all plant parts statistically analyzed separately from each other. Letters (a and b) demonstrate significant ($p \leq 0.05$) differences between treatments as determined by two-way ANOVA with Tukey post hoc test.

2.4 DISCUSSION

Since natural health products containing *Echinacea* often differ in efficacy and concentration between brands, and even between lot, there is a need to optimize and standardize products. This study allows for industry to select for certain compounds at their peak concentrations, whether it be alkylamides or CADs. It is important to note that although some of these compounds were lower later in the season, there is a yield trade-off as biomass was lowest in early growth. Therefore, regardless of trends in compound concentration per gram, there will still be high content in large plants later in the season and it is reasonable for NHP companies to harvest plants at the end of season regardless of the time of peak concentrations.

The most drastic difference in yields was a greater biomass in leaves of the control group than all other treatments (Figure 2.16). This finding can be explained by stress induced by the other treatments, namely those with cytokinin. Increased cytokinin production causes plants to expend less energy on root and leaf development and more on flowering so that the plant can rapidly produce seed (Riefler et al., 2006; Lopez-Bucio et al., 2007). This was more notable in leaves than in roots – where we see no significant reduction – as the plants, regardless of treatment, was already expending more energy into aerial growth. Thus, root differences would not be as noticeable at the end of the season.

Similar to the leaves in this study, this stress phenomenon has been seen in fruits of other plants undergoing similar treatments, where the fruits increased in biomass with the treatment of cytokinin (Illera-Vives et al., 2015; Wiens & Reynolds 2008). In terms of harvesting and overall suggestions for industry, we can conclude that although the control treatment (irrigation water) resulted in increased leaf yield, most natural health products rely on roots which were unaffected by treatment, which are associated with added cultivation costs.

Since recent research interest focuses on the medicinal effects of alkylamides from roots, we can optimize their concentrations using the results of this research. Because treatment had no affect on root yield, no particular treatment can be suggested solely from yields. Due to increased CAD concentrations during flowering – as well as increased size of the plant – harvesting at flowering may result optimal CAD-related products. Considering that 400ppm cytokinin treatments resulted in the highest concentration of alkylamides in the roots – particularly 8/9 – organic farms could alter their protocols to incorporate this fertilizer in the field for product optimization. However, if farmers seek to use whole plants rather than just roots, then implementing fish oil foliar sprays may aid in increasing overall plant biomass and decrease costs for industry (Figure 2.16).

While many results presented a trend without significance, these findings can be attributed to natural variation between individuals that, with a low sample size (n=3-4), may have exceeded seasonal variability. Overall, seasonal trends of both alkylamides and CADs were similar to findings in previous studies with only minor variations. While separation of parts is inconsistent between studies, both Stuart & Wills, 2000 and Brovelli et al. 2005 found no significant differences in concentration of cichoric acid throughout growth, this finding was most notable in leaves of our study.

Trends in alkylamides were also mirrored by previous studies as roots, leaves and stems were highest in alkylamides concentration as the organs earliest development and flowers being highest at the end of first year growth (Stuart & Wills, 2000). This decrease throughout the growing season for roots, leaves and stems may be a result of a number of biological factors such as protecting young roots from infection. The decrease could also reflect localization if the alkylamides were concentrated in tissues that represent a decreasing proportion of root mass over

the season. For example, if they were localized in the endodermis which expands as the root grows, but relative to cortex, phloem and xylem, represent a smaller fraction of larger roots than smaller roots; the endodermis is suberized providing a lipophilic environment for alkylamides (Barberon, 2017). Similarly, alkylamides may increase in flowers as seeds develop and represent a higher proportion of the flower weight and are more likely to contain suberized structures that petals, for example. Differences in this study can be explained by uses of organic treatments and climate – our study occurred in north-western United States and the Stuart and Wills study occurred in Australia.

This study can be beneficial to the scientific community as the results will provide further information on the overall chemistry of *E. purpurea*, as well as the effects of industrial practices on the concentration of plant organ phytochemistry. The control data provides an anatomical and seasonal map of alkylamide and CAD distribution in *E. purpurea*, which can be compared to other *Echinacea* species as well as other alkylamide and CAD producing plants such as *Achillea millefolium* and *Spilanthes acmella* (Veryser et al., 2017; Spelman et al., 2011).

Further research on organic fertilizer effects should include a mixture of kelp and fish oils as this combination has shown to increase biomass in other plant species rather than individual use (Wiens and Reynolds 2008). These methods can also be applied to crops used for a range of popular natural health products to optimize both yields and phytoceutical concentrations. Moreover, continued research will allow for consistency in the natural health product market, as well as offering consumers safe and reliable alternatives to pharmaceutical drugs.

CHAPTER 3: ASSESSEMENT OF ANTIBACTERIAL ACTIVITY OF *ECHINACEA PURPUREA* (L.) MOENCH

3.1 INTRODUCTION

The bioactivity of *Echinacea purpurea* has been extensively studied in models of plant and human disease and include antibacterial, antiviral and antifungal activities (Hudson, 2011). Recent studies have demonstrated the therapeutic potential of *E. purpurea* treatments in their ability to combat illnesses associated with the throat and mouth (Oto-rhino-laryngological, OTO). In a study done by Merali et al. (2003), *Echinacea angustifolia* extracts and their principle alkylamide isomers dodeca-2E,4E,8Z,10Z/E-tetraenoic acid (alkylamide 8/9, also found in *E. purpurea*) were shown to inhibit the growth of *Candida albicans*, one of the leading causes of fungal throat infections. Complementing the ethnobotanical evidence supporting the use of *Echinacea* for OTO-related symptoms and conditions treatment is apparent, *Echinacea*'s ability to inhibit growth of microorganisms causing respiratory infections has also been reported, such as *Legionella pneumophila, Streptococcus pyogenes,* and *Mycobacterium smegmatis* (Sharma et al., 2010). Yet, one of the most studied respiratory microorganisms – *Pseudomonas* – has not been as thoroughly investigated.

While there are eight species of *Pseudomonas*, research mainly focusses on *Pseudomonas aeruginosa* due to its ability to colonize soil, water, plants and animals alike (Hardalo & Edberg 1997; de Bentzmann & Plésiat 2011). In addition to its ability to colonize and replicate within the natural environment, *P. aeruginosa* is an opportunistic pathogen that often affects immunocompromised individuals and those with chronic respiratory diseases such as cystic fibrosis (CF) (Williams et al., 2010; Oliver et al., 2008). This pathogen is of particular concern

with the current rise in antibiotic resistance (Cabot et al., 2016; Stover et al., 2000). Continued research into natural health product medicines can provide an alternative to prescription antibiotics that can potentially lead to the prevention and treatment of *P. aeruginosa* infections meanwhile lowering rates of antibiotic drug use and related development of resistance.

