Evaluating the chemical diversity and biological activity of plant extracts for commercialization

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Dedication

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Abstract

Perennial plants are a manageable natural resource with the potential to provide both highly valuable biologically active chemicals and ecosystem services. Ecosystem services include various benefits that are provided by an ecosystem such as food, fuel, recreation, as well as water, air, and land quality for society. Biologically active chemicals from plants have a long history of use by humans in botanical medicines and pharmaceuticals, food and dietary supplements, agricultural inputs, and home and personal care products. There are different strategies that can be used to incorporate plants into an economic and ecosystem service role. Method development and application studies were used to facilitate use of plant derived bioactive compounds for commercial use. Methodological studies, using the technique of metabolic fingerprinting, resulted in the determination of extraction conditions that maximize chemical diversity and yield. Maximum chemical diversity in a plant extract was most efficiently approached if solvent partitioning was performed on an extract made with 70 percent ethanol. Additionally, strategies to integrate extract chemical analysis with information regarding extract quality, such as cytotoxicity measurements, were developed and used to evaluate commercial kava samples obtained from multiple sources. These approaches were then applied to two different perennial plant species (*Comptonia peregrina*, and *Glycyrrhiza lepidota*) with the aim of developing their commercial value based on their extractable chemical composition. These studies resulted in the isolation of two small molecules from *C. peregrina* with strong antimicrobial activity and the identification of two *G. lepidota* populations with the potential to be developed into a cultivar with optimal characteristics for the cultivation of biologically active compounds.

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Appendix A

List of Abbreviations

^{1}H NMR – Proton nuclear magnetic resonance
^{13}C NMR – Carbon thirteen nuclear magnetic resonance
CD_3OD – Deuterated methanol
$CDCl_3$ – Deuterated chloroform
cv – column volume
DCM – Dichloromethane
DHK – Dihydrokawain
DHM – Dihydromethysticin
DMSO – Dimethyl sulfoxide
EtOAc – Ethyl Acetate
EtOH – Ethanol
FLK A – Flavokawain A
FLK B – Flavokawain B
H_2O – Water
HPLC – High-pressure liquid chromatography
HTS – High-throughput screening
IC_{50} – Concentration inhibiting microbial growth by 50%
IpOH – Isopropanol
K – Kawain
LC-MS – Liquid chromatography-mass spectrometry
M – Methysticin
MeOH – Methanol
MS^2 – Tandem mass spectrometry
MVA – Multivariate statistical analysis
m/z – mass-to-charge ratio
PC – Principal component
PCA – Principal components analysis
ppm – Parts per million
RP-UPLS-ESI(-)-HRAM-MS – C_{18}-reversed-phase ultra-performance liquid chromatography-negative electrospray ionization-high resolution/accurate mass-mass spectrometry
RP-UPLC-ESI(+)-HRAM-MS – C_{18}-reversed-phase ultra-performance liquid chromatography-positive electrospray ionization-high resolution/accurate mass-mass spectrometry
t_R – Retention time
TLC – Thin layer chromatography
UMN – University of Minnesota
UPLC-ESI(-)-SQ-MS – C_{18} reversed-phase-ultra-performance liquid chromatography-negative electrospray ionization-single quadrupole mass spectrometry
UPLC-ESI(+)-TOF-MS – Ultra-performance liquid chromatography-positive electrospray ionization-time-of-flight mass spectrometry
U.S.D.A – United States Department of Agriculture

Chapter 1

Introduction

Natural resources provide humans with basic necessities such as food, fiber building materials and energy. These resources should be managed in such a way to maximize the yield of marketable commercial commodities while simultaneously minimizing negative impacts on the environment. By increasing the utilization of perennial plants in upper Midwestern agricultural systems, significant improvements can be made in nitrogen utilization and soil conservation in addition to other benefits (Randall *et al*., 1997). This dissertation explores methods to identify highly valuable biologically active chemicals to provide economic incentive for increased placement of perennial plant species on to the landscape, for example in agricultural settings.

Ecosystem services

The term "ecosystem services" describes the broad range of benefits that humans obtain from their environment (Boody *et al.*, 2005; Egoh *et al.*, 2007; Jordan *et al.*, 2007). Broadly, ecosystem services provide food, fuel and recreation. Though less tangible, but arguably equally important, these services also include improvements in environmental quality for society as a whole. This concept of environmental quality includes water quality, air quality, and land (soil/fertility) quality. Because perennial plants are present on the landscape year-round, they are engaged in many functions that help to maintain ecosystem quality (Jordan *et al.*, 2007). Some of these functions include: carbon sequestration; erosion control; nutrient retention; wildlife habitat; water filtration and stabilization, and enhancing value within eco/agro-tourism industries (Boody *et al.*, 2005; Egoh *et al.*, 2007; Jordan *et al.*, 2007). The functions that help promote a high quality environment have been shown to be very valuable (Sullivan *et al.*, 2004). For example, the 34-million-acre Conservation Reserve Program, which retires environmentally sensitive land from crop production to perennial cover for 15 years per enrolled acre, has been estimated to produce erosion and wildlife viewing benefits valued at $500 million and $737 million per year, respectively (Jordan *et al.*, 2007, Sullivan *et al.*, 2004). However, these estimated values do not capture the tangible economic gains as increased annual income for individual farmers, but rather it captures those gains diffusely, shared

by society as a whole (Boody *et al.*, 2005; Egoh *et al.*, 2005). This situation creates an economic barrier to transitioning from annual to perennial cropping systems that ultimately blocks the realization of optimized economic potential (Boody *et al.*, 2005). Annual cropping systems provide income based on commodity market value, government payments, and crop insurance (Jordan *et al.*, 2007). The placement of perennial plants on the landscape, which will result in added, but intangible, environmental benefits, may proceed more quickly if linked with the realization of increased income by individual farmers, landowners, and communities through the identification of perennial plant phytochemicals of economic value.

Biologically active compounds

Plants have evolved to produce a vast diversity of chemicals that often help to mediate interactions with their environment. For plants, these chemicals act as signaling molecules, defense compounds against herbivory and microbial attack, pollinator/seed disperser attractors, and protection from UV radiation and other abiotic factors (Ortholand and Ganesan, 2004). Plant chemicals have a long history of use by humans in botanical medicines and pharmaceuticals, food and dietary supplements, agricultural inputs, and home and personal care products (Cragg and Newman, 2013; Duke *et al.*, 2000; Fabricant and Farnsworth, 2001; Newman and Cragg, 2012; Raskin *et al.*, 2002; Starmans and Nijhuis, 1996). For example, the familiar analgesic, morphine, is an alkaloid isolated from *Papaver somniferum* L. (opium poppy) where it acts as a defense compound (Morimoto *et al.*, 2001).

In addition to their human utility, botanically sourced biologically active molecules can have a high monetary value (Raskin *et al.*, 2002). The plant defense stilbene, resveratrol, synthesized by *Vitis* spp. (grapes) and many other plant species, has been reported to have human disease prevention and therapeutic activity for a number of age-related conditions including cancer, neurodegenerative diseases, and cardiovascular disease (Kiselev, 2011; Pawlus *et al.*, 2012). The natural supplements company Reservage™ Organics, which was the 2012 recipient of the Nutrition Business Journals Business Achievement Award (Nutrition Business Journal, 2013), sells bottles of 100 mg of pure resveratrol (30 capsules) for $14.49 each (September 2014 price). Perennial plants have been shown to be a source of highly promising biologically active molecules

(Borchardt *et al.*, 2008a&b, Fabricant and Farnsworth, 2001; Gillitzer *et al.*, 2012, Martin *et al.*, 2014; Raskin *et al.*, 2002). The extraction of biologically active compounds may provide the economic incentive to help promote the cultivation and conservation of perennial plants on the landscape.

Perennial plants on the landscape

There is increasing interest in the use of perennial crops in agricultural settings because of the role they play in maintaining healthy ecosystems (Jordan *et al.*, 2007). Biologically active chemicals extracted from plants are the type of marketable commercial commodities that can be used to incentivize the addition of ecosystem services to a landscape through the addition of perennial plants. Several strategies can be used to incorporate plants into an economic and ecosystem service role through plant collection and cultivation.

Plant collecting involves collecting individuals from existing populations in the wild. Sufficient numbers of individuals must be available in a sampled population to allow for collection without causing harm to its native habitat. Benefits of the collection strategy include low initial inputs for land, labor, or germplasm development and associated costs, the ability to target invasive plant species, and the provision of support for conservation efforts of intact ecosystems where the target plant species are present. A potential drawback of collecting plants in the wild includes the need for maintaining a sustainable source for the material that thereby excludes the use of endangered, rare, and/or species occupying critical-habitats (Dharmananda, 2000). Additionally, the lack of control over growing conditions and biotic mutualistic interactions may result in yearly or site specific variation in plant chemical composition (Fenwick *et al.*, 1990; Hayashi and Sudo 2009; Isbrucker and Burdock, 2006). Thirdly, society may negatively regard the collection practice as an exploitative practice such as ‘bio-piracy’.

The plant cultivation strategy involves the incorporation of selected species into an agronomic setting through domestication or native landscape reclamation. Multifunctional agricultural landscapes balance the production of standard commodities such as food and fiber with the concurrent creation of broader environmental benefits (ecosystem services; Jordan *et al.*, 2007). Developing perennial plant species as alternative crops may allow them to be incorporated into multifunctional agricultural

landscapes (Boody *et al.*, 2005; Egoh *et al.*, 2007). Alternatively, select sub-regions (such as those with high erosion or riparian zones) might be selected for ecosystem reclamation projects. Ecologically sensitive areas could then be better protected from damage (erosion or nutrient leaching) and provide a habitat for commercially valuable perennial plant species harboring valuable phytochemicals. Many perennial plant species can be grown on steep slopes, river banks, and other marginal land not suitable for row crop production that would only add value to a farm through its inclusion (Dharmananda, 2000; Hooper *et al.*, 1984; Sylvestre *et al.*, 2007). Some plant species could co-exist with and enhance traditional row crops, such as corn and soybean, by attracting pollinators, beneficial insects, and increasing macro-level biodiversity to decrease pressure from pests and diseases. Additional benefits to using a perennial crop cultivation strategy include increased control over the entire growing and harvesting process, optimization of germplasm for target production, and the potential for improving a farms public image and for developing agro-tourism (Dharmananda, 2000; Jordan *et al.*, 2007; Ozaki and Shibano, 2014). The major drawback of this strategy is the high initial time, labor, and capital costs required (including land and agricultural equipment acquisition) for perennial crop germplasm development, and labor. Some of these costs may possibly be absorbed by established farms, for example, harvesting biologically active chemicals could be a value added activity to a perennial biomass/biofuels operation. For both the wild-collection and domestication (cultivation) approaches, an increase in the valuation of perennial plants may provide incentive to maintain intact populations and add additional perennial material to the landscape through the preservation and reestablishment of native ecosystems.

Discovery optimization and practice

An early step in the process to increase the use of perennial plants as source material for biologically active compounds includes the optimization of discovery methods. Two aspects of this process include solvent extraction and extract evaluation methods for quality control. Plant extracts are composed of a vast array of different chemicals and their composition is largely dependent upon the solvent extraction conditions employed. Therefore, an analysis tool capable of approaching an evaluation of the complete composition of plant extracts would be beneficial. Plant metabolomics, the

comprehensive study of all small molecules within a biological system, includes the technique of metabolic fingerprinting, which, when paired with multivariate statistical analysis (MVA), facilitates the examination of global molecular diversity within plant species and their extracts (Fiehn, 2002; Hegeman, 2010).

Metabolic fingerprinting facilitated an unbiased evaluation of extract chemical diversity (**Chapter 2**). Extracts were generated using different solvent extraction systems and three different species of perennial plants. Results of these analyses were used to identify efficient extraction conditions that would generate high yielding and chemically diverse plant extracts. Extract yield was defined as the amount of dissolved solids extracted from a discrete amount of starting material and chemical diversity was defined as the number and variety of different chemical structures present in an extract. Metabolic fingerprinting was also used to measure the chemical variability of a popular and controversial commercially available botanical extract (kava, **Chapter 3**). In an effort to understand potential health risks of this botanical therapy, variation in extract chemistry was linked to cytotoxicity measurements. The results of these methodological studies were then used to inform plant extractions and the subsequent evaluation of their bioactivity and chemical compositions.

Perennial plants in the state of Minnesota have shown great potential to be used as a source of biologically active molecules (Borchardt *et al.*, 2008a&b, Gillitzer *et al.*, 2012, Martin *et al.*, 2014). Within this group of plants there are species that grow in abundance and are suitable for the collecting strategy (*Comptonia peregrina*; **Chapter 4**). Conversely, that have the potential to produce useful compounds, but that do not have prolific populations. These species are better suited for the cultivation strategy (*Glycyrrhiza lepidota*; **Chapter 5**). Both the collection and cultivation methods were employed to obtain appropriate plant material to test techniques developed using plant materials collected from a variety of families and with different ecological roles.

Chapter 2

Evaluating solvent extraction systems using metabolomics approaches[1]

Summary

Metabolic fingerprinting was performed on a set of botanical extracts to compare the extraction efficiency of different solvents to inform the construction of phytochemical libraries. We compared the extraction efficiency, examining both yield and chemical diversity, of eight single-solvent extractions prepared in parallel and using solvent-solvent partitioning. Three-dimensional data were reduced into features, which were used as unbiased metrics to identify solvents that would produce botanical extracts with the greatest chemical diversity. Chemical diversity and extract yield did not necessarily increase together. For each species and tissue, the total number of observable chemical features closely approached maximum values when three different single-solvent extractions were performed in parallel. The dynamic range of detectable compounds in plant extracts was increased significantly by performing solvent partitioning**.** Overall, maximum chemical diversity in a plant extract was most efficiently approached if solvent partitioning was performed on an extract made with 70% ethanol. We have shown that using metabolic fingerprinting is a useful for assessing compound diversity in complex plant extracts.

Introduction

One might think, given the tremendous importance of plant natural products in medicine and commercial product formulation (Balasundram *et al.*, 2006; Li and Vederas, 2009) that broadly applicable procedures for natural product extraction would be well defined and firmly supported by methodological experimentation.

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ACM contibutions included: Designing research study, performing research, analyzing data, generating tables and figures, and writing the paper.

It is surprising to see how little information is available concerning optimized extraction protocols that provide maximal chemical diversity in plant extracts given the theoretical importance of sampled chemical diversity for compound discovery through high-throughput screening approaches. Extracting natural products from plant material to funnel into high-throughput screens (HTS) is an effective strategy for testing a broad range of bioactivities nearly simultaneously. Through HTS, valuable chemicals may be uncovered from libraries composed of extract fractions or pure compounds. Plant natural products have a wide variety of physicochemical properties and may be present across a huge range of concentrations. There are a number of different methods available for plant extractions, some requiring specialized equipment such as: supercritical fluid extraction, Soxhlet extraction, pressurized solvent extraction, microwave-assisted, steam/hydro-distillation, decoction, infusion, percolation, pressing, and boiling (Chemat *et al.*, 2012). Solvent impurities and their tendency to form artifacts, such as the condensation products formed with acetone, also must be considered during the selection of extraction solvents (Hunchak and Suffet, 1987). Extraction requires efficient compound solubilization from a diverse set of plant tissue matrices making the optimization of generalized extraction protocols quite challenging. To date, this challenge has been the subject of many studies that attempt to determine ideal extraction conditions for detection of compounds by either monitoring a specific biological activity (Gillitzer *et al.*, 2012; Jones and Kinghorn, 2006; Marzoug *et al.*, 2011; Nostro *et al.*, 2000; Singariya *et al.*, 2011; Sultana *et al.*, 2009), a targeted compound class, (Huie, 2002; Johansen *et al.*, 1996; Julkunen-Titto and Sorsa, 2001; Lapornik *et al.*, 2005; Martinis *et al.*, 2011; Muanda *et al.*, 2011; Sultana *et al.*, 2009) or individual molecules (Saric *et al.*, 2012). One consequence of these extraction optimization strategies is that they ultimately bias the chemical diversity of the resulting extracts toward whichever selection criteria were imposed. This is potentially damaging to the success of high-throughput chemical library screening approaches that depend on the availability of maximal compound diversity within screened populations. In this study, we have used an LC-MS metabolic fingerprinting approach in an attempt to

minimize this bias in evaluating extract chemical diversity to enable a more inclusive assessment of chemical extraction efficiency. In so doing we acknowledge the usefulness of measurements of both known chemicals and the utility of observable yet unknown chemical entities for assessing total extract chemical diversity.

Metabolomics, the comprehensive study of all small molecules within a biological system, includes the technique of metabolic fingerprinting, which when paired with multivariate statistical analysis (MVA), facilitates an examination of the global molecular diversity within a whole extract (Fiehn, 2002; Hegeman, 2010). Metabolic fingerprinting has been carried out using a variety of analytical platforms including liquid chromatography-mass spectrometry (LC-MS) (Allwood and Goodacre, 2010; Kim and Verpoorte, 2010). LC-MS is particularly well suited for the analysis of botanical extracts because they are composed of diverse array of chemical species that range widely in concentration (Kim and Verpoorte, 2010; Saric *et al.*, 2012). In a metabolic fingerprinting experiment, individual chemical fingerprints are collected by LC-MS from replicate samples. Continuous LC-MS data are simplified into discrete sets of *features* for each sample through a process called data reduction. Each feature is made up of a unique retention time (t_R), a monoisotopic mass, and a relative intensity value that varies from feature to feature from sample to sample and must be greater than zero counts (Trygg *et al.*, 2007). Thus, the total feature set provides a reasonable approximation of chemical composition of a sample without requiring the laborious process of chemical structural characterization for all of the sample's components. While some representational bias is present in LC-MS due to competition for ionization energy amongst coeluting chemical species, this bias will be significantly less than other common general analytical techniques such as NMR, which is much less sensitive, GC-MS or GC-FID, which require volatile analytes, or LC-UV/Vis, which requires the presence of a detectable chromophore in each analyte (Dettmer *et al.*, 2007).

Plant materials for this study were selected from a large number of local (Minnesota, USA) plant species shown to display antimicrobial and antioxidant activities in previous work (Gillitzer *et al.*, 2012; Borchardt *et al.*, 2008a &

2008b). We used three of those plant species, *Rhus typhina* L. (staghorn sumac), *Lythrum salicaria* L. (purple loosestrife), and *Monarda fistulosa* L. (wild bergamot or bee-balm), to provide a diverse set of plant materials for this study that would, taken *in toto*, be fairly representative of plant materials in general. Multiple parallel single-solvent extractions and three-part extraction partitions using solvents of variable selectivity such as water, ethanol, and dichloromethane were compared (Allwood and Goodacre, 2010; Jones and Kinghorn, 2006). Extract concentrations were calculated and overall percent yields were determined from residual mass measurements following solvent evaporation. Chemical diversity was evaluated using metabolic fingerprinting by ultra-performance liquid chromatography-electrospray ionization-single quadrupole mass spectrometry (UPLC-ESI-SQ-MS) paired with MVA. Overall, we evaluated extract reproducibility, yield, and the number and uniqueness of detected metabolite features among the different extraction methods.

Materials and Methods

Plant Material. Aerial tissue from three species, *Rhus typhina* L. (staghorn sumac), *Lythrum salicaria* L. (purple loosestrife), *Monarda fistulosa* L. (wild bergamot or bee-balm), were collected from central and southern Minnesota, USA (93.25ºW, 46.25ºN) into cloth bags and dried at 30ºC for three days. Species authentication was performed (by DLW) and voucher specimens were deposited for *R. typhina* (AV0001 stems, AV0002 berries, and AV0003 leaves), *L. salicaria* (AV0017) and *M. fistulosa* (AV0022) in the Department of Horticultural Science University of Minnesota, Saint Paul, MN, USA. Prior to drying *R. typhina* berries were separated from the leaves and stems; these three tissues were treated separately from one another. The dry material was ground in a Thomas Wiley laboratory mill model 4 (Thomas Scientific, Swedesboro, New Jersey, USA) using a 6 mm screen and then stored in sealed opaque containers kept at room temperature until extraction.

Chemical Reagents. HPLC grade solvents purchased from Sigma Aldrich (St. Louis, MO, USA) were used including: acetonitrile, dichloromethane, ethanol (95%), ethyl acetate, formic acid, hexanes, isopropanol, and methanol. Reverse osmosis deionized glass distilled water was obtained in house using a Thermo Scientific Barnstead B-pure™ filter and Distinction water still model D4000 (Bibby Scientific Limited, Stone, Staffordshire ST15 0SA, UK).

Sample Preparation. Two different sets of extraction experiments were performed on dry ground material (Fig. 1). Experiment one consisted of eight single solvent extractions performed in parallel and experiment two was a series of single solvent extractions followed by partitioning with hexanes and dichloromethane. Experiment one provided an initial assessment of eight solvents systems: hexanes, dichloromethane, ethyl acetate, methanol, isopropanol, water, aqueous ethanol (ethanol: water, 70:30 v/v), and a dichloromethane/methanol mix (dichloromethane:methanol, 1:1 v/v). We used aerial tissue, consisting of stems, leaves, flowers, and buds, from *L. salicaria* and *M. fistulosa*; the set of extracts generated from a single species were compared with one another. Briefly, a recorded exact weight between 100-200 mg of dry ground plant material was placed into 2 mL polypropylene microcentrifuge tubes and 1.5 mL of solvent was added. The tubes were individually mixed using a Fisher Scientific fixed speed mini vortexer (Scientific Industries Inc. Bohemia, NY, USA) and then turbo-mixed using a Fisher Scientific vortex Genie 2™ (Scientific Industries Inc., Bohemia, NY, USA) for 15 min. This step was repeated and then the tubes were centrifuged using an Eppendorf 5415C centrifuge (Brinkman Instruments, Westbury, NY, USA) at 12,000 rpm for 5 min. The extract supernatant was removed to a clean tube and placed at 4ºC in the dark. Each extraction was replicated 4 times.

Experiment two was a single-step extraction followed by two solvent partitioning steps using the most effective solvents from experiment one. We focused on three different tissue types from one species, namely, *R. typhina* leaves, berries, and stems. The single-step extraction was prepared using the same general method as in experiment one with methanol, 70% ethanol, dichloromethane,

hexanes, water, and dichloromethane:methanol. The extract partitioning was performed on an initial extract prepared from 70% ethanol in water, 100% water, or 100% methanol, followed by a two-step partitioning with hexanes and then dichloromethane (Fig. 1B). The amount of starting material was increased to 300 mg, to ensure adequate quantities after partitioning, and the shaking was performed on a 2010 Geno/Grinder® (SPEX Sample Prep, Metuchen, NJ, USA) using a 15 min shaking program (5 min at 500 rpm followed by 10 min at 700 rpm). After centrifugation the prepared extract supernatant (about 900 μL) was removed to a clean tube and 900 μL of hexanes was added. After being mixed on the Geno/Grinder for 3 min at 700 rpm the two immiscible layers were allowed to separate for 60 min. After phase separation, the nonpolar hexanes layer (Fig. 1B: E1, W1, M1) was removed from the polar layer (Fig. 1B: E3, W3, M3) and 900 μL of dichloromethane was added to the polar layer for partitioning using the same procedure to generate a medium polarity partition (Fig. 1B: E2, W2, M2). All three partitions were separated into clean tubes, centrifuged at 12,000 rpm for 5 min, and stored at 4ºC in the dark. All extract partitions were replicated 4 times.

Evaluation of Extract Yield. Absolute extract yield was determined by weighing the residue remaining after evaporating 500 μL of extract to dryness (using a Savant model SVC-200H SpeedVac concentrator; Farmingdale, NY, USA). Extract residue yield was calculated as a percent of the initial dry weight of plant material used to produce 500 µL of extract. Additionally, all extracts were digitally photographed and assessed visually for color, clarity, and similarity.

Statistical Analysis. Four replicates were prepared for each extraction. Each sample was analyzed individually and data is reported as mean (n = 4) ± standard error. Analysis of variance (ANOVA) was performed on the yield data using 'R' version 2.15.2. Means were compared using Tukey's HSD and a p-value < 0.01 was considered to be significant ('R' package Agricolae 1.1-4).

Metabolic Fingerprinting

UPLC-ESI(-)-SQ-MS. The C_{18}-reversed-phase ultra-performance liquid chromatography-negative electrospray ionization-single quadrupole mass spectrometry [UPLC-ESI(-)-SQ-MS] was carried out on a UPLC-SQ detector mass spectrometer fitted with an autosampler where sample vials were held at 4ºC (Acquity, Waters, Milford, MA, USA). The following MS conditions were used: full scan mass range of 100-1000 *m/z*, 250 ms scan time, desolvation temperature 350 °C, desolvation flow rate (nitrogen) of 6.5 L/min, capillary voltage of 3000 V, sample cone voltage of 30 V, source temperature of 150°C. Separation was carried out on a C_{18} reversed phase HSS T_3 1.8 μm particle size, 2.1x100 mm column (Waters). Column temperature was 30°C, mobile phase flow rate 0.45 mL/min, injection volume 2 μL. A 28-minute gradient using mobile phases A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile was run according to the following gradient elution profile: initial, 2% B; 2 min, 2% B; 5 min, 18% B; 20 min, 98% B; 22 min, 98% B; 23 min, 2% B; 28 min, 2% B (5-min re-equilibration). MassLynx version 4.1 (Waters) was used to record the chromatograms and spectra. Replicate extractions were organized into four batches and sample analysis order was randomized within batches.

Feature Detection. LC-MS data was subjected to feature detection in order to permit comparison of extract chemical diversity. A custom workflow for feature detection was designed using *Refiner MS* version 7.5 software (GeneData, Lexington, MA, USA). Feature detection was performed on a sub-set of the 28-minute gradient from 2-25 min. The following were data clean-up activities: $5e^4$ intensity thresholding; chemical noise reduction using a retention time (t_R) window of 51 scans with minimum t_R length 3 scans and minimum *m/z* length 3 points; t_R alignment with *m/z* window = 0.1 Da, t_R window 0.2 s, and t_R search interval 30 scans. The following activities were carried out on the aligned data: chromatogram summed peak detection with minimum peak size of 4 scans and derivative based peak detection; maximum missing peaks = 0, first allowed gap position = 3, t_R tolerance = 0.5 s, *m/z* tolerance = 0.8 Da, signal-to-noise ratio of extracted mass features was ≥ 3. All four replicates from each solvent extraction for each

plant/plant part were analyzed together so that for example all 32 *R. typhina* leaf extracts were analyzed in the same run.

Multivariate Statistical Analysis (MVA). Feature lists were transported to *Analyst* version 7.5 software (Genedata) for MVA. The feature lists were inspected and a feature was considered to be real if it was present in greater than 75% of replicate samples with similar intensity in all replicates**.** Once highly confident feature lists were obtained, principle component analysis (PCA) was performed on extracts made from a particular plant or plant tissue. Venn diagrams were then generated to determine how many unique features each solvent extraction contained. These data were converted into bar graphs to facilitate viewing and analysis.

Results and discussion

Extraction Yield. The extraction efficiencies of the eight parallel single-solvent systems used to prepare extracts from *L. salicaria* and *M. fistulosa* aerial tissues were evaluated (Fig. 2). The six best solvents from experiment one were then used to extract *R. typhina* leaves, berries, and stems, and the extraction efficiencies were assessed (Fig. 3). All five sets of extracts show similar trends in the variation of overall percent yield, although absolute extract concentration differed greatly (Fig. 2 & 3). A visual inspection shows marked differences in the appearance of the extracts with 70% ethanol, methanol, and dichloromethane:methanol producing similar looking, dark extracts; water and dichloromethane producing slightly lighter and variably colored extracts, and isopropanol, ethyl acetate, and hexanes producing very light yellow extracts with hexanes being almost colorless (Fig. 8). Extracts prepared with 70% ethanol and, despite its light appearance, water had the highest percent yield for all plant materials except for *R. typhina* berries and stems, where methanol produced higher yielding extracts than water. The extracting solvents, methanol, dichloromethane:methanol, dichloromethane, and hexanes produced moderately yielding extracts, although for *R. typhina* berries extracted by dichloromethane yields were high. Both isopropanol and ethyl acetate extracts had

low yields of less than two percent for all plant materials. Although the trends in percent yield were similar among the solvent systems, the absolute concentration of the extracts varied greatly according to the type of botanical material used. Extract concentrations ranged from less than 2 mg/mL to greater than 40 mg/mL, where extracts prepared from *M. fistulosa* aerial parts and *R. typhina* stem tissue had the lowest concentrations overall. This absolute difference in extract concentration is similar to results from a study by Johansen *et al.*, 1996 where differences in overall yield were found when using an identical extraction method on field peas, toasted soybean meal, cotton seed meal, and a feed mixture. Therefore, it is important to test a range of raw materials when evaluating extraction efficiency. Due to the low yields of samples prepared with isopropanol and ethyl acetate, these two solvents were eliminated from the more in depth study with *R. typhina* leaves, berries, and stems where we performed single solvent extractions followed by partitioning to evaluate the advantages, if any, that partitioning would lend to chemical diversity or yield.

Conclusions drawn solely from yield measurements indicate that in decreasing order of efficiency, 70% ethanol, water, methanol, and dichloromethane:methanol are the most efficient extraction solvents. This result is similar to a study by Sultana *et al.*, 2009 who found that aqueous organic extracts had higher yields of extractable solids than absolute organic extracts. While percent yield measures crude extraction efficiency in terms of quantity, it does not indicate extract chemical diversity. For instance, a solvent system may result in a high yielding extract of primarily tannins and sugars and, therefore, lack molecular diversity. Thus, a solvent that is high yielding may produce extracts of low overall quality due to limited chemical diversity.

Extract Chemical Diversity. The second aspect of extraction efficiency is chemical diversity. We have attempted to find a maximally inclusive way to assess this factor. Previous research has used biological activity (Marzoug *et al.*, 2011; Singariya *et al.*, 2011), levels of specific classes of compounds (e.g. tannins, flavonoids, alkaloids, saponins, etc.), marker compounds (e.g. quercetin or

emodin), or major constituents to evaluate extraction efficiency (Lapornik *et al.*, 2005; Muanda *et al.*, 2011; Sultana *et al.*, 2009). These methods may bias results towards highly abundant common molecules rather than systematically evaluating the total extractable chemical diversity from each species and plant part (Huie, 2002; Raskin *et al.*, 2002). Metabolic fingerprinting favors the analysis of whole extract chemical diversity rather than single components or known compound classes, making it particularly suitable for the inclusion of unknowns when assessing extraction efficiency.

Metabolic Fingerprinting. The acquisition of LC-MS generated fingerprints provides a visual way to qualitatively evaluate chemical diversity (Nostro *et al.*, 2000). Chemical diversity can be quantified by applying MVA to LC-MS fingerprints to generate features that can be used as unbiased metrics for comparison. While collecting LC-MS fingerprints it is essential to maintain a high level of reproducibility among the factors describing a feature for any given sample, those factors being *m/z*, intensity, and t_R. Reproducibility of the first two factors, *m/z* and intensity, are a function of the mass spectrometer. Using unidentified features as metrics made it possible to obtain nominal mass measurements with a single quadrupole instrument. Run-to-run t_R reproducibility is also important. We designed an LC-MS gradient and wash cycle that would provide high peak capacity and t_R reproducibility without being prohibitively long since metabolomics typically requires the acquisition of many repeated measurements.

Although there has been a move towards using very short gradient times, other reports show that increasing the gradient time results in increased numbers of detected features and better separation, particularly later in the gradient; therefore we used a 20 min one-step gradient (Guy *et al.*, 2008; Nordström *et al.*, 2006; Want *et al.*, 2006). Schellinger *et al.*, 2005 showed that run-to-run retention time reproducibility can be achieved by using higher flow rates, higher initial solvent strength, and a 2 column volume (*cv*) wash. This very short wash provides an opportunity to reduce overall cycle time. We included a 2 min hold at initial

conditions (2% B) to decrease the heterogeneity effect on early eluting compounds. We increased the number of re-equilibration *cv*s from the suggested 2 to 6 to compensate for our moderate flow rate and initial solvent composition, but remained well below the commonly used 10-15 *cv*s (Schellinger *et al.*, 2005). This short 5 min re-equilibration is less than half our gradient time, facilitating the analysis of more samples per unit time. All solvent extractions were run using our optimized LC-MS parameters.

Principal Components Analysis. While LC-MS chromatograms provided a visually accessible overview of the chemical diversity, there is too much information to fully evaluate the chemical diversity of the extracts by manually examining chromatograms. By performing feature detection and MVA, it is possible to quantify extract chemical diversity, enabling an unbiased comparison among extracts. Each plant species has a unique set of chemicals, in terms of compound types and concentration, and this is reflected in the feature set for each set of extracts. Each feature set is made up of the total number of unique features reproducibly detected in a given set of extracts. Features had to be present in three out of four replicate samples to be included in the final feature set. Certain solvents generated extracts with fewer features than other solvents where the relative intensity of any given feature is zero counts. For example, hexanes extractions typically had more features with an intensity of zero than 70% ethanol extracts (Fig. 2 & 3). This reduction in detected features may be due to the narrower selectivity and poor cell penetration of hexanes resulting in extracts with fewer features. Alternatively, the low number of features observed for non-polar extracts prepared with hexanes or dichloromethane may also be partly attributable to the detection bias of RPLC-ESI-MS, which favors the detection of more polar metabolites.

In general, we found 70% ethanol extracts to have the highest number of features for all plant material; this finding is in contrast to those in a study by Want *et al.*, where methanol extracts of human serum had the highest feature numbers (Want *et al.*, 2006). Methanol has been shown to be very effective at precipitating

proteins, a key factor for animal and human based metabolomics studies. Additionally, it has been shown that esterification reactions in the presence of methanol can degrade polyphenolics, saponins, and lipids in plant extracts (Lindroth and Pajutee *et al.*, 1987; Tava *et al.*, 2003). We acknowledge that isopropanol, a secondary alcohol, would result in fewer artifacts resulting from esterification reactions. However, the longer alkyl chain of ethanol provides a significant decrease in esterification rates over methanol. Using ethanol as an extracting solvent for plants has additional benefits including its low cost and usefulness in USDA certified "organic" food, medicinal, and cosmetic products (Borchardt *et al.*, 2008a). Even with the differences in sample types and optimal extraction solvent the size of the complete feature sets for the plant material evaluated, 3,790 features for *L. salicaria*, 781 features for *M. fistulosa*; and 1,645 for *R. typhina* leaves; 1,378 for berries; and 1,179 for stems were similiar to the 2000 features detected in methanol/acetone extracts of human serum (Want *et al.*, 2006). The variation in the total number of features reflects the chemical variability of the different species and tissue types.

In addition to quantity, qualitative characteristics of features are also an important comparison metric. Visualizing how the features from a particular solvent extract are distributed across chromatographic and mass spectral space provides information about the elution time and mass of detected molecules. The feature set of *L. salicaria,* represented by black dots is well distributed across this space (Fig. 4). The distribution of features detected in each of the subsequent solvent extractions is also plotted. The extracts prepared from methanol, 1:1 dichloromethane:methanol, and 70% ethanol appear to contain features distributed most similarly to the entire feature set indicating that these solvents more completely extract the chemical diversity of the plant material. The feature distribution of extracts prepared with water is heavily weighted towards early retention times and small *m/z*, indicating that smaller, polar molecules were mostly present in these extracts. Conversely, hexanes and dichloromethane extracts have feature distributions that indicate the extraction of well-retained, non-polar molecules. Additionally, these two solvent extracts show a feature rich area

centered around 20 minutes and *m/z* 700 that is largely absent from the other solvent extract feature distributions. Extracts prepared using isopropanol and ethyl acetate show a decreased number of features and an absence of any unique distribution coverage, further supporting the elimination of these two solvents from consideration for maximizing extraction efficiency.

To aid in showing how solvent extracts are globally related to each other, a principal components analysis (PCA) was carried out. PCA plots display information about sample sets including: 1) LC-MS fingerprinting reproducibility through tight clustering of identical extractions, 2) similarity between different solvents via secondary groupings of clusters, and 3) where the secondary groupings form in relation to the principal components (PC) (Fig. 5). Extract clusters located away from the PCA plot origin are enriched in a particular set of metabolites. Predictions can be made about how secondary groupings might form based on prior knowledge of the chemical selectivity of the extraction solvents.

The effect of the solvents on final extract chemical composition was significant, where PC 1 & 2 explained between 31-50% of the variation for all of the sample sets. This level of variation was driven solely by the extraction solvents. The highly reproducible and unique solvent parameters resulted in tight clusters of replicate extracts prepared with a single solvent. A pairwise plot of PC 1 & 2 for the *L. salicaria* sample set shows the distribution of replicate extract clusters forming secondary groups that separate well across both PCs to form three distinct groups (Fig. 5). Group I contains extracts prepared with methanol, 70% ethanol, and water; Group II contains extracts prepared with hexanes, isopropanol, ethyl acetate, and dichloromethane; and extracts prepared with the dichloromethane:methanol mix form an isolated group III. The isolated location of the dichloromethane:methanol extract indicates that it contains unique features that are either not present or at undetectable levels in the other extracts. These secondary grouping trends are common among all sets of extracts from all plants and plant parts. The formation of the secondary groupings is reasonable based on the similarities and differences among the solvents used. The plot provided a quick method to visualize and compare the chemical diversity of the different

solvent extractions. Typically, the next step would be to use the PC loadings plot to identify the main features responsible for the variation in the different extraction conditions (Bowen and Northen, 2010). Here, however, we continued to use the entire feature-set to assess whole extract chemical diversity.

Feature comparison. Our aim was to find a set of solvents used to prepare separate single-solvent extracts in parallel that would approach maximum chemical diversity for any plant material. Metabolomics fingerprinting experiments generate large datasets that approach a thorough characterization of whole extract chemical diversity. An examination of the features from any two separately prepared parallel single-solvent extractions enables a comparison of both unique and shared features between the two (Fig. 6a). Increases in chemical diversity are seen when examining combinations of two parallel extractions that have the tallest overall bar, which corresponds to the greatest total number of features. This bar will also have the smallest gray shaded area, which corresponds to a lower number of shared features. The total number of features and number of shared features differ greatly, depending on which two solvent extractions are compared with one another (Fig. 6a). Two-solvent combinations that include hexanes, ethyl acetate, or isopropanol have fewer total features. Increasing the number of solvent extractions results in a greater total number of features; however, this increase begins to level off when feature sets from three parallel single solvents extracts are compared to each other (Fig. 6b). Although only the analysis of *L. salicaria* extracts is shown, the other extract sets behaved remarkably similarly in that the maximum number of features is approached when three different solvent systems are used to extract one type of plant material.

With the combinations of three parallel solvent extractions it is important to note which ones have the smallest area of shared features. It is these combinations that will maximize chemical diversity while having to perform the least number of parallel extractions. Our results suggest that the most promising three solvent combinations include: 70% ethanol, dichloromethane:methanol, and dichloromethane; 70% ethanol, hexanes, and dichloromethane:methanol;

methanol, hexanes, and water; or 70% ethanol, water, and dichloromethane (Fig. 6b). Each of the resulting extracts fell into separate PCA secondary groupings, indicating that they contain different features (Fig. 5). The inclusion of 70% ethanol in the most efficient combinations is convenient because although flammable, ethanol is relatively safe, readily available, typically of high purity, completely biodegradable, and suitable for use in U.S.D.A. certified organic products (Chemat *et al.*, 2012).

Extract Partitioning. Solvent partitioning has been previously employed for plant extract screening programs (Koehn, 2008). Partitioning separates polar and non-polar compounds to: reduce bioassay interferences, increase relative concentrations of minor compounds to detectable and/or active levels, decrease the prevalence of hydrophobic compounds contaminating chromatography columns, and simplify later compound isolation efforts (Koehn, 2008). Using the same metric to evaluate extract efficiency, initial extractions with water, 70% ethanol, or methanol of the leaf, berry, and stem tissue from *R. typhina* were subjected to solvent partitioning with hexanes followed by dichloromethane. A visual inspection of the extract partitions shows that the aqueous partition from the methanol or 70% ethanol extracts have a similar green color, whereas the water generated extracts appear brown; also notable is the colorless appearance of the hexanes and dichloromethane partitions from an initial water extraction (Fig. 3b).

Feature detection on the extract partitions revealed an interesting trend. When either 70% ethanol or methanol was the initial extracting solvent, hexanes and dichloromethane partition feature numbers surpassed those of the single-solvent extractions with hexanes or dichloromethane (Fig. 7). Moreover, total number of unique features was higher for extract partitions than for single-solvent extraction combinations including hexanes, dichloromethane, and 70% ethanol or methanol. In *R. typhina* leaves, for instance, the number of unique features present in dichloromethane single solvent extraction was 40; the number of unique features in a dichloromethane partition of an initial 70% ethanol extract was 201. Similarly, the number of unique features in a hexanes extraction of *R. typhina* leaves was 25

compared to 70 unique features in the hexanes partition of an initial 70% ethanol extraction. This increase in feature number may be due to enrichment of low abundance metabolites in partitions where they are most soluble, raising their relative concentration to detectable levels (Jones and Kinghorn, 2006; Koehn, 2008). Additionally, compounds subject to ion suppression in the 70% ethanol extract may have ionized better when concentrated in the dichloromethane or hexanes partition resulting in their detection and inclusion as features. Overall, this increased access to low abundance compounds extends the dynamic range of detection methods and biological assays. Generally, this partition advantage was most strongly observed when 70% ethanol was the initial extracting solvent. Although, the total number of features resulting from hexanes and dichloromethane partitioning of an initial extract made with methanol was greater than the total number of features resulting from parallel single-solvent extractions with hexanes, dichloromethane, and methanol, both of these scenarios showed decreased overall feature numbers than when 70% ethanol was replaced by methanol as an extracting solvent. This increase in feature number was the case when 70% ethanol was the initial extraction solvent that was partitioned with hexanes and dichloromethane or if the three solvents were used in parallel single-solvent extractions (Fig. 7). Furthermore, when water was used as the initial extracting solvent, the number of features in both the hexanes and dichloromethane partitions were greatly reduced when compared to the number of features detected from a single-solvent extraction with either organic solvent. These results are similar to previous studies evaluating extracting solvents, which found 100% water to be inferior to methanol or ethanol in total quantity and diversity of compounds in an extract (Johansen *et al.*, 1996; Lapornik *et al.*, 2005; Want *et al.*, 2006). One additional factor to consider is the use of polypropylene tubes for all extractions; if possible glass vials would be preferred as they would reduce potential for artifact formation, particularly with solvent mixtures containing dichloromethane. For consistency in this study polypropylene vials were used for all extractions and we recognize this as a study limitation. However, extracts prepared with dichloromethane did not have significantly increased feature numbers, plasticizers,

or obvious signs of polymeric materials so we are confident that the potential formation of artifacts did not significantly bias the study results.

Although it seems possible to approach maximum chemical diversity by performing three parallel single-solvent extractions, there is an advantage to performing extract partitioning in certain situations. There was a strong increase in chemical diversity (number of features), but not yield, for non-polar solvents (e.g. hexanes, dichloromethane) that were part of a liquid-liquid, partitioning step when an alcohol, but not water, was used to perform the initial extraction (Fig. 7). The partition step, performed on the 70% ethanol extract, sufficiently enriched certain metabolites soluble in particular phases. This concentration effect may translate to detectable biological activity where there may not have been any previously. We generated pairwise plots of PC 1 & 2 for *R. typhina* extraction partitions and single-solvent extracts prepared from hexanes (Fig. 9), dichloromethane (Fig. 10), and water, 70% ethanol, and methanol (Fig 11). In all cases, the single-solvent extract clusters formed different secondary groupings from the extraction partitions indicating that partitioning of an initial extract effectively changes it. For example, hexanes and dichloromethane partitions of 70% ethanol or methanol extracts formed nearly overlapping secondary groupings that were more similar to their respective single-solvent extracts along PC 1, but differed along PC 2 indicating a difference in chemical composition between the single-solvent extract and partitions. (Fig. 9 and 10). When plotted together with the single-solvent extract prepared with water, the 70% ethanol or methanol single-solvent extracts formed an overlapping secondary grouping that was separated from the polar extract cluster, polar partition of a water extract, and polar partitions of 70% ethanol and methanol extracts, which also formed a secondary grouping (Fig. 11).

Conclusions

A complete assessment of extraction efficiency comprises measurements of both extract yield and chemical diversity (Figs. 2 & 3). This study shows that the dynamic range of detectable compounds in a plant extract can be increased significantly by performing solvent partitioning. This increase in detectable

compounds equates to an observed increase in chemical diversity. No single solvent extract can provide a complete feature set (Fig. 4), so maximum chemical diversity requires parallel single-solvent extractions with multiple solvent systems. In general, a good starting point for selecting a solvent is to choose one that contains greater than 50% of the total number of features distributed evenly over the chromatographic and mass spectral space and has greater than 5% yield.

