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Chemotypic variation in oshá (*Ligusticum porteri*) in Colorado, USA

Brittany Smith[a], Luis Lowe[b], Janel Owens[b], Emily Mooney[b,*]

[a] Colorado State University, Fort Collins, CO 80523, United States
[b] University of Colorado Colorado Springs, 1420 Austin Bluffs Parkway, Colorado Springs, CO 80918, United States

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ABSTRACT

Ligusticum porteri (oshá) is an important wild-harvested medicinal plant. Populations of this plant occur in meadows and forest understories in the subalpine and montane zones of the Rocky Mountains. We examined variation in the bioactive compounds of 44 methanolic extracts of *L. porteri* roots harvested from eleven populations in Colorado, USA during 2012 and 2015. Populations were found in two distinct regions of Colorado, the Front Range and Western Slope. We also classified population light environment as either meadow or understory based on the mean percent of full-sun photosynthetically active radiation above plants. We used high performance liquid chromatography (HPLC) to quantify variation in five secondary metabolites: *Z*-ligustilide, *trans*-ferulic acid, (Z/E)-3-butylidenephthalide, isovanillin and elemicin. Variation between sampling years was found for *trans*-ferulic acid, elemicin and (*E*)-3-butylidenephthalide. Root extracts from the Front Range had more than double the mean relative concentrations of (*Z*)-ligustilide than root extracts from plants on the Western Slope of Colorado. Our results demonstrate distinct chemotypes in *L. porteri*, and this could have therapeutic effects because (*Z*)-ligustilide is the major bioactive compound in this species.

1. Introduction

Demand for many medicinal plant species continues to be met by harvest from the wild due to either consumer preference for wild-harvested materials or insufficient cultivation to meet demand (Hamilton, 2004; Schippmann et al., 2006). Harvesters, practitioners and consumers are interested in the safety and efficacy of herbal medicines. Authenticity of wild-harvested plants can affect safety and efficacy when a species has a look-alike that is either toxic or ineffective, and such is the case when other species of *Actaea* are harvested and sold as black cohosh (*Actaea cimicifuga*) (Baker, 2012). Even when a wild-harvested species is authentic, plants of the same species can show marked variation in the specific secondary metabolites linked to efficacy (Ncube et al., 2012). Both qualitative and quantitative intraspecific variation in secondary metabolites is common (Moore et al., 2014), leading to distinct chemotypes within species (Wallaart et al., 2000). This can occur when plants of similar genotype vary the content or composition of secondary metabolites in response to an environmental cue, i.e. phenotypic plasticity (Briskin, 2000). For example, stress at high altitude can induce the production of greater amounts of secondary metabolites (Arief et al., 2016). Likewise, genotypes with greater fitness in a particular environment may display unique chemical profiles, i.e. local adaptation (Moore et al., 2014). Both phenotypic plasticity and local adaptation allow plants to persist alongside environmental variation, but they create variability in therapeutic effects of wild-harvested species.

Variation in secondary metabolites within a single species may be the product of biotic and abiotic factors. Given their roles in defense, herbivory often drives the creation and maintenance of intraspecific variation in secondary metabolites (Moore et al., 2014). Abiotic factors may similarly explain variation within species. Plants of the same species often occur in widely different light environments (Valladares, 2003); and in high light environments, plants increase the production of secondary metabolites that protect leaf cells from photo-oxidative damage (Close et al., 2003). As examples, UV-absorbing flavonoids block high energy light wavelengths from causing reactive oxygen series while antioxidants quench reactive oxygen series once produced (Brunetti et al., 2015). Many of these light-protective secondary metabolites are well known for their medicinal and therapeutic effects (Ncube et al., 2012). Response to shade among medicinal plants in the wild is not well documented, but shading of tea (*Camellia sinensis*) plants in cultivation decreases undesirable astringency by reducing flavonoid concentration (Ku et al., 2010). Shade may also reduce secondary metabolites in tissues outside of leaves as well. For example, kavapyrone levels in cultivated kava (*Piper methystichum*) roots decrease when shaded (Lebot et al., 1999). Biotic or abiotic conditions

* Corresponding author.
E-mail address: emooney@uccs.edu (E. Mooney).

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that co-vary with light environment can lead to variation in secondary metabolites. For example, heat stress or low soil moisture associated with high light environments can induce secondary metabolite production (Ramakrishna and Ravishankar, 2011; Selmar and Kleinwächter, 2013a).

In this study, the variation in therapeutically important secondary metabolites in populations of the North American medicinal plant *Ligusticum porteri* (Apiaceae) was examined by high performance liquid chromatography with UV-detection (HPLC-UV/Vis) to test the hypothesis that metabolite levels are driven by harvest region and light environment. *L. porteri* is common throughout the Rocky Mountain region, where it grows in both the forest understory as well as in open meadows. The common name for this species is 'bear root' referring to the use of this species by bears, who will ingest and apply roots to fur for their anti-parasitic properties (Jain et al., 2008). Cultivation is uncommon in this species, so most commercial L. *porteri* preparations are derived from wild-harvested roots (Terrell and Fennell, 2008). Although a variety of other uses appear in the ethnobotanical record (Turi and Murch, 2004), this species is commercially harvested for use primarily as an herbal cold and flu remedy (Terrell and Fennell, 2008). *L. porteri* roots contain a number of secondary metabolites, with the phthalides (*Z*)-ligustilide and butylidenepthalide commonly reported for their therapeutic effects (Brindis et al., 2011; Rivero et al., 2012; Turi and Murch, 2004).

We considered variation in secondary metabolites as driven by harvest year, harvest region and light environment. Previous work with *L. porteri* demonstrated greater antibacterial activity in extracts from plants in the sun versus shade (Mooney et al., 2015). Notably, insect herbivory co-varies with light environment in this species: seven years of insect population monitoring shows 11-fold greater colonization by aphids on plants in meadows than in the understory (Mooney et al., 2016). Past research with L. *porteri* roots indicates variation between roots sampled from two wild populations and three accessions from medicinal herb markets (Rivero et al., 2012).

