FINAL REPORT:

Developing Cultural Practices for Organically Grown Medicinal Plants

John Cardina, Bizhen Hu, Lisa Robbins, and Joseph Scheerens
Department of Horticulture & Crop Science, Ohio State University

Burdock has been proposed as a treatment for burn wounds by the Amish communities when used as a topical bandage simultaneously with an organic salve. After salve is applied, burdock leaves are used to wrap skin wounds, replacing conventional bandages. This treatment reportedly speeds the healing process, reduces inflammation and bacterial infections, and in some cases eliminates the need for skin grafts. Currently, this treatment is not recognized in conventional medical practice 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 and efforts to understand its mechanism in burn healing and develop its practical application are lacking. In these studies, we were aimed to understand the secondary metabolites in burdock which might be responsible for burn healing, and to develop techniques for producing these compounds for medicinal usage.

Burdock collection. Our first step was to collect burdock seeds from throughout the world, to sample the potential genetic variation in the species. We collected 17 accessions, which were then grown in Ohio (Table 1). Two species, *Arctium lappa* and *Arctium minus*, were studied in this project due to their prevalence globally. Of the 71 accessions, 24 were *A. lappa* and 47 were *A. minus*.

Metabolite studies. We conducted studies to distinguish the secondary metabolites in burdock leaves which may be responsible for the anti-inflammation, anti-bacterial, and anti-pain properties experienced when using this bandage. Due to the inherent variability among burdock plants, one leaf may be more beneficial than another. Until this work was undertaken, the extent of variability in chemical constituents among burdock plants was unknown.

We evaluated genetic and environmental variability influencing the production of phenolic compounds (hydroxycinnamic acids and flavonols) in burdock leaves. Burdock has high levels of phenolic constituents, and this compound class has previously been shown to promote health by acting as antioxidants and anti-inflammatory agents, both of which are involved with burn healing processes. The extraction protocol used was specific for polyphenols; however, two non-phenolics were consistently found to be present in each plant. Comparison of UV-Visible spectra of the unknowns to that of standards revealed that these compounds are probably sesquiterpene lactones. These compounds are also known for health beneficial properties, including healing of burn wounds.

Table 1. Accession number and origin of burdock seeds; accessions beginning with L are *Arctium lappa*; those beginning with M are *Arctium minus*.

No.	**Seed Origin**	**No.**	**Seed Origin**	**No.**	
L_01	Homerville, Ohio	M_01	Holmes County	M_32	Hayesville, Ohio
L_02	Wooster, Ohio	M_02	Holmes County	M_33	Wooster, Ohio
L_03	Wooster, Ohio	M_03	Pittsgrove, New Jersey	M_34	Mansfield, Ohio
L_04	Wooster, Ohio	M_04	Syracuse, New York	M_35	Mansfield, Ohio
L_06	Oregon	M_07	Dayton, Ohio	M_36	Wooster, Ohio
L_07	Japan (grown in Oregon)	M_08	Dayton, Ohio	M_37	Orono, Maine
L_08	United Kingdom	M_09	Toronto, South Dakota	M_38	Sanford, Michigan
L_09	Japan	M_10	Fitchburg, Wisconsin	M_39	Fremont, Michigan
L_10	United Kingdom	M_12	Morris, Minnesota	M_40	Oakley, Michigan
L_11	Wooster, Ohio	M_13	Wooster, Ohio	M_42	Owosso, Michigan
L_12	Wooster, Ohio	M_14	Munich, Germany	M_43	Morrice, Michigan
L_14	Loudonville, Ohio	M_15	West County, West Virginia	M_44	Plymouth, Michigan
L_15	Sunbury, Ohio	M_17	Ellenburg, New York	M_45	Okemos, Michigan
L_16	Dublin, Ohio	M_18	Mount Gilead, Ohio	M_46	Belleville, Michigan
L_18	Homerville, Ohio	M_19	Wooster, Ohio	M_48	Ashland, Ohio
L_19	Trumansburg, New York	M_20	Millersburg, Ohio	M_49	McGregor, Iowa
L_21	Columbus, Ohio	M_21	Mount Gilead, Ohio	M_50	East Lansing, Michigan
L_22	Medina, Ohio	M_22	Fredericksburg, Ohio	M_51	Wolfville, Nova Scotia
L_23	Medina, Ohio	M_24	Creston, Ohio	M_54	East Lansing, Michigan
L_24	Muskingum, Ohio	M_25	Seville, Ohio	M_55	Caro, Michigan
L_25	Wooster, Ohio	M_26	Medina, Ohio	M_56	Bancroft, Michigan
L_26	Norwalk, Ohio	M_27	Wooster, Ohio	M_57	Campbellsville, Kentucky
L_27	Ashland, Ohio	M_28	Wooster, Ohio	M_58	Carlisle, Kentucky
L_29	Nuremburg, Germany	M_31	Painesville, Ohio		

Chemical composition. We evaluated the variability in bioactivity among burdock plants. Total phenolic content was colorimetrically (ferric reducing ability of plasma), and preliminary identification and quantification of phenolic compounds and sesquiterpene compounds were measured by HPLC. Using these laboratory techniques, 16 major chromatographic compounds were identified and quantified to assess the variability among plants. The 16 major peaks (Figures 1 and 2) were preliminarily identified as seven hydroxycinnamic acids, seven flavonoids, and two 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 the LC-MS or GC-MS, the majority of these compounds are assumed to be phenolic compounds with the exception of two sesquiterpene lactones.

