Natural Variability in Phenolic and Sesquiterpene Constituents Among Burdock (*Arctium lappa* L. and *Arctium minus* L.) Leaves for Potential Medicinal Interests

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

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Abstract

*Arctium lappa* and *Arctium minus*, commonly known as burdock, are introduced, feral, biennial species found throughout the United States. Native to Europe, these plants have been valued for centuries for their medicinal properties and/or as part of a healthy diet by both western and eastern cultures. In the United States, burdock is used to treat burns by holistic medical practitioners (i.e. the Amish communities) as it is believed to relieve pain, reduce scarring, prevent infection, speed healing, and minimize the need for narcotics, antibiotics, and skin grafting.

Since burdock is cross-pollinated, genetic diversity among burdock individuals is likely to result in substantial levels of phenotypic variability among and within species, as well as among and within accessions within species with respect to many traits, including levels of potentially health-beneficial compounds found in medicinal or edible plant parts. Moreover, because burdock thrives in diverse habitats, its production under conditions that might affect these compounds is probable. Constituent variation may also occur among different sized leaves, between first and second year plants, and between rosette leaves and stalk leaves. Therefore, the objectives of this research were to: a) develop techniques to evaluate biochemical components of burdock leaves; b) characterize sources of variability in leaf composition; c) and evaluate the effects of horticultural management techniques (i.e., irrigation, irradiance, and temperature levels)
on the biochemical profile of leaves. The chemical composition of these leaves was analyzed spectrophotometrically for their phenolic content and antioxidant power and then quantified on an HPLC-DAD instrument.

The variability in potentially bioactive chemical constituents among burdock individuals within the population was substantial. *Arctium lappa* and *A. minus* had high levels of variability for flavonoids and hydroxycinnamic acids among accessions and among plants within accessions. In general, *A. minus* plants contained more quantifiable chromatographic compounds (peaks) than those of *A. lappa*. *Arctium lappa* individuals produced primarily hydroxycinnamic acids, but *A. minus* individuals also synthesized high levels of specific hydroxycinnamic constituents along with several flavonoid compounds. Larger leaves contained higher amounts of phenolic compounds than did smaller ones, and second year rosettes contained levels of chemical constituents three-fold higher than these found in first year rosettes. Environmental conditions of high light, frequent irrigation, and cooler temperatures increased phenolic levels in burdock leaves. The data acquired from this project justify further efforts in burdock domestication and be used to develop future ideas and proposals on the commercialization and domestication of burdock and its use in the medical field.
Dedicated to Jane Robbins, and to Justin and Patricia Besancon.
Acknowledgments

I would like to ultimately thank my advisor Dr. Joseph C. Scheerens. I could not have wished for a better mentor in my life. Without his countless hours of help and patience, this project would not have been finished. I’d also like to thank my co-advisor, Dr. John Cardina and my committee member, Dr. Joshua Blakeslee for their encouragement and guidance. I would like to thank Dr. Ann Chanon for her continuous dedication and reassurance. I’d like to acknowledge Dr. Mark Finneran, MD, for this project idea, and Bizhen Hu, a fellow graduate student who worked innumerable hours working with burdock along my side. My gratitude goes to Sarah McNulty, for her constant support and comfort, Kesia Hartzler for her help in the greenhouse and growth chambers, Andrew Glaser, Scott Wolfe, and Griffin Bates for their continuous optimism, Catherine Herms and Jenny Moyseenko for their help with the field and sorting and collecting seed, Lynn Ault for his expertise in the field, Dr. Matthew Kleinhenz for always having the answer, Eun Hyang Han and Lu Zhao for their help with sesquiterpenes, and to my brother, Mark Robbins, for his fabulous artwork. A huge thank you goes to all of the student workers and visiting scholars who have had a hand in the burdock project: Whitney Miller, Brooke Mowrer, Mark Bricker, Abbey VanTyne, Kelsie Herring, Jozi Kohli, Abby Evans, Jebidiah Beeman, Peter McDonough, Alex Foster, Chris Huck, Lourdes Arrueta, Sahari Nunez, Rena Mejia, Delmy Sanchez, and Megan Phyillaier.
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Major Field: Horticulture and Crop Science
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Chapter 1: Introduction

*Arctium lappa* L. and *Arctium minus* L.

*Arctium lappa* and *Arctium minus*, species from the *Asteraceae*, are native to Eurasia. These species have spread throughout North America and Asia. They are typically biennial plants that germinate in the spring and flower in their second year. *Arctium lappa* and *A. minus* were chosen for this study due to their prevalence in the environment, the abundant availability of seeds collected from both species, and experience concerning their medical effectors gathered by holistic practitioners (i.e., Ohio Amish populations). *Arctium tomentosum* and *Arctium nemorosum* have also been introduced to the United States from Europe; however, they are fairly uncommon (Duistermaat, 1996; Strausbaugh and Cole, 1977).

Field-cultivated burdock plants reproduce by out-crossing and exhibit significant phenotypic variability, likely due to the environmental differences and their genetic variation (Gross et al., 1980). In the first year of life, the burdock plant is a rosette of 12 to 40 leaves and a deep taproot. The heart-shaped leaves on the rosette can grow up to 80 cm in length and have a thick layer of trichomes on the bottom surface. The second year burdock plant produces a stalk and branches up to 2 meters in height. The thick leaf
petiole can be an indicator of whether the plant is *Arctium lappa* (solid petiole) or *Arctium minus* (hollow petiole). The plant produces a corymbose inflorescence and the seed heads contain jagged edges which leads to seed dispersal by animals when they fasten on to fur, feathers, or clothing (Gross et al., 1980). Both of these species are often referred to by the common plant name, burdock.

Burdock is commonly known as a nutraceutical vegetable in Japanese and Korean cuisine (Duistermaat, 1996). The leaves, roots, and seeds are consumed as food or used as traditional medicines. Both are edible, and *A. lappa* roots are often used in the Japanese cuisine as a vegetable referred to as ‘gobo’, typically served in sushi bars. Customarily, the roots are served as a grilled vegetable, but they are also served in soups as well (Duh, 1998). This vegetable generally contains a bitter taste; however, Native Americans prepared burdock root as a form of candy. They thinly sliced the roots, coated them with brown sugar or maple syrup, and served them as a sweet candy chip (http://www.herballegacy.com). *Arctium* leaves are also commonly used in herbal refreshments such as tea, soda, or alcoholic beverages. The stringent taste is commonly paired with dandelions and served as a burdock and dandelion drink combination. Traditionally, burdock is used to treat rashes, boils, and general skin disorders (Chan et al., 2010). *Arctium* species also help enhance the immune system of the body and improve metabolism (Lin et al., 2002). Because of this plant’s anti-diabetic, anti-bacterial, anti-cancer, anti-viral, and anti-inflammatory effects, domestication of burdock would provide a valuable source of health-beneficial crops.
Burdock as a source of health-beneficial compounds

Consistent with their use in traditional medicine, *Arctium lappa* and *Arctium minus* contain antioxidants, antibacterial components, fungicidal activities, and antitumor constituents used for the treatment of asthma, blood disorders, and skin conditions. They are also believed to serve as a body detoxifiers, cancer preventatives, and rheumatoid arthritis pain relief (Gross et al., 1980).

Burdock roots and stems contain many constituents contributing to the medicinal bioactivity of these plants. Unique lignans, plant compounds known to exert anti-proliferative effects on cancer cells via induction of apoptosis, were found in burdock, particularly (-)-arctigenin and arctiin, as well as(-)-matairesinol and (+)-7,8-didehydroarctigenin (Liu et al., 2005; Matsumoto et al., 2006). Chlorogenic acid, para-coumaric acid, rutin, and caffeic acid are all additional phenylpropanoid antioxidants that have been isolated from burdock (Lou et al., 2010A). Further, inulin and benzoic acid from burdock have been reported to act as anti-bacterial agents (Lou et al., 2010B); while fructooligosaccharide and luteolin have been demonstrated to have prebiotic (Imahori et al., 2010), and anti-inflammatory (Ferracane et al., 2010) effects, respectively.

In addition to the above compounds, the leaves of *Arctium* species have been hypothesized to possess multiple bioactive constituents efficacious in the healing of burn wounds. Preliminary reports also indicate that burdock leaves possess phenolic, antioxidant, and anti-inflammatory properties which further aid in the healing process.
(Ferracane et al., 2009; Liu et al., 2005; Zhao et al., 2009). Specific medicinal compounds of interest in this area include lignans, sesquiterpene lactones, COX II enzyme inhibitors, and steroidal based constituents.

Burdock leaf phenolics as potential bioactive constituents for the treatment of burns

Phytochemicals are plant produced bioactive nonessential nutrients which may play beneficial roles in human health. Phenolic compounds, a particular sub-class of compounds, serve many roles in plants. They can act as defense mechanisms against predators, pathogens, and parasites. They also can add to the plants color, scent, and even taste (Baidez et al., 2007). Many phenolic compounds found in plants have been found to make or have been linked to antioxidant activities, anticancer, antibacterial, anti-aging, skin-protective, antiviral, and anti-inflammatory properties (Baidez et al., 2007; Han et al., 2007; Owen et al., 2000; Veeriah et al., 2006). Specific phenolic compounds identified in conjunction with these activities include phenolic acids, flavonoids, tannins, coumarins, lignans, stilbenes, curcuminoids, and quinoes (based on the glycosylation or the number and placement of the hydroxyl groups) (Huang et al., 2010). The health benefits of phenolics are thought to be attributed in part to their antioxidant activity (Balasundram et al., 2003).

Over 8000 natural structural phenolic variants have been reported each of which possesses one or more aromatic rings with one or more hydroxyl groups (Fresco et al.,
Phenolic acids comprise a major class of the plant-derived phenolic compounds. Two major subclasses of phenolic acids are the hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids generally are considered aromatic with a one-carbon side chain, C₆-C₁ structure, whereas hydroxycinnamic acids are aromatic compounds with a three-carbon side chain, C₆-C₃ structure. Flavonoids are another group of widely studied phenolic compounds, containing more than 4000 constituents (Huang et al., 2010). The skeleton structure of these compounds generally consist of a phenylbenzopyrone structure (C₆-C₃-C₆) consisting of two aromatic rings linked by three carbons, sometimes found in a third ring, an oxygenated central pyran ring (Cai et al., 2004). These classes are commonly associated with anti-inflammatory properties.

Though nonsteroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs are the current treatments for inflammation, these drugs are not always successful, especially with long-lasting inflammation, and can produce unwanted side effects. Therefore, there is a tremendous need for safer and effective treatments (Garcia-Lafuente, et al., 2009). Burdock plants, especially burdock leaves, exhibit high concentrations of antioxidants and phenolic compounds, particularly hydroxycinnamic acids and flavonoids (Duh, 1998; Lou et al., 2010B). These compounds possess antimicrobial and anti-inflammatory activities (Shetty and McCue, 2003; Shetty and Wahlqvist, 2004) and may be accountable for the usefulness of burdock leaves when used as bandages to treat skin ailments. Unfortunately, there has been little published literature on the quantification of phenolic compounds in burdock leaves is lacking. One study, however, measured the antioxidant activity of burdock leaves and correlated this with the
quantification of caffeic acid, chlorogenic acid, rutin, and cynarin. The authors also found identical phenolic compounds in the seeds and roots of burdock (Ferracane et al., 2010). In addition to phenolic acids, lignans, particularly arctiin and arctigenin, have also been discovered in burdock leaves and are thought to contribute to the anti-inflammatory effects of burdock (Liu et al., 2005). However, the variability of these compounds in burdock plants has yet to be reported burdock leaves contain pharmalogical compounds (Liu et al., 2005), it is unclear whether or not these leaves were from first year rosettes or second year stalk leaves, where the plants originated from, or from what size of leaves. Due to the biennial nature of this plant, it is possible that burdock stores most of its nutrients during the first year in their roots (Morita et al., 1993), which may lead to different phenolic compounds accumulating in first year leaves versus second year leaves.

The medical impetus for domestication of burdock for its leaves

According to the Centers for Disease Control and Prevention (CDC), the National Center for Health Statistics states that each year there are 2.1 million burn victims in the United States. Out of the 2.1 million, 100,000 are hospitalized and approximately 10,000 to 15,000 die (www.cdc.gov/nchs/nvss.htm). Treatment techniques for burns have changed minimally throughout the past fifty years, and are in great need of improvement. Currently, burns are scrubbed twice a day until the bleeding bed is prepared for a skin
 graft. Skin grafts are painful and take weeks to heal, often leaving the patient with multiple complications, which may include infection, dehydration, organ failure, disabilities, deformities, emotional scarring, and even death (Atiyeh et al., 2005).

Current holistic procedures for the treatment of burns involve applying a natural product-based salve (Table 1.1) to the wounded area and then covering it with an organic dressing composed of rehydrated burdock leaves. The salve lubricates the wound, alleviating the need to scrub or scrape the burned area, thus lessening a great deal of unneeded pain for the patient. This salve eliminates the need for antibiotics and narcotics due to its antibacterial properties and the covering of free nerve endings, reducing the pain. The burdock leaf, covered with dense wooly trichomes, aids in treatments of burn wounds by acting as a durable, flexible, hydrophilic, gauze-like bandage, absorbing wound exudates and gently removing dead tissue (Figure 1.1). The durability and texture of this leaf and its complement of bioactive constituents are thought to be properties which aid in the healing of many skin ailments.

According to Dr. Mark Finneran M.D., a physician associated with the practice of healing by the use of burdock, leaves of burdock plants are harvested in the summer and stored dry. When the application of these bandages are necessary, the leaves are rehydrated in boiling water and slightly dried before applied to skin. The rehydrated flexible leaf adheres to the wound easily and is changed and reapplied every 12 hours. With this treatment protocol, burn patients have been relieved of excruciating pain associated with burns, cleansing of the wounds, and skin grafts. The salve and bandage in
this treatment technique reduces pain for the wounds within thirty minutes of the first application. Dr. Finneran observed that burn victims treated with burdock leaves have re-grown new skin without skin grafting and with very minimal scaring (Figure 1.2). Burdock treatments not only eliminated the need of most skin grafts, but they also reduced the recovery time for complete healing to a short 20-40 days compared to 60-90 days with conventional treatments (Finneran, personal communication).

Economic benefits of burdock domestication

Even though the burdock treatments are used by the Amish Community, they are to date not permitted as conventional treatments for burns in the United States. The metabolic profiles of the compounds in these leaves, used as bandages, are unknown, and there are currently no established techniques to analyze the metabolites and their bioactivity. More information of the compounds produced in the burdock leaves is needed before this treatment protocol can be considered for medical practice.

The replacement of current burn treatments with burdock therapy would significantly decrease treatment costs (Anonymous, 2008), which according to Herndon (2007) can exceed $250,000 to treat one burn victim with a typical 30% body surface burn. This treatment would also reduce the use of narcotics, antibiotics, and I.V. fluids up to 90%. Burdock therapy could theoretically eliminate the use of burn tanks, decrease the
need for skin grafting by 80%, and reduce the time used in the intensive care unit by 25%.

Burdock domestication would not only benefit burn victims, but also would provide farmers, particularly Ohio farmers, with economic benefits by allowing the opportunity to grow a value-added crop, while at the same time increasing crop diversity. Ideally burdock would serve as both a high value medicinal crop as well as a food-crop. Not only would farmers profit off of the medical uses of burdock leaves, but they would benefit from burdock by providing vegetable foods and teas.

