**Title:**

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**Footnotes**

**Abstract**

The *Scutellaria* genus contains multiple plant species used extensively in traditional medicines due to their various anti-inflammatory, sedative, and neuroprotective effects. *S. baicalensis* is one of the most well-known of these species, and previous works have identified its accumulation of bioactive flavones as a primary source of these effects. Specifically, the biosynthetic pathway of *S. baicalensis* … root-specific accumulation of 4´-deoxyflavones

**OUTLINE**

1. INTRODUCTION
   1. Background of medicinal plants
      1. Importance in traditional medicines
      2. Limitations – endangerment of native populations, production efficiency
      3. Necessity of biotechnology
   2. Introduction to *Scutellaria* and well-known *Scutellaria* species
      1. *S. barbata*, *S. lateriflora*, *S. baicalensis*
   3. 4´-hydroxyflavones and 4´-deoxyflavones
      1. Medicinal activities
      2. Organ-specific pattern in *S. baicalensis*
      3. Current state of knowledge of flavone pathway in *Scutellaria*
   4. Gaps in knowledge
      1. 470+ species in genus, only several studied
      2. Focus in *S. baicalensis* has been on 4´-deoxyflavones, not hydroxyflavones
      3. F8H for deoxyflavones has been identified in *S. baicalensis*, but unknown if analogous pathway exists for hydroxyflavones
   5. Goal of present study
      1. Identify medicinally valuable species that were previously unrecognized
      2. Assess how well organ-specific accumulation pattern is conserved
      3. Unexpectedly, we have identified a novel 4´-hydroxyflavone in *Scutellaria* which shows organ- and species-specific accumulation patterns. We also identify the enzyme responsible, and quantify it in various species
2. RESULTS
   1. Organ-specific flavone profiling
      1. 4´-deoxyflavone pathway is well conserved
      2. Relative abundance of glycone vs aglycone forms
      3. 4´-hydroxyflavone pathway is less well conserved, but is largely specific to aerial parts
      4. Root specificity of 4´-deoxyflavones as observed in *S. baicalensis* is not well conserved
      5. Accumulation of baicalein + baicalein by *S. wrightii*
      6. Accumulation of oroxylin A + oroxyloside by *S. racemosa*
   2. Identification of unknown peak as isoscutellarin
      1. HPLC chromatogram comparison – peak is present in aerial parts (aerial part specificity of 4´-hydroxyflavones) but only in some species (not *S. racemosa* or *S. wrightii*)
      2. Fractionation of unknown peak
      3. LC-MS and MS/MS results (compare with scutellarin) – identical fragmentation pattern, but different retention time
      4. NMR results to elucidate structure
   3. Establish apigenin as a precursor to isoscutellarin
      1. Apigenin feeding in *S. barbata*
   4. Identification of enzyme responsible for 8-hydroxylation
      1. Discuss previous studies with RTO (Zhao et al., 2018; Berim et al., 2014)
      2. Yeast activity test + *N. benthamiana* and *A. thaliana* infiltration
      3. Detection of new peak in yeast / infiltration data – isoscutellarein (aglycone) OR reduce isoscutellarin to isoscutellarein
      4. Confirm aglycone structure with LC-MS, MS/MS, and NMR (figures go in appendix?)
   5. Organ-specific quantification of isoscutellarein and isoscutellarin
3. DISCUSSION
   1. Overall difference in conservation of 4´-hydroxyflavone and 4´-deoxyflavone pathways across species
      1. Physiological roles – herbivory defense?
      2. 4´-deoxyflavones more common – *Scutellaria* has evolved to use 4´-deoxyflavones instead of 4´-hydroxyflavones
   2. Flavone accumulation is organ-specific in majority of species analyzed
      1. Substrate specificity of enzymes in flavone pathway is conserved and/or gene expression is organ-specific
      2. Pattern of organ-specificity differs in multiple species from that of *S. baicalensis*
   3. Patterns of 4´-hydroxyflavone and 4´-deoxyflavone accumulation with respect to native environment
      1. *S. baicalensis + S. barbata* – cool climate, little 4´-deoxyflavones in aerial parts (isoscutellarin is present)
      2. *S. racemosa + S. wrightii* – warm climate, significant 4´-deoxyflavones in aerial parts (isoscutellarin is not present)
   4. Significance of isoscutellarin discovery
      1. Potential medicinal effects
      2. Isolated in other plant species? – biosynthesis route similar or different?
   5. Species as targets for further medicinal development
      1. *S. racemosa* – oroxylin A + oroxyloside (and overview of previous work with 6-OMTs)
      2. *S*. *wrightii* – baicalein + baicalin
4. MATERIALS AND METHODS
   1. Plant growing conditions
   2. Flavone extraction and quantification
   3. HPLC fractionation to isolate isoscutellarin
   4. LC-MS and MS/MS
   5. NMR
   6. Apigenin feeding
   7. Yeast activity
   8. *N. benthamiana* and *A. thaliana* infiltration