2. Methods

2.1. Field collection of roots and extract preparation

Whole roots were collected from four replicate plants in each of eleven populations in Colorado, USA. In 2012, samples were taken near the Rocky Mountain Biological Laboratory in Gothic, CO USA (Table 1). We harvested four plants from each of five additional populations in the second week of August 2015. The 2015 sampling included two adjacent populations near Kebler Pass, CO and four populations on the Front Range of the Rocky Mountains in Colorado. We identified plants using the morphological criteria outlined in Weber and Wittmann's *Colorado Flora* (Weber and Wittmann, 2001). We collected a voucher specimen from one population (Lower Mt. Herman) which was deposited in the herbarium at Colorado College (COCO) in Colorado Springs, CO, USA. We also marked population locations with GPS, but the coordinates are not published here due to the perception that this species is at risk of overexploitation (Weber and Wittmann, 2001). For all harvested plants, we measured light as photosynthetically active radiation (PAR) at plant height between 10:00 and 15:00 h with using a light meter and sensor (LI-250 A & LI-191R, LI−COR, Lincoln, NE). Details of root collection and extraction procedure are outlined in a previous study (Mooney et al., 2015). In brief, roots were stored at 4 °C until crude methanol extracts were made in late August of 2015 (Mooney et al., 2015), after which the methanol extracts were stored in a freezer at −20 °C. We filtered the crude extracts with a sterile 0.45 µm cellulose-acetate syringe filter prior to HPLC analyses.

2.2. Standard preparation

We selected five standards with documented bioactive roles within *L. porteri* (Fig. 1; Rivero et al., 2012) or related species within the *Ligusticum* genus (Sieniawska et al., 2013; Turi and Murch, 2013). The standards included **(1)** isovanillin (Purity > 99%, Fluka Analytical Sigma-Aldrich, Germany, **(2)** *trans*-ferulic acid (Purity > 99%, Sigma Aldrich, St. Louis, MO), **(3)** elemicin (Purity > 98%, Combi-Blocks Sigma-Aldrich, San Diego, CA), **(4)** *Z*-ligustilide (Purity > 98%, Chengdu Biopurify Phytochemicals Ltd, China) and **(5)** (*E/Z*)-3-butylidenephthalide (Purity > 99%, SAFC Sigma-Aldrich, St. Louis, MO). We prepared 5000 µg/mL stock solutions by dissolving standards 1–5 in acetonitrile (ACN) in a 50 mL volumetric flask. All standards readily dissolved upon inversion except for *Z*-ligustilide, which was placed in an ultrasonic bath for 15 min. Prior to injection in the HPLC, we prepared dilutions to 1500 µg/mL and vortexed each standard solution for 30 s.

2.3. HPLC instrumentation and chromatographic conditions

To assess variation in five compounds, we adapted a previously published HPLC method developed for *L. porteri* (Rivero et al., 2012). An Agilent 1100 HPLC outfitted with a binary pump, degasser, autosampler, and multi-wavelength detector was utilized for separation and detection. A Restek Ultra II C18 column (5 cm × 4.6 mm i.d. × 3 µm packing) with mobile phases of (A) acetonitrile + 0.1% (v/v) formic acid and (B) water + 0.1% (v/v) formic acid was utilized for separation of compounds in all analytical standards and methanolic extracts of plant samples. Gradient conditions were as follows: 95% B from 0 to 1 min; ramp to 60% B at 15 min (hold for 5 min) and return to 95% B at 25 min. The flow rate was 1.25 mL/min. Five µL of standard or sample were injected and the resulting signal at 260 and 280 nm were recorded. Quantitation was completed using area comparison to a single standard (1500 µg/mL) to determine relative concentrations at parts-per-million (ppm) levels in the methanolic extracts.

2.4. Data analysis

The data were analyzed in such a way as to reduce the extent to

Table 1
Elevation, habitat and light environment for eleven populations of *L. porteri* sampled in 2012 and 2015 from the Western Slope and Front Range of Colorado.

Population Name	Harvest Year	Region	Elevation (m)	Habitat	Mean % Full Sun (PAR) ± SE
RMBL Sun 1	2012	Western Slope	2881	Subalpine meadow	0.97 ± 0.03
RMBL Sun 2	2012	Western Slope	3021	Subalpine meadow	0.77 ± 0.03
RMBL Meadow	2012	Western Slope	2902	Subalpine meadow	0.54 ± 0.07
RMBL Aspen 1	2012	Western Slope	2894	Aspen understory	0.41 ± 0.18
RMBL Aspen 2	2012	Western Slope	3030	Aspen understory	0.10 ± 0.04
Gibson Creek	2015	Front Range	3096	Conifer understory	0.50 ± 0.25
Upper Mt. Herman	2015	Front Range	2519	Conifer understory	0.26 ± 0.17
Lower Mt. Herman	2015	Front Range	2512	Conifer understory	0.25 ± 0.20
Duckett Creek	2015	Front Range	2505	Aspen understory	0.36 ± 0.17
Kebler Pass Sun	2015	Western Slope	3045	Subalpine meadow	0.93 ± 0.05
Kebler Pass Shade	2015	Western Slope	3183	Conifer understory	0.05 ± 0.01