**Potential environmental impacts of burdock domestication**

The use of burdock leaves as a novel burn therapy is of great interest. However, the domestication of burdock comes with many risks. Burdock not only has been shown to effect the surrounding vegetative environment, but this weed has also been known to trap and kill native birds and bats (Nealen and Nealen, 2000; Wilkinson, 1999). According to Brewer (1994) and McNicholl (1994), burdock has been documented to kill hummingbirds, kinglets, warblers, and other avian taxa. Information pertaining to avian mortalities may be uncommon, but this characteristic of burdock is not rare.

Burdock is highly adapted to North American environments, and can be routinely found in pastures, roadsides, swamps, waste areas, and sometimes in sandy areas (Gross
et al., 1980). Among potential environmental consequences, burdock’s putative impact on cropland is perhaps most economically germane. Although the results of other alien weed infestations have been well documented, little is known specifically about burdock and its interaction with other species. Research has shown that burdock is a competitive weed, though not a noxious weed, and is present on arable lands. *A. minus* occurs in all of the contiguous U.S. except for Florida (USDA, 2010). Burdock species often, but not always, depend on mobile occupants in its environment for reproduction and seed dispersal (such as animals, people, etc.). Burdock has not only been found throughout natural and wild environments, but has also been found in cultivated farmland and urban areas, likely due to the spread of its seeds as it adheres to and travels with many pets (Gulezian and Nyberg, 2010). The thick taproots and mature root bark often monopolize resources from neighboring vegetation and are harmful to surrounding plant species (American Society of Plasticulture, 2002). Burdock’s fecundity makes it aggressive and it could therefore become a great weed pest. There has been little success with approved chemical controls to diminish the fecundity of burdock, and its seeds retain their viability for approximately 20 years in the soil. According to Gross and Werner (1983), although burdock is considered to be a biennial weed, it can persist as a rosette for up to four years before flowering. This does not affect the medicinal interest because the juvenile leaves are currently of most interest in this study, but it may impact land utilization and production. The domestication of this plant would provide a vegetable, tea, and an innovative bandage aiding greatly in the health and medicinal fields. If contained
properly, breeding and domesticating this weed would outweigh the potential environmental risks.

Summary and research objectives

In summary, burdock could provide an alternative, natural burn treatment. The leaves are known to contain phenolic compounds that are likely responsible for its pharmacological effects. Both the differential genetic makeup of burdock populations and the varying environmental conditions experienced between individual plants make it likely that the levels of medicinal compounds in burdock leaves will vary. For medicinal use, practitioners need to know which leaves to harvest, as varying sizes of rosette leaves may differ in the chemical constituents, as might first year and second year rosette leaves. Before the application of burdock treatment can be approved in the United States, more information about the types and levels of these compounds present in burdock leaves must be obtained.

This project was created to support the interest in commercializing burdock leaves for treatment of severe skin wounds. The overall goal of the research performed was to agronomically and compositionally evaluate burdock as a potential domesticate and to characterize the major bioactive constituents in burdock leaves that aid in the healing and treatment of burns. Specific objectives were 1.) to develop techniques to identify and evaluate biochemical components of both A. lappa and A. minus, 2.) to measure the variation in leaf biochemical profiles among 71 total accessions (i.e. due to accessions,
plants, age, and leaf position), and 3.) to evaluate differences in leaf biochemical profiles resulting from different environmental conditions that affected photosynthetic active irradiance levels, growing temperatures, and irrigation levels. In Chapter 2, the variability in phenolic composition in field grown *Arctium* species will be described. In Chapter 3, studies on the effect of different environmental conditions on the phenolic compositions will be discussed. In these studies, the variation within plants and between plants was reported by measuring the phenolic composition in each. Chapter 4 provides a summary and conclusions drawn from this research, and suggests future studies which can be performed to advance the state of the filed on this innovative and natural burn treatment.
References


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<thead>
<tr>
<th>Ingredients</th>
<th>Cyto PRO (Liquid Bandage)</th>
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<tr>
<td>Honey</td>
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<tr>
<td>Lanolin</td>
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<tr>
<td>Olive oil</td>
<td></td>
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<tr>
<td>Wheat germ oil</td>
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<tr>
<td>Marshmallow root</td>
<td></td>
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<tr>
<td>Aloe vera gel</td>
<td></td>
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<tr>
<td>Wormwood</td>
<td></td>
</tr>
<tr>
<td>Comfrey root</td>
<td></td>
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<tr>
<td>White oak bark</td>
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<tr>
<td>Lobelia</td>
<td></td>
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<tr>
<td>Myrrh</td>
<td></td>
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<tr>
<td>Vegetable glycerin</td>
<td></td>
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<tr>
<td>Bees wax</td>
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Table 1.1. Ingredients listed for Cyto Pro (Liquid Bandage). The ointment used as the natural product based salve simultaneously with the burdock leaves to treat burns (www.cytropro.us).
Figure 1.1. Burdock leaves used as bandages during the treatment of third degree burns. (Photo courtesy of Dr. Mark Finneran, MD. January 15, 2008).
Figure 1.2. Case study of burdock treatment. This child’s burned foot contained second and third degree burns, in need of immediate attention, and was treated with the burdock treatment procedure. After three dressings, 36 hours, a new skin layer started to form and infections/blisters were healed. After a short four days, this child did not require any medical attention. (Case study and photo courtesy of Dr. Mark Finneran, MD. Summer 2011.)
Chapter 2: Variability in phenolic composition in field grown *Arctium* species

Summary

*Burdock* (*Arctium*) species are of great interest in herbal medicine, particularly in the Amish communities, where burdock leaves are used as bandages on burn wounds to alleviate pain and inflammation. The identity of important burdock phytochemicals and the bioactivity by which they contribute to wound healing is unknown. In this study, phenolic compounds were chosen as the target constituents in these leaves. These secondary metabolites have been shown to contain multiple beneficial properties linked to the healing of wounds. This study was created to distinguish the differences among burdock accessions, among plants within accessions, among leaves within a plant, and between first and second year burdock plants. The composition of leaves was analyzed by multiple laboratory procedures. Total phenolic content by the Folin-Ciocalteu assay was used to spectrophotometrically document the complete phenolic profile of these leaves, the Ferric-Reducing Antioxidant Power assay was used to quantify the ferric reducing activity of the leaves, and a reverse-phase HPLC-DAD was used to chromatographically depict and preliminarily identify phenolic compounds. In the process of identifying phenolic content, preliminary data indicated that putative sesquiterpene lactone compounds were present in the chemical compositions of leaf extracts as well. The study
of 16 major chromatographic peaks putatively identified as hydroxycinnamic acids, flavonoids, and sesquiterpene lactones, revealed that overall, *A. minus* accessions generally produced more hydroxycinnamic acids and flavonoids than *A. lappa* accessions. However, *A. lappa* produced more sesquiterpene lactone compounds than *A. minus*. Variability was present among plants within accessions, but only showed differences among the means of compounds in *A. lappa* accessions. Within first year rosette plants, larger leaves contained higher amounts of phenolic compounds on a per gram basis than smaller leaves. Second year pre-bolting rosette plants exhibited higher concentrations of compounds when compared to the first year rosette leaves. No significant differences between stalk leaves or rosette leaves on a second year, flowering burdock plants were shown.

**Introduction**

The biennial weed, burdock, is native to Eurasia and has spread across the world and colonized in North America. Burdock plants cross-pollinate, presumably leading to substantial genetic diversity among individuals (Duistermaat, 1996). Field grown or wild plants exhibit significant phenotypic variability (Gross et al., 1980), but nothing is known about the possible variability in chemical compositions.

The leaves of *Arctium* species have been hypothesized to possess multiple bioactive constituents efficacious in the healing of burn wounds, but more information
about burdock chemical profiles, particularly the phenolic compounds, and the identification of these metabolites is needed before the innovative burdock burn treatment can be developed and applied in the medical field. Burdock leaves have been demonstrated to contain high levels of phenolic compounds (Lou et al., 2010B), which are likely to aid in the healing of burn wounds due to their anti-inflammatory, anti-biotic, and antioxidant activities (Duh, 1998; Shetty and McCue, 2003; Shetty and Wahlqvist, 2004).

Spectrophotometric and chromatographic techniques have been used in order to assay phenolic profiles of burdock leaves. The colorimetric Folin-Ciocalteu assay has been used to determine the amount of total phenolic content in *Arctium* leaves according to a modified version of Singleton and Rossi (1965). Folin-Ciocalteu (FC) reagent causes a color reaction, changing the sample solution from yellow to blue when it is reduced by phenolic compounds. FC reagent consists of sodium tungstate and sodium molybdate. The color change is due to the transfer of electrons at basic pH provided by the sodium carbonate in the reaction buffer. The intensity of the blue color indicates the amount of the phenolic power contained in the leaves to reduce the FC reagent and is quantified using a standard curve of gallic acid (Slinkard and Singleton, 1977).

The ferric reducing activity of the leaves was spectrophotometrically measured by the Ferric Reducing Ability of Plasma assay (FRAP), slightly modified from Benzie and Stain (1996). This method was used to assess the total antioxidant capacity of burdock leaves. Values of FRAP are obtained by comparing the absorbance change with reaction
mixtures and ferrous ions in known concentrations. The assessment of a sample depends on the amount of Fe$^{3+}$ that is converted to Fe$^{2+}$. The larger the absorbance, the more Fe$^{2+}$ is produced in the reaction. When Fe$^{2+}$ is formed, the antioxidants present in the sample via the transfer of the electron to the iron. The reduced ferrous ions formed in this reaction then complexes with TPTZ (2, 4, 6-tris(2-pyridyl)01, 3, 5, triazine) present in the buffer, resulting in a bright blue color. The higher the intensity of this color, the higher levels of antioxidants contained in the burdock leaves.

High Performance Liquid Chromatography (HPLC) was used to quantify and preliminarily identify individual compounds extracted from burdock leaves. HPLC is a widely used tool in biochemistry and analytical analyses (Allwood and Goodacre, 2010), and separates compounds from complex mixtures based on the relative affinities of sample molecules for the stationary phase (column) and mobile phase (solvent) of the chromatographic system (Kirkland, 1971). HPLC coupled to a diode-array detector (DAD) can be used to identify putative molecules or compounds based on their UV-Spectra and retention times (compared to internal standards), quantify compounds based on the areas of each peak (compared to a standard curve), and can also isolate and collect individual peaks/compounds separately. To examine the UV-Spectra of particular peaks of a chromatograph the DAD is necessary. Compounds are detected at specific wavelengths based on the detector settings, and the spectrum of a compound can be correlated with its retention time (both compared to a known standard) to increase the reliability of identifying compounds (Vrsaljko et al., 2012).
In our previous studies, we developed an optimized procedure for examining the phenolic content of burdock leaves (Appendix A). Leaves were harvested and maintained in a -20°C freezer until desiccated by multiple drying treatments including an incubator at room temperature, a forced-air tissue drier at 40°C, as well as a freeze-drier in order to optimize the phenolic content in leaf extracts. The three drying methods were subjected to two different solvent extractions, 1% acidified methanol and a solvent of acetone, water, and acetic acid (70:29.5:0.5 by volume) on two different burdock leaf tissue types (uniformly ground burdock powder and a residual fluff-like tissue of burdock leaves). The optimal drying method, extraction solvent, and leaf tissue were used for further research. From the results of these preliminary tests, maximal phenolic compounds were found in freeze-dried burdock powder, extracted with an acetone extraction solvent.

Previous preliminary studies have provided evidence that burdock is an inhibitor of the cyclooxygenase (COX) -II enzyme. Cyclooxygenase is a lipid metabolizing enzyme that catalyzes the oxygenation of polyunsaturated fatty acids. This process forms prostanoids, specifically eicosanoids, which are known to be potent cell signaling molecules connected to inflammatory processes (Charlier and Michaux, 2003). Phenolic compounds are suspected to be the primary inhibitors of this enzyme, thereby inhibiting the inflammation process, alleviating wound irritation and swelling. A COX inhibitor screening assay kit was purchased from Cayman Chemical Company (Ann Arbor, Michigan) to assay the ability of burdock leaf extracts to inhibit COX-2 enzyme. Burdock samples inhibited COX-2 activity by an average of 72% compared to controls. The results from this enzyme assay kit indicated that burdock contains compounds with
inhibitory effects against the COX-2 enzyme; however the exact compounds which inhibit this enzyme have yet to be identified.

In order for burdock leaves to be used in the medicinal field, an understanding of the variability of the bioactive constituents in these leaves is needed. The goal of this study was to assess and examine the variability among multiple burdock plants.

The specific objectives of this study were to:

1) Assess the phenolic variability among 71 accessions
2) Evaluate the phenolic variances among plants within accessions
3) Analyze the phenolic differences among leaves of different sizes within a plant
4) Distinguish the chemical composition differences between leaves harvested from first year rosette plants and leaves harvested from the same plant in its second year of life, before bolting
5) Determine differences between rosette leaves and stalk leaves on a second year plant post bolting and flowering

The determination of the variability among these burdock plants will be able to aid in the medicinal field. Specific species, accessions, or leaves can be manipulated through breeding processes to produce the optimal chemical constituents needed for ideal bandages for burns. Domestication of burdock as a crop could supply the medical field with burdock bandages and may provide far-reaching benefits to burn victims. The domestication of this plant could offer farmers a benefit of a marketable product with options from several burdock plant parts.
Materials and Methods

Seed collection and preparation

*Arctium lappa* and *Arctium minus* germplasm collection began in the spring of 2009. Seeds were collected throughout the world, particularly the United States and Europe (Table 2.1). Collectors were instructed to select ten burs from a single plant, where each bur contained over twenty seeds. Habitat conditions were noted, along with the geographical location of each plant. Seeds were stored at 4°C, 45% relative humidity. Transplant seedlings for field experiments were grown in the greenhouse since direct seeding proved unsuccessful. Through observation, it was noted that seeds of burdock species required light and high moisture for germination. Seedlings were transplanted to the field at OARDC’s Schafer Farm after the plants had developed two true leaves.

Field design and sample collection

*Variability among accessions.* To determine variability among plants of different accessions from different locations, a total of 71 previously collected burdock accessions were planted in the spring of 2011 (Appendix B, Figure B.1). This included 24 *Arctium lappa* accessions and 37 *Arctium minus* accessions collected from all over the world (Table 2.1). To study the variation among these accessions, each accession was planted in
three field replications containing five half-sibling plants in each. Among the five plants, three were chosen at random in each replication for sampling. Since the larger leaves from burdock rosettes are used as bandages, three mature leaves were harvested from each plant, creating a composite sample of nine leaves per accession per replicate. Analytical values associated with the three-plant accession field replications tended to vary widely (presumably due to the abundant genetic variability among half-siblings). Therefore, to obtain a more representative value for each compound, replicate values were averaged to obtain single megapixel quantifications for each peaks based on the nine plants harvested within the accession. A total of 1,917 leaves were chosen for harvest (71 accessions x 3 field replications x 3 plants per accession x 3 leaves per plant) creating a total of 426 samples (in laboratory replicates) for chemical analyses to compare the variation among accessions as described above.

Variability among plants within accessions. In order to describe the variability among half-sibling plants within accessions, chemical compositions of individual plants were studied. To reduce the number of samples, six Arctium lappa and six Arctium minus accessions were chosen to represent germplasm collected from environmentally and internationally diverse locations (Table 2.2). These six accessions were chosen from the 2011 planted field used for the ‘variability among accessions’ study, listed above (Table 2.1). Out of the five plants in field replication, three plants were chosen at random, and all leaves were harvested from each plant (Appendix B, Figure B.2). Leaves from each
individual plant constituted a plant sample. Assuming each plant contained 10 leaves (which did vary), a total of 1,080 leaves were harvested (six accessions x two species x three field replications x three plants per accession x 10 leaves per plant) creating a total of 108 (in laboratory replicates) samples for lab analyses (as described above) to determine the various bioactive profiles of each individual half-sibling plant within each accession.