It is important to consider that low chemical diversity solvent extracts (those having smaller feature numbers) may still provide novel detectable bioactivities in some high throughput screens. For example, extracts generated from hexanes showed unique distributions patterns of features indicating the presence of different subsets of chemical entities (Fig. 4). If multiple parallel single-solvent extractions are to be performed, solvents from separate secondary groupings on the PCA plot, or those with unique distributions of features should be used in combination. Performing extract partitioning enhances the relative concentrations of low abundance compounds to detectable and potentially active levels, greatly extending the dynamic range of detection methods and biological assays. Obtaining maximum chemical diversity in a plant extracts is most efficiently approached if solvent partitioning is performed using an extract made with 70% ethanol or a comparable high efficiency solvent system (Fig. 7).

Using metabolomics-generated features provided a way to more globally assess the chemical diversity of plant extracts. A set of extraction parameters has been defined that can be used to build a phytochemical library that will approach a complete sampling of the chemical diversity contained in raw plant material. Metabolomics generated datasets are very large; we were able to make use of this large amount of information to thoroughly characterize the samples, without the need for rigorous metabolite identification. Metabolic fingerprinting combined with feature detection and MVA is a tool for global analysis that has the potential to be applied for quick evaluation of whole botanical extracts in initial chemical screens, aiding in natural product dereplication efforts and/or for quality control.

Acknowledgements

Thank you to the botanicals research team in Estée Lauder R&D for helpful discussions, Stephen Brockman and Mary Musielewicz for technical assistance, and the Minnesota Supercomputing Institute (UMN MSI) at the University of Minnesota for software and computational support. This work was funded by the NSF Plant Genome Research Program grants IOS-0923960 and IOS-1238812, the NSF Graduate Research Fellowship Program (00006595), and the UNCF/Merck Science Initiative.

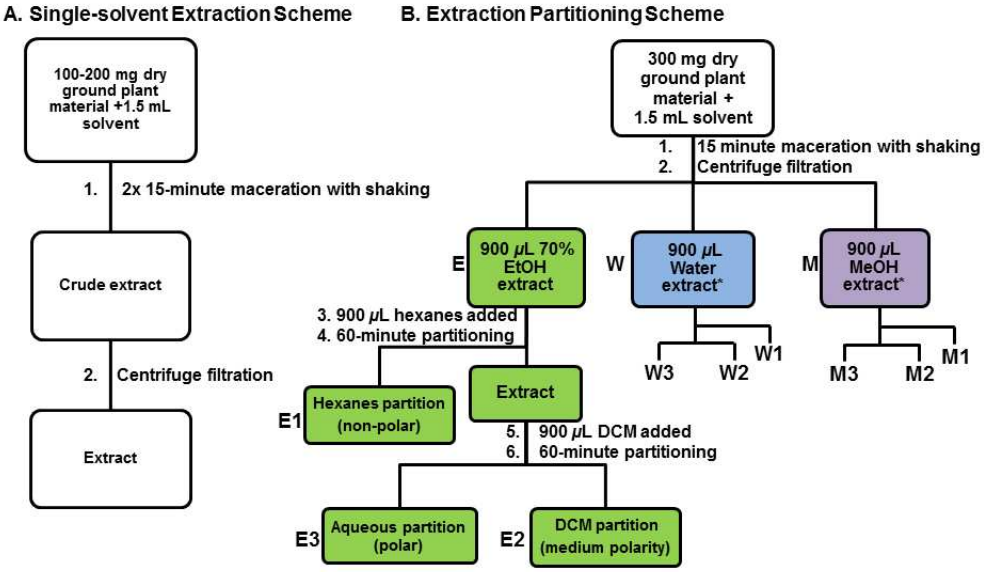


Figure 1. Schematic of workflows for A) parallel single-solvent extractions and B) extraction partitioning. Single solvent extractions with hexanes, dichloromethane, ethyl acetate, methanol, isopropanol, water, aqueous ethanol (ethanol: water, 70:30 v/v), or a dichloromethane/methanol mix (dichloromethane:methanol, 1:1 v/v) follow the simple linear workflow shown in A. For the more complicated extract partitioning workflow shown in B, 300 mg of dry plant material was extracted with 1.5 mL of either 70% ethanol in water (E, green), pure water (W, blue), or pure methanol (M, violet). Each of these extracts was then partitioned against an equal volume of first hexanes (E1, W1, M1), and then DCM (E2, W2, M2), which leaves a residual polar alcohol or aqueous phase (E3, W3, M3). The complete schematic is illustrated in detail for the 70% ethanol in water extract partitioning (green boxes) and is abbreviated for the water (blue) and methanol (violet) extracts.

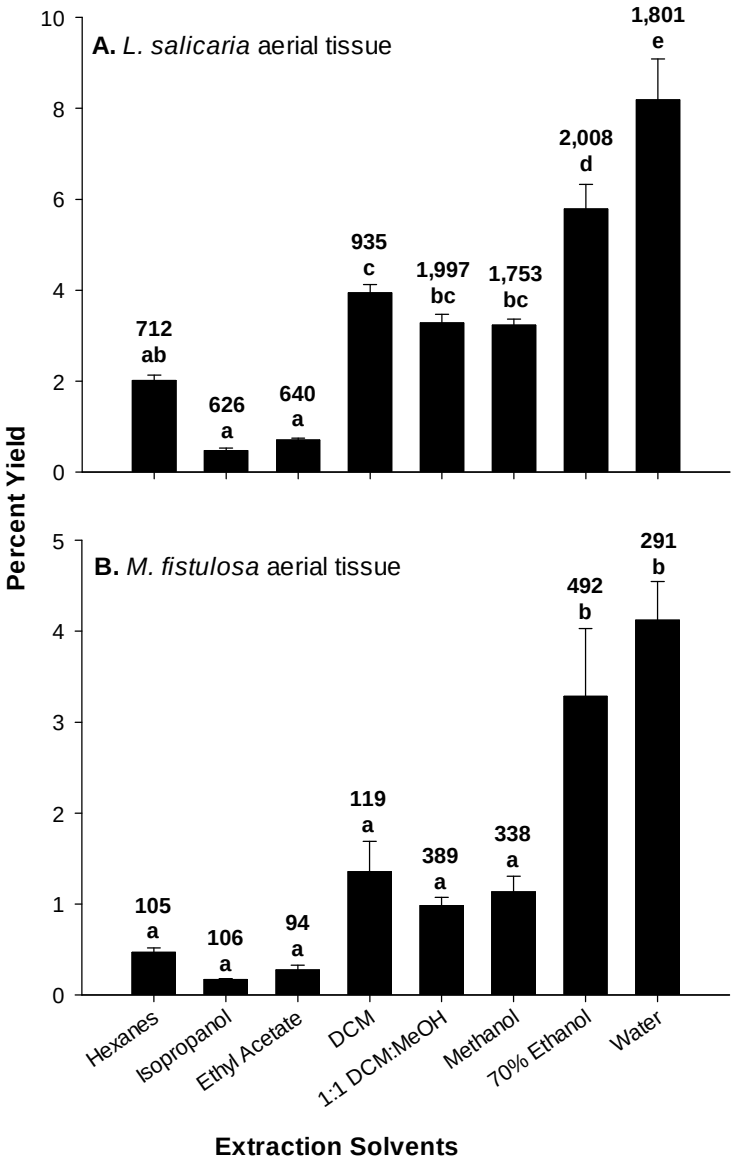


Figure 2. Comparison of extraction efficiency for extracts prepared from 8 parallel single-solvent extractions. Aerial tissue from A) *L. salicaria* and B) *M. fistulosa* was used. Bars show the average percent yield of 4 replicate extractions, as related to the initial dry weight of plant material used, the error bars represent standard error. Letter superscripts indicate statistically different groups at a *p*-value ≤ 0.01 according to Tukey's HSD test. The number of features detected from metabolic fingerprints of the different solvent extractions is displayed above the letter superscripts.

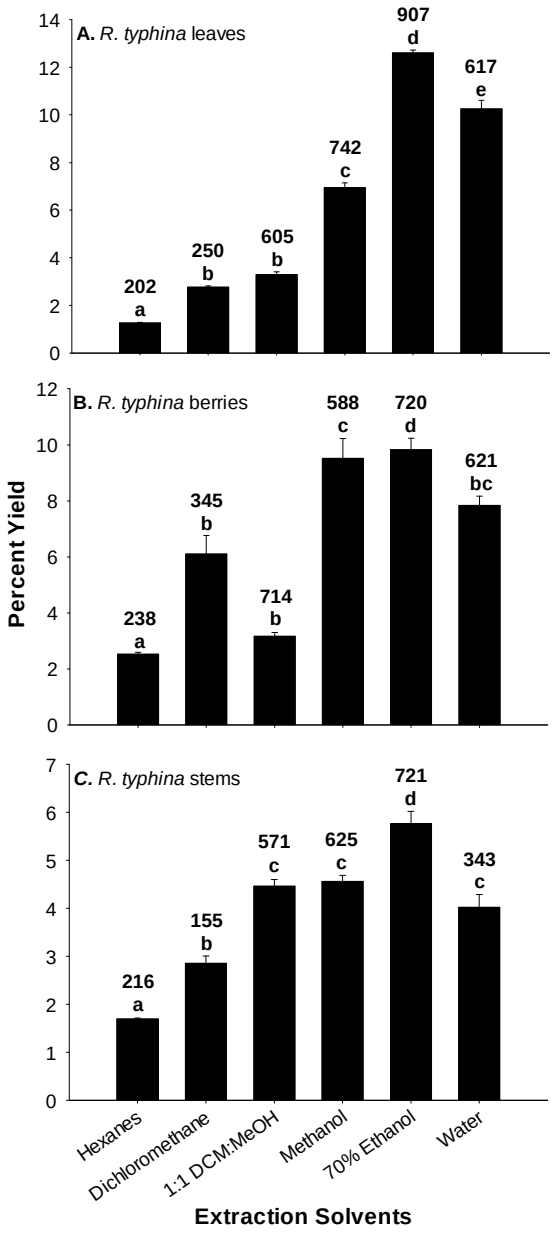


Figure 3. Comparison of extraction efficiency for extracts prepared from 6 parallel single-solvent extractions. *Rhus typhina* A) leaf, B) berry, and C) stem tissue was used. Bars show the average percent yield of 4 replicate extractions, as related to the initial dry weight of plant material used, the error bars represent standard error. Letter superscripts indicate statistically different groups at a *p*-value ≤ 0.01 according to Tukey's HSD test. The number of features detected from metabolic fingerprints of the different solvent extractions is displayed above the letter superscripts.

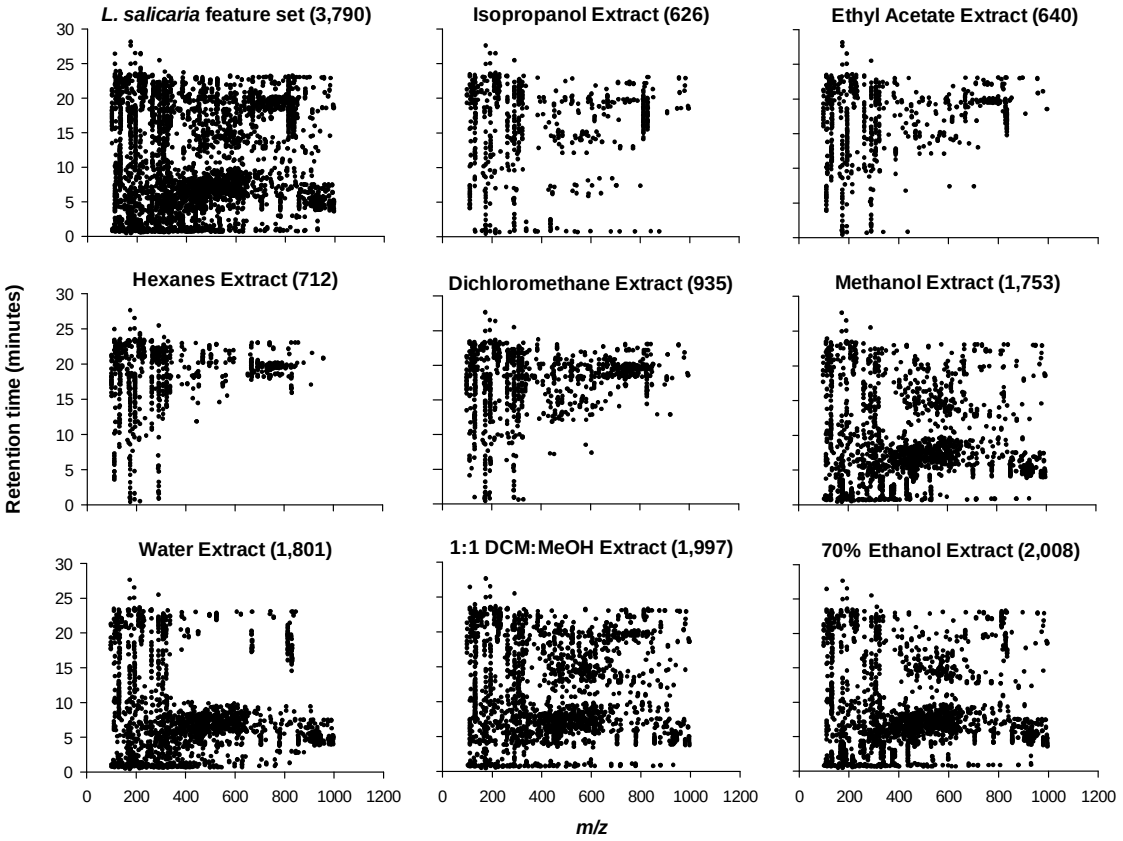


Figure 4. Scatterplots showing the distribution of features detected from metabolic fingerprints of extracts. *L. salicaria* aerial tissue was used to prepare 8 parallel single-solvent extractions. Individual features are represented by black dots and the total number of features detected from each solvent extract is listed parenthetically. This feature set consists of 3,790 features in all.

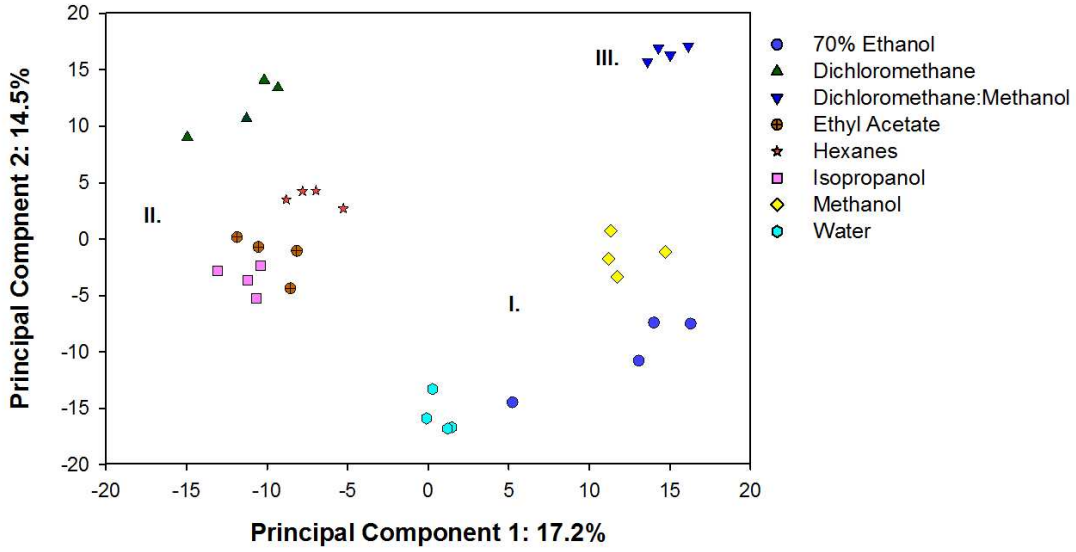


Figure 5. Representative principal components analysis of L. salicaria parallel single-solvent extractions. Principal component (PC) 1 and 2 are shown and together account for 31.7% of the variation in this sample set. Additional PCs 3, 4, 5, and 6 explain 9.6%, 6.6%, 5.0% and 4.1% of the variation, respectively. No single principal component is able to capture a large amount of the variation of the whole set of extraction solvents because of the significant differences between each of solvents individually. Each solvent system has a large amount of highly reproducible unique parameters resulting in the tight cluster groups of replicate extracts produced with a single solvent; however the variation of the sample set as a whole cannot be easily mapped into a single PC. Over 50% of the variance is explained by the first 5 PCs. Secondary groupings I, II, and III are composed of replicate extract clusters that are similar to each other.

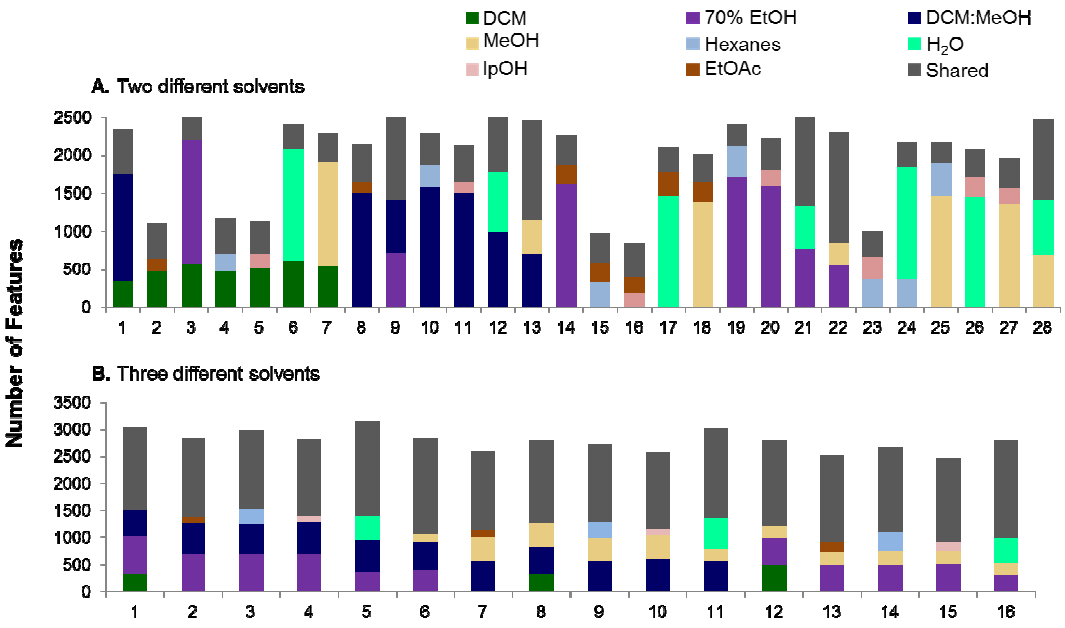


Figure 6. Comparison of extraction efficiency via chemical complexity for combinations of two and three parallel single-solvent extractions. The total numbers of unique observed features from *L. salicaria* aerial tissue single solvent extractions are shown for combinations of two different solvents (in A) and three different solvents (in B). The total numbers of features unique to a particular combination of solvent extractions are shown by the heights of each bar. The fraction of features uniquely found in a single solvent from each combination is shown by each colored bar segment (shaded according to the key). The fraction found in two (or more) solvents in each combination is indicated by the gray segment of each bar. All possible pairwise comparisons of the eight solvents are shown in A.

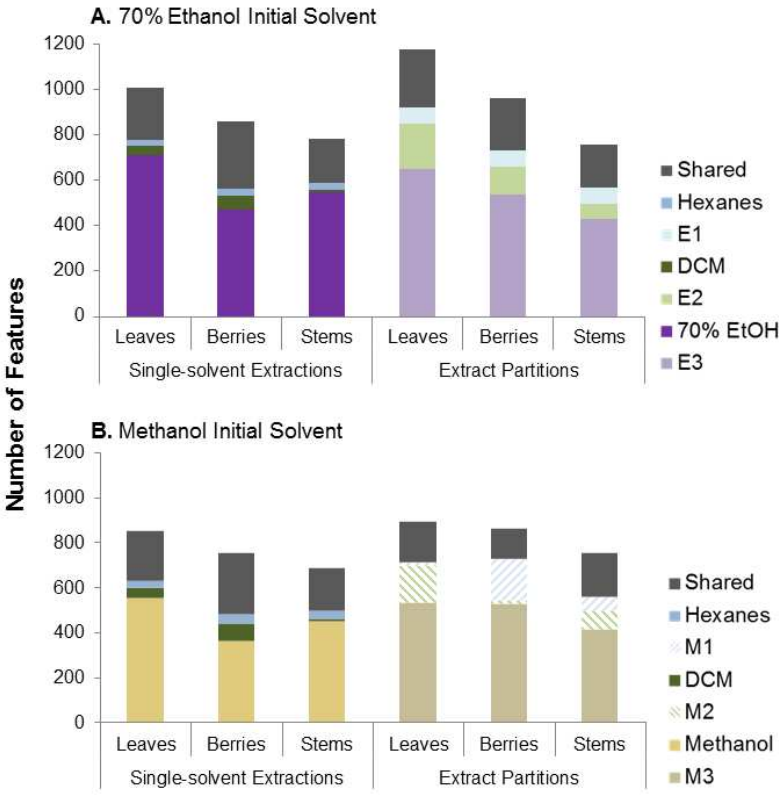
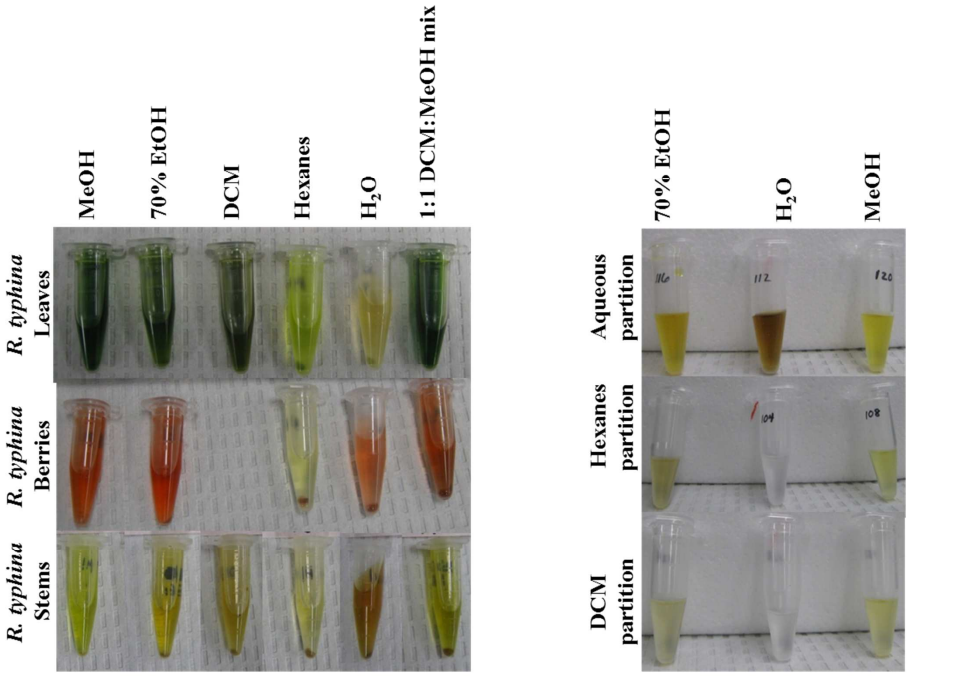


Figure 7. Comparison of extraction efficiency via chemical complexity of combinations of three parallel single-solvent extractions and extraction partitions of *R. typhina* leaf, berry, and stem tissue. The total numbers of features unique to a particular combination of solvent extractions are shown by the heights of each bar. The fraction of features uniquely found in a single solvent or partition from each combination is shown by each colored bar segment (shaded according to the key). The fraction found in two (or more) solvents or partitions in each combination is indicated by the dark gray segment of each bar. The chemical complexity of three independent single-solvent extractions using 70% ethanol, hexanes, and dichloromethane is directly compared against solvent partitions generated from an initial extracting solvent of 70% ethanol followed by partitioning with hexanes and dichloromethane to generate partitions E1 (hexanes), E2 (dichloromethane), and E3 (polar alcohol) partitions (in A). The same comparison is shown in B, where methanol replaces 70% ethanol.



A. *R. typhina* single solvent extractions **B.** *R. typhina* stems partitioning

Figure 8. Photographs of representative extractions. Panel A) single solvent extractions of *R. typhina* tissue from top to bottom: leaves, berries, and stems. Solvent used for the extraction is listed on top. Panel B) representative partitions from *R. typhina* stem tissue. Both leaf and berry tissue looked very similar, with the exception of berry extracts being red rather than green.

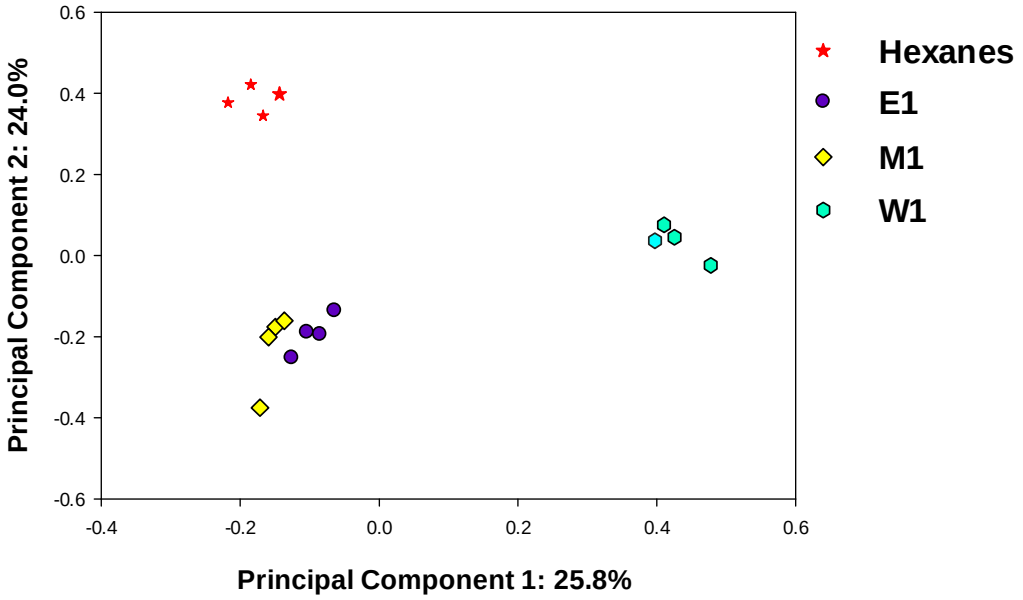


Figure 9. Representative principal components analysis of *R. typhina* berry extraction partitions and hexanes single-solvent extraction. The percent of variation explained by each principal component is shown along the appropriate axis. The data are labeled such that they follow the extraction workflow labels described in Fig. 1 where E1, M1, and W1 are the non-polar hexanes partition from an initial extract made with 70% ethanol, methanol, and water, respectively.

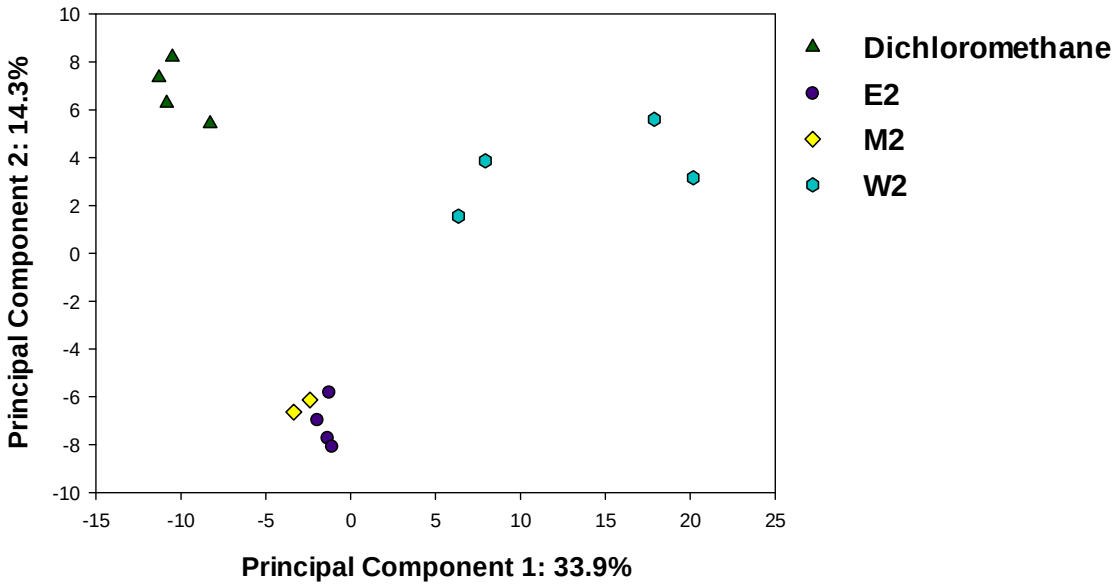


Figure 10. Representative principal components analysis of *R. typhina* berry extraction partitions and dichloromethane single-solvent extraction. The percent of variation explained by each principal component is shown along the appropriate axis. The data are labeled such that they follow the extraction workflow labels described in Fig. 1 where E2, M2, and W2 are the medium polarity dichloromethane partition from an initial extract made with 70% ethanol, methanol, and water, respectively.

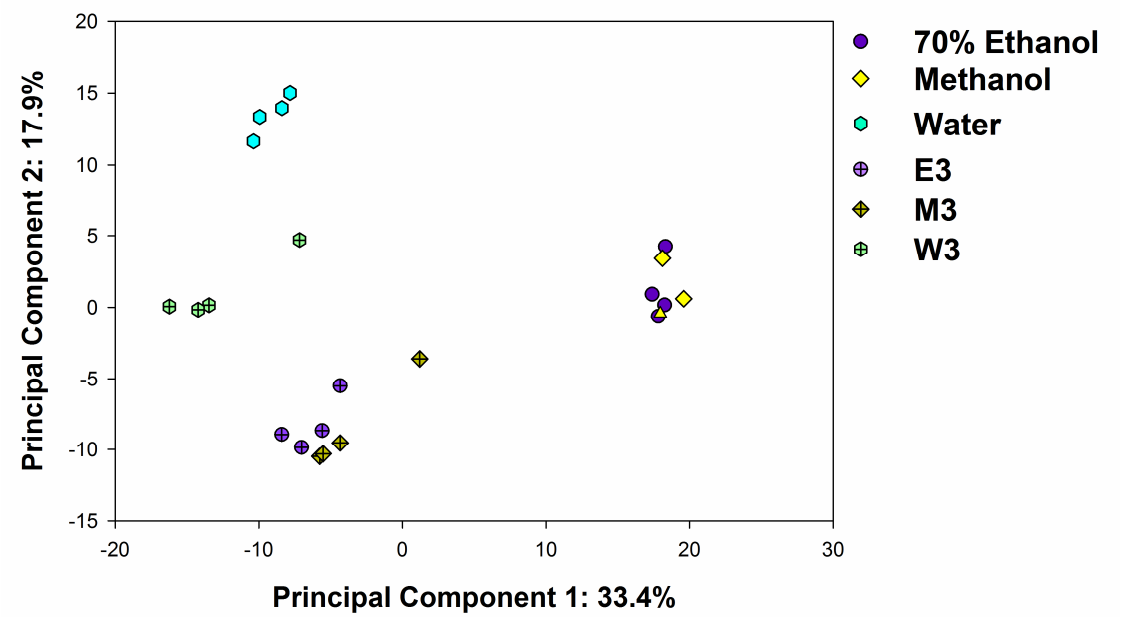


Figure 11. Representative principal components analysis of *R. typhina* berry extraction partitions and 70% ethanol, methanol, and water single-solvent extraction. The percent of variation explained by each principal component is shown along the appropriate axis. The data are labeled such that they follow the extraction workflow labels described in Fig. 1 where E3, M3, and W3 are the residual polar alcohol or aqueous phase after partitioning with hexanes and dichloromethane from an initial extract made with 70% ethanol, methanol, and water, respectively.

Chapter 3

Measuring the chemical and cytotoxic variability of commercially available kava (*Piper methysticum* G. Forster)[2]

Summary

Formerly used world-wide as a popular botanical medicine to reduce anxiety, reports of hepatotoxicity linked to consuming kava extracts in the late 1990s have resulted in global restrictions on kava use and have hindered kava-related research. Despite its presence on the United States Food and Drug Administration consumer advisory list for the past decade, export data from kava producing countries implies that US kava imports, which are not publicly reported, are both increasing and of a fairly high volume. We have measured the variability in extract chemical composition and cytotoxicity towards human lung adenocarcinoma A549 cancer cells of 25 commercially available kava products. Results reveal a high level of variation in chemical content and cytotoxicity of currently available kava products. As public interest and use of kava products continues to increase in the United States, efforts to characterize products and expedite research of this potentially useful botanical medicine are necessary.

Introduction

Kava (*Piper methysticum* G. Forster) is the name of a plant and drink that is prepared traditionally by macerating its roots in cool water or coconut water (Johnston and Rogers, 2006). It has been used for many centuries in the South Pacific and Hawaii for social ceremonies, relaxation, medicine, and a multitude of other purposes (Johnston and Rogers, 2006). More recently, standardized kava extracts, containing 30% active constituents, have been used globally as an anxiolytic (He et al., 1997; Sarris et al., 2011). Additionally, a tight inverse correlation between high rates of kava consumption and low incidences of cancer for populations in the South Pacific has been reported

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ACM contibutions included: Designing research study, performing research, analyzing data, generating tables and figures, and writing the paper.

(Steiner, 2000). Subsequent studies have shown that kava displays cancer preventive properties (Johnson et al., 2008; Johnson et al., 2011; Leitzman et al., 2014; Zi and Simoneau 2005).

There are about 200 different cultivated varieties of kava (Teschke et al., 2011a), each with a unique chemotype that produces specific physiological and psychoactive effects (Dinh et al., 2001; Jokhan et al., 2004; Lebot and Siméoni, 2004; Lebot et al., 1999). The active constituents are chemically classified as kavalactones and six (kawain, dihydrokawain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin) constitute the primary chemicals that are responsible for individual cultivars' unique chemotypes (Dasgupta and Hammett-Stabler, 2011; Johnston and Rogers, 2006; Shao et al., 1998; Siméoni and Lebot, 2002).

A 2002 the Kava Act passed in Vanuatu established four classes of kava cultivars: noble, which have a long history of safe use as traditional drink; medicinal, which have long been used by traditional herbalists in the South Pacific and are banned as export commodities; 'Tu dei', which have a very strong effect that lasts two days; and 'Wichmanni' or wild varieties (Lebot et al., 2014; Teschke et al., 2011a & b). Cultivars from the noble class are typically used to prepare kava extract as they have the optimal therapeutic chemotype. Cultivars belonging to other classes have been reported to have overpowering and unpredictable effects causing symptoms such as nausea and headaches (Lebot, 2006; Teschke et al., 2009). Kava's active constituents are primarily located in its roots; other plant parts such as stems and leaves should not be used in extract preparations (Johnston and Rogers, 2006). Traditional kava is prepared using a 100% aqueous solvent, which results in a drink containing an average of 0.3-20% kavalactone content (Johnston and Rogers, 2006). Commercial manufacturers use up to 100% ethanol or acetone in the extraction process resulting in up to 70% kavalactone content in the final product (Johnston and Rogers, 2006; Teschke et al., 2009). Studies have shown a difference between traditionally prepared extracts and those prepared with ethanol both in cytotoxicity and chemical composition (Jhoo et al., 2006; Johansen et al., 1996; Lapornik et al., 2005; Shaik et al., 2009; Zhou et al., 2010). In addition to those described above, other potential sources of variation in kava products include, contamination of raw kava materials, impurities, post-harvest handling and storage procedures (drying, whole vs.

ground material, humidity, temperature), age of harvested kava plants, mixture and quality of cultivar(s) used (Dasgupta et al., 2011; Teschke et al., 2003; Teschke et al., 2011b, c & d; Teschke and Lebot, 2011; U.S. Food and Drug Administration, 2002; Zhang et al., 2011). Kava is distributed in variable forms, including dry powder, freeze-dried, liquid tincture, and capsule, making it difficult to know exactly which cultivar(s), plant part(s), extraction solvent(s), and other factors were used in the preparation (Teschke et al., 2011c).

Due to reports linking modern kava consumption to individual cases of hepatotoxicity, kava was banned in the European Union and Canada in 2003, voluntarily recalled in Australia in 2003, and included on the United States Food and Drug Administration (US FDA) consumer advisory list in March of 2002 (Dasgupta et al., 2011; Teschke et al., 2003; Teschke et al., 2011; U.S. Food and Drug Administration, 2002; Zhang et al., 2011). These bans and advisories have hindered research on kava as an alternative anti-anxiety and cancer preventive medicine (Teschke et al., 2011b). Despite its presence on the US FDA consumer advisory list for the past decade, the extrapolation of export data from the kava producing nations Fiji, The Republic of Vanuatu, and Tonga to the US (Table 1) indicates that kava imports to the US, which are not publicly reported, are presumably both increasing and of a fairly high volume.

There are many hypothesized mechanisms potentially linking kava consumption to hepatotoxicity (Dasgupta and Hammett-Sabler, 2011; Sarris et al., 2011; Zhou et al., 2010; Teschke, 2011c; Zhang et al., 2011; Johnson et al., 2003; Whitton et al., 2003; Olsen et al., 2011; Anke and Ramzan, 2004; Behl et al., 2011; Zou et al., 2004; Yang and Salminen, 2011). We intended to measure the overall variation in cellular toxicity and chemical composition among the large volume of diverse kava products currently available. Only six kavalactones have been intensively studied (He et al., 1997; Lebot et al., 2014; Meissmer and Haberlein, 2005; Shao et al., 1998; Siméoni and Lebot, 2002; Smith et al., 1984) making it necessary to assess the complete pool of extracted compounds. We performed metabolic fingerprinting; a metabolomics technique that facilitates comparisons based on global metabolite patterns of whole extracts (Hegeman, 2010). We used ultra-performance liquid chromatography-electrospray ionization-time-of-flight-mass spectrometry (UPLC-ESI-TOF-MS) to fingerprint replicate aqueous and

95% ethanolic extracts of 25 commercial kava products (Table S1). We also quantified six compounds found in kava that may be associated with either the medicinal or negative cytotoxic effects of modern kava usage: kawain (K); dihydrokawain (DHK); methysticin (M); dihydromethysticin (DHM); flavokawain A (FLK A); and flavokawain B (FLK B) (Shaik et al., 2009; Johnson et al., 2003; Whitton et al., 2003; Olsen et al., 2011) (Figure 1). Absolute quantification was performed using pure standards and a UPLC-single quadrupole mass spectrometer (MS). Finally, we determined the cytotoxicity levels of each extract in cell viability assays towards human lung adenocarcinoma A549 cancer cell line.

Materials and Methods

Solvents and reagents. HPLC grade solvents from Sigma Aldrich (St. Louis, MO, USA) were used including: acetonitrile, dimethyl sulfoxide (DMSO), formic acid, ethyl acetate, 95% ethanol, and hexanes. Reverse osmosis deionized glass distilled water was obtained in house using a Thermo Scientific Barnstead B-pure™ filter and Distinction water still model D4000 (Bibby Scientific Limited, Stone, Staffordshire ST15 0SA, UK). Standard kava compounds were purified from Gaia Herb (Brevard, NC, USA) commercial Kava extract. Commercial kava samples were obtained from a variety of sources (Table 2).

Kava extraction. Kava samples from 25 different sources were classified as either powder (P) or liquid (L) (Table S1). Four extraction methods were used; methods I and II for powder samples and methods III and IV for liquid samples. The method details are as follows: Method I: 10 mL of room temperature water was added to 5 grams of powdered kava, shaken for 2 hrs, centrifuged to remove insoluble material and the supernatant evaporated to dryness and re-dissolved in water at a concentration of 1.5 mg of residue per mL. Method II: the same as I, except 95% ethanol was used in place of water. Method III: 200 µL of liquid kava sample was dried in vacuo, reconstituted in 500 µL of water and then adjusted to a concentration of 1.5 mg of residue per mL with additional water. Method IV: samples were directly diluted to 1.5 mg/mL with 95% ethanol. For each extraction method four replicates per sample were prepared for

analysis. Extract yield was determined gravimetrically by evaporating 500 µL of extract to dryness using a Savant model SVC-200H SpeedVac concentrator (Farmingdale, NY, USA). Extracts were normalized to 1.5 mg/mL for experiments and stored at 4ºC in the dark for no more than a week prior to LC/MS analysis. All extractions were carried out at room temperature (approximately 25ºC).

Metabolic fingerprinting. Metabolic fingerprints were generated using C18-reversed-phase ultra-performance liquid chromatography-positive electrospray ionization-time-of-flight mass spectrometry (UPLC-ESI(+)-TOF-MS) carried out on a UPLC-TOF LCT Premier XE mass spectrometer fitted with an autosampler with a sample vial block maintained at 4ºC (Acquity, Waters, Milford MA, USA). The following MS conditions were used: full scan mass scan range: 100-1000 m/z, W analyzer mode, extended dynamic range, 0.1 s scan time, desolvation temperature 350°C, desolvation nitrogen flow rate: 7.0 L/min, capillary voltage: 2900 V, sample cone voltage: 30 V, source temperature: 120°C. Separations were carried out on a reversed-phase C18 HSS T3 1.8 µm particle size, 2.1x100 mm column (Waters). Column temperature was 50°C, solvent flow rate 0.3 mL/min, injection volume 5 µL. A 14-minute gradient using mobile phases A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile was run according to the following gradient elution profile: initial, 10%; 3 minutes, 50% B; 8 minutes, 60% B; 13 minutes, 98% B; 14 minutes, 98% B. A 7-minute wash cycle was run between every sample and monitored for the absence of carryover. MassLynx version 4.1 (Waters) was used for data collection and visualization. Sample analysis order was randomized across the entire sample set.

Feature detection and multivariate statistical analysis. LC-MS files were processed using MarkerLynx version 4.1 software (Waters) for feature detection using the following parameters: mass tolerance: 0.01 Da; peak width at 5% height: 0.2 s; intensity threshold: 2000 counts; mass window: 0.05 Da; retention time window: 0.20 s. Following feature detection the feature lists were imported into Analyst version 7.5 software (Genedata, Lexington, MA, USA). Feature lists were inspected and a feature was considered to be real if it was present in greater than 75% of replicate samples with similar intensity in all

replicates. Once highly confident feature lists were obtained, principal components analysis (PCA) was performed.

Absolute quantification. Absolute quantification was performed using a UPLC-single quadrupole mass spectrometer (Waters). Independent standard curves were generated for six compounds (K, DHK, M, DHM, FLKA, and FLKB) found in kava. Mixtures of pure standards were made in seven concentrations from 0.05 ppm to 100 ppm; four technical replicates were completed to account for chromatographic drift and ionization variability. After LC method optimization, standard curves were generated in selected ion recording (SIR) mode with the following retention time windows: 0-3.5 min: scan 220-700 m/z; 3.5-6.5 min: 275±2 m/z; 3.5-6.5 min: 277±2 m/z; 4.0-7.0 min: 231±2; 4.5-7.5 min: 233±2 m/z; 7.5-8.0 min scan 220-700 m/z; 8-11 min: 315±2 m/z; 9-12: 285±2 m/z; 12.0-14.0 min: scan 220-700 m/z. A cone voltage of 40 V was used to disfavor non-covalent compound dimerization in ESI+ mode. The liquid chromatography and column parameters are identical to those used for the metabolic fingerprinting. Standard curves were linear up to 50 ppm. Samples were analyzed using the same LC-MS method with three or four replicates in most cases, although for six samples (N, IV; X, III; Y, IV; BB, III&IV; CC, II) and four samples (J, IV; M, IV; O, IV; X, IV) only two or one replicates were suitable for the final quantification, respectively. The limit of detection was set at concentrations corresponding to a signal-to-noise ratio of 3 to 1; peaks occurring below this threshold are not detected (ND). The limit of quantification was set at a signal-to-noise ratio of 10 to 1. The MassLynxTM application manager QuanLynxTM (Waters) was used to assist with automatic integration and of this large dataset. All integrations were visually inspected and manually adjusted to ensure consistent and accurate quantification.

Kava fractionation and characterization. Fractionation of commercial kava purchased from Gaia Herb (Brevard, NC, USA) was performed as described previously (Leitzman et al., 2014) with normal phase silica gel chromatography generating three modalities – fraction A (hydrophilic), B (medium polarity), and C (lipophilic). Briefly, 300 mL, net weight, of kava residue was mixed with silica gel (300 g). Ethanol and water were removed by vacuum. This silica gel with adsorbed kava residue was subjected to coarse

chromatographic separation using a 750-gram pre-packed silica gel cartridge. The elution method was 28% ethyl acetate and 72% hexane 5 column volumes, followed by 90% ethyl acetate and 10% hexane, 4.1 column volumes, and then 35% methanol and 65% ethyl acetate, 5.5 column volumes. Different eluents were analyzed by TLC and the desired eluents were combined with solvent removed to generate fractions A, B, and C. The fractionation process was automated and monitored by the Biotag Separation System. Each individual fraction was analyzed by 1H-NMR and HPLC to confirm the success of fractionation.

