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Iridoid and phenylethanoid/phenylpropanoid metabolite profiles of *Scrophularia* and *Verbascum* species used medicinally in North America

Korey J. Brownstein[1], **Mahmoud Gargouri**[1], **William R. Folk**[2,*], **David R. Gang**[1,*]

[1]Washington State University, Institute of Biological Chemistry, Pullman, Washington, 99164, USA

[2]University of Missouri, Department of Biochemistry, Columbia, Missouri, 65211, USA

Abstract

Introduction—Botanicals containing iridoid and phenylethanoid/phenylpropanoid glycosides are used worldwide for the treatment of inflammatory musculoskeletal conditions that are primary causes of human years lived with disability (YLDs), such as arthritis and lower back pain.

Objectives—We report the analysis of candidate anti-inflammatory metabolites of several endemic *Scrophularia* species and *Verbascum thapsus* used medicinally by peoples of North America.

Methods—Leaves, stems, and roots were analyzed by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) and partial least squares-discriminant analysis (PLS-DA) was performed in MetaboAnalyst 3.0 after processing the datasets in Progenesis QI.

Results—Comparison of the datasets revealed significant and differential accumulation of iridoid and phenylethanoid/phenylpropanoid glycosides in the tissues of the endemic *Scrophularia* species and *Verbascum thapsus*.

Conclusions—Our investigation identified several species of pharmacological interest as good sources for harpagoside and other important anti-inflammatory metabolites.

Keywords

Harpagoside; iridoid; phenylethanoid/phenylpropanoid; *Scrophularia*; verbascoside; *Verbascum*

1. Introduction

Plant species within the genera *Scrophularia* and *Verbascum* (Scrophulariaceae) produce significant amounts of iridoid and phenylethanoid/phenylpropanoid glycosides believed to be responsible for anti-inflammatory and other pharmacological properties that are highly

*Corresponding authors William R. Folk folkw@missouri.edu, David R. Gang gangd@wsu.edu.
Present address of Mahmoud Gargouri is the Center of Biotechnology of Borj Cedria, Laboratory of Plant Molecular Physiology, Tunisia.

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valued by humans (reviewed in Dembitsky 2006, Tundis et al. 2008, and Viljoen et al. 2012). Historically, Native Americans, Canada's First Peoples, and European colonists of the Americas utilized endemic *Scrophularia* species and imported *Verbascum thapsus* for medicinal treatments, including inflammatory conditions (Herrick 1977; Hough 1849; Moerman 2003; USDA ARS 1992). Botanicals and dietary supplements prepared from these plants continue to be marketed, but with scientifically unsubstantiated claims of efficacy and little understanding of composition. We have analyzed the content of iridoid and phenylethanoid/phenylpropanoid glycosides in the leaves, stems, and roots of these species by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS), and have identified levels of harpagoside, verbascoside, and other potential anti-inflammatory metabolites comparable to those found in botanicals marketed widely in Africa, Asia, Europe, and North America, for which there is evidence of medicinal and pharmacological value.

2 Materials and methods

2.1 Plant growth

Seeds of the study plants (Table 1) were sown directly on Sunshine Mix LC1 soil (sphagnum peat moss and perlite), or for *Scrophularia lanceolata*, were first soaked in a 1.0 mg/mL gibberellic acid (GA3) (Sigma-Aldrich, St. Louis, MO, USA) solution for 4 days at 4°C in complete darkness and then sown. The germinated plants were grown under the following growth chamber conditions—photoperiod: 16 h/8 h (day/night), average temperatures: 25°C/18°C (day/night), average humidity: 70%, and fertilized twice a week with Peters 20-20-20 (N-P-K) supplemented with iron chelate, magnesium sulfate, and trace elements. Voucher specimens were collected, dried, and filed in the Marion Ownbey Herbarium, Washington State University, Pullman, WA, USA. Dried secondary tubers of *Harpagophytum procumbens* (*Hp*) harvested in Namibia in late 2014 were purchased from Parceval Herbals, LLC (Wellington, South Africa) and milled dry, then shipped to the U.S by courier and stored refrigerated.

2.2 Extraction, analysis of metabolites, and data processing

After growth for two to three months, tissues were collected, ground with a mortar and pestle in liquid nitrogen and then lyophilized. It has been shown in field-grown plants that the production of metabolites, including harpagoside, declines after two to three months potentially due to floral development (Brownstein et al. 2016). Approximately 40 mg of lyophilized tissue was extracted with 1.0 mL of cold methanol (4°C) containing the internal standard, 100 μM of 6-chloro-4-hydroxycoumarin (Sigma-Aldrich, St. Louis, MO, USA), which is chemically similar to the metabolites analyzed, but not present in the Lamiales. The samples were sonicated continuously in cold water for 2 h and then centrifuged at 5,000 g for 10 min at 4°C. The supernatant was removed and diluted with cold water : acetonitrile [1:1]/0.10% formic acid (4°C) to a concentration that fit within the developed standard curve. This solution was filtered through a 0.20 μm filter into a sample vial for analysis.