Genetic variation. For burdock leaves to be useful in medicine, practitioners need to know whether the active chemicals are similar among all burdock plants. Therefore, we evaluated the population variability among 71 burdock accessions. There was a large degree of variation among accessions, but we found no consistent pattern associated with the geographic origins of the accessions (Figure 3). Ttwo *A. lappa* accessions (L_18 and L_22) contained high amounts of phenolic compounds. To determine the variability contained *within* accessions, a subsample of

accessions was studied for each species. Three plants within each accession were studied separately to determine the level of variability in bioactive content. *A. lappa* accessions showed more variability within accessions than within *A. minus* accessions. Findings indicate that peaks 1, 3, 6, and 14 were more concentrated in *A. lappa* plants, and peaks 4, 5, 7, 11, and 16 were more concentrated in *A. minus* plants. These studies showed that the *A. lappa* accession L_03 contains significant variability within hydroxycinnamic acid levels, and produced the highest concentration of sesquiterpene lactone (peak 14). *A. lappa* plants in general displayed significantly more variability within accessions than *A. minus*, where accession L_19 was highly variable for flavonoid compounds. Accessions L_18 and L_22 had the highest amounts of hydroxycinnamic acids among all accessions, even though *A. minus* plants generally contained higher amounts than *A. lappa* plants. Accession M_19 had the lowest level of flavonoids among *A. minus* accessions, and M_15 had the highest amount of sesquiterpene lactones.

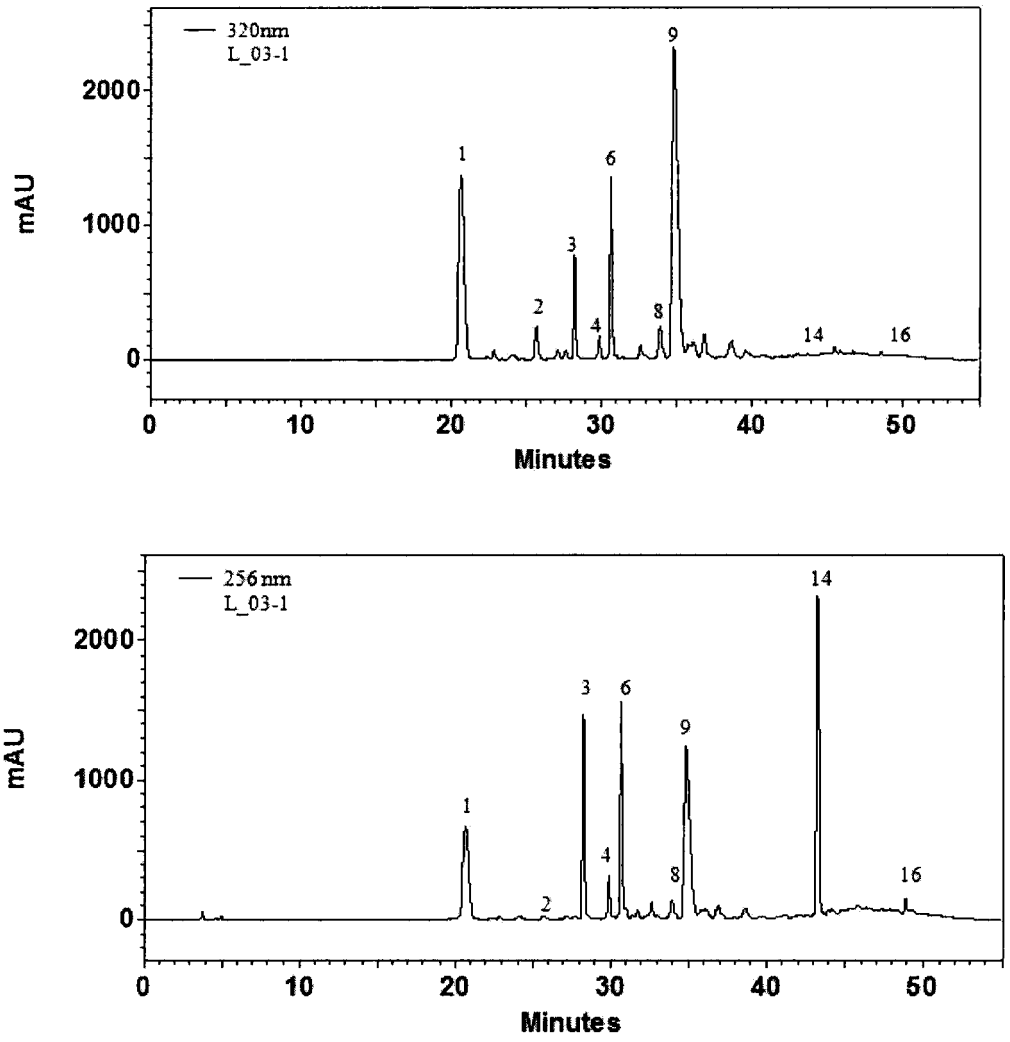


Figure 1. A typical chromatogram of an *A.lappa* plant at 320 nm and 256 nm.