Although previous studies have tested the inhibitory effects of *E. purpurea* on *P. aeruginosa,* the results of these studies were inconsistent with some reporting inhibition of growth and others reporting no effects. *In vivo* studies supporting the efficacy of *E. purpurea* include the work by Bany et al. (2003), which found that feeding mice *E. purpurea* extracts reduced the colony counts of *P. aeruginosa* in the liver of the mice. Other studies which show no or limited activity against *Pseudomonas* are difficult to use as a comparison as there are inconsistencies in plant parts and bacterial strains used for these studies (Stanisavljević et al., 2009; Sharma et al., 2008; Bachir Raho et al., 2015).

3.1.2 *Rationale and research objectives*

The overarching objective of this study was to investigate the antibacterial potential of *E. purpurea* with respect to OTO and respiratory infections and to clarify inconsistencies in the literature. Three specific objectives were pursued: i) determine if *E. purpurea* extracts can inhibit biofilm development through the disruption of quorum sensing; ii) determine if *E. purpurea* extracts can inhibit the growth of different *P. aeruginosa* strains; and lastly, iii) determine if *E. purpurea*'s antibacterial activity varies between plant part and developmental growth stages.

3.2 METHODS

3.2.1 Echinacea purpurea *extract preparation*

The extracts used for these assays were originally prepared for phytochemical analysis (see Chapter 2). The selected samples were the control group plants (no organic treatment) including all parts (roots, leaves, flowers and stems). Extracts generated during flowering (July 26th, 2016) were tested in quorum sensing (Objective 1) and strain sensitivity (Objective 2) assays whereas extracts generated throughout first year growth, with an added collection date at the end of season (September 30th, 2016), were assayed for Objective 3. For each collection date, 10 mL of each replicate were pooled and processed as described below. These pooled extracts were used for the seasonal minimum inhibitory concentration (MIC) assay. Figure 3.1 shows the methodology of this chapter through choice of antimicrobial assay.

3.2.2 E. purpurea *extract preparation for quorum sensing assay*

High and low final concentrations (500 µg/ml and 100 µg/ml, respectively) were prepared to provide a comparison for quorum sensing (QS) disruption. Extracts of *E. purpurea* were weighed and dissolved in 70% EtOH solvent. Extract (25 µg) was later added to sterile paper disks (Whatman No. 40; 6.0 mm in diameter) for agar disk diffusion QS assay.

3.2.3 E. purpurea *extract preparation for MIC assay*

Extracts were filtered using 0.4 µm PTFE filters into 50 mL centrifuge tubes. They were then placed in a centrifugal vacuum concentrator for approximately 6 hours and then freeze-dried

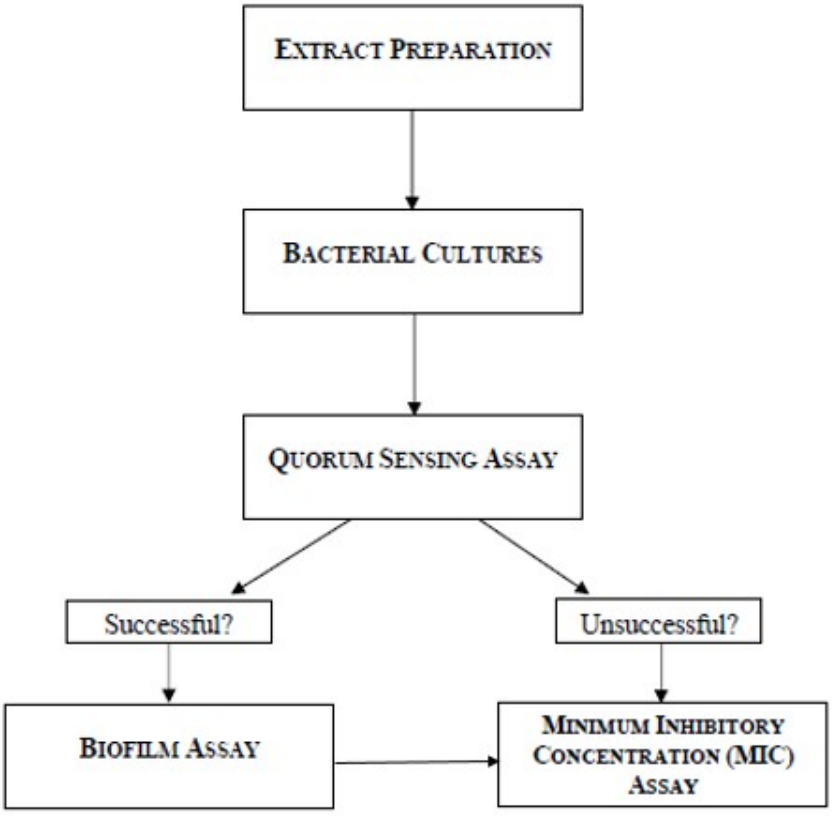

Figure 3.1. Flow chart representing the workflow for investigating the antibacterial activity of *E. purpurea* extracts toward *P. aeruginosa*.

overnight. The dried extracts were weighed and resolubilized with the appropriate amount of 30% EtOH to obtain the desired final concentration of 100 mg/mL.

3.2.4 *Bacterial cultures*

3.2.4.1. CHROMOBACTERIUM VIOLACEUM

Freezer stock of *Chromobacterium violaceum* (stored at -80˚C) was inoculated into LB for overnight incubation at 35˚C on an automatic shaker. *C. violaceum* was used due to its bright violet colouring for visual measurement of inhibition. The following day, 1-2 drops of the overnight culture were inoculated onto Standard Methods Agar (SMA) and streaked for purity assessment (Wiegand et al., 2008). The agar plate was then placed in an incubator overnight at a temperature of 35˚C. After overnight incubation and purity assessment, 3-5 colonies were placed in LB for overnight incubation (35˚C) in the automatic shaker at 200 rpm to make the suspension. Overnight suspensions were then diluted with LB to reach a turbidity of 1.0 McFarland standard.

3.2.4.2 *P. AERUGINOSA* CELL CULTURE

The first part of this experiment was performed by Xinran Wang, an Honour's student under my mentorship in 2017-2018. The sensitivity of 21 strains of *Pseudomonas aeruginosa* to *E. purpurea* was assessed using a single representative extract of each plant part. These clinical and environmental isolates of *P. aeruginosa* were generously provided by Dr. Rees Kassen (Appendix 1.1; Appendix 1.2). Following the streaking protocol outlined in LaBauve and Wargo (2015), bacterial cultures were streaked from frozen 50% glycerol stocks onto an LB agar plate

and incubated overnight at 37ºC. One colony was inoculated into LB broth, and then placed on the shaker at 200 rpm at 37ºC for overnight incubation. The cells were grown to an optical density of 0.1 (OD_{600}), and then diluted to obtain approximately 100 colony forming units (CFU) per plate.