Cytotoxicity test. Cytotoxicity tests were performed on fractions A, B, & C and different kava extracts. Extracts were dried and reconstituted in DMSO at a concentration of 10 mg/mL. From these stock solutions working solutions of 150, 75, and 37.5 µg/mL were prepared from each extract. Their cytotoxicity against human lung adenocarcinoma A549 cancer cells (American Type Culture Collection CCL-185) were evaluated by following our established procedures (Warmka et al., 2012). Briefly, A549 cells were plated in a 96-well plate (2.5 × 103 cells/well). The cells were treated with kava extracts with 0.5% DMSO in the final cell media (cells treated with media containing 0.5% DMSO served as a control). After 48 h of treatment, the relative cell viability in each well was determined by using CellTiter-Blue cell viability assay kit (Promega, CA). Two biological repeats with three replicates per experiment were performed.

Results and Discussion

Metabolic fingerprinting experiments measured three aspects of chemical variation: reproducibility of replicate extractions of individual products; differences between using 100% water or 95% ethanol as the extraction solvent; and the overall variation among the set of kava products tested. Similarly to previous quantitative studies of compounds from kava, we observed a high level of reproducibility of replicate extractions of material from individual kava sources (Meissmer and Haberlein, 2005). Principal component analysis showed that replicate extractions from the same kava source are tightly clustered (Figure S1). Moreover, there were small standard errors (average standard error 12.6%) from the absolute quantification measurements of K,

DHK, M, DHM, FLKA, and FLKB (Tables S2 and S3). These results provide evidence that there is consistency in the material contained within a single batch of kava from any given source.

Extract chemical composition was strongly influenced by extraction solvent. Metabolic fingerprints from aqueous and ethanolic extracts plotted in principal component space formed two distinct groups driven by extraction solvent where the use of either 100% water or 95% ethanol was responsible for 71.1% of the variation among all samples explained by PC1 (Figure S2). The detected ion m/z, retention time pairs that contribute the most to the loadings for PC1 were 315.1132 m/z, 9.1823 min and 285.1021 m/z, 9.4699 min, which correspond to the masses and retention times of FLKA and FLKB, respectively. Compound quantification showed that extracts prepared with 95% ethanol resulted in higher yields and greater consistency among replicates, compared with extracts prepared with 100% water. This result is similar to previous studies that found water produced kava extracts with decreased compound concentrations compared to extracts prepared with ethanol (Johansen et al., 1996; Lapornik et al., 2005). Specifically, K, DHK, M, and DHM concentrations were 1.5-5x higher in samples extracted with 95% ethanol than in those extracted with 100% water. The concentrations of FLKA & FLKB were up to fifty times higher in samples extracted by 95% ethanol than in those extracted with 100% water although a significant number of the water extracts contained concentrations of FLKA or FLKB that were below detectable limits (Figure 1). Extracts prepared with 95% ethanol consistently contained greater quantities of FLKA and FLKB than corresponding water extracts, and were highly variable across kava products (ranging from undetectable concentrations up to 14.7 ppm; Figure 1).

While extraction solvent was the most influential variable affecting the observed chemical composition, significant variation in the concentrations of K, DHK, M, and DHM for identically prepared extracts was observed from different source materials. This variation was even more dramatic in regard to the concentration of FLKA and FLKB. The variation in chemical composition was further reflected by the differences in cytotoxicity observed for each commercial kava product extract.

Cytotoxicity assays against human lung adenocarcinoma A549 cancer cell line with aqueous extracts from all 25 commercial kavas showed no toxicity at any

concentration measured up to 500 µg/mL. This result is similar to previous studies indicating that aqueous extracts have low to no cytotoxic effect (Teschke et al., 2009). In contrast, identically prepared ethanol extracts from different commercial sources varied greatly in their relative cytotoxicity at all concentrations measured 37.5, 75 (shown in Figure 2 top), and 150 µg/mL. Ethanol extracts prepared from commercial kava sources K, M, N, O, W, Y, DD, and EE exhibited very low cell toxicity at all concentrations, indicated by a relative cell viability level of greater than 90%. In contrast, ethanolic extracts from G, H, P, Q, R, S, V, Z, and BB, displayed the highest levels of toxicity, with a relative cell viability level of less than 30%. Cytotoxicity levels at these three discreet extract concentrations varied over a wide range similarly to the variation observed in extract chemical composition, especially in regard to FLKA and FLKB.

We observed a moderate correlation between the concentrations of FLKA & FLKB (log2 normalized) and the relative cytotoxicity across the sampled kava products. High concentrations of the flavokawains generally mirrored lower relative cell viability (Figure 2). Some samples, however, deviated from this correlation, specifically N and BB, which have medium concentrations of FLKA and FLKB and display low and high cytotoxcity, respectively. Scatterplots of these data revealed that concentrations of FLKA and FLKB correlated similarly to cytotoxicity level with R2 values equal to 0.68 and 0.69, respectively for extracts prepared at 75 µg/mL and 0.78 and 0.77, respectively for extracts prepared at 150 µg/mL (Figures S3 and S4). Based on these correlation values, clearly, FLKA and FLKB are likely the major but not the solely compounds responsible for the extract toxicity. However, additional reports of flavokawain toxicity, including measured IC50 levels for FLKA and FLKB of 13 ± 1.1 and 6.6 ± 0.1 ppm, respectively against Hepa 1c1c7 liver cells (Shaik et al., 2009), and 57% growth inhibition of bladder T24 tumor cells by FLKA (Zi and Simoneau, 2005) warrant further exploration of the link between flavokawains and kava's cytotoxicity (Jhoo et al., 2006; Johansen et al., 1996; Zhou et al., 2010).

We also generated a non-polar flavokawain enriched kava fraction to determine the IC50 values of 48-hour toxicities against hepatocytes from mouse, rat, and monkey. We measured IC50 values for this FLK rich fraction of 57 ± 9, 45 ± 4 and 49 ± 6 µg/mL, for mouse, rat, and monkey hepatocytes, respectively. In contrast polar and medium

polarity fractions and whole traditionally prepared kava had non-detectable IC50 values greater than 400 µg/mL in all three cell types.

For each compound, K, DHK, M, and DHM there was no obvious association between concentration and relative cell viability, although extracts with higher overall concentrations of all six compounds resulted in lower relative cell viability. This trend suggests that K, DHK, M, and DHM are less likely to be involved in the specific mechanism(s) of cytotoxicity.

Additional reports of cytotoxic compounds found in kava describe one additional flavokawain, distinct from FLKA and FLKB with the following chemical formula and exact mass C17H16O5, 300.0998 (FLK C) (Olsen et al., 2011) and three alkaloids found in kava leaves with the following chemical formulae and exact masses: C14H17NO2, 231.1259 (awaine); C16H17NO4, 287.1157 (pipermethystine); C16H17NO5, 303.1106 (3α,4α-epoxy-5β-pipermethystine) (Dragull et al., 2003). These alkaloids may have been present in commercial kava products produced by European companies leading up to the European ban of kava in 2003 (Lebot et al., 2014). We looked for patterns between cytotoxicity level and the presence of these potentially toxic compounds using M+H extracted ion chromatograms. Overall, only a peak corresponding to FLKC was detectable above the limit of detection (s/n > 3), where the relative intensity of this peak was higher in extracts with higher toxicities. Additional experiments are necessary to understand how these compounds interact with FLKA and FLKB to produce extract cytotoxicity (Lebot et al., 2014; Teschke and Lebot, 2011; Teschke et al., 2011b & c). Regardless of the precise cause of cytotoxicity it is clear that tremendous variation exists in the chemical composition and resulting toxicity of commercially available kava products.

Conclusions

Kava export data show that in spite of bans and warnings, consumption of unregulated kava products appears to be increasing. Our analysis shows that the assortment of commercially available kava products varies widely in chemical composition and cytotoxicity level. Certain kava cultivars and preparation methods may produce products that vary broadly in both their toxicity and their efficacy and thus a rapid and easily

applied method to characterize and classify kava products would be beneficial to the consumer. Disregarding kava and its potential use as an anxiolytic or for cancer preventive ignores the great potential societal benefits of the rational and informed medicinal use of this plant.

Acknowledgments

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Table 1. Kava exports from Fiji, Tonga and Vanuatu: 2008 through 2013[a]

	Total metric tons exported (subset exp. to US)		
Year	from Fiji[b]	from Tonga[c]	from Vanuatu[d]
2008	184 (93)	27[e]	356[e]
2009	212 (123)	38.9 (0.7)	485[e]
2010	244 (91)	61.6 (29.2)	498
2011	276 (95)[f]	68.6 (42)	734
2012	NA	117 (80)	643
2013	NA	NA	558[g]

[a]Kava exports are reported in metric tons where available from 2008 through 2013. The subset of exports to the United States is given parenthetically next to each total export figure where available. NA indicates that the data were not available for that year from the sources cited.

[b] Fiji Bureau of Statistics, 2013

[c] Tonga Statistics Department, 2008-2012

[d] Vanuatu National Statistics Office, 2013

[e] Discussion paper on the development of a standard for kava products, 2012

[f]Represents exports for January through November of 2011.

[g]Represents exports for January through August 2013.

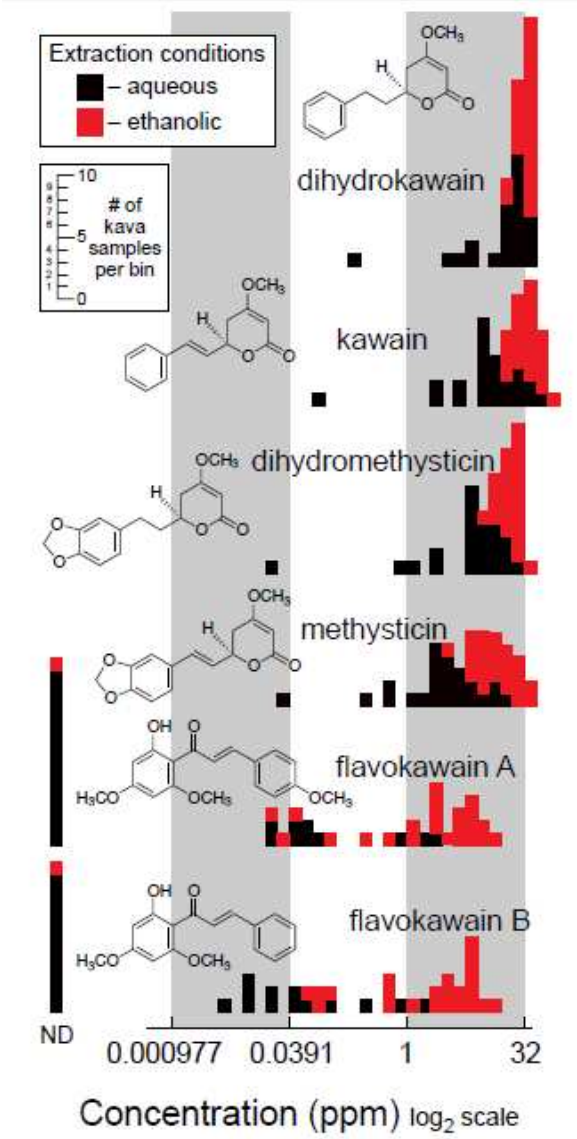

Figure 1. Histograms showing the distribution of concentrations of compounds found in commercial kava preparations. Kava samples were extracted with both 100% water (black) and 95% ethanol (red). The six compounds shown were quantified in each extract by LC-MS and the resulting concentrations in part per million are displayed histogramatically. Distributions were normalized by display on a $\log_2$ scale. The inset y-axis scale indicates the numbers of kava samples in each bin. Measurements designated as not detected (ND) were below the limits of detection (LOD ($s/n < 3$) for each analysis, which were typically 0.0005 to 0.001 ppm depending on variation in signal to noise from sample to sample.

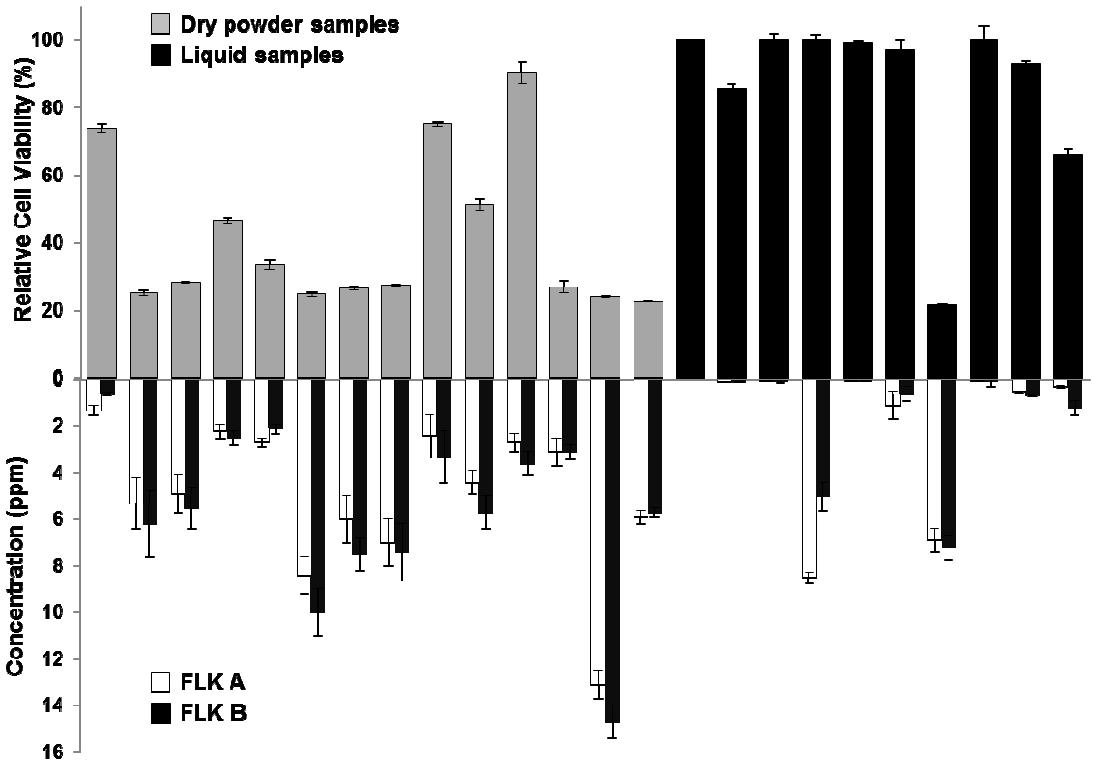

Figure 2. Comparison of relative cell viability to flavokawain (FLK) A and B concentrations. Top. Relative cell viability of human lung adenocarcinoma A549 cancer cell line after 48-hour incubation with ethanolic kava extracts at 75 μg/mL. Samples are organized according to kava preparation type with the gray bars representing the dry power samples organized from coarse grind on the left to very fine grind on the right with the last three dry powder samples (P, Z, and V) being instant freeze-dried kava. Black bars represent liquid samples. **Bottom.** Concentration of two potentially cytotoxic compounds found in kava (white bars: FLKA and black bars: FLKB respectively). Error bars represent standard error of 3-4 replicates.

Table 2. Commercial Kava Sources

Name	Type[A]	Source, harvest date, origin, and notable properties
Black Sand Kava Nakamal Grade	P	Nakamal@Home, June 2011 Republic of Vanuatu; fine grind
Kava powder P.E. 30%	P	December 2010; very fine grind
Freeze-dried Borogoru kava	P	Ed Johnston, Hawaii, USA; freeze-dried[B]
Tanna Kava	P	Tanna Kava Kava from the Jungle; coarse grind
Fire Island Kava Instant Kava, 100% dried kava juice	P	Nakamal@Home, Republic of Vanuatu; freeze-dried[B]
Kava Kava extract with glycerin and grain alcohol	L	Now Foods, Republic of Vanuatu/Fiji; glycerin extract
Solomon Kava Nambawan (#1) grade	P	Nakamal@Home, June 2011, Solomon Islands; very fine grind
Stone Kava Stone grade	P	Nakamal@Home, June 2011 Republic of Vanuatu; fine grind
Big Island Grown Kava	P	Paradise Kava, June 2011, Hawaii, USA; fine grind
Kava Kava Root with grain alcohol, glycerin, and water	L	Gaia Herbs, Republic of Vanuatu; medium to high alcohol extract
Kava Kava critical CO_2 extract	L	Mr. Jay Stopper
Pharma Kava® Liquid Extract with grain alcohol	L	Herb Pharm, Republic of Vanuatu; "organic" grain alcohol extract
Fiji Kava Bula Grade	P	Nakamal@Home June 2011, Fiji; fine grind
Whole kava root	P	Medium fine grind
Kava Kava Root with grain alcohol and water	L	Gaia Herbs, Republic of Vanuatu; high alcohol extract
Kava extract with 95% ethanol	L	Kennin Garrett, August 2001
Kava professional Gaia Herb	L	Medium to high alcohol
Pentecost Pride kava	P	Vanuatu Kava Store, Republic of Vanuatu; medium fine grind
Wow! Kava Connoisseur Grade	P	Nakamal@Home, June 2011 Republic of Vanuatu; fine grind
Fire Island Kava Instant Kava, 100% dried/ground kava roots	P	Nakamal@Home, Republic of Vanuatu; freeze-dried[B]
Dry kava root	P	Kennin Garrett; coarse grind
Kava Kava Root with glycerin, water, and <8% grain alcohol	L	Gaia Herbs; low alcohol extract
Kava water, homemade, 100% aqueous	L	Ed Johnston, Hawaii, USA; alcohol-free
Kava Tincture with 97% ethanol	L	Hilo Natural Health Clinic, Hawaii, USA

All products were obtained in fall of 2011, the harvest date and country of origin is indicated for products where this information was available. (A) Type describes the

starting state of the source material P- dry powder and L-liquid. (B) Extracted kava was freeze-dried and pulverized to form a water soluble instant drink mix.

Table 3. Average concentration (ppm) of compounds from dry powder commercial kava sources

Code	Extraction method	K	DHK	M	DHM	FLK A	FLK B
C	I	9 ±2	22 ±1	2.0 ±0.2	6.7 ±0.6	ND	ND
	II	30 ±1	36 ±1	17.3 ±1.8	26.9 ±0.7	2.7 ±0.2	2.1 ±0.2
G	I	13.3 ±0.6	23.4 ±0.4	2.9 ±0.2	8.1 ±0.3	ND	ND
	II	45 ±3	35 ±1	21 ±3	25 ±1	5.3 ±1.1	6.2 ±1.4
H	I	13 ±1	22.9 ±0.5	2.7 ±0.2	8.1 ±0.4	0.04 ±0.01	0.02±0.01
	II	42 ±1	34.9 ±0.5	13.2 ±1.6	25 ±1	4.9 ±0.8	5.5 ±0.9
P	I	47.2 ±2.4	36.8 ±0.3	19.3 ±2.1	17.3 ±0.1	ND	ND
	II	25.6 ±0.3	34.0 ±0.8	8.1 ±0.2	19.1 ±0.9	3.1 ±0.6	3.1 ±0.3
Q	I	11 ±1	24.6 ±1.0	1.9 ±0.2	8.2±0.8	0.02 ±0.01	0.01±0.008
	II	51 ±2	38.1 ±0.5	30.4 ±3.6	30 ±1.0	8.4 ±0.8	10±1
R	I	11.2 ±0.7	22.4 ±0.8	2.4 ±0.2	7.8 ±0.5	0.04 ±0.01	0.04±0.004
	II	51 ±1	37 ±1	32.2 ±1.5	28.5 ±0.4	6 ±1	7.5 ±0.7
S	I	15 ±2	28.3 ±1.9	3.7 ±0.7	12 ±2	0.02±0.01	0.02±0.008
	II	50 ±1	37.3 ±0.9	29 ±5	30.1 ±0.7	7 ±1	7.4 ±1.2
T	I	10.9 ±0.2	22.8 ±0.3	3.3 ±0.1	6.5 ±0.2	ND	0.005±0.003
	II	48.3 ±3.6	38 ±1	24 ±2	22.1 ±0.6	4.4 ±0.5	5.7 ±0.7
U	I	11.3 ±0.3	21.7 ±0.4	2.6 ±0.1	6.1 ±0.2	ND	ND
	II	41 ±6	34.5 ±0.5	21.2 ±4.5	22 ±1	2.4 ±0.9	3.3 ±1.1
V	I	18.4 ±0.2	30.6 ±0.3	5.2 ±0.1	12.8 ±0.2	ND	ND
	II	65.3±4.3	38.2 ±0.7	41.4 ±3.3	27 ±1	5.9 ±0.3	5.7 ±0.2
W	I	12 ±1	17.4 ±0.7	4.1 ±0.6	6.4 ±0.6	0.05 ±0.006	0.1 ±0.01
	II	45±2	36.7 ±0.5	25 ±4	24.7 ±0.3	2.7 ±0.4	3.6 ±0.5
Z	I	26.4 ±0.4	35.7 ±0.4	6.4 ±0.2	20.8 ±0.3	ND	0.01±0.001

	II	45 ±1	38.3 ±0.4	6.4 ±0.2	33 ±1	13.1 ±0.6	14.7±0.7
AA	I	11 ±2	20 ±2	2.4 ±0.4	6.7 ±0.9	ND	ND
	II	26.5 ±0.2	25.5 ±0.4	13.4 ±1.0	19.7 ±0.4	1.3 ±0.2	0.6 ±0.07
CC	I	23.2±6.9	27 ±3	7.5 ±2.8	13.8 ±4.0	1.6 ±0.6	1.6 ±0.8
	II	41 ±1	33.9 ±0.5	14.3 ±2.1	23.0 ±0.7	2.2 ±0.3	2.5 ±0.3

Values represent the mean of four extraction replicates with standard error reported. ND indicates that the concentration was below the level of detection. Extraction method I used water and method II used 95% ethanol. Compounds are abbreviated as follows: K - kawain, DHK - dihydrokawain, M - methysticin, DHM - dihydromethysticin, FLK A, - flavokawain A, FLK B - flavokawain B.

Table 4. Average concentration (ppm) of compounds from liquid commercial kava sources

Code	Extraction method	K	DHK	M	DHM	FLK A	FLK B
K	III	4.2 ±1.2	6.6 ±1.4	1.4 ±0.5	2.1 ±0.6	ND	0.01±0.005
	IV	31 ±1.0	29.3 ±0.1	11.3 ±0.0	16.9 ±0.2	0.5 ±0.01	0.7 ±0.007
L	III	2.1 ±0.8	3.2 ±1.1	0.3 ±0.2	0.8 ±0.4	ND	ND
	IV	37.5 ±0.1	32.3 ±0.2	9.6 ±0.4	18.6 ±0.3	0.09±0.01	0.1 ±0.01
M	III	0.08±0.1	0.2 ±0.0	0.03±0.0	0.02 ±0.00	ND	ND
	IV	19±3.4*	21±3*	6±1.3*	9.4±1.5*	0.04±0.01*	0.07±0.06*
N	III	2.3 ±0.4	4.3 ±0.5	0.5 ±0.1	1.1 ±0.2	ND	ND
	IV	16±6	18.2 ±9.7	7 ±3	12 ±2	8.5 ±0.2	5.0 ±0.6
O	III	9 ±3	12.5 ±2.8	5 ±2	6 ±2	0.06±0.03	0.06±0.02
	IV	26±4.7*	31±4.3*	10±2.1*	18±2.6*	0.03±0.004*	0.06±0.01*
X	III	32±6	34 ±1	12 ±7	19 ±2	0.9 ±0.09	0.3 ±0.1
	IV	40±7.2*	44±6.1*	14±3*	26±4.1*	0.3±0.04*	1.2 ±0.3*
Y	III	27 ±1	28 ±1	9 ±1	14.2 ±0.6	0.07 ±0.1	0.04±0.02
	IV	20±13	24 ±4	8 ±6	12 ±6	1.1 ±0.6	0.6 ±0.3
BB	III	33±9	36.2 ±0.4	13 ±6	28.3 ±0.1	2.1 ±0.05	0.9 ±0.009
	IV	49.4 ±0.7	33 ±4	17±1	24 ±6	6.9 ±0.5	7.2 ±0.5
DD	III	5 ±2	6.4 ±2.0	0.5 ±0.2	2 ±1	ND	ND
	IV	24±4.3*	27±3.8*	3.8±0.8*	13±2.1*	0.02±0.003*	0.07±0.2*
EE	III	17.0 ±0.1	23.9 ±0.2	3.5 ±0.1	10.0 ±0.1	ND	ND
	IV	22.4 ±0.2	27.0 ±0.1	5.9 ±0.1	14.4 ±0.1	ND	ND

Values represent the mean of four extraction replicates with standard error reported. *Single replicate analysis with error estimates based on calculated average relative error. ND indicates that the concentration was below the level of detection. Extraction method III used water and method IV used 95% ethanol. Compounds are abbreviated as follows: K - kawain,

DHK - dihydrokawain, M - methysticin, DHM - dihydromethysticin, FLK A, - flavokawain A, FLK B – flavokawain B.

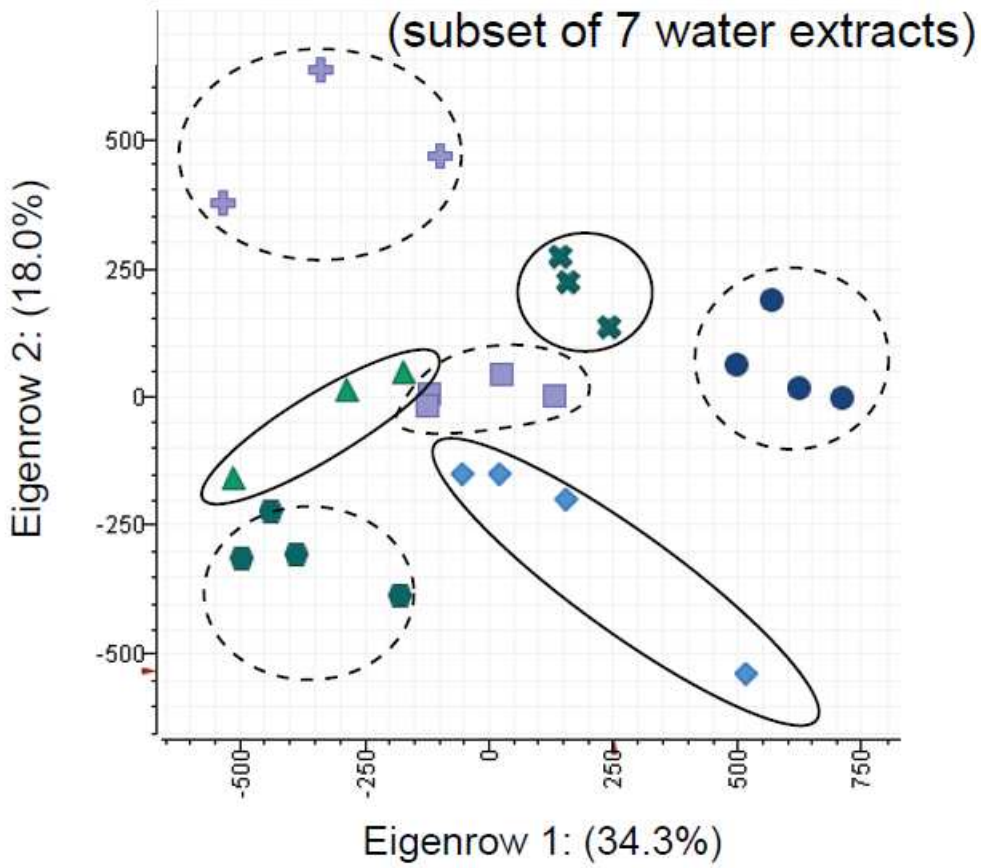

Figure 3. Principal components analysis (PCA) of commercial kava preparations. Dry ground kava was extracted with water. Replicate extractions of the same material form clusters identified by drawn circles. The percent of variation explained by each principal component is shown along the appropriate axis.

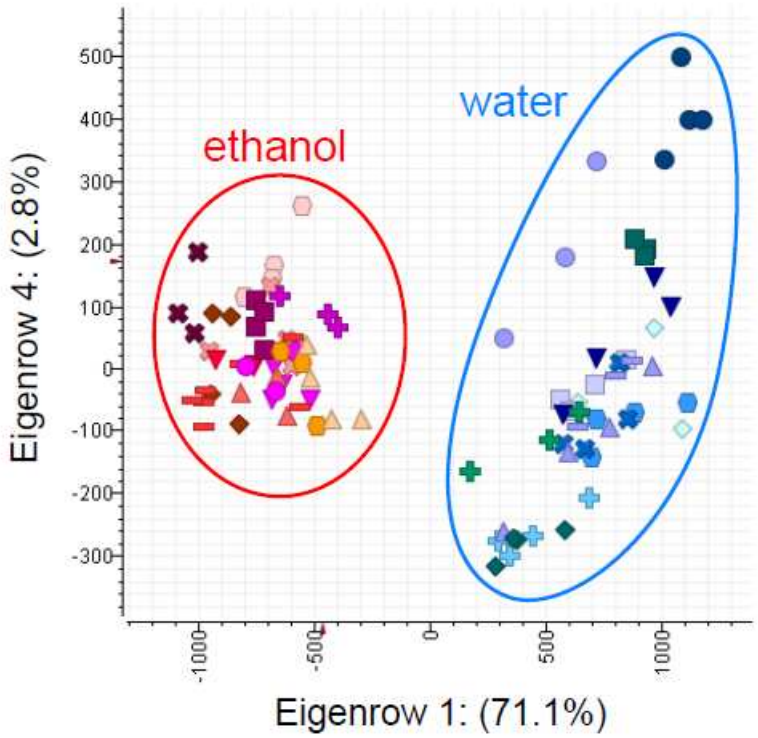

Figure 4. Principal components analysis (PCA) of commercial kava preparations. Dry ground kava was extracted with water (blue) and ethanol (red). Replicate extractions of the same material form tight clusters. Secondary groups identified by drawn circles are formed based on the extraction solvent used; where the large amount of variation explained by Eigenrow 1 (PC1) is due to the use of either water or ethanol. The percent of variation explained by each principal component is shown along the appropriate axis.

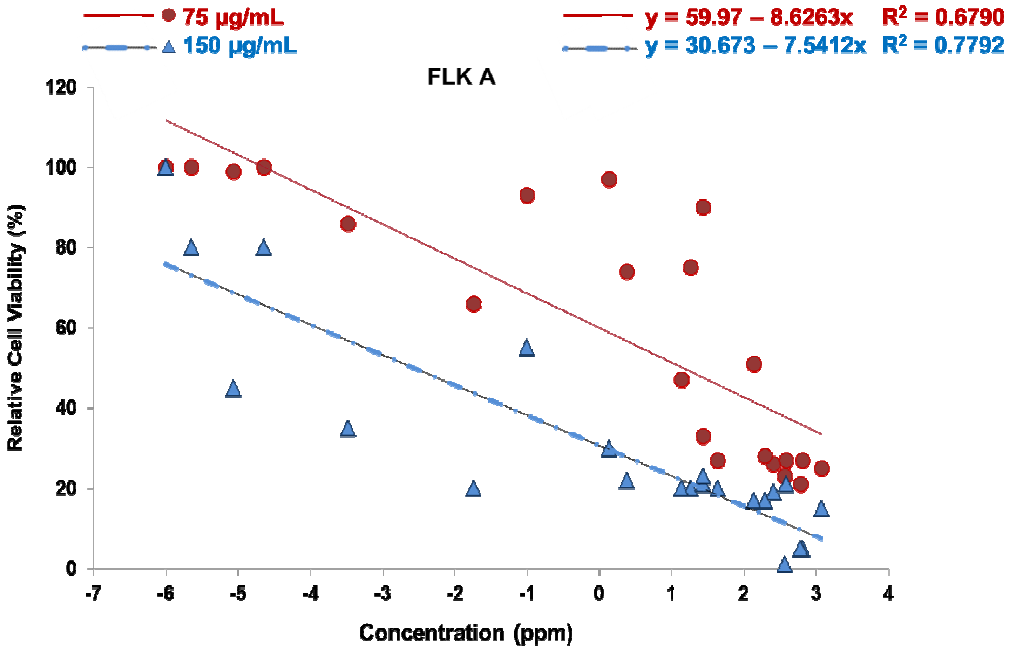

Figure 5. Correlation between relative cell viability and Flavokawain A concentration. Relative cell viability of human cancer cells after 48-hour incubation with kava extracts at 75 μg/mL (red, circles) and 150 μg/mL (blue squares) is plotted to sample FLK A concentrations ($\log_2$ normalized) with R^2 values shown at the top for each extract concentration.

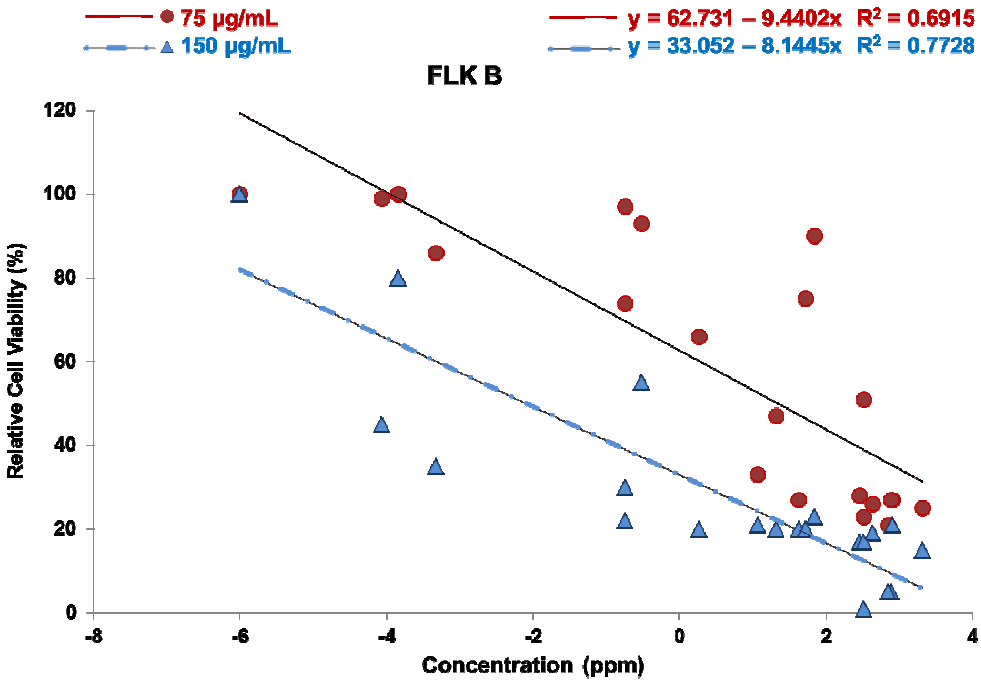

Figure 6. Correlation between relative cell viability and Flavokawain B concentration. Relative cell viability of human cancer cells after 48-hour incubation with kava extracts at 75 μg/mL (red, circles) and 150 μg/mL (blue squares) is plotted to sample FLK B concentrations ($\log_2$ normalized) with R^2 values shown at the top for each extract concentration.

Chapter 4

Antimicrobial constituents of *Comptonia peregrina* (L.) J.M. Coulter (Sweet Fern)

Summary

This study was conducted to investigate the potential antimicrobial activity of *Comptonia peregrina* (sweet fern) aerial parts collected in Minnesota. Activities against both fungi and Gram-positive bacteria were found in the dichloromethane partition of a methanol extract with complete growth inhibition occurring at concentrations of 250 and 125 μg/mL, respectively. Further bioassay guided fractionation led to the identification of pinosylvin monomethyl ether with IC_{50} values of 10.2 and 17.4 μg/mL for *Candida albicans and Staphylococcus aureus*, respectively. Additionally, pinocembrin was found to have antimicrobial activity with IC_{50} values of 65.9 and 79.0 μg/mL for *C. albicans and S. aureus*, respectively. Our results indicate the potential to increase the economic value of sweet fern by using it as a source for antimicrobial agents.

Introduction

Plant and traditional uses. Sweet fern, syn. meadow fern (*Comptonia peregrina* (L.) J.M. Coulter; Family: Myricaceae) is the only plant in its genus (Bell and Curtis, 1985; Halim and Collins, 1973). Sweet fern is not a fern but rather a small, deciduous, aromatic shrub with long narrow leaves that have rounded rolled back edges and fern-like divisions. As a non-legume nitrogen fixer it forms a symbiotic relationship with actinomycetes belonging to the *Frankia* genus (Goforth and Torrey, 1977; Lechevalier and Ruan, 1984; Mishra *et al.*, 2010; Popovici *et al.*, 2010). Sweet fern is drought tolerant, can grow on marginal soil, and is typically found in open woodlands from southern Quebec, Canada to the extreme north of Georgia, and west to Minnesota, USA (Hooper *et al.*, 1984; Sylvestre *et al.*, 2007). Sweet fern displays a low occurrence of insect damage, possibly attributable to the oily yellow colored essential oils secreted from visible glandular trichomes present on its stems and leaves (Bell and Curtis, 1985).

Traditionally, sweet fern was used by indigenous peoples of North America and European settlers to treat a variety of dermatological disorders, including skin cancer (Lau-Cam and Chan, 1973; Hooper *et al.*, 1984; Sylvestre *et al.*, 2007). Leaves were used by the Canadian Maritime and northern Wisconsin and Minnesotan Ojibwe Indian Nations as: an astringent; blood purifier; expectorant; tonic; and as a remedy for: diarrhea; headache; fevers; catarrh (mucous membrane inflammation); vomiting of blood; rheumatism; toothaches; sprains; stings; ringworm and swelling and inflammation caused by poison ivy or poison sumac (Hooper *et al.*, 1984; Monte *et al.*, 2008; Sylvestre *et al.*, 2007). A concoction of sweet fern roots has been reported to be useful for the treatment of psoriasis and eczema (Hooper *et al.*, 1984). This report shows the bioassay-guided isolation of two antimicrobial compounds and one, abundant, but inactive compound from the aerial parts of *C. peregrina* collected from established populations in Minnesota. The presence of bioactive compounds in sweet fern may increase the economic potential of this ecologically important plant.

Previously isolated constituents. Qualitative studies have shown that *C. peregrina* contains compounds belonging to a variety of chemical classes including: alkaloids, terpenes, and polyphenolics including, phenolic acids, flavonoids, and *C*-methylchalcones (Chandler and Hooper, 1982; Fang *et al.*, 2011; Hooper and Chandler, 1984; Hooper *et al.*, 1984; Jankowski and Gilles, 2002; Sylvestre *et al.*, 2007; Wollenweber *et al.*, 1985). Most of the phytochemical reports on *C. peregrina* have concentrated on the composition of its volatile essential oils (Collin *et al.*, 1988; Halim and Collins, 1973; Lawrence and Weaver, 1974; Sylvestre *et al.*, 2007). Studies have reported the presence of of the monoterpenes 1,8-cineole and γ-terpinene, as major constituents of *C. peregrina* essential oil (Collin *et al.*, 1988; Halim and Collins, 1973). Other main terpene constituents of sweet fern essential oil include *cis*-ocimene, *trans*-ocimine, *E*-2-hexenal linalool, β-caryophyllene, and α-humulene (Halim and Collins, 1973; Lawrence and Weaver, 1974; Sylvestre *et al.*, 2007) sabinene, copaene, and α-muurolene (Collin *et al.*, 1988), thujone, and 3-thujen-2-one (Jankowski and Gilles, 2002). Flavonoids isolated from *C. peregrina* leaves include myricetin, galangin (Lau-Cam and Chan, 1973), alpinone (Wollenweber, 1985), myrigalon B (Carlton *et al.*, 1992),

pinocembrin (5,7-dihydroxyflavanone), and its 6-*C* and 8-*C*-methyl derivatives, strobopinin and cryptostrobin, respectively (Williams *et al.*, 1997; Wollenweber *et al.*, 1985). Additionally, the flavanone comptonin (7-hydroxy-5-methoxy-6-*C*-methyl flavanone), along with trace amounts of 2',4'-dihydroxy-6'-methoxy-5'-*C*-methyl chalcone, and the phenolic acid, gallic acid, were isolated from leaf exudates (Wollenweber *et al.*, 1985).

Reported activities. Some chemical constituents isolated from *C. peregrina* have been shown to exhibit various biological activities. Methyl *p*-coumarate isolated from sweet fern roots and stems was cytotoxic in *in vitro* assays against mouse TLX5 lymphoma cells (Hooper *et al.*, 1984). One study reported that the sesquiterpene enriched essential oil fraction displayed cytotoxicity against human lung carcinoma cell line A-549 and human colon carcinoma cell line DLD-1; this activity was partially attributed to the presence of α-humulene and (*E*)-nerolidol (Sylvestre *et al.*, 2007). The stilbene (*E*)-3-hydroxy-5-methoxystilbene, isolated from *C. peregrina* leaves, was found to have antimicrobial activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (minimum inhibitory concentration (MIC) 32 μg/mL), vancomycin resistant enterococci (16 μg/mL), *Mycobacterium bovis* (26 μg/mL), and several *Bacillus* species including an avivrulent strain of *Bacillus anthracis* (MIC 8 μg/mL) (Kabir *et al.*, 2008). Activity against the Gram-negative bacteria, *Esherichia coli* and *Pseudomonas aeruginosa*, was not found (Kabir *et al.*, 2008). The activity of this naturally occurring stilbene prompted the synthesis of several stilbenoid analogs, which displayed similar activity when at least one phenolic moiety was present (Kabir *et al.*, 2008).

Materials and Methods

Chemical reagents. HPLC grade solvents, NMR solvents, and reagents purchased from Sigma Aldrich (St. Louis, MO, USA) were used including: acetonitrile, chloroform, dichloromethane, deuterated methanol (CD_3OD), deuterated chloroform ($CDCl_3$), ethanol (95%), ethyl acetate, formic acid, hexanes, resazurin sodium salt, concentrated sulfuric

acid, and vanillin. Germall™ was obtained from Fisher Scientific (Pittsburgh, PA, USA), dimethyl sulfoxide (DMSO) from J.T. Baker (Center valley, PA, UA), and tryptone soy broth (TSB) from BD (Franklin Lakes, NJ, USA). Thin layer chromatography (TLC) silica gel 60 F_{254} glass plates were obtained from Merck (Whitehouse Station, NJ, USA). Standard reverse osmosis deionized glass distilled water was obtained in house using a Thermo Scientific Barnstead B-pure™ filter and Distinction water still model D4000 (Bibby Scientific Limited, Stone, Staffordshire ST15 0SA, UK).

Bioassay-guided fractionation. *C. peregrina* aerial tissues were collected in north central Minnesota by Donald L. Wyse, Professor, University of Minnesota, Saint Paul, MN, USA on September 23, 2012 and dried at 30°C for 24 hours and then crushed by hand prior to extraction. Species authentication was performed (by DLW) and a voucher specimen, assigned the code AV0010, was deposited in the Department of Horticultural Science, University of Minnesota. The dry crushed plant material (520 g) was extracted by maceration with methanol (3 x 3000 mL) at room temperature, for 24 hours each. After filtration using Whatman filter paper and evaporation of the solvent *in vacuo* at less than 35 °C, the combined crude methanolic extract was diluted to 10% methanol in water, and then partitioned in turn with *n*-hexane (3 x 300 mL) and dichloromethane (3 x 300 mL). The *n*-hexane (dissolved solids; 6.8 g, **A**), dichloromethane (9 g, **B**), aqueous (39.6 g, **C**) soluble partitions were submitted for testing in the antimicrobial assays against *Candida albicans* (American Type Culture Collection (ATCC) 10231), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 6538), *and Staphylococcus aureus* (ATCC 6538) (Fig. 1).

The dichloromethane partition, **B** (8 g), was dissolved in a solution of 12 mL chloroform: methanol (5:1 *v/v*), filtered through Whatman filter paper and fractionated using a Revelaris flash system (Grace, Columbia, Maryland, USA) equipped with a 40 g silica column (Grace). Prior to sample loading, the column was equilibrated with 100% chloroform. The 24 minute gradient, from chloroform to methanol, was manually optimized in real time with a constant flow rate of 25 mL/min. Both an evaporative light scattering detector (threshold 3 mV, carrier solvent: isopropanol) and an ultraviolet detector (threshold 0.02 AU, 254 and 320 nm), were used to monitor column effluent.