Leaf size. Practitioners also need to know which leaves on the plant to choose, and whether leaf age or position might influence biological activity. Leaves chosen for bandages from a burdock plant are generally large leaves from the bottom of the rosette. We sampled plants to determine whether these mature leaves contain different levels of these 16 compounds than other leaves found in the rosette. Four sizes of leaves were chosen and their chemical content studied. Leaf sizes were: < 6 inches in length, 6 to 8 inches, 8 to 10 inches, and >10 inches in length. Large, mature leaves contained significantly higher levels of phenolic and sesquiterpene lactone compounds than smaller leaves.

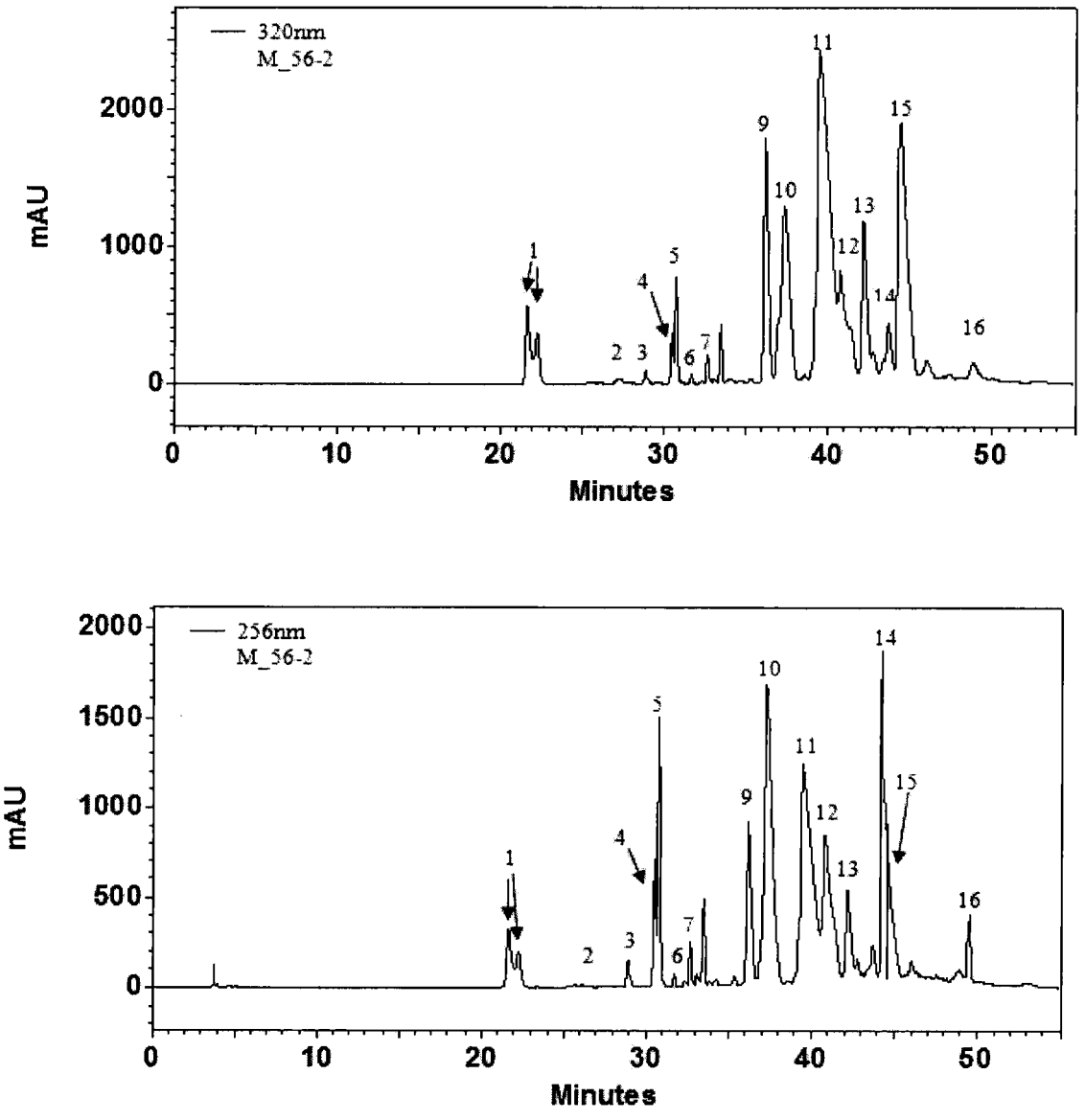


Figure 2. A typical chromatogram of an *A. minus* plant at 320 nm and 256 nm.