*Compound variability as influenced by leaf developmental stage within first year plants.* In order to determine whether or not a newly produced leaf differs in its phenolic profile from that of a mature leaf, two common accessions of each species, found in Wooster, Ohio, were chosen for this study and planted in separate field plots with three field replications. Ten plants of each species were planted in each replication (Appendix B, Figure B.3). Three out of the ten plants were chosen from each field replication, and all of their leaves were harvested. Once harvested, these leaves were combined and then separated into four categories based on the leaf diameter: below 15 centimeters, between 15 and 20 centimeters, between 20 and 25 centimeters, and above 25 centimeters. With three field replications, two species, and assuming each plant contained approximately 10 leaves, a total of 180 leaves were harvested (two species x one accession x three field replications x three plants per accession x 10 leaves per plant), providing 24 samples (two species x four size categories x three field replications) for chemical laboratory analyses.
Variability among years for plants within accessions. Large and mature leaves are generally harvested for bandages from a first year rosette burdock plant; however, second year plants may contain very different and potentially more beneficial bioactive profiles. Plants analyzed in the 2011 ‘variability among plants within accessions’ study were reharvested in the summer of 2012 as second year rosettes, before a stalk appeared on the plant. Every leaf was harvested from these regenerated rosette plants and treated as described above. The leaves from each individual plant constituted a plant sample. Estimating that each plant contains 10 leaves, a total of 1,080 leaves were harvested (six accessions x two species x three field replications x three plants per accession x 10 leaves per plant) creating a total of 108 (in laboratory replicates) samples for lab analyses (as described above) for chemical analyses of each individual plant.

Variability between stalk and rosette leaves within second year plants. This preliminary study consisted of four plants: two Arctium lappa and two Arctium minus plants. These plants were harvested after the stalk formed, and the flowers were in full bloom. For an individual plant, all rosette leaves were harvested as one sample, and all of the stalk leaves were harvested for comparison (Appendix B, Figure B.4). The rosettes generally consisted of 10 to 20 leaves over 25 centimeters in diameter, whereas the stalk leaves contained up to 100+ leaves varying in sizes. Each of the four plants only contained two samples, generating a total of eight field samples and 16 laboratory extraction samples, performed in laboratory duplicates.
Materials, solvents, and reagents

Acetone, water, acetic acid, acetonitrile, and ethyl acetate solvents were purchased from Fisher Scientific and were all of HPLC-grade quality. Gallic acid, sodium carbonate (99.95-100.05% dry basic), sodium acetate (anhydrous), Folin-Ciocalteu’s phenol reagent (2N), 6-hydroxy-2,5,7,8-tetramethylchroman-22-carboxylic acid, iron (III) chloride, and 2,4,6-Tris(2-pyridyl)-s-triazine were all purchased from Sigma Aldrich. Chromatographic vials with septum caps, 0.45um filters, and 3ml syringes were purchased from Fisher Scientific.

Sample preparation and methods for phenolic extractions

Composite samples of burdock leaves were harvested from 24 A. lappa accessions and 47 A. minus accessions to distinguish the variability among accessions. Burdock leaves were also collected from individual plants within six A. lappa accessions and six A. minus accessions to determine the variability among plants within accessions. To analyze the differences within a plant, composite samples of specific leaf sizes from within an A. lappa accession and an A. minus accession were collected. Second year plants were studied the following year to compare to first year plants, and to distinguish variability within second year stalk leaves versus rosette leaves. Each sample was
analyzed for their phenolic and antioxidant contents via spectrophotometric assays and HPLC quantification.

During individual leaf harvest, collected leaves were placed in labeled bags and stored in a 4°C cooler for less than three hours. Each leaf within a sample was washed to remove any soil or contaminants and towel dried to remove residual surface water. The total area of each leaf was recorded and measured with a LI-COR, Inc. LI-3100C Area Meter. Leaves were also placed on a scale for their individual fresh weights. After these two measurements, midveins were removed and the remaining blade tissues were placed back into labeled bags and stored in a -20°C freezer until further preparation.

According to optimized sampling procedures in the preliminary studies (Appendix A), each set of leaves was lyophilized with a Labconco FreeZone® 12 Liter Freeze Dry System equipped with a Stoppering Tray Dryer. When dried, the leaves were ground in an analytical mill (IKA, A11 basic) and passed through a 177 micron sieve. The resulting fine-particled powder was collected and stored in multiple polypropylene tubes and placed into a -20°C freezer and used in following extractions.

Prior to extractions, each sieved powder sample was weighed into two replicate polypropylene tubes, measuring 0.250 g each. Phenolic constituents were extracted using a method modified from Ozgen et al. (2008), where 30ml of acetone, water, and acetic acid solution (70:29.5:0.5 v/v) was added to each sample tube and agitated to a suspension every five minutes for 30 minutes. A Thermo Scientific Sorvall® Legend™ T/RT Centrifuge was used to separate the particulate tissue from the samples for 15
minutes at 7800 g to create a pellet. The supernatant was pooled into a 250 ml suction filtration flask equipped with a Buchner funnel and passed through a Whatman No. 1 filter paper. The pellet was resuspended in 30 ml of the acetone extraction solvent three more times. After the final supernatant was pooled in the flask, the 120 ml of burdock extract was transferred to a 500 ml round bottom flask and concentrated using a BUCHI RII Rotovaporator System equipped with a V-700 vacuum pump and a water bath temperature of 35°C, also equipped with a Brinkmann cooling unit. The extracts were reduced in volume until the sample was free of acetone and acetic acid as determined by free form olfactory inspection, leaving the burdock extracts in predominantly water. The remaining sample was transferred into a 25 ml volumetric flask and brought to a standard volume of 25 ml with water. These extracts were divided into two samples: one portion (5 ml) was used for FRAP and total phenolic assays, and the second portion (20 ml) was used for further phenolic extraction using ethyl acetate.

To reduce the number of chromatographic samples, the two acetone extraction replications were combined, extracted with ethyl acetate, and used as a representative sample for the HPLC analysis. Ethyl acetate extractions on acetone extract fractions were performed in a 50 ml polypropylene tube with 22.5 ml of combined burdock extract, 7.5 ml of 0.4 M sodium acetate, and 20 ml of ethyl acetate. After agitating the sample, the solution was phase partitioned and the upper (organic) phase was collected in a 50 ml glass tube. The lower phase was re-extracted sequentially with 20 ml and then 10 ml of ethyl acetate. The combined organic phase of ethyl acetate extracts (approximately 50 ml) containing burdock phenolics was dried under a stream of nitrogen at 35°C with an
OA-SYS Nitrogen evaporator system until dryness. The dried ethyl acetate fraction was stored in a -20°C freezer for less than two weeks prior to HPLC analyses.

*Measurement of total phenolic content*

A modified procedure from Singleton et al. (1999) was used to determine the amount of total phenolics present in leaf samples. An aliquot of 1 ml of burdock acetone extract was diluted with 23 ml of double distilled water (ddH₂O) and mixed with 1 ml of FC reagent and allowed to react for 8 min. Following this, 10 ml of 7% sodium carbonate solution was added to the sample to neutralize the reaction, along with 20 ml of ddH₂O. This mixture was incubated at room temperature for 2.0 h before reading the absorbance at 750 nm on a Beckman Coulter DU730 UV/Visible Spec spectrophotometer. A standard curve was prepared with concentrations of gallic acid ranging from 0-500 mg/L. Total phenolic content was calculated as gallic acid equivalents according to a daily standard curve performed in tandem with sample analyses (R² = ≤0.999), and expressed as milligrams of gallic acid equivalents per gram of fresh leaf tissue. Two laboratory replications were performed for each acetone extract.
Analysis of antioxidant power

An adapted method from Benzie and Strain (1996) was used to obtain values of FRAP by comparing the absorbance change at wavelength 593 nm with reaction mixtures and ferrous ions in known concentrations. A working solution was prepared and consisted of 30 mM sodium acetate (pH 7.0) mixed with 20 mM FeCl₃ and 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (10:1:1). An aliquot of 2.97 ml of this working solution was combined with 30 µl of burdock extract and mixed vigorously. This solution was allowed to react at room temperature for 1 hour before the absorbance of the resulting purple color was measured at 593 nm. A standard curve was created of 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (trolox) ranging from 0-0.1 µM trolox molecules ($R^2 = \leq 0.999$). Burdock samples were compared to this standard curve, and the amount of antioxidants present in these leaves were calculated. Results were reported in trolox equivalents per gram fresh weight of leaf tissue. Two laboratory replications were performed for each acetone extract.

Determination of individual phenolic compounds by HPLC

Phenolic content of burdock was examined and quantified by a reversed-phase HPLC System Gold 406A liquid chromatograph (Beckman Coulter, Inc., Fullerton, CA) equipped with an autosampler (model 508) and a diode array detector (model 168).
interfaced to an IBM computer with Beckman Coulter, Inc. 32 Karat V.8.0 software. The dried ethyl acetate extractions described above were re-dissolved in 1 ml (HPLC-grade) 30% Acetonitrile (CH$_3$CN). This solution was filtered using 3 ml disposable luer-lock syringe attached to a disposable 0.45 µm nylon filter. In order to adequately quantify peaks of highly variable content, it was necessary to run samples at this standard concentration, but also at a 10 fold dilution. These filtered extracts were transferred to labeled amber chromatographic sample vials. Each sample was analyzed on a Phenomenex Gemini (C6-phenyl) column at a constant temperature of 30°C. The mobile phase of this program consisted of the first solution (solvent A) 0.2% acetic acid in HPLC grade water, and the second solution (solvent B) of 100% HPLC grade CH$_3$CN. The injection volume of the sample was 50 µl and the program had a flow rate of 0.7 ml/min. The 55 min HPLC program consisted of a solvent (mobile phase) gradient starting at 9% B held for 10 min, then ramped to 22% B over the next 10 min with a transition to 30% B from 20 min to 35 min. The program changed to 60% B from 35 to 40 min, was held at 60% B from 40 to 45 min, and transitioned to 9% from 45 to 50 min and was consistently held at that concentration until 55 min. The quantifying detection wavelengths were 256 nm and 320 nm. The UV/Visible spectra of peaks and their retention times were used to categorize and in some cases, putatively identify compounds. Individual peaks were quantified using peak area reported by the Beckman Coulter 32 Karat software. Individual phenolic content was expressed as megapixels.
Statistical analysis

Prior to analyses, data were transformed to a normal distribution \((\bar{x} = 0, \sigma^2 = 1)\) using the PROC STANDARD procedure in SAS 9.2 software. Variability among accessions was analyzed by the FASTCLUS procedure. The 16 major chromatographic peaks were analyzed and the accessions were clustered into 4 groups. Box plots were provided to show the variability between accessions for the 16 major peaks and also to show the variances among plants within 12 accessions for the 16 major peaks. The PROC CORR procedure was used to create correlation graphs to show the relationship between TP and FRAP, and the relationships between the peaks and FRAP. Variability among plants within accessions compared the coefficient of variances among accessions of each species using the PROC GLM procedure. The variability in mean values within leaf sizes, between first and second year plants, and within second year plants was also studied by the PROC GLM procedure. Significant differences among means were determined by Tukey’s Procedure. Significance was measured with alpha=0.05.
Results and Discussion

Compounds of interest

Sixteen major chromatographic peaks were examined and quantified from *A. lappa* and *A. minus* when present. Average chromatograms of both species are shown (Figures 2.1 and 2.2) to indicate the relative retention times and differential solubilities of each peak. Although these compounds have yet to be identified via LC-MS and/or GC-MS, their UV/Vis spectra were used to tentatively classify each as a hydroxycinnamic acid, a flavonoid, or a sesquiterpene lactone. Overall, 16 major chromatographic peaks were identified throughout both species (Figure 2.3). The putative hydroxycinnamic acids, flavonoids, and sesquiterpene lactones in the burdock profile produce spectra containing characteristics that are similar to, but not identical to, the standards shown below (Figure 2.4). Slight differences observed between the peaks and their spectra and the standards may be due to relatively minor differences in the structure. However, since spectra are influenced by compound concentration, they may indeed be identical compounds.

Hydroxycinnamic acids are a class of polyphenols containing a C6-C3 skeleton. Para-coumaric acid is similar in structure to caffeic acid, but lacks the hydroxyl group at the #3 position. There is a relatively drastic shift in light absorbance with just this minor change. Ferulic acid is also very similar in structure to caffeic acid, but the hydroxyl group at the #3 position is replaced with a methoxy group. Ferulic acid is a precursor in
the formation of aromatic compounds. Chlorogenic acid is formed through the esterification of caffeic acid to the hydroxybenzoic acid, quinic acid. The spectra of ferulic and chlorogenic acid more closely resemble that of caffeic acid than they do para-coumaric acid.

Flavonoids are a class of compounds containing ketone structures. The spectra of the preliminarily identified flavonoid peaks in burdock closely resemble those of flavonoid glycosides, in particular quercetin-3-rutinoside, quercetin-3-galactoside, and quercetin-3-glucoside. Other compounds found in burdock appear to resemble aglycone flavonoids such as, isorhamnetin (a methylated quercetin), myricetin (a flavonol which contains a 3-hydroxyflavone backbone and 6 hydroxyl groups), and apigenin-7-glucoside (an aglycone of several glycosides).

Due to the complexity of identifying sesquiterpene lactones on the HPLC, only one member of this family, parthenolide, was able to be putatively identified which matched the retention time of burdock peak 16. Peak 14 was also preliminarily identified as a parthenolide derivate.

The phenolic profile in the *Arctium minus* plants contains the same compounds found in *Arctium lappa*. However, in some instances, the apparent concentrations are drastically different. The compound represented by Peak 14 appears to be the most “standard” between the two species. Proportionally the compound in Peak 1 (putatively caffeic acid) was drastically reduced in the *A. minus* profile compared to that of the *A. lappa* profile. On average, *A. lappa* contained fewer quantifiable peaks than *A. minus*. In
order to identify what each compound is, each peak will be isolated from the HPLC and analyzed using LC-MS and GC-MS procedures.

Variability among accessions

Among accession variability was explored to determine the concentration limits of each potential bioactive compound within the overall population and to determine patterns among accession groups based on species, accession origin, compound class, or other common factors. Each of the 71 accessions was analyzed for their variability within each of the 16 major chromatographic peaks (Figure 2.5). The box plot represents all 71 accessions and the variability of concentrations within each peak. This box plot demonstrates the variability of peaks with the population. The boxes are comprised of the values in which fall within the 25th and 75th percentiles. Peak 11 contains the largest box, indicating that this percentage range for this particular peak is larger than others. The whiskers denote the range of values within the 10th and 90th percentile and the dots represent values outside of these ranges. Peak 11 also shows a large whisker which represents a large amount of variability among the 10th and 90th percentile range of this peak. Peak 16 shows a small number of dots, indicating that most of the variability of this peak throughout the 71 accessions is within the range of 10 and 90 percent. The most prominent outlier is in chromatographic peak 14, a putatively identified sesquiterpene lactone compound. One accession in particular produced this peak in abundance
compared to the other accessions. Aside from peak 14, the more variable peaks among these accessions were peaks 1, 9 and 11, all preliminarily identified as hydroxycinnamic acids.

A cluster analysis was performed to group individual accessions within populations based upon the degree of their commonality. Initially, the statistical procedure was limited to four clusters in order to uncover factors underlying the strongest relationships among accessions (Table 2.3). Cluster 1 contained 20 accessions consisting of mainly A. lappa (18 A. lappa and two A. minus). This cluster was perhaps driven by lack of peak 10 and the high amounts of peak 1. Cluster 2 contained 41 accessions (five A. lappa and 36 A. minus). Peak 16 was higher in this cluster than any of the others, possibly being the main contributing factor of this cluster. Cluster 3 contained one A. lappa accession (L_03) due to the high amount of chromatographic peak 14 (a putative sesquiterpene lactone) it produced. Cluster 4 contained nine A. minus accessions and no A. lappa accessions. The amounts of peaks 4, 7, 10, 11, 12, and 15 in this cluster were substantially higher than the other clusters. This table shows the general trend of accessions within a species clustering with each other.

