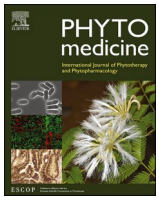
Phytomedicine 123 (2024) 155259

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed



Original Article

Unraveling spatial metabolome of the aerial and underground parts of *Scutellaria baicalensis* by matrix-assisted laser desorption/ionization mass spectrometry imaging

Peipei Zhou [a,b,1], Lihua Zuo [a,b,1], Chang Liu [c], Baolin Xiong [d], Zhuolun Li [a,b], Xiaoguang Zhou [f], Heying Yue [a,b], Qingquan Jia [a,b], Tianyuan Zheng [a,b], Jing Zou [a,b], Shuzhang Du [a,b,*], Di Chen [e,**], Zhi Sun [a,b,*]

[a] *Pharmaceutical Department, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China*
[b] *Precision Clinical Pharmacy Laboratory of Henan Province, Zhengzhou, China*
[c] *Department of Oral Emergency, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China*
[d] *School of Biomedical Engineering, Division of Life Sciences and Medicine, University of Science and Technology of China, Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Science, Suzhou, China*
[e] *School of Pharmaceutical Sciences, Zhengzhou University, Zhengzhou, China*
[f] *Intelligene Biosystems (QingDao) Co. Ltd., Shangdong Province, China*

ARTICLE INFO

Keywords:
Scutellaria baicalensis
The aerial part
The underground part
MALDI-MSI

ABSTRACT

Background: *Scutellaria baicalensis* Georgi, a traditional Chinese medicine, is clinically applied mainly as the dried root of *Scutellaria baicalensis*, and the aerial parts of *Scutellaria baicalensis*, its stems and leaves, are often consumed as "*Scutellaria baicalensis* tea" to clear heat, dry dampness, reduce fire and detoxify, while few comparative analyses of the spatial metabolome of the aerial and underground parts of *Scutellaria baicalensis* have been carried out in current research.

Methods: In this work, Matrix-assisted laser desorption ionization-mass spectrometry imaging (MALDI-MSI) was used to visualize the spatial imaging of the root, stem, and leaf of *Scutellaria baicalensis* at a high resolution of 10 μm, respectively, investigating the spatial distribution of the different secondary metabolites in the aerial and underground parts of *Scutellaria baicalensis*.

Results: In the present results, various metabolites, such as flavonoid glycosides, flavonoid metabolites, and phenolic acids, were systematically characterized in *Scutellaria baicalensis* root, stem, and leaf. Nine glycosides, 18 flavonoids, one organic acid, and four other metabolites in *Scutellaria baicalensis* root; nine glycosides, nine flavonoids, one organic acid in *Scutellaria baicalensis* stem; and seven flavonoids and seven glycosides in *Scutellaria baicalensis* leaf were visualized by MALDI-MSI. In the underground part of *Scutellaria baicalensis*, baicalein, wogonin, baicalin, wogonoside, and chrysin were widely distributed, while there was less spatial location in the aerial parts. Moreover, scutellarein, carthamidin/isocarthamidin, scutellarin, carthamidin/isocarthamidin-7-O-glucuronide had a high distribution in the aerial parts of *Scutellaria baicalensis*. In addition, the biosynthetic pathways involved in the biosynthesis of significant flavonoid metabolites in aerial and underground parts of *Scutellaria baicalensis* were successfully localized and visualized.

Conclusions: MALDI-MSI offers a favorable approach for investigating the spatial distribution and effective utilization of metabolites of *Scutellaria baicalensis*. The detailed spatial chemical information can not only improve our understanding of the biosynthesis pathways of flavonoid metabolites, but more importantly, suggest that we need to fully exert the overall medicinal value of *Scutellaria baicalensis*, strengthening the reuse and development of the resources of *Scutellaria baicalensis* aboveground parts.

Abbreviations: CHCA, α-cyano-4-hydroxycinnamic acid; 1,5-DAN, 1,5-diaminonaphthalene; 9-AA, 9-aminoacridine; DHB, 2,5-dihydroxybenzoic acid; MALDI-MSI, matrix-assisted laser desorption ionization-mass spectrometry imaging; TOF, time of flight; TIC, total ion chromatogram.

* Corresponding authors at: Pharmaceutical Department, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China.
** Corresponding author.
E-mail addresses: dszyxb@163.com (S. Du), dichen@zzu.edu.cn (D. Chen), sunzhi2013@163.com (Z. Sun).

[1] The first authors, Peipei Zhou and Lihua Zuo contributed equally to this work.

https://doi.org/10.1016/j.phymed.2023.155259
Received 13 October 2023; Received in revised form 20 November 2023; Accepted 4 December 2023
Available online 5 December 2023

Introduction

Scutellaria baicalensis Georgi, a plant belonging to the genus *Scutellaria*, was first described in " Shen Nong's Herbal Classic, and the underground root is commonly used as a traditional Chinese medicine (Liao et al., 2021). As a medicinal plant, *Scutellaria baicalensis* root has a long history in China due to its extensive biological and pharmacological activities, which is used to treat diseases such as diarrhea, dysentery, high blood pressure, bleeding, insomnia, inflammation, and respiratory infections. In modern pharmacological research, the active components in *Scutellaria baicalensis* root exhibit good antibacterial, anti-tumor, antioxidant, hypoglycemic, and liver protective effects (Zhao et al., 2019). The aerial parts, stems and leaves of *Scutellaria baicalensis*, as "*Scutellaria baicalensis tea*," have a history of nearly thousands of years. It is a folk beverage for preventing sun-stroke, clearing heat, moistening dryness, anti-inflammatory, and promoting digestion (Shen et al., 2020). However, compared with the abundant *Scutellaria baicalensis* aerial resources, the effective utilization of the aerial parts of *Scutellaria baicalensis* only accounts for a small proportion. With the comprehensive utilization of plant resources becoming the trend of green development, the value and development potential of the aerial parts of *Scutellaria baicalensis* have been paid more and more attention. What's more, modern pharmacological studies have shown that the aerial parts of *Scutellaria baicalensis* are rich in flavonoids (Yan et al., 2017), which have antiviral, antioxidant (Li et al., 2022), anti-myocardial ischemic (Cai et al., 2023) and other pharmacological effects (Gao et al., 2021; Shengkai et al., 2022), suggesting that the aerial parts of *Scutellaria baicalensis* have the potential as a new food and medicinal resource.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a soft ionization tool with significant advantages in detecting proteins, lipids, drugs, and small molecules (Hermann et al., 2020). In recent years, MALDI-MS technology has been applied in many fields (Fasih Ramandi et al., 2022; Huang et al., 2023), including the analysis of traditional Chinese medicine components and the application of medicinal plants (Qin et al., 2018). *Scutellaria baicalensis* is extremely rich in flavonoid metabolites with diverse chemical structures, which play a crucial role in maintaining plant growth and development and resisting various adversities and stresses. Traditional metabolomics aims to elucidate the overall changes and patterns of metabolites in plant tissues or cells (Villate et al., 2021). Thin-layer chromatography, high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) (Selegato et al., 2019), liquid chromatography-mass spectrometry (Artati et al., 2019), gas chromatography-mass spectrometry (Choudhury et al., 2022), and other techniques have been applied to the study of metabolic changes in plants (Matich et al., 2019), while information lacking spatial resolution is incomplete for these analyses. The synthesis and accumulation of components and metabolites in plants often have precise spatial distributions, and their physiological functions are generally closely related to their spatial distributions in tissues (Costine et al., 2022). Therefore, the accurate localization of metabolic products in tissues with high spatial resolution is of great significance for the synthesis, accumulation, and metabolism of metabolites in *Scutellaria baicalensis*. Mass spectrometry imaging technology, with its advantages of no probe labeling, non-specific detection, and one-time simultaneous imaging of hundreds of metabolite molecules, can directly capture the spatial distribution of the target metabolites in plant tissues, breaking through the shortcomings of traditional metabolomics in terms of information depth, expanding tissue ology information to two dimensions, and significantly improving our knowledge of plant tissues (Kaspar et al., 2011; Schulz et al., 2019). Compared with traditional imaging methods, MALDI-MSI has significant advantages in the field of pharmaceutical plants, which has gradually become one of the new trends in mass spectrometry.

The types and contents of active constituents of *Scutellaria baicalensis* varied considerably according to the extraction sites (Liu et al., 2011; Qiao et al., 2016). The root is the central main part of *Scutellaria baicalensis* (Cui et al., 2021), while the aerial parts, the stems and leaves, are mainly used for a folk tea drinking. Current research on *Scutellaria baicalensis* focuses more on the underground part but less on the aerial parts (Sun et al., 2020). The aerial parts of *Scutellaria baicalensis*, with a huge biomass, are not yet fully and effectively utilized. To reveal and understand the differences in metabolic mechanisms between the aerial and the underground parts of *Scutellaria baicalensis* from a more intuitive and scientific perspective, laying the foundation for the quality evaluation and secondary development of the aerial parts of *Scutellaria baicalensis*, MALDI-MSI was used not only to conduct spatial characterization of the main constituents in the underground part of *Scutellaria baicalensis,* but also to precisely locate and visually analyze the aerial parts with a spatial resolution of 10 μm. Further exploitation of the aerial resources of *Scutellaria baicalensis* is of great practical and long-term significance for the green development of traditional Chinese medicine resources.