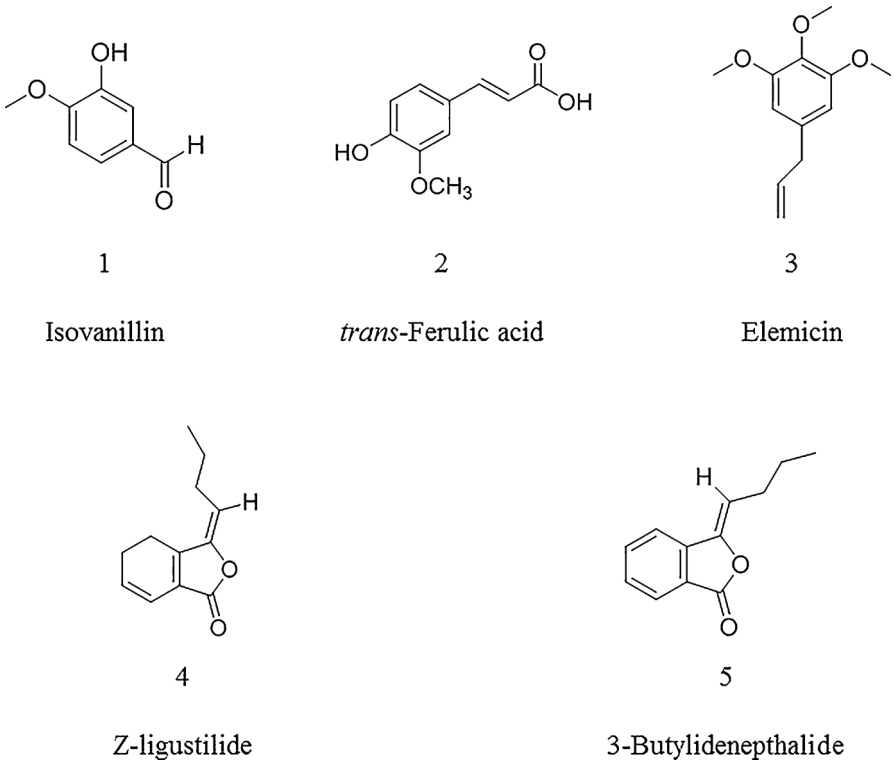

Fig. 1. Isolated compounds from *Ligusticum porteri* sampled from Colorado, USA.

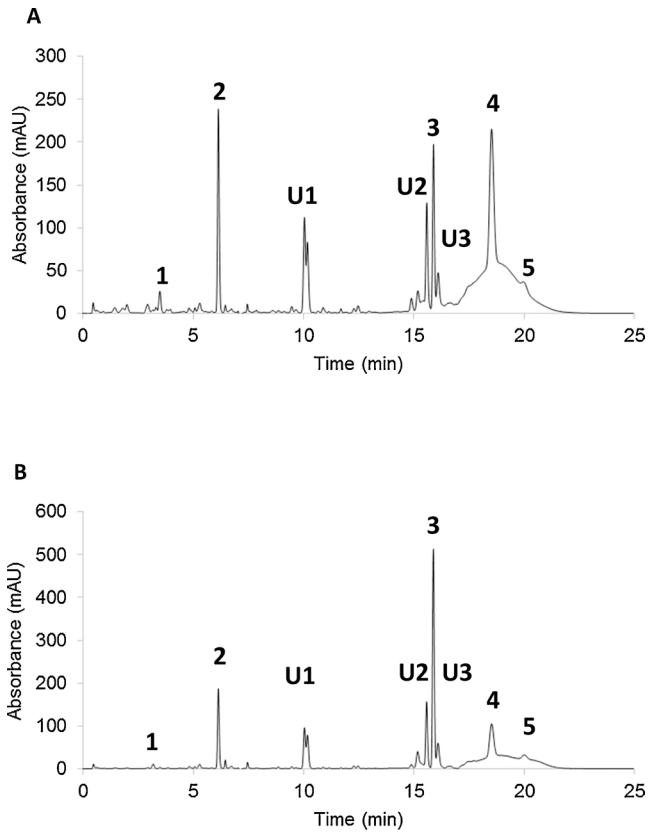

Fig. 2. Representative chromatograms of root extracts from plants in (A) understory and (B) meadow populations; numbers above peaks represent the standards (1) isovanillin, (2) *trans*-ferulic acid, (3) elemecin, (4) *Z*-ligustilide and (5) (Z)-3-butylidenephthalide. Unknown compounds shown are unknown 1 (U1), unknown 2 (U2) and unknown 3 (U3).

which sampling year might be confounded with the potential effects of population, sampling region and light environment. Using only the plants sampled from 2012 ($n = 20$), we first tested for population-to-population variation among Western Slope populations. Separate general linear models included population as an explanatory variable with the relative concentrations (ppm) of each compound as the response variable. For those compounds that did not vary by population ($P > 0.05$), we tested for variation in relative concentration of compounds between sampling years (2012 versus 2015) only using plants harvested from populations on the Western Slope. To account for uneven sample sizes between sampling years, we compared plants sampled in 2015 to a random sample of the same dimension ($n = 8$ plants in two populations) from 2012. We used a general linear mixed effects model with sampling year as a fixed effect and population nested within year as a random effect. Because the same populations were not sampled in 2012 and 2015, we could not test for sampling year-by-population interactions.

For any compound that showed a lack of sampling year effect ($P > 0.05$), we then tested for variation by light environment and region, pooling all samples ($n = 44$). Populations from both sampling years were grouped into either meadow ($\bar{x}_{PAR} = 73.9\%\ of\ full\ sun$) or understory ($\bar{x}_{PAR} = 23.5\%\ of\ full\ sun$) categories. We repeated the same analyses using region—Front Range versus Western Slope—as the explanatory variable. Because we sampled no meadow populations on the Front Range, we could not test for light environment-by-region interactions. We used a general linear mixed effects model with either habitat or sampling location as a fixed effect and population nested within year as a random effect. The mixed models were created using 'lmer' function from the 'lme4' package (Bates et al., 2015). We obtained *p*-values from the 'Anova()' function from the 'car' package (Fox and Weisberg, 2011).We performed all of the analyses in R Version 3.4.1 (R Development Core Team, 2017).

Table 2
Retention times and concentrations of seven compounds analyzed by HPLC; mean concentrations include all plants ($n = 44$) and the standard error is the standard error of this mean.

Compound	Retention time (min)	Mean concentration (ppm)	Standard error
trans-Ferulic acid	7.5	935.5	145.3
Unknown **1**	11.4	1065.6	161.5
Unknown **2**	16.2	460.9	42.8
Elemicin	16.9	1535.0	441.4
Unknown **3**	17.4	2370.6	258.4
(*E*)-3-butylidenepthalide	17.9	681.5	81.8
(*Z*)-Ligustilide	21.9	3163.7	271.5

3. Results

3.1. Variation in compounds identified by HPLC

We quantified variation in the amounts of *Z*-ligustilide, *trans*-ferulic acid and elemicin among the 44 plant extracts (Fig. 2). The (*E/Z*)-3-butylidenephtalide standard produced two peaks (RT: 17.9 min and RT: 19.0 min) reflecting its mixture of isomers. Previous HPLC analysis of the congener *Ligusticum chuanxiong* showed (*E*)-3-butylidenepthalide to have a lower retention time relative to (*Z*)-3-butylidenepthalide (Yi et al., 2007), thus we tentatively identified the first peak (RT: 17.9 min) as (*E*)-3-butylidenepthalide. Isovanillin was detected in very few (< 10%) of the plant extracts, and (Z)-3-butylidenephthalide co-eluted with unknown compounds; thus, we only present results for relative concentrations of *trans*-ferulic acid, elemicin, (*Z*)-ligustilide and (*E*)-3-butylidenepthalide. We also consistently detected large peaks (> 50 mAU) for three unknown compounds, which we sequentially numbered 1–3 by their relative retention times. Elemicin showed the largest amount of plant-to-plant variation, followed by *Z*-ligustilide (Table 2). Unknown 2 had the least amount of plant-to-plant variation of the seven compounds we quantified.

