**Title:**

Bryce Askey1, Dake Liu2, Ru Dai1, Andrew Kunik1, Yeong Hun Song1, Yousong Ding2,\* and Jeongim Kim1,3,\*

1Horticultural Sciences Department, University of Florida, Gainesville, FL, 32611

2Department of Medicinal Chemistry, University of Florida, Gainesville, FL, 32610

3Plant Molecular and Cellular Biology Graduate Program, University of Florida, Gainesville, FL, 32611

**\*Corresponding Authors:** Jeongim Kim; jkim6@ufl.edu, Yousong Ding; yding@cop.ufl.edu

**ORCIDs:**

Bryce Askey: 0000-0002-4449-6891

Ru Dai: 0000-0002-2040-7049

Jeongim Kim: 0000-0002-5618-3948

**Footnotes**

**Abstract**

*Scutellaria* is a genus of plants containing multiple species with well-documented medicinal effects. *S. baicalensis* and *S. barbata* are among the most well-studied *Scutellaria* species, and previous works have established flavones to be the primary source of their bioactivity. Recent genomic and biochemical studies with *S. baicalensis* and *S. barbata* have advanced understanding of flavone biosynthesis in *Scutellaria.* However, as over several hundreds of *Scutellaria* species occur throughout the world, the biochemical properties of most are poorly understood. In this study, we analyze organ-specific flavone profiles of seven *Scutellaria*species. We found that in contrast to the flavone profiles of *S. baicalensis* and *S. barbata*, *S. racemosa* and *S. wrightii* accumulated high levels of 4'-deoxyflavones in their aerial parts. We also found most species to accumulate 4'-hydroxyflavones exclusively in their aerial parts. Our metabolomics and NMR study identified the accumulation of isoscutellarein 8-glucuronide, a rare 4'-hydroxyflavone in the stems and leaves of several *Scutellaria* species including *S. baicalensis* and *S. barbata*.Distinctive organ-specific metabolite profiles between *Scutellaria* species indicates the selectivity of biosynthetic enzymes … and differential expression … **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 biotechnology-based mass production systems for these medicinal compounds is desirable. Development of effective biotechnology for chemical production requires an understanding of the biochemistry behind the compounds of interest. In this work, we analyze biochemical properties of 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). *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).

One class of bioactive compounds which has received considerable research attention in *Scutellaria* is flavones (Karimov & Botirov, 2017, Q. Zhao et al., 2019). Most *Scutellaria* species produce two classes of flavones: 4´-hydroxyflavones and 4´-deoxyflavones. 4´-hydroxyflavones, including apigenin and its derivatives, are relatively common across the plant kingdom whereas 4´-deoxyflavones, which include chrysin and its derivatives, are relatively rare outside of *Scutellaria* with the exception of several plant species not in the genus (Kato et al., 1992; V. M. Rao et al., 2009; Y. K. Rao et al., 2002). Recent works in *S. baicalensis* and *S. barbata* have identified multiple enzymes responsible for flavone biosynthesis in *Scutellaria*, and have described the differential activity of specific enzymes towards either 4´-hydroxyflavones or 4´-deoxyflavones. This differential activity leads to the development of an organ-specific pattern of flavone accumulation in *Scutellaria* (Q. Zhao et al., 2016, 2018, 2019) (Fig. 1, Appendix S1). 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 (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). Thus, 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 this work, we aimed to expand the current knowledge of flavone diversity in *Scutellaria* by analyzing metabolite profiles of seven species, several of which were previously uncharacterized at the time of this study. During this analysis, we unexpectedly identified a 4´-hydroxyflavone which has not been included in recent biosynthetic studies of *S. baicalensis*. We elucidated the structure of this 4´-hydroxyflavone and quantified in the seven species which we analyzed previously. Our results revealed diversity in site and type of flavone accumulated across the species we selected.

**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 (1;apigenin, 2; apigenin 7-glucuronide (apigenin 7-G), 3; scutellarein, 4; scutellarin, 5; hispidulin, 6; hispiduloside) and eight 4´-deoxyflavones (7; chrysin, 8; chrysin 7-glucuronide (chrysin 7-G), 9; baicalein, 10; baicalin, 11;oroxylin A, 12; oroxyloside, 13; wogonin, 14; wogonoside) in these samples (Fig. 2, Table 1).

