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Cell type-specific qualitative and quantitative analysis of saikosaponins in three  
Bupleurum species using laser microdissection and liquid chromatography–quadrupole/  
time of flight-mass spectrometry

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1 **Title: Cell type-specific qualitative and quantitative analysis of saikosaponins in three**  
2 ***Bupleurum* species using laser microdissection and liquid chromatography–**  
3 **quadrupole/time of flight-mass spectrometry**

4  
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19 **ABSTRACT:** Cell type-specific metabolite analysis is a promising method for understanding  
20 plant metabolite production, function, transport and storage. In the present study, laser  
21 microdissection (LMD) and ultra-high performance liquid chromatography quadrupole/time of  
22 flight-mass spectrometry are combined to determine where secondary metabolites are  
23 accumulated in the roots of *Bupleurum scorzonerifolium* Willd, *B. chinense* DC. and *B. falcatum*  
24 L.. Four tissues, namely cork, cortex, phloem and xylem, were microdissected by laser  
25 microdissection, and their chemical profiles were analyzed. The main metabolites are  
26 saikosaponins. Different tissues contained different saikosaponins. Generally, the cork and  
27 cortex from all three species contained more types of saikosaponins and higher contents of  
28 saikosaponins a, c and d than did the phloem and xylem. Interestingly, in the roots of *B.*  
29 *scorzonerifolium* and *B. falcatum*, the cork contained much higher contents of saikosaponins a, c  
30 and d than did the cortex; while in the root of *B. chinense*, the cortex contained higher contents  
31 of saikosaponins a, c and d than the cork. Explanation and application of the results are discussed.  
32 The present findings yield valuable insights into the quality evaluation of Bupleuri Radix by  
33 morphological features.

34 *Keywords:* Bupleurum, Tissue specific, Saikosaponin, Metabolite profiling, Laser  
35 microdissection, UHPLC-QTOF-MS

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## 40 1. Introduction

41 Plants produce a high diversity of secondary metabolites, and many of these have been used  
42 by mankind for thousands of years as therapeutic agents. These compounds are synthesized in a  
43 tissue-, organ- and developmental-specific way by specific biosynthesis enzymes, and are then  
44 stored, sometimes in high concentrations in the plants producing them. If sites of synthesis are  
45 not the sites of storage, then long-distance transport occurs-- by xylem, phloem, or apoplast [1,  
46 2]. Analyzing single-cell or tissue chemical profiling is a promising method for understanding  
47 secondary metabolite production, function, transport and storage in plants.

48 To detect the metabolites in single cells or tissues, various techniques have been developed  
49 [3-6]. Among these techniques, laser microdissection (LMD) can isolate specific single cells,  
50 while mass spectrometry-based methods are the most straightforward way to identify metabolites  
51 in a single cell or tissue. The method combining LMD and ultra-high performance liquid  
52 chromatography quadrupole/time of flight-mass spectrometry (UHPLC-QTOF-MS) can achieve  
53 both qualitative and quantitative analysis of metabolites in a single cell or tissue [7, 8]. However,  
54 the method has not been applied to analyze the tissue-specific chemical profiles in different  
55 species from the same genus.

56 Currently, the quality evaluation of herbal medicines mostly depends on analytical  
57 techniques with modern instruments for determination the contents of marker compounds.  
58 However, when the public goes to buy herbal medicines in the retail shops, it is not possible for  
59 them to evaluate the quality by modern instruments. In practice, characteristics of herbal  
60 medicines such as shape, size, color, texture, and cross-section are usually used to evaluate their  
61 quality. But the morphological investigation for evaluating the quality of herbal medicines lacks

62 objective data and hasn't been validated by modern analytical methods. Bupleuri Radix is a  
63 commonly used herbal medicine for the treatment of fevers and colds, malaria, cholecystitis,  
64 hepatitis, pancreatitis, and menstrual disorders in China. According to the Chinese  
65 Pharmacopoeia (2010 edition), Radix Bupleuri is derived from the dried roots of *Bupleurum*  
66 *scorzonerifolium* Willd or *B. chinense* DC. [9]. In the Japanese Pharmacopoeia (16<sup>th</sup> edition), the  
67 official botanical origin of Bupleuri Radix (pronounced "saiko" in Japanese) is the roots of *B.*  
68 *falcatum* L. [10]. In the traditional morphological descriptions used for quality evaluation of  
69 Bupleuri Radix, samples of larger sizes are considered to have higher quality for medicinal use.  
70 However, how the pharmaceutical quality being linked to the sizes is not known.