Ultra-performance liquid chromatography (UPLC) was conducted on a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) with photodiode array (PDA)

detection ranging between 210 and 400 nm. One microliter of sample was injected through a 2.0 μL sample loop using the full loop injection mode, and analyzed with a Waters Acquity UPLC column (BEH C18, 1.7 μm, 2.1 x 50 mm) using a flow rate of 0.45 mL/min with water/0.10% formic acid (A) and methanol : acetonitrile [2:3]/0.10% formic acid (B) in a slightly concave gradient elution mode. The gradient elution was applied as follows—97% A : 3% B to 15% A : 85% B from 0.00 to 12.00 min, changed to 3% A : 97% B in 0.10 min, maintained at 3% A : 97% B until 14.00 min, returned to the initial conditions of 97% A : 3% B in 0.10 min, and then, before the next injection, maintained at 97% A : 3% B until 16.00 min. The analysis time was 16.00 min. The autosampler chamber and column temperature were 8°C and 30°C, respectively.

A Waters Synapt G2-S HDMS Q-TOF with lockspray ionization was operated in the ESI negative and resolution mode. The scan range was from 50 to 750 *m/z* with a scan time of 0.3 s. Mass spectral data were collected in profile mode using MS^E with high collision energy (ramp 15 to 40 V) for fragmentation. The capillary voltage, sampling cone voltage, and source offset voltage were 2.5 kV, 60 V, and 60 V, respectively. The source temperature was 120°C with a cone gas (nitrogen) flow rate of 100 L/h. The desolvation temperature was 300°C with a desolvation gas (nitrogen) flow rate of 900 L/h. The nebulizer gas (nitrogen) flow was 4.0 bar and the lock mass compound was leucine enkephalin with a reference mass of 554.2615 $[M-H]^-$ *m/z*. All solvents used for extractions and analyses were of liquid chromatography-mass spectrometry grade.

Harpagide, 8-*O*-*p*-coumaroylharpagide, verbascoside (synonyms: acteoside and kusaginin), and isoverbascoside were purchased from PhytoLab GmbH & Company KG (Vestenbergsgreuth, Germany), and harpagoside and 8-*O*-acetylharpagide were purchased from ChromaDex (Irvine, CA, USA). Under our UPLC-MS conditions harpagide (363.13 $[M-H]^-$ *m/z*), 8-*O*-acetylharpagide (405.14 $[M-H]^-$ *m/z*), verbascoside (623.19 $[M-H]^-$ *m/z*), isoverbascoside (623.19 $[M-H]^-$ *m/z*), 8-*O*-*p*-coumarylharpagide (509.16 $[M-H]^-$ *m/z*), and harpagoside (493.17 $[M-H]^-$ *m/z*) eluted at about 1.25, 2.55, 3.65, 3.90, 4.60, and 5.55 min, respectively. From the PDA raw data, an 11-point standard curve was developed for harpagoside at 280 nm. The peak areas of harpagoside were processed in TargetLynx (Waters Corporation, Milford, MA, USA).

The datasets were processed in Progenesis QI (Nonlinear Dynamics, Newcastle, UK) using the following parameters: the automatic sensitivity method value was set at 1 (fewer), peak widths at and less than 0.19 min were ignored, ions after 12 min were ignored, and each sample was normalized to the internal standard. With these parameters, about two thousand compounds were detected. The datasets were exported to Microsoft Office Excel 2010 and statistical analysis was performed in MetaboAnalyst 3.0 after selecting Mean Intensity Value and then Pareto Scaling (Xia et al. 2015). Venn diagrams were drawn in Venny v2.1 (Oliveros 2015). Molecular formulas were determined in the Identify Compounds section of Progenesis QI. In Elemental Composition, the elemental composition calculation parameters were set with element H from 0 to 100; element C from 0 to 50; element N from 0 to 5; and element O from 0 to 40. The tolerance for mass and isotope similarity was 5 ppm and 95%, respectively. Predicted molecular formulas were sorted by mass error (ppm).