Fractions were collected automatically into 15 mL test tubes as peaks were detected according to detector thresholds. Afterwards, fractions were evaluated by silica gel TLC developed in chloroform: methanol (4:1 *v/v*), and the fractions were grouping into 11 fractions (fractions **D-N**). TLC plates were visualized using long and short UV light exposure followed by staining with sulfuric acid-vanillin reagent (15 g vanillin, 250 mL ethanol, and 2.5 mL concentrated sulfuric acid) and heating to 220°C. All fractions **D-N** were then tested for antimicrobial activity against *C. albicans* and *S. aureus*. Activity was only observed only in fraction **D**. Antimicrobial activity was not detectable at the highest concentration tested for inactive fractions**.** Although inactive, methanol soluble fraction **E** appeared to be mostly pure and further purification by recrystallization with hexanes yielded galangin (**1**). Fraction **D** (3 g) was further fractionated by flash chromatography after being dissolved in a minimal amount of hexanes: ethyl acetate (7:3 v/v) and manually injected onto a 40 g silica column (Grace). Prior to loading, the column was equilibrated with 100% hexanes. The 38-minute gradient from hexanes to ethyl acetate was manually optimized in real time, with a constant flow rate of 40 mL/min. An ultraviolet detector (threshold 0.02 AU, 254 and 320 nm), was used to monitor the separation. Fractions were collected automatically by volume, evaluated using TLC (eluted with hexanes: ethyl acetate 3:1, v/v) and tested for antimicrobial activity. The biologically active fraction **D1** (0.6 g) was then isocratically separated on 160g of Sephadex LH-20 (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) with methanol as the eluting solvent to yield 12 fractions (**D1-1** to **D1-12**), of which **D1-2** (200 mg) was the largest by volume.

Analysis by C_{18} reversed-phase-ultra-performance liquid chromatography-negative electrospray ionization-single quadrupole mass spectrometry (RP-UPLC-ESI(-)-SQ-MS) showed that fraction **D1-2** required further separation. About 190 mg of fraction **D1-2** was dissolved in 76% methanol and filtered through a 25 mm syringe filter fitted with a 0.2 μm pore membrane (Arodisc, Supor, Pall Corporation, Ann Arbor, MI) and further separated using a semipreparative HPLC (Agilent, Saint Clara, CA, USA) equipped with a 19 x 150 mm Xbridge Prep 18 column, with 5 μm particle size packing (Waters, Milford, MA, USA). Prior to loading, the column was equilibrated with 60% methanol. The 17-minute linear gradient from water to methanol was performed with a

flow rate of 15 mL/min. Eluting peaks were monitored by ultraviolet detection (threshold 0.02 AU, 254, 280, and 320 nm) and the pure compounds pinocembrin (**2**) and pinosylvin monomethyl ether (**3**) were collected manually. Compounds **1**, **2** and **3** were identified via spectrometric analysis (HRAM-MS, MS^2, UV-VIS, ^1H- and ^{13}C-NMR) and submitted to bioassay.

RP-UPLC-ESI(-)-SQ-MS. Low-resolution nominal mass analysis was carried out using a UPLC-SQ detector mass spectrometer fitted with an autosampler where sample vials were held at 4°C (Acquity, Waters, Milford, MA, USA). The following MS conditions were used: full scan mass range of 100–1000 *m/z*, 250 ms scan time, desolvation temperature 350°C, desolvation flow rate (nitrogen) of 6.5 L/min, capillary voltage of 3000 V, sample cone voltage of 30 V, source temperature of 150°C. Separation was carried out on a C_{18} reversed phase HSS T3 1.8 mm particle size, 2.1 x 100 mm column (Waters). Column temperature was 40°C, mobile phase flow rate 0.45 mL/min, 1 injection volume 5 μL. A 29-minute gradient using mobile phases A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile was run according to the following gradient elution profile: initial, 20% B; 2 min, 20% B; 20 min, 98% B; 22 min, 98% B; 28 min, 20% B; 29 min, 20% B. MassLynx version 4.1 (Waters) was used to record the chromatograms and spectra.

***RP-UPLC-PDA-ESI(+/-)-HRAM-MS* and $-MS^2$**. High-resolution accurate mass measurements (HRAM) were obtained via C_{18}-reversed-phase ultra-performance liquid chromatography-photodiode array-positive/negative electrospray ionization-hybrid quadrupole-orbitrap mass spectrometry fitted with an autosampler maintained at 4ºC (Ultimate® 3000 HPLC, Q Exactive™, Thermo Scientific). Separations were carried out on a reversed-phase C_{18} HSS T_3 1.8 μm particle size, 2.1x100 mm column (Waters). Column temperature was 40°C, solvent flow rate 0.45 mL/min, injection volume 1 μL. An eight-minute gradient using mobile phases A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile was run according to the following gradient elution profile: initial, 50%B; 1 min., 50% B; 5 minutes, 75% B; 5.5 minutes, 98% B; 6 minutes, 98% B; 6.5 minutes, 50% B; 8 minutes, 50% B. Ultraviolet absorbance between 220 and 800 nm

was measured using an inline PDA detector. The following MS conditions were used: full scan mass scan range: 150-1000 *m/z*, resolution: 70,000, data type: profile, desolvation temperature 350°C, capillary voltage: 3800 V (+), 3300 V (-); data-dependent MS^2: resolution: 17,500, ACG target: 1e5, maximum IT: 50 ms, loop count: 10, MSX count: 1, fixed first mass: 50.0 *m/z*, intentsity threshold: 2.0e4. Xcaliber™ software version 2.1 (Thermo Scientific) was used to record the chromatograms and spectra.

Nuclear Magnetic Resonance (NMR). A 400 MHz (^{1}H) and 100 MHz (^{13}C) NMR Spectrometer (Varian Unity Inova, Palo Alto, CA, USA) was used for all NMR experiments. The NMR spectrometer was operated using VnmrJ 2.2D software and 5 mm NMR tubes. ACD/NMR processor software was used for off-line processing.

Antimicrobial bioassay. Antimicrobial activity against *C. albicans, E. coli, P. aeruginosa, and S. aureus* was measured using a 96-well plate broth dilution method. Rapid evaluation of antimicrobial activity of early fractions was completed using a qualitative resazurin assay. The extract concentration at which bacterial growth was inhibited by 50% (IC_{50} value) was determined using a method adapted from Weigland *et al.*, 2008. Briefly, extract fraction residue was dissolved in 100% DMSO and working stock solutions of 1 mg/mL (0.1%) for *S. aureus* and *C. albicans* and 2 mg/mL (0.2%) for *E. coli* and *P. aeruginosa* were prepared in TSB. Extract solutions were serially diluted with TSB, to concentrations ranging from 500 μg/mL to 0.98 μg/mL for *S. aureus* and *C. albicans* or 1 mg/mL to 1.95 μg/mL for *E. coli* and *P. aeruginosa*, and 100 μL of each solution was added to an empty well of a 96-well plate. For screening pure compounds a working stock solution was prepared at a concentration of 0.250 mg/mL (.025%) in DMSO and 100 μL was added to an empty well of the 96-well plate at concentrations ranging from 125 μg/mL to 0.24 μg/mL. Microbes were grown overnight with agitation at 32°C and diluted into TSB to an optical density of approximately 0.5 using a MacFarland standard (Becton Dickinson and Company, Microbiology Systems, Sparks, MD). This solution was further diluted 1:100 with TSB to give a bacterial concentration of ~10^6 colony-forming units (cfu)/mL and 50 μL was added to the extracts in each well of the 96-well plate for a final concentration of 5×10^5 cfu/mL. Dilutions of Germall™,

extract free DMSO were used as positive and negative control, respectively. All bacterial, extract, and chemical solutions were used within 30 minutes of their preparation.

For qualitative resazurin analysis, after 24 hours of incubation at 32°C, with shaking during the last hour, 10 μL of resazurin (at 20 μg/mL) was added to each well of the 96-well plate. Plates were further incubated at 37°C for 15 minutes for *S. aureus* and *E. coli*, two hours for *P. aeruginosa*, and four hours for *C. albicans*. After the specified incubation times individual wells were visually assessed for the presence of the color blue, which indicated no growth of microorganisms (presumably due to the additional of an antimicrobial extract) or the present of pink, which indicated microbial growth. For IC_{50} determination, prior to the addition of resazurin, the optical density of each individual well was measured at 520 nm. IC_{50} values were determined by non-linear regression analysis in GraphPad Software (LaJolla, California) using a custom designed program to subtract blanks, log transform the concentration values, and normalize the optical density values. All assays were performed in triplicate.

Results and Discussion

Evaluation of all partitions from bioassay-guided fractionation of methanolic extract from *C. peregrina* aerial parts showed activity against *C. albicans* and *S. aureus*. The dichloromethane soluble partition, **B**, demonstrated the most potent activity and was therefore selected for bioassay-guided fractionation to isolate and identify the compound(s) responsible for this activity (Table 1). All other extract partitions were stored at -20°C.

The following three dichloromethane soluble compounds were isolated and identified via spectral and physical data reported below to be, pinosylvin monomethyl ether (**3**) and pinocembrin (**2**) as the active constituents and galangin (**1**) as the inactive constituent against the fungi, *C. albicans* and the gram-positive bacteria, *S. aureus* (Fig. 2)

The flavonol galangin syn. chrysin (3,5,7-trihydroxyflavone, **1**, 22.56 mg) was obtained as a yellow powder after five purification steps including, extraction, partitioning, normal-phase flash chromatography, and crystyllation/recrystallyzation.

PDA measurements in methanol showed λ_{max} values of 267 and 359 nm. High resolution accurate mass (HRAM) measurements using a hybrid quadrupole-Orbitrap mass spectrometer (Thermo) showed molecular ion peaks at 271.063 *m/z* $[M+H]^+$ and 269.045 *m/z* $[M-H]^-$ consistent with a molecular composition of $C_{15}H_{10}O_5$. Using an HCD collision energy of 60.0, data-dependent MS^2 fragmentation showed characteristic fragments of galangin in both positive (271.1 [100], 153.02 [66], 105.0 [46], 165.0 [21], 215.1 [11] *m/z*) and negative (269.0 [100], 169.1 [32], 171.0 [30], 143.0 [25], 213.1 [21], 197.1 [15] *m/z*) ESI modes (Appendix A., Fig. A1 & A2) (Medana *et al.*, 2008; Samart, 2007; Smith *et al.*, 2005). Percent intensities, relative to base peaks, are noted in brackets. ^{1}H-NMR δ (ppm) values in CD_3OD are 8.17 (dd, 2H, *J* = 6.7, 1.6 Hz, 2', 6'-H); 7.46 (m, 3H, 3', 4', 5'-H); 6.40 (d, 1H, *J* = 2.08 Hz, 8-H); 6.18 (d, 1H, *J* = 2.07 Hz, 6-H). ^{13}C-NMR δ (ppm) values, in CD_3OD, are 170 (4-C); 165.9 (7-C); 162.6 (5-C); 158.4 (9-C); 146.9 (2-C); 138.5 (3-C); 130.9 (1'-C); 129.4 (3', 5'-C); 128.7 (2', 4', 6'-C); 104.7 (10-C); 99.4 (8-C); 94.5 (6-C) (Appendix A., Fig. A3 & A4). The spectral data of galangin are in agreement with the earlier published data (Medana *et al.*, 2008; Pretsch *et al.*, 2009; Rubens *et al.*, 2005; Samart, 2007).

The flavanone, pinocembrin ((2*S*)-5,7-dihydroxyflavanone, **2**, 26.48 mg) was obtained as white powder after eight purification steps including, extraction, partitioning, normal-phase flash chromatography, separation over Sephadex LH-20, C_{18} reversed-phase HPLC, and recrystallyzation. PDA measurements in methanol showed a λ_{max} value of 280 nm. HRAM measurements showed molecular ion peaks at 257.083 *m/z* $[M+H]^+$ and 255.066 *m/z* $[M-H]^-$ consistent with a molecular composition of $C_{15}H_{12}O_4$. Data-dependent fragmentation using an HCD collision energy of 50.0 showed characteristic fragments of pinocembrin in both positive (153.0 [100], 131.1 [50], 103.1 [18], 257.1 [5.3] *m/z*) and negative (151.0 [100], 255.1 [91], 107.1 [88], 213.1 [74], 145.1 [62] *m/z*) ESI modes (Appendix A., Fig. A5 & A6) (Horai, 2010; Medana *et al.*, 2008; Smith *et al.*, 2005). ^{1}H-NMR δ (ppm) values in CD_3OD are 7.49 (d, 2H, *J* = 9.2 Hz, 2', 6'-H); 7.39 (m, 3H, 3', 4', 5'-H); 5.94 (d, 1H, *J* = 2.16 Hz, 8-H); 5.90 (d, 1H, *J* = 2.15 Hz, 6-H); 5.46 (dd, 1H, *J* = 12.8, 3.05 Hz, 2-H); 3.10 (dd, 1H, *J* = 17.1, 12.8 Hz, 3-H); 2.77 (dd, 1H, *J* = 18.3, 2.0 Hz, 3-H). ^{13}C-NMR δ (ppm) values, in CD_3OD, are 197.3 (4-C); 168.4 (7-C); 165.5 (5-C); 164.7 (9-C); 140.4 (1'-C); 129.65 (3', 5'-C); 127.3 (2', 4', 6'-C); 103.4 (10-C);

97.2 (8-C); 96.2 (6-C); 80.5 (2-C); 44.2 (3-C) (Appendix A., Fig. A7 & A8). The spectral data of pinocembrin are in agreement with the earlier published data (Chen *et al.*, 2010; Hanawa *et al.*, 2001; Horai, 2010; Kuroyanagi *et al.*, 1983; Medana *et al.*, 2008; Pretsch *et al.*, 2009). Although the stereochemistry of pinocembrin isolated from *C. peregrina* has not been shown here or elsewhere, the stereochemistry of pinocembrin isolated from *Pinus strobus* bark has been reported and is indicated above (Hanawa *et al.*, 2001).

The stilbene, pinosylvin monomethyl ether (3-hydroxy-5-methoxystilbene, **3**, 53.62 mg) was obtained as a brown oil after seven purification steps including, extraction, partitioning, normal-phase flash chromatography, separation over Sephadex LH-20, and C_{18} reversed-phase HPLC. Attempts to obtain pure crystals of this compound via recrystallization were unsuccessful. PDA measurements in methanol showed λ_{max} values of 220 and 300 nm, HRAM measurements showed molecular ion peaks at 227.109 *m/z* $[M+H]^+$, 225.091 *m/z* $[M-H]^-$ consistent with a molecular composition of $C_{15}H_{14}O_2$. Data-dependent fragmentation using an HCD collision energy of 50.0 showed characteristic fragments of pinosylvin monomethyl ether in both positive (91.1 [100], 121.1 [94], 117.1 [68], 149.1 [45], 167.1 [43] *m/z*) and negative (210.1 [100], 209.1 [35], 225.1 [15] *m/z*) ESI modes (Appendix A., Fig. A9 & A10). ^{1}H-NMR δ (ppm) values in $CDCl_3$ are 7.49 (dd, 2H, *J* = 6.86, 1.04 Hz, 2'-H, 6'-H); 7.35 (dd, 2H, *J* =6.28, 1.37 Hz, 3'-H, 5'-H); 7.26(tt, 1H, *J* = 5.88, 0.97, 4'-H); 7.06 (d, 1H, *J* = 13.03 Hz, 8-H); 6.99 (d, 1H, *J* = 13.03 Hz, 7-H); 6.64 (dd, 1H, *J* = 1.48 Hz, 2-H); 6.62 (dd, 1H, 6-H); 6.35 (dd, 1H, *J* = 1.72 Hz 4-H); 3.80 (s, 3H, 5-OCH_3). ^{13}C-NMR δ (ppm) values, in $CDCl_3$, are 55.37, –OCH3; 101, (7-C); 104.8 (6-C); 106 (4-C); 125.9 (2-C); 126.6 (2', 6'-C); 127.7 (4'-C); 128.8 (3', 5'C); 129.3 (1-C); 137.0 (8-C); 139.6 (1'-C); 157 (5-C); 161(3-C) (Appendix A., Fig. A11 & A12). The spectral data of pinosylvin monomethyl ether are in agreement with the earlier published data (Ngo and Brown, 1998; Pretsch *et al.*, 2009; Smith *et al.*, 2005; Suga *et al.*, 1993).

The stability of the flavonoid compounds, galangin and pinocembrin was demonstrated by the relatively high HCD collision energy that was required to facilitate adequate fragmentation for structural analysis and database searching (Medana *et al.*, 2008). Moreover, the MS^2 spectra show the molecular ion peak as a predominant peak for these compounds, sometimes occurring as the base peak even after being exposed to

high-energy fragmentation conditions (Appendix A, Fig. A1 & A2). The hydroxyl group present at the C-3 position provided extra stability for galangin so that a very high HCD collision energy of 60.0 was required to produce any fragmentation. Both MS^2 spectra for galangin and pinocembrin also show ions corresponding to the commonly observed retro Diels-Alder reaction fragmentation pathway products for flavonoids, which include 153 *m/z* positive ions and 151 *m/z* negative ions (Medana *et al.*, 2008, Tureček and Hanuš, 1984) (Appendix A, Fig. A1, A2, A5 & A6).

The dichloromethane partition of the methanolic crude extract of *C. peregrina* aerial parts was found to be more than two-times and four-times more active than the crude extract against *C. albicans* and *S. aureus*, respectively (Table 1). *C. peregrina* activity against gram-positive bacteria has previously been shown; however this is the first report of sweet fern activity against *C. albicans* in the literature (Kabir *et al.*, 2008). All three isolated compounds were tested for antimicrobial activity against *C. albicans* and *S. aureus*. Although galangin was found to be inactive, pinocembrin was found to be almost eight-times more active against *C. albicans* and greater than six-times more active against *S. aureus* than the crude extract. Even more active, pinosylvin monomethyl ether was found to be almost 50-times more active against *C. albicans* and 29-times more active against *S. aureus* than the crude extract.

The antimicrobial activity of bee propolis extract, which includes a complex mixture of polyphenolic compounds including pinocembrin and galangin, has been reported (Koo *et al.*, 2000; Park *et al.*, 1998). In particular, antimicrobial activity against oral microorganisms such as *C. albicans*, *S. aureus*, and *Steptococcus mutans* has been attributed to the extract's flavonoid constituents (Koo *et al.*, 2000; Park *et al.*, 1998). These findings are consistent with the activity reported here for pinocembrin, but contrary to the lack of activity demonstrated by galangin. The fungicidal activity of pinosylvin monomethyl ether towards wood-destroying fungi has previously been demonstrated, where fungicidal activity is moderate towards white-rot fungi and low towards brown-rot fungi (Celimene *et al.*, 1999). The observed activity was attributed to the hydrophobic properties of the stilbene facilitating rapid wood dehydration, and thereby limiting water access for the fungi, although this hypothesis was not tested (Celimene *et al.*, 1999). Pinosylvin monomethyl ether has also been shown to possess

cytotoxic activity at concentrations as low as 20 μg/mL, towards human A549, DLD-1 carcinoma and WS1 normal skin fibroblast lines (Simard *et al.*, 2008).

Conclusions

The antimicrobial activity of the dichloromethane partition of the methanolic extract of *C. peregrina* above-ground tissues has been demonstrated. The potent activity of pinosylvin monomethyl ether, almost 50-times more active against the yeast *C. albicans* and 29-times more active against the Gram-positive bacteria *S. aureus* than the crude extract, makes sweet fern a potentially valuable source of compounds with preservative properties. Moreover, sourcing biologically active compounds from this non-legume nitrogen fixer that is well adapted to marginal soil would provide economic incentives to protect its natural habitat. As an economically important plant, sweet fern has the potential to provide not only valuable bioactive compounds, but also improvements to the nitrogen content and soil quality where it is grown.

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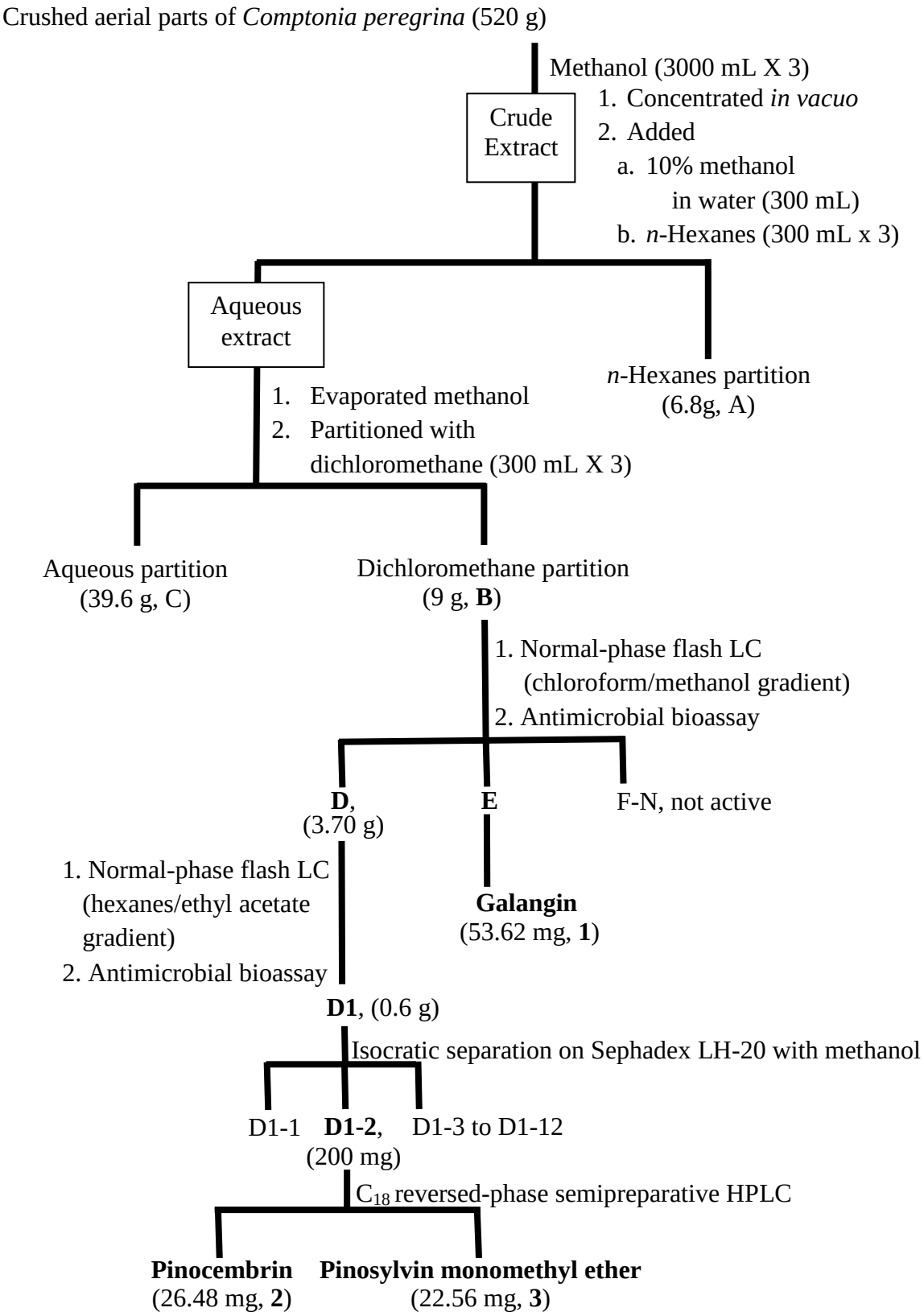


Figure 1. Solvent extraction scheme used for the aerial tissues of *Comptonia peregrina*.

Table 1. Antimicrobial activity of methanolic *Comptonia peregrina* extract partitions, fractions and isolated compounds

Partition/ fraction/compound	Weight (g)	*C. albicans*	*E. coli*	*P. aeruginosa*	*S. aureus*
DMSO (negative control)	--	NA	NA	NA	NA
Germall™ (positive control)	--	500†	125†	62.5†	46.9†
Crude extract	55.4	>500†	NA	NA	>500†
Partition **A**	6.8	>500†	NA	NA	>500†
Partition **B**	9.0	250†	NA	NA	125†
Partition **C**	39.6	500†	NA	NA	500†
Fraction **D**	3.7	125†	NA	NA	125†
Fraction **D1**	0.6	62.5†	NA	NA	31.25†
Galangin (**1**)	0.054	NA	NA	NA	NA
Pinocembrin (**2**)	0.026	65.88 ± 0.92‡	NA	NA	78.96 ± 9.47‡
Pinosylvin monomethyl ether (**3**)	0.023	10.15 ± 3.47‡	NA	NA	17.43 ± 6.91‡

†Activity is reported as the minimum amount needed for complete kill (in μg/mL), as determined by resazurin viability assay. ‡$IC_{50,}$ concentration inhibiting microbial growth by 50%, reported as μg/mL NA, antimicrobial activity not detectable at the highest concentration tested.

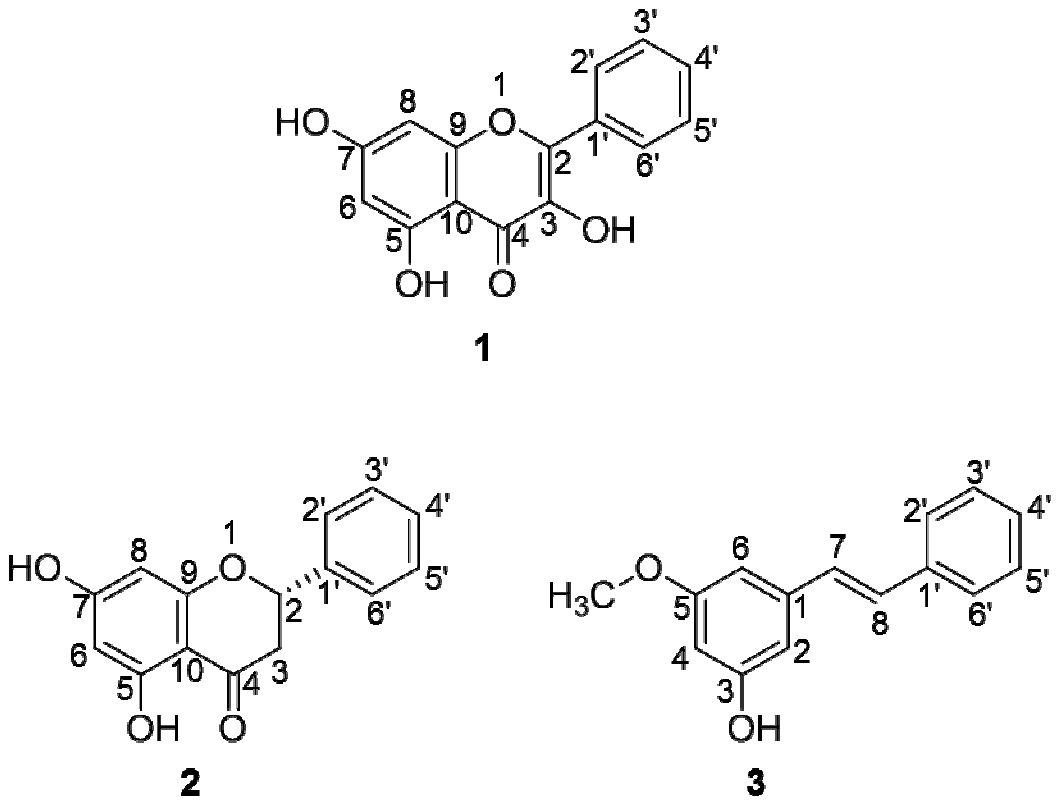


Figure 2. Structures of the compounds isolated from *C. peregrina* above ground parts. **1**) galangin, **2**) pinocembrin, and **3**) pinosylvin monomethyl ether.

Chapter 5

Developing native Minnesota American licorice (*Glycyrrhiza lepidota* (Nutt.) Pursh) germplasm for the cultivation of biologically active compounds

Summary

The genus *Glycyrrhiza* (licorice) contains many closely related species that have a long history of use as botanical medicines across the globe. Recently, there has been interest in cultivating these plants for medicinal uses rather than collecting them. Although there has been interest, licorice plants have never been cultivated commercially in the United States. There is only one licorice species native to the U.S., *Glycyrrhiza lepidota* (American licorice). American licorice thrives in marginal soil over a large part of the Northwestern U.S. and Canada. This three-year common garden study provides a first step towards the development of optimized *G. lepidota* germplasm to be grown in an agricultural setting for medicinal purposes. Nine populations of *G. lepidota* native to Minnesota were evaluated for 1) plant traits facilitating success in an agricultural setting, 2) high levels of antimicrobial activity against *Staphylococcus aureus* and, 3) the potential for additional biological activities via chemical diversity. A collection period to maximize antimicrobial activity occurring between mid-July and early August was established. Furthermore, two populations possessing desirable traits were identified as potential source material for the development of a further optimized *G. lepidota* cultivar.

Introduction

Cultivation of medicinal plants in an agricultural setting is an alternative strategy to collecting plants from the wild. This strategy has several advantages including: the development of optimized cultivars and conditions for bioactive compound production, increasing farmland biodiversity, sustainable sourcing of medicinal plants, creating opportunities for agro-tourism, and improved public image (Dharmananda 2000; Hayashi and Sudo 2009; Jordan *et al.*, 2007). Disadvantages of cultivation include a high initial cost for inputs such as land, germplasm development, agricultural equipment (including specialized equipment), and labor costs. Overall, the long term benefits of cultivating

medicinal plants are very worthwhile; therefore this strategy was modeled using a native Minnesota plant, *Glycyrrhiza lepidota* (Nutt.) Pursh (American licorice, Fabaceae) belonging to the medicinally popular licorice genus *Glycyrrhiza*.

Licorice Taxonomy

Plants of the *Glycyrrhiza* genus are commonly known as licorice, liquorice, sweet root, *gancao*甘草(Chinese), *kanzou* 漢方医学 (Japanese), and *yasti-madhu* (Sanskrit) (Nassiri and Hosseinzadeh, 2008; Zhang and Ye, 2009). The genus name is derived from the ancient Greek word *γλυκός* (*glykos) ρίζα (rhiza)* or 'sweet root', in reference to the highly active, sweet tasting triterpene saponin, glycyrrhizin (**A),** which is abundant in the roots of licorice plants (Fig. 1). The Greek name was later Latinized to *liquiritia* and eventually evolved into the present day name licorice (Fiore *et al.*, 2005; Isbrucker and Burdock, 2006).

As members of the Fabaceae family, plants belonging to the *Glycyrrhiza* genus are nitrogen fixing, leguminous shrubs, 70-200 cm in height. They occur mainly in sub-tropical regions including Greece, Turkey, Spain, Iraq, Caucasian and Transcapsian Russia, and northern China (Davis and Morris, 1991) growing wild or under cultivation, where little care is needed (Fenwick *et al.*, 1990). There are about 20 species of *Glycyrrhiza* including *G. aspera* Pall., *G. acanthocarpa* (Lindl.) J.M. Black, G. *bucharica* Regel, *G. echinata* L., *G. eglandulosa* X.Y. Li, *G. eurycarpa* P.C. Li, *G. foetida* Desf., *G. foetidissima* Tausch, *G. gontscharovii* Maslenn., *G. iconica* Hub.-Mor., *G. inflata* Batal., *G. korshinskyi* Grig., *G. lepidota, G. macedonica* Boiss, *G. pallidiflora* Maxim., *G. squamulosa* Franch., *G. triphylla* Fisch C.A. Mey, G. *yunnanensis* P.C. Li, and the two most important medicinally and economically, *G. uralensis* Fisch., and *G. glabra* (L.).

Traditional and Modern Use of Licorice

Licorice has a long history of being an economically and medicinally important plant (Davis and Morris, 1991; Fenwick *et al.*, 1990; Fiore *et al.*, 2005; Fukai *et al.*, 2002; Hayashi and Sudo, 2009; Isbrucker and Burdock, 2006). Licorice roots were found in ancient tombs of Egyptian pharaohs, including that of King Tutankhamun (Davis and Morris, 1991). The earliest written reference to licorice as a medicine is in the Assyrian Tablets *Codex Hummurabi* dating from 2100 $_{BC}$ (Davis and Morris, 1991; Fenwick *et al.*

1990). Ancient references to the medicinal properties of licorice include its healing effects on: ulcers, Hippocrates (430 $_{BC}$); asthma and wounds, Theophrastus (322 $_{BC}$); stomach, liver, and kidney diseases, Dioscurides '*De Materia Medica*' ($_{AD}$ 40-90) (Fukai *et al.*, 2002); and its use as a soothing throat lozenge and thirst quencher, Pliny the Elder ($_{AD}$ 79) (Davis and Morris, 1991; Fenwick *et al.*, 1990; Fiore *et al.*, 2005; Isbrucker and Burdock, 2006). Starting in the Middle Ages grocer-apothecaries combined licorice juice with honey and sugar to make sweets (Fenwick *et al.*, 1990; Hayashi and Sudo 2009).

Licorice has long been imported into the US mostly for use in the tobacco industry to flavor and cure tobacco for cigars, pipe tobacco, cigarette, snuff, and chewing tobacco (Brinckmann, 2003; Davis and Morris, 1991; Whitman, 1885). As early as 1884 almost 18 million kg of licorice roots, valued at $800 thousand USD, were imported into the US (Hunter, 1885). Slightly increasing about 19 million kg of licorice have been reported to be imported to the USA in the year 1952 from the Middle East, Spain, Russia, and East Africa (Davis and Morris, 1991). More recently, data from 2002 show licorice imports to the US, from Turkmenistan, Uzbekistan, Afghanistan, Azerbaijan, China, and Israel, to be more than 12 million kg of licorice root, valued at greater than 58 million USD and about 5 million kg of licorice root extract, valued at greater than 123 million USD (Brinckmann, 2003).

Although the tobacco industry remains the main user of licorice there are many other common modern uses, particularly of its below ground parts. In the pharmaceutical industry, licorice is used as an expectorant and demulcent in over the counter medicines and as filler for pills to enhance their consistency and surface coating (Davis and Morris, 1991). Minophagen Pharmaceutical Co. Ltd., located in Japan, has been producing a prescription drug to treat liver and allergy diseases prepared from licorice roots for over 60 years. This drug, widely used in Japan, China, Korea, India, and Mongolia, is available as an injection (Stronger Neo-Minophagen®) and tablet (Glycyron®) (Hayashi and Sudo 2009). In the confectionary and food industries, licorice is used as a flavoring and coloring for jam, marmalade, sauces, chewing gum, sweet sauces, diet cola (to mask bitter aftertaste of saccharin), reconstituted vegetable proteins, gelatin, pudding, cream, herbal tea, health drinks, chocolate (where it may replace up to 25% of cocoa), and beer and spirits (to improve foam stabilization and head formation) (Davis and Morris, 1991

Fenwick *et al.*, 1990; Hayashi and Sudo 2009; Isbrucker and Burdock, 2006). Other industrial uses of licorice include as an insecticide adhesive agent, an industrial process surfactant, a fire extinguisher component, fiberboard insulation, and in animal feedstuffs (Fenwick *et al.* 1990; Isbrucker and Burdock, 2006).

The long history of licorice use as a safe and effective traditional medicine has been globally validated (Fenwick *et al.*, 1990; Isbucker and Burdock 2006). Licorice and licorice derivatives, including ammoniated glycyrrhizin, are affirmed as "Generally Recognized as Safe" (GRAS) by the Flavor and Extract Manufacturers' Association (FEMA) and approved for use in foods by the U.S. FDA (21 CFR 184.1408) the Council of Europe, the UK Food Additive and Contaminants Committee, and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Licorice extract and its derivatives are approved for use in some over-the-counter drugs (21 CFR 310.528; 310.544; 310.545), and licorice is included as a GRAS ingredient in animal feeds (21 CFR 582.10; 582.20) (Fenwick *et al.*, 1990; Isbrucker and Burdock, 2006).

Licorice Chemistry and Biological Activity

The historical use of the below ground parts of licorice for their medicinal properties has been largely substantiated by modern scientific studies (Davis and Morris, 1991; Fiore *et al.*, 2005). In particular, the triterpene saponin, glycyrrhizin, has been shown to accumulate in the roots and has been reported to have potent biological activities including anti-viral, anti-ulcer, anti-inflammatory (Fig. 1) (Ammosov and Litvinenko, 2003; Beasley *et al.*, 1979; Fenwick *et al.*, 1990; Hayashi *et al.*, 2005; Hiraga *et al.*, 1984; Isbrucker and Burdock, 2006; Montoro *et al.*, 2010; Ozaki and Shibano, 2014). Licorice root remains to be one of the most widely used plants in traditional medicine practices including, Traditional Chinese Medicine (TCM), Ayruveda, and Japanese Kampo, where it is commonly combined with other medicinal plants to increase the distribution and absorption of botanical therapies in the body (Davis and Morris, 1991; Hayashi and Sado, 2009).

Studies have also shown that the above ground parts of licorice are rich in biologically active chemical constituents (Fenwick *et al.*, 1990; Scherf *et al.*, 2012; Siracusa *et al.*, 2011). Several bibenzyls have been isolated from *G. glabra* leaves and are reported to have anti-inflammatory, anti-oxidant, and antimicrobial activity (Fig. 1, **B-E**)

(Asl and Hosseinzadeh, 2008; Biondi *et al.*, 2005; Siracusa *et al.*, 2011). Additionally, the antimicrobial and anticancer flavanone glucoside, liquiritin (**F**), has been isolated from *G. glabra* and *G. inflata* aerial parts (Fig. 1) (Fukai *et al.*, 2002; Hiraga *et al.*, 1984; Isbrucker and Burdock, 2006; Liao *et al.*, 2012; Nomura and Fukai, 1998).

Developing a use for licorice leaves, which are currently a composted or discarded byproduct of root harvesting, would be logistically and economically attractive. Bioactive compounds could be extracted from plants already being processed for other purposes. Even though certain species, for example *G. glabra*, are well studied, new biologically active compounds are being discovered in plants from the same genus (Ammosov and Litvinenko, 2003). For example, it has been shown that *G. glabra* and *G. lepidota* have different active constituents, including the antimicrobial prenylated bibenzyls, glepidotin C (**G**) and D (**H**) and prenylated flavanols glepidotin A (**I**) and B (**J**), isolated from *G. lepidota* aerial parts (Fig. 1). (Biondi *et al.*, 2005; Gollapudi *et al.*, 1989, Manfredi *et al.*, 2001, Mitscher *et al.*, 1987 & 1993) In general, licorice plants include chemical constituents belonging to a variety of classes, but are particularly rich in polyphenolics and terpenes, especially prenylated compounds. Appendix C summarizes the main chemical constituents found in licorice, including the species and plant part of discovery and any notable biological activities.

Agricultural Use of Licorice

Cultivating medicinal plants may provide a way to have a steady supply of plant material for extracting compounds of interest in a controlled setting (Hayashi and Sudo 2009). A controlled setting is important since the composition of plant extracts depends upon a variety of factors including, genetics, processing, plant cultivar, and growing climate (Fenwick *et al.*, 1990; Isbrucker and Burdock, 2006). Globally, there has been interest in the cultivation of licorice as a medicinal plant (Dharmananda, 2000; Hayashi and Sudo 2009; Hunter, 1885; Ozaki and Shibano, 2014; Whitman, 1885). Recently, in Japan, a new high performing cultivar of *G. uralensis* (Chinese licorice) was developed by crossbreeding two proprietary strains with the complementary characteristics of high glycyrrhizin content (strain A-19) and vigorous growth (strain G-6) (Ozaki and Shibano, 2014). The transition to cultivation from collection of *G. uralensis* in China was initiated in the early 2000s as an alternative to the overharvesting of the wild population to ensure

that a steady supply of high quality licorice would be maintained (Dharmananda, 2000; Hayashi and Sudo, 2009). A dispersion cultivation method, which distributed the land planted in licorice, the growing responsibly, and the risk over a large area and among many people was employed. Specifically, about 600 households transitioned a quarter of their five-acre lots to licorice production. Collection standards were put in place to ensure that harvested mature roots meet the *Chinese Pharmacopoeia* 2000 Edition standards, including for example, minimum root diameter and length 0.7 x 30 cm and the following root preparation instructions: 10 mm oblique slices with brown bark, yellow cortex and pith (Dharmananda, 2000). In addition to environmental benefits, the positive consequences of this program included supply stabilization, species and cultivar homogeneity, and uniformity of harvested plant material. The approach also helped standardize harvest time and post-harvest processing methods, while increasing yields helped to support developing markets (Dharmananda, 2000).

To maximize economic and ecological benefits, a plant species targeted to produce extractable biologically active compounds must undergo germplasm optimization (Hayashi and Sudo 2009). For example, researchers in Japan are developing licorice cultivars with the following characteristics: underground parts that grow vigorously, high glycyrrhizin content, and erect aerial parts (Ozaki and Shibano, 2014). Other plant traits may be evaluated such as biomass production, flowering time, multi-year survival rate, and seed viability to ensure that a selected cultivar will thrive in a cultivated system. Additionally, localization of active compounds to specific plant parts, optimized cultivation conditions, harvest time, and post-harvest processing procedures must also be determined. Bioactivity screens can be used to select a specific cultivar whose extracts are maximally active. Chemical screens are important to ensure consistent production of target compounds. They can also be used to search for novel compounds in chemically diverse plant extracts.

American Licorice

As the only North American native licorice species, *G. lepidota*, is an ideal plant to use in a Minnesotan agricultural setting for the cultivation of bioactive compounds. Although similar to the other more commonly used species including *G. glabra* and *G. uralensis*, American licorice is currently not widely used. Interest in cultivating licorice

in the US go back as far as 1885 when representatives from the US Department of State, noting the large amount of licorice being imported into the US, sent out circulars and reports inquiring about such topics as: whether imported licorice grows wild or is cultivated; the soil type and climate best suited to it growth; the mode and manner of its cultivation (Hunter, 1885; Whitman, 1885). In spite of this early interest in growing licorice domestically, a 2003 International Trade Centre US Market Brief, noted the lack of commercial cultivation of licorice in the US (Brinckmann, 2003). Botanically, *G. lepidota* is an erect perennial growing up to 90 cm in height. It has a stem covered with minute, sticky hairs, cream-colored flowers crowded on a terminal spike, and pinnately compound leaves. Its brown fruit is covered with hooked spines and resembles a cocklebur. American licorice is flood tolerant and can be found in prairies, stream valleys, and roadsides across western USA and Canada.

The historical use of American licorice plant by indigenous peoples of North America is well documented (Gilmore, 1997; Kindscher, 1987; Manfredi *et al.*, 2001; Munson, 1981; Weiner, 1990). It has been shown that American licorice was in the diet of prehistoric American populations (Moore, 1979). The earliest indication of its use in North America is via the detection of its odors from coprolites (fossilized feces) dating back to before 4000 $_{BC}$ possibly from the ingestion of *G. lepidota* (Moore *et al.*, 1984). Various traditional medicinal uses of American licorice have been reported. A tea of its peeled dried roots or leaves was used to relieve diarrhea and upset stomach, coughs, chest pain, and sore throat (Kindscher, 1987). Licorice root tea was also taken to speed the delivery of the placenta (Weiner, 1990). It was given as a cooling drug for fevers to relieve thirst and burning sensations (Muson, 1981). Chewing the root or holding it in the mouth was used for toothache (Kindscher, 1987). Leaves steeped in water were used for earache (Weiner, 1990). Licorice root and seed powder were taken with milk for use as a body tonic and aphrodisiac (Weiner, 1990). Externally, it was applied with honey for cuts and wounds (Munson, 1981). The powdered root was also used as a sweetener in other herbal remedies and teas (Gilmore, 1997; Kindscher, 1987).

Although there are few modern day studies reporting on the biological activity of constituents found in *G. lepidota*, compounds belonging to a variety of chemical classes have been isolated from this plant including flavanones, prenylated flavanols, prenylated

bibenzyls, and triterpene saponins (Ammosov and Litvinenko, 2007; Gollapudi *et al.*, 1989, Manfredi *et al.*, 2001, Mitscher *et al.*, 1983 Siracusa *et al.*, 2011). One group reported high to moderate antioxidant activity for ethanolic extracts of American licorice roots; however, no further attempt to identify the anti-oxidant constituents was carried out (Amarowicz *et al.*, 1999 & 2004). They measured the antioxidant activity a number of ways including *β*-carotene linoleate, reducing power and free radical scavenging assays and electron paramagnetic resonance spectroscopy. A study by Manfredi *et al.*, (2001) reported that extracts of *G. lepidota* leaves and stems showed moderate activity in the US National Cancer Institute *in vitro* anti HIV-1 bioassay. In this study a novel diprenylated bibenzyl, glepidotin D (**H**), (Fig. 1) was discovered and shown to have anti-HIV activity that was an order of magnitude greater than the organic extract (dichloromethane: methanol (1:1)), where the aqueous extract was not active (Manfredi *et al.*, 2001).

When compared to other *Glycyrrhiza* species, *G. lepidota* is a particularly interesting case, both chemically and phylogenetically. In the mid-1960s, the Russian researcher, Kruganova, proposed to subdivide the *Glycyrrhiza* genus into true licorice (*EuGlycyrrhiza Boiss.*) and pseudolicorice *(PseudoGlycyrrhiza Regel. Krug.)*, based on chemical differences; this systematic delineation persists today (Ammosov and Litvinenko, 2003). The species *G. uralensis*, *G. glabra*, *G. inflata*, and *G. aspera*, are all classified as true licorices. These true licorices are characterized by the presence of oleanane-type triterpenes saponins with a *β*-amyrin biosynthetic intermediate, including such compounds as glycyrrhizin comprising 10-15% or more in underground parts and licorice-saponin H2 (Hayashi *et al.*, 2005) The species *G. foetidissima*, *G. echinata*, *G. pallidiflora*, and *G. macedonica*, are classified as pseudolicorices. These pseudolicorices are characterized by the absence of glycyrrhizin and the presence of triterpenoic acids such as macedonic acid (macedonoside A & C) and echinatic acid (Ammosov and Litvinenko, 2003; Hayashi *et al.*, 2005).