71 The bioactive components of herbal medicines generally belong to the category of plant  
72 secondary metabolites. For Bupleuri Radix, saponins, especially saikosaponins a and d have been  
73 demonstrated to be responsible for its main pharmacological effects [11]. To date, more than 100  
74 saikosaponins have been isolated and elucidated from *Bupleurum* species [12]. A previous  
75 histochemical study on the species of *B. chinense* has demonstrated that saikosaponins are  
76 mainly distributed in the pericycle and primary phloem of young roots but in the vascular  
77 cambium and secondary phloem of mature roots [13]. Another similar study of *B. chinense* also  
78 indicated that saikosaponins were abundant in the cortex outside the cambium but rare in the  
79 xylem of the root [14]. The two reports localized the position of saikosaponins using 5%  
80 vanillin-glacial acetic acid-perchloric acid solution and 99% ethanol-sulphuric acid solution, for  
81 the cortex and xylem, respectively. However, these studies couldn't definitively elucidate the  
82 chemical profiles of various tissues. Moreover, no comparative investigation of the various  
83 specific tissues of the three official *Bupleurum* species has been carried out. In the present study,  
84 the combination of LMD and UHPLC-QTOF-MS was used for analyzing the chemical profiles

85 of cork, cortex, phloem and xylem from the roots of *B. scorzonerifolium*, *B. chinense* and *B.*  
86 *falcatum*. The study reveals where different saikosaponins accumulate in these three *Bupleurum*  
87 species. This information is directly relevant to the quality evaluation of Bupleuri Radix by  
88 morphological features.

89

## 90 **2. Experimental**

91

### 92 *2.1. Plant materials*

93

94 Six batches of the roots of *B. scorzonerifolium*, *B. chinense* and *B. falcatum* were collected.  
95 Details of the samples are shown in Table 1. The diameter of roots for tissue preparation was  
96 about 0.7 cm. All the herbal samples were authenticated by Dr. Liang Zhitao from the School of  
97 Chinese Medicine, Hong Kong Baptist University and deposited in the Bank of China (Hong  
98 Kong) Chinese Medicines Centre of Hong Kong Baptist University.

99

### 100 *2.2. Chemicals and reagents*

101

102 Chemical markers of saikosaponins a, c and d were isolated by the author [15]. Ginsenoside  
103 Rb<sub>1</sub> (GS) was purchased from the National Institute for the Control of Pharmaceutical and  
104 Biological Products (Beijing, People's Republic of China). The purity of each chemical was  
105 above 98%, as determined by high performance liquid chromatography (HPLC) analysis. The

106 solvents, acetonitrile and methanol, were of HPLC grade from E. Merck (Darmstadt, Germany).  
107 Formic acid with a purity of 96% was also of HPLC grade (Tedia, U.S.A.). Water was obtained  
108 from a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

109

### 110 *2.3. Preparation of tissue and sample solutions*

111

112 After softening by wrapping with water-soaked non-cellulose paper, the roots were cut into  
113 small sections, embedded in cryomatrix<sup>TM</sup> (Thermo Shandon Limited, U.K.), and then placed on  
114 a cutting platform in the cryobar of a cryostat (Thermo Shandon As620 Cryotome, U.K.) at -20  
115 °C. Serial slices of 40  $\mu\text{m}$  in thickness were cut at -10 °C. Each sectioned tissue slice was

116 mounted directly to a non-fluorescent PET microscope steel frame slide (76 mm  $\times$  26 mm, 1.4  
117  $\mu\text{m}$  thick, Leica Microsystems, Germany). The slide was observed with a Leica LMD 7000  
118 microscope system (Leica, Bensheim, Germany) in fluorescence mode with a dichromatic mirror.  
119 Microdissection was conducted by a DPSS laser beam at 349 nm wavelength, aperture of 10,  
120 speed of 12 and power of 50-60  $\mu\text{J}$  under a Leica LMD-BGR fluorescence filter system at 10X  
121 magnification. Tissue parts within an area of approximately  $1 \times 10^6 \mu\text{m}^2$  were determined as the  
122 investigated size and dissected separately under fluorescence inspection mode. The



123 microdissected tissues fell into caps of 500  $\mu\text{L}$  microcentrifuge tubes (Leica, Germany) by  
124 gravity.