Based on our root-specific flavone profile results (Fig. 2C), the 4´-deoxyflavone pathway appears to be very well-conserved across all species we selected. We detected at least six unique 4´-deoxyflavones in the roots of all species we analyzed (Table 1). 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. *S. baicalensis* and *S. wrightii* stand out due to their high accumulation of 4´-deoxyflavones, and specifically, baicalin (Fig. 2C). Finally, the absence of 4´-hydroxyflavones in the roots of all but one species (*S. leonardii*) indicates their specificity to the aerial organs of the plant in most species we selected.

Although root-specific flavone profiles were relatively consistent across the selected species, aerial tissue-specific profiles were more varied (Fig. 2A, B). 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.

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 in its leaves, but little of any other 4´-deoxyflavone (Fig. 2, Table 1). Both *S. racemosa* and *S. wrightii* accumulated high concentrations of both oroxylin A and oroxyloside in their stems, and *S. racemosa* also in its leaves (Fig. 3). This finding is especially remarkable considering the relative rarity of these 4´-deoxyflavones in *S. baicalensis* and *S. barbata*, two well-studied species. (Fig. 3). Overall, our detection of chrysin in the leaves of all species analyzed and baicalein in stems and leaves of most species suggests that 4´-deoxyflavones aren’t root-specific to the same degree that 4´-hydroxyflavones are aerial tissue-specific.

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

During our metabolite 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 (Fig. 4, Table S1). In our HPLC chromatograms, we detected the peak corresponding to this metabolite in the aerial parts of *S. baicalensis* and *S. barbata*, but not in *S. racemosa* and *S. wrightii*. The peak was absent in root chromatograms collected from all seven species. The aerial specificity of the metabolite led us to hypothesize that it 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). Interestingly, LC/MS analysis revealed that its mass and mass fragmentation pattern were same as scutellarin although they were eluted at different retention times (Fig.5). We further analyzed its structure using nuclear magnetic resonance spectrometry (NMR), which confirmed it as isoscutellarein 8-glucuronide (isoscutellarin, isoscutellarein 8-G) (Fig. S2).

After confirming the identity of the unknown metabolite as isoscutellarein 8-G, we then quantified its relative abundance in all organ-specific tissue samples we had collected **(Fig. 6, Table S2)**. Isoscutellarein 8-G was accumulated only in the aerial parts of all species we analyzed, matching the pattern which we had previously observed for 4´-hydroxyflavones. *S. barbata* accumulated the greatest overall concentrations of isoscutellarein 8-G, as the flavone was relatively abundant in both its leaves and stems. *S. baicalensis*, *S. altissima*, and *S. tournefortii* also accumulated isoscutellarein 8-G in their stems. In contrast, *S. leonardii*, *S. racemosa*, and *S. wrightii* accumulated no isoscutellarein 8-G in their aerial parts.

Apigenin feeding **(Fig. 8)**

Transient expression of RTO in *S. barbata* + *S. baicalensis* + *N. benthamiana*; Yeast transformation **(Fig. 9)**

**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 (Fig 2). 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 enzyme isoforms 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 aerial parts as compared to their roots. These species included *S. racemosa* and *S. wrightii*. Also, all seven species, including *S. baicalensis*,accumulated chrysin and/or chrysin 7-glucuronnide in their leaves (Fig 2a). This suggests that the expression of 4´-deoxyflavone enzyme isoforms is not perfectly root-specific, and some enzymes having activities toward 4´-deoxyflavone precursors such as SbCLL-7 and SbCHS-2 may be active in both roots and aerial parts at least under our growth conditions. It is also possible that some fraction of 4´-deoxyflavones being synthesized in the roots are being transported to the aerial parts. The fact that 4´-hydroxyflavones were not detected in roots of most species 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.