To further investigate this cluster supposition, concentrations of compounds within classes were used separately to cluster accessions (Tables 2.4, 2.5, 2.6). The analysis of the hydroxycinnamic acid concentrations (Table 2.4) also indicated the separation based on species. Cluster 1 of the hydroxycinnamic acids grouped an A. lappa accession (L_18) by itself due to the lack of peaks 11, 13, and 15; however, this cluster
seemed to contain a substantially higher amount of peak 2. Cluster 2 contained eight *A. minus* plants with the addition of one *A. lappa* accession (L_22). This cluster seemed to be driven by the high amounts of peaks 8, 11, and 13. Cluster 3 was the largest grouping and generally contained 36 *A. minus* accessions, but it also contained nine *A. lappa* accessions. Peak 15 showed the highest mean value, though there was no considerable difference in these concentrations. Cluster 4 contained 13 *A. lappa* accessions and four *A. minus* accessions. The clusters containing mainly *A. minus* plants reported higher concentrations of hydroxycinnamic acid peaks than those of the clusters containing *A. lappa* accessions, with the exception of L_18 from cluster 1. The *A. lappa* accession L_22 also stood out with the *A. minus* plants in cluster 2 which reported the highest averages for each hydroxycinnamic acid peak. The peaks which appeared to be the primary factors when clustering were peaks 2, 11, and 13; however, many accessions lacked the production of these peaks.

When looking at flavonoid compounds alone, the 4 clustered groups appeared more strongly grouped than the hydroxycinnamic acids (Table 2.5). Cluster 1 only contained four *A. minus* accessions, and cluster 4 consisted of only eight *A. minus* accessions. Clusters 2 and 3 were the only clusters which contained *A. lappa* accessions, where cluster 3 contained 20 *A. lappa* accessions and cluster 2 only had four. Cluster 1 was grouped due to the highly concentrated flavonoids (peaks 4, 7, 10, and 12) produced in these four *A. minus* accessions. Cluster 4 grouped these *A. minus* accessions perhaps due to the high amounts of peak 5, and the considerably low concentrations of peak 6. Cluster 2 contained the most amounts of accessions, and also possessed no sizeable
differences in concentrations of each peak. Cluster 3 contained mainly A. lappa plants, with the exception of one A. minus accession (M_19). This grouping was due to the lack of peaks 10 and 12, the high amount of peak 1 and 6, and the very low amounts of peaks 5 and 7. The clusters, according to flavonoid peaks, grouped the 71 accessions largely by species. A. minus accessions, with the exception of M_19, had consistently higher concentrations of flavonoids than A. lappa accessions. The peaks that seemed most responsible for the clustering of these accessions were peaks 3, 6, and 7; however, peaks 6 and 7 were not always present throughout all accessions.

When distinguishing the differences in variability in regards to the putative sesquiterpene lactone peaks 14 and 16, the pattern of species grouping continued; however A. lappa accessions appeared to produce more of these two peaks than A. minus (Table 2.6). Cluster 1 contained the highest amount of accessions with no substantial differences in either peaks. Cluster 2 contained only A. minus accessions which produced high amounts of peak 16. Cluster 3 contained one A. lappa accession (L_03) which produced an exceedingly high amount of peak 14. Cluster 4 contained eight A. lappa accessions along with one A. minus accession (M_15) and these tended to have higher concentrations of peak 14. These results indicate that A. lappa accessions, and the particular M_15 accession, produce higher amounts of sesquiterpene lactones than A. minus accessions. Peak 14 appeared to be the strongest driver peak of the putative sesquiterpene lactones.
Figure 2.6 displays the relationship between the FRAP and TP results. Both techniques tend to follow the same trend, indicating that they are highly correlated with each other. Though FRAP and TP are strongly correlated, FRAP is a more general test that can detect many other compounds in addition to phenolics containing antioxidant power.

When the three classes of compounds were analyzed individually, total megapixel areas of each class were obtained and averaged within each accession. Each of the 71 accessions were correlated with antioxidant power (Figure 2.7). The hydroxycinnamic acids reported to be the most predictive of antioxidant power, with an $R^2$ value of 0.46. Chromatographic peaks 1, 2, 8, 9, 11, 13, and 15 megapixels were summed and compared to the FRAP values for each accession. This class of compounds showed the most tightly correlated representation with antioxidant power. The flavonoids showed a significant correlation to FRAP values, with an $R^2$ of 0.17; however, the predictive values of this compound class were weak according to the $R^2$ value. Peaks 3, 4, 5, 6, 7, 10, and 12 were all analyzed for the flavonoid correlation with antioxidant power. Though flavonoids showed less correlation with FRAP than the hydroxycinnamic acids, they still showed a higher correlation with antioxidant power than did sesquiterpene lactones. In this case, only two suspected sesquiterpene lactones are reported in burdock leaves. These two peaks were compared with the FRAP values for each accession and conveyed little compatibility with FRAP. The $R^2$ value for this particular correlation was <0.01.
In summary, the resulting identifications of the variability among these 71 accessions failed to indicate apparent differences between the parent plant origins. The results did show that *A. minus* accessions tend to produce more hydroxycinnamic acids (with the exception of two *A. lappa* accessions, L_18 and L_22). This class of compounds showed the highest correlation with antioxidant activity. In contrast, *A. minus* plants exhibited higher flavonoid concentrations. Sesquiterpene lactone compounds tended to be produced at higher levels in *A. lappa* accessions, especially in L_03, than *A. minus*.

*Variability among plants within accessions*

To determine the variability within accessions, a subsample of six *A. lappa* (L_03, L_07, L_09, L_15, L_19, and L_25) and six *A. minus* (M_12, M_13, M_14, M_24, M_49, and M_51) accessions were chosen for study. These 12 accessions were chosen based on the diverse locations of the parent plants. In order to compare original location differences and to distinguish the variability within plant accessions, three *A. lappa* and two *A. minus* accessions were selected from local Ohio environments, whereas the other seven were chosen from other states or countries (Table 2.2). To represent the variability within each accession, box plots were created for each of the 16 major chromatographic peaks (Figure 2.8). Chromatographic values for the nine plants chosen for study in each accession were represented in an individual box plot for each chromatographic peak.
Peaks 9 and 10 are not shown because not one plant in this study contained either of these compounds. Peak 8 was found in only one data point from one A. lappa accession, L_15, in this study. The putative hydroxycinnamic acid, peak 11, showed the most variability in both A. lappa and A. minus accessions. The least variable peak in these accessions was peak 14, the preliminarily identified sesquiterpene lactone. Consistent concentrations of this peak among accessions resulted in less variability than was observed for other peaks.

TP and FRAP values were also analyzed for the variability among plants within accessions and supported the claim above that these two spectrophotometric tests are highly correlated. Box plots were generated to allow a visual comparison (Figure 2.9). The $R^2$ value for the correlation between TP and FRAP in A. lappa accessions was 0.95 with $P=0.0001$. The $R^2$ value for A. minus accessions was 0.97 with $P=0.0001$. These tests showed no significant differences among plants within these 12 accessions.

When comparing A. lappa to A. minus accessions, A. lappa showed more variability within peaks 1, 2, 3, 6, and 8; three putative hydroxycinnamic acids and two flavonoids. Among these peaks, L_15 showed to be the most variable accession in three of the peaks. A. minus accessions showed more variation in peaks 4, 5, 7, 12, 13, 15, and 16; preliminarily identified as four flavonoids, two hydroxycinnamic acids, and one sesquiterpene lactone. The accession, M_49, displayed more variability throughout these peaks over other A. minus accessions. It was also determined from the box plots that along with L_19 and M_49 accessions, L_19 showed enormous variability, especially in peaks 12 and 13. These peaks were more variable within the A. minus accessions, but this
accession showed an abundant amount of variability within these peaks. The other A. 
*lapp*a accessions did not produce these peaks, increasing the contribution of accession 
L_15 to be highly variable.

To estimate differences in overall compound variability within accessions, 
coefficients of variability (CVs) were calculated among the extant values (n = nine, when 
all plants in the accession exhibited the peak) for each peak (Table 2.7). CV values for 
each peak within an accession were considered to be ‘replicate’ values of variability for 
that accession. Differences among accessions for within accession variability were 
determined by analysis of variance for each compound class.

Though the box plots showed *A. minus* accessions to be more variable throughout 
a higher number of peaks than *A. lappa* accessions, *A. minus* showed no significant 
differences among their variability when analyzing the compound classes individually. 
The *A. lappa* accessions were shown to contain significant differences throughout all 
three compound classes. The accession L_09, showed the least amount of variability 
throughout all compounds, whereas L_19 showed to have the most significant variability 
throughout all compound classes. In regards to the individual compound classes, L_03 
was significantly more variable within the hydroxycinnamic acids than the other *A. lappa* 
accessions. L_19 showed the most variability within the flavonoids, and both *A. lappa* 
accessions, L_03 and L_19, were significantly different within the sesquiterpene 
lactones. When referring to the ‘variability among accessions’ study, L_03 contained a 
major outlier in the sesquiterpene lactone, peak 14. These results indicate that the plants
in this specific *A. lappa* accession contain high amounts of variability of this particular compound.

*Compound variability as influenced by leaf developmental stage within first year plants*

When harvesting burdock leaves for bandages, large mature leaves are generally selected to treat burns. However, the variability within a single plant and the differences in leaf chemical compositions among leaf sizes was unknown. To investigate phytochemical levels between various leaf sizes, four size categories were chosen: size class 1 consisted of leaves smaller than 15 centimeters in length, size class 2 contained leaves from 15 to 20 centimeters, size class 3 contained leaves from 20 to 25 centimeters, and size class 4 was contained leaves above 25 centimeters. One accession each of *A. lappa* (L_03) and *A. minus* (M_13) was chosen for this study. Leaves were studied separately in each species according to the categories of size. Table 2.8 shows the mean values among the sizes in each species and the significant differences determined by Tukey’s procedure. In this study, chromatographic peaks 2, 8, 9, 10, and 13 were not present in the samples. These plants were harvested from a separate field plot from the ‘variability among accessions’ study and the ‘variability among accessions within plants’ study. Plants from this alternative plot showed a slower growth rate, and were harvested approximately two weeks after the other studies. Explanation of the numerous missing peaks in the chromatography analysis of this study could be attributed to the new half-
sibling plants and their genetic differences, as well as an unknown environmental determinant.

In both species, the peaks generally contained elevated concentrations as leaves increased in size. In the *A. lappa* accession, chromatographic peak 1, a putative hydroxycinnamic acid, and two flavonoid peaks, peak 3 and peak 6, contained significantly different means from the smallest size leaf to the largest. Total phenolic analysis and FRAP analysis also showed significant differences from the small to large leaves.

In *A. minus* accessions, only one hydroxycinnamic acid compound, peak 15, showed a significant difference between the different sized leaves, while three flavonoids (peaks 3, 4, and 7), and one sesquiterpene lactone (peak 14) showed significant differences from small to large leaves. Total phenolic analysis and FRAP analysis also showed significant differences between small to large leaves. The TP and FRAP analyses were shown to highly correlate with each other in both species.

The general conclusion from this study states that larger leaves, particularly leaves above 25 centimeters in length, contain higher concentrations of compounds than smaller leaves. Leaves analyzed in this study were normalized to a standard weight for analysis for equal comparison. As leaves grow larger, hydroxycinnamic acids, flavonoids, and sesquiterpene lactones appear to continue increasing. These results also agree with the previous two studies, wherein the *A. minus* accessions generally contained larger
chromatographic compounds than the A. lappa accession. A. lappa also follows the same pattern and contains higher concentrations of the putative sesquiterpene lactone, peak 14.

Variability among years for plants within accessions

In order to investigate whether leaves from second year rosettes differ from first year rosettes, specific plants from the ‘within accession variability’ study were chosen to analyze the chemical composition in their second year of life. Three plants from each of the 12 accessions were chosen for study. Table 2.9 shows the mean separations for each species and year. In year one, A. lappa and A. minus plants did not contain the chromatographic peaks 8, 9, or 10. However, in the second year, these plants produced generally high amounts of each of these peaks.

Higher amounts of all compounds were shown in year two in A. lappa and A. minus accessions. Significant differences between years were shown in peaks 1, 2, 3, 4, 6, 8, 9, and 14 in A. lappa accessions and in peaks 1, 3, 7, 8, 9, 10, 11, 12, and 13 in the A. minus accessions. When comparing species to one another, A. lappa contained higher amounts of peaks 1, 2, 3, 6, and 14, whereas A. minus contained higher amounts of the other 11 major peaks. The TP and FRAP values also support this claim, showing significantly higher values in the second year plants than the first year plants.
To conclude this study, *A. minus* plants have still shown to contain higher amounts of compounds than *A. lappa*. Furthermore, in both species, burdock leaves harvested from the second year rosettes, before bolting, contained higher amounts of the 16 major compounds and possess significantly higher antioxidant power than first year rosette leaves.

*Variability between stalk and rosette leaves within second year plants*

In addition to the comparison of the age difference between rosette leaves, differences in phytochemical composition between second year rosettes and second year stalk leaves may also be of interest. For example, if the stalk leaves were found to produce more bioactive compounds than rosette leaves, this could change the choice of leaf selection for burn treatment significantly. Because rosette leaves and those associated with inflorescence develop in physiologically distinct environments (i.e., sink strengths, nutrition, carbohydrate or water levels, changes in the metabolic conditions of the leaves due to multiple stress factors, etc.) the secondary compounds present in the rosette may be different than those present in leaves produced from the stalk. A preliminary study was performed to investigate the chemical composition of the stalk leaves of the second year plants after flowering. Four plants, two *A. lappa* and two *A. minus*, were chosen for a preliminary study to determine whether gross differences between stalk and rosette leaves exist after flowering. Table 2.10 show the mean separations between rosette leaves and
stalk leaves for each species. The one peak that showed a significant difference between leaves of different positions on the plants was putative sesquiterpene lactone, peak 14 in *A. minus*. It was higher in concentration throughout the stalk leaves than the rosette leaves in *A. lappa*, but was significantly higher in rosette leaves than the stalk leaves for *A. minus*.

Concentrations of these 16 major chromatographic peaks were not nearly as high as those found in second year pre-bolting rosettes. When the plant produces a stalk, the compounds seem to spread throughout the leaves, not concentrating in the rosette leaves as was observed in the pre-bolting rosette study above. Little differences in phytochemical levels were observed between stalk and rosette leaves following bolting and flowering. The TP and FRAP values in *A. lappa* support this observation and show no significant differences between the stalk and rosette leaves. Interestingly, *A. minus* plants possessed significant differences in FRAP values between stalk and rosette leaves. Overall, *A. lappa* was found to have larger peak areas in the rosette leaves compared to the stalk leaves, while *A. minus* exhibited larger peak areas in the stalk leaves compared to the rosette leaves. This observation further supports the claim that *A. lappa* accessions are significantly different than *A. minus* accessions.