**INTRODUCTION**

Medicinal plants have played an important role in the traditional medicines of indigenous populations for thousands of years. Due to this widespread usage, modern research techniques are being applied to identify the specific compounds responsible for these medicinal properties and better characterize their method of action (Shang et al., 2010). A negative consequence of increased attention to and demand for medicinal plants is the endangerment of native plant populations resulting from overharvesting (I. B. Cole et al., 2007). Therefore, development of mass production systems for these medicinal compounds is extremely desirable. As chemical synthesis methods can be limited by their expense and relative inefficiency, biotechnology-based methods are a promising alternative for mass production of more structurally complex compounds (Yang et al., 2016). Development of effective biotechnology for chemical production requires an understanding of the biochemistry behind the compounds of interest. In this work, we chemically analyze multiple species from the *Scutellaria* genus to identify candidates for biotechnology improvement, and investigate a previously uncharacterized step in a chemical pathway.

Part of the mint family Lamiaceae, *Scutellaria* is a genus of plants containing multiple species with well-documented medicinal effects. Extracts from the aerial parts of *S. barbata* are commonly applied in Eastern medicines to treat swelling, inflammation, and cancer (G. Tao & Balunas, 2016). These activities, and especially its anticancer effects, have drawn research attention to *S. barbata*, and early phase clinical trials of aqueous extracts have demonstrated its selective cytotoxicity towards breast cancer cells (Chen et al., 2012). In addition, *S. barbata* extracts have exhibited remarkable activity towards multi-drug resistant strains of bacteria (Tsai et al., 2018). A reference genome for the species has recently been published (Xu et al., 2020). *S. baicalensis* is another species extensively applied in Eastern medicines, with extracts of its roots being prescribed to treat diarrhea, dysentery, hypertension, inflammation, and a variety of other diseases (T. Zhao et al., 2019). Numerous clinical studies have demonstrated the neuroprotective, antibacterial, antitumor, antioxidant, and other beneficial health effects of these extracts (Saralamma et al., 2017; Y. Tao et al., 2018; Zhu et al., 2016). A reference genome has also been published for *S. baicalensis*, and a unique biochemical pathway responsible for its synthesis of a variety medicinally active metabolites has been described (Q. Zhao et al., 2019).

This pathway is that of flavones, which *S. baicalensis*, *S. barbata*, and other medicinally active *Scutellaria* species accumulate in high concentrations (Karimov & Botirov, 2017). Most *Scutellaria* species produce two classes of flavones: 4´-hydroxyflavones and 4´-deoxyflavones. Biosynthesis of 4´-hydroxyflavones, which include apigenin and its derivatives, is relatively common across the plant kingdom. In contrast, biosynthesis of 4´-deoxyflavones, which include chrysin and its derivatives, is relatively rare outside of *Scutellaria*, and 4´-deoxyflavones have only been identified in several plant species not in the genus (Kato et al., 1992; V. M. Rao et al., 2009; Y. K. Rao et al., 2002). As mentioned previously, the flavone biosynthetic pathway has been most well studied in *S. baicalensis*. Multiple works have identified the enzymes of the pathway, and described the differential activity of specific enzyme isoforms towards either 4´-hydroxyflavones or 4´-deoxyflavones (Q. Zhao et al., 2016, 2018, 2019) (Fig. 1). It is this differential activity which leads to the development of an organ-specific pattern of accumulation in *S. baicalensis*. In this pattern, 4´-hydroxyflavones accumulate in the aerial parts of the plant at higher concentrations than in the roots, and 4´-deoxyflavones accumulate at higher concentrations in the roots as compared to the aerial parts. A similar organ-specific accumulation pattern occurs in *S. barbata*, but the specifics of the biochemical basis of this pattern are less well studied (G. Tao & Balunas, 2016; Xu et al., 2020).