One of the most notable species we analyzed was *S. racemosa*, which we found to accumulate high concentrations of oroxylin A, and its 7-glucuronide, oroxyloside, in its leaves. These concentrations exceeded that which we detected in any organ of all other species included in our organ-specific profiling. Oroxylin A is a 4´-deoxyflavone which has been demonstrated to exhibit memory enhancement and neuroprotective effects in rat models (Jeon et al., 2011, 2012). The most likely route for oroxylin A biosynthesis is methylation of baicalein at its 6-OH group (Elkin et al., 2018). Although previous works have identified a variety of O-methyltransferases (OMTs) in plants, OMTs with high specificity for the 6-OH group in flavonoids are rare, as the reaction is biochemically unfavorable (Zhang et al., 2016). The absence of another species in our analysis which accumulated comparable amounts of oroxylin A and oroxyloside indicates the possible evolution of a highly regioselective OMT in *S. racemosa*. Work in sweet basil (*Ocimum basilicum*), a species also in the Lamiaceae family with *Scutellaria*, identified a methyltransferase capable of specific methylation of the 6-OH group of scutellarein (Berim et al., 2012). Scutellarein is a 4´-hydroxyflavone identical in structure to baicalein apart from its 4´-OH group. To ensure the proper orientation of its substrate, and thus its regioselectivity, the *O. basilicum* OMT uses a Threonine residue to hydrogen bond with the 4´-OH group of scutellarein. However, as baicalein has no 4´-OH group, it would be impossible for a regioselective OMT in *S. racemosa* to rely on this interaction during the methylation of baicalein. Research by Zhang et al. (2016) in a liverwort species (*Plagiochasma appendiculatum*) identified a methyltransferase that is capable of regioselective methylation of the 6-OH group in baicalein. As this OMT has not yet been structurally characterized, the method by which it achieves its specificity remains unknown. The evolutionary distance separating *S. racemosa* and *P. appendiculatum* suggests an occurrence of convergent evolution, possibly indicating the physiological importance of oroxylin A and oroxyloside in these species. Future work in *S. racemosa* should be directed towards characterizing its biosynthesis of oroxylin A, with specific attention paid to the potential specialization of OMTs in the pathway. Overall, *S. racemosa* is a promising target for biotechnology improvement due to the significant bioactive effects of oroxylin A and oroxyloside.

Isoscutellarein 8-G in any plant was first detected in the liverwort species *Marchantia berteroana* (Markham & Porter, 1975). Following this initial report, Miyaichi et al. (1988; 1988) detected the flavone in the aerial parts of *S. indica* and *S. baicalensis*. Aside from these works by Miyaichi et al., few other studies have reported isoscutellarein 8-G in *Scutellaria*, though several have detected its aglycone and 7-O-glycosylated forms (Karimov & Botirov, 2017). This rarity in detection may be a result of its low abundance relative to other glycosylated flavones in *Scutellaria*. A potential reason for this low abundance is its unique glycosylation at the 8-O position. Flavone 7-O glycosylation is more common in *Scutellaria* due to the presence of a hydroxyl group at the 7-O position in all flavones synthesized via the core flavone pathway (Fig. 1). On the other hand, 8-O glycosylation first requires the activity of an 8-hydroxylase to add the free hydroxyl group to which the carbohydrate will be attached. As the purpose of glycosylation is typically to increase the stability of the flavone for long term storage (Slámová et al., 2018), it’s possible that 8-O glycosylation provides slightly greater stability as compared to 7-O glycosylation. Therefore, it would be preferrable to glycosylate isoscutellarein at the 8-O position, even though a free hydroxyl group is also present at the 7-O position. Several species may have evolved regioselective glycosyltransferase enzymes for this purpose. Researchers working with a glycosyltransferase from *Bacillus cereus* demonstrated that a single amino acid substitution could alter the primary site of quercetin glycosylation with high specificity (Chiu et al., 2016). Perhaps a similar mutation occurred in several *Scutellaria* species to allow for the biosynthesis of isoscutellarein 8-G. Alternatively, it’s possible that the glycosyltransferase enzymes of these species which accumulate isoscutellarein 8-G have less strict regioselectivity, and are capable of glycosylation at both 7-G and 8-G positions. Quantification of isoscutellarein 7-G alongside isoscutellarein 8-G would provide valuable insight regarding these theories.