3.2.5 *Bacterial assays*

3.2.5.1 QUORUM SENSING ASSAY

Plant extract quorum sensing assays are typically done through pigmentation inhibition. For these assays *C. violaceum* is widely used as a test organism due to its dark lavender colour, making inhibition visually evident (Thornhill & McLean, 2018). 100 µL of *C. violaceum* was inoculated onto SMA agar. An even lawn of growth was obtained by using a metal spreader. The suspension was then air dried in a biosafety cabinet for 15-20 minutes. Disks with *E. purpurea* extract were placed face down on agar in triplicate. A separate control plate was used with the positive control being *Delisea pulchra* – a deep-sea red alga with quorum sensing inhibiting furanones – and negative control being 70% EtOH in the same concentrations as the high and low extracts (see section 2.1.1). Quorum sensing was measured by the distance from the disks, across clear agar, to the beginning of purple uninhibited *C. violaceum*.

3.2.5.2 MINIMUM INHIBITORY CONCENTRATION ASSAY

The antibacterial activity of *E. purpurea* extracts was assessed using a broth microdilution method (Wiegand et al., 2008). Using clear, flat-bottom, 96-well microtiter plates, 100 µgL of plant extract and carrier control (30% EtOH) were loaded into the first column, with the rest of the columns being loaded with 50 µL of LB, as shown in Figure 3.2. A 10-step two-

fold serial dilution was carried out using 50 µL, with the final column serving as a growth control. 150 µL of diluted bacterial cells were added to each well of either extract or carrier control row. No cells were added to the sterility row and the positive control was the antibiotic gentamycin. An LB agar plate was also streaked with 150 µL of each cell solution to verify the approximate number of CFUs in each well, and to ensure the regularity of colonies. The agar plates and the microtiter plates were then incubated overnight at 37ºC. The agar plate colonies were counted manually, whereas the bacterial growth in the wells were measured by reading the OD_{600} of the microtiter plates using Cytation 3 plate reader (BioTek Instruments, Vermonta).

3.2.6. *Minimum inhibitory concentration (MIC) determinations*

The minimum inhibitory concentration 50 (MIC50) is the lowest concentration at which 50% of the bacterial growth is inhibited. All OD_{600} plate readings by Cytation 3 were exported to an Excel spreadsheet and blanked against sterility. The following protocol was used to determine the MIC50 consistently across all strains:

1. To account for well-to-well variability in growth, uninhibited growth was calculated as the average of all OD measures within 20% of the highest value.
2. To find the MIC50, the value from step 1 was divided by two and the lowest concentration at which the optical density falls below this number was recorded as the MIC50. The concentration can be directly deduced from the dilution concentration, since the extract concentration at 100% was 100 mg/mL. Reported values represent the mean and standard of error of the mean from three independent replicates.

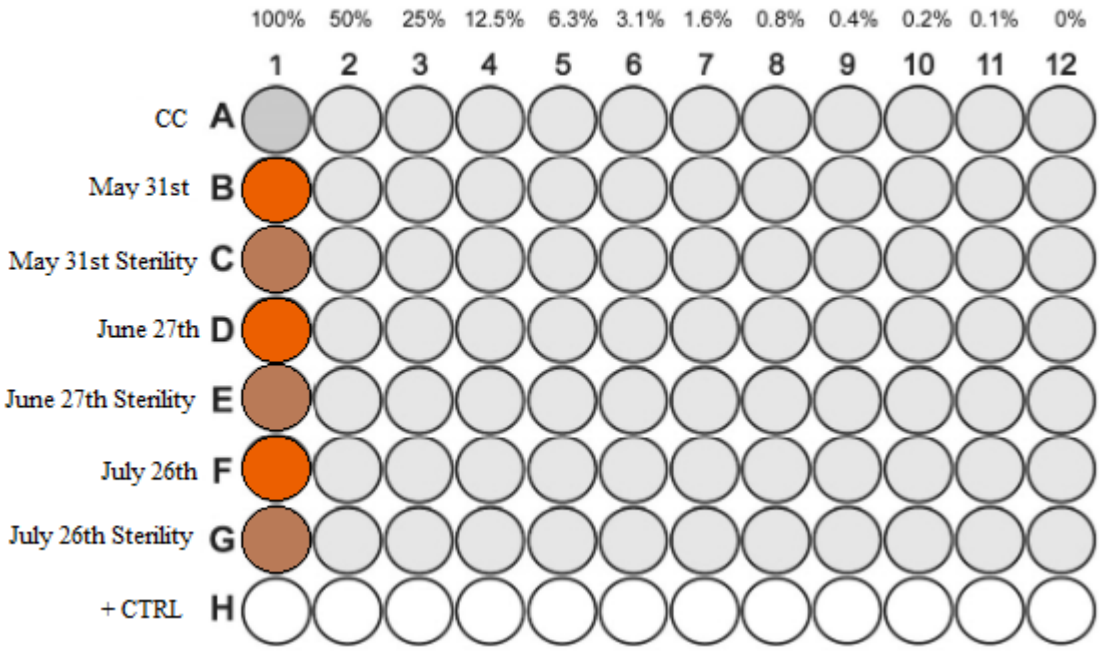

Figure 3.2. Set-up of 96-well microtiter plate for antibacterial assay. The orange wells represent 100 µL of the plant part extract pertaining to that plate (one plant part extract per plate). The brown wells indicate 50 µL of LB broth. Row A was 100 µL of the carrier control ("CC"), which was 30% EtOH in this protocol. Row H (white wells) indicating positive control (gentamycin).

3.2.7. *Data analysis*

The MIC50 was obtained for both the extract and the carrier control. The carrier control was used as a reference point for the uninhibited bacterial growth control as variability was within rows. After MIC50 was measured for each replicate across all plant parts and collection dates means and SEMs were calculated. For the first part of this experiment (comparing strains) the inhibition of each strain was tested using a T-test between the plant extract and the carrier control (Appendix 1.3) since sensitivity to the EtOH vehicle varied across *P. aeruginosa* strains.