Taxonomically, pseudolicorice species are more primitive or unchanged from their original, ancestral form than true licorice species (Ammosov and Litvinenko, 2003; Hayashi *et al.*, 2005). The split between true and pseudolicorice species is thought to have occurred at the beginning of the Neogene period (23.03mya – 2.58mya) when a

protoGlycyrrhizae species, formed during the Paleogene period (66mya-23.03mya), began to differentiate into the two subdivisions. *G. lepidota* is closely related to the primary *proto* species due to its Neogene migration (Ammosov and Litvinenko, 2003). A study by Hayashi *et al.*, (2005) showed that *G. lepidota* is chemotaxonomically an intermediate species between true and pseudo licorices. This finding was based on phylogenetic analysis of chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcL*)) nucleotide sequence data and a comparison of chemical profiles. Both licorice-saponin H2 and small amounts of glycyrrhizin (saponins produced by true licorice species) as well as macedonoside A and small amounts of macedonoside C (saponins produced by pseudo licorice species) were isolated from *G. lepidota* (Hayashi *et al.*, 2005). The location of *G. lepidota* as an intermediate species between true and pseudo licorice further supports the potential for this species to contain a wealth of chemical constituents that may be useful in diverse industries. As a first step towards developing optimized germplasm for cultivation of biologically active compounds in an agricultural setting, six geographically isolated populations of Minnesota native *G. lepidota* were evaluated for traits important for cultivation, biological activity, and chemical diversity in a common garden setting.

Materials and Methods

Chemical Reagents. HPLC grade solvents and reagents purchased from Sigma Aldrich (St. Louis, MO, USA) were used including: acetonitrile, dichloromethane, ethanol (95%), ethyl acetate, formic acid, hexanes, 2,3,5-triphenyl-tetrazolium chloride (tetrazolium). Germall™ was obtained from Fisher Scientific (Pittsburgh, PA, USA), dimethyl sulfoxide (DMSO) from J.T. Baker (Center valley, PA, UA), and tryptone soy broth (TSB) from BD (Franklin Lakes, NJ, USA). Standard reverse osmosis deionized glass distilled water was obtained in house using a Thermo Scientific Barnstead B-pure™ filter and Distinction water still model D4000 (Bibby Scientific Limited, Stone, Staffordshire ST15 0SA, UK).

Common Garden. A common garden on the University of Minnesota, Saint Paul campus was established to facilitate the growth and evaluation of nine different populations of *G. lepidota* in an agricultural setting.

Common garden design. A randomized complete block design with six replicates was used. Nine individual plants from each population were placed 30.48 cm apart on all sides in the center of each 1.5 m^2 subplot for a total of 54 plants from each population planted into the entire block. The 0.76 m wide alleys and 3 m wide borders were planted with matrix grasses using seed acquired from Prairie Moon Nursery® (PMN, 32115 Prairie Lane Winona, MN 55987). The matrix grasses included *Bouteloua curtipendula* (Michx.) Torr. (sideoats grama), *Bromus kalmia* A. Gray (Kalm's brome), *Panicum oligosanthes* Schult. (Scribner's rosette grass), and *Koeleria macrantha* (Ledeb.) Schult. (Prairie June Grass)

Plant Collection. Scissors and clippers were used to collect *G. lepidota* seedpods from established prairies into location specific brown paper bags in fall of 2010 from nine locations distributed north to south in western Minnesota (Table 1). The prairies were located on Minnesota Department of Natural Resources, Scientific and Natural Areas (SNAs), The Nature Conservancy Areas (TNC), and roadside with high quality prairie ecosystems. All required permits, which fully described environmentally sustainable collection practices, were acquired prior to the collection of plant material and included, for example, that no permanent objects would be used to mark plant population locations, but instead only written descriptions and GPS coordinates were used (Appendix C).

Plant Propagation. All subsequent activities were carried out in such a way as to isolate the nine different populations from one another. This isolation facilitated confident assignment of biological and chemical observations to plants from specific populations so that population level differences could be identified. Species authentication was performed by Kevin Betts, Senior Scientist, Department of Agronomy and Plant Genetics, University of Minnesota, Saint Paul, MN, USA (UMN) and population specific voucher specimens were assigned the

specimen code AV0030 and deposited in the Department of Horticultural Science UMN.

Immediately following collection, the samples were placed in a 30°C drying oven for three days after which the seed was manually separated from the chaff. The cleaned seed was scarified using a modified belt grinder fitted with coarse sand paper, mixed with inoculum for *Glycyrrhiza spp.* (PMN), placed on moistened blotter paper in plastic petri dishes, and cold stratified for thirty days at 4°C. Following cold stratification, rhizobia-inoculated seeds in petri dishes were placed in a greenhouse maintained at 30°C, where they were kept moist until they germinated. Germinated seeds were placed on soil filled RLC4 "Cone-tainers"™ (Ray Leach, Stuewe & Sons, Tangent, Oregon), covered with wet butcher paper, and watered daily until seedlings were established. The plants were allowed to grow in the "Cone-tainers"™ until their roots were sufficiently established to allow removal of the whole plant without significant root damage. In June of 2011, these plants were manually transplanted into an outdoor common garden located on the UMN Saint Paul field station, where they were hand watered and hand weeded until they were established. The plot was maintained as a pesticide and herbicide free plot with hand weeding and rain water supplemented by irrigation when necessary from establishment in 2011 to summer 2014.

Common Garden Treatments. The licorice growing in the common garden was collected according to population at several time points in 2012 and 2013. The time points included **A**: June 24, 2013; **B**: July 18, 2013; **C**: August 5, 2013; **D**: August 26, 2013; **E**: September 14, 2012; **F**: September 16, 2013; and **G**: October 15, 2013. This material was kept separated according to population and sample date and the following were evaluated: I. individual populations sampled over time; II. all populations sampled at a single time point; and III. Above ground material compared to below ground material collected at time point **G**. The evaluations carried out include plant traits, antimicrobial activity assays, and metabolic fingerprinting, and are described below.

Plant Traits evaluation

Three-year survival rates. On September 4, 2013, the specific spots where transplants were placed in the soil in 2010 were located and the presence or absence of living plants was recorded. There were originally nine transplants planted in 2010. The following equation was used to calculate the three-year survival rate: (Number of living plants in 2013÷9) ×100.

Total biomass evaluation. Above ground biomass was measured from material collected at time points **E** and **G**. All *G. lepidota* material present above ground in each subplot was collected into a paper bag resulting in six replicates per population. Care was taken to remove any soil, insects, or other plant material. Dry weights were measured after the material was dried in a 30°C drying oven for three days. Below ground biomass was measured from material collected at time point **G**. Prior to collecting above ground material the location of each *G. lepidota* plant was flagged to facilitate removal of below ground parts. Using a garden shovel the subplots were completely dug out and all rhizomes, stolons, and roots from an individual subplot were collected into large paper bags. This material was then thoroughly washed with water to remove all soil and insects and placed into clean paper bags. Dry weights were measured after the material was dried in a 30°C drying oven for three days.

Number of inflorescences and seedpods. The number of inflorescences and seeds pods was manually counted for all of the plants each specific subplot. Counts were carried out on the following dates: June 25, 2013; July 9, 2013, and July 19, 2013.

Seed-mass evaluation. The seedpods for each subplot was collected into a paper bag at time point **F** and dried in a 30°C drying oven for three days. The biomass of the intact seedpods was measured. Afterwards each seed pod was opened and the number of seeds contained within was counted. The ratio of individual seed biomass to total biomass was calculated by combining the measurements for below and above ground biomass and

dividing the approximate weight of an individual seed (population average seed biomass ÷ average number of seeds per seedpod) by this number.

Seed viability. Tetrazolium bioassays were carried out to evaluate the viability of seed collected at time point **F** and processed as described above. A 1.0% solution of tetrazolium in water was freshly prepared (pH 6.0) for each experiment. Seeds bulked according to population were randomly placed into three replicates of 50 seeds each. The seeds were mechanically scarified three times before being submerged in 3.0 mL of 1.0% tetrazolium solution and placed in the dark at 40°C for 24 hours. Following incubation and removal of the tetrazolium solution the seeds were rinsed three times with distilled water. The seeds were carefully cut in half using a razor to facilitate visual evaluation of stain pattern and intensity. Viable seeds cotyledons, embryos, and emerging radicles stained pink or dark red. Non-viable seeds showed no stain or a gray color (Patil and Dadlani, 2009).

Statistical Analysis. For each plant trait evaluation, the individual plants from each subplot were combined resulting in a total of six replicates per population. Each population was analyzed individually and data is reported as mean (n = 6) ± standard error. Analysis of variance (ANOVA) was performed using 'R' version 2.15.2. Means were compared using Tukey's HSD and a p-value < 0.05 was considered to be significant ('R' package Agricolae 1.1-4).

Antimicrobial activity evaluation. The antimicrobial activities of above and below ground material according to population and collection time were evaluated.

Extract preparation. For each population, above ground material was collected at all-time points and below ground material was collected at time point **G** and processed as described above (*Biomass evaluation.)* The dry material was ground in a Thomas Wiley laboratory mill model 4 (Thomas Scientific, Swedesboro, New Jersey, USA) using a 6 mm screen and then stored in sealed opaque containers kept at room temperature until extraction. Dry ground plant material in the amount

of 250 mg was placed into 2 mL microcentrifuge tubes and 1.5 mL of aqueous ethanol (ethanol: water, 70:30 v/v) was added. The tubes were individually mixed using a Fisher Scientific fixed speed mini vortexer (Scientific Industries Inc. Bohemia, NY, USA). After which extraction was allowed to proceed for four hours with agitation using a 2010 Geno/Grinder® (SPEX Sample Prep, Metuchen, NJ, USA) set at 700 RPM. Following extraction, the tubes were subjected to centrifugation using an Eppendorf 5415C centrifuge (Brinkman Instruments, Westbury, NY, USA) at 4000 g for 5 min. The supernatant was removed to a clean tube and concentrated to dryness *in vacuo* (using a Savant model SVC-200H SpeedVac concentrator; Farmingdale, NY, USA). Extract residue was placed in sealed vials at 4ºC in the dark prior to bioassay. For each sampling date there were six biological replicate extractions.

Antimicrobial bioassay. The antimicrobial activities of the above extracts against *Staphylococcus aureus* (ATCC 6538) were measured using a 96-well plate broth dilution method. The extract concentration at which 50% of the bacterial growth was inhibited (IC_{50} value) was determined using a method adapted from Weigland *et al.*, 2008. Briefly, extract residue was dissolved in 100% DMSO and working stock solutions of 1 mg/mL (0.1%) were prepared in TSB. Extract solutions were serially diluted with TSB to concentrations ranging from 500 μg/mL to 0.98 μg/mL, and 100 μL of each solution was added to an empty well of a 96-well plate. *S. aureus* bacteria were grown overnight with agitation at 32°C and diluted into TSB to an optical density of approximately 0.5 using a MacFarland standard (Becton Dickinson and Company, Microbiology Systems, Sparks, MD). This solution was further diluted 1:100 with TSB to give a bacterial concentration of ~10^6 colony-forming units (cfu)/mL and 50 μL was added to the extracts in each well of the 96-well plate for a final *S. aureus* concentration of 5×10^5 cfu/mL. Dilutions of Germall™, extract free DMSO, and TSB were used as positive, negative, and blank controls, respectively. All bacterial, extract, and chemical solutions were used within 30 minutes of their preparation. After 24 hours of incubation at 32°C, with shaking during the last hour, bacterial growth was

evaluated by measuring the optical density of the individual wells at 520 nm. IC_{50} values were determined by non-linear regression analysis in GraphPad Software (LaJolla, California) using a custom designed program to subtract blanks, log transform the concentration values, and normalize the optical density values. Six biological replicates were carried out for each sampling date and data were reported as mean values (n = 6) ± standard errors. Analysis of variance (ANOVA) was performed using 'R' version 2.15.2. Mean IC_{50} values were compared using Tukey's HSD and a *p*-value < 0.01 was considered to be significant ('R' package Agricolae 1.1-4). Bulk population IC_{50} values at each time point were also reported.

Metabolic fingerprinting. The effects of population and sampling date on chemical diversity were evaluated by liquid-chromatography mass spectrometry (LC-MS) based metabolite fingerprinting.

Extract preparation. Eight individual replicates of above ground material were collected from each of the six surviving populations at each time point **A** to **G**. Additionally, at time point **G**, eight individual replicates were prepared from the below ground material collected from each population. The identical method described above was used to process the collected material and prepare the extracts for metabolic profiling. The only exception is that extracts for metabolic profiling were not dried *in vacuo* instead they were diluted 1:10 with 70% ethanol (ethanol: water, 70:30 v/v).

LC-MS data acquisition. Metabolic fingerprints were generated using C_{18}-reversed-phase ultra-performance liquid chromatography-electrospray ionization-hybrid quadrupole-orbitrap mass spectrometry fitted with an autosampler maintained at 4ºC (Ultimate® 3000 HPLC, Q Exactive™, Thermo Scientific). Separations were carried out on a reversed-phase C_{18} HSS T_3 1.8 μm particle size, 2.1x100 mm column (Waters). Column temperature was 40°C, solvent flow rate 0.45 mL/min, injection volume 1 μL. A 20-minute gradient using mobile phases A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile was run according to the following gradient elution profile:

initial, 15%B; 1 min., 15% B; 2 minutes, 50% B; 15 minutes, 98% B; 16 minutes, 98% B; 16.5 minutes, 15% B; 20 minutes, 15% B. The following MS conditions were used: full scan mass scan range: 130-1000 *m/z*, resolution: 35,000, data type: profile, desolvation temperature 350°C, capillary voltage: 3800 V (+), 3300 V (-). Xcaliber™ software version 2.1 (Thermo Scientific) was used to record the chromatograms and spectra. To check for precision and accuracy throughout this analysis of this large batch of samples, control samples were prepared including: solvent blanks, periodic quality control (QC) samples, containing compounds that generate several known ions, and a mixed sample pool, consisting of a small amount of extract from all 490 samples. Additionally, because of the large number of experimental replicates the following randomized complete block design was used to organize this analysis:

Sequence	**Run Order**	**Sample Description**
Opening sequence	1	Solvent blank 1
	2	Solvent blank 2
	3	Solvent blank 3
	4	QC 1
	5	Mix 1
	6	Mix 2
Block sequence	7	Solvent blank 4
	8-37	Randomized licorice extract samples
	38	Solvent blank 5
	39	Mix 3
	40-70	Randomized licorice extract samples
	71	QC 2
Main run	72-528	Block 2- Block 8
End sequence	529	Solvent blank post run
	530	Solvent blank end
	531	Wash n' store (10 minute 98% acetonitrile wash)

Feature Detection. LC-MS data were subjected to feature detection for comparison of extract chemical diversity. A custom workflow for feature detection was designed using *Refiner MS* version 7.5 software (GeneData, Lexington, MA, USA). The following data processing steps were performed: $10e^5$ intensity thresholding; chemical noise reduction using a retention time (t_R) window of 51 scans with minimum t_R length 4 scans and minimum *m/z* length 3 points; t_R

alignment with *m/z* window = 5 points, t_R window 5 scans, and t_R search interval 50 scans. Feature extraction was carried out on the aligned dataset using the indicated parameters: chromatogram summed peak detection with minimum peak size of 4 scans and curvature-based peak detection; t_R tolerance = 0.1 s, *m/z* tolerance = 0.05 Da, signal-to-noise ratio of extracted mass features was ≥ 3.

Multivariate Statistical Analysis (MVA). Feature lists were transported to *Analyst* version 7.5 software (Genedata) for MVA. The feature lists were inspected and a feature was included in the analysis if present in greater than 75% of replicate samples with similar intensity in all replicates for each population or collection date**.** High confidence feature lists were exported to Microsoft Excel 2010 software where scatterplots showing the distribution of features detected from extracts made from different populations and from different collection time points were generated.

Results and Discussion

Three-year survival rates. Initially, nine populations of *G. lepidota* collected in fall 2010 from established prairie ecosystems were germinated in a greenhouse and transplanted into a field plot in Saint Paul, MN (Table 1). Similar to previous studies showing that, depending on the collection year, field collected *G. lepidota* seeds can have high germination rates; greenhouse germination generated at least 54 seedlings per 100 seeds for each population for transplantation into the common garden (Whitman, 1979). The seedlings, initially planted in the spring of 2011 developed slowly and during summer 2012 only produced vegetative material. The survival rate of each of the nine populations was measured in the spring of 2013 and of the nine populations only six maintained a three-year survival rate over 50 percent (min. 52 percent, max. 80 percent) (Fig 2.)

Seeds from the six successfully established populations had been collected from sites distributed widely across the geographic sampling area. The population from Faribault County roadside (21) exhibited the maximum survival rate for all nine populations of about 80 percent, although this rate was not significantly

different from any of the other five populations with survival rates greater than 50 percent. Only two populations, collected from Glynn Prairie SNA (28) and Lyon County Roadside (31), had zero percent survival, where no living plants could be detected. Seeds from these unsuccessful populations were collected from both a protected SNA site and roadside site located in southwest Minnesota (Table 1). It has been previously shown that greenhouse emergence and subsequent field establishment of *G. lepidota* is highly variable (Boe and Wynia, 1985, Whitman, 1979; Wynia *et al.*, 1981). Different populations of field collected *G. lepidota* seed native to North Dakota exhibited a range in greenhouse emergence from 0 to 97 percent (Boe and Wynia, 1985). Once transplanted into the field, certain populations that at first established quickly, later became chlorophyll deficient and died (Boe and Wynia, 1985). In this study, all subsequent measurements were performed on plants from populations with survival rates greater than 50 percent. Although the survival rate of plants collected from Brown county roadside (24) was not significantly different from several other more successful populations, this population was omitted from further analysis due to the insufficient number of live plants available.

Total biomass evaluation. Total biomass differences were evaluated for the six successfully established populations. Above ground biomass was measured in 2012 and 2013 and below ground biomass was measured only in 2013. While there were no significant differences in total above or below ground biomass among the *G. lepidota* populations a trend based on geographic origin is apparent. Those populations originating from more northern collection sites (2,7, and 8) tended to have higher average shoot biomass than those originating from southern collection sites (16, 20, and 21) (Fig 3.) This trend for increased shoot biomass in northern collected populations was consistent between 2012 and 2013. Because biologically active compounds would potentially be sourced from above ground materials, a larger shoot biomass may lead to increased yield of valuable extractable chemicals. All six populations increased their average shoot biomass in 2013, after an additional year of growth. The range of average shoot biomass in 2012 was 25 to 31 g and in 2013, 37 to 64 g. The increased range of shoot biomass

values may be due to the fact that by the third year of growth, differences according to specific populations (2, 7, 8, 16, 20, and 21) were more pronounced.

Total root biomass was more consistent regardless of differences in above ground biomass. Plants sourced from Ottertail Prairie SNA (7) had the highest average shoot biomass in 2012 and 2013, 31 g and 64 g, respectively and the highest average root biomass of 84 g, although these measurements were not statistically higher than shoot or root biomass for any of the other five populations. Extensive root systems in *G. lepidota* have been noted to help prevent erosion and nutrient leaching, even where the soil quality is marginal (Weaver, 1954; Allen and Allen, 1981; Duke, 1981; Whitman, 1979). Populations that most quickly establish large root systems may be better able to take in soil nutrients potentially leading to better field establishment. Regardless of differences in above ground biomass, all six of the populations had very similar average root biomass.

Number of inflorescences and seedpods. During the common garden establishment year in 2011 and in the first year of growth in 2012 only vegetative shoot material was observed. This observation is similar to results reported by Wynia *et al.*, (1981) where very little flowering and subsequent seedpod set were observed in field planted *G. lepidota* seedlings in their second year of growth. In spring and summer 2013, the plants were observed frequently to record the occurrence of any flowering or seed set, which would indicate that the plants had reached reproductive maturity. Inflorescences were observed in late July on plants from all populations, although plants from population 7 showed significantly higher numbers than plants from any other population (Fig 4). The transition from flowering to seed-set occurred over about a one-month time period, where seedpods were present on plants from all six populations by mid-July.

Seed-mass evaluation. To increase population size a plant must allocate a significant portion of resources to generate seeds. These resources may, however, be in direct competition with those required for production of phytochemicals. *G. lepidota*, like other *Glycyrrhiza* species, produces seeds enclosed in clusters of small burrs (spiked seed pods) that act to protect and transport the seeds away from their parent plant as they attach like Velcro to animal fur or clothes of people passing closely by (Duke, 1981). We

observed significant differences in the average biomass of intact seedpods and in the average number of seeds contained in each seedpod (Fig. 5). In general, average seedpod biomass increased with the average number of seeds indicating that the increase in seedpod biomass was from the number and not the size of seeds. Furthermore, we observed a large amount of insect infestation in seedpods collected from population 16 (Redwood County Roadside) plants, which also had the largest average seedpod biomass and seed count, although many of the seeds appeared to be unviable. Desiccated seeds, from population 16 plants, with obvious insect damage, including holes, were omitted from subsequent seed viability tests. Insect predation is a potential threat to the success and expansion of licorice populations (Boe *et al.*, 1988; Boe and Wynia, 1985). *G. lepidota* seedpods are susceptible to infestation, which can greatly reduce the number of viable seeds for a population (Boe and Wynia, 1985). The size of individual seeds remained fairly constant among seed from all observed populations. However, the ratio of individual seed mass to total biomass showed that plants from population 20 (Martin County Roadside) allocated a larger percentage of the total biomass to each individual seed than any other population (Fig. 5c). Seed collected from population 20 consistently produced smaller plants and fewer but proportionally larger seeds.

Seed viability. Population differences in percent seed viability were similar to the differences in average number of seeds per seedpod. All populations exhibited greater than 60 percent seed viability, however, seed from plants sourced from population 2 (Pembina Trail Preserve SNA) had significantly lower percent seed viability (62 percent) than all other populations as well as the lowest seed count of 28 seeds per seedpod (Fig. 6). Seed collected from plants sourced from population 20 had the second lowest seed count (66 seeds per seedpod), proportionally the largest size, and showed the highest percent seed viability of 88 percent, among the six populations, although not significantly different from the percent viable seed from population 16 plants. Only population 16 seeds without obvious insect damage were tested for viability; including all seeds collected from population 16 plants for viability testing, regardless of visible damage, may have resulted in lower percent viable seeds from population 16 plants. Collecting and storing viable seed is a convenient way to save genetic material of important

populations. However, *G. lepidota* can also reproduce vegetatively. A study by Boe and Wynia (1985) reported low rates of flowering and seedpod set in populations of field planted *G. lepidota*, but prolific rhizome production. Vegetative reproduction of optimized plants would ensure that selected traits would be passed on in the genetically identical offspring.

Antimicrobial activity evaluation. Initial screening of bulk 70 percent ethanol *G. lepidota* extract made from above and below ground material showed good antimicrobial activity against *S. aureus*. IC_{50} values were used to measure differences in antimicrobial activity among populations, between shoot and root materials, and across sampling dates. *G. lepidota* root material from all populations showed no activity at any concentration measured up to 500 μg/mL. Crude 70 percent ethanolic extract of above ground *G. lepidota* material from all populations showed good antimicrobial activity ranging from 58 to 178 μg/mL (Fig. 7). The observed difference in activity between roots and shoots provides evidence that the antimicrobial compound(s) active against *S. aureus* are mostly distributed into above ground material. It has been previously shown that chemical defense compounds can be differentially distributed among roots and shoots (Kaplan *et al.*, 2008; Parker *et al.* 2012). Extracts made from plants sourced from populations 7 and 20 displayed the lowest IC_{50} values of 63 and 58 μg/mL, respectively. The observed differences in activity level for each population were not statistically significant. This is likely due to the large amount of variation in activity level among plants collected from the same population over time.

The antimicrobial activity of *G. lepidota* shoot extract increased from late spring to early August, when the maximum activity was observed. Antimicrobial activity subsequently decreased in late summer and remained at a constant level through fall; however the activity never decreased to the low levels measured in the spring (Fig. 7). The antimicrobial activities measured in the fall at collections points **E** and **F** are remarkably consistent. This year-to-year consistency provides evidence that the pattern of activity level measured throughout the collection season is reproducible. Low IC_{50} values ranging from 49 to 15 μg/mL were measured from extracts prepared from plants collected at time point **C**; plants from population 20 displayed the lowest IC_{50} value of 15

μg/mL. Decreasing values ranging from 195 to 23 μg/mL were measured from plants collected at the proceeding time point **B**, where again population 20 exhibited the lowest IC_{50} value at that time point.

The observed increase in antimicrobial activity followed a timeline similar to that of seed development. Maximum antimicrobial activity was measured once seedpods were present on all plants. The antimicrobial constituents present in the shoot material may act to deter insect herbivory of seeds (Parker *et al.*, 2012). Plants sourced from population 20 exhibited the lowest number of the proportionally largest seeds and the highest antimicrobial activity. Larger seeds with increased amounts of stored nutrients may exhibit higher germination and establishment rates. Plants sourced from population 20, which were smaller in overall size, invested a relatively larger amount of resources into producing fewer, larger seeds and the chemical defenses to protect them. Parker *et al.*, (2012) reported that slower growing genotypes of *Oenothera biennis* L. (common evening primrose) allocated more resources to above ground chemical defenses possibly to protect against herbivory.

The observed strategy employed by population 20, relative to the other five populations, can be somewhat explained by the resource availability hypothesis (RAH). The RAH predicts that slower growing plants will have higher amounts of constitutive defenses and support lower herbivory rates (Endara and Coley, 2011). Although the RAH provides rational for the life strategy of *G. lepidota* plants sourced from population 20 it cannot be applied to all of the populations. For example, fast growing, large plants sourced from population 7, exhibited an average number of average sized seeds and also a relatively low average IC_{50} value of 63 μg/mL. Likewise, plants sourced from population 16, which produced the largest amount of seeds, exhibited moderate antimicrobial activity (average IC_{50} value, 99 μg/mL), but displayed a high level of insect infestation. Additionally, seeds from population 16 plants had a high percent seed viability of 81 percent, second only to seeds from population 20 plants (88 percent), but this number only accounts for healthy looking seeds collected from population 16 plants. The production of a large amount of viable seeds susceptible to infestation combined with the relatively good antimicrobial activity exhibited by plants from population 16 is in contrast to the RAH. In general, the optimal plants for extracting bioactive compounds

would include a combination of traits from population 7 and population 20. These traits include, the accumulation of large amounts of bioactive compounds, evidenced by low IC_{50} values, combined with fast growing, large plants that do not allocate proportionally large amounts of energy to seed production.

Metabolic fingerprinting. Ideally, a cultivar of *G. lepidota* targeted for extraction of biologically active compounds will possess three elements: 1) superior plant traits allowing it to thrive in an agricultural setting, 2) a high level of antimicrobial activity *and*, 3) the potential for additional biological activities. An extract containing a wide variety of chemical constituents may include those chemical entities with biological activities that can be applied to known or yet unknown biological problems (Koehn, 2008). Evaluating the chemical diversity of a plant extract provides a way to estimate the potential capacity for additional biological activities a particular plant cultivar may have.

LC-MS based metabolic fingerprinting and subsequent analysis of features can be an efficient and unbiased way to measure the chemical diversity of many plant extracts at the same time (Martin *et al.*, 2014). A feature is composed of a unique retention time, monoisotopic mass, and relative intensity of a detected ion (Trygg *et al.*, 2007). The chemical composition of an extract can be estimated using its complete feature set. Previously, we have shown that 70 percent ethanol generates plant extracts with chemical entities possessing a wide-variety of physio-chemical properties (Martin *et al.*, 2014); therefore, we used this solvent to prepare extracts both for antimicrobial testing and metabolic fingerprinting.

A large number of biological replicate analyses per sample were required (8 per sample) to elucidate differences within this highly similar sample set. Typically three to five replicates are used in metabolic fingerprinting experiments (Martin et al, 2014; Want *et al.*, 2013), however for experiments measuring very similar samples, higher degrees of replication are required to detect differences (Glauser *et al.*, 2012).

A qualitative assessment of the distribution of features across chromatographic and mass spectral space shows a high level of similarity between extracts generated from different *G. lepidota* populations and collection times (Fig. 8 & 9). This result was expected since the material used to generate the extracts was so similar. There is a high

density of features shown to elute early in the gradient, between three to seven minutes with mass-to-charge ratios between 200-400 *m/z*. These features correspond to the large number of low molecular weight polyphenolic compounds, including flavonoids, coumarins, and bibenzyls known to be synthesized in licorice (Appendix 2). Although the relative distribution of features is similar regardless of population or collection date a quantitative evaluation shows that the number of features detected changes depending on these factors.

The entire feature set for *G. lepidota* shoot extracts contained 1019 reproducible features. No set of extracts produced from a single population or collection date contained the entire set of 1019 features. The number of features detected in different populations and sample dates ranged between, 759 to 941 and 571 to 827, respectively. This difference provides evidence that certain populations and collection dates result in extracts with an increased number of chemical entities. This increase in chemical diversity indicates a greater potential for useful biological activities to be discovered form extracts made from certain populations collected at optimal times.

G. lepidota roots, collected at time point **G**, showed significantly lower numbers of features than shoot material collected at the same time, 382 compared to 648 features, respectively. This lower number of features detected from root tissue, indicates decreased chemical diversity and may provide evidence for the lack of antimicrobial activity exhibited by *G. lepidota* roots, under the conditions tested. In contrast, extracts made from *G. lepidota* shoot material from population 20, showed the highest number of features (941) with those made from population 8, 16, and 7 following with feature numbers of 831, 812, and 805, respectively.

Differences in the number of detected features according to collection date followed a similar pattern as changes in antimicrobial activity according to collection date (Fig. 9). Extracts made from material collected in late spring at collection point **A** had the lowest feature number (608 features) and the lowest activity against *S. aureus* (average IC_{50} value 231 μg/mL). In contrast, extracts made from material collected in mid-summer at collection points **B** and **C** had the highest number of features, 827 and 809 features, respectively and the highest activity against *S. aureus* with IC_{50} values of 62 and 32 μg/mL, respectively. As noted earlier, this increase in antimicrobial activity level

(and feature number) coincided with seedpod development and maturation. Additionally, extracts made from material collected at similar times in different years, 2012 and 2013, (collection points **E** and **F**), showed very similar antimicrobial activity levels and almost identical feature numbers of 798 detected features in 2012 and 797 in 2013. This consistency in feature number provides further evidence that year-to-year variation for a particular collection point is small.

Conclusions

Interest in cultivating commercially important species of *Glycyrrhiza* in the United States has existed since the 1800s (Hunter, 1885; Whitman, 1885). *G. lepidota* is adapted to the growing conditions of the U.S. and was used in traditional medicine for purposes similar to those of the economically important *Glycyrrhiza* species such as *G. glabra* and *G. urlanensis* (Kindsher, 1987; Hayahsi and Sudo, 2009). To maximize the value that could result from cultivating *G. lepidota* nine populations were evaluated for agronomically important plant traits, biological activity, and chemical diversity.

The results of this study are a first step towards developing an optimal cultivar for the cultivation of biologically active compounds from *G. lepidota*. Nine populations were initially included in this common garden experiment. They were evaluated based on their performance in an agricultural settings, antimicrobial activity, and potential for additional applications via their chemical diversity. The results indicate that a combination of traits from two populations, 7 and 20, would generate a high performing cultivar. Extracts made from plants from population 20 displayed the highest antimicrobial activity level and feature number, but the lowest average biomass. In contrast, plants from population 7 had higher average biomass, the second highest antimicrobial activity, and a large number of features. It would be ideal to have a high shoot biomass producing cultivar that exhibited high antimicrobial activity and feature number. An optimal collection period for increased antimicrobial activity occurring between mid-July and early August was identified. It is important to recognize that these optimized parameters have only been tested in a single common garden setting. It will be important to verify these conditions in each location that may be used for the cultivation of *G. lepidota* for biologically active compounds. The results of this common garden study provide valuable information that

can be used to breed and select a more optimized *G. lepidota* cultivar using germplasm from populations 7 and 20. Moreover, information regarding collection times can be tested immediately using naturally occurring populations of *G. lepidota* growing in cultivation or in the wild.

Although the initial investment is large as evidenced by the three-year common garden experiment reported here, the potential for long-term benefits is very high. Moreover, the large investment in effort, time, land, collection, and cultivation necessary for establishment of any *G. lepidota* population makes the use of optimized germplasm essential.

Acknowledgements

We thank the botanicals research team in Estée Lauder R&D for helpful discussions, assistance. This work was funded by the NSF Plant Genome Research Program grants IOS-0923960 and IOS-1238812, the NSF Graduate Research Fellowship Program (00006595), and the UNCF/Merck Science Initiative.

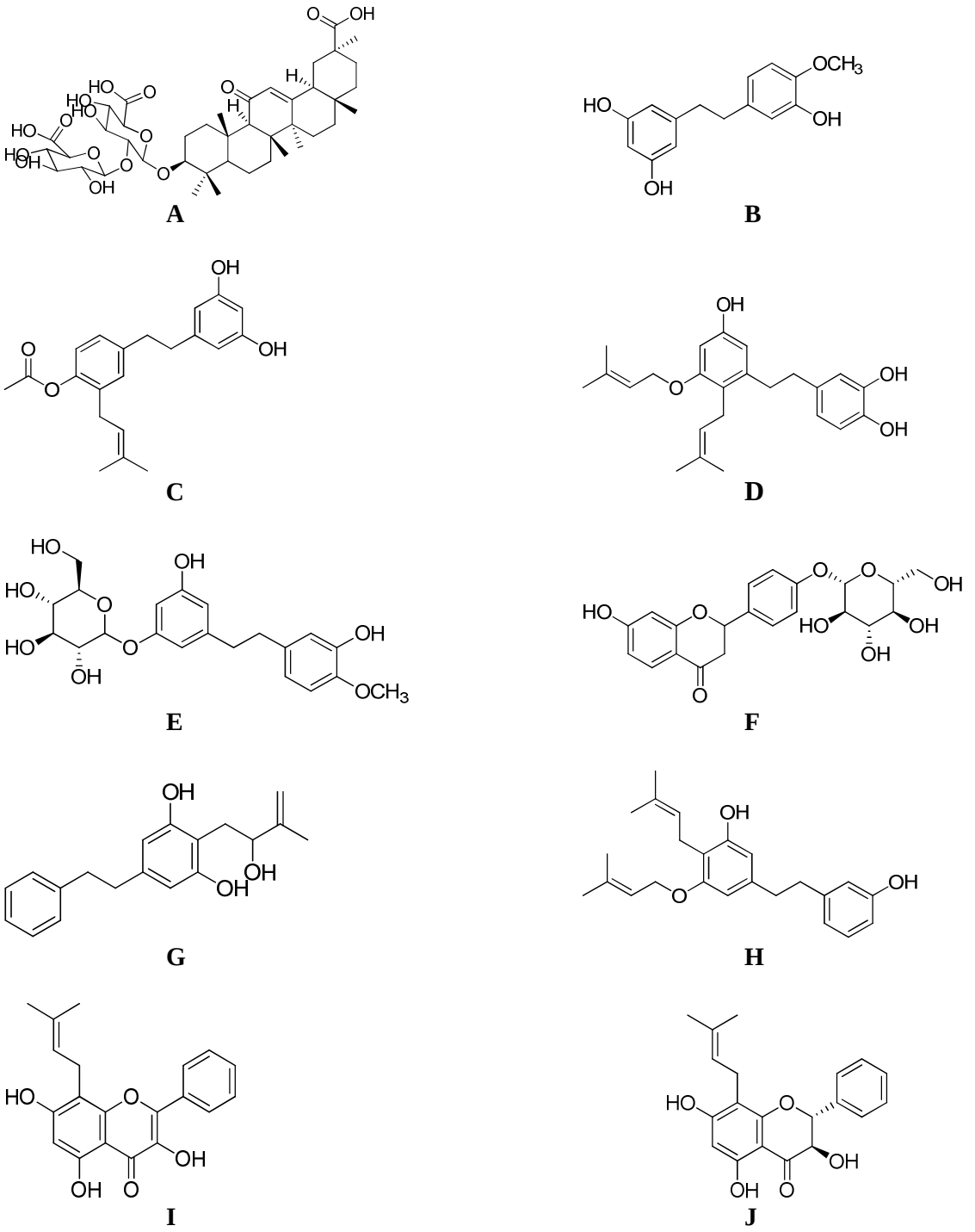
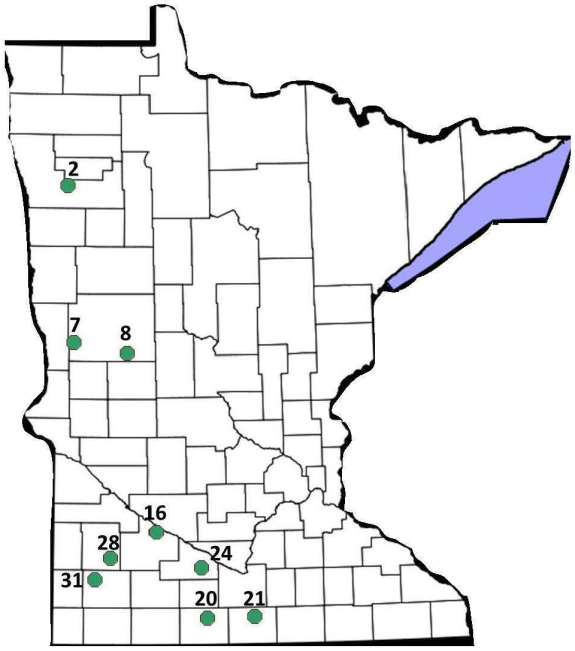


Figure 1. Compounds isolated from plants belonging to the genus *Glycyrrhiza*. **A)** Glycyrrhizin, 97; **B)** 5-(3-hydroxy-4-methoxyphenethyl)benzene-1,3-diol, 10; **C)** 4-(3,5-dihydroxyphenethyl)-2-(3-methylbut-2-en-1-yl)phenylacetate, 47; **D)** 4-(5-hydroxy-2-(3-methylbut-2-en-1-yl)-3-((3-methylbut-2-en-1-yl)oxy)phenethyl)benzene-1,2-diol, 66; **E)** 2-(3-hydroxy-5-(3-hydroxy4methoxy-phenethyl)phenoxy)-6-(-(hydroxymethyl) tetrahydro-2-pyran-3,4,5-triol, 83; **F)** Liquiritin, 79; **G)** Glepidotin C, 26; **H)** Glepidotin D, 60; **I)** Glepidotin A, 40; **J)** Glepidotin B, 45. The number following the compound names refers to Appendix B.

Table 1. Minnesota *Glycyrrhiza lepidota* seed collection locations

Code	Location	Latitude	Longitude	Date
2	Pembina Trail Preserve SNA	47° 42' 13" N	96° 19' 57" W	Sept. 9, 2010
7	Ottertail Prairie SNA	46° 09' 13" N	96° 13' 50" W	Sept. 11, 2010
8	Inspirational Peak State Park	46° 08' 21" N	95° 35' 01" W	Sept. 11, 2010
16	Redwood County Roadside	44° 13' 46" N	95° 14' 29" W	Oct. 5, 2010
20	Martin County Roadside	43° 51' 26" N	94° 27' 07" W	Oct. 6, 2010
21	Faribault County Roadside	43° 50' 01" N	94° 04' 19" W	Oct. 6,2010
24	Brown County Roadside (New Ulm)	44° 20' 51" N	94° 32' 22" W	Oct. 5, 2010
28	Glynn Prairie SNA	44° 15' 88" N	95° 41' 67" W	Oct. 3, 1010
31	Lyon County Roadside (Balaton)	44° 13' 36" N	95° 50' 43" W	Oct 3, 2010



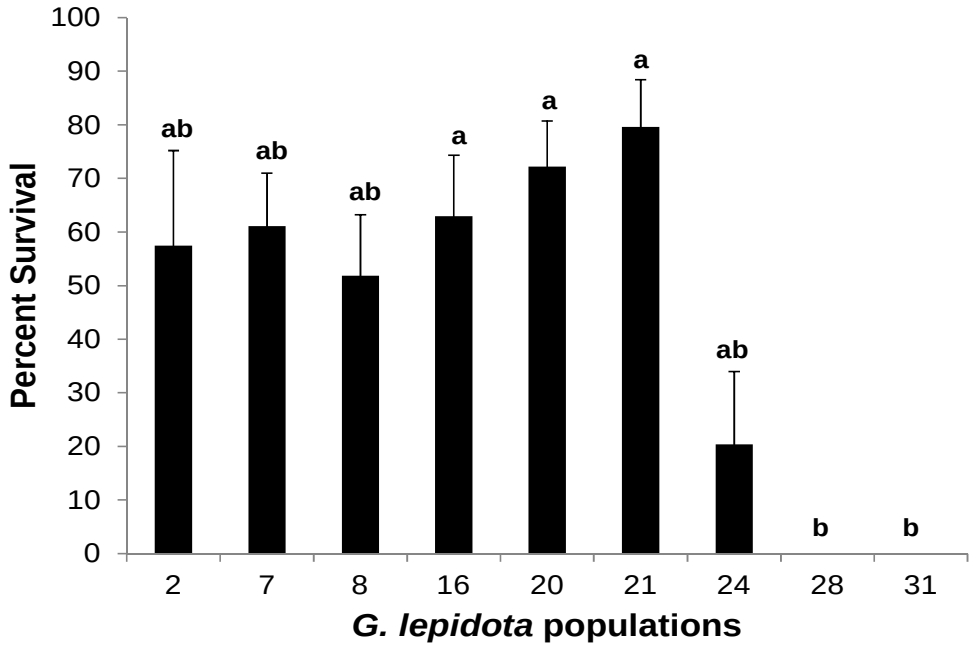


Figure 2. Three-year survival rate for nine Minnesota native populations of *Glycyrrhiza lepidota*. Bars show the average percent survival of plants growing in six individual subplots per population. Subplots were planted with nine seedlings each. Error bars represent standard error (n=6). Letter superscripts indicate statistically different groups at a p-value ≤ 0.05 according to Tukey's HSD test.

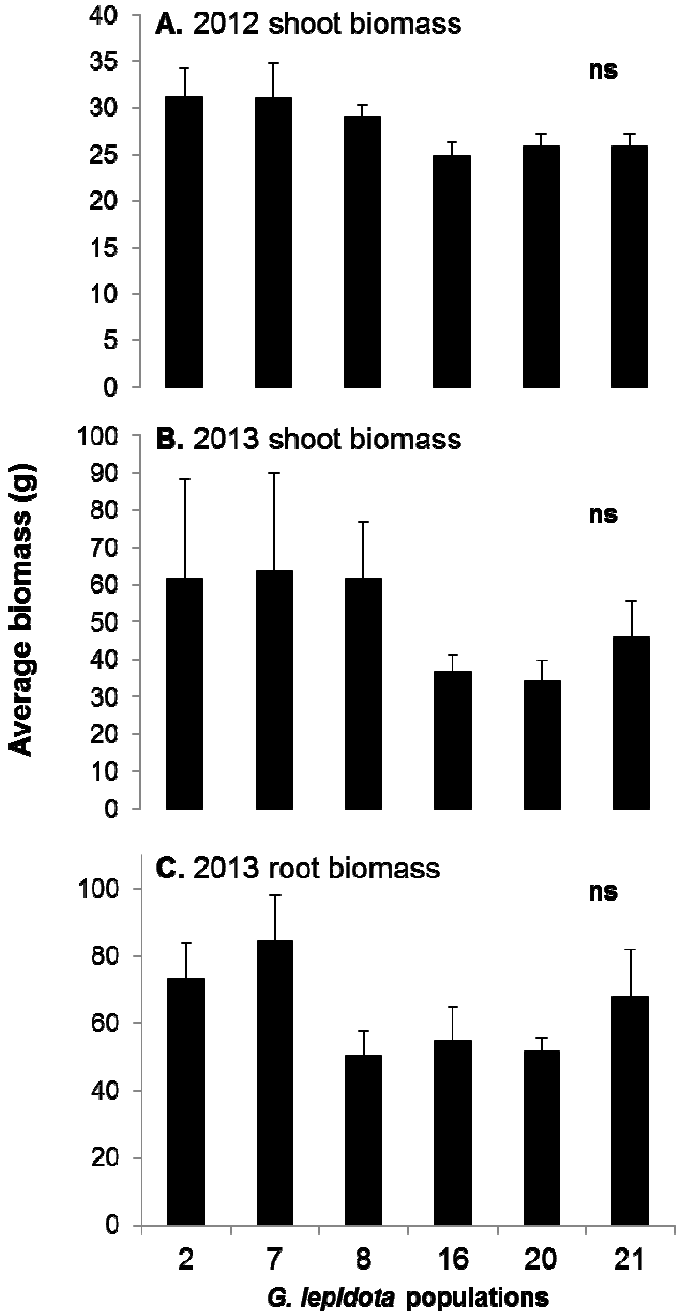


Figure 3. Total above and below ground biomass for six Minnesota native populations of *Glycyrrhiza lepidota*. A) average above ground biomass in 2012, **B)** average above ground biomass in 2013, **C)** average below ground biomass in 2013. Bars show the average above or below ground biomass of plants collected from six individual subplots for each population. All above or below ground material from each subplot was collected and weighed *en masse*. Error bars represent standard error (n=6). ns; not significant at a p-value ≤ 0.05 according to Tukey's HSD test.