3 Results

3.1 Major known anti-inflammatory metabolites accumulate differentially in the tissues of *S. californica*, *S. lanceolata*, *S. marilandica*, and *V. thapsus*

Harpagoside, a major anti-inflammatory metabolite, has been reported in a variety of taxa, and the *Harpagophytum* species after which it is named are collected for the preparation of anti-inflammatory botanicals and dietary supplements used worldwide (Georgiev et al. 2013; Mncwangi et al. 2012). Our analyses revealed that harpagoside accumulated in the leaves of *Scrophularia* species and the roots of *Vt* (Fig. 1) at levels considered high (1.20-4.37%) by Mncwangi et al. (2014) for *Hp*. Previously, Grabias and Swiatek (1987) had identified harpagoside in *Vt* by thin-layer chromatography and we (Brownstein et al. 2016) had studied accumulation of this metabolite in *Sl* and *Sm* tissues under field and greenhouse cultivation. Here, we extend these observations and report the presence of harpagoside in *Sc*. The biosynthetic pathway(s) for harpagoside are not well elucidated, but harpagide is believed to be an intermediate (Georgiev et al. 2013), and is pro-inflammatory in some assays (Abdelouahab and Heard, 2008). We observed the highest percentage of harpagide in the stems of *Scrophularia* species and the young leaves of *Sc* (Fig. 2). *Sc* stems and *Sm* young leaves also accumulated the highest percentage of 8-*O*-acetylharpagide (Fig. 2).

Under our growth chamber conditions, *Sc* produces more harpagoside in its young leaves, old leaves, and stems in comparison to *Sl*, *Sm*, and *Vt* (Fig. 1), and the amount in the old leaves (0.94%) were near the high levels reported in *Hp* tubers (Mncwangi et al. 2014). Fig. S1 shows the morphological differences between the genera *Scrophularia* and *Verbascum*. After analyzing a few *Verbascum* species, Georgiev et al. (2011) detected harpagoside in the leaves of only two *Verbascum* species. As revealed in Fig. 1, *Vt* produces low amounts of this metabolite in its leaves. Since *Vt* and other *Verbascum* species grow in a rosette-form, this may cause harpagoside to accumulate in the roots rather than the aerial tissues. This differential accumulation in *Vt* can also be observed with other iridoid glycosides, including harpagide, 8-*O*-*p*-coumaroylharpagide, and 8-*O*-acetylharpagide (Fig. 2).

Sc and *Vt* roots and *Sl* and *Sm* young leaves contained the highest levels of 8-*O*-*p*-coumaroylharpagide (Fig. 2), and this metabolite is similarly low in *Hp*, ranging between 0.04-0.12% (Baghdikian et al. 1997). Two common phenylethanoid/phenylpropanoid glycosides that are anti-inflammatory, verbascoside and isoverbascoside, were detected in *Sc*, *Sl*, *Sm*, and *Vt* (Fig. 2). Verbascoside is not only a common metabolite in *Hp* and the genera *Scrophularia* and *Verbascum*, but it is also present throughout the Lamiales (Alipieva et al. 2014a; Stevens 2001). It is apparent that the *Scrophularia* species produced verbascoside and isoverbascoside primarily in their roots, while *Vt* produced these metabolites equally in its tissues (Fig. 2).

3.2 *S. californica, S. lanceolata, S. marilandica,* and *V. thapsus* and their tissues have unique metabolite profiles

After analyzing the entire metabolite profile of each species, partial least squares-discriminant analysis (PLS-DA) of *Sc*, *Sl*, *Sm*, and *Vt* of their tissues indicated *Sl* and *Vt* are more distinctly separate in comparison to the other species (Fig. 3). The young and old

leaves, and old leaves and roots of *Sc* and *Sm*, respectively, can be seen forming a larger cluster. In Fig. 4, the roots of *Sl*, and *Sm* clustered together implying a large number of shared metabolites. The old leaves of *Vt* separated from the *Scrophularia* species because the lack of a stem may distribute metabolites differently. To some extent, clustering of other metabolite profiles can also be observed for *Sl* and *Sm* young leaves and *Sc* and *Sl* old leaves (Fig. 4). This indicates that the tissues of these species may share some metabolite similarities.

Comparing the PLS-DA of roots of *Hp* to the roots of *Sc*, *Sl*, *Sm*, and *Vt* revealed four separate groups: *Hp*, *Sc*, *Sl*/*Sm*, and *Vt* (Fig. 4). The roots of *Sl* and *Sm* were most similar among one another (Fig. 4b) and this was also shown in Fig. 4a. In addition, the metabolite profiles of the *Scrophularia* species and *Vt* roots were most similar to one another even when *Hp* was included in the PLS-DA.

The genera *Scrophularia* and *Verbascum* are thought to have diverged 46.86 mya. *Verbascum* species were recently introduced from Europe to North America, and New World *Scrophularia* species may have a common Asian ancestor and migrated across the Bering Land Bridge between 22.27-8.32 mya (Scheunert and Heubl 2011). The morphology of *Scrophularia* and *Verbascum* species are quite different, but when compared to *Hp*, the metabolite profile of these two sister taxa are most similar.