125 The separated tissue part in each cap was transferred to the bottom of the tube through  
126 centrifugation (Centrifuge 5415R, Eppendorf, Hamburg, Germany) at 10,000 rpm for 5 minutes.  
127 100  $\mu\text{L}$  methanol was added into each microcentrifuge tube. The tube was sonicated for 30 min  
128 (CREST 1875HTAG ultrasonic processor, USA). The microcentrifuge tube was centrifuged again  
129 for 10 min at 10,000 rpm, and 4 °C. 90  $\mu\text{L}$  of the supernatant was transferred to a glass insert with  
130 plastic bottom spring (400  $\mu\text{L}$ , Grace, HK) in a 1.5 mL brown HPLC vial (Grace, HK) and stored  
131 at 4°C for analysis.

132

#### 133 *2.4. UHPLC-QTOF-MS analysis*

134

135 UHPLC-QTOF-MS analysis was performed on an Agilent 6540 ultra-high definition accurate  
136 mass quadrupole time-of-flight spectrometer with UHPLC (UHPLC-QTOF-MS, Agilent

137 Technologies, USA). A UPLC C<sub>18</sub> analytical column (2.1mm × 100 mm, I.D. 1.7μm, ACQUITY  
138 UPLC<sup>®</sup> BEH, Waters, U.S.A.) was used for separation, coupled with a C<sub>18</sub> pre-column (2.1mm ×  
139 5mm, I.D. 1.7μm, VanGuard<sup>TM</sup> BEH, Waters, U.S.A.) at room temperature of 20 °C. The  
140 mobile phase was a mixture of water (A) and acetonitrile (B), both containing 0.1% formic acid,  
141 with an optimized linear gradient elution as follows: 0–5 min, 10–35 % B; 5–25 min, 35–55 % B;  
142 25–28 min, 55–85 % B; 28–30 min, 85–100 % B. The injection volume was 4 μL. The flow rate  
143 was set at 0.35 mL/min. The mass spectra were acquired in negative mode by scanning from 100  
144 to 1700 in mass to charge ratio (*m/z*). The MS analysis was performed under the following  
145 operation parameters: dry gas temperature 300°C, dry gas (N<sub>2</sub>) flow rate 5 L/min, nebulizer  
146 pressure 30 psi, Vcap 3000, nozzle voltage 500V, and fragmentor voltage 200V.

147 Data analysis was performed on Agilent MassHunter Workstation software-Qualitative  
148 Analysis (version B.04.00, Build 4.0.479.5, Service Pack 3, Agilent Technologies, Inc. 2011).

149

150 *2.5. Method validation*

151

152 Linearity, repeatability, stability, recovery, limits of detection (LODs), and limits of  
153 quantification (LOQs) were carried out under the above mentioned conditions. Chemical markers  
154 of saikosaponins a, c and d were accurately weighed and dissolved individually in methanol to  
155 produce standard stock solutions. Working solutions were prepared by diluting the stock  
156 solutions with methanol to yield a series of standard solutions in the concentration range of 60–  
157 3000 ng/mL, 30-1500 ng/mL and 110-5500 ng/mL for saikosaponins a, c and d, respectively.  
158 The internal standard ginsenoside Rb<sub>1</sub> was dissolved in methanol and added in sample solution  
159 with a concentration of 185 ng/mL.

160 The cortex was selected for testing the method repeatability, stability and recovery.  
161 Repeatability was evaluated by three replicated analyses of cortex at locations close to each other  
162 in the same tissue slice. Stability testing was performed on a sample solution after it had stood, at  
163 room temperature, for 0, 2, 4, 8, 12, 24 and 48h. Recovery study was conducted on a sample  
164 spiked with approximately 100% of known amounts of saikosaponins a, c and d in the samples,  
165 with three replicated analyses.

166

### 167 **3. Results and discussion**

168

#### 169 *3.1. Laser microdissection of tissues*

170

171 The anatomical features of the roots from *B. scorzonerifolium*, *B. chinense* and *B. falcatum*  
172 were found to be fundamentally identical, being composed of cork, cortex, phloem, cambium  
173 and xylem. Cork consisted of several layers of flat cells. Cortex was narrow; a few oil canals  
174 could be found in *B. chinense*. Phloem showed scattered oil canals, often with clefts. Cambium  
175 was in a ring. Xylem was broad, occupying more than half of the radius of the root; vessels were  
176 single or grouped; xylem fibres were well developed. Investigated by fluorescence mode, the  
177 cork showed brown or reddish-brown fluorescence. The cortex, phloem and xylem sometimes  
178 showed the same fluorescence, such as sample CH-12 of *B. scorzonerifolium* showing slight blue  
179 fluorescence. Sometimes they showed different fluorescence. For example, sample CH-15 of *B.*  
180 *chinense*, cortex showed bluish-yellow fluorescence; phloem showed slight bluish-purple  
181 fluorescence and xylem showed yellowish-green fluorescence (Fig. 1). According to their tissue  
182 structures and fluorescence characteristics, we separated cork, cortex, phloem and xylem tissues  
183 for analysis of cellular metabolites.