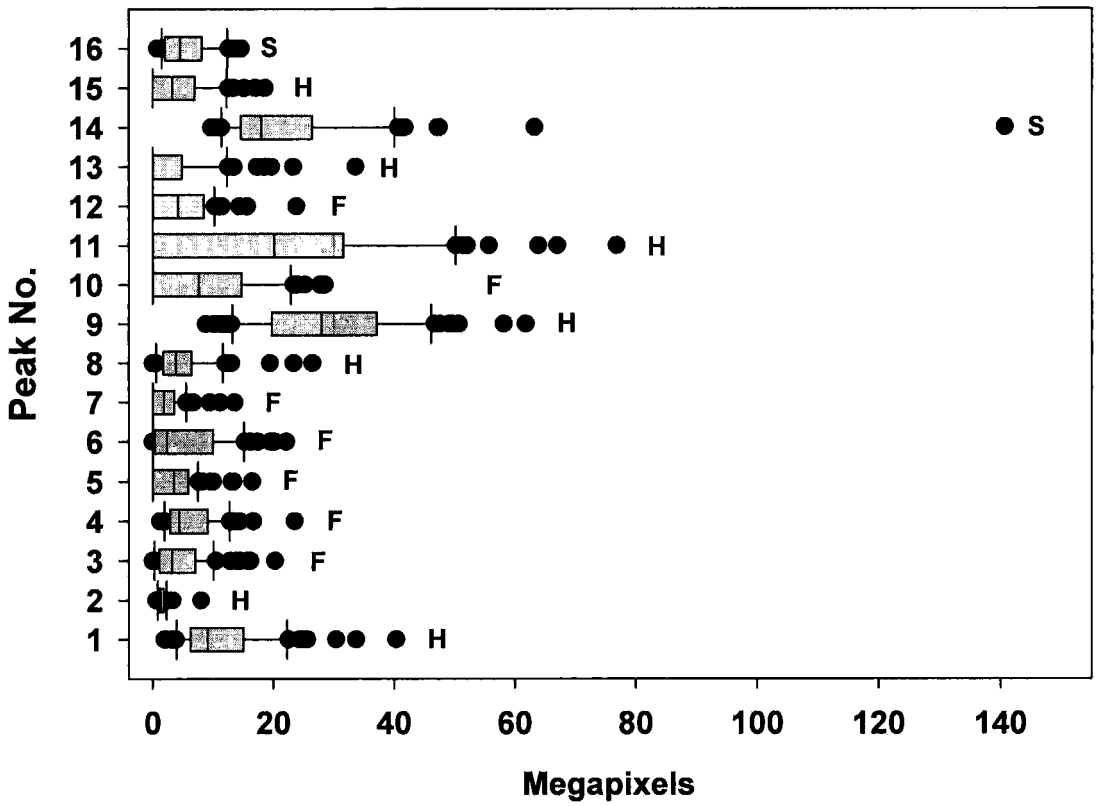
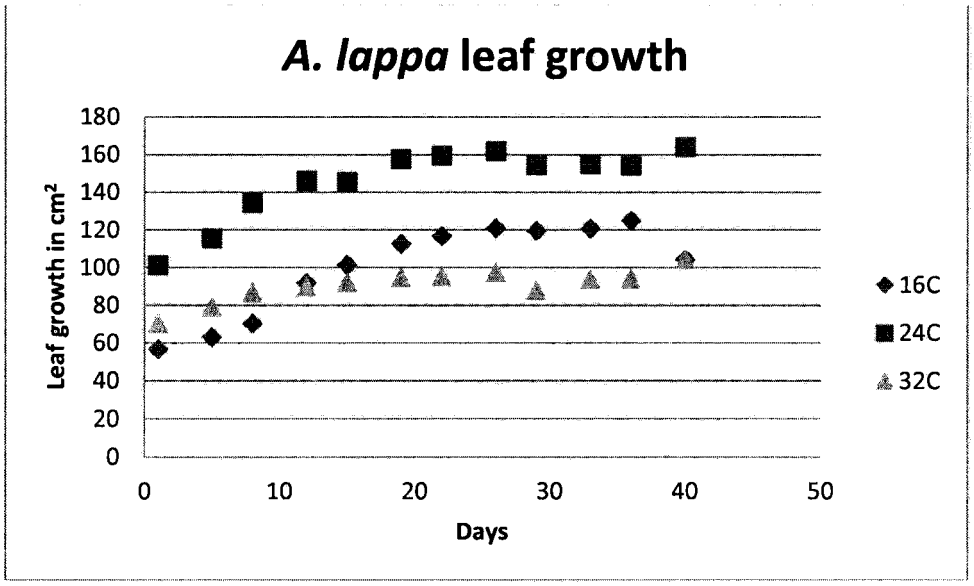


Figure 3. Variability among 71 accessions for chromatographic peaks 1-16; F = flavonoid, H = hydroxycinnamic acid, S = sesquiterpene lactone. Box plots: boxes encompass values that fall within the 25th and 75th percentiles, the vertical line within the boxes indicate median values, whiskers denote range of values within the 10th and 90th percentiles, circles indicate values that are beyond the 10th or the 90th percentile.

Leaf age. Another source of variation is plant age. Burdock is a biennial species that produces only a rosette of leaves the first year and basal and 'stalk' leaves the second, seed producing year. Most bandages used to treat burn wounds are probably chosen from first year rosette burdock plants, but early in the second year the plant resembles the first year rosette. We conducted a study to determine whether phenolic levels differ between first and second year burdock plants. Before the two year old plants produced a stalk, leaves were harvested from the rosettes and compared to the first year rosette plants. For both species there was a significant increase (average of three fold increase) in the phenolic levels and antioxidant power within leaves of second year plants compared to first year plants. The one exception is peak 12, the only compound more concentrated in first year leaves than second year leaves. Peak 9 was never produced in first year leaves, and was the most abundant compound found in second year plants. Peak 1 averaged 7.8-times higher in second-year plants than the first-year plants. At this time we

do not know the medical significance, if any, of these individual compounds or their possible interactions.