3.2. Variation in compounds by sampling year and population

Relative concentrations *trans*-ferulic acid ($F = 0.25$, $P = 0.907$), unknown 1 ($F = 0.63$, $P = 0.65$), unknown 2 ($F = 1.26$, $P = 0.327$), elemicin ($F = 0.45$, $P = 0.771$), unknown 3 ($F = 0.99$, $P = 0.443$) (*Z*)-ligustilide ($F = 2.89$, $P = 0.059$) and (*E*)-3-butylidenepthalide ($F = 0.14$, $P = 0.9652$) did not vary among populations sampled in 2012 on the Western Slope. Relative concentrations (*Z*)-ligustilide ($\chi^2 = 2.06$, $P = 0.152$) and unknown 2 ($\chi^2 = 2.94$, $P = 0.086$) did not vary in root extracts from plants harvested in 2012 and 2015 from the Western Slope. However, relative concentrations of *trans*-ferulic acid, unknown 1, elemicin, unknown 3 and (*E*)-3-butylidenepthalide varied between sampling years (Fig. 3). *Trans*-ferulic acid ($\chi^2 = 54.73$, $P < 0.001$), unknown 1 ($\chi^2 = 64.15$, $P < 0.001$) elemicin ($\chi^2 = 6.83$, $P = 0.008$), and unknown 3($\chi^2 = 4.55$, $P = 0.033$) were all relatively greater in samples collected in 2012 than from 2015. (*E*)-3-butylidenepthalide showed the opposite pattern with relative concentrations being greater in samples from 2015($\chi^2 = 20.15$ $P < 0.001$).

3.3. Variation in compounds by sampling year by habitat and region

Neither the relative concentrations of (*Z*)-ligustilide or unknown 2 showed light environment-based differences in relative concentration ($P > 0.05$). Root extracts from populations on Colorado's Front Range had 126% higher mean relative concentrations of (*Z*)-ligustilide than those from the Western Slope (Fig. 4; $\chi^2 = 43.26$, $P < 0.001$). Relative concentration of unknown 2 in root extracts did not vary by region ($\chi^2 = 1.66$, $P = 0.197$).

4. Discussion

We found evidence of distinct chemotypes of *L. porteri* in different regions of Colorado for the major bioactive compound (*Z*)-ligustilide. In their previous HPLC analysis of six extracts of *L. porteri* roots, Rivero et al. (2012) reported similar magnitudes of variation in (*Z*)-ligustilide content: root extracts from a plant harvested from Rabbit Ears Pass, Colorado, USA had 96% less (*Z*)-ligustilide than an extract of roots obtained in a Mexico City, Mexico market (Rivero et al., 2012). (*Z*)-ligustilide is an important bioactive constituent of *L. porteri* and other related plants with a variety of therapeutic effects (Yang et al., 2012). Because of the magnitude of regional differences, chemotypic variation may have therapeutic implications for users of *L. porteri*. Our results indicate that future studies of the bioactive compounds in *L. porteri* should take into account where root extracts originate as well as sample year variation. Recent studies have examined the effects of *L. porteri* root extracts on human leukemia cells (Nguyen et al., 2017), human peripheral blood lymphocytes (Nguyen et al., 2016) and pain responses in mice (Juárez-Reyes et al., 2014), and our results indicate that such experimental results could be influenced by these factors.

Regional variation in chemotypes is a common feature of medicinal and aromatic plants (Moore et al., 2014), and the mechanisms underlying this variation are diverse. For example, Schlag and McIntosh (2006) found distinct regional differentiation in American ginseng (*Panax quinquefolius*) saponins. This differentiation persisted in cultivation in a common environment, indicating that chemotypes of American ginseng were more likely the products of genetic differentiation than environmental variation. Genetic differences in biosynthetic pathways provide an underlying mechanism. In *Artemesia annua* (sweet wormwood), chemotypes result from a bottleneck in the biosynthetic pathway for artemesinin (Wallaart et al., 2000). Chemotypes with low levels of artemisinin and dihydroartemisinic acid contained high levels of artemisinic acid, which is a direct precursor of dihydroartemisinic acid in the biosynthetic pathway of artemisinin. Other chemotypes showed the reverse pattern, and the authors inferred that an "artemisinic acid reductase" encoding gene could be responsible for these regional chemotypes (Wallaart et al., 2000). Geographic isolation ultimately drives population differentiation, as in the case of *Helichrysum microphyllum* subsp. *tyrrenichum* found in the La Maddalena Archipelago of Sicily (Ornano et al., 2015; Venditti et al., 2016). The Tyrrhenian Sea isolates these islands from other Sardinian populations, and this isolation is evidenced by distinctive chemotypes of *H. microphyllum* subsp. *tyrrenichum* (Ornano et al., 2015) and other species (Ornano et al., 2013). For *L. porteri*, the topography of the Rocky Mountains create isolation among populations as evidenced by

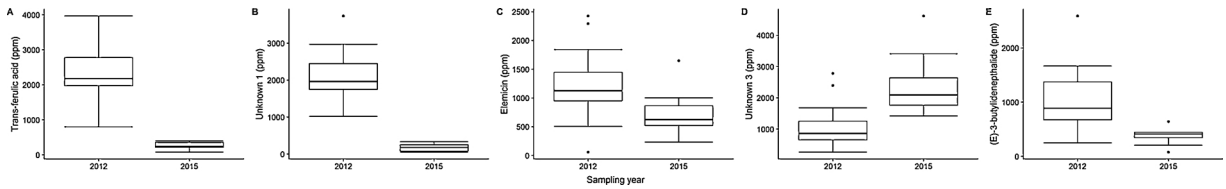

Fig. 3. Variation by sampling year in mean relative concentrations (ppm) of five compounds *trans*-ferulic acid (A), unknown 1 (B), elemicin (C), unknown 3 (D) and (*E*)-3-butylidenephthalide between root extracts from plants harvested on the Western Slope of Colorado, USA.