Variable importance in projection (VIP) scores from the PLS-DA results revealed 73, 25, 18, and 71 significant metabolites specific to *Sc*, *Sl*, *Sm*, and *Vt*, respectively (Fig. 5). There were 21 metabolites common to *Sc*, *Sl*, *Sm*, and *Vt*, and four of these were identified as harpagoside, harpagide, verbascoside, and isoverbascoside (Fig. 5). The *Scrophularia* species shared 34 metabolites, including 8-*O*-*p*-coumaroylharpagide and 8-*O*-acetylharpagide (Fig. 5). Comparisons of high VIP scoring metabolites identified two known metabolites, 8-*O*-*p*-coumaroylharpagide and 8-*O*-acetylharpagide, as potential metabolites that distinguish the *Scrophularia* species from *Vt*. Harpagide was the most important feature in *Sc*, *Sl*, *Sm*, and *Vt*, while 8-*O*-acetylharpagide was the most important feature shared with *Sc*, *Sl*, and *Sm* (Table S1).

4 Discussion

Historically, *Scrophularia* species were widely used by Native Americans. The Ohlone and Kashaya Pomo of Northern California prepared a poultice of *Sc* leaves to treat boils, swellings, and infections. The Ohlone also used this plant to treat sore eyes and as an eyewash for poor vision (Moerman 2003). *Sl* had been used by the Iroquois to treat sunburns, sunstroke, edema, frostbite, and for gynecological and obstetrical complications (Herrick 1977). *Sm* was widely used in women's health, but Native Americans and colonists also used this species to treat fevers, hemorrhoids, insomnia, restlessness, skin diseases, tuberculosis, wounds, and as an anthelmintic and diuretic (Hough 1849; Moerman 2003; USDA ARS 1992).

Verbascum species were historically used in European folk medicine to treat various ailments, including respiratory disorders, pain and inflammation, and infections, with *Vt*

being the most widely used species (Alipieva et al. 2014b). European settlers introduced the seeds of *Vt* in the early 18th century (Wilhelm 1974). Over time this species spread westward and is now common throughout North America. Due to this species being widespread and commonly used in Native American communities, it was initially thought to be endemic to North America. Indigenous peoples throughout North America used *Vt* to treat a variety of ailments, including colds, coughs, fevers, toothaches, wounds, and for relieving pain and swelling. The Winnebago applied heated leaves of *Vt* to swellings (Kindscher and Hurlburt 1998). The leaves were also smoked by the Menominee, Nlakapamuk, and Tiwa as a ceremonial tobacco as well as by the Iroquois, Maliseet, Mohegan, Navajo, Penobscot, and Potawatomi for asthma, catarrh, and to treat sore throats (Herrick 1977; Moerman 2003; USDA ARS 1992). The metabolites present in these species used for pain mitigation and to combat infections enable more informed understanding of the potential medicinal and pharmacological value of species' anti-inflammatory properties. Metabolites with high VIP scores unique to *Sc*, *Sl*, *Sm*, and *Vt* are potential biomarkers for pharmacological activities (Fig. 5).

Harpagoside was first isolated from the tubers of *Hp* (Tunman and Lux 1962) and is thought, together with one or more iridoid and phenylethanoid/phenylpropanoid glycosides to be responsible for the anti-inflammatory properties of the various extracts, salves, and tinctures made from this and related plants (Abdelouahab and Heard 2008; Anauate et al. 2010; Galindez et al. 2002; Garcia et al. 1996; Georgiev et al. 2013; Gyurkovska et al. 2011; Mncwangi et al. 2012; Tundis et al. 2008; Viljoen et al. 2012). Whether harpagoside alone may not be sufficient for anti-inflammatory activity toward many conditions, it is a useful molecular marker required by European pharmacopoeia for Devil's Claw products. *Sc*, *Sl*, and *Sm* leaves produce up to 1.2% harpagoside (Fig. 1), which is similar to amounts reported in the leaves of *Scrophularia nodosa* (Sesterhenn et al. 2007) and *Scrophularia scorodonia* (Diaz et al. 1998). Sesterhenn et al. (2007) and Diaz et al. (1998) proposed that these *Scrophularia* species could be used as sources for harpagoside, and Garcia et al. (1996) found that extracts from the aerial parts of *Scrophularia frutescens* reduced inflammation in the rat hind paw carrageenan-induced edema model; however, more research is necessary to study the safety and efficacy of using tissues from these species.