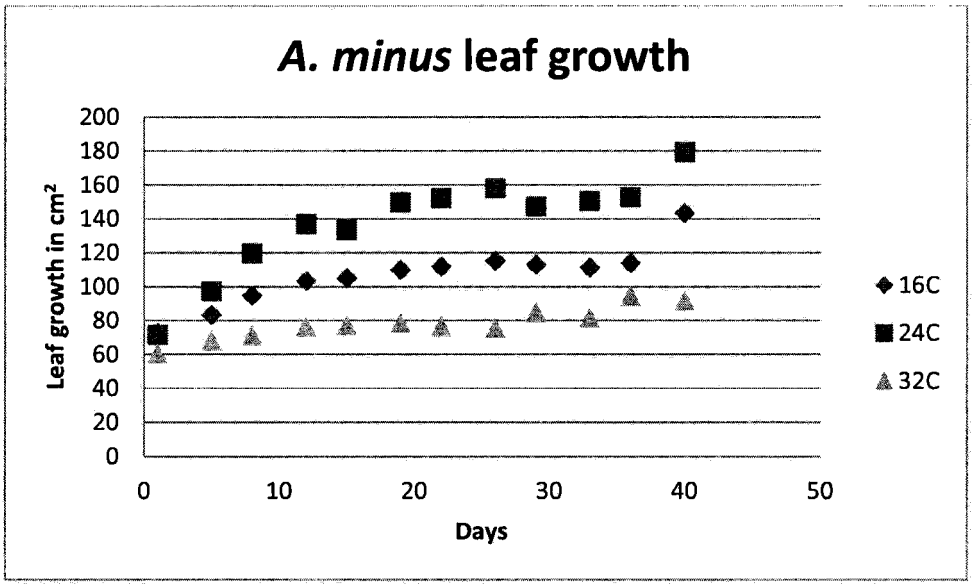


Figure 4. Average leaf growth (cm^2) for each species at 16, 24, and 32 C.

Leaf position. A further question is whether chemical composition varies with leaf position on the plant. Once second year plants produce a stalk, hundreds of leaves are produced per plant. We analyzed the chemical compositions of stalk leaves versus the rosettes leaves. On average, no significant differences were found. Differences in species were present, with *A. lappa* accessions having higher quantities of compounds in the rosette leaves compared to their stalk leaves, and *A. minus* accessions produces higher levels of compounds in stalk leaves than rosette leaves.

When comparing stalk leaves and rosette leaves throughout the second year plants, peak 9 again produced in high levels compared to first year plants. The only significant difference was for peak 14. In addition, *A. lappa* had higher mean percentages of these compounds in the rosette leaves than the stalk leaves, and *A. minus* had higher levels in the stalk leaves than the rosette leaves.

Growing conditions. Burdock plants are found all over the world and in contrasting environments, including full sun dry sites and shaded stream banks. Therefore, we were interested to know whether light and water interact to influence the chemical composition of burdock leaves. Controlled environment experiments were conducted in a greenhouse facility and growth chambers to determine effects of light levels, soil moisture, and temperature. Treatments inlcuded full light, 45% shade, full irrigation (daily), and restricted irrigation (once per week). The highest levels of phenolic compounds were found in both species treated with full light and frequent irrigation. The influence of temperature was studied in growth chambers programmed at 16, 24, and 32 °C.

For both species, leaf growth was greatest at 24 C and lowest at 32 C (Figure 4). This helps explain the distribution of these species, which are found mostly in temperate climates. In North America, they are not commonly found south of 35'north latitude or in most western states, but are widespread as far north as Ontario. Nevertheless, the pattern of temperature suggests a restricted range of adaptation that was unexpected.

In both species, higher phenolic levels were found in plants grown in 16 °C, whereas sesquiterpene lactones were highest at 32 °C. Peak 4 had the most variability, with significant differences in all interactions. Peaks 3 and 11 showed significant responses to temperature.

Callus culture. Due to the high level of variation between species, among accessions, and with different growing conditions, we believe that leaves of consistent quality will be difficult to produce in a field crop setting. There might be medically important chemical constituents that we did not measure, but it is unlikely that they will be more uniform than the metabolites that were measured here. Therefore, we conducted further studies to develop methods for callus culture, which would allow us to produce genetically uniform material and metabolites of interest.

We used burdock callus as the materials for medicinal metabolite production, and focused on phenolic compounds. The reason to use callus culture was that it is more controllable, stable, and efficient for plant-derived metabolite production compared with intact plants growing in the field. We focused on phenolics as the target compounds for two reasons: 1) phenolics possess medicinal functions such as antioxidant and anti-inflammatory activities, which are involved in the burn healing process; and 2) burdock contains high amounts of phenolics. Both reasons indicate that phenolics in burdock are likely to be the compounds responsible for the

effectiveness of burdock for treating burns. Therefore, we produced phenolic compounds in burdock callus culture, and analyzed the profiles of these phenolics.

We first developed the procedures for inducing and maintaining callus culture from burdock. We evaluated different explants, including burdock cotyledons, leaves, petioles, and stems, cut into different sizes, cultured in dark or in light, on media supplemented with different concentrations and combinations of growth regulators including BA, KT, TDZ, NAA, IBA, and 2, 4-D. A suitable procedure for burdock callus culture was developed, which showed high callus induction frequency and maintained fast and continually growing callus.