The results of these studies indicated that 16 major chromatographic peaks that are produced in *Arctium* species. Seven of these peaks were preliminarily identified as hydroxycinnamic acid derivatives, seven were putatively assumed as flavonoids, and two were postulated to be sesquiterpene lactone derivatives based on their retention times and
their UV/Visible spectra (Figure 2.3 and 2.4). Confirmation of these preliminarily identified compounds is needed and will be performed by the use of a Gas Chromatograph Mass Spectrometer and a Liquid Chromatograph Dual Mass Spectrometer. However, our results are generally consistent with previous studies, which report the detection of various phenolic acids from burdock, along with sesquiterpene lactones (Ferracane et al., 2010; Lou et al., 2010A, B; Chen et al., 2004; Rustaiyan et al., 1986).

Hydroxycinnamic acids are commonly found in plants, and are known to be beneficial to human health (Gallardo et al., 2006; Korkina, 2007; Shahidi and Chandrasekara, 2010). When studying the variability among accessions, it was determined that the relationship between hydroxycinnamic acids and FRAP was most pronounced (Figure 2.7). In previous research, hydroxycinnamic acids have been found to act as free radical scavengers and possess antioxidant activities (Chen and Ho, 1977; Nardini et al., 1995). They have also been shown to inhibit the growth of harmful microorganisms (Harris et al., 2010; Stead, 1993), reduce keloid and hypertrophic scars (Phan et al., 2003), and serve as various skin protectors and wound healers (Graf, 1992; Phan et al., 2001). These properties associated with hydroxycinnamic acids could potentially allow them to serve as the major healing compound in the burdock leaves. Oxidative stress is important in pathophysiological alterations, including inflammation and proliferation reduction, along with tissue remodeling, which are all essential in the healing of burns. The antioxidant properties of hydroxycinnamic acids could be beneficial in burn healing as they might function to counteract toxic oxygen and reactive
nitrogen species that impair the wound healing process (Liu et al., 2008). In addition to the antioxidant properties of hydroxycinnamic acids, the anti-inflammatory and antimicrobial characteristics of these compounds could also contribute to burn healing. These specific mechanisms could be responsible for the pain relief, reduction in swelling, and decrease in infections that burn victims have experienced with the burdock treatments (Mark Finneran, personal communication).

Flavonoids are a polyphenolic class of plant secondary metabolites. This subclass of compounds is characterized by two or more aromatic rings, each of which possess at least one aromatic hydroxyl group and connected with a heterocyclic pyran ring (Beecher, 2003). These compounds are widely distributed among the plant kingdom and are known to exert many biological effects including anticancer, antiviral, and anti-inflammatory activities (Garcia-Lafuente, 2009). The use of plant extracts containing flavonoids has been a popular remedy to reduce inflammation in Traditional Chinese Medicines (TCM) for centuries.

Several mechanisms explaining the anti-inflammatory activities of these flavonoids have been proposed, including: antioxidative and radical scavenging activities, similar to those described in association with hydroxycinnamic acids (Korkina and Afanas’ev, 1997); the regulation of cellular actions in inflammation-related cells (Middleton et al., 2000); the adjustment of arachidonic acid metabolism enzyme activities (Chi et al., 2001); and the regulation of proinflammatory molecules and gene expressions (Garcia-Lafuente et al., 2009; Santangelo et al., 2007).
Flavonoids are powerful antioxidants able to scavenge a wide range of free radical species produced during inflammation and these antioxidants can inhibit their functions, thus reducing inflammation. Several flavonoids have been shown to affect enzyme arrangements specifically involved with the early stages of inflammatory responses, preventing inflammatory cells such as T cells, B cells, macrophages, neutrophils, mast cells, or basophils from functioning, resulting in the reduction of inflammation (Middleton et al., 2000; Rudd, 1990). Flavonoids have also been shown to reduce the activity of arachidonic acid (AA) metabolizing enzymes including phospholipases, cyclooxygenases, and lipoxygenases. The inhibition of these enzymes inhibits the production of AA, prostaglandins, leukotrienes, and nitric oxide, all of which mediate the inflammation process (Chi et al., 2001). Flavonoids are currently studied to determine how these compounds affect proinflammatory molecules accumulating and gene expression. The exact mechanism which changes proinflammatory gene expression is unknown, but it is known that in response to inflammation, flavonoids affect mRNA levels with transcriptional activity suppression (Santangelo et al., 2007). Along with hydroxycinnamic acids, flavonoids show great promise in contributing to the pain relief and reductions in swelling and infection provided by burdock treatments.

Sesquiterpene lactones are a class of chemical compounds that are found in many plants. Currently, over 3000 sesquiterpene lactones have been discovered from various species (Chaturvedi, 2011). The highest concentrations of sesquiterpene lactones in plants are generally found in the leaves and flowering heads of the organisms. Large concentrations of these compounds in leaves have been found in glandular trichomes on
the leaf surface (Rodriguez et al., 1976). The relative amounts of sesquiterpene lactones present in a given plant vary from species to species, ranging from 0.001-5% dry weight. Species in the Asteraceae family, in particular, are known to contain these compounds and are commonly used as medicinal treatments of multiple anti-inflammatory diseases (Rustaiyan, et al., 1986). Along with anti-inflammatory properties, sesquiterpene lactones have also been demonstrated to possess antitumor, anti-infection, anti-bacterial, anti-fungal, and anti-helminthic properties. Specific studies provide evidence that individual sesquiterpene lactones inhibit growth of the bacteria *Staphylococcus aureus*, the yeast *Candida albicans*, and the fungi *Trichophyton mentagrophytes*, *T. acruminatum*, and *Epidermophyton* (Char and Shankarabhat, 1975; Mathur et al., 1975; and Olenchnowicz and Stepien, 1963). Both the anti-inflammatory and anti-microbial properties of these compounds could attribute to the ability of burdock to function in burn treatment.

The variability discovered among burdock plants could potentially pose a problem in the medicinal field. If plants contain this amount of variability, it will be almost impossible to standardize treatments simply picking from wild plants. However, if domestication occurs, this information could contribute to breeding an optimal plant. With 71 accessions, the population mean was collected through studying large numbers of plants and indicating specific accessions with variations in concentrations among specific compounds. These studies provided information to help breed and select for particular traits within these plants. For instance, L_03 had a unique amount of peak 14, the putative sesquiterpene lactone. If breeders determine that high amounts of this compound are needed, this particular accession would be ideal to use. There is no
evidence to indicate what levels of hydroxycinnamic acids, flavonoids, and sesquiterpene lactones are required for burn healing; maximum levels may or may not be optimal for treatment. However, the information collected from this study could provide ranges of phenolic compounds found throughout multiple accessions, ages of plants, sizes of leaves, and the position of the leaves on the plant. This information could be used when choosing plants for breeding.
References


Accessions of both species were sequentially numbered by the receiving order of the collection of seed; L = *Arctium lappa*, M = *Arctium minus*.

<table>
<thead>
<tr>
<th>Accession&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Location of Seed Collection</th>
<th>Accession&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Location of Seed Collection</th>
<th>Accession&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Location of Seed Collection</th>
</tr>
</thead>
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<td>M_01</td>
<td>Holmes County</td>
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<td>M_02</td>
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<td>M_33</td>
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<td>M_36</td>
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<td>M_08</td>
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<td>M_37</td>
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Table 2.1. Listed are 71 burdock accessions and the location of where the seed was originally collected. Accessions were labeled with an L for *Arctium lappa* and an M for *Arctium minus*. Accessions were numbered sequentially by the time of arrival to our laboratory.
<table>
<thead>
<tr>
<th>Accession&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Location of Seed Collection</th>
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<td>L_03</td>
<td>Wooster, Ohio</td>
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<td>L_07</td>
<td>Originally from Japan. Locally grown in Oregon</td>
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<tr>
<td>L_09</td>
<td>Grown in Japan.</td>
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<td>Sunbury, Ohio</td>
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<td>M_51</td>
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<sup>a</sup> L = Arctium lappa, M = Arctium minus

Table 2.2. Listed are 12 burdock accessions chosen to study the variability within accessions. Six *Arctium lappa* and six *Arctium minus* accessions were chosen to represent germplasm collected from environmentally and internationally diverse environments.
Table 2.3. Cluster analysis to determine relationships among 71 *A. lappa* and *A. minus* accessions using 16 chromatographic peaks (1-16) representing putative hydroxycinnamic acids (H), flavonoids (F), and sesquiterpene lactones (S). Values in “#Accessions” in parentheses indicate the number of *A. lappa* and *A. minus* accessions (respectively) that fall within each cluster. Values represent the means (top) and standard deviations (bottom) of megapixels associated with each peak for members in that cluster.
Table 2.4. Cluster analysis to determine relationships among 71 *A. lappa* and *A. minus* accessions using 7 chromatographic peaks (1, 2, 8, 9, 11, 13, and 15) representing putative hydroxycinnamic acids. Values in “# Accessions” in parentheses indicate the number of *A. lappa* and *A. minus* accessions (respectively) that fall within each cluster. Values represent the means (top) and standard deviations (bottom) of megapixels associated with each peak for members in that cluster.
Table 2.5. Cluster analysis to determine relationships among 71 *A. lappa* and *A. minus* accessions using 7 chromatographic peaks (3, 4, 5, 6, 7, 10, and 12) representing putative flavonoids. Values in “# Accessions” in parentheses indicate the number of *A. lappa* and *A. minus* accessions (respectively) that fall within each cluster. Values represent the means (top) and standard deviations (bottom) of megapixels associated with each peak for members in that cluster.
Cluster analysis to determine relationships among 71 *A. lappa* and *A. minus* accessions using 2 chromatographic peaks (14 and 16) representing putative sesquiterpene lactones. Values in “#Accessions” in parentheses indicate the number of *A. lappa* and *A. minus* accessions (respectively) that fall within each cluster. Values represent the means (top) and standard deviations (bottom) of megapixels associated with each peak for members in that cluster.

<table>
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<td></td>
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<td>S</td>
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<td>5.59</td>
<td>1.88</td>
</tr>
<tr>
<td>2</td>
<td>18 (0,18)</td>
<td>19.88</td>
<td>11.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.23</td>
<td>1.90</td>
</tr>
<tr>
<td>3</td>
<td>3 (1,0)</td>
<td>140.66</td>
<td>2.06</td>
</tr>
<tr>
<td>4</td>
<td>9 (8,1)</td>
<td>42.54</td>
<td>2.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.20</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Table 2.6. Cluster analysis to determine relationships among 71 *A. lappa* and *A. minus* accessions using 2 chromatographic peaks (14 and 16) representing putative sesquiterpene lactones. Values in “#Accessions” in parentheses indicate the number of *A. lappa* and *A. minus* accessions (respectively) that fall within each cluster. Values represent the means (top) and standard deviations (bottom) of megapixels associated with each peak for members in that cluster.
<table>
<thead>
<tr>
<th>Species</th>
<th>Accession</th>
<th>H</th>
<th>F</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lappa</td>
<td>L_03</td>
<td>86.34 a</td>
<td>27.42 b</td>
<td>40.33 a</td>
</tr>
<tr>
<td>Lappa</td>
<td>L_07</td>
<td>59.19 ab</td>
<td>49.09 ab</td>
<td>28.40 ab</td>
</tr>
<tr>
<td>Lappa</td>
<td>L_09</td>
<td>33.51 b</td>
<td>30.64 b</td>
<td>20.88 ab</td>
</tr>
<tr>
<td>Lappa</td>
<td>L_15</td>
<td>53.84 ab</td>
<td>48.81 ab</td>
<td>9.61 b</td>
</tr>
<tr>
<td>Lappa</td>
<td>L_19</td>
<td>72.45 ab</td>
<td>77.28 a</td>
<td>35.61 a</td>
</tr>
<tr>
<td>Lappa</td>
<td>L_25</td>
<td>35.24 b</td>
<td>37.78 ab</td>
<td>16.14 ab</td>
</tr>
<tr>
<td>Minus</td>
<td>M_12</td>
<td>72.72</td>
<td>105.31</td>
<td>26.15</td>
</tr>
<tr>
<td>Minus</td>
<td>M_13</td>
<td>56.79</td>
<td>53.08</td>
<td>44.81</td>
</tr>
<tr>
<td>Minus</td>
<td>M_14</td>
<td>86.94</td>
<td>75.20</td>
<td>51.86</td>
</tr>
<tr>
<td>Minus</td>
<td>M_24</td>
<td>76.50</td>
<td>74.57</td>
<td>41.92</td>
</tr>
<tr>
<td>Minus</td>
<td>M_49</td>
<td>85.45</td>
<td>44.22</td>
<td>57.72</td>
</tr>
<tr>
<td>Minus</td>
<td>M_51</td>
<td>49.39</td>
<td>43.22</td>
<td>55.84</td>
</tr>
</tbody>
</table>

Table 2.7. Means of the coefficient of variability (CV) for each accession separated by hydroxycinnamic acid (H), flavonoid (F), and sesquiterpene lactone (S) peaks. As determined by Tukey’s Procedure, significant differences of the means are displayed by ‘a’ and ‘b’.
Table 2.8. Mean separations in different size classes in each species for the 16 major peaks, TP and FRAP. Peaks 2, 8, 9, 10, and 13 were not present in this study. Size 1= 15 centimeters and below in length, 2= 15 to 20 centimeters in length, 3= 20 to 25 centimeters in length, and 4= 25 centimeters and above in length. As determined by Tukey’s Procedure, significant differences of the means are displayed by ‘a’, ‘b’ and ‘c’. H = hydroxycinnamic acids, F = flavonoids, and S = sesquiterpene lactones.
Table 2.9. Mean separations in each species for the 16 chromatographic peaks, TP and FRAP between rosettes in year one (2011) and in year two (2012). As determined by Tukey’s Procedure, significant differences of the means are displayed by ‘a’ and ‘b’. H = hydroxycinnamic acids, F = flavonoids, and S = sesquiterpene lactones.