Abdelouahab and Heard (2008) reported that 8-*O*-*p*-coumaroylharpagide had comparable antiinflammatory properties to harpagoside; therefore, identifying plants producing significant amounts is of interest. However, this metabolite is produced at low amounts in *Sc*, *Sl*, *Sm*, and *Vt*. Harpagide has a similar iridoid core structure to harpagoside and 8-*O*-*p*-coumaroylharpagide, but some evidence suggests it might be pro-inflammatory (Abdelouahab and Heard, 2007). This metabolite accumulated in the stems of *Sc*, *Sl*, and *Sm* (Fig 2).

Vt roots produced higher amounts of 8-*O*-acetylharpagide compared to *Sc*, *Sl*, and *Sm* roots (Fig. 2). In bioassays, 8-*O*-acetylharpagide isolated from *Scrophularia deserti* and *Scrophularia scorodonia* has been shown to possess weak to moderate anti-inflammatory activities in comparison to harpagoside (Tundis et al. 2008). Verbascoside isolated from *Hp* tubers also possesses similar anti-inflammatory properties to harpagoside (Abdelouahab and Heard 2008; Gyurkovska et al. 2011).

This is the first study to comprehensively compare the chemical composition of North American medicinal plants known to produce significant amounts of harpagoside and related anti-inflammatory metabolites (Figs. 3 and 4). Our findings indicate that the North American *Scrophularia* species produce significant amounts of harpagoside in leaves and may possibly be alternatives to *Hp*, whose extensive use places it at risk of becoming endangered (Bairu et al. 2011; Stewart and Cole 2005). However, further research is necessary to test the bioactivity of these species and their tissues. Identifying alternative species should not only require analysis of what is thought to be the primary bioactive metabolites (for instance, harpagoside and 8-*O*-*p*-coumaroylharpagide), but also potential pro-inflammatory metabolites; i.e., a whole metabolite profile analysis.

5 Conclusions

It has been shown that related plants from particular regions of the world have similar ethnobotanical uses. Different cultures will not encounter the same species, genera, or even families; however, overlaying phylogenetic trees of medicinal plants endemic to these particular regions have shown that these plants are found in the same lineages (Saslis-Lagoudakis et al. 2012). Comparing metabolite profiles can identify which species have similar medicinal properties, regardless of their native ecosystem. For instance, *H. procumbens* has a history of use in Africa for relieving pain and inflammation and numerous studies point to the significance of its iridoid and phenylethanoid/phenylpropanoid glycoside content (reviewed in Mncwangi et al. 2012). Herein, we have shown that several *Scrophularia* species endemic to North America have potential pharmacological activities because of their production of similar iridoid and phenylethanoid/phenylpropanoid glycosides. Omics (or in this case, metabolomics) provides insights into why people preferred certain species of plants to treat particular ailments (Ngo et al. 2013). Both *H. procumbens* and the North American *Scrophularia* species have a history of treating pain and inflammation and our PLS-DA revealed that these medicinal properties might have been due to these species possessing similar metabolites. The results presented here demonstrate that medicinal uses of *S. californica*, *S. lanceolata*, and *S. marilandica* have the potential to be rediscovered for the benefit of all.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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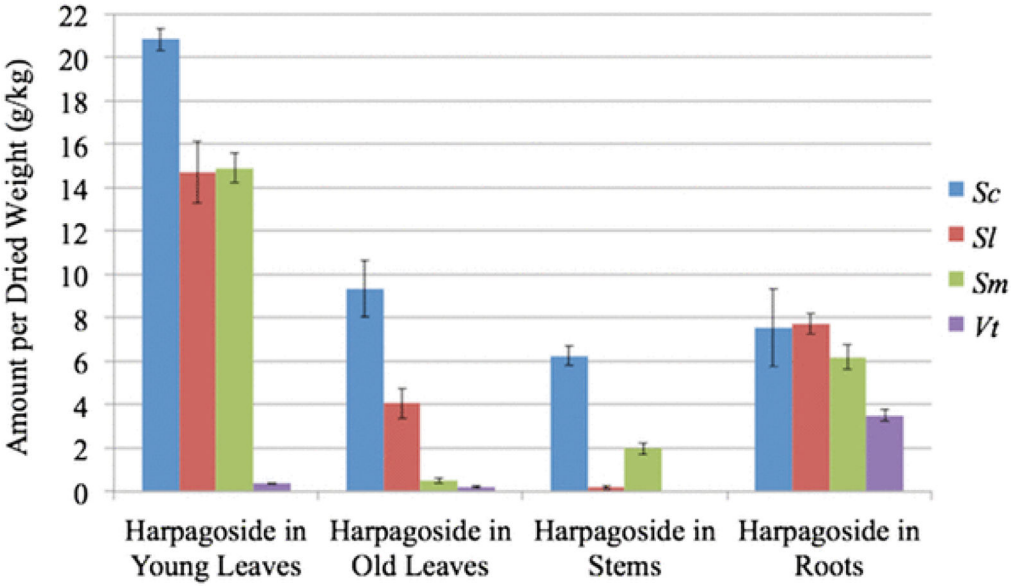

Fig. 1.