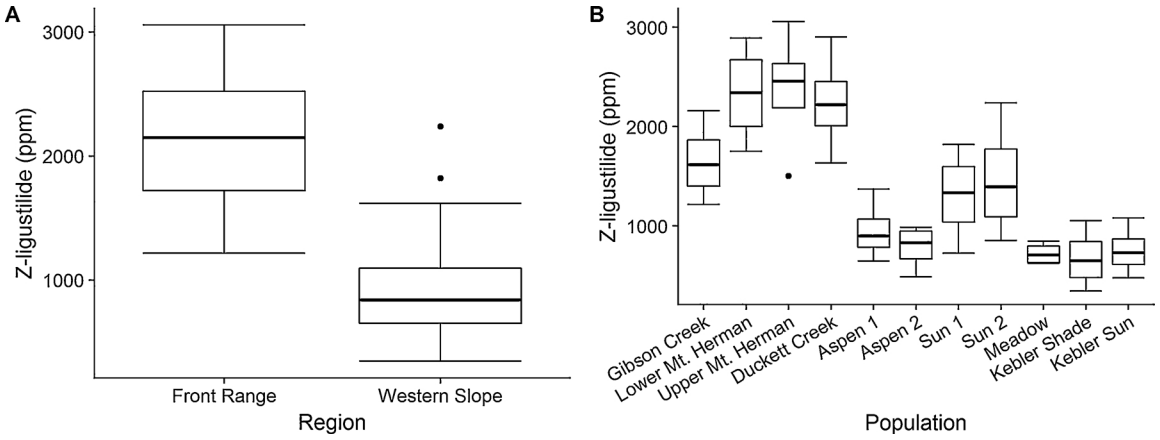

Fig. 4. Variation in mean relative concentrations (ppm) of (Z)-ligustilide in root extracts from plants harvested on the Western Slope and Front Range of Colorado, USA (A) and among populations (B).

phylogeographic studies of other montane species (Massatti and Knowles, 2014). Fungal endophytes provide yet another mechanism for chemotypic variation driven by genetic distance. For example, population-to-population variation in alkaloid content of *Achnatherum robustum* (sleepygrass) is associated with distinct genotypes of fungal endophytes (Shymanovich et al., 2015).

A similar variety of genetic and environmental factors may drive the regional variation in chemotypes of *L. porteri* that we observed. Despite much research on their therapeutic roles, no studies have explored the ecological or physiological roles of secondary metabolites in *L. porteri* (Terrell and Fennell, 2008; Turi and Murch, 2004). Functional studies of similar secondary metabolites in related species provide some insight for our results in *L. porteri*. (*E*)-3-butylidenepthalide and *(Z)*-ligustilide are members of the chemical class known as phthalides, and plants in the Apiaceae family have a particularly rich diversity of phthalides (Beck and Chou, 2007). Despite their diversity, complex phthalides in apiaceous plants share lactone as their core chemical structure (Donkor et al., 2016). The lactone moiety of phthalides in particular may underlie their noted repellency to herbivores (Fung et al., 1988; Meade et al., 1994). The phthalides in *L. porteri* may also protect roots against underground herbivores. Pocket gophers (Family Geomyidae) are common root-feeding herbivores in the subalpine and lower-montane habitats where we harvested *L. porteri* roots (Miller, 1964). Gophers consume roots of *L. porteri* and even small amounts of gopher grazing can affect *L. porteri* abundance (Laycock and Richardson et al., 1975). Western Slope *L. porteri* plants encounter one species of pocket gopher (mountain pocket gopher—*Thomomys talpoide*) while Front Range plants would overlap with as many as three other species (the valley pocket gopher—*T. bottae*, the plains pocket gopher—*Geomys bursarius*, and the yellow-faced pocket gopher – *Cratogeomys castanops*) (Miller, 1964). Resistance to pocket gopher herbivory may underlie the differences that we observed in (*Z*)-ligustilide between Front Range and Western Slope populations.

The relative concentrations secondary metabolites from root extracts sampled on the Western Slope of Colorado showed significant year-to-year differences. Although population-to-population variation was not significant, variation by year should be interpreted cautiously since the same populations were not sampled in 2012 and 2015. At least some of this variation is likely due to year of sampling. Past studies of the secondary metabolites of *L. porteri* have not explicitly tested for variation among sampling years (Collin et al., 2014; Delgado et al., 1992; León et al., 2011; Rivero et al., 2012). Rivero et al. (2012) compared two wild collected samples from 2005 with dried roots obtained from markets in 2008 and 2009. Qualitative comparisons of the ratio of (*Z*)-ligustilide to (Z)-6,6′,7,3′ α-diligustilide showed that this ratio varied from 2:1 to 9:1 across these five samples. Some of this variation may be due to sampling year, but it would be confounded by location and drying technique in market samples. Using one plant divided between fresh and dried portions, León et al. (2011) found drying to coincide with increases in ferulic acid alongside decreases in coniferyl ferulate. We observed root extract made from 2012 samples to have elevated amounts of *trans*-ferulic acid and (*E*)-3-butylidenephthalide relative to 2015 samples. The physiological role of (*E/Z*)-3-butylidenephthalide in plant roots is unknown, but León et al. (2011) found evidence for the direct transformation of (*Z*)-ligustilide to (*Z*)-3-butylidenephthalide in the one dried root they examined. In contrast, *trans*-ferulic acid is a nearly ubiquitous secondary metabolite with many established physiological and ecological roles (Rosazza et al., 1995). Experimental studies show that plants accumulate ferulic acid in leaves and roots during drought stress (Amarowicz and Weidner, 2009; Hura et al., 2009), and this may be modulated by the intensity of drought (Bettaieb et al., 2011). Both 2012 and 2015 were drier than average years in terms of snow pack and summer precipitation (Berkelhammer et al., 2017), but water limitation was likely greatest in 2012. The weather station nearest to the *L. porteri* populations in Gothic, CO recorded 53.09 mm total precipitation in August of 2012 and 77.72 mm total precipitation in August of 2015 (WRCC, 2018). Very dry conditions in 2012 may have led *L. porteri* roots to accumulate *trans*-ferulic acid. Drought has produced similar changes in secondary metabolites in other medicinal plants, and the inductions of drought stress can be used to manipulate levels of desired compounds (Selmar and Kleinwächter, 2013a, 2013b).