<table>
<thead>
<tr>
<th>Species&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Year</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>TP</th>
<th>FRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8.5b</td>
<td>1.2b</td>
<td>6.2b</td>
<td>1.7b</td>
<td>0.8</td>
<td>8.2b</td>
<td>0.2</td>
<td>0.0b</td>
<td>0.0b</td>
<td>0.0</td>
<td>8.5</td>
<td>1.0</td>
<td>1.2</td>
<td>24.3b</td>
<td>1.1</td>
<td>3.1</td>
<td>15.4b</td>
<td>82.3b</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>62.5a</td>
<td>2.3a</td>
<td>12.0a</td>
<td>5.4a</td>
<td>0.0</td>
<td>14.9a</td>
<td>2.0</td>
<td>7.0a</td>
<td>98.7a</td>
<td>0.3</td>
<td>13.1</td>
<td>0.9</td>
<td>8.6</td>
<td>21.5a</td>
<td>0.0</td>
<td>3.1</td>
<td>36.9a</td>
<td>174.4a</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.3b</td>
<td>0.7</td>
<td>0.9b</td>
<td>8.4</td>
<td>3.8</td>
<td>0.5</td>
<td>2.5b</td>
<td>0.0</td>
<td>0.0b</td>
<td>0.0b</td>
<td>15.3b</td>
<td>19.6a</td>
<td>35.9b</td>
<td>22.7</td>
<td>13.5</td>
<td>6.4</td>
<td>14.1b</td>
<td>76.4b</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35.9a</td>
<td>1.1</td>
<td>2.9a</td>
<td>13.9</td>
<td>4.1</td>
<td>3.3</td>
<td>8.7a</td>
<td>7.3</td>
<td>217.5a</td>
<td>35.0a</td>
<td>42.7a</td>
<td>2.8b</td>
<td>155.0a</td>
<td>27.5</td>
<td>22.9</td>
<td>4.8</td>
<td>29.0a</td>
<td>199.3a</td>
</tr>
</tbody>
</table>

<sup>a</sup>L = *Arctium lappa*, M = *Arctium minus*
| Part of Plant | Species | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | TP  | FRAP |
|--------------|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|              |         |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|              |         |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Rosette      | *Lappa* | 67.02 | 1.23 | 23.97 | 2.78 | 0.00 | 39.05 | 0.00 | 17.01 | 95.56 | 0.00 | 16.79 | 0.00 | 119.60 | 3.81 | 22.75 | 285.48 |
| Stalk        | *Lappa* | 45.56 | 1.45 | 19.99 | 2.55 | 0.00 | 32.54 | 0.00 | 10.99 | 71.89 | 0.00 | 13.43 | 0.00 | 91.96 | 3.86 | 18.20 | 232.73 |
| Rosette      | *Minus* | 19.23 | 0.00 | 1.18 | 11.87 | 19.04 | 0.00 | 7.98 | 0.00 | 183.99 | 13.56 | 11.70 | 46.60 | 32.82 b | 7.60 | 22.25 | 206.07 b |
| Stalk        | *Minus* | 21.97 | 0.00 | 18.38 | 40.26 | 20.93 | 15.88 | 14.33 | 0.00 | 214.87 | 27.75 | 39.48 | 30.26 | 40.78 a | 8.97 | 29.22 | 295.78 a |

Table 2.10. Mean separations for the 16 major peaks, TP and FRAP between the second year plant rosettes and stalks in both species. Peaks 12 and 15 were not present in this study. As determined by Tukey’s Procedure, significant differences of the means are displayed by ‘a’ and ‘b’. H = hydroxycinnamic acids, F = flavonoids, and S = sesquiterpene lactones.
Figure 2.1. A typical chromatogram of an *A. lappa* plant at 320 nm and 256 nm. Not all of the 16 major chromatographic peaks were present.
Figure 2.2. A typical chromatogram of an *A. minus* plant at 320 nm and 256 nm. Not all of the 16 major chromatographic peaks were present.
Figure 2.3. UV/Visible spectra of the 16 major chromatographic peaks identified in burdock plants. Peaks 1, 2, 8, 9, 11, 13, and 15 are putative hydroxycinnamic acids, peaks 3, 4, 5, 6, 7, 10, and 12 have preliminarily identified as flavonoids, and peaks 14 and 16 are assumed sesquiterpene lactones. Continued.
Figure 2.3 continued.
Figure 2.3 continued.
Figure 2.3 continued.

[Graphs showing UV-Vis spectra for Peak 10, Peak 11, and Peak 12.

Continued.]
Figure 2.3 continued.
Figure 2.3 continued.
Figure 2.4. UV/Visible spectra of the tentative standards which are used to compare with the 16 major chromatographic peaks discovered in burdock plants. Standards of hydroxycinnamic acids consist of caffeic acid, para-coumaric acid, ferulic acid, and chlorogenic acid. Standards of flavonoids are quercetin-3-rutinoside, quercetin-3-galactoside, quercetin-3-glucoside, myricetin, isorhamnrtin, and apigenin-7-glucoside. The sesquiterpene lactone standard provided is parthenolide. Continued.
Figure 2.4 continued.

- Chlorogenic acid

- Quercetin-3-rutinoside

- Quercetin-3-galactoside

Continued.
Figure 2.4 continued.
Figure 2.4 continued.
Figure 2.5. Variability among 71 accessions for chromatographic peaks 1-16; F = flavonoid, H = hydroxycinnamic acid, S = sesquiterpene lactone. Box plots: boxes encompass values that fall within the 25th and 75th percentiles, the vertical line within the boxes indicate median values, whiskers denote range of values within the 10th and 90th percentiles, circles indicate values that are beyond the 10th or the 90th percentile.
Figure 2.6. Relationship between ferric reducing antioxidant power (FRAP) and total phenolic content (TP) among 24 *A. lappa* and 47 *A. minus* accessions.
Figure 2.7. Relationships between ferric reducing antioxidant power (FRAP) and megapixel areas for all (A) hydroxycinnamic peaks, (B) flavonoids, and (C) sesquiterpene lactones.
Figure 2.7 continued.
Figure 2.8. Variability within six *Arctium lappa* (L) and six *Arctium minus* (M) accessions for chromatographic peaks 1-16; H = hydroxycinnamic acid, F = flavonoid, S = sesquiterpene lactone. Box plots: boxes encompass values that fall within the 25th and 75th percentiles, the vertical line within the boxes indicate median values, whiskers denote range of values within the 10th and 90th percentiles. Peaks 9 and 10 were not pictured because they were not present in any of the samples. Continued.
Figure 2.8 continued.
Figure 2.8 continued.
Figure 2.8 continued.
Figure 2.9. Variability within six *Arctium lappa* (L) and six *Arctium minus* (M) accessions for total phenolic content (TP) and ferric reducing antioxidant power (FRAP). Box plots: boxes encompass values that fall within the 25th and 75th percentiles, the vertical line within the boxes indicate median values, whiskers denote range of values within the 10th and 90th percentiles.
Chapter 3: Effect of Differential Environmental Growing Conditions on Phenolic Composition

Summary

*Arctium* species have been used in traditional medicinal practices for centuries. Phenolic compounds contained in these plants are likely responsible for the effectiveness in treating multiple illnesses, diseases, and skin ailments. The leaves from burdock plants are of interest in the medical field particularly for use on burns and skin diseases. The leaves are harvested, dried, rehydrated, and applied directly to the skin wound. The bioactivity of these leaves is unknown as well as the variability in effectiveness of leaves from different sources. If these plants are to be domesticated to supply the medical field with innovative, natural bandages, more about the influence of growing conditions on bioactive constituents is needed.

Burdock has been found in wet, dry, shaded, sunny, cold, and warm environments; however, the chemical composition in these leaves, especially the phenolic content, may change with varying environmental conditions. In this study, 14 putative phenolic compounds and two putative sesquiterpene lactones were measured in multiple growing environments including treatments with differential photosynthetic active
irradiance levels, irrigation levels, and three different growing temperature regimens. This study was conducted to determine and measure the changes of constituent compounds in plants grown under different environmental conditions. Leaf chemical profiles were analyzed by three laboratory procedures. The Folin-Ciocalteu assay was used to spectrophotometrically document the complete phenolic profile of these leaves, the quantification of antioxidant capacity contained in these leaves was performed by the Ferric-Reducing Antioxidant Power assay, and a reverse-phase HPLC-DAD chromatography was used to preliminary identify and quantify the phenolic compounds contained in the leaves of these plants. Results showed that burdock plants generally produce more phenolic compounds under full light, frequent irrigation, and cooler temperatures. *A. minus* plants exhibited a higher production of phenolic compounds than *A. lappa* plants, but both species contained higher concentrations of phenolic compounds in these conditions. Sesquiterpene lactone contents were unaffected by light levels and irrigation frequency. However, in contrast to the effect of temperature on phenolic constituents, the synthesis of these compounds was apparently stimulated at higher temperatures.

Introduction

Burdock species are considered a dietary vegetable in Japanese and Korean cuisine (Duistermaat, 1996). Burdock roots, leaves, and seeds are used in nutritional...
soups, vegetable combinations, teas, and multiple beverages (Duh, 1998). These feral species are also used as medicinal plants and health foods by many cultures. They have been applied as skin treatments in Eurasia and Amish cultures, and have often been used as a traditional herbal medicine in China. Burdock species are thought to contain strong antioxidant, anti-bacterial, anti-inflammatory, and anti-fungal properties (Duistermaat, 1996). The leaves of *Arctium* species have been suspected to possess multiple chemical constituents which are thought to improve the healing of burns (Ferracane et al., 2009; Liu et al., 2005; Zhao et al., 2009). There is a specific interest in the variability of chemical constituents contained in burdock leaves. The use of burdock leaves as a natural bandage for the novel burn therapy is of great interest. Domesticating burdock as a crop to supply the medical field with burdock bandages may provide far-reaching benefits to burn victims while simultaneously offering farmers the economic benefits of increased crop diversity and marketable product options from several burdock plant parts.

Two burdock species, *Arctium lappa* and *Arctium minus* originated in Eurasia, but have now spread throughout the world (Table 2). They are highly adapted to North American environments, and can be routinely found in pastures, roadsides, swamps, waste areas, and sometimes in sandy areas (Gross et al., 1980). Because burdock thrives in many different environments, variability among and within species may also be influenced by growing conditions. These species produce seed heads which have jagged edges and easily fasten to animal fur, feathers, and even human clothing. The attachment of seed heads on traveling objects leads to seed dispersal and the spread of burdock throughout the world.
These biennial herbs cross-pollinate, which promotes high levels of phenotypic and genetic variability among and within the species (Duistermaat, 1996; Gross et al., 1980). Chapter 2 reported variability among and within burdock accessions. However, these plants were produced under relatively uniform field conditions. Experiments in Chapter 3 were designed to explore these environmental effects on phenolic and sesquiterpene leaf constituent levels. The objectives of this study were to:

1) Assess the phenolic and sesquiterpene variability due to differential irrigation treatments and irradiance treatments.

2) Analyze the differences in phenolic and sesquiterpene levels attributed to three temperature regimens.

The irrigation and irradiance treatments were performed in a greenhouse facility, whereas temperature regimens were administered in three separate growth chambers. In both studies, samples from each study were assessed for their total phenolic and antioxidant contents by spectrophotometric assay; a chromatographic profile analysis of phenolic and sesquiterpene constituents was also performed for each.
Materials and Methods

Seedling preparation

Select accession seedstocks that had been collected for the studies in Chapter 2 were used for the greenhouse and growth chamber studies. Transplant seedlings were grown in the greenhouse in plug flats and were transplanted into individual 2 gallon pots when the plant established two true leaves.

Materials, equipment and reagents

Conviron growth chamber systems were used to control temperature treatments, and a greenhouse facility was used for the light and irrigation treatments. Filtered irradiance was attained through the use of a color filter purchased from Rosco Laboratories. The filter blocked specific wavelengths that effect photosynthesis. Soil was prepared with a 50:50 Pro-Mix BX mycorrhizae™: Wooster silt loam (fine-loamy, mixed mesic Typic Fragiudef) combination. The same solvents, equipment, and standards were used as discussed in Chapter 2.

Physical measurements were used in these studies to stabilize treatments by a Li-Cor® LI-250A light meter, a Li-Cor® LI-1600 steady state porometer, and a ThermoWorks IR-Gun-S.
Sample preparation and storage conditions

In the greenhouse experiment, tissues from replicate plants within accessions receiving the same treatments were composited together at harvest. In the growth chamber experiment, tissues from each plant were analyzed separately. All leaves were harvested from each plant and immediately immersed into liquid nitrogen and placed into the -80°C freezer in labeled tubes. Tubes were weighed to determine how much leaf tissue was in each sample. An aliquot of frozen leaf tissue was weighed and placed into 50 ml polypropylene tubes for freeze-drying. The remainder of the leaf tissue was stored at -80°C for future enzymatic work not completed in this project. After freeze drying, the samples were prepared and extracted as in Chapter 2. Due to the limited amount of tissue collected, 0.100g of sieved powder was weighed for two extraction laboratory replications. Samples were all subjected to the total phenolic assay and the antioxidant power measurement in a manner identical to that employed in Chapter 2. They were also chromatographically quantified for the effects of each treatment on their bioactive profiles.
**Quantification of total phenolic content and antioxidant power**

Total phenolic content was calculated as gallic acid equivalents by reference to a standard curve performed daily in tandem with the sample analyses ($R^2 = \leq 0.999$), and expressed as milligrams of gallic acid equivalents per gram of fresh leaf tissue.

Ferric reducing antioxidant power was calculated as trolox equivalents by reference to a stand curve performed daily in tandem with the sample analyses ($R^2 = \leq 0.999$), and was expressed as milligrams of trolox equivalents per gram of fresh leaf tissue.

**Determination of phenolic and sesquiterpene content via HPLC**

Phenolic extractions were performed using freeze dried burdock powder and an acetone extraction solvent (acetone, water, acetic acid: 70:29.5:0.5 by volume) and further extracted with ethyl acetate. The dried ethyl acetate extractions were re-dissolved in 1 ml (HPLC-grade) 30% acetonitrile ($\text{CH}_3\text{CN}$). This solution was filtered by the use of a 3 ml disposable luer-lock syringe attached to a disposable 0.45 µm nylon filter. The remaining, filtered extract was transferred to a labeled amber chromatographic sample vial. An aliquot of 100 µl of each sample was diluted with 900 µl of (HPLC-grade) 30% CH$_3$CN to perform a 10 fold dilution of each sample. Each sample was analyzed on a Phenomenex Gemini (C6-phenyl) column at a stable temperature of 30°C. The mobile
phase of this program (solvent A) was 0.2% acetic acid in HPLC grade water, and the second solution (solvent B) contained 100% HPLC grade CH$_3$CN. The injection volume of the sample was 50 µl and the program had a flow rate of 0.7 ml/min. The solvent program was modified from that used in Chapter 2 in order to improve the separation of burdock leaf constituents. The new 50 minute HPLC program consisted of a solvent (mobile phase) gradient starting at 20% B changing to a rate of 30% B in 10 minutes. There was then a transition to 60% B over the following 15 minutes. The solvent system remained at 60% B for 10 minutes and was then returned to 20% B in 10 minutes. It was held at 20% B until the run stopped at 50 minutes. The detection wavelengths were 256 nm and 320 nm. When the program finished, the peaks were preliminary identified by comparing their UV-Visible Spectra and their retention times with commercial standards. Quantification of individual peaks was analyzed by the area reported by the Beckman Coulter 32 Karat software. Individual phenolic sesquiterpene contents were expressed as megapixels.

Irrigation and irradiance treatments

In order to determine whether irrigation or irradiance levels affect the phenolic and sesquiterpene levels of burdock leaves, a greenhouse-based experiment was conducted from May 2012 to June 2012 and then replicated in time from June 2012 to July 2012. During both replications-in-time, the greenhouse environment was set at a
range temperature of 70 to 76°F during the day and 60 to 65°F during the night. The data in Appendix C reports the daily humidity levels and natural sunlight levels within the greenhouse room from the months of May 2012 to July 2012, when the two replicates of this study were performed.

Two *Arctium* accessions were chosen, L_03 and M_13, to study the main and interactive effects of irradiance levels and irrigation treatments on leaf constituents. A split split plot design was used (Figure 3.1). To provide a framework for shading materials, cages were built with PVC pipes. For the shaded treatments, a green color film was attached to the pipes to filter 52% natural light according the Rosco Laboratories (Figure 3.2). For the natural light treatments, no film was used. Six plants, three *A. lappa* and three *A. minus*, were placed in each cage (Figure 3.3). Within each cage, three plants were irrigated daily with 250 ml of water and three plants were irrigated with 250 ml of water only once a week. Temperature was measured twice a week under each cage to verify that the film was not trapping extra heat in the cages to inflict more stress on the plants. A light intensity meter was used to determine the amount of light the covered cages were filtering. A porometer was used to measure the amount of diffusion conductance through the stomatal apertures between the frequently irrigated plants and the minimally irrigated plants. All leaves from each plant were harvested, providing a total of 64 samples from each treatment and each time. Tissue weight at harvest was used as an indicator of overall plant growth throughout the experiment.
Temperature treatments

Growth chambers were used to control three stable temperatures, 16°C, 24°C, and 32°C to determine their effects on the growth rate of burdock leaves and on the phenolic and sesquiterpene content within *A. lappa* and *A. minus* accessions. Five accessions, L_03, L_07, M_13, M_14, and M_49, were chosen for this study (Figure 3.4). Five plants of each accession were placed at random in each of the three different temperature chambers. Each chamber was programmed to deliver an average summer day’s light cycle, maintain 40% humidity levels, and hold a consistent temperature. Each chamber contained three metal halide light bulbs (MH) and three high quality pressure sodium light bulbs (HPS). The standard programs used in these chambers are listed in Appendix C, Table C.3. Irrigation was supplied as needed to each chamber. This study was also repeated twice for replication. Each experimental replicate was performed for five weeks; the first replication occurred from February 2012 to March 2012, and the second replication from April 2012 to May 2012. During each experimental replication, leaf growth rate was estimated in square centimeters twice a week by ascertaining the length and width of two leaves per accession in each temperature chamber and comparing these results with standard curves. Standard curves developed from 50 leaves of each species were generated by comparing L X W measurements with leaf areas as determined on a LiCor Li-3100C leaf area meter. All leaves from each plant were harvested, and each accession was composited into one sample per temperature, creating a total of 30 samples.
after both studies. Tissue weight at harvest was used as an indicator of overall plant growth throughout the experiment.