Materials and methods

Chemicals and reagents

Acetonitrile (LC-MS grade) and methanol (LC-MS grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). The LC-MS grade's formic acid (FA) was obtained from Aladdin Industrial Co., Ltd. (Shanghai, China). Trifluoroacetic acid (TFA; LC-MS grade) was obtained from J&K Scientific Technology Co., LTD. (Beijing, China). 2,5-Dihydroxy benzoic acid (DHB), α-Cyano-4-hydroxycinnamic acid (CHCA), and 1,5-diaminonaphthalene (DAN) were acquired from TCI Development Co., Ltd (Tokyo, Japan). 9-Aminoacridine (9-AA) was purchased from Sigma-Aldrich (ST. Louis, USA). The 2-year-old *Scutellaria baicalensis* plants were collected from the *Scutellaria baicalensis Georgi* cultivation site in Yuncheng, Shanxi, China.

Sample preparation for MALDI-MSI and matrix coating

Tissue sectioning

The fresh roots, stems, and leaves were prepared and embedded in 10% gelatin (wt/vol) solutions as quickly as possible. *Scutellaria baicalensis* roots and leaves were placed in tissue cryomolds (25 × 25 × 5 mm), and stems were kept in 7 × 7 × 5 mm molds. The gelatin was poured into molds for tissue embedding and placed at −80 °C for freezing. Before tissue sectioning, roots, stems, and leaves of *Scutellaria baicalensis* were transferred to a −20 °C refrigerator to equilibrate for 10 min and then sliced into 20 μm, 30 μm, and 30 μm sections, respectively, on a cryostat microtome (Leica CM1950, Germany).

Preparation of tissue extracts

Fresh *Scutellaria baicalensis* roots, stems, and leaves were taken as 2 g, 1 g, and 1 g, crushed and placed in a 100 ml conical flask, respectively, and 70% ethanol was added to 40 ml, 20 ml, and 40 ml, respectively (the samples came from the same parts of roots, stems of the sliced tissue. The leaf samples were derived from the same plant from which the tissues were sliced), subsequently weighed and extracted by ultrasonic extraction for 1 h, then the extract was taken out and cooled down to room temperature, and weighed again to make up for the loss of weight with 70% ethanol. The supernatant was filtered by a 0.22 μm micropore filter membrane.

Evaluation of matrix candidate

To image more metabolites with stronger ion intensities, 1,5-DAN (10 mg/ml) dissolved in ACN/H_2O/FA (3.5:1.49:0.01, v/v/v), 9-AA (10 mg/ml) dissolved in methanol: water (9:1, v/v), and CHCA (10 mg/ml) dissolved in ACN/0.1 %TFA (7:3, v/v) were successively tried as MALDI matrices to measure. The dried-droplet sample preparation method was conducted as follows: extracts obtained from the roots, stems, and leaves of *Scutellaria baicalensis* were mixed with 1,5-DAN, 9-

AA, and CHCA substrate solutions at a ratio of 1:5 (v/v) respectively, and 3 μl of the mixture taken was then pipetted on the stainless steel, drying at room temperature for analysis.

Matrix coating

The solution of 1,5-DAN was finally chosen for substrate spraying. HTX TM-Sprayer™ (HTX Technologies, LLC, Carolina, USA) was used to perform matrix coating. The flow rate of the sprayer was set to 0.1 ml/min, the spray temperature at 40 °C, the pressure set to 5 psi, and the track speed was set at 1200 mm/min with a total of 6 times spraying performed.

MALDI-MSI

MALDI MSI measurements were performed using QuanIMAGE MALDI TOF mass spectrometer (Intelligene Biosystems Co. Ltd) with an Nd: YAG laser (5000 Hz, 349 nm) at the spatial resolution of 10 μm per pixel in the negative-ion mode. Ions with *m/z* values in the range of 50–1000 were acquired. The *m/z* values were calibrated using the exact adduct peaks of 1,5-DAN. Other parameters were set as follows: Pulse Frequency 3000 Hz; Detector Voltage −0.55 kv; Laser Pulse Current 2.06 Amps; Source Voltage 10 kV; Sample Rate 800 ps; Motion Scanning Speed 3.00 mm/sec; Averaged shots per spectrum 10; Laser Pulse Energy 5.5 μJ; QuanViewer (v1.0, Intelligene Biosystems Co. Ltd) software was used for MSI and tissue image reconstruction. Metabolites were identified in a combination of exact mass measurements, MS/MS experiments.

LC-MS/MS analysis for metabolite identification

The supernatants of 200 μL of *Scutellaria baicalensis* root, stem, and leaf extracts taken were separately injected into the vials for LC-MS/MS analysis. LC-MS/MS was performed by adopting a UHPLC Dionex Ultimate 3000 with a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer system (Thermo Scientific, San Jose, USA). Chromatographic peaks were separated on a Waters ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) at a flow rate of 0.20 ml/min with water containing 0.1% formic acid (v/v) (A) and acetonitrile (B). For the root extracts, the mobile phase condition is: 0.0–2.0 min, 5% B; 2.1 – 4.0 min, 5% − 15% B; 4.1 – 20.0 min, 15% − 30% B; 20.1 – 24.0 min, 30%− 100% B; 24.1 – 27.0 min, 100% B; 27.1 – 27.2 min, 100% − 5% B. The mobile phase condition for the stem and leaf extracts is: 0.0 – 2.0 min, 5% B; 2.1 – 24.0 min, 5% − 100% B; 24.1 – 27.0 min, 100% B; 27.1 – 28.0 min, 100% − 5% B. The injection volume was 5 μl for analysis. A Q-Exactive hybrid quadrupole-orbitrap mass spectrometer equipped with heat electrospray ionization (HESI) was employed. The optimized parameters of mass spectrometry were as follows: Spray voltage: +3.5 kV or −2.8 kV; Capillary temperature: 320 °C; Sheath gas flow rate (Arb): 40 in positive mode or 38 in negative mode; Full scan resolution: 70,000; dd-MS2/dd-SIM Resolution: 17,500; Scan range: 80 – 1200 *m/z*; NCE/stepped: 20, 30, 40; Aux gas pressure: 10 arb. All data collected in profile mode were acquired and processed using Thermo Xcalibur 4.0.27 software (Thermo Scientific, San Jose, USA).

Results

Characterizing endogenous molecules of Scutellaria baicalensis Georgi by MALDI TOF/MS

Primary and secondary metabolites in plant tissues are closely related to the maintenance of their growth, reproduction, nutrition, and physiological functions. Therefore, the precise localization of metabolites in plant tissues is essential for elucidating the mechanisms of metabolite synthesis and regulation. With the optimized MALDI parameters, flavonoids, flavonoid glycosides and organic acids in the underground part of *Scutellaria baicalensis* were successfully visualized at a high spatial resolution of 10 μm, and the spatial metabolic distributions of metabolites in the aboveground parts of *Scutellaria baicalensis* were also accurately localized. Different matrices were used to identify metabolites in *Scutellaria baicalensis* extracts during the method testing stage. The total ion chromatograms (TIC) of *Scutellaria baicalensis* root extract, stem extract, and leaves extract are provided in supplementary materials S1. In the results, the 1,5-DAN matrix screened more components of *Scutellaria baicalensis* compared to 9-AA and CHCA. Therefore, 1,5-DAN was reckoned as a suitable matrix for high-resolution imaging analysis of *Scutellaria baicalensis* (Fig. 1). The results of 9-AA and CHCA are shown in supplementary materials S2 and S3. It should be noted that the isomers were not distinguished by full scan MALDI MSI. The spatial distribution patterns of various secondary metabolites were visualized in this work, and the identity of these ions was further verified by LC-MS/MS (Tables S4, S5, and S6).

In *Scutellaria baicalensis* roots, in addition to the spatial analyses of baicalein (*m/z* 269.00), baicalin (*m/z* 445.05), wogonin (*m/z* 283.09), and wogonoside (*m/z* 459.11), which are the main active constituents of *Scutellaria baicalensis*, the distinctive spatial distributions of various metabolites were summarized as shown in Fig. 2. Fig. 2 displays two-dimensional ion intensity maps of flavonoid aglycones, which largely accumulate in cortex and periderm of the root, and flavonoid glycosides, highly detected in the root of *Scutellaria baicalensis*, are primarily located in xylem, pith and cortex of the root, while the organic acids exist in the root of *Scutellaria baicalensis* distributed in the periderm or scattered irregularly. Flavonoids such as chrysin (*m/z* 253.00), pinocembrin (*m/z* 255.09), tetrahydroxyflavone (*m/z* 285.07), carthamidin/isocarthamidin (*m/z* 287.09), trihydroxy-methoxyflavone (*m/z* 299.11), trihydroxy-methoxyflavanone (*m/z* 301.07), pentahydroxyflavanones (*m/z* 303.06), tetrahydroxy-methoxyflavone (*m/z* 315.05), tetrahydroxy-methoxy flavanone (*m/z* 317.06), tenaxin I or skullcapflavone (*m/z* 343.12), viscidulin III (*m/z* 345.11), trihydroxy-trimethoxyflavone (*m/z* 359.14), skullcapflavone II (*m/z* 373.12), dihydroxy-tetramethoxyflavanone (*m/z* 375.13), and seven flavonoid glycosides such as chrysin-7-O-β-D-glucuronide (*m/z* 429.10), baicalein 7-O-β-D-glucoside (*m/z* 431.09), dihydrobaicalin (*m/z* 447.07), trihydroxy-methoxyflavone-7-O-glucoside (*m/z* 461.11), trihydroxy-methoxyflavone-O-glucuronide (*m/z* 475.14), chrysin 6-C-β-D-glucoside-8-C-β-arabinoside (*m/z* 547.18) are observed in this work.