Callus growth. High callus induction frequency and fast, healthy callus growth are the two criteria for selecting callus induction media. Callus induction frequency from burdock cotyledon sections was 100% on all three media (M 1, M 2, and M 3), but the growth of callus varied with the media. Callus growth was quantified as the fresh weight of cotyledon sections over the first 5 weeks after inoculation, which was measured weekly. On medium M 1, callus exhibited a sigmoidal growth pattern (Figure 5). Following a slow increase in fresh weight in the first week after inoculation, callus quickly grew (0.08 g/week) from the second week to the fourth week, and then the increase in fresh weight slowed (0.06 g/week) in the fifth week.

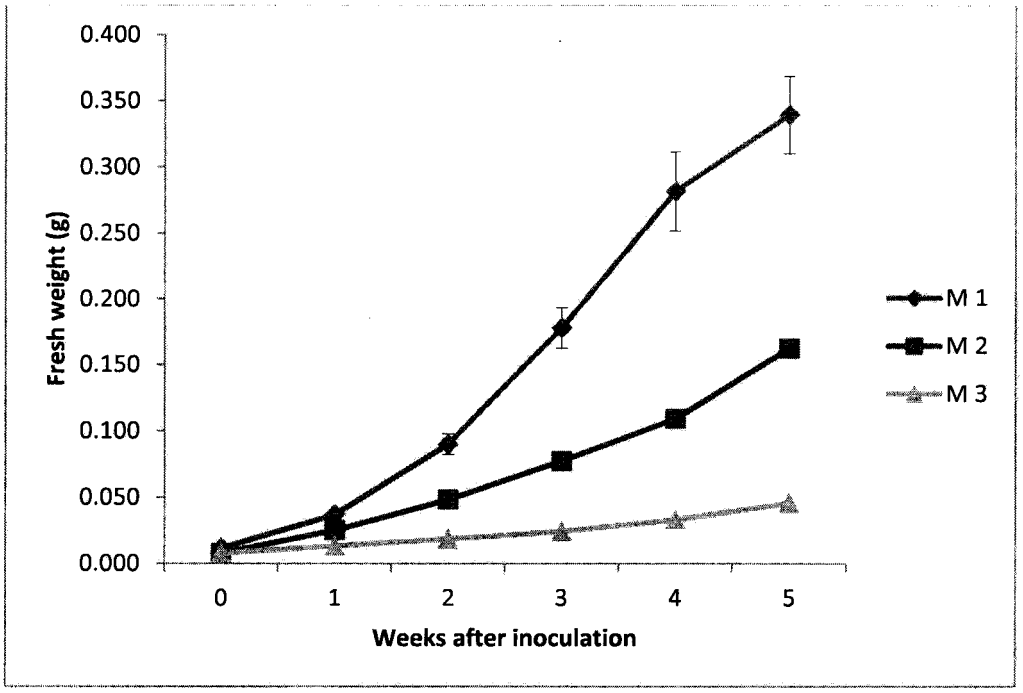


Figure 5. Growth curve of burdock callus on the three media (M 1, M 2, and M 3) based on fresh weight. Data represented average of three replications; bars indicate standard error.

After the method for burdock callus culture was developed, we modified the culture media by adding different concentrations of methyl-jasmonate (MeJA) and sucrose, aiming at enhancing phenolic productivity. We then analyzed phenolic compounds in burdock callus by determining

total phenolic content through the Folin-Cuicaktey assay, and attempted to identify and quantify individual phenolic compounds by HPLC.

When MeJA and high concentrations of sucrose were added to culture media, callus growth was inhibited with the increase of concentrations of MeJA and sucrose. However, MeJA up to 1 mM and 60 and 90 g/L sucrose increased both total phenolic content and major individual phenolic content. Optimum concentrations of MeJA and sucrose for total phenolic production were determined by considering both the effects on callus growth and total phenolic content, and calculating the product of callus relative growth and total phenolic content.

Phenolics in culture. Phenolic compounds accumulated in burdock callus were preliminarily identified by HPLC as hydroxycinnamic acid derivatives based on their spectra compared with commercial standards. All of the three major peaks detected from HPLC had similar spectra but different retention times, which suggested that they are likely to be different hydroxycinnamic acid derivatives.

Total phenolic content was reported as mg gallic acid equivalent/g fresh weight of callus. Methyl jasmonate ranging from 0.01 mM to 1 mM enhanced total phenolic content in burdock callus (Figure 6). As the concentration of MeJA increased up to 1 mM, the total phenolic content increased. However, 10 mM MeJA reduced total phenolic content. Over the four weeks after treatment, total phenolic content increased within the first two weeks, and decreased in the latter two weeks, meaning two weeks after treatment was the best time to harvest callus for the highest total phenolic content. Callus cultured on media with 1 mM MeJA and harvested two weeks after treatment contained a higher total phenolic content than all the other treatments ($p=0.05$).