It is more difficult to attribute underlying processes to the variation we observed in the unknown compounds. We can only speculate on the identity of unknown 1 and unknown 3 which also showed significant differences in relative concentration between years. *Ligusticum* congeners have a diversity of monomeric and dimeric phthalides as well as alkaloids, organic acids, ceramides, cerebrosides and saccharides (Donkor et al., 2016). That being said, the genus *Ligusticum* is now recognized as being polyphyletic, and it and other genera in the Apiaceae are in the process of revision (Downie et al., 2010; Jiménez-Mejías and Vargas, 2015). Despite the taxonomic revision taking place, the abundance of bioactive compounds in *Ligusticum sensu lato* explains their use in pharmacopeias around the world (Venditti et al., 2016).

Differentiation in chemotypes for plants sampled within the same year can also be the product of environmental variation. For example, intraspecific variation in the flavonoid phloridzin is associated with altitude in wild *Malus* species (Tang et al., 2015). We were interested in the role of light environment in particular because of past research showing that both antibacterial activity and herbivory co-vary with light environment (Mooney et al., 2016, 2015). We did not detect variation in secondary metabolites in root extracts of *L. porteri* between

meadow and understory populations. *L. porteri* and its congeners have a great diversity of bioactive secondary metabolites (Delgado et al., 1992; Turi and Murch, 2013). (*Z*)-ligustilide is not antibacterial itself but it can potentiate the activity of the antibiotic norfloxacin in resistant strains of *Staphylococcus aureus* (Cégiéla-Carlioz et al., 2005). In *Ligusticum officinale*, phthalides showed little activity against mycobacteria relative the sesquiterpene alcohol α-prethapsenol (Guzman et al., 2013). It is likely that the compound responsible for antibacterial activity was not among the seven compounds analyzed here. In the case of herbivory, experimental studies show that interactions with mutualist ants—and not secondary metabolites— underlie the greater abundance of aphids in sun populations of *L. porteri* relative to shade populations (Mooney et al., 2016).

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References

Amarowicz, R., Weidner, S., 2009. Phenolic compounds and properties of antioxidants in grapevine roots *Vitis vinifera* L. Under drought stress followed by recovery. Acta Societatis Botanicorum Poloniae 78, 389–405. https://doi.org/10.1007/978-90-481-2305-6_14.

Arief, Z.M., Shawl, A.S., Munshi, A.H., 2016. Altitudinal variation in pharmacologically active compounds of wild and cultivated populations of Epimedium elatum. Journal of Applied Research Medicinal Aromatic Plants 3, 48–51. https://doi.org/10.1016/j.jarmap.2016.01.001.

Baker, D.A., 2012. DNA barcode identification of black cohosh herbal dietary supplements. Journal of AOAC International 95, 1023–1034. https://doi.org/10.5740/jaoacint.11-261.

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67, 1–48. https://doi.org/10.18637/jss.v067.i01.

Beck, J.J., Chou, S.-C., 2007. The structural diversity of phthalides from the Apiaceae. Journal of Natural Products. 70, 891–900. https://doi.org/10.1021/np0605586.

Berkelhammer, M., Stefanescu, I.C., Joiner, J., Anderson, L., 2017. High sensitivity of gross primary production in the Rocky mountains to summer rain. Geophysical Research Letters 44, 3643–3652. https://doi.org/10.1002/2016GL072495.

Bettaieb, I., Hamrouni-Sellami, I., Bourgou, S., Limam, F., Marzouk, B., 2011. Drought effects on polyphenol composition and antioxidant activities in aerial parts of *Salvia officinalis* L. Acta Physiologiae Plantarum 33, 1103–1111. https://doi.org/10.1007/s11738-010-0638-z.

Brindis, F., Rodríguez, R., Bye, R., González-Andrade, M., Mata, R., 2011. (Z)-3-butylidenephthalide from *Ligusticum porteri*, an α-glucosidase inhibitor. Journal of Natural Products 74, 314–320. https://doi.org/10.1021/np100447a.

Briskin, D.P., 2000. Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. Plant Physiology 124, 507–514. https://doi.org/10.1104/pp.124.2.507.

Brunetti, C., Guidi, L., Sebastiani, F., Tattini, M., 2015. Isoprenoids and phenylpropanoids are key components of the antioxidant defense system of plants facing severe excess light stress. Environmental Experimental Botany 119, 54–62. https://doi.org/10.1016/j.envexpbot.2015.04.007.

Cégiéla-Carlioz, P., Bessière, J.-M., David, B., Mariotte, A.-M., Gibbons, S., Dijoux-Franca, M.-G., 2005. Modulation of multi-drug resistance (MDR) in *Staphylococcus aureus* by osha (*Ligusticum porteri* L., Apiaceae) essential oil compounds. Flavour and Fragrance Journal 20, 671–675. https://doi.org/10.1002/ffj.1584.

Close, D., McArthur, C., Paterson, S., Fitzgerald, H., 2003. Photoinhibition: a link between effects of the environment on eucalypt leaf chemistry and herbivory. Ecology 84, 2952–2966.

Collin, G., Gagnon, H., St-Gelais, A., Turcotte, M., 2014. Composition of the essential oil and the hydrosol of the roots of Ligusticum porteri. American Journal of Essential Oils and Natural Products 1, 4–10.

Delgado, G., Reza-Garduño, R.G., Yolandarios, M., 1992. Phthalides and monoterpenes of the hexane extract of the roots of *Ligusticum porteri*. Planta Medica 58, 570–571.