In *Scutellaria baicalensis* stems, the spatial distribution patterns of multiple flavonoids were detected, namely apigenin (*m/z* 269.02), naringenin (*m/z* 271.04), scutellarein (*m/z* 285.03), carthamidin/isocarthamidin (*m/z* 287.07), trihydroxy-methoxyflavone (*m/z* 299.08), pentahydroxyflavone (*m/z* 301.05), pentahydroxyflavanone (*m/z* 303.05), dihydroxy-dimethoxyflavone (*m/z* 313.12), and spatial distribution of 7 flavonoid glycosides, specifically, apigenin-7-O-glucuronide (*m/z* 445.07), tetrahydroxyflavone-O-glucoside (*m/z* 447.05), scutellarin (*m/z* 461.04), carthamidin/isocarthamidin-7-O-glucuronide (*m/z* 463.04), trihydroxy-methoxyflavone-7-O-glucuronide (*m/z* 475.13), pentahydroxy flavanone-7-O-glucuronide (*m/z* 479.07), schaftoside (*m/z* 563.17) were displayed.

In *Scutellaria baicalensis* leaves, we successfully achieved spatial imaging of the main flavonoids in leaf cross sections, mapping the locations of functional metabolites in *Scutellaria baicalensis* leaves, such as apigenin, scutellarein, Carthamidin, naringenin, pentahydroxyflavones, and flavonoid glycosides apigenin-7-O-glucuronide, scutellarin, pentahydroxyflavanone-7-O-glucuronide, schaftoside, chrysin 6-C-β-D-glucoside-8-C-β-arabinoside and other metabolites.

In situ metabolite profiling in Scutellaria baicalensisGeorgi root, stem, leaf by MALDI MS

To further characterize the expression of functional metabolites in different tissue micro-regions of aerial and the underground parts of *Scutellaria baicalensis*, specific mass spectra of pith, xylem, phloem, cortex and periderm of the root and stem were extracted, as well as the view analyses of the leaf cross-section based on the optical-MSI overlay

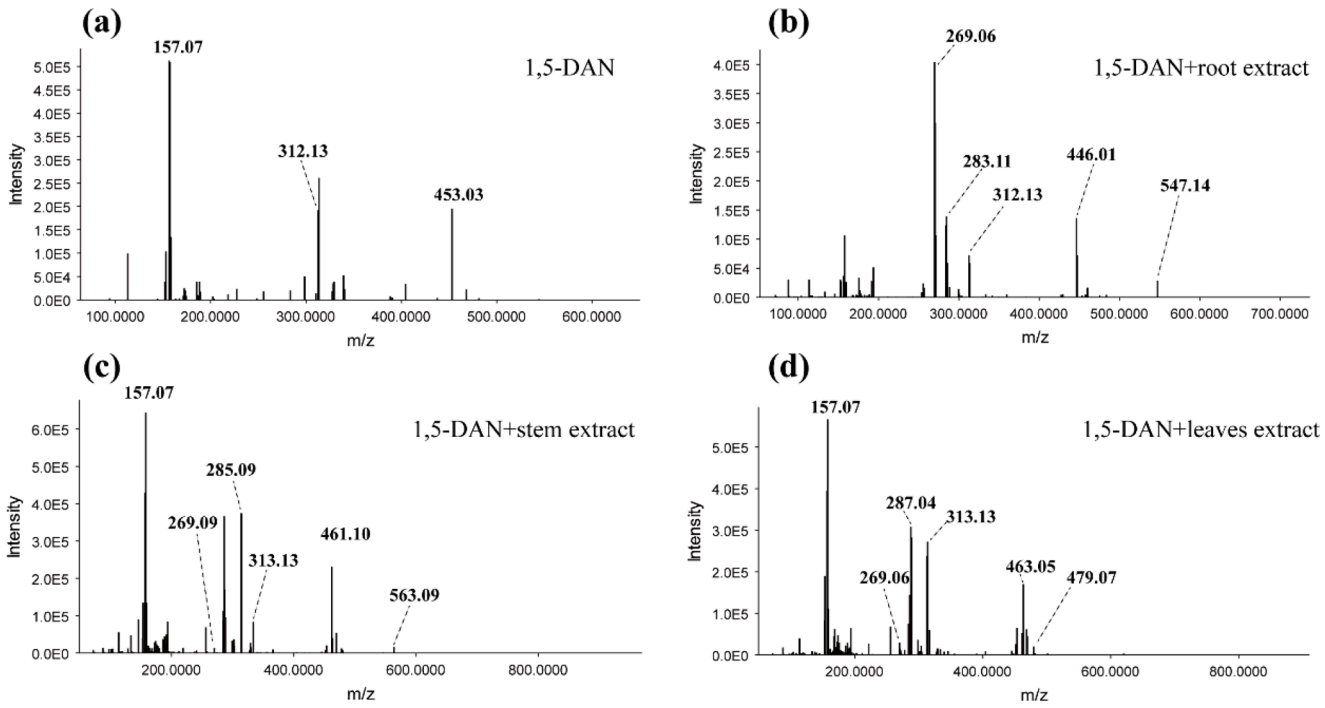


Fig. 1. MALDI-TOF MS spectra of *Scutellaria baicalensis* extracts analyzed in negative ion mode by using 1,5-DAN (a) 1,5-DAN (b) 1,5-DAN + root extract, (c) 1,5-DAN + stem extract (d) 1,5-DAN + leaves extract.

image. As shown in Fig. 2 and Fig. 3, chrysin (*m/z* 253.00) and pinocembrin (*m/z* 255.09) are widely scattered in the *Scutellaria baicalensis* roots, which is abundant in the pith and phloem. Baicalein (*m/z* 269.00) and dihydronorwogonin (*m/z* 271.04) are distributed in all parts of the roots of *Scutellaria baicalensis*, especially highly located in the pith, xylem, and phloem. Wogonin (*m/z* 283.09) found is primarily clustered in the periderm, xylem, and pith, while rarely distributed in the phloem-cambium interface. Tetrahydroxyflavones (*m/z* 285.07) are largely gathered in the pith and periderm of roots; carthamidin/isocarthamidin (*m/z* 287.09) mainly accumulates in the phloem and periderm in the roots. Trihydroxymethoxyflavones (*m/z* 299.11) are located higher in the phloem and periderm of the roots. Trihydroxy-methoxyflavanones (*m/z* 301.07), pentahydroxyflavanones (*m/z* 303.05), tetrahydroxyflavones (*m/z* 315.05), we found that the periderm contained more constituents than those in the inner portion of the root. The flavonoids' secondary metabolites viscidulin III (*m/z* 345.11), trihydroxy trimethoxyflavones (*m/z* 359.14), skullcapflavone II (*m/z* 373.12), dihydroxy-tetramethoxyflavanones (*m/z* 375.13), are mainly distributed in the periderm of the roots, and the inner portion barely contains; Flavonoid glycoside metabolites chrysin-7-O-β-D-glucuronide (*m/z* 429.10), baicalein-7-O-glucoside (*m/z* 431.09), baicalin (*m/z* 445.05), dihydrobaicalin (*m/z* 447.07), wogonoside (*m/z* 459.11), trihydroxy-methoxyflavone-7-O-β-D-glucoside (*m/z* 461.11), trihydroxy-methoxyflavone-O-glucuronide (*m/z* 475.14), chrysin 6-C-β-D-glucoside-8-C-β-arabinoside (*m/z* 547.18) distributed are more plenteous in the xylem, pith and cortex in the *Scutellaria baicalensis* roots.

The stem structure of *Scutellaria baicalensis* is periderm, cortex, phloem, xylem, and pith from outside to inside. In the results shown in Fig. 4, apigenin (*m/z* 269.02), naringenin (*m/z* 271.04), pentahydroxyflavone (*m/z* 301.05), and pentahydroxyflavanone (*m/z* 303.05) in the stem of *Scutellaria baicalensis* are largely located in phloem and periderm. The scutellarin (*m/z* 285.03) and carthamin (*m/z* 287.07) are plentiful in the stem and widely agglomerated in the xylem, phloem, and periderm. The distributions of trihydroxy-methoxyflavone7-O-glucuronide (*m/z* 475.13), pentahydroxy flavanone-7-O-glucuronide (*m/z* 479.07), and tetrahydroxyflavone-O-glucoside (*m/z* 447.05) in the stem are less abundant than those in the root, mainly accumulating in the phloem and cortex in the stem. However, scutellarin (*m/z* 461.07), isocarthamidin/carthamidin-7-O-glucuronide (*m/z* 463.04) and schaftoside (*m/z* 563.17) are plenteous in the stem of *Scutellaria baicalensis*, which are amassed in the xylem, phloem and periderm of the stem. The compounds *m/z* 671.52 and *m/z* 833.59, which may be flavonoid glycosides, are mostly distributed in the xylem and phloem of the stem, and the compound *m/z* 671.52 has a high distribution in the stem. To reveal the nature and function of these unknown compounds, further studies are needed. Since the xylem occupies a large proportion of the stem structure, the vast majority of metabolites are distributed in the xylem of the stem. Some substances, such as scutellarin, isocarthamidin/carthamidin-7-O-glucuronide, are also distributed in the phloem and periderm. However, due to the small percentage of volume, the xylem is still analyzed as the site with high levels of accumulated metabolites. The results of representative constituents in the stem of *Scutellaria baicalensis* were analyzed and enumerated below.