Although flavone profiles of *S. baicalensis*, *S. barbata*, and several other species of *Scutellaria* have been described, the genus contains approximately 350 species, distributed nearly worldwide (Shang et al., 2010). As metabolite profiles for most of these species have not been documented, it is unknown if the overall flavone pathway, and the organ-specific accumulation patterns of *S. baicalensis* and *S. barbata*, are well-conserved across the genus. In addition, the limited number of *Scutellaria* species that have been chemically profiled presents the possibility of a species with high flavone accumulation going uncharacterized. Studying the biochemistry of such a species could be extremely valuable for biotechnology efforts targeting mass production of medicinal flavones. Another limitation facing studies of biochemistry in *Scutellaria* is the sheer number of flavone structures that can be potentially generated through different sequences of hydroxylation, methylation, and glycosylation. The biochemical steps which have been elucidated in *S. baicalensis* thus far may only be representative of a portion of the flavone pathway, with multiple steps still undiscovered or not yet well described.

In this work, we aimed to expand our current knowledge of flavone diversity in *Scutellaria* by chemically analyzing seven species, several of which were previously uncharacterized at the time of this study. From the results of this profiling, we identified two species with medicinally significant flavone profiles which could serve as valuable biotechnology targets. During this analysis, we also unexpectedly identified a novel 4´-hydroxyflavone. We quantified this 4´-hydroxyflavone in the seven species which we analyzed previously, and attempted to identify the enzyme responsible for its biosynthesis in the *S. baicalensis* reference genome.

**RESULTS**

***Organ-specific flavone diversity across seven Scutellaria species –***

We selected seven species of *Scutellaria* for organ-specific flavone profiling with High Performance Liquid Chromatography (HPLC). These species included *S. altissima*, *S. baicalensis*, *S. barbata*, *S. leonardii*, *S. racemosa*, *S. tournefortii*, and *S. wrightii*. We grew plants of each species from seed in climate-controlled conditions, and harvested tissue samples from the roots, stems, and leaves of mature plants in biological triplicate. We then quantified concentrations of six 4´-hydroxyflavones and eight 4´-deoxyflavones in these samples **(Fig. 2, Appendix S1)**.

Based on our root-specific flavone profile results, the 4´-deoxyflavone pathway appears to be very well-conserved across all species we selected. We detected at least six 4´-deoxyflavones in the roots of all species we analyzed. Interestingly, although chrysin is proposed to serve as a precursor for all 4´-deoxyflavones we quantified, we detected chrysin in the roots of none of the seven species, and detected its glycosylated form, chrysin 7-G, in only three. This rarity in detection is possibly a result of chrysin rapidly being converted into downstream products before it accumulates to a level detectable by our HPLC method. *S. baicalensis* and *S. wrightii* stand out due to their high accumulation of 4´-deoxyflavones, and specifically, baicalin. The root-specific accumulation of baicalin by *S. baicalensis* is well documented, so this result aligns well with published data (I. Cole et al., 2008; T. Zhao et al., 2019). However, as no chemical data for *S. wrightii* had been published at the time of this study, this result was intriguing. Finally, the absence of 4´-hydroxyflavones in the roots of all but one species (*S. leonardii*) indicates that the specificity of the 4´-hydroxyflavone biosynthetic pathway to aerial tissues as described in *S. baicalensis* may also be well-conserved in other *Scutellaria* species (Q. Zhao et al., 2016).

Although root-specific flavone profiles were relatively consistent across the selected species, aerial tissue-specific profiles were more varied. 4´-hydroxyflavones were present in the aerial tissues of all species analyzed, but the pathway seemed to be conserved to more varying degrees than that of 4´-deoxyflavones in the roots. We were unable to detect hispidulin, or its glucoside, hispiduloside, in the leaves or stems of two species: *S. altissima* and *S. tournefortii*. Hispiduloside was particularly rare, and out of all tissue samples taken, we only detected it in the stems of *S. racemosa*. Although these more advanced steps in the pathway may not be well-conserved, our detection of scutellarin in the aerial tissues of all seven species indicates at least partial retention of 4´-hydroxyflavone biosynthesis in these species. As a precursor to all 4´-hydroxyflavones analyzed, apigenin was surprisingly scarce, and we detected it in the aerial tissues of only two species: *S. baicalensis* and *S. leonardii*. This pattern is analogous to that which we observed with chrysin in our root tissue samples, and suggests rapid conversion of apigenin into downstream products.