Discussion of isoscutellarein 8-G biosynthesis (**Fig. 10)**

Our quantification of isoscutellarein 8-G across the seven *Scutellaria* species we analyzed revealed an intriguing pattern. Isoscutellarein 8-G was entirely absent in the species of *S. leonardii*, *S. racemosa*, and *S. wrightii*, all of which we had previously noted to accumulate high concentrations of 4´-deoxyflavones in their aerial parts. This specific example is representative of a broader pattern - species with high accumulation of 4´-deoxyflavones in their aerial parts accumulated low concentrations of 4´-hydroxyflavones. This substitution of 4´-hydroxyflavones with 4´-deoxyflavones potentially indicates an evolution to utilize 4´-deoxyflavones to fulfill the physiological roles which 4´-hydroxyflavones do in other species. Works in species outside of *Scutellaria* have demonstrated the anti-herbivory effects of several of 4´-hydroxyflavones we quantified here (Gallon et al., 2019; Sosa et al., 2004). However, little is known about the physiological role that 4´-deoxyflavones play in plants. Further research should be devoted to exploring the role of 4´-deoxyflavones in plant growth and stress response to better understand the evolutionary advantage their accumulation offers.

**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 vouchers of all species were submitted to the University of Florida Herbarium, and accession numbers are provided in Appendix S3.

***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 –***

***Transient expression via infiltration –***

***Yeast transformation and feeding –***

**FIGURE LEGENDS**

**Figure 1.** Proposed 4´-hydroxyflavone and 4´-deoxyflavone pathway. Structures of glycosylated flavones are not shown to save space but are included in Appendix S1. Enzyme names in blue are specific isoforms that have been identified in *S. baicalensis*, and enzyme names in black are general names. Flavones that were quantified have names in bold and are numbered to match the labeling of Figure 2.

**Figure 2.** Metabolite data collected from the (a) leaves, (b) stems, and (c) roots of 7 *Scutellaria* species via High Performance Liquid Chromatography (HPLC). Samples were taken in biological triplicate, and the average concentration of each metabolite calculated. Metabolites are numbered to match their order of occurrence in the flavone pathway, shown in Figure 1.

**Figure 3.** Organ-specific (a) oroxylin A and (b) oroxyloside concentrations in 7 *Scutellaria* species, as determined via High Performance Liquid Chromatography (HPLC). Concentrations were averaged from tissue samples taken from 3 biological replicates, and error bars represent standard error.

**Figure 4.** Comparison of chromatograms collected via HPLC from *S. barbata, S. baicalensis,* and *S. racemosa* stems. Time interval displayed was selected to center the unknown peak in the chromatograms.

**Figure 5.** MS/MS data collected from a scutellarin standard and the unknown metabolite.

**Figure 6.** NMR data used to elucidate structure of unknown metabolite.

**Figure 7.** Organ-specific isoscutellarein 8-glucuronide peak areas in 7 *Scutellaria* species, as determined via High Performance Liquid Chromatography (HPLC). Peak areas were averaged from tissue samples taken from 3 biological replicates, and error bars represent standard error.

**Figure 8.** Apigenin feeding data used to establish apigenin as a precursor to isoscutellarein 8-G.

**Figure 9.** Yeast transformation / infiltration data used to elucidate activity of SbRTO.

**Figure 10.** Proposed pathway for biosynthesis of isoscutellarein 8-glucuronide in *Scutellaria*.

**TABLES**

**Table 1.** Organ-specific flavone concentrations collected from 7 *Scutellaria* speces via High Performance Liquid Chromatography (HPLC). Units for all flavones are µmol / g fresh weight. Data is presented as mean ± standard error, as calculated from samples taken in biological triplicate.

**APPENDICES**

**Appendix S1.** Proposed 4´-hydroxyflavone and 4´-deoxyflavone pathway with structures of glycosylated flavones included. Enzyme names in blue are specific isoforms that have been identified in *S. baicalensis*, and enzyme names in black are general names. Flavones that were quantified have names in bold and are numbered to match the labeling of Figure 2.

**Appendix S2.** Organ-specific isoscutellarein 8-G peak areas collected from 7 *Scutellaria* species via High Performance Liquid Chromatography (HPLC). Data is presented as mean ± standard error, as calculated from samples taken in biological triplicate.

**Appendix S3.** FLAS herbarium voucher accession numbers.

**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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