Donkor, P.O., Chen, Y., Ding, L., Qiu, F., 2016. Locally and traditionally used *Ligusticum* species – a review of their phytochemistry, pharmacology and pharmacokinetics. Journal of Ethnopharmacology 194, 530–548. https://doi.org/10.1016/J.JEP.2016.10.012.

Downie, S.R., Spalik, K., Katz-Downie, D.S., Reduron, J.-P., 2010. Major clades within Apiaceae subfamily Apioideae as inferred by phylogenetic analysis of nrDNA ITS sequences. Plant Diversity and Evolution 128, 111–136. https://doi.org/10.1127/1869-6155/2010/0128-0005.

Fox, J., Weisberg, S., 2011. An R Companion to Applied Regression.

Fung, S.Y., Herrebout, W.M., Verpoorte, R., Fischer, F.C., 1988. Butenolides in small ermine moths,*Yponomeuta* spp. (Lepidoptera: Yponomeutidae), and spindle-tree, *Euonymus europaeus* (Celastraceae). Journal of Chemical Ecology 14, 1099–1111. https://doi.org/10.1007/BF01019338.

Guzman, J.D., Evangelopoulos, D., Gupta, A., Prieto, J.M., Gibbons, S., Bhakta, S., 2013. Antimycobacterials from lovage root (*Ligusticum officinale* Koch).Phyto. Phytotherapy Research 27, 993–998. https://doi.org/10.1002/ptr.4823.

Hamilton, A.C., 2004. Medicinal plants, conservation and livelihoods. Biodiversity Conservation 13, 1477–1517. https://doi.org/10.1023/B:BIOC.0000021333.23413.42.

Hura, T., Hura, K., Grzesiak, S., 2009. Possible contribution of cell-wall-bound ferulic acid in drought resistance and recovery in triticale seedlings. Journal of Plant Physiology 166, 1720–1733. https://doi.org/10.1016/J.JPLPH.2009.04.012.

Jain, C., Dashora, A., Garg, R., 2008. Animal self-medication through natural sources. Natural Product Radiance 7, 49–53.

Jiménez-Mejías, P., Vargas, P., 2015. Taxonomy of the tribe Apieae (Apiaceae) revisited as revealed by molecular phylogenies and morphological characters. Phytotaxa 212, 57–79. https://doi.org/10.11646/phytotaxa.212.1.2.

Juárez-Reyes, K., Angeles-López, G.E., Rivero-Cruz, I., Bye, R., Mata, R., 2014. Antinociceptive activity of *Ligusticum porteri* preparations and compounds. Pharmaceutical Biology 52, 14–20. https://doi.org/10.3109/13880209.2013.805235.

Ku, K.M.O., Choi, J.N., Kim, J., Kee Kim, J., Yoo, L.G., Lee, S.J., Hong, Y.S., Lee, C.H., 2010. Metabolomics analysis reveals the compositional differences of shade grown tea (*Camellia sinensis* L.). Journal of Agricultural and Food Chemistry 58, 418–426. https://doi.org/10.1021/jf902929h.

Laycock, W.A., Richardson, B.Z., 1975. Long-term effects of pocket gopher control on vegetation and soils of a subalpine grassland. Journal of Range Management 28, 458–462. https://doi.org/10.2307/3897222.

León, A., Chávez, M.I., Delgado, G., 2011. 1H and DOSY NMR spectroscopy analysis of Ligusticum porteri rhizome extracts. Magnetic Resonance Chemistry 49, 469–476. https://doi.org/10.1002/mrc.2767.

Massatti, R., Knowles, L.L., 2014. Microhabitat differences impact phylogeographic concordance of codistributed species: genomic evidence in montane sedges (*Carex* L.) from the Rocky Mountains. Evolution 68, 2833–2846. https://doi.org/10.1111/evo.12491.

Meade, T., Daniel Hare, J., Midland, S.L., Millar, J.G., Sims, J.J., 1994. Phthalide-based host-plant resistance to *Spodoptera exigua* and *Trichoplusia ni* in Apium graveolens. Journal of Chemical Ecology 20, 709–726. https://doi.org/10.1007/BF02059608.

Miller, R.S., 1964. Ecology and distribution of pocket gophers (Geomyidae) in Colorado. Ecology 45, 256–272. https://doi.org/10.2307/1933839.

Mooney, E.H., Martin, A.A., Blessin, R.P., 2015. Effects of light environment on recovery from harvest and antibacterial properties of oshá *Ligusticum porteri* (Apiaceae). Economic Botany 69, 72–82. https://doi.org/10.1007/s12231-015-9302-2.

Mooney, E.H., Phillips, J.S., Tillberg, C.V., Sandrow, C., Nelson, A.S., Mooney, K.A., 2016. Abiotic mediation of a mutualism drives herbivore distribution. Ecology Letters 19, 37–44. https://doi.org/10.1111/ele.12540.

Moore, B.D., Andrew, R.L., Külheim, C., Foley, W.J., 2014. Explaining intraspecific diversity in plant secondary metabolites in an ecological context. New Phytologist 201, 733–750. https://doi.org/10.1111/nph.12526.

Ncube, B., Finnie, J.F., Van Staden, J., 2012. Quality from the field: the impact of environmental factors as quality determinants in medicinal plants. South African Journal of Botany 82, 11–20. https://doi.org/10.1016/j.sajb.2012.05.009.

Nguyen, K., Sparks, J., Omoruyi, F., 2017. Effects of *Ligusticum porteri* (osha) root extract on human promyelocytic leukemia cells. Pharmacognosy Resesearch 9, 156–160. https://doi.org/10.4103/0974-8490.204641.

Nguyen, K., Sparks, J., Omoruyi, F.O., 2016. Investigation of the cytotoxicity, antioxidative and immune-modulatory effects of *Ligusticum porteri* (osha) root extract on human peripheral blood lymphocytes. Journal of Integrative Medicine 14, 465–472.

Ornano, L., Venditti, A., Ballero, M., Sanna, C., Quassinti, L., Bramucci, M., Lupidi, G., Papa, F., Vittori, S., Maggi, F., Bianco, A., 2013. Chemopreventive and antioxidant activity of the chamazulene-rich essential oil obtained from *Artemisia arborescens* L. growing on the Isle of La Maddalena, Sardinia, Italy. Chemistry & Biodiversity 10, 1464–1474. https://doi.org/10.1002/cbdv.201200435.