UPLC-UV quantification of harpagoside content in various tissues of *S. californica*, *S. lanceolata*, *S. marilandica*, and *V. thapsus*. *Sc* = *Scrophularia californica*, *Sl* = *Scrophularia lanceolata*, *Sm* = *Scrophularia marilandica*, and *Vt* = *Verbascum thapsus*. Vertical bars represent standard errors of five different plant replicates (*n* = *5*). The harpagoside content in the stems of *Vt* is absent because this species lacks a stem

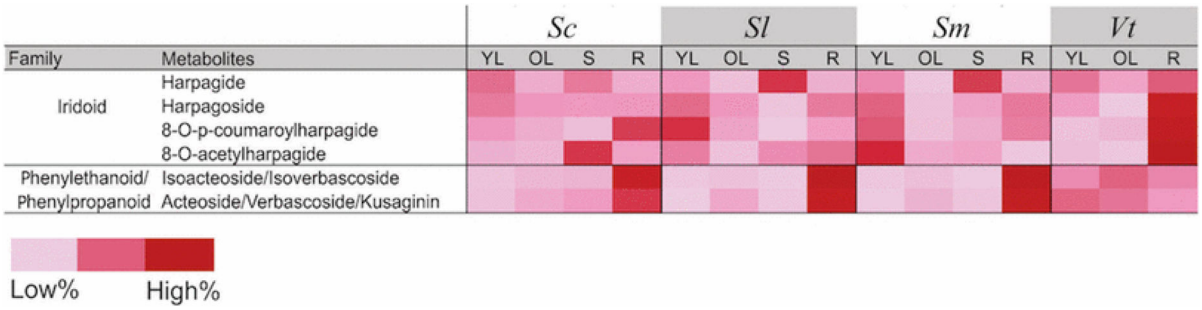

Fig. 2.

Heat map comparing tissues of each species for the concentration of a particular metabolite. Low, middle, and high are 0% (not detected), 50%, and 100%, respectively. *Sc* = *Scrophularia californica*, *Sl* = *Scrophularia lanceolata*, *Sm* = *Scrophularia marilandica*, *Vt* = *Verbascum thapsus*, YL = young leaves, OL = old leaves, S = stems, and R = roots