The basic structure of a leaf cross-section includes the upper epidermis, lower epidermis, mesophyll tissue, xylem, and phloem (Fig. 5). In this work, a high spatial resolution visual analysis was carried out on a cross-section of the *Scutellaria baicalensis* leaf. As shown in Fig. 6 and Fig. 7, apigenin (*m/z* 269.02), scutellarein (*m/z* 285.01), and naringenin (*m/z* 271.03) are mainly accumulated in the mesophyll and epidermis of the leaf, with less distribution in the xylem and phloem parts. Scutellarin (*m/z* 461.03), isocarthamidin/carthamidin-7-O-glucuronide (*m/z* 463.07), and schaftoside (*m/z* 563.11) have a high content located in various parts of the leaf, including in the mesophyll, xylem, phloem, and epidermis parts. The distribution of scutellarin in the mesophyll on both sides of the leaf is higher than that in the middle mesophyll. Apigenin-7-O-glucuronide (*m/z* 445.06) is observed in the mesophyll, and chrysin 6-C-β-D-glucoside-8-C-β-arabinoside (*m/z* 547.14) is predominantly detected in the upper epidermis of *Scutellaria baicalensis* leaves, not much plentiful. This work intuitively shows and reveals the material basis and scientific connotation of the long history of Scutellaria tea.

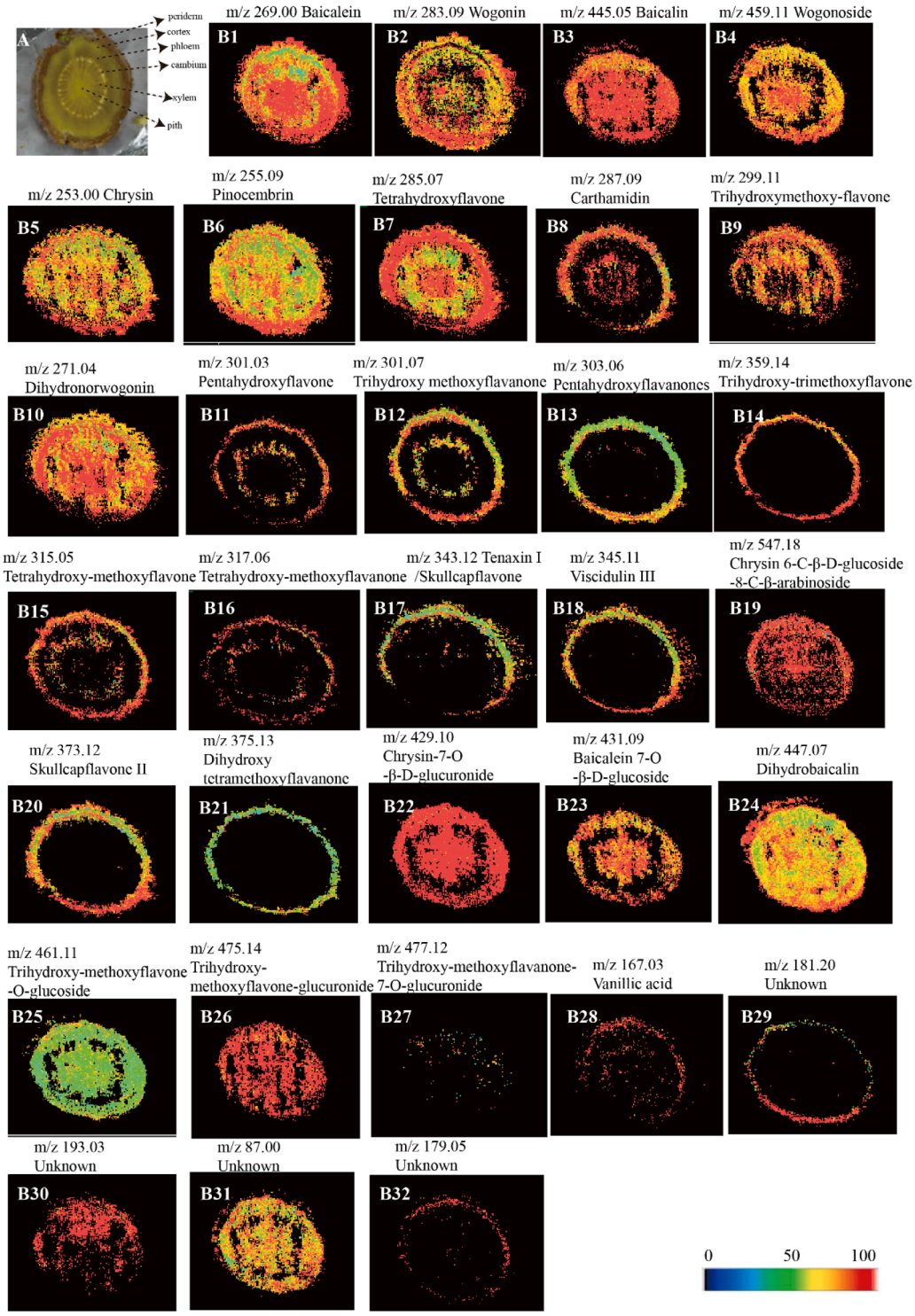


Fig 2. (A) Optical image of the root section (B) MS images of representative metabolites in *Scutellaria baicalensis* root sections. Color scales encode arbitrary ion relative strength.

Comparative analysis of the spatial distribution of other metabolites in the aerial and underground parts of Scutellaria baicalensis by MALDI MSI

Other metabolites, such as organic acids in *Scutellaria baicalensis*, are trace active components in the biosynthesis of *Scutellaria baicalensis* and play an integral role in the synthesis of major secondary metabolites and nutrient supply. The distribution of organic acid components in the aerial and underground parts of *Scutellaria baicalensis* differed. Vanillic acid (*m/z* 167.03), the compounds at *m/z* 87.00, *m/z* 179.05, *m/z* 181.20 and *m/z* 193.03 are detected in root. The compounds at *m/z* 179.05 and 181.20 observed are mainly gathered in the periderm. Vanillic acid is mainly clustered in the cortex and periderm of the root, while the compound observed at *m/z* 193.03 is distributed widely, mostly located in the cortex and periderm of the *Scutellaria baicalensis*