Statistical analysis

Prior to analyses of variance, all data were transformed to a normal distribution ($\bar{x} = 0$, $\sigma^2 = 1$) using PROC STANDARD in SAS 9.2 software. Irrigation and irradiance treatments (greenhouse study) and temperature treatments (growth chamber study) were analyzed by the PROC GLM. Significant differences were determined by Tukey’s Procedure measured with alpha=0.05.

Results and Discussion

Irrigation and irradiance treatments

To establish the equality of irradiance and irrigation treatments, physical measurements were performed to distinguish how much light was filtered by the green film layers, whether the filmed cages trapped extra heat, and how the water uptake affected the amount of diffusion conductance through the stomatal apertures of the leaves. The measurements reported that the film filtered out 45% of the natural light in
the greenhouse. The cages with no filter measured an average of 431.34 ± 15.31 μmoles m⁻² s⁻¹, whereas the cages containing the green filter reported an average of 283.23 ± 644 μmoles m⁻² s⁻¹. The mean temperatures under the shaded cages (25.33 ± 0.10°C) were similar to those found in the cages with no film (24.27 ± 0.35°C). The porometer readings for the plants irrigated daily averaged 23.83 ± 0.33 cm⁻² s⁻¹, and the plants irrigated once a week averaged 15.67 ± 0.15 cm⁻² s⁻¹, suggesting that irrigation frequency may have affected the water status of plants.

Preliminary observations showed that burdock seedlings grew with high light and high moisture. In agreement with this supposition, average leaf weights among plants grown with frequent irrigation were 61.11 ± 3.97 grams of fresh weight (gfw) whereas those watered once weekly produced only 7.03 ± 0.98 gfw. However, leaf weights of plants grown in light averaged 31.80 ± 1.31 gfw whereas those grown in shaded conditions were 36.33 ± 3.59 gfw. Plants of A. lappa produced slightly greater leaf weights (35.69 ± 4.97 gfw) than did A. minus (32.44 ± 2.71 gfw) leaves per plant.

Sixteen major chromatographic peaks were observed in this study; peaks 2 and 12 were not produced in leaves of greenhouse-grown plants, and levels of peaks 10 and 15 were not significantly different among treatments or accessions. However, irradiance levels had a pronounced effect on most phenolic constituents, with five hydroxycinnamic acids (peaks 1, 8, 9, 11, and 13), and four flavonoids (peaks 4, 5, 6, and 7) showing significant decreases in mean concentration in response to shade (Table 3.1). The values for TP and FRAP were also significantly depressed in shaded treatments.
Both species of burdock plants that were irrigated every day produced higher amounts of phenolic compounds than the plants that were irrigated once a week. However, only four of these compounds showed significant differences between the irrigation treatments, two hydroxycinnamic acids (peaks 8 and 13), and two flavonoids (peaks 4 and 6). The TP and FRAP analyses also showed higher values correlating with more irrigation, but only FRAP contained a significant difference between the irrigation treatments.

*A. lappa* and *A. minus* plants significantly differed in chemical composition. In this experiment, *A. lappa* produced more of five compounds (peaks 1, 3, 6, 9, and 14) and TP and FRAP values. *A. minus* displayed higher amounts of peaks 4, 5, 7, 8, 10, 11, 13, 15, and 16. Species differed in all peaks except 9, 10, and 15. Species differences with respect to these compounds reflected the exact pattern uncovered in Chapter 2. TP values were shown to be significant between these species, but FRAP values were not.

Analysis of variance revealed significant interactions for the effects of irradiance and species (Light X Species) for peaks, 4, 5, 6, 7, 8, 13 (Figure 3.5). Mean separations for the interactions in each peak revealed that the overall reduced performance in shade was species-driven, specifically by *A. minus* for peaks 4, 5, 7, 8 and 13 and by *A. lappa* for peak 6. In each case, the corresponding species performed similarly in both irradiance regimes, primarily due to limited and/or inconsistent production of the compound among plants within the treatment. The interactive patterns for irrigation regimen and species (Water X Species) for peaks 4, 5, and 6 were similar to those of Light X Species,
suggesting that reductions in compound level associated with reduced irrigation frequency were also species-driven (Figure 3.6). The lack of significance for the peak 5 irrigation main effect likely resulted from the intermediate value associated with *A. minus* irrigated weekly. The significant interaction for irradiance vs. irrigation regimens (Light X Water) for peak 4 was also likely conditioned by the intermediate value associated with plants receiving full light and infrequent irrigation.

_Temperature treatments_

The three growth chambers were programed at their appropriate temperatures, along with a light program (Appendix C, Table C.3). The average growth rate of the burdock leaves were measured biweekly (Figure 3.7). Leaves of both species exhibited the most rapid increase in size when plants were in chambers held at 24°C. Leaf growth rates were lower for plants at 16°C and substantially lower when grown at 32°C. Harvested leaf weights from plants at 24°C (126.55 ± 34.65 gfw for *A. lappa* and 121.68 ± 16.10 gfw for *A. minus*) were substantially greater than those grown at 16°C (63.49 ± 22.69 gfw and 63.92 ± 4.55 gfw, respectively) or at 32°C (56.91 ± 11.60 gfw and 40.22 ± 0.30 gfw, respectively).

The sixteen major chromatographic peaks observed in the greenhouse study described above were considered for the growth chamber study, although peaks 2, 10, 12, and 15 were not produced in these plants, and peaks 6, 7 and 9 were not significantly
different among temperature regimens or between species (Table 3.2). In general, burdock plants produced higher concentrations for all phenolic compounds regardless of significance levels (i.e., including peaks 6, 7 and 9) when grown at 16°C. However, main effects for temperature regimen were only significant for phenolic peaks 1 and 11. The values for TP and FRAP also showed higher mean values for the 16°C temperatures, but neither exhibited a significant main effect for temperature treatments. Conversely, putative sesquiterpene lactone (peaks 14 and 16) concentrations were highest among plants grown in 32°C chambers; the concentration of peak 16 was significantly elevated in plants grown at this temperature.

To further support earlier findings, burdock species were found to be significantly different in their production of six phenolic compounds, and in both sesquiterpene lactone constituents. The association of individual peaks with specific accessions in this experiment follows very closely with patterns found in Chapter 2 and in the greenhouse experiment reported above. On average, the TP values were higher in A. lappa accessions, and the FRAP values were larger in the A. minus accessions, however, neither of these spectrophotometric tests showed significant differences between the species.

Interactions between species and temperature regimens were significant for peaks 3, 4 and 11 and for values of TP. Although temperature main effects were not significant for peaks 3 and 4, mean separation patterns for the interaction effects clearly indicated that growth at 16°C elevated levels of these compounds in A. lappa and A. minus, respectively (Figure 3.8), Interactions associated with the corresponding species were
unaffected by temperature regimen, partly conditioned by limited and inconsistent production of these compounds among plants. Similarly, *A. minus* plants produced higher levels of peak 11 at 16°C whereas those of *A. lappa* did not; *A. lappa* plants produced more total phenolic levels at 16°C whereas those of *A. minus* did not.

As burdock plants are noted to thrive in diverse environments, experiments in Chapter 3 examined the influence of growing conditions (specifically, levels of irradiance, irrigation regimens and growing temperatures) on the levels of phenolic and sesquiterpene constituents produced in burdock leaves. In addition, differences among species with respect to the production of specific phenolic or sesquiterpene components were studied and compared with those uncovered in field-grown plants characterized in Chapter 2.

The results of the greenhouse and growth chamber experiments showed that *A. lappa* and *A. minus* were different with respect to the production of specific phenolic and sesquiterpene constituents in leaves. Productions of specific species-associated constituents were strongly influenced by irradiance levels and, to a lesser extent, to irrigation frequency. These results suggest that burdock species produce more phenolic compounds when grown in sunny areas than shaded areas, and that regular patterns of rainfall increase the biosynthesis of these constituents. Sesquiterpene lactones, however, were not influenced by light levels or irrigation frequency. Whether the lack of response to treatments indicates that plants can produce these compounds at consistent levels even in putatively stressful environments remains to be evaluated. The growth chamber studies
suggested that phenolic constituents are likely higher in leaves developed in cooler, northerly environments, or early in the season when temperatures are relatively low. On the other hand, sesquiterpene lactone production may be favored in plants grown at elevated temperatures or during the heat of mid-summer.

Environmental influences on the production of potentially bioactive burdock leaf constituents may have consequences for its use as an effective treatment of burns. There may be an optimal environment from which to collect leaves and dry them for future use. Moreover, information about the potential influence of environment on the production of phenolics and sesquiterpene lactones may be relevant to breeding efforts as these species undergo domestication. The importance of the findings will be clarified only after compounds reported herein are positively identified and their bioactivity ascertained through further research.
References


Table 3.1. ANOVA and main effects mean separations for the irradiance and irrigation studies performed in the greenhouse. L = *Arctium lappa*, and M = *Arctium minus*. H = hydroxycinnamic acids, F = flavonoids, and S = sesquiterpene lactones.
Table 3.2. ANOVA and main effects mean separations for the temperature treatments studied in the growth chambers. L = *Arctium lappa*, and M = *Arctium minus*. H = hydroxycinnamic acids, F = flavonoids, and S = sesquiterpene lactones.

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Table 3.2. ANOVA and main effects mean separations for the temperature treatments studied in the growth chambers. L = *Arctium lappa*, and M = *Arctium minus*. H = hydroxycinnamic acids, F = flavonoids, and S = sesquiterpene lactones.
Figure 3.1. The setup of the greenhouse study in order to examine the effects of irrigation and irradiance levels on the chemical compositions in burdock. Solid green rectangles represent the shaded cages with the green filter and the rectangles with no color represent cages in which were not shaded. Circles represent the six plants within each cage; the blue circles represent the three plants irrigated daily and the no colored circles are the three plants in which were irrigated one a week. L = *A. lappa* and M = *A. minus*. Red rectangles represent one block.
Figure 3.2. Technical data sheet for the green color filter purchased for the light shading from Rosco Laboratories. (http://www.rosco.com/us/index.cfm)
Figure 3.3. Pictorial portrayal of the greenhouse study. Three *A. lappa* and three *A. minus* plants were fit in one cage; three with daily irrigation, three with weekly irrigation. Shaded treatments consisted of cages covered with the green film to provide an average of 45% filtered light.
Figure 3.4. Growth chamber study setup to examine three different growing temperatures on five different accessions of burdock plants. The different colored circles represent one accession; red = L_03, yellow = L_07, green = M_13, blue = M_14, and purple = M_49.
Figure 3.5. Means, standard errors and mean separations for significant irradiance X species interactions for constituents of greenhouse-grown burdock leaves: black bars = *Arctium lappa*; gray bars = *Arctium minus*. Continued.
Figure 3.5 continued.
Figure 3.6. Means, standard errors, and mean separations for significant irradiance X irrigation interactions (top left panel) or irrigation X species interactions for constituents of greenhouse-grown burdock leaves: top left panel; black bars = irrigated daily; gray bars = irrigated weekly; top right and bottom panels; black bars = *Arctium lappa*; gray bars = *Arctium minus*.
Figure 3.7. Average leaf growth (cm\(^2\)) for each species in the three temperature treatments.
Figure 3.8. Means, standard errors and mean separations for significant species X temperature interactions for constituents of growth chamber-grown burdock leaves: black bars = *Arctium lappa*; gray bars = *Arctium minus*. 
Chapter 4: Summary, Conclusions, and Future Work

Burdock has been proposed as an effective treatment for burn wounds by the Amish communities when used as a topical bandage with an organic salve. After salve is applied, burdock leaves are used to wrap skin wounds, replacing conventional bandages. This treatment has been shown to speed the healing process, reduce inflammation and bacterial infections, and in some cases eliminate the need for skin grafts. Though this treatment has shown to be effective compared to the conventional treatments for burns, it is not recognized in the United States due to the lack of literature on the mechanisms of how burdock leaves affect these benefits. Efforts to understand the bioactivity of burdock leaves are in process, but still at an elementary stage.

The aim of this study was to extract and isolate the secondary metabolites in burdock leaves that may be responsible for anti-inflammation, anti-bacterial, and anti-pain properties demonstrated by burdock bandages. Due to the inherent variability among burdock plants, leaves might differ in effectiveness. Until this work was undertaken, the extent of variability in chemical constituents among burdock plants was unknown.
The genetic and environmental variability influencing the production of phenolic compounds (hydroxycinnamic acids and flavonols) in burdock leaves was also investigated in this study. Burdock possesses high levels of phenolic constituents which have been shown to promote health by acting as antioxidants and anti-inflammatory agents, both of which are involved in the burn healing process. While the protocol used to extract putative bioactive constituents was specific for polyphenols, two non-phenolics were consistently found to be present in each plant. After comparison of UV-Visible spectra of the unknowns to that of standards, these compounds were preliminarily identified as sesquiterpene lactones, which are also known to exhibit health beneficial properties, and could also attribute to the healing of burn wounds.

To evaluate the variability of the bioactivity among burdock plants, total phenolic content was measured spectrophotometratically through the Folin-Ciocalteu assay, antioxidant power was measured colorimetrically with the ferric reducing ability of plasma assay, and preliminary identification and quantification of phenolic compounds and sesquiterpene compounds were measured by the HPLC. Sixteen major chromatographic compounds were discovered, and each peak was quantified and used to evaluate the variability among plants. The 16 major peaks were preliminarily identified as seven hydroxycinnamic acids, seven flavonoids, and two putative sesquiterpene lactones based on their UV/Visible spectra and retention times compared to those of commercial standards. Until further analysis is performed to identify the exact structure of these compounds by use of LC-MS and/or GC-MS, most of these compounds are assumed to be phenolic compounds with the exception of two sesquiterpene lactones.
An evaluation of 71 burdock accessions was performed to measure the population variability. Seeds from plants were collected throughout the world, and were grown in Ohio. Two species, *Arctium lappa* and *Arctium minus*, were studied in this project due to their prevalence globally. Out of the 71 accessions, 24 were *A. lappa* and 47 were *A. minus*. No patterns associated with the geographic origins of accessions were found. However, two *A. lappa* accessions, L_18 and L_22, contained the highest amounts of phenolic compounds. To determine the variability within each accession, a subsample of accessions was studied of each species. Three plants within each accession were studied separately to measure variability in bioactive content of each plant. There was more variability among *A. lappa* accessions than *A. minus* accessions. Peaks 1, 3, 6, and 14 were consistently larger in *A. lappa* plants, and peaks 4, 5, 7, 11, and 16 were always larger in *A. minus* plants. Results showed that *A. lappa* accession L_03 contained significant variability within its hydroxycinnamic acid levels, and produced the largest amounts of putative sesquiterpene lactone, peak 14. *A. lappa* accessions in general had more variability within their accessions than *A. minus* plants. Accession L_19 had the most amount of variability, especially within its flavonoid compounds. L_18 and L_22 were two *A. lappa* accessions which demonstrated the largest amounts of hydroxycinnamic acids among any accession, even though *A. minus* plants generally contained higher amounts than *A. lappa* plants. M_19 had the lowest amounts of flavonoids among *A. minus* accessions, and M_15 had the highest amount of putative sesquiterpene lactones throughout the *A. minus* accessions, causing both of these accessions to closely resemble *A. lappa* accessions.
Generally, leaves chosen for use as bandages from a burdock plant are large, mature leaves from the bottom of the rosette. In order to determine whether these mature leaves contain different amounts of these 16 compounds than other leaves found in the rosette, four different sizes of leaves were chosen and studied for the chemical content. Leaves ranged from below 15 centimeters in length, 15 to 20 centimeters, 20 to 25 centimeters, and 25 centimeters in length and above. Large mature leaves contained significantly higher levels of both phenolic and putative sesquiterpene lactone compounds.