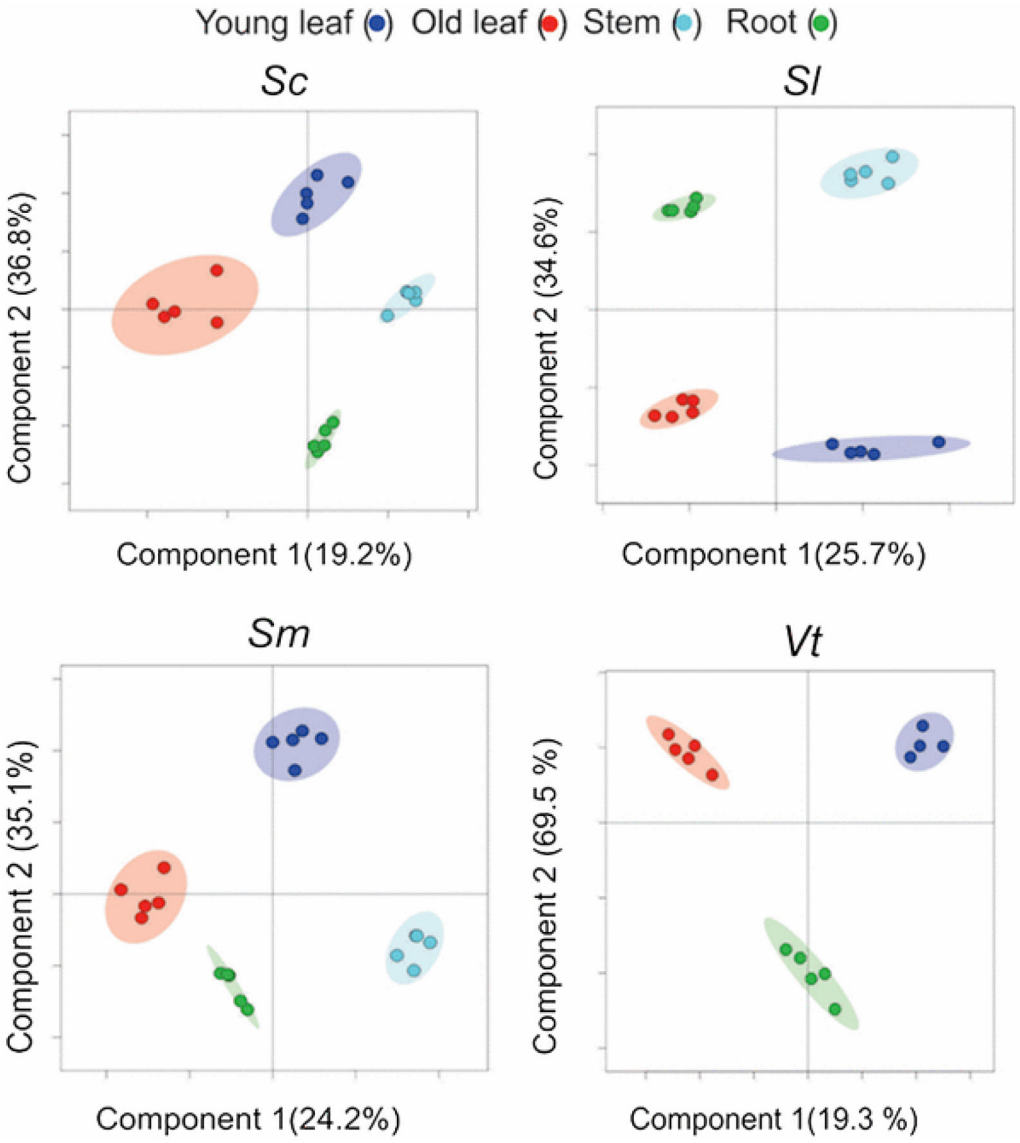

Fig. 3.

Partial least squares-discriminant analysis (PLS-DA) of tissues within each species. The young leaves, old leaves, and roots were compared in *V. thapsus* (*Vt*) because this species lacks a stem. *Sc* = *Scrophularia californica*, *Sl* = *Scrophularia lanceolata*, *Sm* = *Scrophularia marilandica*, and *Vt* = *Verbascum thapsus*. Each dot represents five different plant replicates (*n* = *5*)