Results from the next experiment, which looked as seasonal variability of bioactivity against PA14, were analyzed through comparisons of means using a one-way ANOVA to determine seasonal changes in activity, followed by *post-hoc* Tukey test. MIC data were also analyzed for significant activity relative to carrier control. Control data were averaged across all plant parts and collection dates (n=15) then tested for normality and significant differences. Since no significant differences were observed, this mean value was accepted as the CC for all plant parts. This was followed by *post-hoc* Dunnett's test as all extracts were compared to CC.

3.3 RESULTS

3.3.1 *Quorum sensing*

Bacterial QS plays a role in biofilm formation. Disruption of quorum sensing can prevent the establishment of biofilms or weaken established biofilms. Accordingly, anti-QS activity is an indication that a given antibiotic may have the ability to inhibit biofilm formation (Li and Tian 2012). If quorum sensing is not disrupted, then the antibiotic will be less likely to inhibit biofilm formation. Treatment of bacterial cultures with extracts of *E. purpurea* root, leaf, stem and flower led to no disruption of quorum sensing at a high concentration (500 ug/mL) (0 mm distance from disk to *C. violaceum,* n=3), which suggested poor potential as biofilm inhibitors.

3.3.2 *Minimum inhibitory concentration 50 (MIC50) – Strain sensitivity*

The first experiment analyzed the inhibition of 21 different *P. aeruginosa* strains using all plant part extracts from a single *E. purpurea* collection date (July 26th, 2016). These assays revealed only two cases in which bacterial growth was significantly inhibited in comparison to the carrier control: PA14 by the flower extract and JD312 by the root extract (Figure 3.3A; Figure 3.3D). Although strains PA111 and PA140 showed sensitivity across parts, they were not significantly different to the carrier control. Due to the inconsistent growth between control replicates of JD312, PA14 was the only strain chosen to test seasonal variability. Since this strain showed sensitivity to all plant part extracts (but only significantly for flowers), the impacts of seasonal changes in *Echinacea* chemistry (Chapter 2) on MIC50 were investigated using PA14 cultures.

3.3.3 *Minimum inhibitory concentration 50 (MIC50) – Seasonal variability*

The observed MIC50 values varied over 3- and 4-fold for leaf and root extracts, respectively, with flower and stem extracts exhibiting more consistent effects across the growing season (Table 3.1). However, with considerable variability within and between samples, no significant differences were observed between mean MIC50 and CC across all parts and collection dates (Table 3.1). In terms of seasonal variability of the bioactivity, there were no significant changes in bioactivity among extracts of the leaves, stems or flowers. Seasonal variability was found among root extracts, with the weakest activity at flowering (July 26th, 2016) which was significantly lower than the highest inhibition at maturity and end of season (August 26th and September 30th, 2016) (Table 3.1).

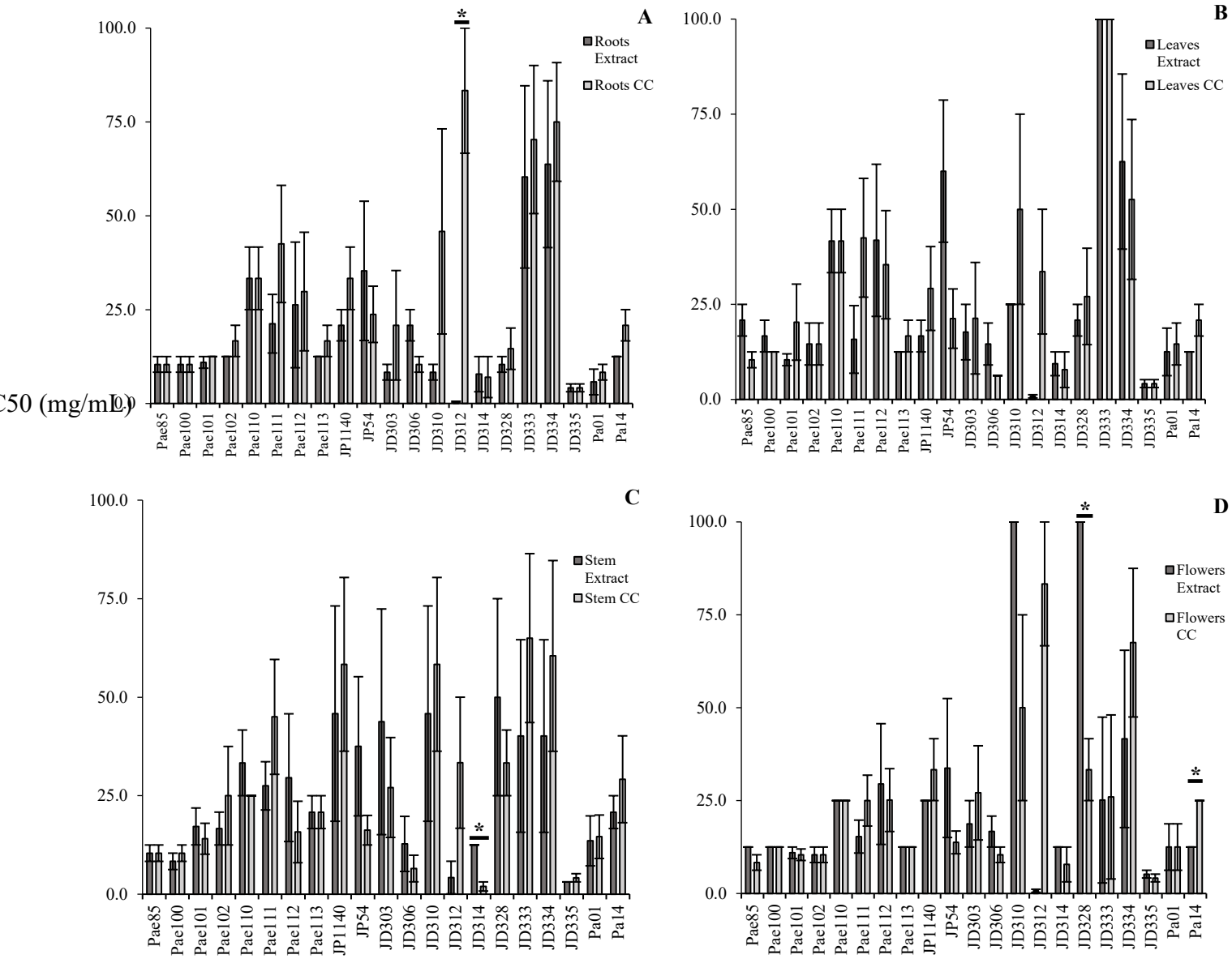

Figure 3.3. Mean (±SE) MIC50 of *E. purpurea* extracts vs 21 strains of *P. aeruginosa*. **A**: Roots, **B**: Leaves, **C**: Stems and **D**: Flowers. MIC50 was the concentration (mg/mL) at which 50% of the bacteria growth was inhibited. Extract refers to the MIC50 of the plant part and CC refers to the MIC50 of the carrier control (30% EtOH). For visualization purposes, a MIC50 of 100 in this graph means there was no point at which the bacterial growth dropped below the 50% of the growth control. Asterisks ("*") indicates a p-value ≤ 0.05 between means, as analyzed with an unpaired t-test. n = 3-6.