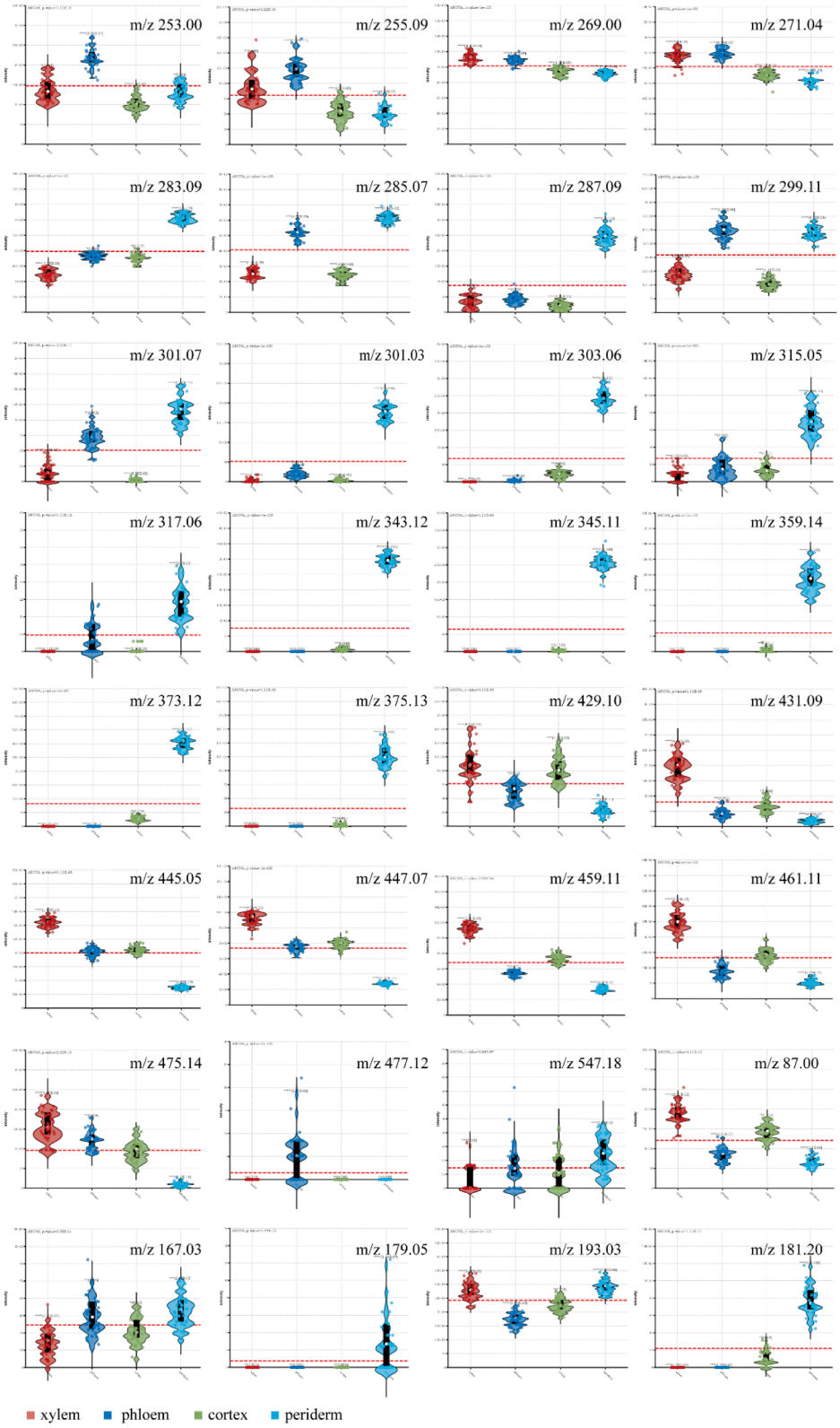


Fig. 3. The analysis of representative components in the root of *Scutellaria baicalensis* in different micro-regions of the xylem, phloem, cortex, and periderm tissues.

root. The compound at m/z 87.00 was detected situated generously in the xylem and cortex in the root, and the identification work still needs in-depth study. The compounds 4-hydroxybenzoic acid (*m/z* 137.02), the compounds at *m/z* 146.04 and 193.03 are observed in the stem of *Scutellaria baicalensis*. Among them, 4-hydroxybenzoic acid and the compound at *m/z* 193.03 are found to be highly distributed in the xylem and periderm of the stem; the compound at *m/z* 146.04 is gathered chiefly in the xylem. In root, the distribution of organic acids is mainly synthesized in the periderm, for organic acids analogs play an essential protective role in plant resistance to pathogen attack. Therefore, it may be assigned to these protective functions in the periderm.

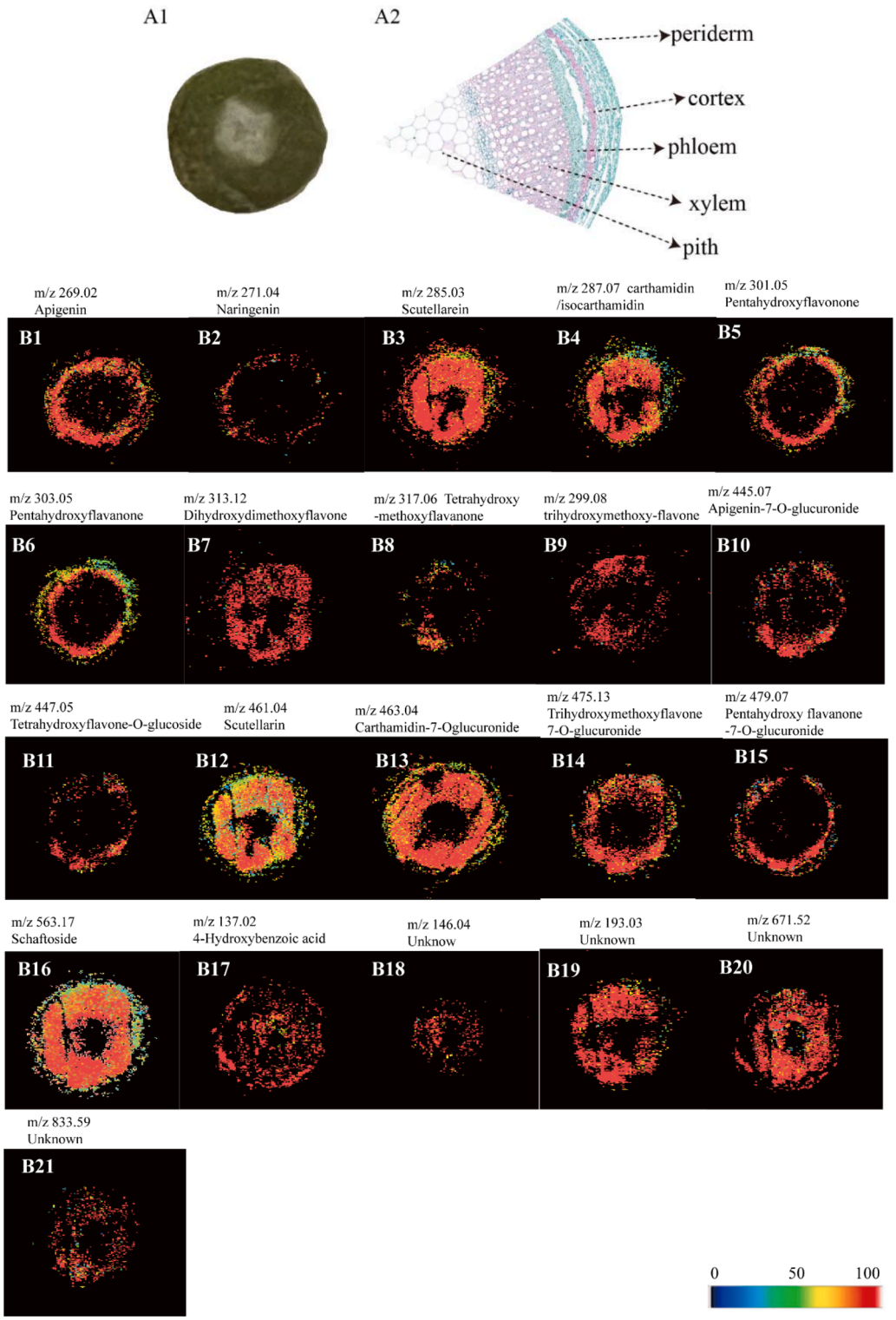


Fig. 4. (A) Optical image of the stem section and the structure diagram of *Scutellaria baicalensis* stem (B) MS images of representative metabolites in *Scutellaria baicalensis* stem sections. Color scales encode arbitrary ion relative strength; (C) The analysis of representative components in the stem of *Scutellaria baicalensis* in different micro-regions of the pith, xylem and phloem, and periderm tissues.

Metabolic biology of Scutellaria baicalensis

There are two flavonoid synthesis pathways in *Scutellaria baicalensis*: the aerial and underground parts. Scutellarein and scutellarin were synthesized mainly from the aerial stems and leaves, the classic flavonoid synthesis pathway (Liu et al., 2021). The underground part, the root of *Scutellaria baicalensis*, mainly synthesizes baicalein, wogonin, baicalin, and wogonoside. At present, the flavonoid synthesis pathway of the underground part is only found in *Scutellaria baicalensis*, which is a unique root flavonoid synthesis pathway (Xu et al., 2020). Based on