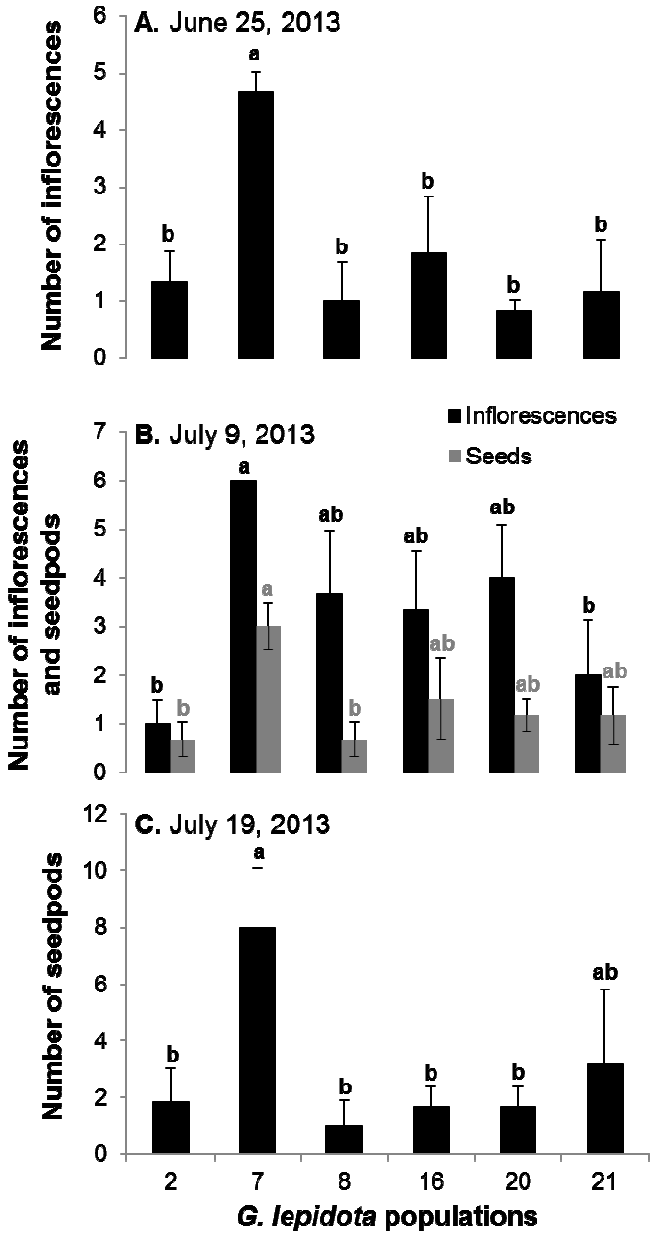


Figure 4. Average number of inflorescences and seedpods for six Minnesota native populations of *Glycyrrhiza lepidota*. A) average number of inflorescences observed on June 25, 2013; **B)** average number of inflorescences and seedpods observed on July 9,2013; **C)** average number of seedpods observed on July 19, 2013. Bars show the average number of inflorescences or seedpods counted from plants growing in six individual subplots for each population. Error bars represent standard error (n=6). Letter superscripts indicate statistically different groups at a p-value ≤ 0.05 according to Tukey's HSD test.

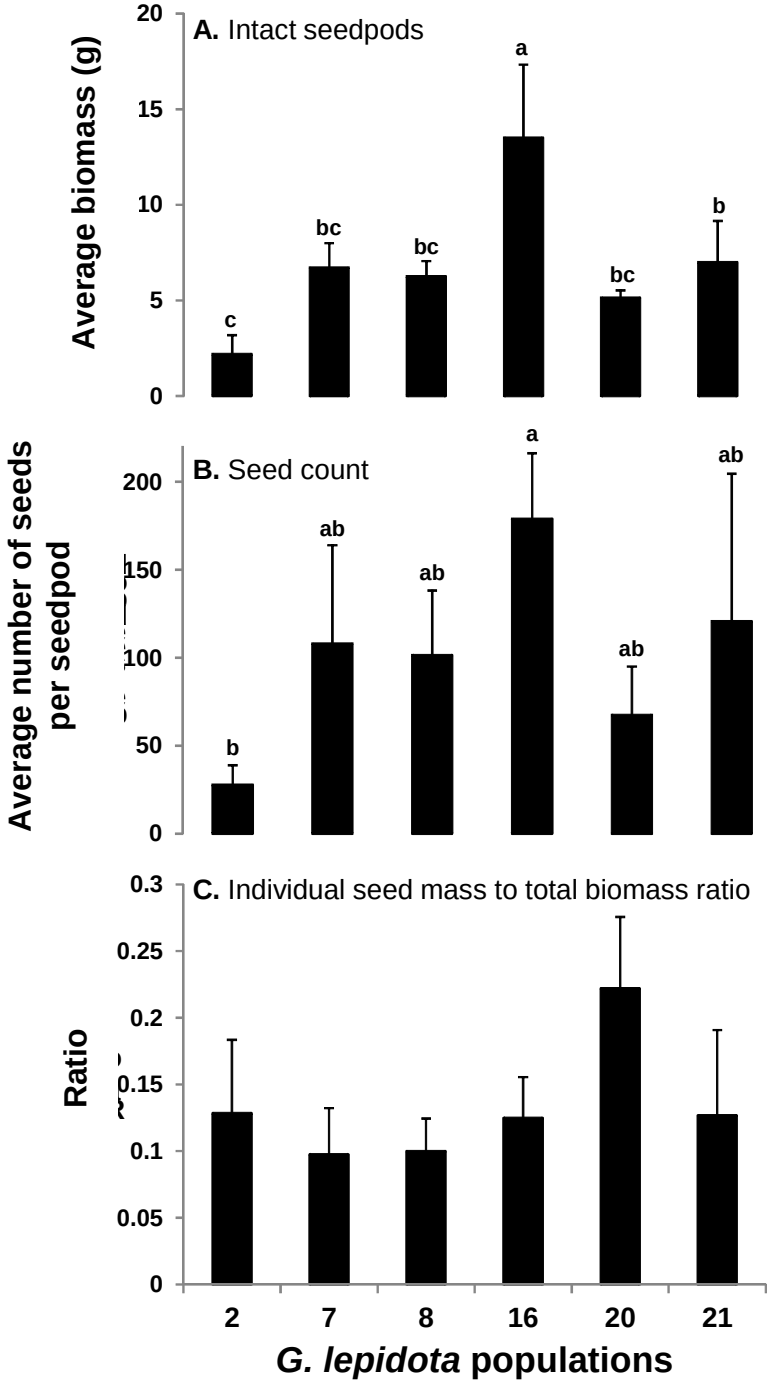


Figure 5. Average seed traits for six Minnesota native populations of *Glycyrrhiza lepidota*. A) average biomass of intact seedpods; **B)** average number seeds per seedpod; **C)** ratio of individual seed-mass to total above and below ground biomass. Bars in panel **A** and **B** show the averages measured from plants growing in six individual subplots for each population. Error bars represent standard error (n=6). Letter superscripts indicate statistically different groups at a p-value ≤ 0.05 according to Tukey's HSD test.

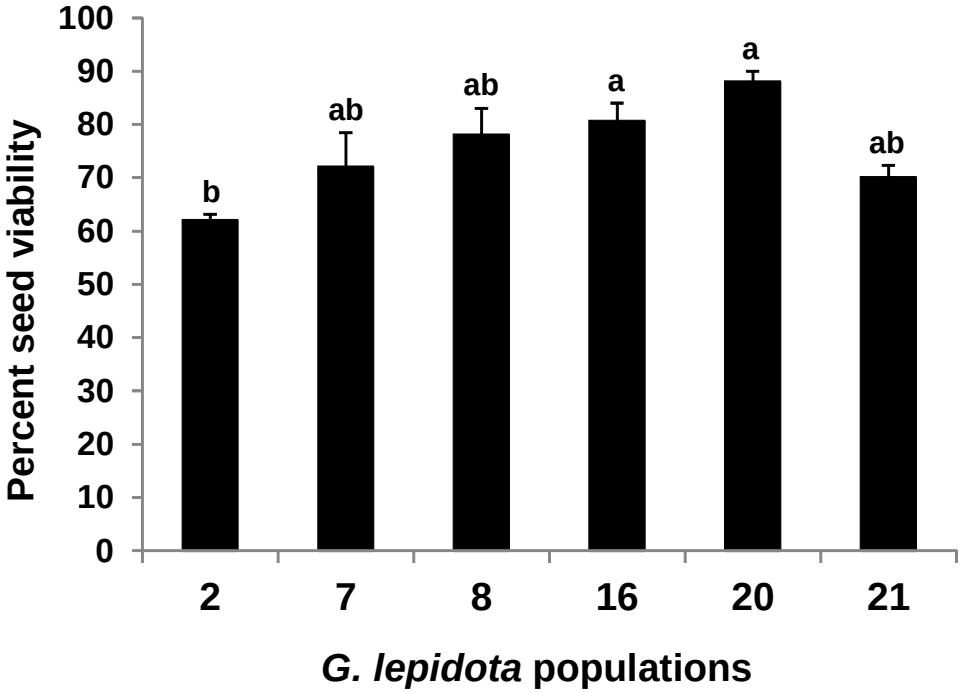


Figure 6. Average percent seed viability for six Minnesota native populations of *Glycyrrhiza lepidota*. Bars show the average percent seed viability for seed collected from each population; three replicates of 50 seeds each were tested. Error bars represent standard error (n=3). Letter superscripts indicate statistically different groups at a *p*-value ≤ 0.05 according to Tukey's HSD test.

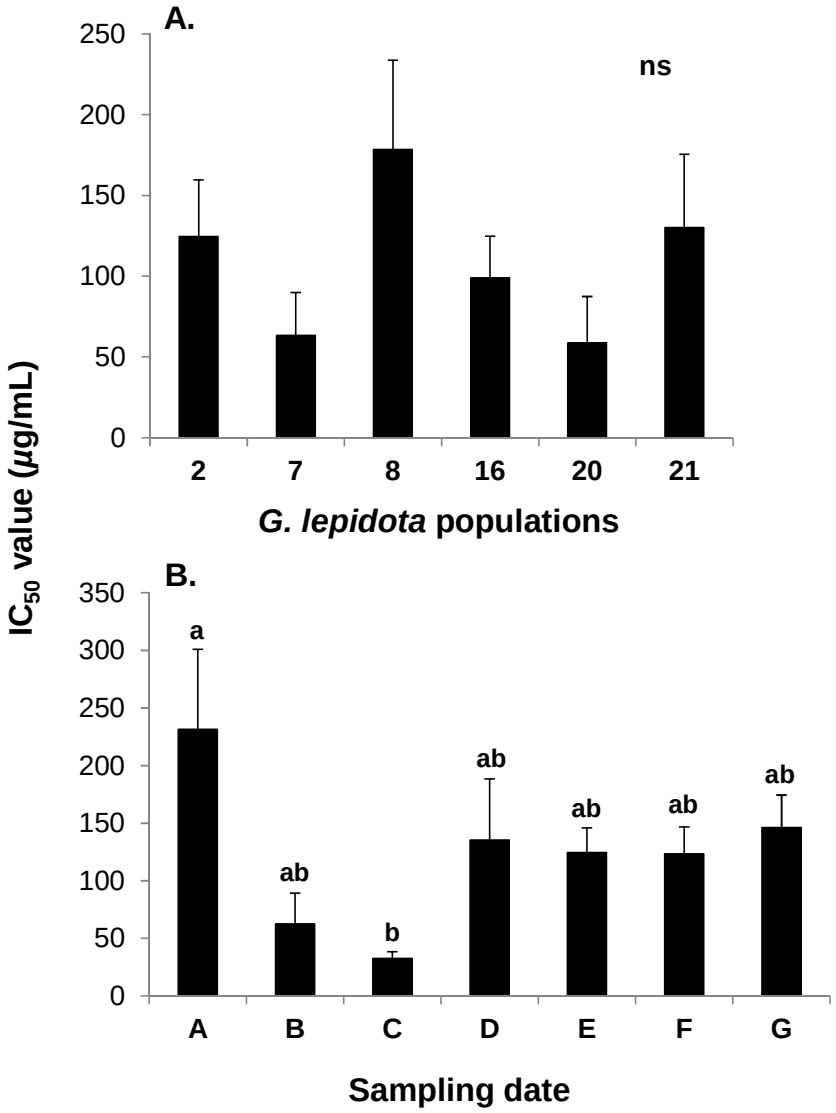


Figure 7. Average antimicrobial activity for six Minnesota native populations of *Glycyrrhiza lepidota* against *Staphylococcus aureus*. A) average activity of each population measured overtime; **B)** average activity of all populations according to collection date. Collection dates are as follows: **A)** June 24, 2013; **B)** July 18, 2013; **C)** August 5, 2013; **D)** August 26, 2013; **E)** September 14, 2012; **F)** September 16, 2013; and **G)** October 15, 2013. Bars show the average IC_{50} value measured from extracts made from plants collected from six individual subplots for each population. Error bars represent standard error (n=6). Letter superscripts indicate statistically different groups at a *p*-value ≤ 0.05 according to Tukey's HSD test.

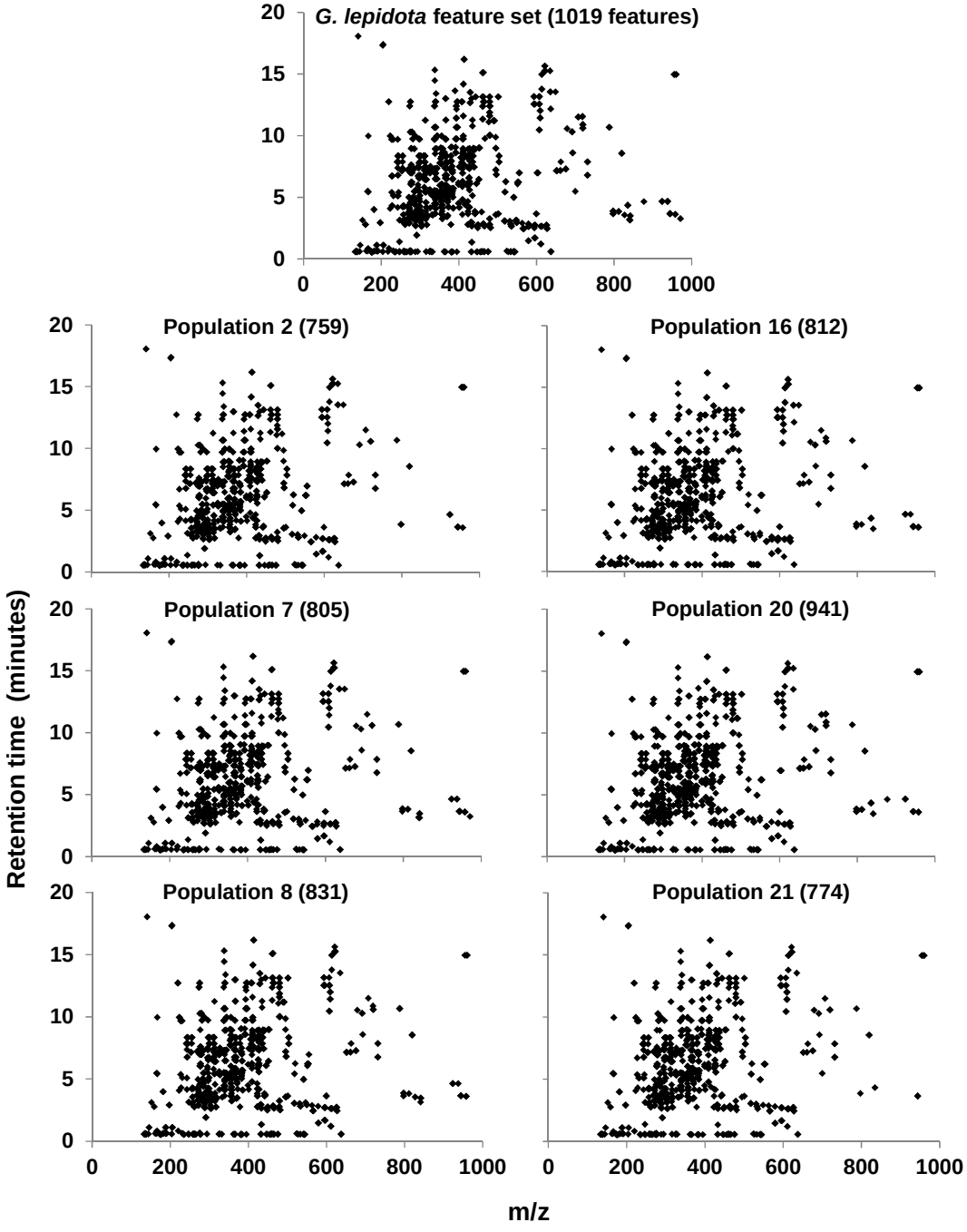


Figure 8. Scatterplots showing the distribution of features detected from metabolic fingerprints of extracts made from different populations of *Glycyrrhiza lepidota*. Individual features are represented by black dots. The total number of features detected from extracts made from each population are listed parenthetically. The entire feature set contains 1019 features in all

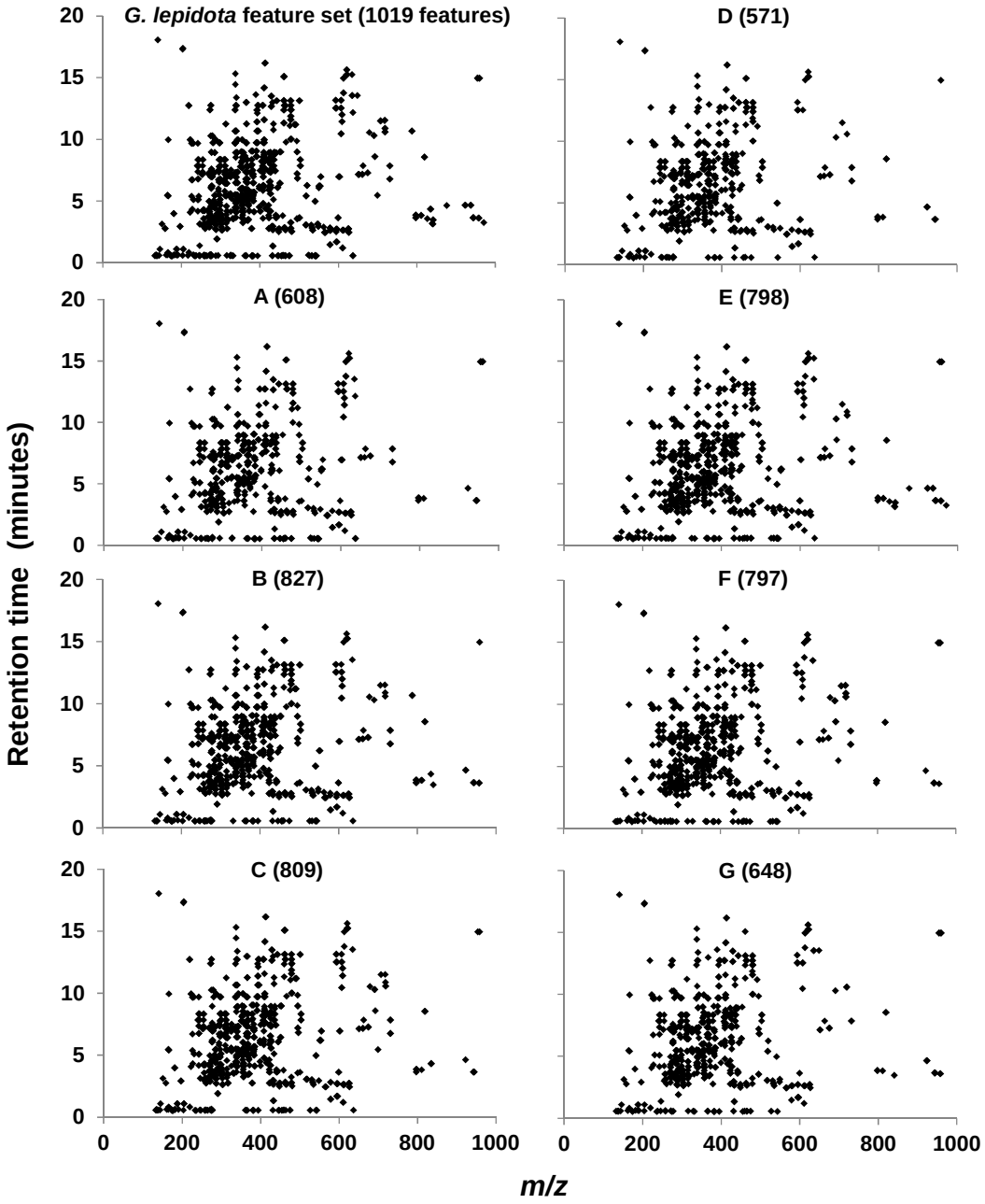


Figure 9. Scatterplots showing the distribution of features detected from metabolic fingerprints of extracts made from *Glycyrrhiza lepidota* collected at different time points. Individual features are represented by black dots. The total number of features detected from extracts made from material collected at different time points are listed parenthetically. Collection dates are as follows: **A)** June 24, 2013; **B)** July 18, 2013; **C)** August 5, 2013; **D)** August 26, 2013; **E)** September 14, 2012; **F)** September 16, 2013; and **G)** October 15, 2013. The entire feature set contains 1019 features in all.

Conclusions

Perennial plants are a natural resource with the potential to be developed as sources of highly valuable biologically active chemicals and ecosystem services. Optimization of bioactive compound discovery and its subsequent application may increase the presence of perennial plants on the landscape. Because novel compounds are increasingly more difficult to discover natural products discovery will be most effective if performed in a maximally unbiased way. It was shown in Chapter 2 that metabolomics fingerprinting is an effective technique to assess plant extract chemical diversity and yield. Using this technique revealed that chemical diversity and extract yield did not necessarily increase together. Furthermore, maximum chemical diversity in a plant extract was most efficiently approached if solvent partitioning was performed on an extract made with 70 percent ethanol. In Chapter 3, metabolic fingerprinting was used as a tool to globally assess extract contents in context with other data (cytotoxicity, efficacy etc.). This integrative approach helped to highlight useful chemical information regarding extract variation and the associated potential risks.

In Chapter 4, information from these methodological studies was applied to bioactive compound discovery from the potentially highly valuable Minnesota native plant *C. peregrina*. Collections were made from this abundantly available plant and subsequent bioassay-guided fractionation yielded the isolation of two small molecules with strong antimicrobial activity. Sourcing biologically active compounds from *C. peregrina* may provide economic incentives to protect its natural habitat to ensure the future availability of source material for these antimicrobial compounds. Finally, in Chapter 5, methods from all of the proceeding studies were combined and extended to perform an intensive evaluation of distinct populations of *G. lepidota* in an agricultural setting. This integrated approach resulted in the identification of two populations of this plant with the potential to be developed into a *G. lepidota* cultivar with optimal characteristics for the cultivation of biologically active compounds.

The results of these methodological and applied studies show that perennial plants have a great potential to be managed in such a way as to maximize the yield of marketable commercial commodities while, simultaneously producing valuable ecosystem services.

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Zou L, Henderson G L, Harkey M R, Sakai Y, Li A (2004) Effects of kava (Kava-kava, ‘Awa, Yaqona, *Piper methysticum*) on c-DNA-expressed cytochrome P450 enzymes and human cryopreserved hepatocytes. *Phytomedicine* **11**: 285-294.

Appendix A

Spectral data (MS, MS^2, ^1H- and ^{13}C-NMR) for compounds isolated from *C. peregrina*.

Summary

Compounds were isolated from the dichloromethane partition of a methanolic extract of *C. peregrina* above ground parts. Isolated compounds include, galangin (3,5,7-trihydroxyflavone, **1**), pinocembrin (5,7-dihydroxyflavanone, **2**), and pinosylvin monomethyl ether (3-hydroxy-5-methoxystilbene, **3**).

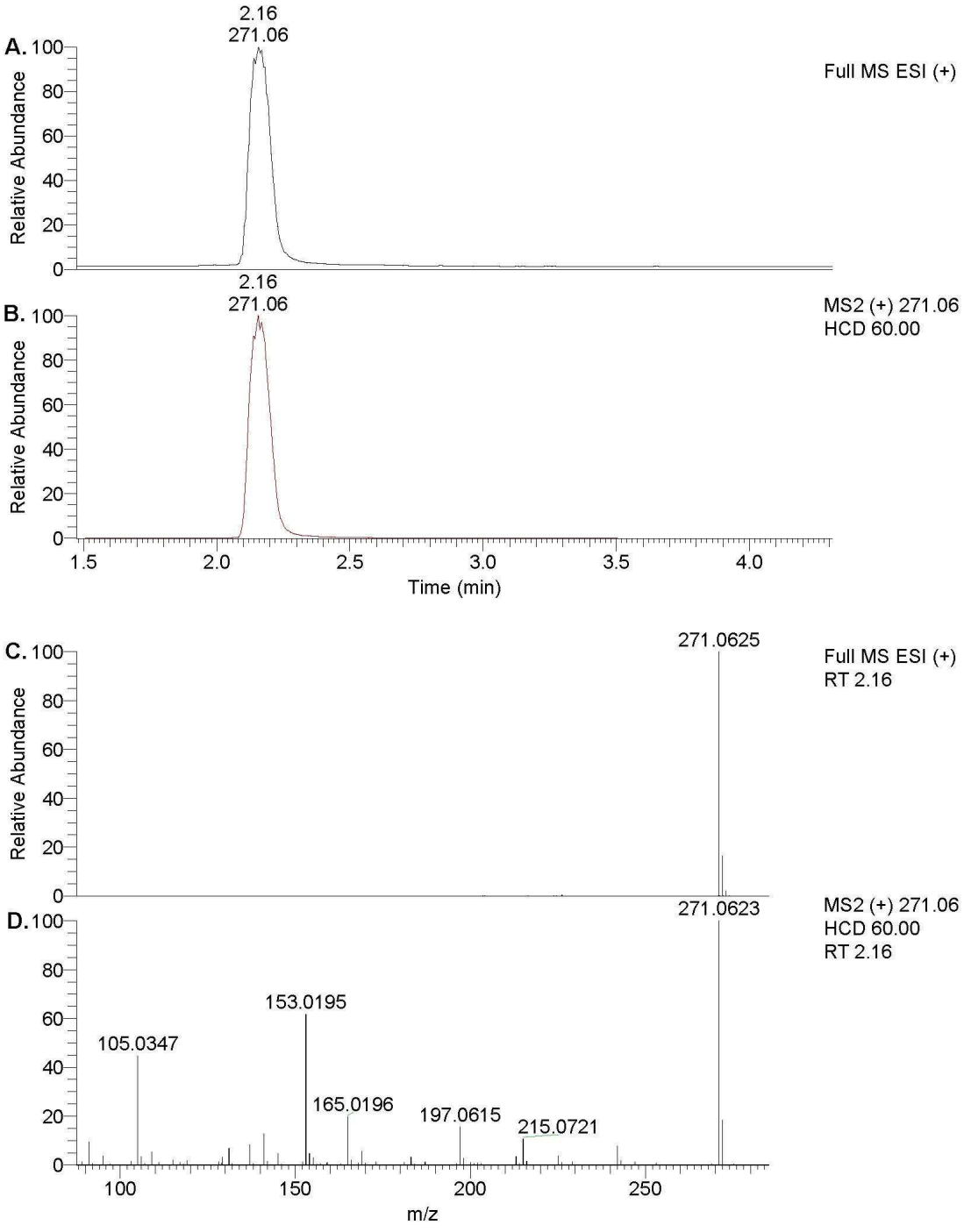


Figure A1. RP-UPLC-ESI(+)-HRAM-MS and -MS^2 spectra of galangin, 1. A) Full MS chromatogram showing the base peak as **1**. **B)** Chromatogram showing peak sampled for MS^2 fragmentation. **C)** MS spectrum showing the base peak as the $[M+H]^+$ ion of **1**. **D)** MS^2 spectrum showing fragment ions with precursor $[M+H]^+$ ion as the base peak.

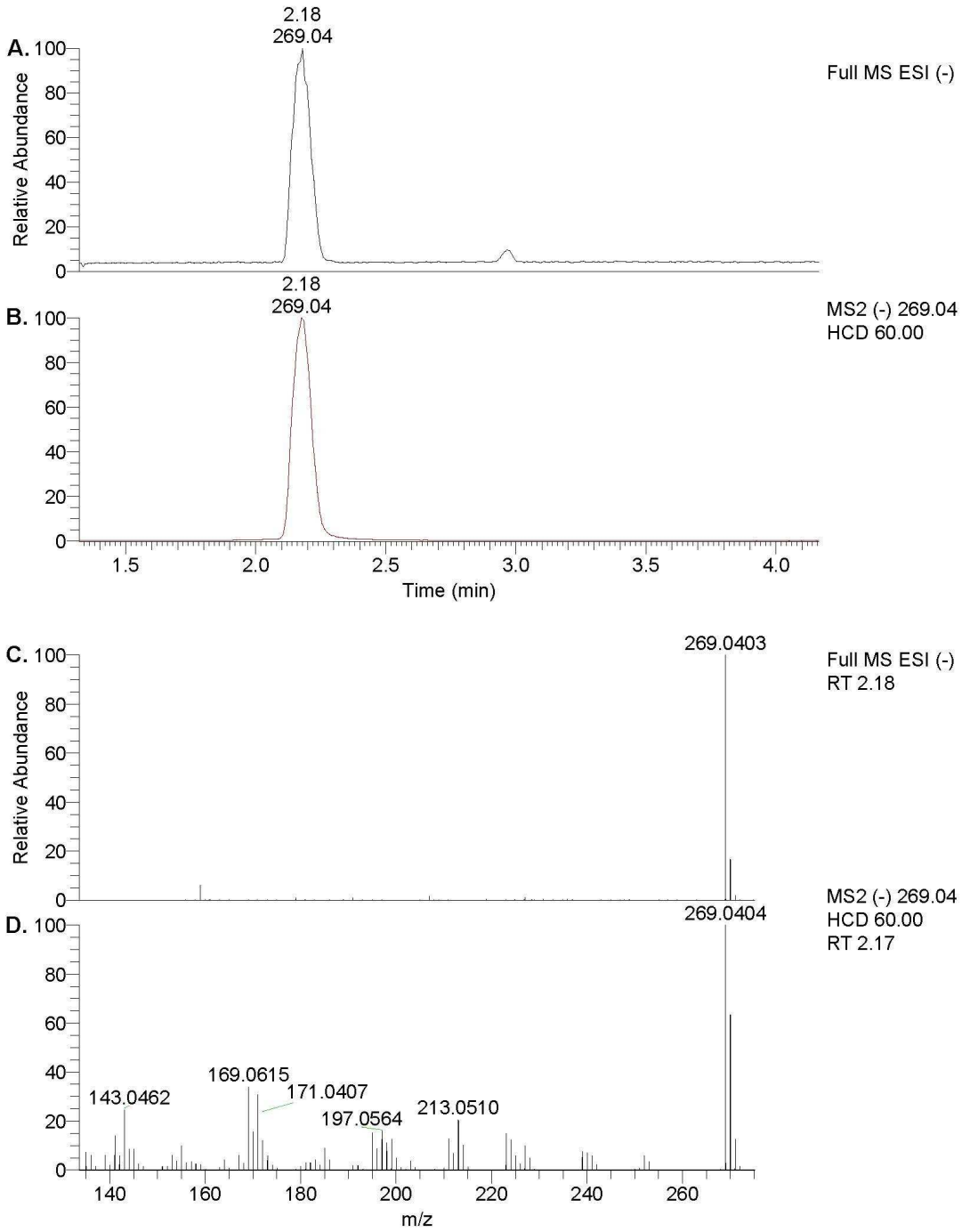


Figure A2. RP-UPLC-ESI(-)-HRAM-MS and -MS^2 spectra of galangin, 1. A) Full MS chromatogram showing the base peak as **1**. **B)** Chromatogram showing peak sampled for MS^2 fragmentation. **C)** MS spectrum showing the base peak as the [M-H]$^-$ ion of **1**. **D)** MS^2 spectrum showing fragment ions with precursor [M-H]$^-$ ion as the base peak.

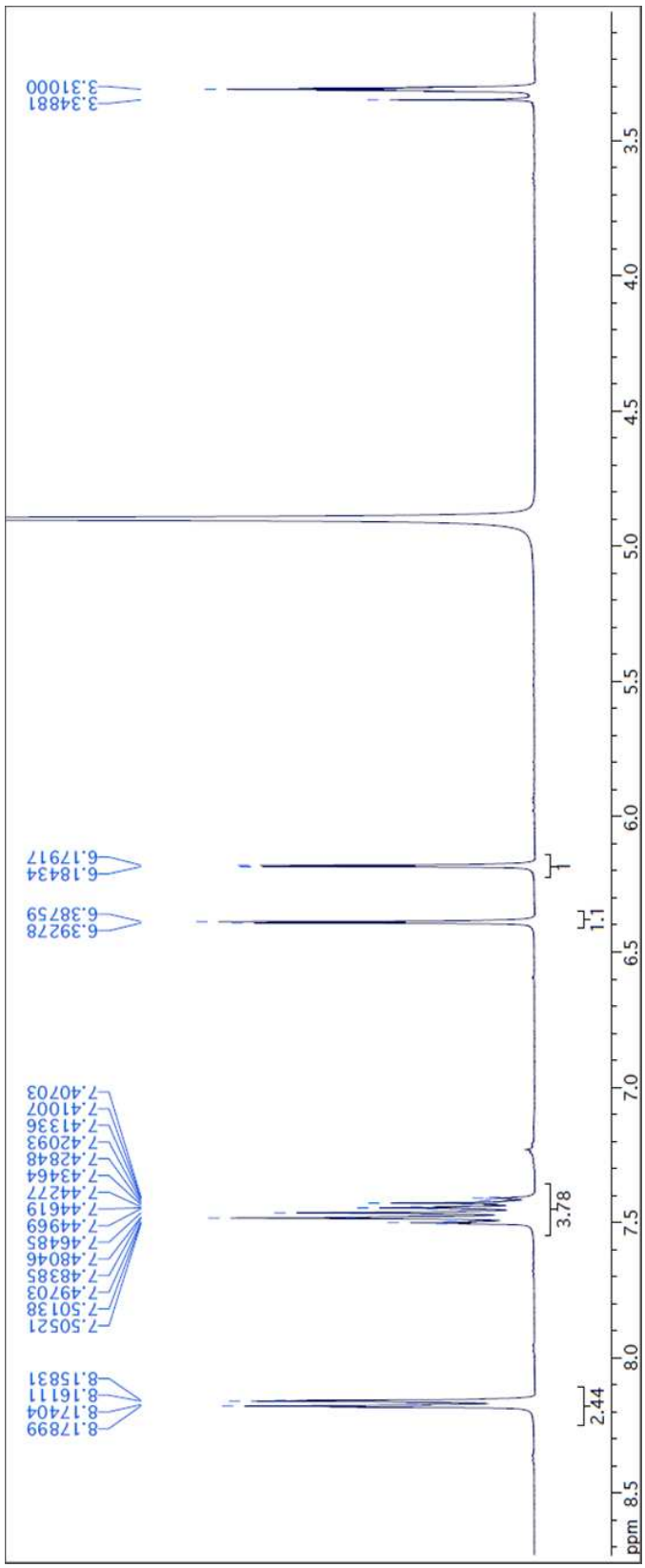


Figure A3. ^{1}H NMR spectrum of galangin, 1.

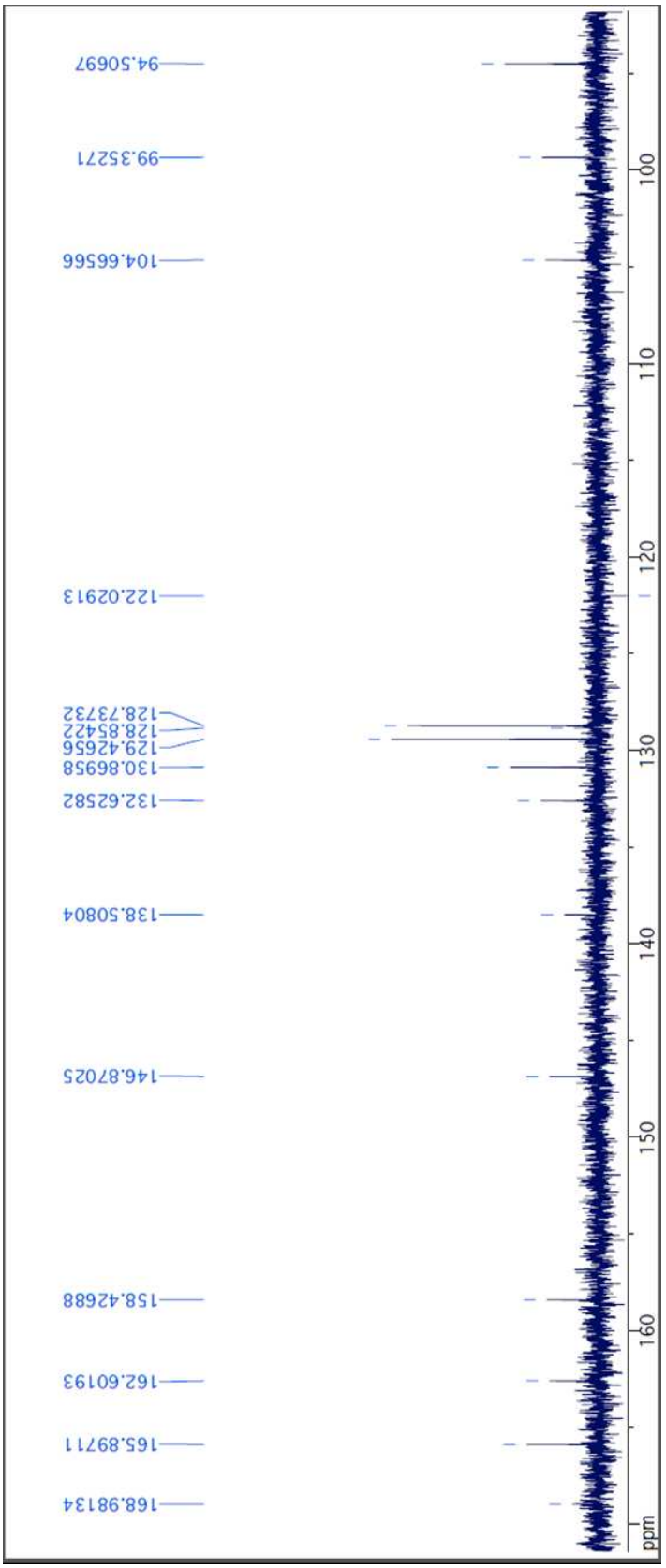


Figure A4. ^{13}C NMR spectrumof galangin, 1.

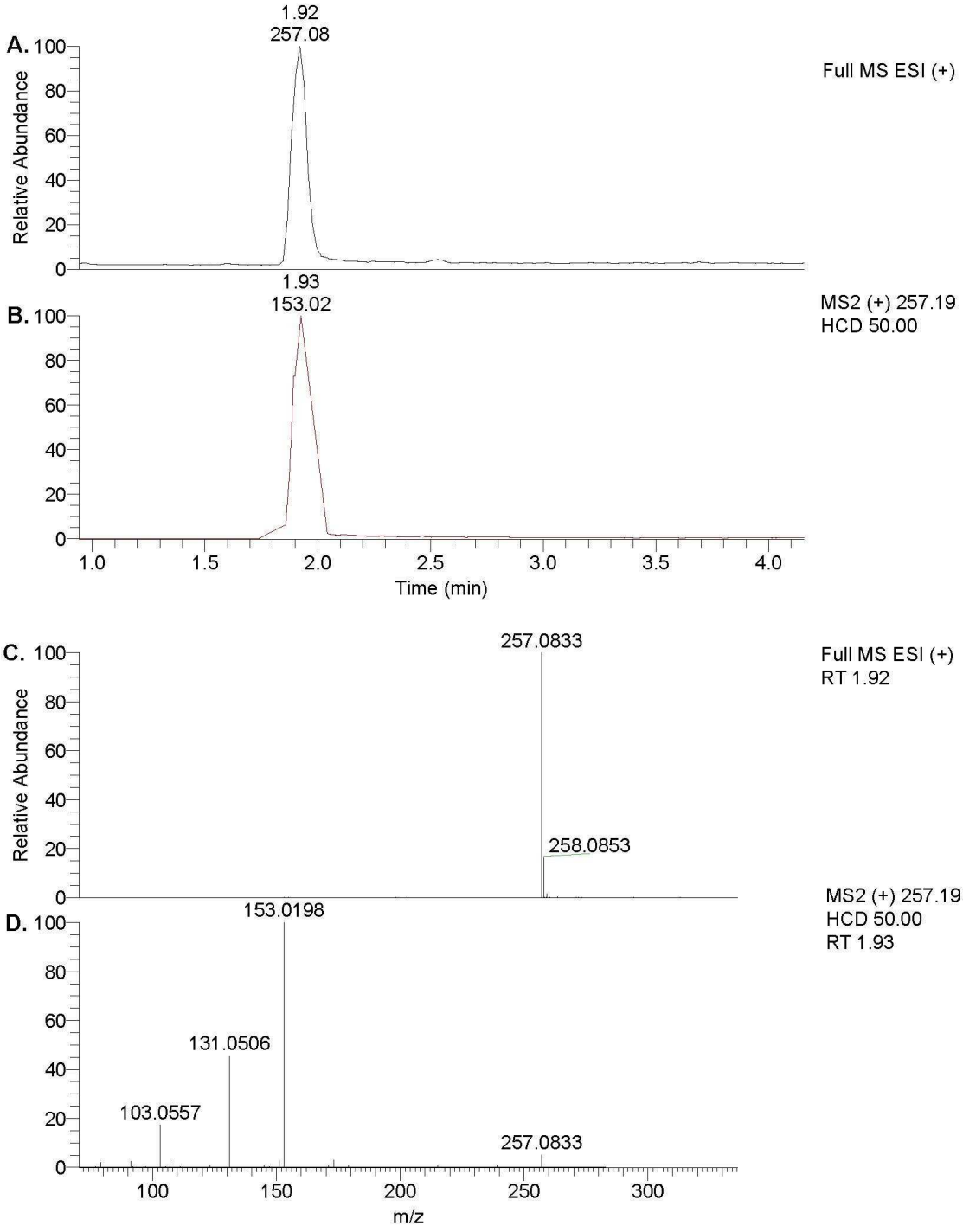


Figure A5. RP-UPLC-ESI(+)-HRAM-MS and -MS^2 spectra of pinocembrin, 2. A) Full MS chromatogram showing the base peak as **2**. **B)** Chromatogram showing peak sampled for MS^2 fragmentation. **C)** MS spectrum showing the base peak as the $[M+H]^+$ ion of **2**. **D)** MS^2 spectrum showing fragment ions with the precursor $[M+H]^+$ ion present.