Ornano, L., Venditti, A., Sanna, C., Ballero, M., Maggi, F., Lupidi, G., Bramucci, M., Quassinti, L., Bianco, A., 2015. Chemical composition and biological activity of the essential oil from *Helichrysum microphyllum* Cambess. ssp. tyrrhenicum Bacch, Brullo e Giusso growing in La Maddalena Archipelago, *Styrrhenicum* bacch, brullo e giusso growing in La maddalena archipelago, sardinia. Journal of Oleo Science 64, 19–26. https://doi.org/10.5650/jos.ess14171.

R Development Core Team, 2017. R: A Language and Environment for Statistical Computing.

Ramakrishna, A., Ravishankar, G.A., 2011. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signaling & Behavior 6, 1720–1731. https://doi.org/10.4161/psb.6.11.17613.

Rivero, I., Juárez, K., Zuluaga, M., Bye, R., Mata, R., 2012. Quantitative HPLC method for determining two of the major active phthalides from *Ligusticum porteri* roots. Journal of AOAC International 95, 84–91. https://doi.org/10.5740/jaoacint.11-151.

Rosazza, J.P.N., Huang, Z., Dostal, L., Volm, T., Rousseau, B., 1995. Review: biocatalytic transformations of ferulic acid: an abundant aromatic natural product. Journal of Indian Microbiology 15, 457–471. https://doi.org/10.1007/BF01570016.

Schippmann, U., Leaman, D., Cunningham, A.B., 2006. A comparision of cultivation and wild collection of medicinal and aromatic plants under sustainability aspects. In: Bogers, R., Craker, L., Lange, D. (Eds.), Medicinal and Aromatic Plants: Agricultural, Commercial, Ecological, Legal, Pharmacological and Social Aspects. Springer, Dordrecht, The Netherlands, pp. 75–95.

Schlag, E.M., McIntosh, M.S., 2006. Ginsenoside content and variation among and within American ginseng (*Panax quinquefolius* L.) populations. Phytochemistry 67, 1510–1519. https://doi.org/10.1016/j.phytochem.2006.05.028.

Selmar, D., Kleinwächter, M., 2013a. Influencing the product quality by deliberately applying drought stress during the cultivation of medicinal plants. Industrial Crops Products 42, 558–566. https://doi.org/10.1016/j.indcrop.2012.06.020.

Selmar, D., Kleinwächter, M., 2013b. Stress enhances the synthesis of secondary plant products: the impact of stress-related over-reduction on the accumulation of natural products. Plant and Cell Physiology 54, 817–826. https://doi.org/10.1093/pcp/pct054.

Shymanovich, T., Saari, S., Lovin, M.E., Jarmusch, A.K., Jarmusch, S.A., Musso, A.M., Charlton, N.D., Young, C.A., Cech, N.B., Faeth, S.H., 2015. Alkaloid variation among epichloid endophytes of sleepygrass (*Achnatherum robustum*) and consequences for resistance to insect herbivores. Journal of Chemical Ecology 41, 93–104. https://doi.org/10.1007/s10886-014-0534-x.

Sieniawska, E., Baj, T., Los, R., Skalicka-Wozniak, K., Malm, A., Glowniak, K., 2013. Phenolic acids content, antioxidant and antimicrobial activity of *Ligusticum mutellina* L. Natural Product Research 27, 1108–1110. https://doi.org/10.1080/14786419.2012.698413.

Tang, J., Tang, L., Tan, S., Zhou, Z., 2015. The study of variation of phloridzin content in six wild malus species. Journal of Food and Nutrition Research 3, 146–151. https://doi.org/10.12691/jfnr-3-3-3.

Terrell, I.B., Fennell, A., 2008. Osha: bear root. Native Plants Journal 10, 111–118.

Turi, C.E., Murch, S.J., 2013. Targeted and untargeted phytochemistry of *Ligusticum canbyi:* indoleamines, phthalides, antioxidant potential, and use of metabolomics as a hypothesis-generating technique for compound discovery. Planta Medica 79, 1370–1379. https://doi.org/10.1055/s-0033-1350618.

Turi, C.E., Murch, S.J., 2004. The genus *Ligusticum* in North America: an ethnobotanical review with special emphasis upon species commercially known as “Osha.”. HerbalGram 89, 40–51.

Valladares, F., 2003. Light heterogeneity and plants: from ecophysiology to species co-existence and biodiversity. Progress in Botany 64, 439–471.

Venditti, A., Lattanzi, C., Ornano, L., Maggi, F., Sanna, C., Ballero, M., Alvino, A., Serafini, M., Bianco, A., 2016. A new glucosidic phthalide from *Helichrysum microphyllum* subsp. *tyrrhenicum* from la Maddalena Island (Sardinia, Italy). Natural Product Research 30, 789–795. https://doi.org/10.1080/14786419.2015.1067619.

Wallaart, T.E., Pras, N., Beekman, A.C., Quax, W.J., 2000. Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annu*a of different geographical origin: proof for the existence of chemotypes. Planta Medica 66, 57–62. https://doi.org/10.1055/s-2000-11115.

Weber, W.A., Wittmann, R.C., 2001. Colorado Flora: Western Slope, third edition. University Press of Colorado, Boulder.

WRCC, 2018. Billy Barr Weather Station (Accessed 23.04.2018) Available at. Rocky Mountain Biological Lab, Colorado. https://wrcc.dri.edu/weather/rbil.html/.

Yang, J., Chen, H., Wu, J., Gong, S., 2012. Advances in studies on pharmacological functions of ligustilide and their mechanisms. Chinese Herbal Medicines 4, 26–32. https://doi.org/10.3969/j.issn.1674-6384.2012.01.005.

Yi, T., Leung, K., Lu, G.-H., Zhang, H., 2007. Comparative analysis of *Ligusticum chuanxiong* and related umbelliferous medicinal plants by high performance liquid chromatography-electrospray ionization mass spectrometry. Planta Medica 73, 392–398. https://doi.org/10.1055/s-2007-967139.