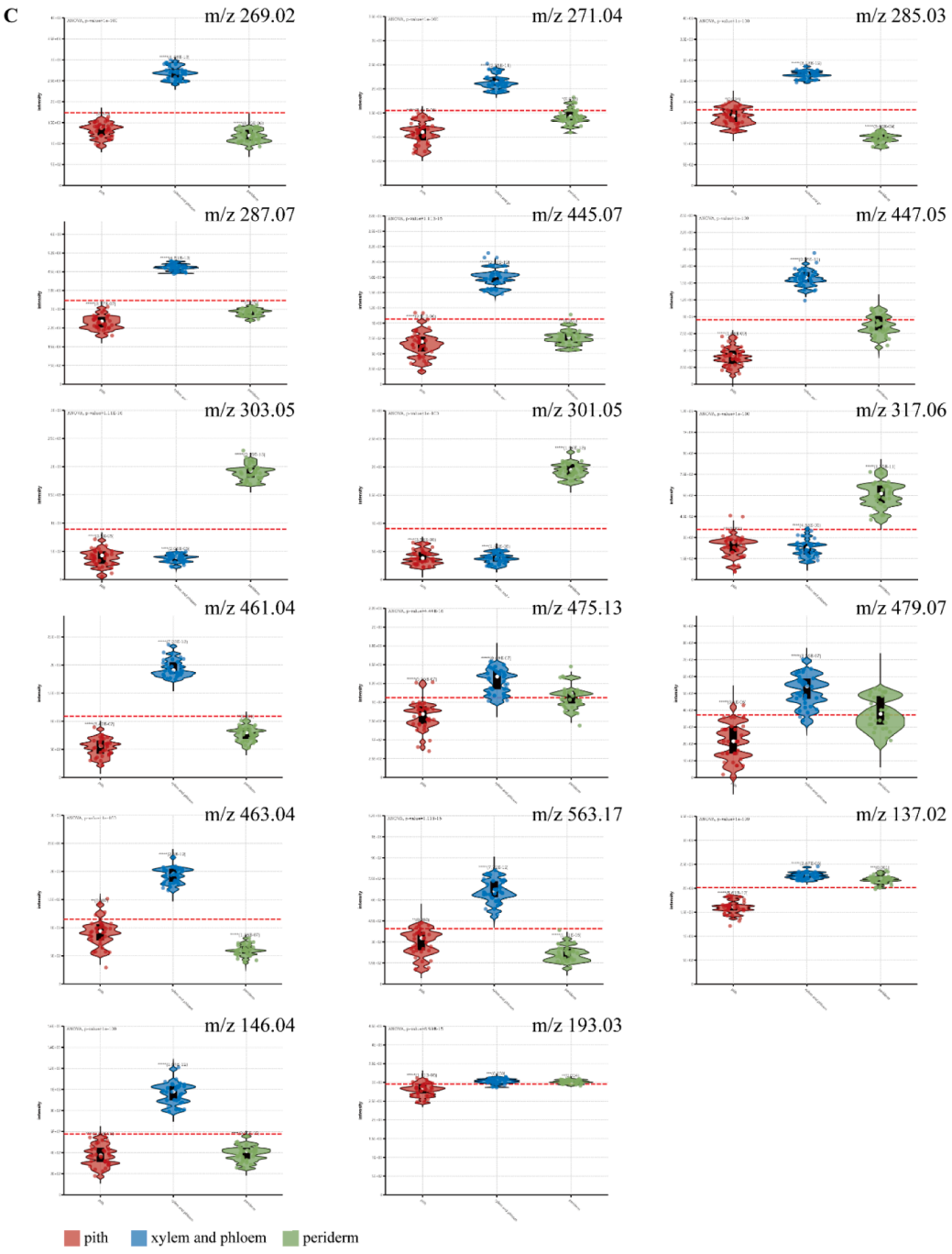


Fig. 4. (*continued*).

mass spectrometry imaging, this study visually analyzed the different biological metabolic pathways of *Scutellaria baicalensis* flavonoids in aboveground and underground anabolism.

The first step in the synthesis of flavonoids in *Scutellaria baicalensis* is through the phenylpropane pathway, where phenylalanine is catalyzed by alanine ammonia lyase (PAL) to form cinnamic acid. It is worth noting that after this step, the biosynthesis of flavonoids in aerial and underground parts of *Scutellaria baicalensis* produces different compounds in the presence of different enzymes. The cinnamic acid formed in the classical flavonoid synthesis pathway in aerial parts of *Scutellaria baicalensis* produces p-coumaric acid by the action of cinnamic acid hydroxylase, and p-coumaric acid builds naringenin chalcone by the enzyme p-coumaroyl coenzyme A (p-coumaroyl CoA), which in turn generates naringenin (Vogt, 2010). Naringin is catalyzed by flavone synthase II (SbFNSII-1) to produce apigenin and finally scutellarin. In aerial parts of *Scutellaria baicalensis*, apigenin and scutellarein are highly distributed in stems and leaves, which in turn generate abundant scutellarin and carthamidin/isocarthamidin-7-O-glucuronide, the main active constituents of the aerial parts of *Scutellaria baicalensis*.

The cinnamic acid produced in the specific flavonoid synthesis pathway in roots is catalyzed by cinnamic acid coenzyme A ligase (SbCLL-7) to form cinnamoyl CoA (Zhao et al., 2016). With pinocembrin-chalcone synthase, cinnamoyl CoA generates pinocembrin chalcone, which in turn produces pinocembrin. Pinocembrin is catalyzed by flavonoid synthase (SbFNSII-2) to generate chrysin, which further produces baicalein or norwogonin, and norwogonin eventually makes wogonin. In the meantime, baicalein and wogonin were catalyzed by the flavonoid 7-O-glucuronosyltransferase (UBGAT) to produce

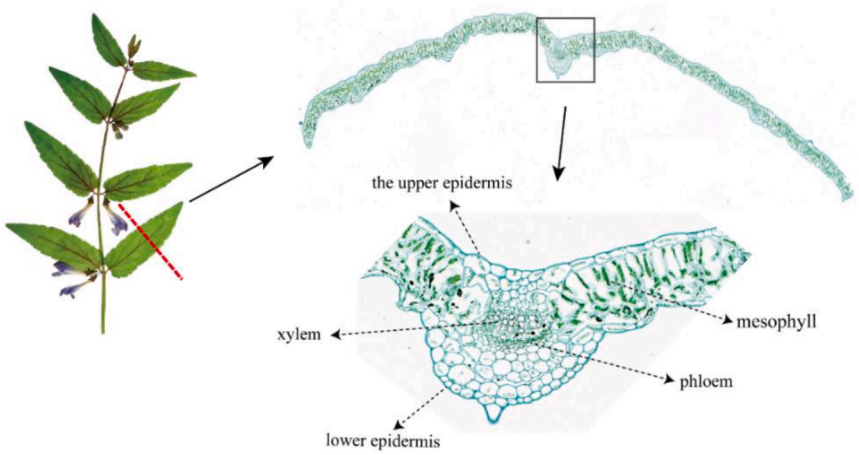


Fig. 5. Safranin O-Fast Green Staining of a cross-section of *Scutellaria baicalensis* leaf and a close-up view of the upper epidermis, lower epidermis and mesophyll tissue.

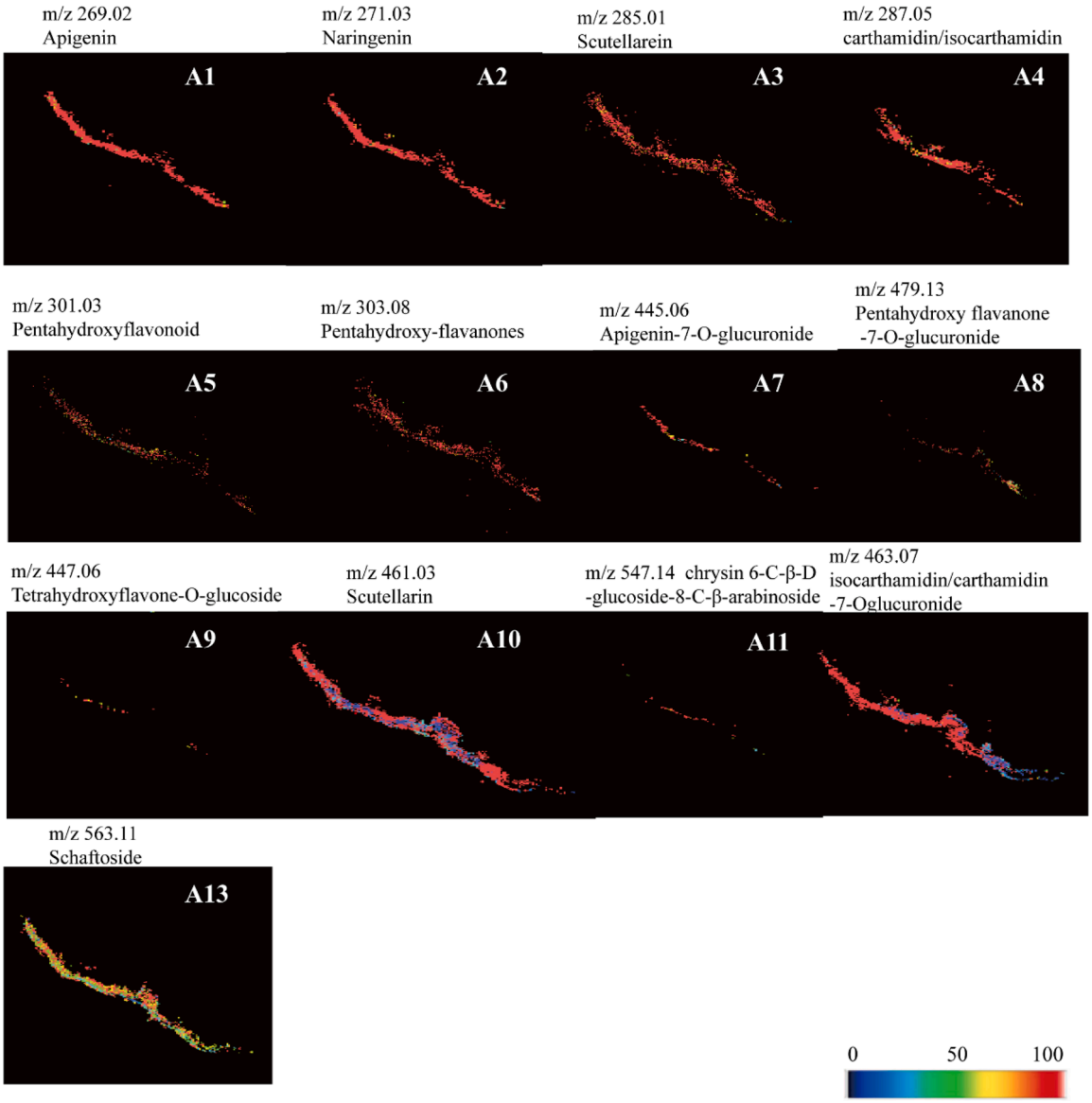


Fig. 6. MS images of representative metabolites in *Scutellaria baicalensis* leaf sections. Color scales encode arbitrary ion relative strength.