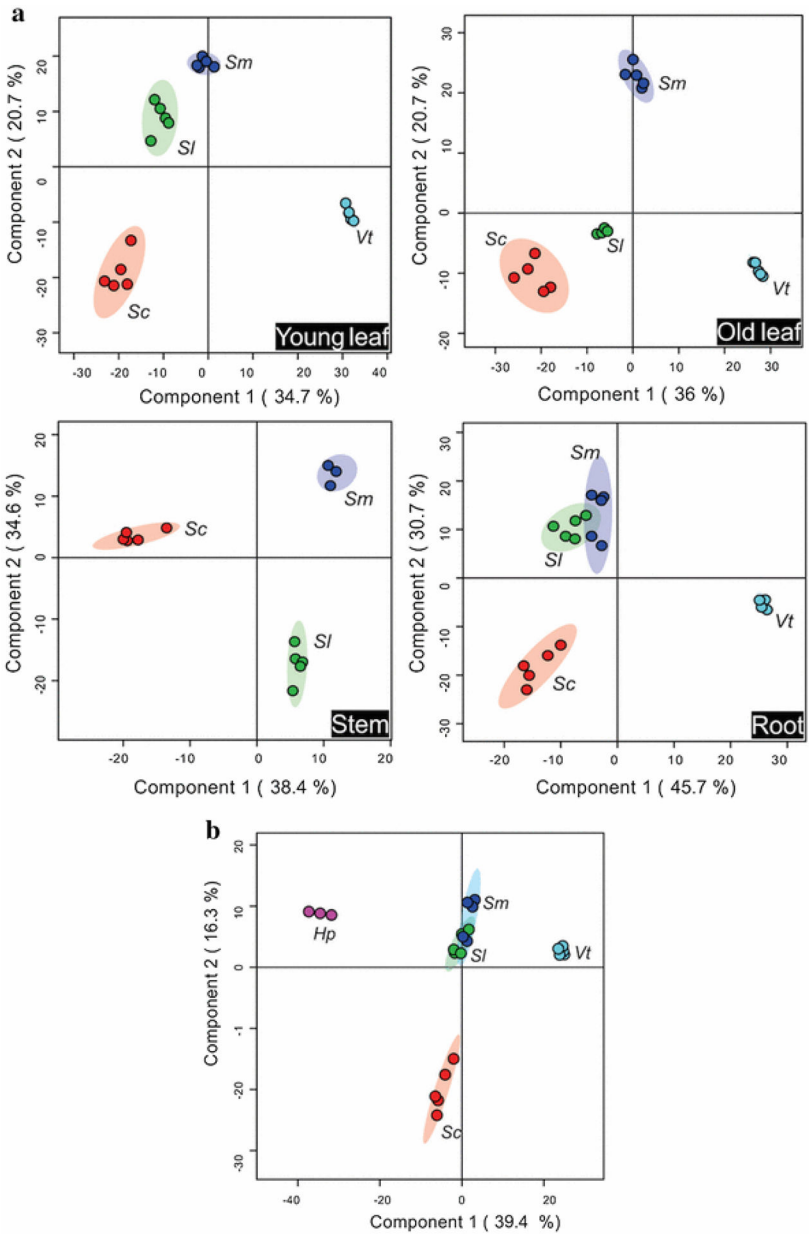

Fig. 4.

Partial least squares-discriminant analysis (PLS-DA) **a** comparing the tissues between each species, excluding *H. procumbens* (*Hp*), and **b** comparing between only the roots of each species, including *Hp*. *V. thapsus* (*Vt*) is absent from the stem PLS-DA because this species lacks a stem. *Hp* = *Harpagophytum procumbens*, *Sc* = *Scrophularia californica*, *Sl* = *Scrophularia lanceolata*, *Sm* = *Scrophularia marilandica*, and *Vt* = *Verbascum thapsus*. Each dot represents five different plant replicates (*n* = *5*)

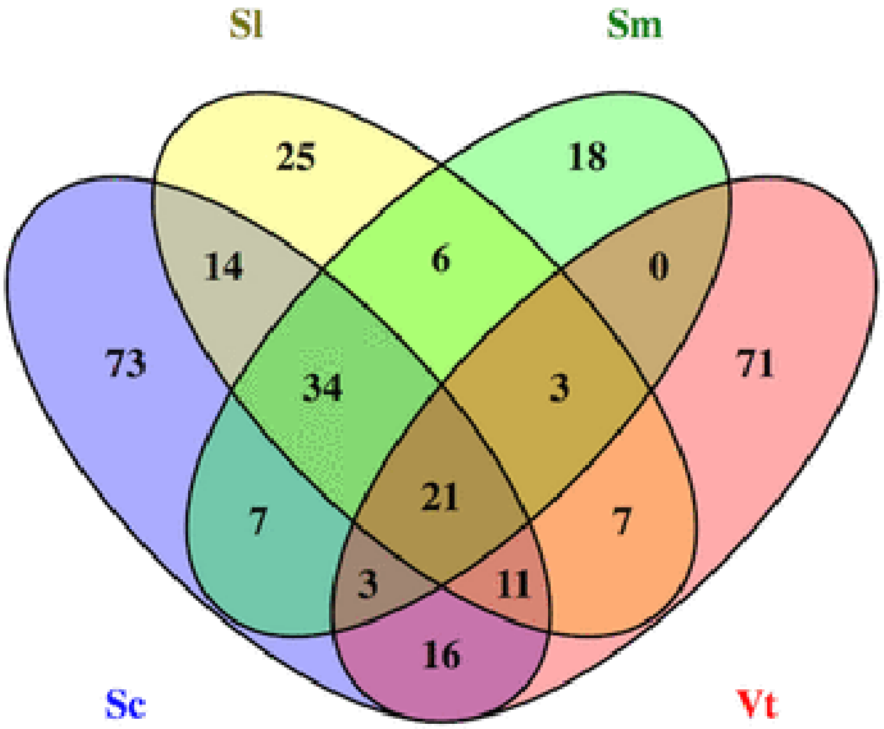

Fig. 5.

Venn diagram showing which metabolites are shared and unique among *S. californica*, *S. lanceolata*, *S. marilandica*, and *V. thapsus* with variable importance in projection (VIP) scores greater than one. *Sc* = *Scrophularia californica*, *Sl* = *Scrophularia lanceolata*, *Sm* = *Scrophularia marilandica*, and *Vt* = *Verbascum thapsus*

Table 1:

Plants studied

Species (Abbreviation)	Native Region	Seed Source
Scrophularia californica Cham. & Schldl. (*Sc*)	North America, coastal west	Larner Seeds, Bolinas, CA, USA
Scrophularia lanceolata Pursh (*Sl*)	North America	Prairie Moon Nursery, Winona, MN, USA
Scrophularia marilandica L. (*Sm*)	North America	Prairie Moon Nursery, Winona, MN, USA
Verbascum thapsus L. (*Vt*)	Europe (introduced to North America)	Companion Plants, Athens, OH, USA