**Title**:

**Authors:**

**Author affiliations:**

**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 (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 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 more than 470 species, distributed nearly worldwide (Yoonkyung & Kim, 2021). 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 in the *S. baicalensis* reference genome, identified the enzyme responsible for its biosynthesis.

**RESULTS**

**DISCUSSION**

**MATERIALS AND METHODS**

**REFERENCES**