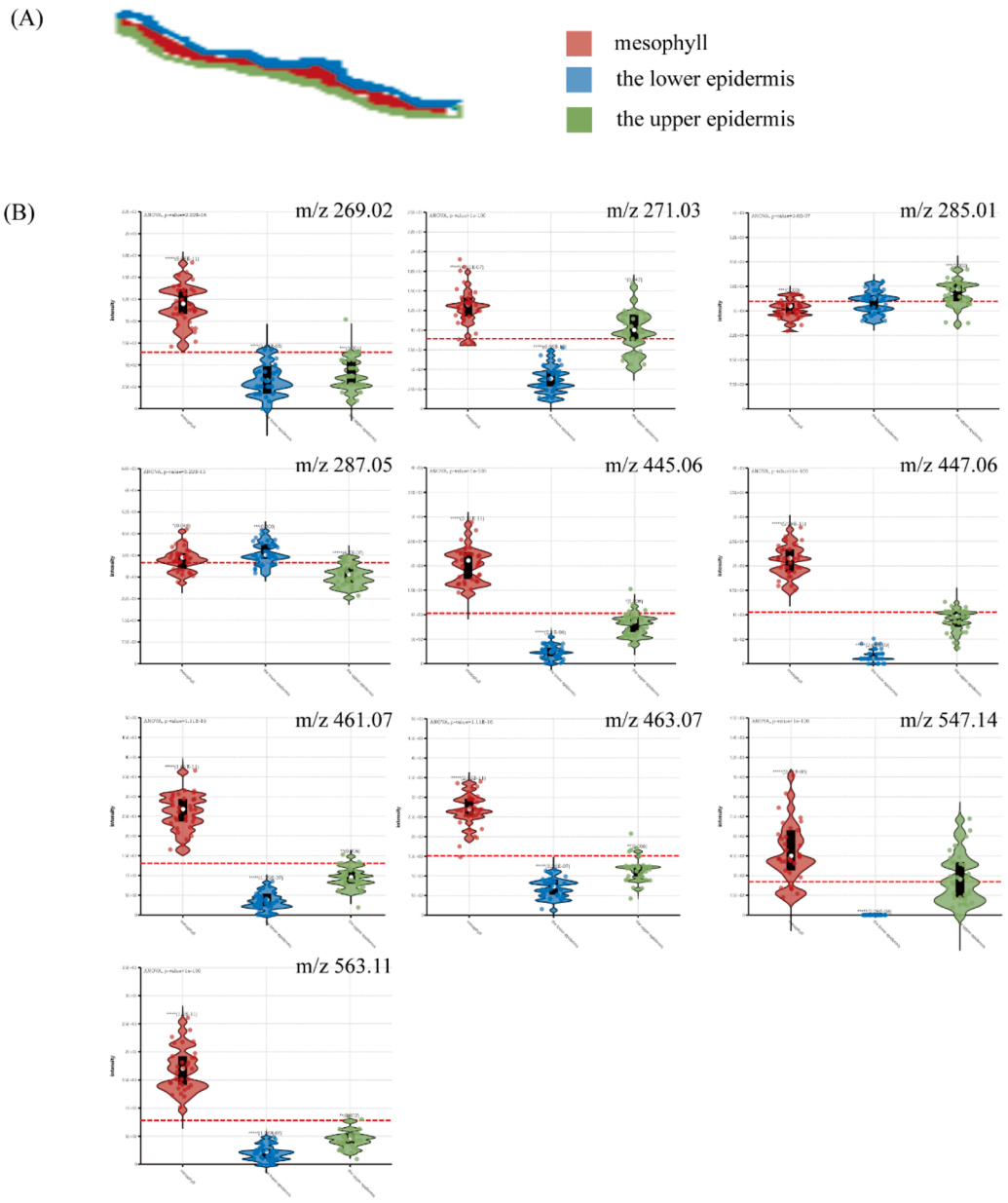


Fig. 7. (A) Division of tissue area in cross-section of *Scutellaria baicalensis* leaf; (B) The analysis of representative components of *Scutellaria baicalensis* leaf in different tissue compartments, mesophyll and epidermis.

baicalin and wogonoside. In *Scutellaria baicalensis* root, chrysin and pinocembrin are accumulated mainly in the root, especially in the xylem and pith, generating high levels of baicalein and wogonin, leading to the eventual synthesis of abundant baicalin and wogonoside. The regional distribution of the key components of the flavonoid synthesis pathway is graphically and intuitively shown in Fig. 8.

Discussion

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, as a soft ionization mass spectrometry technique, has been widely used in the analysis of macromolecules such as proteins, peptides, and nucleic acids. Due to the matrix-related peak interferences and poor homogeneity of matrix/analyte co-crystallization, detecting the metabolites in low molecular weight (LMW) region using MALDI MS is still a challenging task. Therefore, selecting a suitable matrix is crucial for low molecular weight metabolite mass spectrometry imaging performed by MALDI-MSI. Flavonoids are the main active components in the aerial and underground parts of *Scutellaria baicalensis*. Since most of the flavonoids have conjugated groups, which have a better ionic response in the negative ion mode, three matrices, 1,5-DAN, 9-AA, and CHCA, were used to detect and analyze the active constituents of *Scutellaria baicalensis* in the negative ion mode respectively. As shown in Fig. 1, more and higher ion signal intensities were observed when 1,5-DAN was used as a substrate compared with CHCA and 9-AA, and then 1,5-DAN was finally chosen as a substrate for spatial metabonomics imaging of *Scutellaria baicalensis* in this work.

One challenge we confronted was preparing high-quality cryosections of plant tissues for subsequent MSI measurements, particularly the preparation of tissue sections of *Scutellaria baicalensis* stems and leaves. Owing to the unique structural composition of plant tissues, such as cell walls, vesicles, chloroplasts, phloem, xylem, and other plant-specific cellular structures, the optimal cutting temperature compound (OTC) embedding methods applicable to the routine preparation of animal samples are not well suited for the preparation of plant tissue samples, especially for fragile and thin-layered tissues such as leaves. At

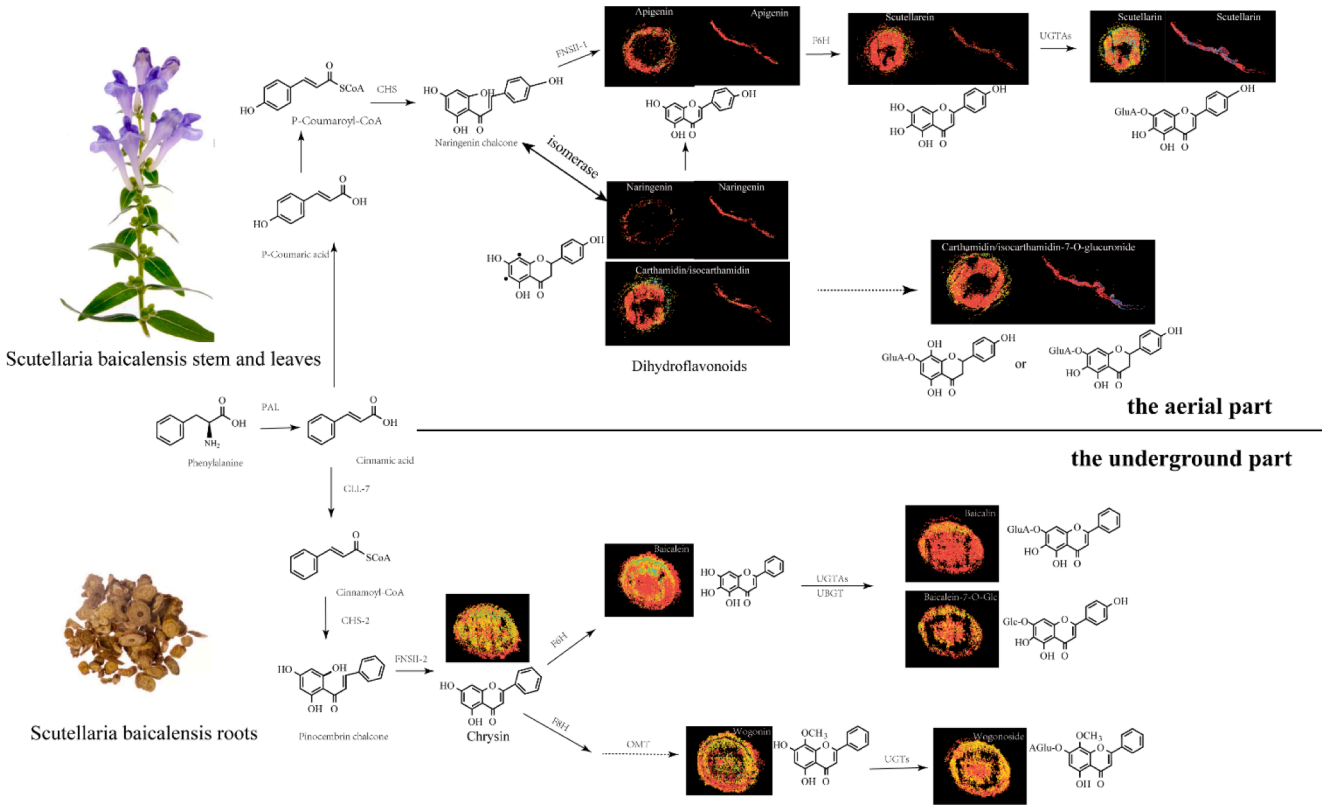


Fig. 8. Two flavonoid biosynthetic synthesis pathways of *Scutellaria baicalensis* in aerial and underground parts.

the same time, the presence of a waxy layer on the surface of plant tissues often leads to the low ionization efficiency of the molecules to be measured, increasing the difficulty of instrument detection. In this work, 10% gelatin was used for embedding and fixing the tissues, which enhanced the adhesion of the plant tissues on indium tin oxide (ITO) coated slides and maximized the protection of the original morphology of the plant tissues. With the suitable tissue section thickness and appropriate MSI instrument parameters, we successfully realized the spatial imaging distribution of endogenous metabolites in the root, stem, and leaf of *Scutellaria baicalensis*.