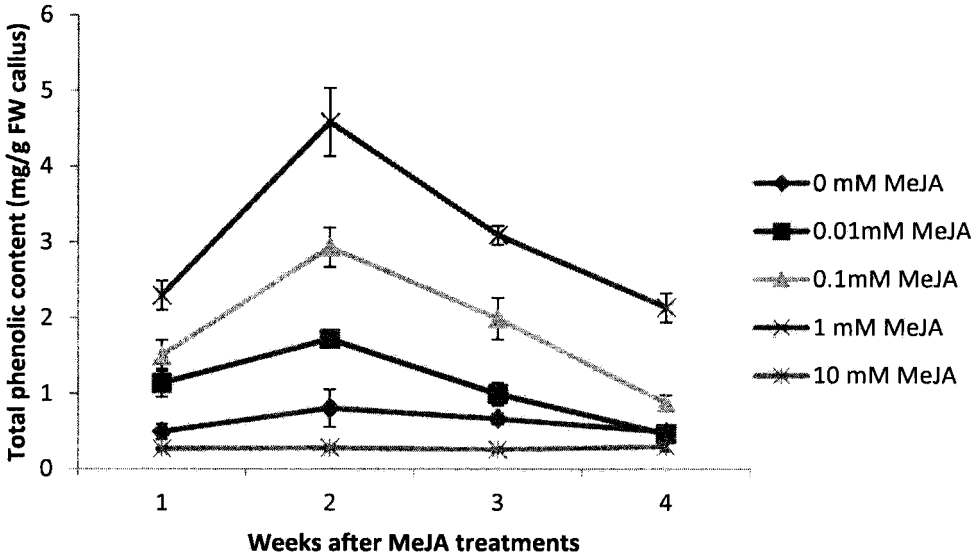


Figure 6. Total phenolic content in burdock callus 1 to 4 weeks after treatment with different concentrations of MeJA. Data are averages of three replications; bars indicate standard error.

Total phenolic production was affected by sucrose concentration and harvest time after treatment (Figure 7). Burdock callus grown on 30 g/L sucrose and harvested 2 or 3 weeks after treatment produced the highest amount of total phenolics. With 30 g/L sucrose, total phenolic content was higher at the end of the second week than that of the third week; however, callus mass was higher at the end of the third week than the second. Therefore, the total phenolic production in burdock callus 2 weeks and 3 weeks after treatment of 30 g/L sucrose did not differ.

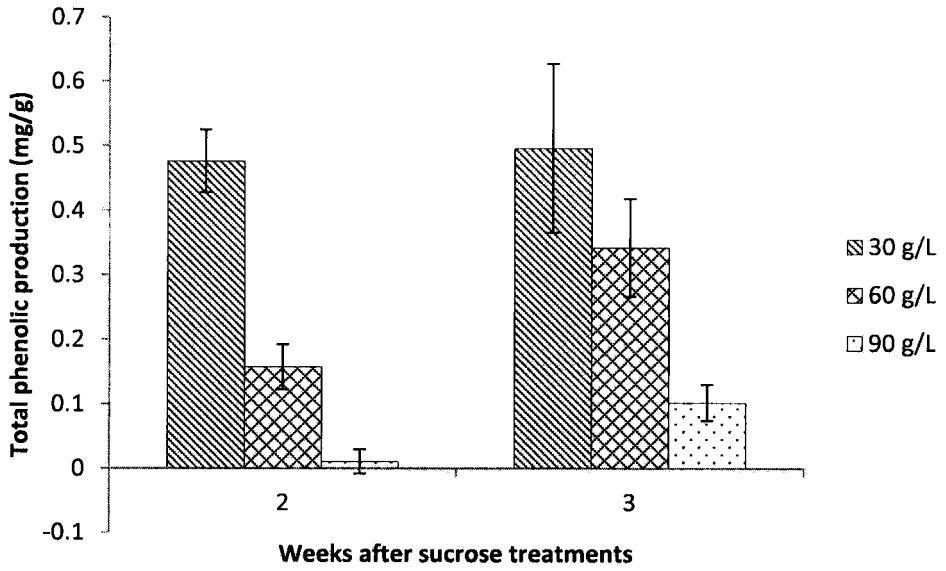


Figure 7. Total phenolic production in burdock callus 2 and 3 weeks after being treated with different concentrations of sucrose. Total phenolic production = phenolic content (mg/g FW) * callus relative growth (g/g). Means in each week with different letters were significantly different according to the Least Squares Means test (p=0.05). Data represent average of three replications.

Identification of phenolics. An HPLC chromatogram of burdock callus extracts in MeJA and sucrose studies is shown in Figure 8. The peaks marked as A, B, C appeared in each sample. Compound A was much more concentrated than the others. The UV spectra of these three peaks seemed to be identical to that of the chlorogenic acid standard (Figure 14), but their retention times were different. Based on their similar UV-spectra and various retention times, we preliminarily identified these three compounds to be hydroxycinnamic acid derivatives. The other small peaks detected by HPLC in this figure also had similar spectra to chlorogenic acid. These results suggest, tentatively, that diverse hydroxycinnamic acid derivatives accumulated in burdock callus.