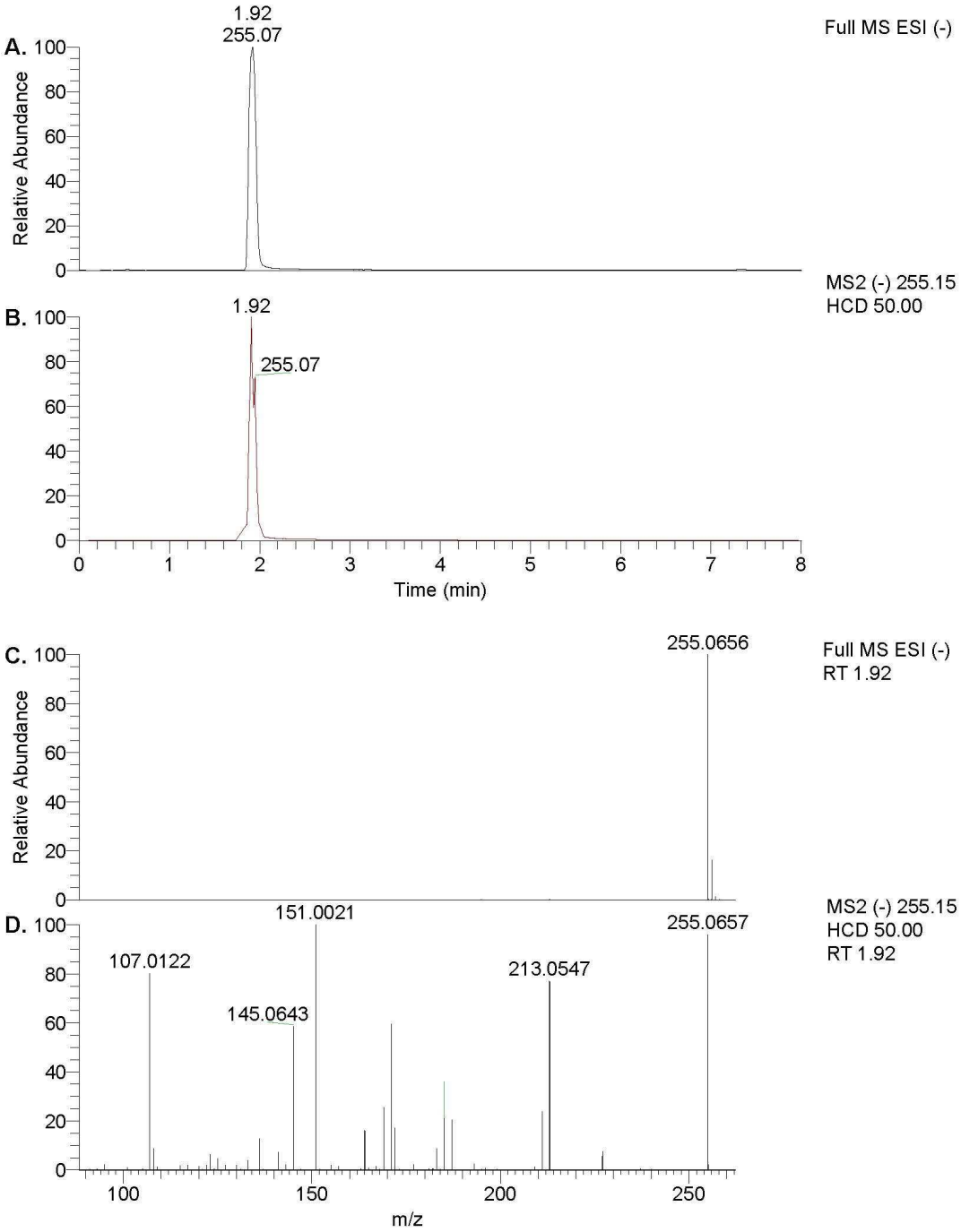


Figure A6. RP-UPLC-ESI(-)-HRAM-MS and -MS^2 spectra of pinocembrin, 2. A) Full MS chromatogram showing the base peak as **2**. **B)** Chromatogram showing peak sampled for MS^2 fragmentation. **C)** MS spectrum showing the base peak as the $[M-H]^-$ ion of **2**. **D)** MS^2 spectrum showing fragment ions with precursor $[M-H]^-$ ion as a predominant peak.

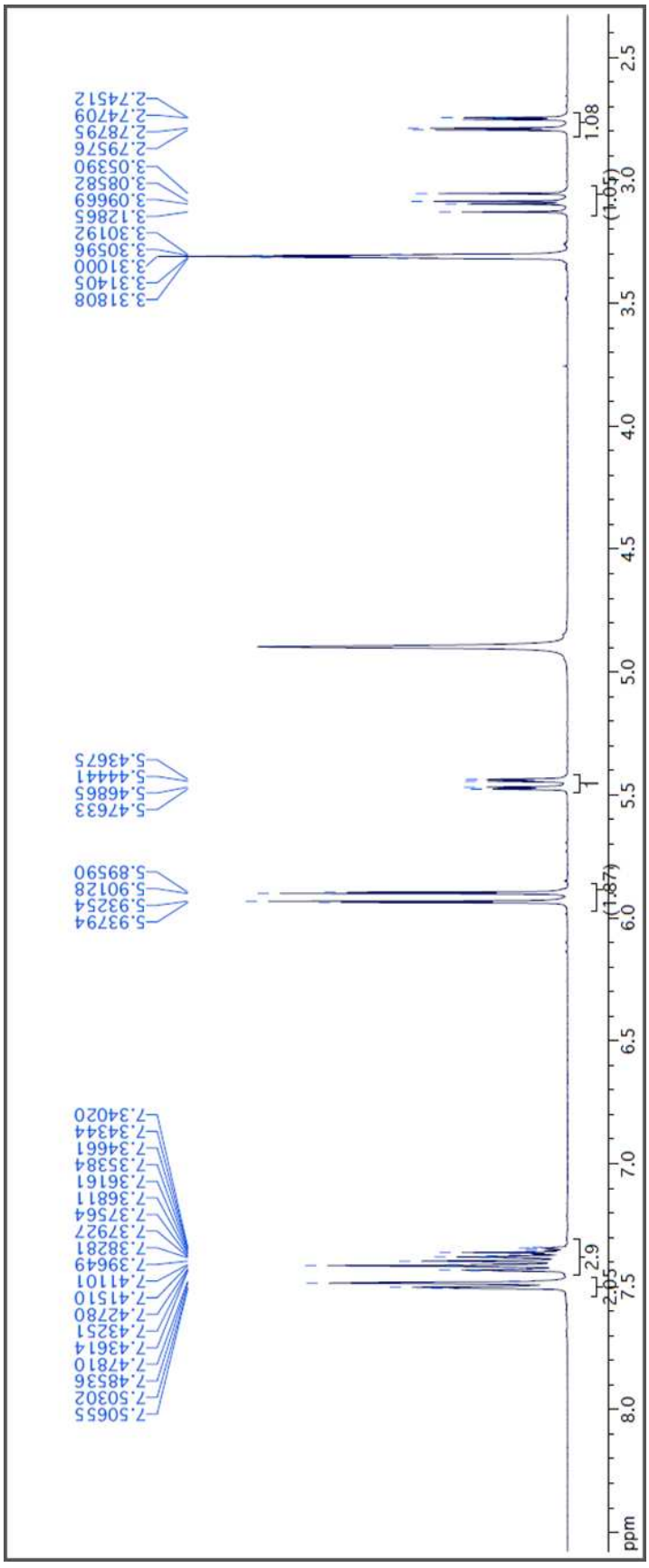


Figure A7. ^{1}H NMR spectrum of pinocembrin, 2.

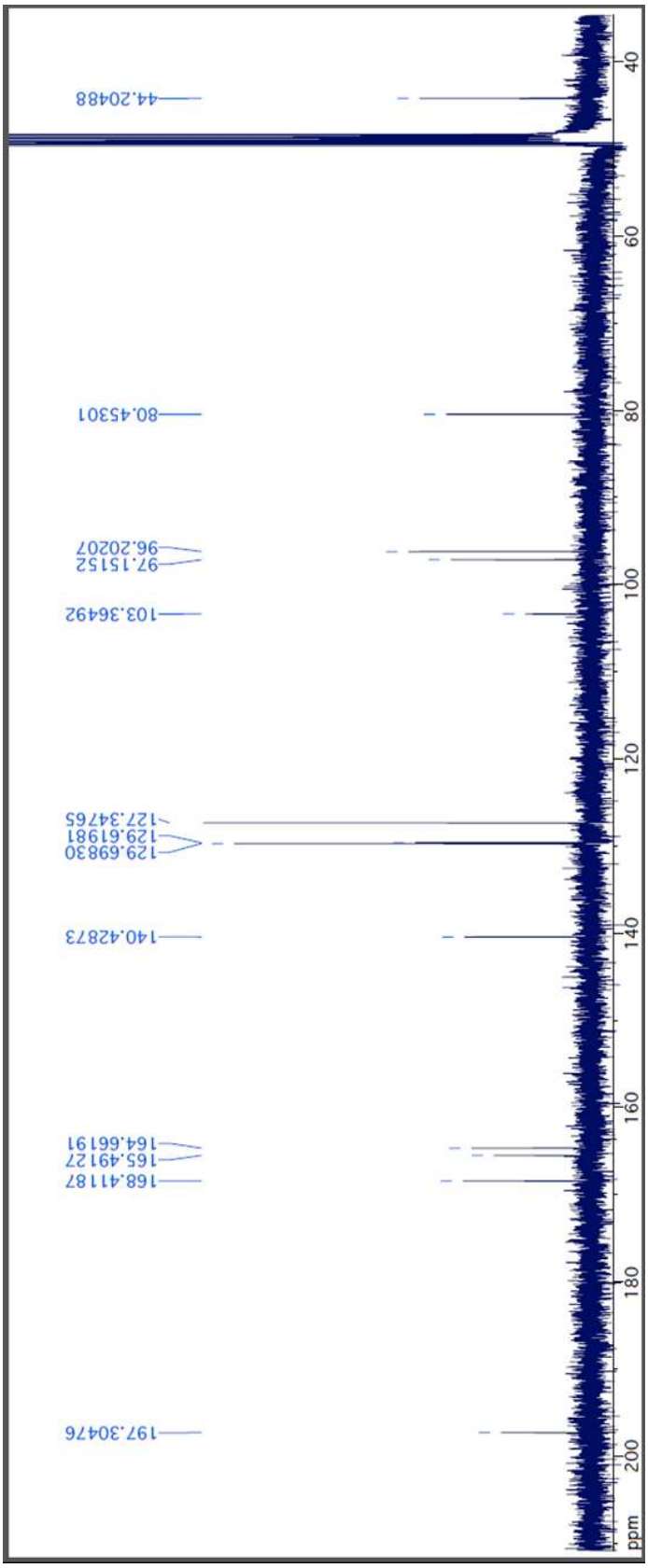


Figure A8. ^{13}C NMR spectrum of pinocembrin, 2.

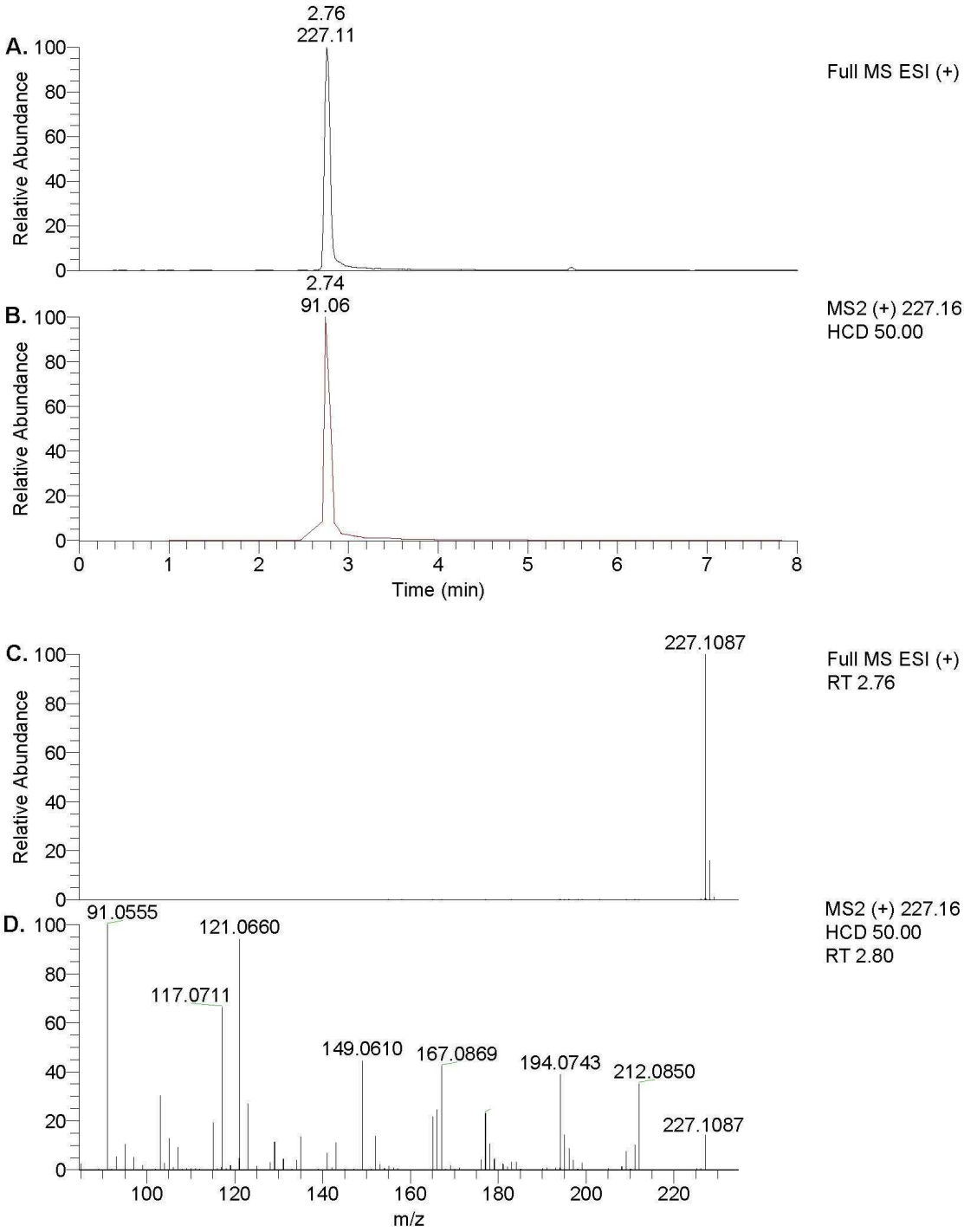


Figure A9. RP-UPLC-ESI(+)-HRAM-MS and -MS^2 spectra of pinosylvin monomethyl ether, 3. A) Full MS chromatogram showing the base peak as **3**. **B)** Chromatogram showing peak sampled for MS^2 fragmentation. **C)** MS spectrum showing the base peak as the $[M+H]^+$ ion of **3**. **D)** MS^2 spectrum showing fragment ions with precursor $[M+H]^+$ ion present.

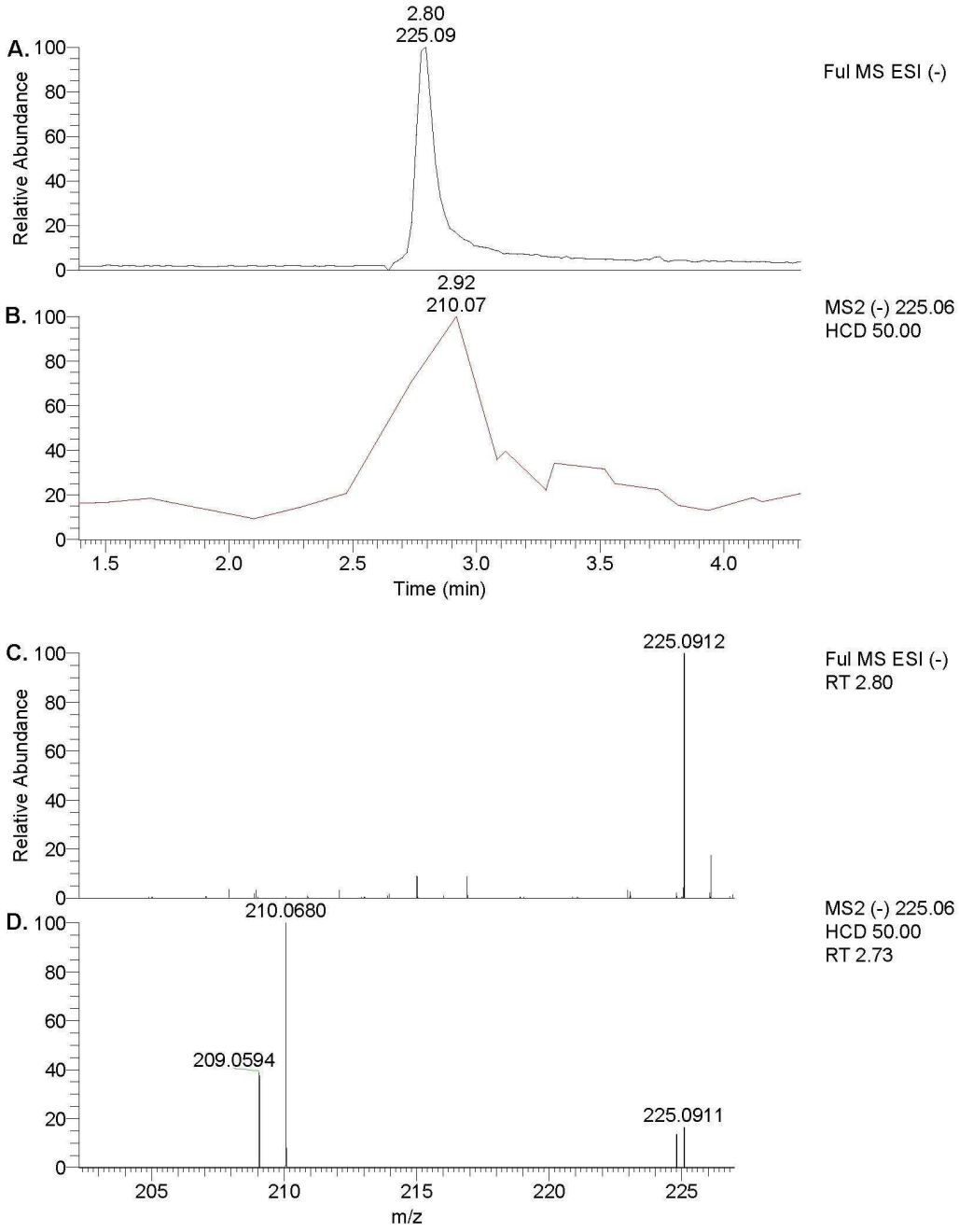


Figure A10. RP-UPLC-ESI(-)-HRAM-MS and -MS^2 of pinosylvin monomethyl ether, 3. A) Full MS chromatogram showing the base peak as **3**. **B)** Chromatogram showing peak sampled for MS^2 fragmentation. **C)** MS spectrum showing the base peak as the $[M-H]^-$ ion of **3**. **D)** MS^2 spectrum showing fragment ions with the precursor $[M-H]^-$ ion present.

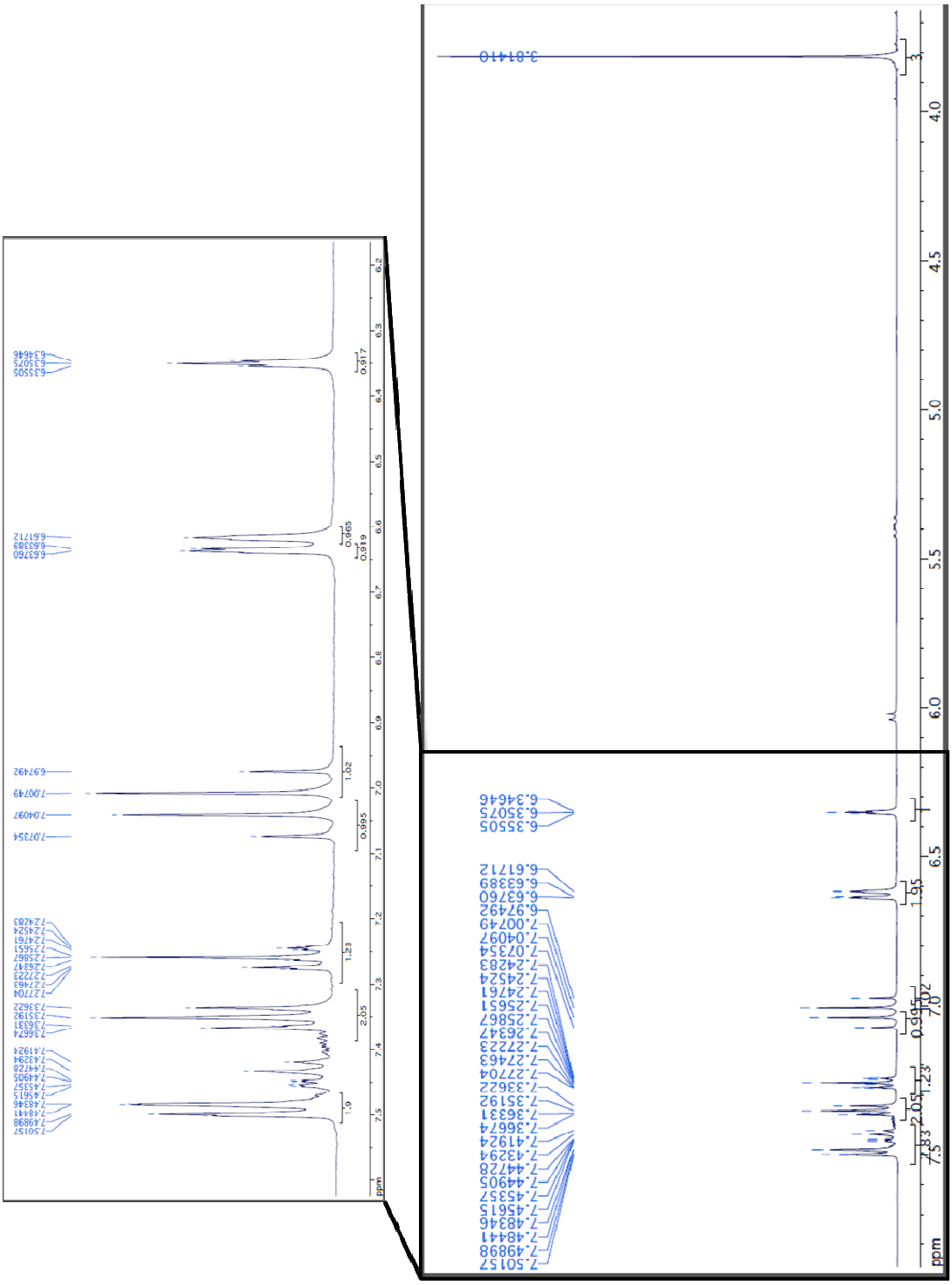


Figure A11. ^{1}H NMR spectrum of pinosylvin monomethyl ether, 3, with magnified inset.

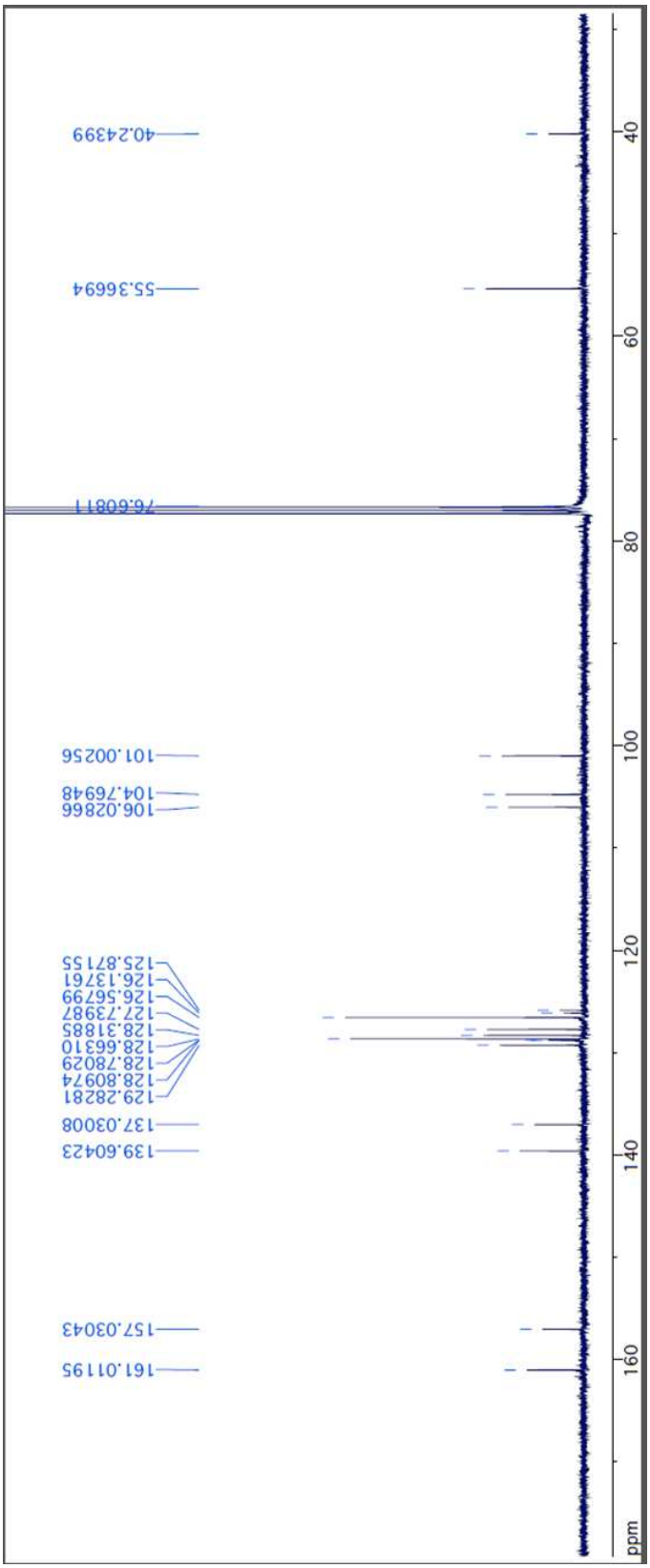


Figure A12. ^{13}C NMR spectrum of pinosylvin monomethyl ether, 3

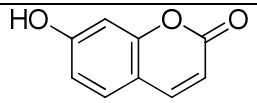
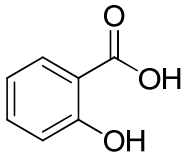
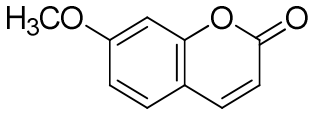
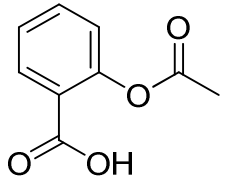
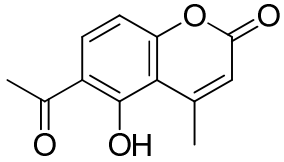
Appendix B

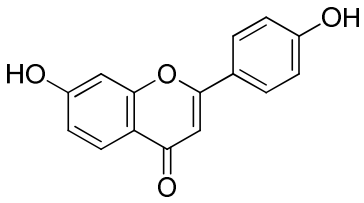
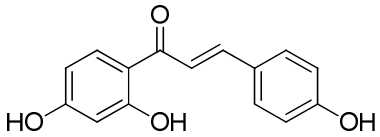
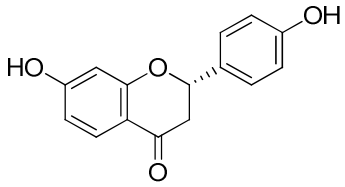
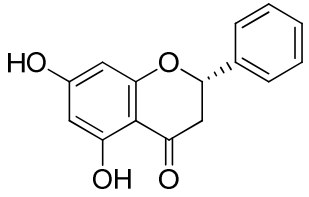
Compounds found in plants of the *Glycyrrhiza* (licorice) genus

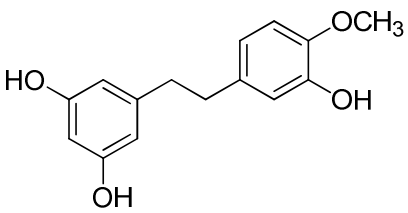
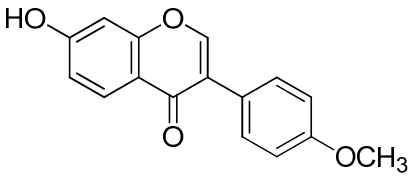
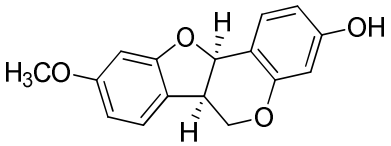
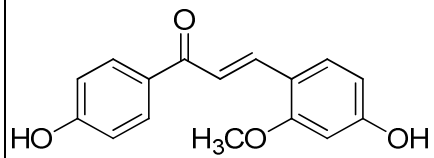
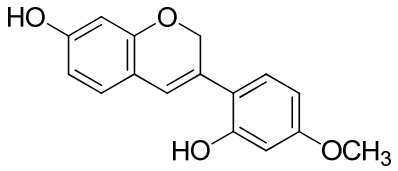
Summary

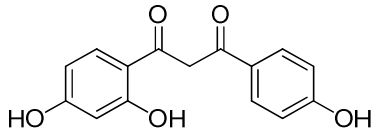
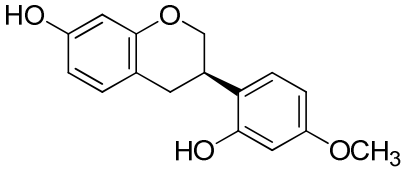
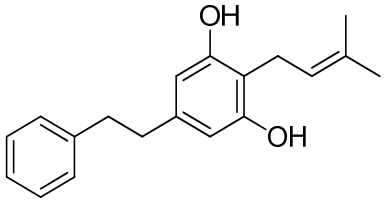
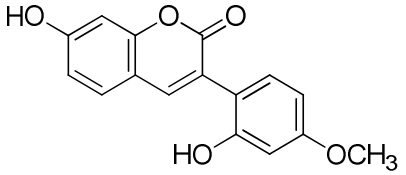
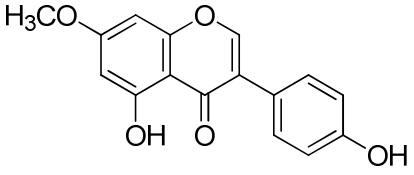
The structure and molecular weight of the main active constituents found in licorice, including the species and plant part of discovery and any notable biological activities. Compounds are organized from lowest to highest molecular weight.

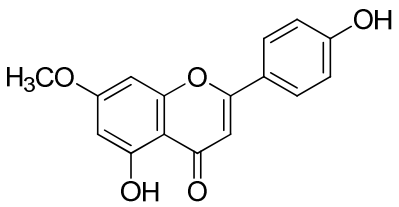
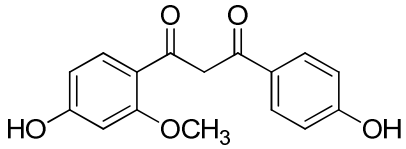
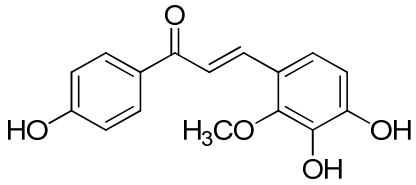
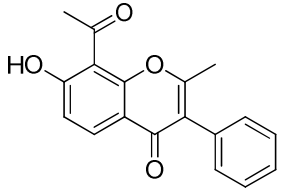
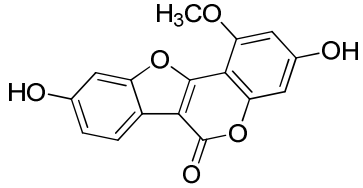
Table 1. Compounds found in plants of the *Glycyrrhiza* (licorice) genus[a]

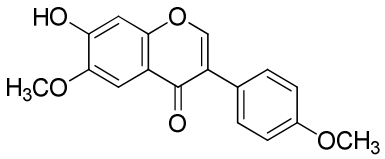
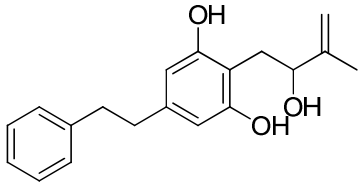
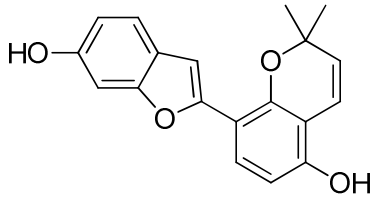
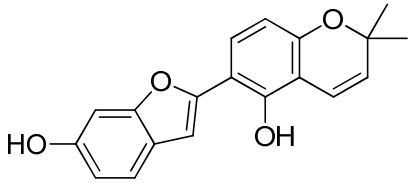
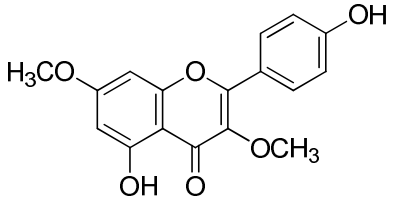
No.	Compound name (class)	Chemical structure	Chemical formula, exact mass	Botanical species, plant part, and biological activity information	References
1	Umbelliferon (7-hydroxycoumarin)		$C_9H_6O_3$ 162.0317	• Isolated from *G. glabra* roots	Ammosov and Litvinenko, 2007; Asl and Hosseinzadeh, 2008; Fenwick *et al.*, 1990
2	Salicylic acid (phenolic acid)		$C_8H_{10}O_4$ 170.0579	• Isolated from *G. glabra* roots • Anti-oxidant activity • Anti-acne activity • Fever and pain reducer	Mitscher *et al.*, 1980
3	Herniarin (methoxy-coumarin)		$C_{10}H_8O_3$ 176.0473	• Isolated from *G. glabra*	Asl and Hosseinzadeh, 2008; Fenwick *et al.*, 1990
4	*O*-acetyl salicylic acid (phenolic acid)		$C_9H_8O_4$ 180.0423	• Isolated from *G. glabra* roots	Mitscher *et al.*, 1980
5	Liqcoumarin (methylcoumarin)		$C_{12}H_{10}O_4$ 218.0579	• Isolated from *G. glabra* roots	Asl and Hosseinzadeh, 2008; Bhardwaj *et al.*, 1976

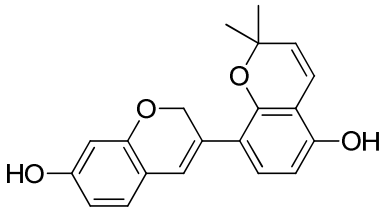
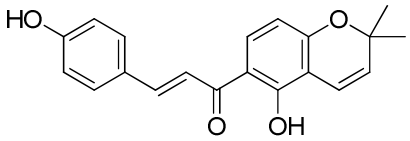
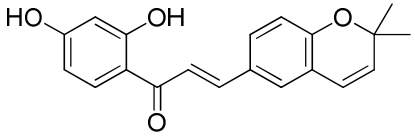
6	4',7-dihydroxyflavone (flavone)		$C_{15}H_{10}O_4$ 254.0579	• Isolated from *G. pallidiflora* roots	Kajiyama *et al.*, 1993
7	Isoliquiritigenin (chalcone)		$C_{15}H_{12}O_4$ 256.0736	• Isolated from *G. glabra* roots • Anti-oxidant activity towards LDL oxidation • Gastic ulcer therapy	Fenwick *et al.*, 1990; Hayashi *et al.*, 1996; Hiraga *et al.*, 1984; Kajiyama *et al.*, 1993; Vaya *et al.*, 1997
8	Liquiritigenin (flavanone)		$C_{15}H_{12}O_4$ 256.0736	• Isolated from *G. glabra* , *G. urlanensis*, and *G. Korshinskyi* roots and aerial parts	Fenwick *et al.*, 1990; Kajiyama *et al.*, 1993; Liao *et al.*, 2012
9	Pinocembrin (flavanone)		$C_{15}H_{12}O_4$ 256.0736	• Isolated from *G. glabra*, *G. uralensis*, and *G. lepidota* whole plant • Antimicrobial activity against: *Staphylococcus aureus*, *Mycobacterium smegmatis*, and *Candida albicans*	Hayshi *et al.*, 1996; Mitscher *et al.*, 1983

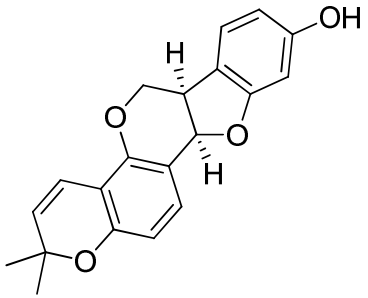
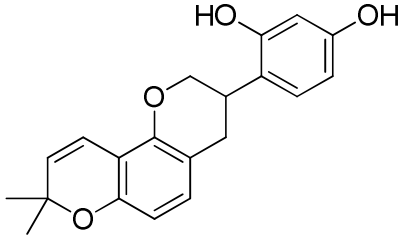
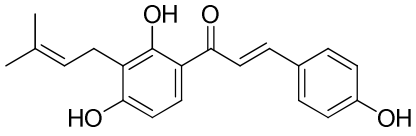
10	5-(3-hydroxy-4-methoxyphenethyl)benzene-1,3-diol (bibenzyl)		$C_{15}H_{16}O_4$ 260.1049	• Isolated from *G. glabra* leaves • Anti-inflammatory activity • Anti-oxidant activity • Antigenotoxic activity	Asl and Hosseinzadeh, 2008; Biondi *et al.*, 2005; Siracusa *et al.*, 2011
11	Formononetin (isoflavone)		$C_{16}H_{12}O_4$ 268.0736	• Isolated from *G. glabra* and *G. pallidiflora* roots • Estrogenic activity	Fenwick *et al.*, 1990; Hayashi *et al.*, 1996; Kajiyama *et al.*, 1993, Mitscher *et al.*, 1980; Vaya *et al.*, 1997
12	(-)-Medicarpin (pterocarpan)		$C_{16}H_{14}O_4$ 270.0892	• Isolated from *G. pallidiflora* roots	Kajiyama *et al.*, 1993
13	Echinatin (retrochalcone)		$C_{16}H_{14}O_4$ 270.0892	• Isolated from *G. echinata, G. inflata,* and *G. pallidiflora* roots • Estrogenic activity	Kajiyama *et al.*, 1993, Asl and Hosseinzadeh, 2008
14	2'-7-dihydroxy-4'-methoxyisoflav-3-ene (isoflavonoid)		$C_{16}H_{14}O_4$ 270.0892	• Isolated from *G. pallidiflora* roots	Kajiyama *et al.*, 1993

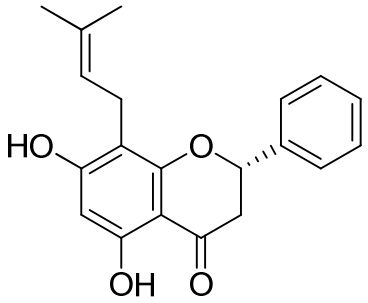
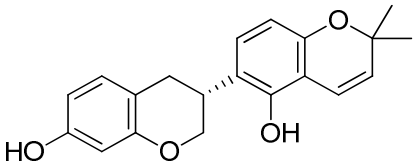
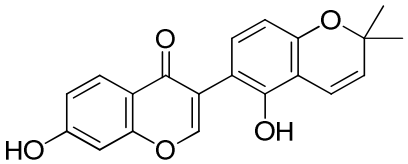
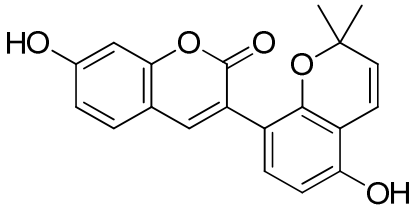
15	Licodione (dibenzoylmethane)		$C_{15}H_{12}O_5$ 272.0685	• Isolated from *G. pallidiflora* roots	Kajiyama *et al.*, 1993
16	(-)-Vestitol (isoflavan)		$C_{16}H_{16}O_4$ 272.1049	• Isolated from *G. pallidiflora* roots	Kajiyama *et al.*, 1993
17	3,5-dihydroxy-4-(3-methyl-2-butenyl)-bibenzyl (prenylated bibenzyl)		$C_{19}H_{22}O_2$ 282.1620	• Isolated from *G. lepidota* whole plant • Antimicrobial activity against: *S. aureus, M. smegmatis,* and *C. albicans*	Mitscher *et al.*, 1983, Gollapudi *et al.*, 1989
18	2'-7-dihydroxy-4'-methoxy-3-arylcoumarin		$C_{16}H_{12}O_5$ 284.0685	• Isolated from *G. pallidiflora* roots	Kajiyama *et al.*, 1993
19	Prunetin (*O*-methyl-isoflavone)		$C_{16}H_{12}O_5$ 284.0685	• Isolated from *G. glabra* aerial parts	Ammosov and Litvinenko, 2007; Fenwick *et al.*, 1990; Hayashi *et al.*, 1996

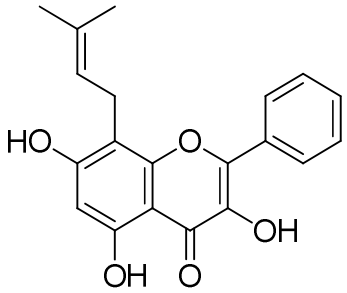
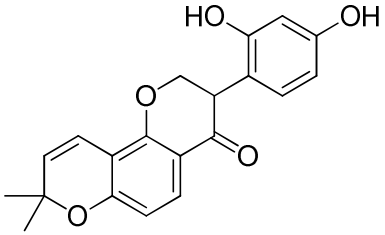
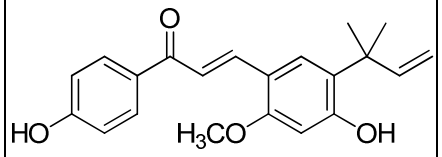
20	Genkwanin (*O*-methylflavone)		$C_{16}H_{12}O_5$ 284.0685	Isolated from *G. glabra* and *G. uralensis* aerial parts	Fenwick *et al.*, 1990
21	2'-*O*-methyl-licodione (dibenzoylmethane)		$C_{16}H_{14}O_5$ 286.0841	• Isolated from *G. pallidiflora* roots	Kajiyama *et al.*, 1993
22	Licochalcone B (retrochalcone)		$C_{16}H_{14}O_5$ 286.0841	• Isolated from *G. inflata*	Asl and Hosseinzadeh, 2008; Fenwick *et al.*, 1990
23	Glyzarin (isoflavone)		$C_{18}H_{14}O_4$ 294.0892	• Isolated from *G. glabra* roots	Ammosov and Litvinenko, 2007; Asl and Hosseinzadeh, 2008
24	Isotrifoliol (coumestan)		$C_{16}H_{10}O_6$ 298.0477	• Isolated from *G. uralensis* underground parts	Ammosov and Litvinenko, 2007; Asl and Hosseinzadeh, 2008

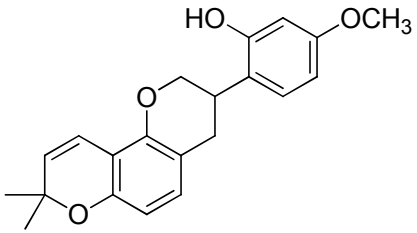
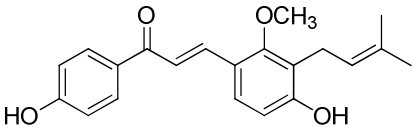
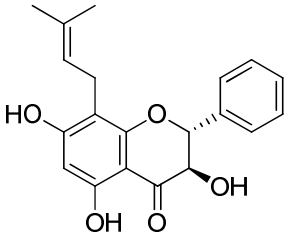
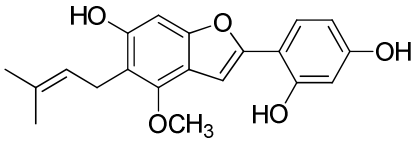
25	Afromosin (isoflavone)		$C_{17}H_{14}O_5$ 298.0841	• Isolated from *G. pallidiflora*	Ammosov and Litvinenko, 2007; Kajiyama *et al.*, 1993
26	Glepidotin C (prenylated bibenzyl)		$C_{19}H_{22}O_3$ 298.1569	• Isolated from *G. lepidota* aerial parts • Weakly antimicrobial against: *M. smegmatis*	Ammosov and Litvinenko, 2007; Gollapudi *et al.*, 1989; Manfredi *et al.*, 2001; Mitscher *et al.*, 1993;
27	Glabrocoumarone A (coumarin)		$C_{19}H_{16}O_4$ 308.1049	• Isolated from *G. glabra*	Asl and Hosseinzadeh, 2008
28	Glabrocoumarone B (coumarin)		$C_{19}H_{16}O_4$ 308.1049	• Isolated from *G. glabra*	Asl and Hosseinzadeh, 2008
29	Kumatakenin (isoflavone)		$C_{17}H_{14}O_6$ 314.0790	• Isolated from *G. spp* roots	Asl and Hosseinzadeh, 2008; Hiraga *et al.*, 1984

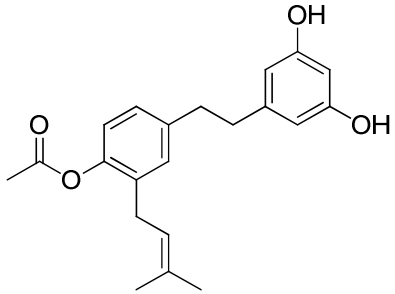
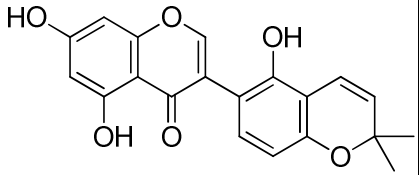
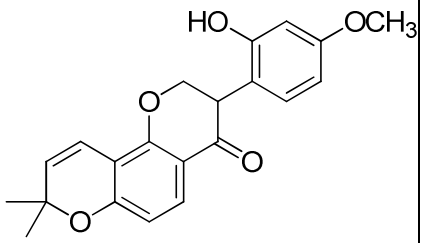
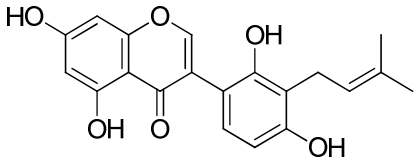
30	Glabrene (isoflavonoid)		$C_{20}H_{18}O_4$ 322.1205	• Isolated from *G. spp* roots • Antimicrobial against*: S. aureus,* MRSA, *M. smegmatis, C. albicans, Micrococcus luteus,* and *Bacillus subtilis*	Asl and Hosseinzadeh, 2008; Fenwick *et al.*, 1990; Fukai *et al.*, 2002; Hiraga *et al.*, 1984; Mitscher *et al.*, 1980
31	4-Hydroxylonchocarpin (chalcone)		$C_{20}H_{18}O_4$ 322.1205	• Isolated from *G. glabra* hairy root cultures • Antimicrobial activity against: *S. aureus, E.coli, C. albicans, P. aeruginosa,* and *B. subtilis*	Li *et al.*, 1998
32	Kanzonol B (chalcone)		$C_{20}H_{18}O_4$ 322.1205	• Isolated from *G. glabra* hairy root cultures • Antimicrobial activity against: *S. aureus, E .coli, C. albicans, Pseudomonas aeruginosa,* and *B. subtilis*	Li *et al.*, 1998