Combined with optical image and mass spectrometry imaging analysis in this work, the results showed that the content of secondary metabolites of *Scutellaria baicalensis* was closely related to its structural characteristics. The distributions of flavonoid glycosides in *Scutellaria baicalensis* are relevant to the thin-walled cells of vascular tissue in nutrient organs. They are mostly gathered in the vascular tissue and accumulated therein, which is the main tissue responsible for plant material transport. Flavonoid aglycones are mostly distributed in the cortex or pericarp in the rhizomes, which may be an adaptation of *Scutellaria baicalensis* growth and development to environmental stresses. Scutellarein, carthamidin/isocarthamidin, scutellarin, carthamidin/isocarthamidin-7-O-glucuronide and schaftoside accumulate in the mesophyll and epidermis of the leaves. The differences between aboveground and underground flavonoid metabolites in *Scutellaria baicalensis* are caused by two different synthetic pathways of aboveground and aboveground flavonoids. In conclusion, the distribution and accumulation of its secondary metabolites are closely related to the structure of *Scutellaria baicalensis.*

The difference in substance composition leads to the difference in pharmacological properties between the aboveground and underground parts of *Scutellaria baicalensis*. As a traditional medicinal part, the root is cold and bitter in nature. It has the effects of clearing heat and drying dampness, reducing fire and detoxification, and stopping bleeding to tranquilize the foetus, which is clinically efficacious in the treatment of real-heat syndromes (Fang et al., 2023). It is commonly used as a combination of proprietary Chinese medicine for the treatment of various diseases. A growing number of recent pharmacological studies have revealed that *Scutellaria baicalensis* root extracts possess antitumor activity, and the pharmacological material basis for its antitumor action is mainly reported to be the constituents of baicalein, wogonin, baicalin and wogonoside. The compounds related to the antipyretic and anti-inflammatory mechanism of *Scutellaria baicalensis* root are likewise mainly baicalein, wogonin, baicalin, and wogonoside, as well as other compounds, e.g., chrysin, which also has anti-inflammatory effects. *Scutellaria baicalensis* has a bitter flavor, and some *in vitro* experiments have confirmed that baicalin has significant inhibitory and killing effects on trichomonas vaginalis. *Scutellaria baicalensis*' foetus-restoring effects are closely connected to its anti-inflammatory, antiviral, immune-enhancing, and uterine contraction-suppressing effects. Baicalein and baicalin from *Scutellaria baicalensis* root affect both embryo adhesion and implantation preprocess (Wen et al., 2020). It is suggested that the efficacy of *Scutellaria baicalensis* is closely related to the high content of baicalein, wogonin, baicalin, and wogonoside in its root.

The aerial parts are also effective in clearing fire and reducing inflammation. According to the results of this work, the distributions of baicalein, wogonin, baicalin, and wogonoside in aerial parts of *Scutellaria baicalensis* are not much more abundant than those in the underground parts, and the drug properties of bitter and cold may be weakened compared with the root. However, the aerial parts of *Scutellaria baicalensis* contain relatively high contents of scutellarein, carthamidin/isocarthamidin, scutellarin, carthamidin/isocarthamidin-7-O-glucuronide, which explains the heat-removing and detoxifying effects of *Scutellaria baicalensis* tea. Pharmacological studies on the aerial parts of *Scutellaria baicalensis* mainly focus on the analysis of total flavonoids in the stem and leaf, and the aerial parts as the tea drink have effects such as clearing heat and drying dampness, laxative and detoxification. Additionally, the tea is found to have the impact of promoting digestion, improving intestinal flora, lowering blood glucose and blood lipids, and

preserving the liver and cardio-cerebral vascular protection (Zhao et al., 2019). Meanwhile, some studies have found that *Scutellaria baicalensis* stem and leaf extracts can enhance memory and improve learning in animals with Alzheimer's disease (Shengkai and Yazhen, 2022). In the current studies on the components of the total flavonoids in stems and leaves of *Scutellaria baicalensis*, scutellarin has been studied more, which has a good effect on protecting endothelial cells, antiplatelet, and antithrombotic to prevent cardiovascular and cerebrovascular diseases. Schaftoside also exhibits a good cerebral neuroprotective effect (Zhou et al., 2019). From the perspective of spatial imaging, this study intuitively illustrates the differences between the aerial and underground parts of *Scutellaria baicalensis* due to the differences in material basis, which determines the differences in their application.

Although several researches have been conducted to investigate the biosynthetic pathways and key metabolic synthases in aerial and underground parts of *Scutellaria baicalensis*, the information on the overall spatial distribution of intermediates in two different flavonoid biosynthetic pathways in the aerial and underground parts of *Scutellaria baicalensis* has not yet been reported. In this work, we demonstrated that MALDI MSI can be used to visualize the accumulation sites of primary intermediates involved in the aerial and underground flavonoid biosynthesis pathways. From Fig. 8, it can be concluded that Naringenin and chrysin are the key metabolites in the aerial and underground parts of *Scutellaria baicalensis*, respectively. Naringenin is converted into abundant scutellarein and scutellarin through a series of biosynthetic pathways in the stems and leaves of *Scutellaria baicalensis*. Meanwhile, the underground part contains a rich amount of chrysin, which, under the action of various enzymes, generates a large number of 4′-deoxy flavonoids, including baicalein, wogonin, baicalin and wogonoside that are specific to *Scutellaria baicalensis*; The difference of active components in aerial and underground parts of *Scutellaria baicalensis* is related to the different expression of intermediate metabolites in the flavonoid synthesis pathway of *Scutellaria baicalensis*.

Flavonoids are the characteristic components of *Scutellaria baicalensis*, and there are apparent differences in the types and contents of flavonoids between the aerial and underground parts of *Scutellaria baicalensis*. Due to the extensive research on the underground part, we mainly focus on the aerial parts of *Scutellaria baicalensis* in this discussion. The aerial parts, stems, and leaves of *Scutellaria baicalensis* are the source of *Scutellaria baicalensis* tea, which contains various characteristic components, including scutellarein, carthamidin/isocarthamidin, scutellarin, and carthamidin/isocarthamidin-7-O-glucuronide. Among them, the component scutellarin is clinically used in the treatment of cardiovascular and cerebrovascular diseases, and the scutellarin products currently in clinical use mostly come from the plant Erigeron breviscapus. In this work, the results of spatial imaging at a high spatial resolution showed that the aerial parts of *Scutellaria baicalensis* contain a large amount of scutellarin. In addition, the aerial parts are relatively large and rich in resources, and only a small portion of the aerial resources of *Scutellaria baicalensis* are used as drinking tea in daily life, resulting in multitudinous *Scutellaria baicalensis* resources are not fully utilized. With the trend of green development of medicine resources, the aerial parts of *Scutellaria baicalensis* are expected to become an important plant source for the clinical application of scutellarin.

Conclusions

In this work, an effective MALDI-MSI method was developed to visualize the spatial imaging of secondary metabolites in aerial and underground parts of *Scutellaria baicalensis*. The spatial distribution of various metabolites, flavonoid glycosides, flavonoid metabolites, and phenolic acids in the root, stem, and leaf of *Scutellaria baicalensis* were clearly mapped. Based on spatial imaging analysis methods, the spatial distribution of key metabolites in two pathways of aerial and underground flavonoid biosynthesis in *Scutellaria baicalensis* was elucidated for the first time. Compared with the underground part of *Scutellaria baicalensis*, the spatial distributions of scutellarin and carthamidin/isocarthamidin-7-O-glucuronide are rich in the stems and leaves of *Scutellaria baicalensis,* which has a high medicinal value. The research facilitates us to further understand the structural development process of *Scutellaria baicalensis* and the accumulation law of flavonoids, providing a scientific basis for the aboveground application of *Scutellaria baicalensis* and the comprehensive utilization and development of *Scutellaria* plant resources.

CRediT authorship contribution statement

Peipei Zhou: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft. **Lihua Zuo:** Validation, Investigation. **Chang Liu:** Investigation, Formal analysis. **Baolin Xiong:** Data curation, Software. **Zhuolun Li:** Formal analysis. **Xiaoguang Zhou:** Resources. **Heying Yue:** Validation. **Qingquan Jia:** Formal analysis. **Tianyuan Zheng:** Validation. **Jing Zou:** Validation. **Shuzhang Du:** Writing – review & editing, Funding acquisition, Supervision. **Di Chen:** Writing – review & editing, Funding acquisition, Supervision. **Zhi Sun:** Supervision, Project administration.

Declaration of Competing Interest

No potential conflict of interest was reported by the authors.

Acknowledgments

This work was financially supported by Henan Provincial Science and Technology Research Project [Grant No. 232102311200] and the China Postdoctoral Science Foundation [Grant No. 2021M702937].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2023.155259.

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