Most burdock bandages used to treat burn wounds are only chosen from first year rosette burdock plants. As a biennial plant, burdock produces many leaves, but in its second year of life, burdock can generate hundreds of leaves. To determine whether phenolic levels present in second year burdock plants are comparable to those present in the leaves of first ear rosette plants, a subsample of plants were studied again in their second year of life. Before the two year old plants produced a stalk, leaves were harvested from the rosettes and compared to those of first year rosette plants. Both species showed a significant increase (an average threefold increase) in the phenolic levels and antioxidant power in the second year leaves, with the exception of peak 12. This particular peak was the only compound to be found more concentrated in first year leaves than second year leaves. Peak 9 was more abundant than any other compound found in second year plants, and was never produced in first year leaves. Peak 1 also
showed a significant difference between years, exhibiting an average 7.8 fold increase over the younger plants.

Once the second year plant produced a stalk, hundreds of leaves were produced per plant. The chemical compositions of these stalk leaves versus the rosettes leaves were of interest. However, no significant differences were shown in the comparison of these leaves. Differences in species were noted where *A. lappa* accessions showed higher quantities of compounds in the rosette leaves compared to stalk leaves, and *A. minus* accessions produced larger amounts of compounds in the stalk leaves versus the rosette leaves. The amounts of peaks produced in the flowered, mature plants were comparable to those of the pre-bolted second year rosette plants, with generally higher amounts of compounds compared to first year rosette plants. However, to measure the variability within a mature second year plant, the differences between stalk leaves and rosette leaves of *A. lappa* and *A. minus* plants were examined. Peak 9 was produced in much higher amounts compared to first year plants. This study only showed a significant difference within peak 14, a putative sesquiterpene lactone. However, the general trend of *A. lappa* plants containing higher mean percentages of its compounds in the rosette leaves over the stalk leaves, and *A. minus* plants containing higher amounts in the stalk leaves versus the rosette leaves was observed.

Since burdock plants are found all over the world, in warm, cold, dry, and wet climates, evaluation of variation among growing environments was of interest. Effects of light levels, soil moisture, and temperature were studied by the use of controlled
environments in a greenhouse facility and growth chambers. When studying light levels and soil moisture, treatments consisted of full light, 45% shaded light, irrigation every day, and irrigation once a week. Maximal levels of phenolic compounds were found in both species following treatment with full light and frequent irrigation. Temperature levels were studied in growth chambers, programmed at three temperature regimens, 16°C, 24°C, and 32°C. In both species, higher phenolic levels were found in plants grown in 16°C, and sesquiterpene lactones were found to be highest following treatments of 32°C. When studying environmental growing conditions, peak 4 appeared to be the most influenced by environmental stimulus, showing significant differences in all interactions. Peaks 3 and 11 also showed significant differences in the temperature interactions.

Conclusions drawn from these discoveries are that A. minus plants generally contain higher amounts of phenolic acids and exhibit less variability within accessions than A. lappa plants. However, A. lappa plants were shown to produce more sesquiterpene lactone compounds. Large mature leaves were shown to have significantly higher levels of phenolic compounds than smaller leaves, and second year rosette leaves (before bolting) were shown to have an average of three fold the amount of compounds in their leaves. The geographic sources of the parent plants of either species appeared to exert no effect on compound accumulation; however, both species produce more of each compound under ideal conditions of high light, generous irrigation levels, and cooler climates.
If future domestication of burdock occurs to develop a standardized burdock leaf bandage, the data generated in this project could be useful in developing a plant with optimal phenolic or sesquiterpene lactone levels.

A major limitation of this project was the selection of metabolites for study. Based on previous research on their medicinal properties, phenolic compounds were chosen as the target chemical constituents to quantify and analyze in this project. However, it is still undetermined whether these particular compounds are the active metabolites in burn wound healing. Pharmacological assistance is needed to determine the exact complement of metabolites responsible in this treatment process. Another limitation in this study was the preliminary identification of individual chemical compounds. HPLC results cannot identify compounds with certainty; therefore, further identification processes will have to be performed in order to determine which compounds were quantified in these leaves.

Further chemical work is required to determine the identity of burdock bioactive compounds, particularly by use of liquid chromatography-mass spectrometry or gas chromatography-mass spectrometry. Work in this area is currently being pursued in the laboratory. The definitive identification of bioactive compounds is needed before burdock leaves can be applied in the medical field. Professional clinical studies will also be essential to determine the exact effects the chemical constituents in burdock leaves have on wounds. Along with future investigation into the phenolic compounds of burdock leaves, the exploration of terpene and perhaps steroid levels in burdock would be advantageous. These compounds also have beneficial medicinal properties, and high
amounts of putative sesquiterpene lactone derivatives have already been observed in burdock leaves. It is hoped that the research presented in this study will provide a foundation for and assist future studies on the domesticating and investigating processes of these potential natural bandages.
Bibliography


Appendix A: Preliminary Studies
Preliminary Studies

Preliminary experiments were conducted throughout October 2010 to February 2011 to assess methods for optimized recovery of burdock leaf phenolic constituents. Variables examined in these experiments included methods of drying, differential leaf tissue types and solvent extraction procedures (Figure A.1).

Materials and Methods

Drying methods

Mature field-grown \textit{A. lappa} and \textit{A. minus} leaves were harvested and dried by three methods. Leaves were dried at room temperature in an incubator (Hoffman Manufacturing) to approximate conditions used by practitioners of herbal medicine to prepare leaves for use as bandages. Additionally, leaves were dried rapidly at 40°C in a forced-air tissue drier (Hoffman Manufacturing) and more slowly, after freezing at -20°C, and also in a Labconco freeze-drier. The latter technique is typically preferred among plant scientists for the extraction and quantification of compounds from dried plant tissues. Leaves were removed from their drying apparatus only when desiccated sufficiently for milling.
Preparation of tissues for extraction

Each dried burdock sample was ground in an analytical mill (IKA, A11 basic) and the rough-ground material was sieved through a 177 micron sieve. The leaf material passing through the sieve consisted of a fine homogeneous powder suitable for chemical analyses whereas the material remaining exhibited a “fluff-like” texture. Microscopic observation of the latter material suggested that it was relatively fibrous and likely contained a substantial portion of glandular trichome tissues. Because burdock glandular trichomes may be a source of bioactive materials, both powder and residual tissues were collected for analysis, partitioned into polypropylene tubes in 0.25 g replicates and stored in a -20°C freezer. Burdock powdered and residual tissues were subjected to two solvent extraction protocols and then analyzed using spectrophotometric and chromatographic procedures to assess differences in phenolic compound concentrations in each tissue type.

Solvent extraction procedures

Two extraction solvents were tested on powdered and residual tissues of each species; an acetone extraction solvent containing acetone, water, and acetic acid (70:29.5:0.5 by volume) and a 1% acidified methanol solution (i.e., 12 N HCl in
methanol in a 1:99 ratio). Both of these techniques are commonly used to extract phenolic materials from a variety of plant sources.

The acetone extraction procedure was modified from that described by Ozgen et al. (2008). Acetone extraction solvent (30 ml) was added to each sample tube; tubes were agitated every five minutes for 30 minutes in order to maintain sample particles in suspension. After the initial extraction period, samples were centrifuged for 15 minutes at 7500 rpm. The supernatant was filtered through a Buchner funnel containing Whatman No. 1 filter paper and collected into a 250 ml suction filtration flask. The pellet was resuspended in 30 ml of the acetone extraction solvent and the extraction process was repeated multiple times until the solution and pellet contained no color. After the final supernatant was pooled in the flask, the 120 ml of burdock extract was transferred to a 500 ml round bottom flask and concentrated using a rotary evaporator system under partial vacuum (via aspirator) with a water bath temperature of 35°C until the acetone and acetic acid was evaporated as determined by olfactory observation, leaving the burdock extracts in only water. The remaining sample was transferred into a 25 ml volumetric flask and brought to a standard volume with water. These extracts were divided into two samples: one portion (5ml) was used for FRAP and total phenolic assays, and the second portion (20 ml) was used for further phenolic extraction using ethyl acetate.

Except for the nature of the extraction solvent, early steps of the 1% acidified methanol extraction protocol (i.e., those used to sequentially extract the dried material) were nearly identical to those described above for use with acetone extraction solvent. However, rather than be subjected to rotary evaporation, the pooled supernatants (≈ 90
ml) were brought to volume in a 100 ml volumetric flask with DDH₂O. These extracts were divided into two samples: one portion (5ml) was used for FRAP and total phenolic assays, and the second portion (95ml) was available for further phenolic extraction using ethyl acetate.

Sample analysis

Sample extracts obtained by either procedure were analyzed spectrophotometrically for total phenolic content and ferric reducing antioxidant power and chromatographically via HPLC-DAD procedures.

A ferric reducing antioxidant power assay (FRAP) method adapted from Benzie and Strain (1996) was used to assess total antioxidant capacity. This assay procedure estimates the antioxidant capacity of extract constituents by measuring their ability to reduce Fe³⁺ to Fe²⁺. The level of reduced iron can be detected at 593 nm after complexing with the chromophore, TPTZ (2,4,6-tris(2-pyridyl)1,3,5, triazine). Absorbance values of samples were compared with those of a standard curve prepared with trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and quantified as trolox equivalents.

Values of total phenolic (TP) levels were determined colorimetrically after reaction with Folin-Ciocalteu’s phenol reagent following the method described by
Singleton et al. (1999). Sample extracts were combined with reagent allowing sample phenolic materials to reduce reagent transition metal ions in the solution. After seven minutes, the reaction was then quenched with the addition of sodium carbonate. After a one hour incubation period, the blue/green color resulting from metal ion reduction was measured at 750 nm. Sample total phenolic values were estimated by comparing the absorbance of each duplicate with those of a response curve generated in triplicate with a commercial standard of gallic acid.

For HPLC analyses, an aliquot of the two extraction replications were mixed with an equal volume of a 0.2 M sodium acetate solution. This mixture was suspended and extracted with ethyl acetate multiple times. The ethyl acetate fraction of this separation was combined, and dried down with a constant stream of nitrogen until completely dry. The dried sample was re-suspended in a 30% acidic acetonitrile solution, filtered through a 0.45 micron nylon filter and was injected into the HPLC. The chromatographic conditions used were described fully in Chapter 2.

Results and Discussion

Drying methods

Chromatograms of *A. lappa* and *A. minus* extracted with the acetone solvent were chosen to represent the effects of drying methods on the constituent profiles of burdock.
(Figure A.2). In general, freeze-dried extracts contained more quantifiable peaks than the other drying treatments and many peaks (e.g., peaks 1, 11, 13 and 15 along with 14 and 16, putative sesquiterpene lactones) were present at higher concentrations in extracts of freeze dried material than in extracts prepared from oven-dried or air-dried leaves. However, this trend was not universal; peaks 4, 6 and 7, for instance appear to be more concentrated in extracts from materials dried differently, but these differences were not consistent across species. Therefore, freeze-drying was chosen for the development of the experimental protocols used in Chapters 2 and 3 herein; more compounds were detected in each species, compounds tended to be more concentrated and the suitability of this method for extraction of dried materials is substantiated in published literature.

**Tissue types**

Chromatograms of freeze-dried *A. lappa* finely-powdered sieved burdock leaves and residual material primarily comprised of fibrous and glandular trichome-derived tissues, extracted with the acetone solvent were chosen to depict differences in extractable constituents among tissue types (Figure A.3). When analyzed by the HPLC, the residual tissue remaining after sieving dried mill-ground burdock leaves displayed three quantifiable peaks, as opposed to the burdock sieved powder which contained six compounds. Interestingly, peak 14, a presumed sesquiterpene lactone, was the predominant compound present in residual tissues; this class of compounds is typically
associated with glandular trichomes. However, because the original focus of this study was to quantify phenolic levels in burdock leaves, the sieved burdock powder was chosen as the optimum material to uncover levels of constituent variability and monitor the effects of growing environments.

*Extraction solvents*

In order to study the differences that extraction solvents produce, freeze dried *A. lappa* and *A. minus* sieved powder was used (Figure A.4.). Extracts prepared with acetone contained a substantially greater number of quantifiable peaks than did those prepared with 1% acidic methanol. Acidic methanol did, however, appear to be an excellent for peak 14, the presumed sesquiterpene lactone. The lack of phenolic methanol-extractable compounds was most pronounced in *A. lappa*. Based on these results, samples prepared for analysis in Chapters 2 and 3 were extracted using the acetone extraction procedure.

*Corroborative TP and FRAP data*

TP and FRAP values were obtained to augment chromatographic data outlining differences in the efficiency of drying methods and solvent protocols for extracting
constituents from burdock sieved powder (Figure A.5). Both of these spectrophotometric assay procedures displayed results corroborating the choice of freeze drying and acetone extraction as a means to maximize phenolic recovery.
References


Figure A.1. Flowchart of preliminary optimization studies conducted with *A. lappa* and *A. minus* plants.
Figure A.2. HPLC chromatograms of three different drying methods on *A. lappa* and *A. minus* leaf tissue.
Figure A.3. HPLC chromatograms displaying the difference between the residual material of *A. lappa* leaf tissue and sieved powder.
Figure A.4. HPLC chromatograms of *A. lappa* and *A. minus* leaves extracted with the acetone extraction solvent and with 1% acidified methanol.
Figure A.5. Bar graphs of total phenolic and ferric reducing plasma assay results when comparing the sieved burdock powder of the three drying treatments and two extraction solvents in *Arctium lappa* and *Arctium minus* plants. Green bars represent the *A. lappa* plants and the blue bars represent *A. minus* plants. The solid filled bars denote the acetone extractions, and the speckled bars denote the 1% acidified methanol extractions.
Appendix B: Field Maps
Figure B.1. Layout of one field replication in the 2011 planting to study the variability among all 71 accessions. Refer to Table 2.1 for accession labeling. The ‘o’ represents each individual plant and the ‘x’ represents any plant which did not survive. The yellow denotes each accession and the orange represents the three plants within each accession which were selected for studying the variability among accessions.
Figure B.2. Layout of one field replication in the 2011 planting to study the variability among plants within the 12 chosen accessions. Refer to Table 2.1 for accession labeling.
Figure B.3. Layout of one field replication in the 2011 planting to study the compound variability as influenced by leaf developmental stage within a first year plant in two species. Refer to Table 2.1 for accession labeling.
Figure B.4. Depiction of how second year plant leaves were compared; stalk leaves and the rosette leaves. Illustration courtesy of Mark Robbins.
Appendix C: Greenhouse and Growth Chamber Data
### Averaged Daily Greenhouse Data from May 2012 to July 2012

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## Growth Chamber Data

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</tr>
<tr>
<td>21:00</td>
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</tr>
</tbody>
</table>

Table C.3. Growth chamber programming data, including temperature, humidity, and light intensity levels.