Table 3.1. Mean (±SE) MIC50 of *E. purpurea* plant parts throughout growth vs *P. aeruginosa* strain PA14, determined using a microtiter plate-based broth microdilution assay. MIC50 was the concentration (mg/mL) at which 50% of the bacteria growth was inhibited (extracts n=3; carrier control n=15; +CTRL n=15). 1.6 mg/mL was the lowest concentration tested. Letters a and b used to mark significance (p-value ≤ 0.05) between means of collections within each plant part, as analyzed with ANOVA and *post-hoc* Tukey. No significant differences were observed within leaves, stems, or flowers.

MIC50 (mg/mL)				
CC 8.6±1.1 +CTRL 1.6±0.0				
	Roots	Leaves	Stems	Flowers
May 31st	ab 4.7±1.6	13.5±6.3	N/A	N/A
June 27th	ab 12.5±0.0	10.4±2.1	N/A	N/A
July 26th	b 14.6±5.5	6.3±0.0	14.6±5.5	5.2±1.1
Aug 26th	a 3.2±1.6	12.5±0.0	10.4±2.1	4.7±1.6
Sept 30th	a 3.1±0.0	3.7±1.4	10.4±2.1	2.6±1.9

3.4 DISCUSSION

Echinacea's medicinal uses extend over a broad spectrum of symptoms and conditions, including OTO and respiratory ailments (Islam & Carter, 2005; Vimalanathan et al., 2017). *Pseudomonas aeruginosa* is one of the most widely studied respiratory pathogens in current microbiology research, whether it be assessing its genome, spontaneous mutations or its ability to resist antibiotics (Turner et al., 2015; Dettman et al., 2016; Vasse et al., 2017). Beyond its clinical importance, *Pseudomonas* is the most abundant endophytic bacterial genus in tissues of *E. purpurea* (Chiellini et al., 2014). Because of the demand for further research on this organism, it was of importance and interest to test the antimicrobial potential of *E. purpurea* to provide a potential alternative to biomedical antibiotics furthering the development of natural health products and evidence for their efficacy.

The first assay tested if *E. purpurea* had the ability to disrupt quorum sensing prior to determining its biofilm inhibitory potential (Simoes et al., 2009). The biofilm assay was of interest as over 80% of microbial infections in the human body are associated with biofilms which includes *P. aeruginosa* in the lungs (Khan et al., 2016). The negative result of the quorum sensing assay was unexpected since a study by Ortiz-Castro et al. (2009) predicted that the signalling system of alkylamides and cytokinin would inhibit quorum-sensing signalling due to plant responses to microbe signaling – although this prediction was not tested. Due to this negative result in quorum sensing disruption, it was assumed that these extracts would not be viable in inhibiting biofilms as the signalling of QS controls biofilm formation; thus, biofilms can not be inhibited without inhibition of QS (Hammer & Bassler, 2003; Singh et al., 2000).

Although *E. purpurea* did not show inhibition against most of the 21 *P. aeruginosa* strains, the significant result against PA14 by flower extracts (and its sensitivity to other extracts)

can be explained by the strain's stability. PA14 was one of the only laboratory strains tested (Appendix 1.1; Appendix 1.2), as most strains were obtained from either the environment or from cystic fibrosis patients (clinical). Laboratory strains are modified for stability, decreased pathogenicity and a have smaller genome, thus they can be more predictable than environmental or clinical strains which have increased capacity for resistance and mutations (Fux et al., 2005). Due to these differences – and as presented in Figure 3.3 – it can be assumed that *Echinacea* would not be as affective against clinical and environmental strains. A study by Mišić et al. in 2009 tested *E. purpurea* against several other clinically important pathogens using MIC assays at concentrations greater than 2560 µg/mL, also resulting in no significant antibacterial activity. This supports the finding that *E. purpurea* is not an ideal antibiotic target for inhibiting this clinically-relevant pathogen.

The seasonal variability in roots was minor since the only significant result was extracts at post-flowering which showed greater inhibition than during flowering. This result was not predicted as the phytochemical trends do not account for this finding (Chapter 2). In Chapter 2, we observed that alkylamides decreased in concentration over the growing season. Caffeic acid derivatives in roots increased over the season but were highest in concentration at flowering and decrease post-flowering – which means caftaric acid and cichoric acid were not responsible for the significant differences in seasonality of bioactivity. While the phytochemicals observed did not account for this finding, there could be other compounds at work in combination with alkylamides and CADs driving the bioactivity against *P. aeruginosa*.

This study may be insightful to natural health product companies in many facets. Most *Echinacea* products contain roots as the active ingredient, with fewer products containing aerial parts; however, this study suggests that flowers were also bioactive. Although seasonal effects

were only present in the roots, there was a significantly greater inhibition at the end of the season. As mentioned in chapter 2, biomass was greatest at the end of the season and thus the increased bioactivity at this time would be further reasoning to harvest when flowers are mature.

Future studies

Although there were few significant results from this study, the bioactivity of *E. purpurea* is still worth investigating. As many tests against fungi, bacteria and viruses have shown promising results (Sharma et al. 2008, Sharma et al. 2010, Vimalanathan et al. 2005 and Pleschka et al. 2009), in depth studies into separation of plant parts across the season should be continued with other organisms. In particular, the bioactivity of *E. purpurea* should be tested more extensively against organisms that affect the Oto-rhino-laryngological tract such as *C. albicans* as that is what *Echinacea* has been traditionally used for (Chapter 1).