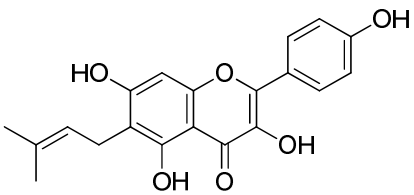
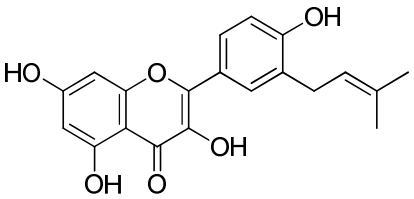
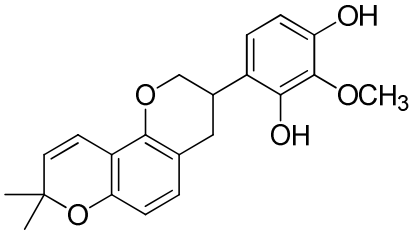
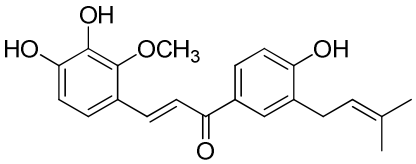
33	Shinpterocarpin (pterocarpans)		$C_{20}H_{18}O_4$ 322.1205	• Isolated from *G. spp and G. glabra* roots • PPAR-γ ligand binding activity • Potential antidiabetes and anti-obesity activity	Ammosov and Litvinenko, 2007; Asl and Hosseinzadeh, 2008; Simmler *et al.*, 2013a
34	Glabridin (isoflavane)		$C_{20}H_{20}O_4$ 324.1362	• Isolated from *G. spp and G. glabra* roots • Anti-oxidant activity towards LDL oxidation • Antimicrobial activity against: *S. aureus, Methicillin-resistant S. aureus, M. smegmatis, C. albicans, Mycobacterium tuberculosis, M. luteus,* and *B. subtilis*	Fukai *et al.*, 2002; Gupta *et al.*, 2008; Hiraga *et al.*, 1984; ; Li *et al.*, 1998; Mitscher *et al.*, 1980; Simmler *et al.*, 2013b; Vaya *et al.*, 1997
35	Isobavachalcone (chalcone)		$C_{20}H_{20}O_4$ 324.1362	• Isolated from *G. glabra* hairy root cultures • Antimicrobial activity against: *S. aureus, E .coli, C. albicans, P. aeruginosa,* and *B. subtilis*	Li *et al.*, 1998

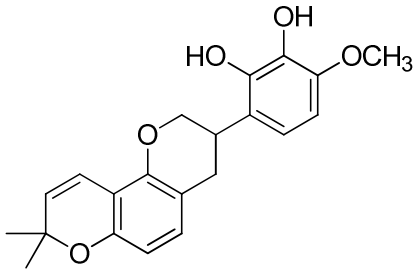
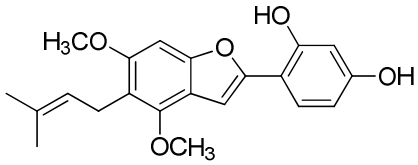
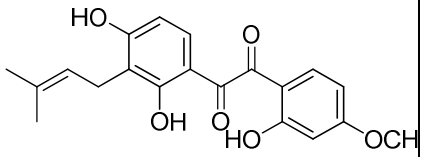
36	Glabranin (flavanone)		$C_{20}H_{20}O_4$ 324.1362	• Isolated from *G. glabra, G. uralensis,* and *G. lepidota* whole plants • Antimicrobial activity against: *S. aureus, M. smegmatis,* and *C. albicans*	Ammosov and Litvinenko, 2007; Siracusa *et al.*, 2011
37	Phaseollinisoflavan (benzopyran)		$C_{20}H_{20}O_4$ 324.1362	• Isolated from *G. glabra* roots • Antimicrobial activity against: *S. aureus, M. smegmatis,* and *C. albicans*	Fenwick *et al.*, 1990, Mitscher *et al.*, 1980
38	Glabrone (isoflavanoid)		$C_{20}H_{16}O_5$ 336.0998	• Isolated from *G. glabra* roots	Ammosov and Litvinenko, 2007; Asl and Hosseinzadeh, 2008; Hiraga *et al.*, 1984
39	Glabrocoumarin (coumarin)		$C_{20}H_{16}O_5$ 336.0998	• Isolated from *G. glabra*	Asl and Hosseinzadeh, 2008

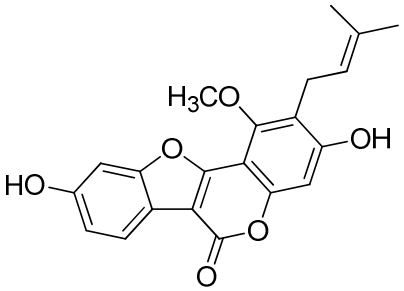
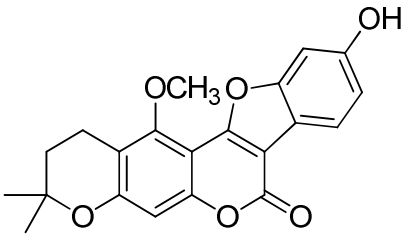
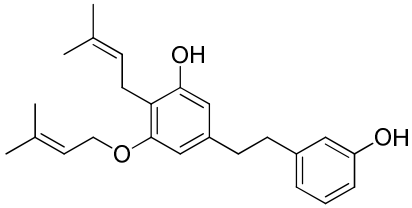
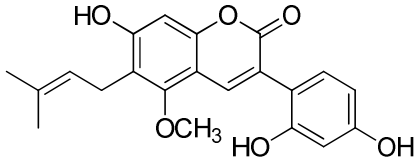
40	Glepidotin A (prenylated flavanol)		$C_{20}H_{18}O_5$ 338.1154	• Isolated from *G. lepidota* leaves and whole plants • Antimicrobial activity against: *S. aureus*, *M. smegmatis*, *C. albicans*	Gollapudi *et al.*, 1989 Manfredi *et al.*, 2001, Mitscher *et al.*, 1983
41	Glabroisoflavanone A (isoflavone)		$C_{20}H_{18}O_5$ 338.1154	• Isolated from *G. spp*	Asl and Hosseinzadeh, 2008
42	Licochalcone A (retrochalcone)		$C_{21}H_{22}O_4$ 338.1518	• Isolated from *G. uralensis*, *G. inflata*, and *G. spp* roots • Antitubercular activity • Antimicrobial activity against: (*S. aureus*, *MRSA*, *M. luteus*, and *B. subtilis*) • Anti-HIV activity	Ammosov and Litvinenko, 2007; Asl and Hosseinzadeh, 2008; Fukai *et al.*, 2002; Gupta *et al.*, 2008; Hiraga *et al.*, 1984; Zhang and Ye, 2009

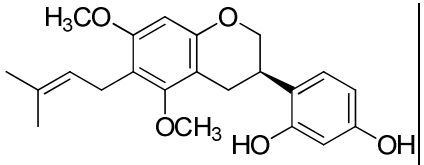
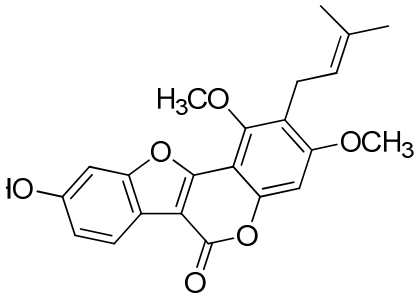
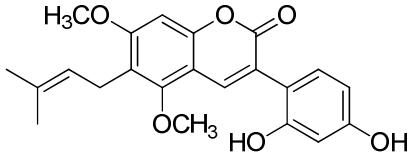
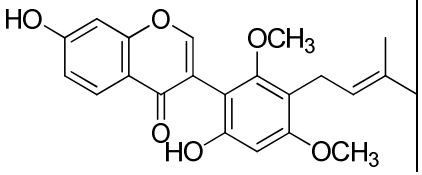
43	4'-*O*-Methylglabridin (isoflavan)		$C_{21}H_{22}O_4$ 338.1518	• Isolated from *G. glabra* roots • Anti-oxidant activity toward LDL oxidation • Antimicrobial activity against: *S. aureus, M. smegmatis,* and *C. albicans*	Li *et al.*, 1998; Mitscher *et al.*, 1980; Vaya *et al.*, 1997
44	Licochalcone C (retrochalcone)		$C_{21}H_{22}O_4$ 338.1518	• Isolated from *G. inflate* roots	Asl and Hosseinzadeh, 2008
45	Glepidotin B (prenylated flavanol)		$C_{20}H_{20}O_5$ 340.1311	• Isolated from *G. lepidota* leaves and whole plants • Antimicrobial activity against: *S. aureus, M. smegmatis,* and *C. albicans*	Manfredi *et al.*, 2001, Mitscher *et al.*, 1983
46	Licocoumarone (benzofuran)		$C_{20}H_{20}O_5$ 340.1311	• Isolated from *G. uralensis* roots	Ammosov and Litvinenko, 2007; Fenwick *et al.*, 1990; Zhang and Ye, 2009

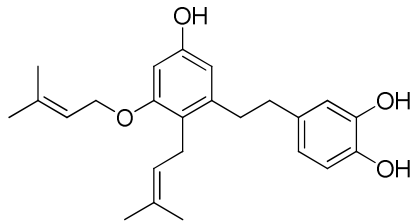
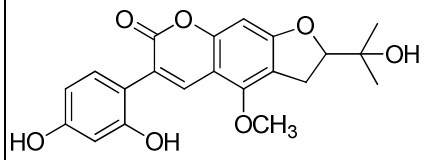
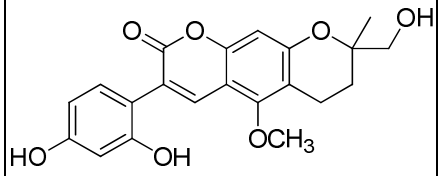
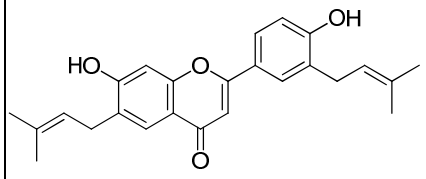
47	4-(3,5-dihydroxyphenethyl)-2-(3-methylbut-2-en-1-yl)phenyl acetate (prenylated bibenzyl)		$C_{21}H_{24}O_4$ 340.1675	• Isolated from *G. glabra* leaves • Anti-inflammatory activity • Anti-oxidant activity • Antigenotoxic activity	Asl and Hosseinzadeh, 2008; Biondi *et al.*, 2005; Siracusa *et al.*, 2011
48	Licoisoflavone B (isoflavone)		$C_{20}H_{16}O_6$ 352.0947	• Isolated from *G. spp.* roots • Antimicrobial activity against: *S. aureus* and MRSA	Asl and Hosseinzadeh, 2008; Fukai *et al.*, 2002; Hiraga *et al.*, 1984
49	Glabroisoflavanone B (coumarin)		$C_{21}H_{20}O_5$ 352.1311	• Isolated from *G. spp.*	Asl and Hosseinzadeh, 2008
50	Licoisoflavone A (prenylated isoflavone)		$C_{20}H_{18}O_6$ 354.1103	• Isolated from *G. spp.* roots • Antitubercular activity	Asl and Hosseinzadeh, 2008; Gupta *et al.*, 2008; Hiraga *et al.*, 1984

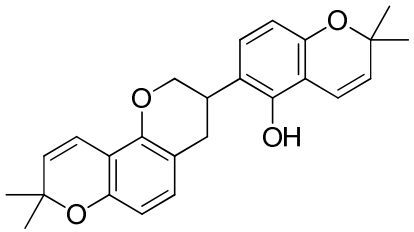
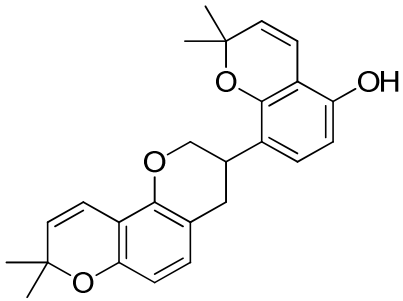
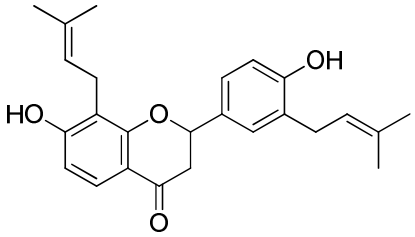
51	Licoflavonol (flavonol)		$C_{20}H_{18}O_6$ 354.1103	• Isolated from *G. spp.* roots	Hiraga *et al.*, 1984
52	Isolicoflavanol (flavonol)		$C_{20}H_{18}O_6$ 354.1103	• Isolated from *G. glabra* and *G. urlanensis* • Moderate antimicrobial activity against: *S. aureus*, MRSA, *M. luteus*, and *B. subtilis*	Fukai *et al.*, 2002; Liao *et al.*, 2012
53	3'-methoxyglabridin (isoflavanoid)		$C_{21}H_{22}O_5$ 354.1467	• Isolated from *G. glabra* roots • Antimicrobial activity against: *S. aureus*, *M. smegmatis*, and *C. albicans*	Fenwick *et al.*, 1990; Mitscher *et al.*, 1980
54	Licochalcone D (retrochalcone)		$C_{21}H_{22}O_5$ 354.1467	• Isolated from *G. inflata* roots	Asl and Hosseinzadeh, 2008

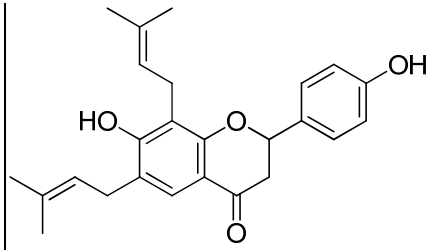
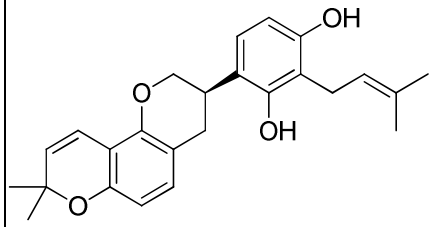
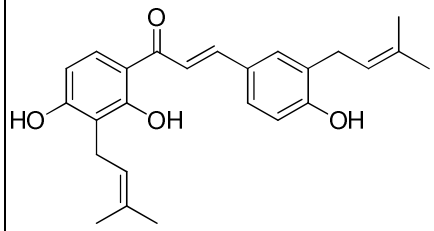
55	3'-Hydroxy-4'-*O*-methylglabridin (isoflavone)		$C_{21}H_{22}O_5$ 354.1467	• Isolated from *G. spp.*	Asl and Hosseinzadeh
56	Gancaonin I (benzofuran)		$C_{21}H_{22}O_5$ 354.1467	• Isolated from *G. uralensis* • Antimicrobial activity against: *S. aureus*, MRSA, *M. luteus*, and *B. subtilis*	Fukai *et al.*, 2002
57	Licoagrodione (prenylated bibenzoyl)		$C_{20}H_{20}O_6$ 356.1260	• Isolated from *G. glabra* hairy root cultures • Antimicrobial activity against: *S, aureus, B. subtilis, E. coli, P. aeruginosa, Klebsiella pneumoniae, C. albicans, Aspergillus niger*, and *Trichophyton mentagrophytes*	Li *et al.*, 1998

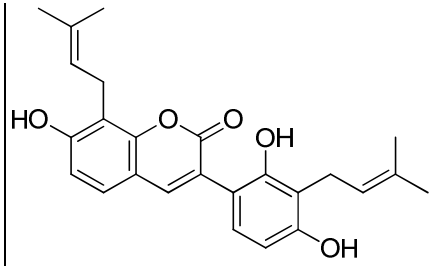
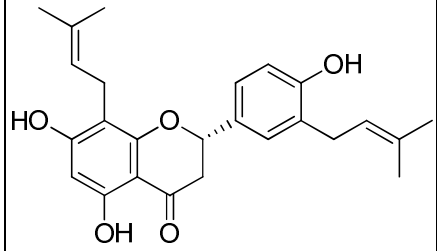
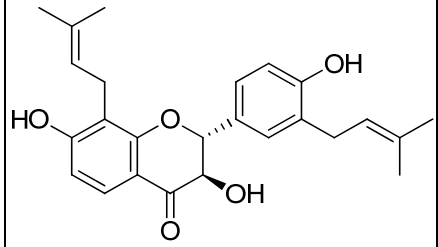
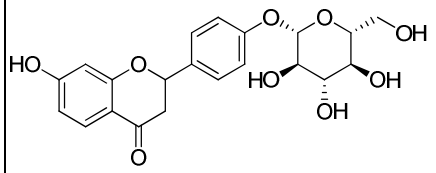
58	Glycyrol (coumestan)		$C_{21}H_{18}O_6$ 366.1103	• Isolated from *G. uralensis* roots and stems • Antimicrobial activity against: *S, aureus*, MRSA, *B subtilis*, and *M. luteus*	Ammosov and Litvinenko, 2007; Fukai *et al.*, 2002; Hiraga *et al.*, 1984; Zhang and Ye, 2009
59	Isoglycyrol (coumestan)		$C_{21}H_{18}O_6$ 366.1103	• Isolated from *G. uralensis* roots and stems	Ammosov and Litvinenko, 2007; Fenwick *et al.*, 1990; Fukai *et al.*, 2002 ; Zhang and Ye, 2009
60	Glepidotin D (prenylated bibenzyl)		$C_{24}H_{30}O_3$ 366.2195	• Isolated from *G. lepidota* leaves • NCI *in vitro* anti-HIV 1 activity	Biondi *et al.*, 2005; Manfredi *et al.*, 2001
61	Glycycoumarin (coumarin)		$C_{21}H_{20}O_6$ 368.1260	• Isolated from *G. uralensis* roots and stems • Anti-HIV activity	Asl and Hosseinzadeh, 2008; Fenwick *et al.*, 1990; Hayashi *et al.*, 2005; Zhang and Ye, 2009

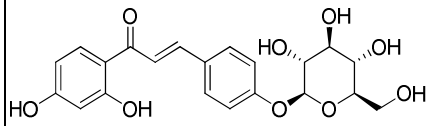
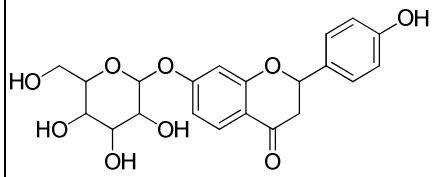
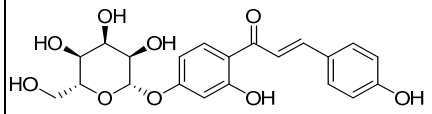
62	Glyasperin D (isoflavanoid)		$C_{22}H_{26}O_5$ 370.1780	• Isolated from *G. uralensis* • Antimicrobial activity against: *S, aureus*, MRSA, *B subtilis*, and *M. luteus*	Fukai *et al.*, 2002
63	3-*O*-methylglycyrol (coumestan)		$C_{22}H_{20}O_6$ 380.1260	• Isolated from *G. uralensis* • Antimicrobial activity against: *S, aureus*, MRSA, *B subtilis, E. coli*, and *M. luteus*	Fenwick *et al.*, 1990; Fukai *et al.*, 2002
64	Glycyrin (coumarin)		$C_{22}H_{22}O_6$ 382.1416	• Isolated from *G. glabra* and *G. uralensis* roots • Moderate antimicrobial activity against: *S, aureus*, MRSA, *B subtilis, E. coli*, and *M. luteus*	Ammosov and Litvinenko, 2007; Asl and Hosseinzadeh, 2008; Hiraga *et al.*, 1984; Fukai *et al.*, 2002; Zhang and Ye, 2009
65	Licoricone (prenylated isoflavonoid)		$C_{22}H_{22}O_6$ 382.1416	• Isolated from *G. glabra* and *G. uralensis* roots	Ammosov and Litvinenko, 2007; Fenwick *et al.*, 1990

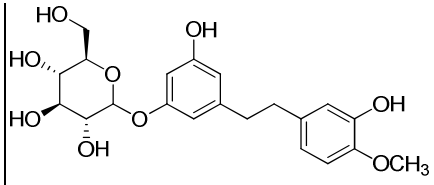
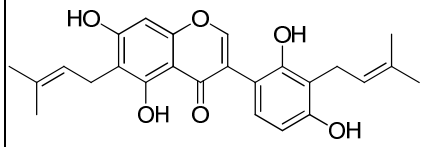
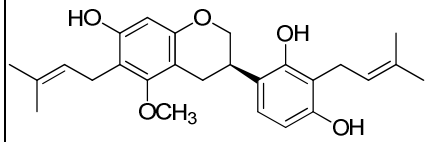
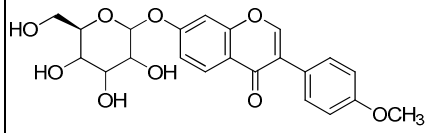
66	4-(5-hydroxy-2-(3-methylbut-2-en-1-yl)-3-((3-methylbut-2-en-1-yl)oxy)phenethyl)benzene-1,2-diol (prenylated bibenzyl)		$C_{24}H_{30}O_4$ 382.2144	• Isolated from *G. glabra* leaves • Anti-inflammatory activity • Anti-oxidant activity • Antigenotoxic activity	Asl and Hosseinzadeh, 2008; Biondi *et al.*, 2005; Siracusa *et al.*, 2011
67	Licofuranocoumarin (coumarin)		$C_{21}H_{20}O_7$ 384.1209	• Isolated from *G. glabra*	Asl and Hosseinzadeh, 2008
68	Licopyranocoumarin (coumarin)		$C_{21}H_{20}O_7$ 384.1209	• Isolated from *G. glabra* and *G. uralensis* • Anti-HIV activity	Asl and Hosseinzadeh, 2008; Zhang and Ye, 2009
69	Licoflavone B (prenylated flavone)		$C_{25}H_{26}O_4$ 390.1831	• Isolated from *G. inflata* and G. *echinata* roots	Ammosov and Litvinenko, 2007; Hiraga *et al.*, 1984

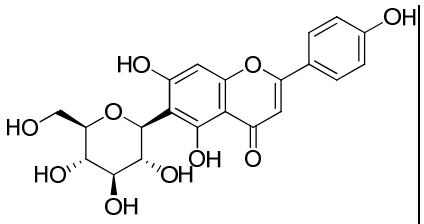
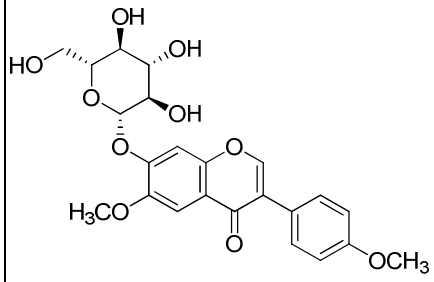
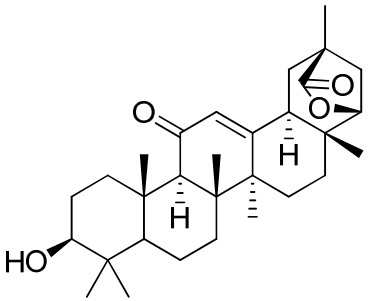
70	Hispaglabridin B (isoflavone)		$C_{25}H_{26}O_4$□3 90.1831	• Isolated from *G. glabra* roots • Anti-oxidant activity towards LDL oxidation • Antimicrobial activity against: *S. aureus, M. smegmatis,* and *C. albicans*	Asl and Hosseinzadeh, 2008; Gupta *et al.*, 2008; Mitscher *et al.*, 1980; Vaya *et al.*, 1997
71	Glyinflanin K (isoflavan)		$C_{25}H_{26}O_4$39 0.1831	• Isolated from *G. glabra* hairy root cultures and *G. inflata* roots	Ammosov and Litvinenko, 2007; Li *et al.*, 1998
72	Glabrol (prenylated flavanone)		$C_{25}H_{28}O_4$□ 392.1988	• Isolated from *G. spp.* roots • Antimicrobial activity against: *S. aureus* and *M. smegmatis*	Fenwick *et al.*, 1990; Hiraga *et al.*, 1984; Mitscher *et al.*, 1980

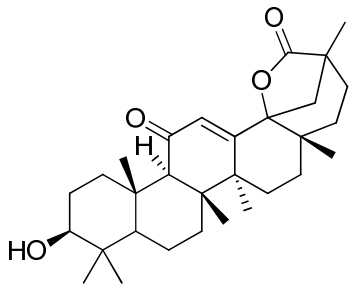
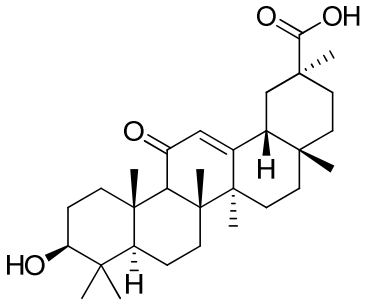
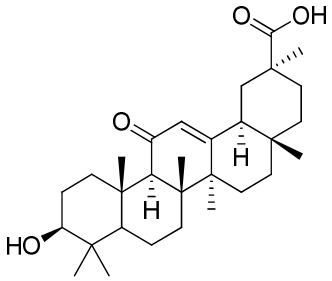
73	4',7-Dihydroxy-6,8-diprenylflavanone (flavanone)		$C_{25}H_{28}O_4$ 392.1988	• Isolated from *G. pallidiflora* roots	Kajiyama *et al.*, 1993
74	Hispaglabridin A (prenylated isoflavone)		$C_{25}H_{28}O_4$ 392.1988	• Isolated from *G. glabra.* roots • Anti-oxidant activity towards LDL oxidation • Antimicrobial activity against: *S. aureus, M. smegmatis,* and *C. albicans*	Asl and Hosseinzadeh, 2008; Mitscher *et al.*, 1980; Vaya *et al.*, 1997
75	1-(2,4-dihydroxy-3-(3-methylbut-2-en-1-yl) phenyl)-3-(4-hydroxy-3-(3-methylbut-2-en-1-yl) phenyl)prop-2-en-1-one (prenylated chalcone)		$C_{25}H_{28}O_4$ 392.1988	• Isolated from *G. glabra.* roots • Anti-oxidant activity towards LDL oxidation	Vaya *et al.*, 1997

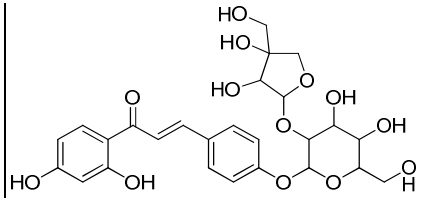
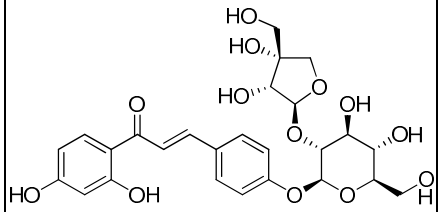
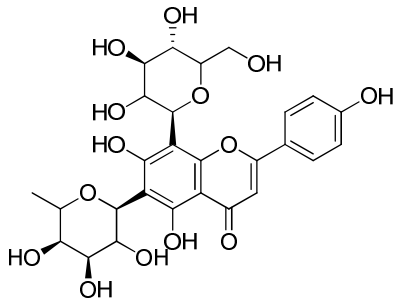
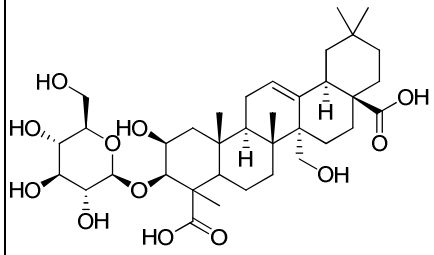
76	Licocoumarin A (prenylated coumarin)		$C_{25}H_{26}O_5$ 406.1780	• Isolated from *G. glabra.* roots and *G. uralensis* stems	Ammosov and Litvinenko, 2007; Fenwick *et al.*, 1990
77	Lespedeza-flavanone B (prenylated flavanone)		$C_{25}H_{28}O_5$ 408.1937	• Isolated from *G. glabra.* hairy root cultures • Antimicrobial activity	Li *et al.*, 1998
78	3-Hydroxyglabrol (prenylated flavanone)		$C_{25}H_{28}O_5$ 408.1937	• Isolated from *G. glabra* roots • Antimicrobial activity against: *S. aureus, M. smegmatis,* and *C. albicans*	Ammosov and Litvinenko, 2007; Li *et al.*, 1998, Mitscher *et al.*, 1980
79	Liquiritin (flavanone glucoside)		$C_{21}H_{22}O_9$ 418.1264	• Isolated from *G. glabra.* and *G. inflata* roots and aerial parts • Moderate antimicrobial activity against: *S,*	Fukai *et al.*, 2002; Hiraga *et al.*, 1984; Isbrucker and Burdock, 2006; Liao *et al.*, 2012; Nomura and Fukai, 1998

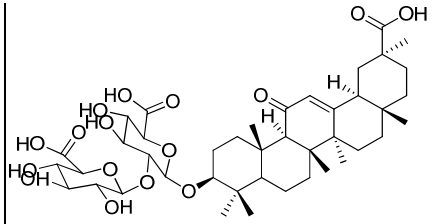
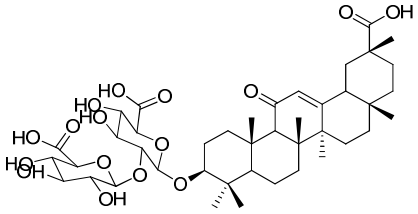
				aureus, MRSA, *B. subtilis*, *E. coli*, and *M. luteus* • Anticarcinogenic activity	
80	Isoliquiritin (chalcone)		$C_{21}H_{22}O_9$ 418.1264	• Isolated from *G. glabra*, *G. uralensis*, and *G. korshinskyi.* roots • Potential depigmenting agent • Anticarcinogenic activity	Ammosov and Litvinenko, 2007; Asl and Hosseinzadeh, 2008; Hayashi *et al.*, 1996; Hiraga *et al.*, 1984; Isbrucker and Burdock, 2006; Nomura and Fukai, 1998
81	Neoliquiritin (flavanone)		$C_{21}H_{22}O_9$ 418.1264	• Isolated from *G. glabra.* roots	Hayashi *et al.*, 1996; Hiraga *et al.*, 1984; Liao *et al.*, 2012
82	Neoisoliquiritin (chalcone glycoside)		$C_{21}H_{22}O_9$ 418.1264	• Isolated from *G. glabra*, *G. uralensis*, and *G. korshinskyi.* roots	Ammosov and Litvinenko, 2007; Hayashi *et al.*, 1996; Hiraga *et al.*, 1984, Liao *et al.*, 2012; Nomura and Fukai, 1998

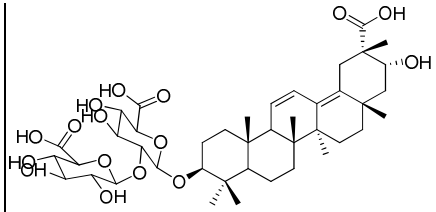
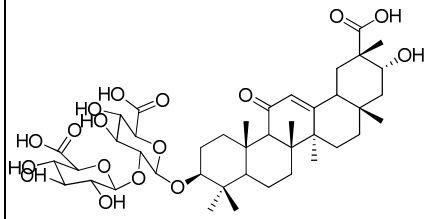
83	2-(3-hydroxy-5-(3-hydroxy-4-methoxyphenethyl)phenoxy)-6-(hydroxymethyl) tetrahydro-2-pyran-3,4,5-triol (bibenzyl glycoside)		$C_{21}H_{26}O_9$ 422.1577	• Isolated from *G. glabra* leaves • Anti-inflammatory activity • Anti-oxidant activity • Antigenotoxic activity	Asl and Hosseinzadeh, 2008; Biondi *et al.*, 2005; Siracusa *et al.*, 2011
84	Glisoflavanone (flavonoid)		$C_{25}H_{26}O_6$ 422.1729	• Isolated from *G. urlanensis.* underground parts	Asl and Hosseinzadeh, 2008
85	Licoricidin (prenylated isoflavonoid)		$C_{26}H_{32}O_5$ 424.2250	• Isolated from *G. glabra and G. urlanensis* • Antimicrobial activity against: *S. aureus*, MRSA, *M. luteus*, and *B. subtilis*	Fenwick *et al.*, 1990; Fukai *et al.*, 2002; Liao *et al.*, 2012
86	Ononin (isoflavone glycoside)		$C_{22}H_{22}O_9$ 430.1264	• Isolated from *G. pallidiflora* roots	Kajiyama *et al.*, 1993

87	Saponaretin (flavone glycoside)		$C_{21}H_{20}O_{10}$ 432.1056	• Isolated from *G. glabra* and *G. urlanensis.* aerial parts	Ammosov and Litvinenko, 2007; Fenwick *et al.*, 1990
88	Wistin (isoflavone glycoside)		$C_{23}H_{24}O_{10}$ 460.1369	• Isolated from *G. pallidiflora* roots	Kajiyama *et al.*, 1993
89	Glabrolide (triterpene)		$C_{30}H_{44}O_4$ 468.3240	• Isolated from *G. glabra, G.uralensis, G. inflata,* and *G. aspera* roots	Ammosov and Litvinenko, 2003; Asl and Hosseinzadeh, 2008; Nomura and Fukai, 1998

90	Isoglabrolide (triterpene)		$C_{30}H_{44}O_4$ 468.3240	• Isolated from *G. glabra.* roots	Ammosov and Litvinenko, 2003; Asl and Hosseinzadeh, 2008; Nomura and Fukai, 1998
91	Glycyrrhetic acid, Glycyrrhetinic acid (triterpene)		$C_{30}H_{46}O_4$ 470.3396	• Isolated from *G. glabra.* roots • Synthetic derivative carbenoxolone is a licensed drug treatment for ulcers and inflammation • Up to 50 times as sweet as sugar	Ammosov and Litvinenko, 2003; Asl and Hosseinzadeh, 2008; Beasley *et al.*, 1979; Isbrucker and Burdock, 2006
92	Liquiritic acid (triterpene)		$C_{30}H_{46}O_4$ 470.3396	• Isolated from *G. glabra* and *G. inflata* roots	Ammosov and Litvinenko, 2003; Asl and Hosseinzadeh, 2008; Nomura and Fukai, 1998; Zhang and Ye, 2009

93	Licuroside (chalcone)		$C_{26}H_{30}O_{13}$ 550.1686	• Isolated from *G. glabra* • Potential depigmenting agent	Asl and Hosseinzadeh, 2008; Fenwick *et al.*, 1990; Nomura and Fukai, 1998
94	Neolicuroside (chalcone)		$C_{26}H_{30}O_{13}$ 550.1686	• Isolated from *G. glabra.* • Potential depigmenting agent	Fenwick *et al.*, 1990; Nomura and Fukai, 1998
95	Isoviolanthin (flavone)		$C_{27}H_{30}O_{14}$ 578.1636	• Isolated from *G. glabra.* and *G. urlanesis* • Anti-oxidant activity	Liao *et al.*, 2012
96	Tenuifolin (triterpene saponin)		$C_{36}H_{56}O_{12}$ 680.3772	• Isolated from *G. glabra.* hairy root cultures • Antimicrobial activity	Li *et al.*, 1998

97	Glycyrrhizin (triterpene saponin)		$C_{42}H_{62}O_{16}$ 822.4038	• Isolated from *G. spp.* • Trade names: Epigen, Glycyron • 5-25% of root content naturally occurs as calcium or potassium salt • Distinct and lingering sweet taste (50-200x sweeter than sucrose) • Anti-inflammatory • Antiviral, including anti-HIV • Anti-allergenic • Anti-ulcer, • Anti-oxidative • Chemopreventativ • Ammoniated salt is used to mask the bitter taste of cough syrups and other medicines	Ammosov and Litvinenko, 2003; Beasley *et al.*, 1979; Fenwick *et al.*, 1990; Hayashi *et al.*, 2005; Hiraga *et al.*, 1984; Isbrucker and Burdock, 2006; Montoro *et al.*, 2010
98	Licorice-saponin H2, Liquiritinic acid diglucoside (triterpene saponin)		$C_{42}H_{62}O_{16}$ 822.4038	• Isolated from *G. lepidota* and *G. uralensis* roots	Hayashi *et al.*, 2005; Nomura and Fukai, 1998

99	Macedonoside C (triterpene saponin)		$C_{42}H_{62}O_{16}$ 822.4038	• Isolated from *G. lepidota G. echinata, G. pallidiflora*, and *G. foetidissima* roots	Ammosov and Litvinenko 2003, Hayashi *et al.*, 2005
100	Macedonoside A (triterpene saponin)		$C_{42}H_{62}O_{17}$ 838.3987	• Isolated from *G. lepidota* roots	Hayashi *et al.*, 2005

[a]Compounds are organized by molecular weight.

Appendix C

MN Plant Collection Permits

Summary

The following collection permits were acquired from the State of Minnesota Department of Natural Resources prior to any collection of plant material from any Minnesota State Parks, Scientific and Natural Areas, or the Nature Conservancy Areas. The sustainable collection practices described in the permit applications were strictly followed.

STATE OF MINNESOTA
DEPARTMENT OF NATURAL RESOURCES
Division of Parks and Trails
SPECIAL PERMIT NO. 201050

DATE: June 27, 2010

PERMISSION IS HEREBY GRANTED TO:
The individual(s) listed below to do a project entitled **201054** as described in the research application. This permit applies only to those lands administered by the Division of Parks and Trails. The applicant is also subject to any other state or federal permits which may apply.

Permittee	**State Park(s)**	**State Park Contact Information**
Amanda C. Martin	Afton	Gene Groebner, 651-436-5391
	Big Stone Lake	Joanne Svendsen, 320-839-3663
	Buffalo River	Brian Nelson, 218-498-2124
	Glacial Lakes	Amy Schnoes, 320-239-2860
	Inspiration Peak c\o Lake Carlos	Elizabeth Murray
	Itasca	Chris Gronewold, 218-699-7208
	Kilen Woods	Phil Nasby, 507-831-2900
	Lake Bronson	Allen Lego, 218-754-2200
	Lake Louise c/o Forestville	Mark White, 507-352-5111
	Wild River	Paul Kurvers, 651-583-2125
	William O' Brien	Steve Anderson, 651-433-0500

1) Applicant must contact park manager to notify them when permitted activities are scheduled to begin.
2) The Park Manager may approve or disapprove where such activities may be carried on.
3) Permit Activity must be carried so as to minimize the potential to introduce, establish or spread invasive species.

4) The site where you will be working may be subject to management actions such as prescribed burning. Unless prior arrangements have been made with PAT, study sites will not be exempt from these actions.
5) It is a condition of this permit that interim progress reports be submitted annually by the expiration date of the permit. A final report is also required at the conclusion of the project. Reports shall be submitted to: Natural Resource Program Consultant, DNR/Parks and Trails, 500 Lafayette Rd., Box 39, St. Paul, MN 55155-4039. It is requested that interim and final reports be submitted electronically in either .docx or .pdf format.
6) You must have a copy of this permit when you are working in the park.
7) All markers, equipment, and other items used during the research must be removed at the end of the study. Marking ribbons, stakes or similar items must be marked with the permit number.
8) You are using the State Park at your own risk. You agree to take all necessary safety precautions to protect yourself, your assistants, and any other State Park visitors.
9) All rules for State Parks and State Trails remain in effect except that portion of 6100.0900 Subp. 1 which is waived to allow the research to be conducted.

This permit is valid from the date of issuance through October 15, 2010 but it may be revoked at any time.

Special Conditions
1. Only above ground parts of native prairie and wetland plants may be collected, no roots.
2. Per the application 100 grams of plant material per species may be collected from areas where numerous individuals are present such that at least several plants in a particular population are not impacted.
3. Equipment & clothing must be cleaned in between parks to reduce the risk of introducing invasive species.
4. No federal or state endangered/threatened species may be collected.

TOM LANDWEHR, COMMISSIONER
DEPARTMENT OF NATURAL RSOURCES

By_____
Edward M. Quinn
State Parks and Trails Resource Management Program Consultant

cc: Regional Resource Specialist
Are/Park Resource Specialist
Park Managers

STATE OF MINNESOTA
DEPARTMENT OF NATURAL RESOURCES
DIVISION OF ECOLOGICAL RESOURCES
SCIENTIFIC AND NATURAL AREAS PROGRAM

SPECIAL PERMIT NUMBER: **2010-28R**

SCIENTIFIC AND NATURAL AREAS: **sites to be selected from among all SNAs statewide**

DATE: August 2, 2010

By virtue of the authority conferred on me by the Commissioner of Natural Resources relative to Scientific and Natural Areas, I grant permission to:
Amanda C. Martin, University of Minnesota, Department of Horticultural Science, Alderman Hall Rm 305, 1970 Folwell Ave., St. Paul, MN 55108, 651-624-5300, mart2406@umn.edu
to enter upon the above Scientific and Natural Areas (SNAs) for the purpose of **collecting and screening native and naturalized Minnesota plants for antimicrobial and antioxidant biological activity** as described in the proposal (dated 7/15/10; with the 7/19/10 revised plant list) and under the conditions listed below.

Since several SNAs are also preserves of The Nature Conservancy, this letter serves as a joint permit with the Conservancy and the MN DNR (TNC permit #2010-13). Please call or e-mail Meredith Cornett, 218-727-6119 or mcornett@TNC.ORG if you have any questions about the TNC portion of the permit

It is understood that the above named persons have a clear understanding of the purpose and long-term goal of state Scientific and Natural Areas. In keeping with this purpose, they shall always conduct their activities in a manner that is least disruptive to the on-going natural processes of these areas. All activities carried out must be in accordance with the proposal submitted. Permission must be received from the SNA Program if the permittee desires or anticipates deviating from this permit. In addition, the following conditions are placed on the proposal submitted:

1. Submit a list of the SNAs where collection is taking place to the SNA program when the locations are known.
2. Collection of state listed endangered or threatened species may be collected is

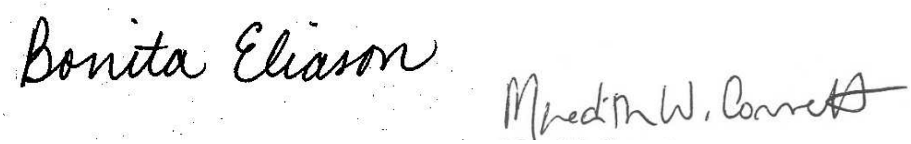
depended upon your having a special permit from the DNR's Division of Ecological Resources through Richard Baker, Minnesota Endangered Species Coordinator, (651-259-5073),(richard.baker@state.mn.us).

3. All markers, equipment, and other items used during the research shall be removed at the end of the study.
4. Equipment and procedures used to collect specimens or mark plots or other features should be placed or used so as not to cause damage to the resources.
5. All work shall be done to prevent the inadvertent transport of invasive species.
6. Please carry this permit while on the SNA and extend courtesy to any other site visitors, explaining this research work when necessary.
7. You are using the SNA at your own risk. You agree to take all necessary safety precautions to protect yourself, your assistants, and any other SNA visitors
8. Please acknowledge the Minnesota DNR, Scientific and Natural Areas Program in any articles and presentations concerning this research.
9. Please submit an electronic copy of a preliminary research summary by December 31, 2010 and a final report upon completion of your work to the DNR – peggy.booth@state.mn.us – and to TNC – mcornett@tnc.org and cseurer@tnc.org. Reports need to include the names of species collected at each site; and if available, please include maps or GPS points where the species collected. We would also appreciate receiving a copy of any future peer-reviewed publications that summarize work conducted on our lands – in pdf format if possible. If for any reason you do not do any work under this research permit, please notify us.

As with all SNAs, the site you have selected may be subject to planned management activities (e.g. brush and tree removal, prescribed burns, seed harvest, etc) during the duration of your permitted activities.

This permit is valid through December 31, 2010 and may be revoked at any time to protect the resources of the SNA upon verbal or written communication. This permit may be renewed for fieldwork in 2011.

By



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Assistant Division Director,
Division of Ecological Resources
500 Lafayette Rd., Box 25
St. Paul, MN 55155-4025

Meredith Cornett
Director of Conservation Science
The Nature Conservancy
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Minneapolis, MN 55415