Our detection of at least one 4´-deoxyflavone in the leaves of all species analyzed suggests that 4´-deoxyflavones aren’t root-specific to the same degree that 4´-hydroxyflavones are aerial tissue-specific. One explanation is provided by considering the overall flavone profiles of *S. altissima, S. baicalensis, S. tournefortii,* and *S. wrightii*. In these species, we detected higher concentrations of many of the same 4´-deoxyflavones in the roots as compared to the leaves. In addition, a mix of 4´-hydroxyflavones and 4´-deoxyflavones were present in the stems of these plants. Therefore, it’s possible that some fraction of 4´-deoxyflavones being synthesized in the roots are being transported to the aerial parts. It’s also possible that the expression of 4´-deoxyflavone enzyme isoforms is not perfectly root-specific, or that these specialized enzyme isoforms have some activity towards 4´-hydroxyflavones.

In contrast to the root-heavy accumulation of 4´-deoxyflavones we observed in *S. baicalensis*, several species included in our study accumulated greater concentrations of 4´-deoxyflavones in their aerial parts as compared to their roots. Interestingly, *S. leonardii* accumulated high concentrations of chrysin 7-G in its leaves, but little of any other 4´-deoxyflavone. *S. racemosa* accumulated high concentrations of both oroxylin A and oroxyloside in its leaves. This finding is especially remarkable when considering the relative rarity of these 4´-deoxyflavones in the six other species we analyzed **(Fig. 3)**.

***Identification of isoscutellarein 8-G, a novel 4´-hydroxyflavone in Scutellaria –***

During our chemical analysis, we detected multiple metabolites which we had no standard for, and therefore were unable to identify. Of these unknown metabolites, one drew our interest because of its pattern of accumulation across the tissue samples we collected. In our HPLC chromatograms, we detected the peak corresponding to this metabolite in the aerial parts of several species, including *S. baicalensis* and *S. barbata*. In contrast, the metabolite was entirely absent in other species, including *S. racemosa* **(Fig. 4)**. Its aerial specificity in the species in which accumulated it led us to hypothesize that the metabolite was a 4’-hydroxyflavone, but its UV absorbance spectra did not resemble that of any 4’-hydroxyflavone which we had a standard for. Therefore, to elucidate its structure, we fractionated the unknown metabolite from our *S. barbata* leaf extracts, and analyzed the fraction with tandem mass spectrometry (MS/MS) and nuclear magnetic resonance spectrometry (NMR).

Explanation of MS/MS and NMR results. Need X-ray crystallography?

***Investigation of isoscutellarein 8-G biosynthesis –***

**DISCUSSION**

From our analysis of organ-specific flavone diversity, we detected profiles for *S. baicalensis* and *S. barbata* which matched closely with previous publications (Xu et al., 2020; Q. Zhao et al., 2016). In these flavone profiles, high concentrations of 4´-deoxyflavones accumulated in the roots, and much lower concentrations of 4´-deoxyflavones and 4´-hydroxyflavones accumulated in the stems and leaves. As described by Q. Zhao et al. (2016), the root-favored accumulation of 4´-deoxyflavones by *S. baicalensis* is due to root-specific overexpression of several enzymes with activity exclusively, or near exclusively in 4´-deoxyflavone biosynthesis. In contrast to the pattern we observed in *S. baicalensis* and *S. barbata,* we identified several species which accumulated higher concentrations of 4´-deoxyflavones in their leaves as compared to their roots. These species included *S. racemosa* and *S. leonardii*. As only trace amounts of 4´-hydroxyflavones were present in the aerial tissues of both species, this result suggests an upregulation of at least one 4´-deoxyflavone specific biosynthetic gene, rather than an upregulation of genes with similar activity in both 4´-deoxyflavone and 4´-hydroxyflavone biosynthesis. For all species we selected, differences in root and aerial tissue flavone profiles indicates the selectivity of enzymes towards either 4´-deoxyflavones or 4´-hydroxyflavones (or their respective precursors), as well as organ-specific regulation of biosynthetic gene expression.