3.5 CONCLUSION

In summary, this chapter profiles the bioactivity of *E. purpurea* against quorum sensing and *P. aeruginosa* strains. Across parts, there was no effect on quorum sensing, few strains sensitive to *E. purpurea* and minor seasonal effects. Due to the negative results, it can be concluded that *E. purpurea* is not an ideal candidate for the inhibition of *P. aeruginosa.*

CHAPTER 4: GENERAL DISCUSSION

4.1 Overview

This thesis investigated the phytochemical profile of all major parts of *Echinacea purpurea* throughout first year growth as well as how these phytochemicals were affected by organic foliar sprays. In addition, the antibiotic potential of *E. purpurea* against *Pseudomonas aeruginosa* was measured to determine if phytochemical fluctuations throughout the first year of growth correlated with bioactivity. This study was novel in several ways, first being that few outdoor field trials of *Echinacea* cultivation have been published and the organic foliar sprays (high cytokinin, low cytokinin from kelp and fish oils) have never been reported for *E. purpurea* for phytochemical and yield optimization. These results are of importance as there is an increasing need for alternative approaches to optimize crop yield and quality for natural health products (NHPs) as they are often used in conjunction with, or as a replacement to, antibiotics (Hemaiswarya, et al., 2008). Also, this study was unique in being the first to test the bioactivity of *E. purpurea* against 21 *P. aeruginosa* strains as well as testing all plant parts across seasonal growth against strain PA14.

In Chapter 2, I analyzed the phytochemistry and biomass of *E. purpurea* samples which were sorted by plant part, collection date and foliar spray treatment. The objective of this chapter was to investigate specific variables that lead to high yield of plant mass for industry purposes and to also determine how these variables effect commercially important phytochemicals. Although studies across growth have been done and affirm the seasonal trends in flowerheads for both alkylamides and cichoric acid, no study has looked at this set of variables in combination with each other – these variables being organic foliar spray use, throughout first year growth across all major plant parts (Letchamo et al., 1999; Letchamo et al., 2002). Despite this study not

resulting in a specific set of recommendations for industry that would result in both high biomass and high concentration of all key chemical constituents, it did result in a better understanding of the resource allocation of phytochemicals across parts of the plant throughout development as well as determining increased flowering with the use of foliar sprays. This finding is useful for industry as minimum product concentrations of alkylamides and cichoric acid are 3 mg/g and 5 mg/g, respectively, for assumed efficacy (Wills & Stuart, 1999). Throughout treatment groups, there was substantial variability between individual plants, generating large SEMs. This variability could have overshadowed seasonal trends, providing less significant differences between collection dates and between organic treatments.

The goal of Chapter 3 was to follow-up on the analysis from Chapter 2 by mirroring the seasonal variability of phytochemistry with seasonal variability of antimicrobial activity. In order to test this, I used the same extracts from Chapter 2 for consistent comparisons – although extract degradation may have occurred from the time of phytochemical tests to the time of the bioactivity assays (Nusslein et al., 2000). The microbe chosen for this was *P. aeruginosa* for its prevalence in public health and variability between strains. The strain sensitivity test only showed significant inhibition in 4 of the 21 strains – this statistical finding is predictable with this number of tests (including all plant parts) and should not be a considered to be substantial bioactivity. Overall, *E. purpurea* was not an ideal antibiotic in inhibiting the growth of *P. aeruginosa* PA14 as there were no significant differences to the carrier control. Since *Echinacea* has been reported to inhibit *P. aeruginosa* growth, our results nonetheless highlight the potential impact of different bacterial strains as a contributing factor toward inconsistencies in the reported antibiotic effects of other medicinal plants.

When correlating the significant bioactivity to phytochemistry, the observed phytochemicals can not explain the bioactivity. However, other CADs and individual alkylamides that were not quantified may account for these trends. The only significant difference in inhibition throughout seasonal growth was the root extract, which displayed greater antibiotic potential (lower MIC) during flowering than post-flowering. In terms of phytochemistry, CADs were highest at flowering and total alkylamides were lowest at the end of the growing season. This means that neither alkylamides nor CADs could explain the highest inhibition happening post-flowering. If an original strain-sensitivity test was done with samples collected at the end of the season, there may have been more strains sensitive to *E. purpurea.* These secondary metabolites are often studied for their bioactivity, yet numerous other secondary metabolites are associated with other pharmacological and immunostimulant effects such as polysaccharides, polyacetylenes, and flavonoids; therefore, they could be contributors to the inhibition demonstrated at the end of first-year growth (Barnes et al., 2005; Bohlmann & Hoffmann, 1983; Steinmüller et al., 1993; Kurkin et al., 2011).

4.2 Future directions

Phylogeny is a useful tool to speculate on a plant's medicinal potential as close relations could present analogous medicinal properties. For those plants that have been tested and used traditionally, observing close phylogenetic relatives could lead to the discovery of similar chemically active species. While it is clear that *E. purpurea* did not display activity against *Pseudomonas*, bioactivity against other clinical pathogens including *Candida albicans* has been reported and could be useful for other clinically relevant bacteria (Merali et al., 2013; Sharma et al. 2010). Since species within the same subtribe and tribe as *E. purpurea* could share much of

the same secondary metabolites, it would be useful to test these plants for similar activity – particularly the roots and flowers, where both CADs and alkylamides are most abundant.

Preliminary work has been done to follow-up with this study by Honour's student Simon Barry under my supervision, through testing the phytochemistry of several other plants that are phylogenetically related to *E. purpurea* including *Spillanthes acmella*, *Ratibida pinnata, Ratibida columnifera* and *Heliopsis helianthoides*. Developing poorly studied target species with phytochemistry and bioactivity related to *Echinacea*'s would benefit industry by providing novel NHPs to increase the current variety of products and applications. While the phytochemistry of these species has yet to be confirmed, the first set of exploratory chromatograms suggests several distinct alkylamides present in these plants, which, like *E. purpurea,* were predominantly detected in roots but also in flowers (Appendix 1.4). By increasing the replicates, confirming the phytochemistry through MS and sampling across first year growth, this study could provide a comparison to phytochemical similarities to *E. purpurea.*

Unpublished results from a colleague's Master's thesis found bioactivity of roots and flowers of *Achillea millefolium*, another Asteraceae species more distantly related from *E. purpurea* yet containing some of the same secondary metabolites, including alkylamides in roots and mature flowers (Kachura, Masters, 2018), revealing a consistent distribution of these bioactive secondary metabolites across the Asteraceae family and suggesting a role in root and seed physiology or chemical defence (Barnes et al., 2005). *In vivo* plant studies comparing the anatomy and pest resistance of high and low-alkylamide producing varieties would provide additional insight into this hypothesis.