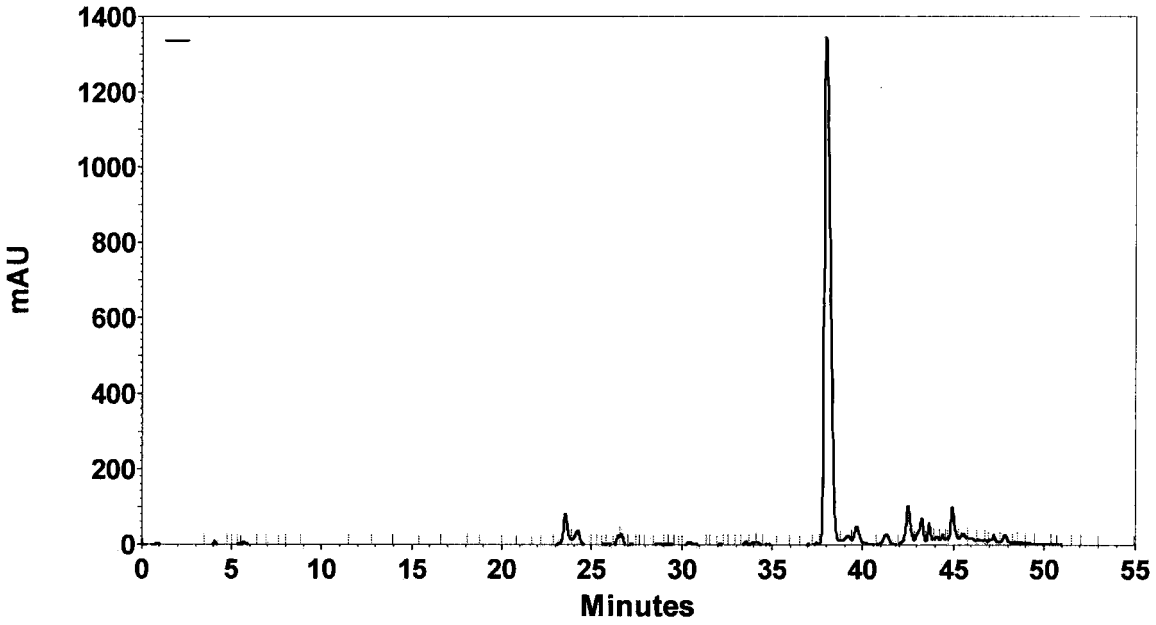


Figure 8. HPLC chromatogram of burdock callus extracts in MeJA and sucrose studies at 320 nm. Marked peaks A, B, C were the three major compounds of interest.

The conclusions drawn from these studies are as follows:

1. *A. minus* plants generally contain higher amounts of phenolic acids and less variability within accessions than *A. lappa* plants.
2. *A. lappa* plants were produced more sesquiterpene lactone compounds than *A. minus* plants.
3. Large mature leaves were have higher levels of phenolic compounds than smaller leaves.
4. Second year rosette leaves (before bolting) have about 3-times the amount of compounds in their leaves.
5. Geographic origin showed no significant differences.
6. Both species produce more of these metabolites under high light, high moisture, and lower temperature conditions.
7. Burdock callus can be used as the material for phenolic production.
8. Phenolic compounds accumulated in burdock callus are probably hydroxycinnamic acid derivatives
9. Appropriate concentrations of MeJA (0.1mM or 1 mM) added to culture medium can increase total phenolic productivity; a commonly used concentration of sucrose (30 g/L) was the optimum concentration for high total phenolic production in burdock callus.

Our study provided useful information about the potential medical compounds in burdock, and developed a new and efficient method for their production. This study laid a foundation for future studies on burdock metabolites that could be used to treat burns. The callus culture system

we developed can be used as the research method for future studies, and it has the potential to be further improved for commercial production of medicinal phenolics from burdock.

We recognize two major limitations in our study. We focused on phenolics as our target compounds to produce and analyze from burdock, based on the hypothesis that phenolics are the metabolites having medically functions for burn treatments. However, other metabolites in burdock could be responsible for or aid in its medical effectiveness. Assistance from the medical community will be necessary to solve this problem, which is beyond the scope of the studies described here. Another limitation was the difficulty in identification of individual phenolic compounds. With only the HPLC results, we could not identify the compounds with certainty since some compounds share very similar retention times and spectra.

Future studies:

For future domestication to develop a standardized burdock leaf bandage, the data generated in this project can be used to guide development of a burdock 'variety' with optimal phenolic or sesquiterpene lactone levels. Based on previous research, phenolic compounds were chosen as the target chemical constituents to quantify and analyze based on their medicinal properties. However, future studies are needed to determine which compounds are the responsible metabolites for assisting the healing of burn wounds. Pharmacological assistance is needed to determine the exact content of metabolites responsible in this treatment process, recognizing that a specific combination of compounds in particular ratios might be functional. HPLC results used here were not able to identify compounds with certainty; therefore, further identification studies are needed to determine with greater certainty the compounds quantified in these studies.

Further biochemical studies to identify these compounds will require liquid chromatography-mass spectrometry or gas chromatography-mass spectrometry. These studies are ongoing in the laboratory. The identification of these compounds and further knowledge of biological activity are needed before burdock can be recommended as a standard medical practice for burn treatment. Professional clinical studies are essential to determine the exact effect the chemical constituents in burdock leaves have on wounds. Along with future work with phenolic content of these leaves, the exploration of the terpene levels and perhaps steroid levels in burdock would be advantageous. These compounds also have beneficial medicinal properties, and high amounts of sesquiterpene lactone derivatives have already been observed in these leaves. The research described here can assist future studies on the development of burdock as a practical wound healing treatment

Clinical studies are essential to determine the effectiveness and mechanism of burdock for treating burns. Such studies should determine the specific metabolites that are associated with the effectiveness of burdock for burns. In addition, the compounds in burdock callus need to be identified using more reliable methods such as liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS). These studies are ongoing in this

laboratory. After the identification of metabolites in burdock callus, they should be further compared with those metabolites accumulated in burdock plants grown in the field to verify that burdock callus culture could be used for medically useful metabolite production. Our system for burdock callus culture can likely be transferred to cell suspension culture systems, to meet commercialization requirements.