**Differences in how well 4´-deoxyflavone and 4´-hydroxyflavone pathways are conserved.**

**MATERIALS AND METHODS**

***Plant growth conditions –***

Plants of 7 *Scutellaria* species were grown from seed at the University of Florida (Gainesville, Florida, USA) in indoor, climate-controlled conditions at 21-23 °C. Fluorescent lighting of intensity 140 µE m-2 s-1 was applied in a 16 hour light / 8 hour dark cycle. Plants were watered every 5-8 days, and root, stem, and leaf tissue samples collected in biological triplicate 6-8 weeks after germination. Seeds of all species were obtained from online retailers, except for those of *S. racemosa* and *S. wrightii*. To collect seeds of *S. racemosa*, mature plants were taken from a field in Hattiesburg, Mississippi, USA, and grown in indoor, climate-controlled conditions at the University of Florida until seeds were ready to harvest. Seeds of *S. wrightii* were collected directly from mature plants grown in outdoor greenhouse conditions at Far South Wholesale Nursery (Austin, Texas, USA). Herbarium voucher submission (Supplemental table).

***Flavone extraction and quantification –***

With High Performance Liquid Chromatography (HPLC), 15 flavones were quantified from root, stem, and leaf tissue samples of plants. The flavones quantified included seven 4´-hydroxyflavones, which were apigenin, apigenin-7-glucuronide (apigenin 7-G), scutellarein, scutellarin, hispidulin, hispiduloside, and isoscutellarein-8-glucuronide (isoscutellarein 8-G). The remaining eight flavones were 4´-deoxyflavones, which were chrysin, chrysin-7-glucuronide (chrysin 7-G), baicalein, baicalin, oroxylin A, oroxyloside, wogonin, and wogonoside. The fresh weight of each tissue sample was determined with an analytical balance immediately after harvesting. An extraction buffer of 50% HPLC grade methanol was added to each so that the following ratio was achieved: 30 mg tissue/1 mL solvent. Samples were then sonicated for 1 hour at room temperature**.** Following sonication, the extraction solution was withdrawn and further diluted with additional 50% methanol to achieve a final ratio of 1 mg tissue/1 mL solvent. To remove any remaining particulate, extractions were centrifuged at 15,000 rpm for 5 minutes, and syringe filtered with a filter having a pore size of 0.45 µm.

Flavones were quantified in this final extraction with a Thermo Scientific (Massachusetts, USA) UltiMate 3000 HPLC system. Flavones were separated with a 3 x 100 mm Acclaim RSLC 120 C18 column, and eluted by a mixture of 0.1% formic acid (A) and 100 % acetonitrile (B) with the following gradient: -8 to 0 min, 5% B; 2 min, 25% B; 2 to 6 min, 25% B; 9 min, 50% B; 9 to 11 min, 50% B; 15 min, 95% B; and 15 to 23 min, 95% B. A flowrate of 0.5 mL/min was used and the column oven temperature set to 40°C. Peak areas were measured at wavelength 276 µm. For all flavones except for isoscutellarein 8-G, calibration mixes of 0.1, 0.5, 1, 5, 10, 25, 50, and 100 ppm were used to convert peak areas to concentrations in ppm. Chemical standards used to prepare calibration mixes were purchased in powedered form from ChemFaces (Wuhan, China) or MilliporeSigma (Massachusetts, USA), and dissolved in dimethylsulfoxide to generate stocks of 1000, 2000, or 4000 ppm. These stocks were then diluted with 50% methanol and mixed to generate calibrations mixes of the varying concentrations. With the peak areas of these calibration mixes and the molecular weight of each metabolite, flavone concentrations in µmol/g fresh weight were calculated. As a chemical standard was not purchased for isoscutellarein 8-G, only peak areas are reported.

***HPLC fractionation to isolate isoscutellarein 8-G –***

***LC-MS, MS2, and NMR to elucidate structure of isoscutellarein 8-G –***

***Apigenin feeding assay –***

***Yeast activity assay –***

***Transient expression via infiltration –***

**FIGURE LEGENDS**

**Figure 1.**

**Figure 2.**

**Figure 3.**

**Figure 4.**

**Figure 5.**

**Figure 6.**

**Figure 7.**

**Figure 8.**

**Figure 9.**

**TABLES**

**Table 1.**

**Author Contributions**

B.A., Y.D., and J.K. designed the research project; B.A., D.L., Y.S., and R.D. performed the experiments and analyzed the data; B.A., Y.D., and J.K. wrote the manuscript.

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