Another avenue to expand on this study is to choose pathogenic microorganisms that affect conditions related to the ears, nose, and throat – including infections as these are the most

common traditional uses of *Echinacea* species among North American Indigenous peoples. The ailments that are considered under the OTO category are related through the tract of the human body of the same name. The first part "otology" referring to the studies of the ears, "rhinology" refers to studies of the nose and "laryngology" referring to studies of the throat. Conditions of the ear are often symptoms of inner ear inflammation which lead to pain and potential hearing loss (McCabe, 1979). The cytokines that are often associated with inner ear inflammation are Tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6) and interleukin 1 beta (IL-1β) (Satoh et al., 2003). These same cytokines are associated with other sources of inflammation such as sinuses and the throat as they are pro-inflammatory cytokines (Prasad et al., 2000). This suggests that the primary source of the conditions of OTO is inflammation as a result of upregulated release of cytokines TNFα, IL-6 and IL-1β (Fast et al., 2015). While these tests have previously been done on a number of the organisms affecting the OTO tract, there are no reports of this study being done using extracts of all *E. purpurea* parts throughout first year of growth. This study could be combined with the multiple plant species experiment previously suggested to create a profile of phytochemistry and bioactivity throughout growth of *Echinacea* and its relatives.

There are multiple factors that industry may implement in the future using the findings of this study. Although foliar spray treatments showed no consistent effect on chemistry and did not affect the yield of the highly-valued roots, foliar sprays increased biomass of stems and flowers, which may be beneficial when harvesting these parts for NHPs or seeds for propagation. Combining the foliar sprays, as seen in the study using cytokinin and fish oils on fruits to increase yield (Illera-Vives et al., 2015; Wiens and Reynolds 2008), may similarly enhance flower and seed production in *Echinacea*. Other practical knowledge gained for industry

cost/benefit analysis of harvesting after the establishment of mature flowers – granted that certain secondary metabolites were lower in concentration at this time, the increased biomass counter-balances this decrease. This knowledge can be applied to further *Echinacea* research to develop a larger profile of its phytochemistry and bioactivity. Also, this study can be applied to related plants to develop novel NHPs through displaying the significance of studying ethnobotany, quantitative phytochemistry and antimicrobial activity.

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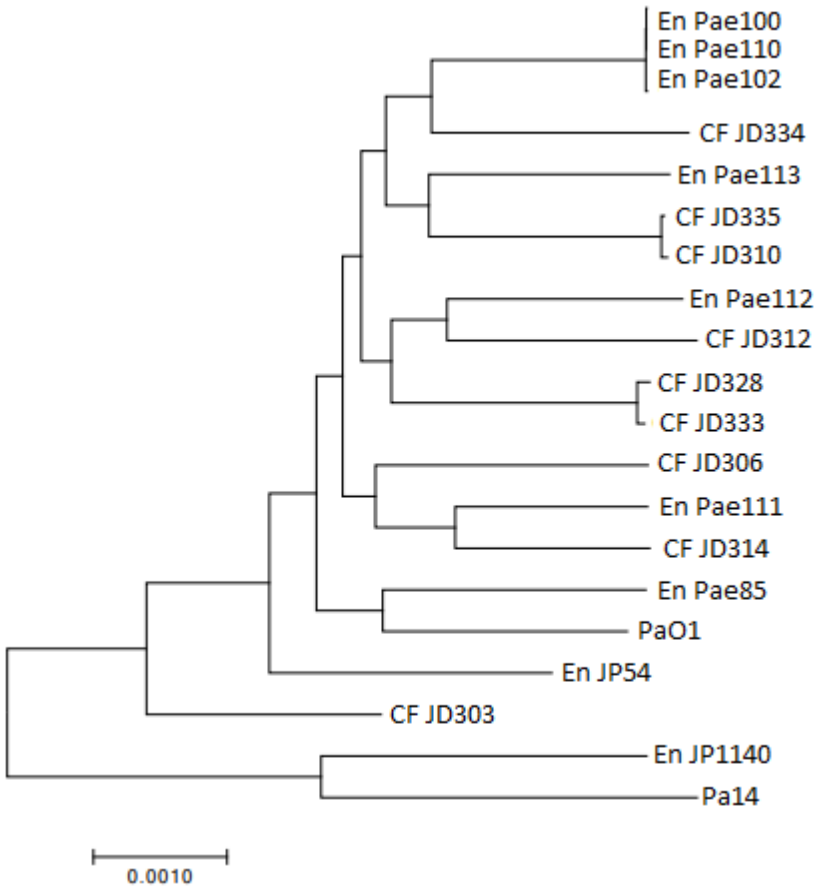
Appendix 1.1

Strains of *Pseudomonas aeruginosa* used for Honour's project as a precursor to Chapter 3.

Niche	Strain	Isolation Location	Country	Isolation Date	Source
Environmental	Pae85	King City, ON	Canada	2004	Soil
	Pae100	King City, ON	Canada	Apr 7/10	Soil
	Pae101	King City, ON	Canada	Apr 7/11	Soil
	Pae102	King City, ON	Canada	Apr 7/12	Soil
	Pae110	Toronto, ON	Canada	Apr 8/13	Soil/Water
	Pae111	Maysville, KY	USA	Apr 8/14	Soil
	Pae112	Maysville, KY	USA	Apr 8/15	Soil
	Pae113	Maysville, KY	USA	Apr 8/16	Soil/Water
	Jp1140	Pacific Ocean	Japan	2003	Sea water
	Jp54	Suruga Bay	Japan	2004	Sea water
Clinical	JD303	Toronto, ON	Canada	Nov/05	CF-patient
	JD306	Toronto, ON	Canada	Nov/05	CF-patient
	JD310	Ottawa, ON	Canada	Feb/06	CF-patient
	JD312	Kitchener, ON	Canada	Nov/05	CF-patient
	JD314	Kitchener, ON	Canada	Jan/06	CF-patient
	JD323	Hamilton, ON	Canada	Mar/06	CF-patient
	JD328	London, ON	Canada	May/06	CF-patient
	JD333	Toronto, ON	Canada	Jan/08	CF-patient
	JD334	Sudbury, ON	Canada	Sep/06	CF-patient
	JD335	Toronto, ON	Canada	Oct/06	CF-patient
	Pa14 *				
	PaO1 *				

*PaO1 and Pa14 are laboratory strains.

Appendix 1.2

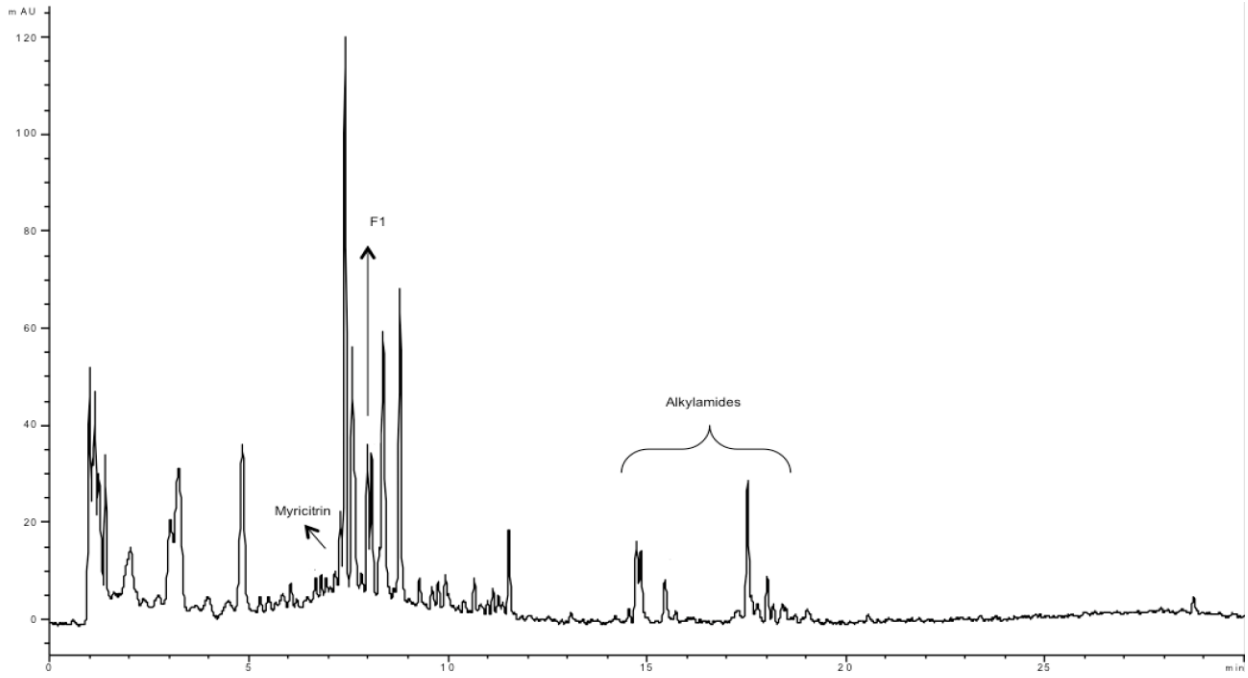

En Pae100
En Pae110
En Pae102
CF JD334
En Pae113
CF JD335
CF JD310
En Pae112
CF JD312
CF JD328
CF JD333
CF JD306
En Pae111
CF JD314
En Pae85
PaO1
En JP54
CF JD303
En JP1140
Pa14
0.0010

Appendix 1.3

Mean (±SE) MIC50 of *E. purpurea* plant parts vs 21 strains of *P. aeruginosa,* determined using microtiter plate-based broth microdilution**.** MIC50 is the concentration (mg/mL) at which 50% of the bacteria growth was inhibited. Asterisks ("*") indicate a p-value < 0.05, as analyzed with an unpaired t-test.

	MIC50 (mg/mL)							
	Flowers		Leaves		Roots		Stem	
	Extract ±SE	CC ±SE	Extract ±SE	CC ±SE	Extract ±SE	CC ±SE	Extract ±SE	CC ±SE
Pae85	12.5 0.0	8.3 2.1	20.8 4.2	10.4 2.1	10.4 2.1	10.4 2.1	10.4 2.1	10.4 2.1
Pae100	12.5 0.0	12.5 0.0	16.7 4.2	12.5 0.0	10.4 2.1	10.4 2.1	8.3 2.1	10.4 2.1
Pae101	11.0 1.6	10.4 1.6	10.4 1.6	20.3 10.0	11.0 1.6	12.5 0.0	17.2 4.7	14.1 3.9
Pae102	10.4 2.1	10.4 2.1	14.6 5.5	14.6 5.5	12.5 0.0	16.7 4.2	16.7 4.2	25.0 12.5
Pae110	25.0 0.0	25.0 0.0	41.7 8.3	41.7 8.3	33.3 8.3	33.3 8.3	33.3 8.3	25.0 0.0
Pae111	15.3 4.4	25.0 6.8	15.8 8.9	42.5 15.6	21.3 7.8	42.5 15.6	27.5 6.1	45.0 14.6
Pae112	29.5 16.3	25.1 8.5	41.8 20.0	35.4 14.2	26.3 16.7	29.8 15.8	29.6 16.2	15.8 7.8
Pae113	12.5 0.0	12.5 0.0	12.5 0.0	16.7 4.2	12.5 0.0	16.7 4.2	20.8 4.2	20.8 4.2
JP1140	25.0 0.0	33.3 8.3	16.7 4.2	29.2 11.0	20.8 4.2	33.3 8.3	45.8 27.3	58.3 22.0
JP54	33.8 18.7	13.8 3.1	60.0 18.7	21.3 7.8	35.3 18.6	23.8 7.5	37.5 17.7	16.3 3.8
JD303	18.8 6.3	27.1 12.7	17.7 7.3	21.4 14.7	8.3 2.1	20.8 14.6	43.8 28.6	27.1 12.7
JD306	16.7 4.2	10.4 2.1	14.6 5.5	6.3 0.0	20.8 4.2	10.4 2.1	12.8 7.0	6.5 3.4
JD310	100.0 0.0	50.0 25.0	25.0 0.0	50.0 25.0	8.3 2.1	45.8 27.3	45.8 27.3	58.3 22.0
JD312	0.7 0.4	83.3 16.7	0.8 0.4	33.6 16.4	0.4 0.2	83.3 16.7	4.2 4.1	33.4 16.6
JD314	12.5 0.0	7.8 4.7	9.4 3.1	7.8 4.7	7.8 4.7	7.0 5.5	12.5 0.0	2.0 1.2
JD328	100.0 0.0	33.3 8.3	20.8 4.2	27.1 12.7	10.4 2.1	14.6 5.5	50.0 25.0	33.3 8.3
JD333	25.1 22.3	26.0 22.1	100.0 0.0	100.0 0.0	60.4 24.3	70.3 19.7	40.2 24.4	65.0 21.4
JD334	41.6 23.9	67.5 20.0	62.5 23.0	52.6 21.0	63.8 22.2	75.0 15.8	40.1 24.4	60.5 24.2
JD335	5.2 1.0	4.2 1.0	4.2 1.0	4.2 1.0	4.2 1.0	4.2 1.0	3.1 0.0	4.2 1.0
Pa01	12.5 6.3	12.5 6.3	12.5 6.3	14.6 5.5	5.7 3.4	8.3 2.1	13.5 6.3	14.6 5.5
Pa14	12.5 0.0	25.0 0.0	12.5 0.0	20.8 4.2	12.5 0.0	20.8 4.2	20.8 4.2	29.2 11.0

Appendix 1.4



Chromatogram of *Heliopsis helianthoides* flowers demonstrating a possibility of alkylamides.