184

### 185 3.2. Identification of saikosaponins in microdissected tissues

186

187 The chemical profiles of microdissected tissues were analyzed by UHPLC-QTOF-MS. The  
188 representative total ions current (TIC) chromatograms of microdissected tissues from *B. chinense*  
189 (CH-13) are showed in Fig.2. The TIC chromatograms of microdissected tissues from other  
190 herbal samples were showed in the supplementary material (Fig. S1-S6). Sixty chromatographic  
191 peaks were detected (Table 2). Peaks of saikosaponins, as the major type of secondary  
192 metabolites in *Bupleurum* species, can be easily recognized by their generated molecular ions of

193 [M-H]<sup>-</sup> and/or [M+HCOO]<sup>-</sup>. Peaks 3, 6 and 15 were identified as saikosaponins a, c and d,  
194 respectively, by their accurate mass weight and corresponding mass ions; identifications were  
195 confirmed by comparison with weight, mass ions and retention times of chemical markers. The  
196 molecular ions of saikosaponins a (779.4566 *m/z*, [M-H]<sup>-</sup> and 825.4678 *m/z*, [M+HCOO]<sup>-</sup>), c  
197 (971.5245 *m/z*, [M+HCOO]<sup>-</sup>) and d (779.4589 *m/z*, [M-H]<sup>-</sup> and 825.4651 *m/z*, [M+HCOO]<sup>-</sup>)  
198 were detected in standard and sample solutions. Other detected peaks were tentatively identified  
199 by their accurate mass data in comparison with reported references [16-20]. The detailed  
200 information is shown in Table 2.

201 In the TIC chromatograms of microdissected tissues, the chemical profiles of the three  
202 *Bupleurum* species varied (Table 3). For examples, peaks 5, 10, 14 were unique to *B.*  
203 *scorzonerifolium* and peaks 52-60 were only detected in *B. falcatum*. Nevertheless, it is distinct  
204 and consistent for all that cork and cortex contain more chromatographic peaks. For example,  
205 sample CH-15, seventeen, sixteen, three and one peaks were detected in the cork, cortex, phloem  
206 and xylem, respectively. Peaks 3, 6, 12, 15, 17 and 18, corresponding to saikosaponin c (peak 3),  
207 saikosaponin a (peak 6), O-acetyl-saikosaponin a or O-acetyl-saikosaponin b<sub>1</sub> (peak 12),  
208 saikosaponin d (peak 15) and O-acetyl-saikosaponin d or O-acetyl-saikosaponin b<sub>2</sub> (peak 17&18)  
209 were detected in the cork from all samples. Peaks 3, 6, 11 and 15 were found in the cortex of all  
210 samples but no common chromatographic peaks were found in the phloem or xylem. A few  
211 chromatographic peaks identified as saikosaponins were found in the phloem, especially from *B.*  
212 *chinense*. For example, peaks 3, 4, 6, 11, 15, 17, 18, 26 and 37 were detected in the phloem from  
213 sample CH-13. For *B. scorzonerifolium* and *B. falcatum*, in samples CH-11 and CH-TL2,  
214 respectively, no saikosaponin peaks were detected; the peaks of saikosaponin a and saikosaponin  
215 c were detected in sample CH-12; and saikosaponin a was found in sample CH-TL1. For the

216 xylem, although a few saikosaponin peaks could be found in the xylem from *B. chinense*, no  
217 saikosaponin peak was detected in the samples of *B. scorzonerifolium* and *B. falcatum*.

218 From Fig. 2, peaks of saikosaponins a (peak 3), c (peak 6) and d (peak 15) appear in  
219 chromatograms of many tissues. Their chemical structures with their calculated accurate mass  
220 weights are showed in Fig. 3. Also, peaks 3, 6 and 15 were found as the common peaks of cork  
221 and cortex. The results distinctly demonstrate that saikosaponins a, c and d are the main  
222 components in the tissues of roots from these three *Bupleurum* species. Therefore, more specific  
223 determinations of saikosaponins a (peak 3), c (peak 6) and d (peak 15) in the four specific tissues  
224 (cork, cortex, xylem, phloem) were carried out by UHPLC-QTOF-MS.

225

### 226 3.3. Method validation

227

228 Method validation parameters included linearity, repeatability, stability, recovery, limits of  
229 detection (LODs), and limits of quantification (LOQs) (Table 4). Linearity was examined with  
230 selected concentration range with 6 levels. The calibration curves were constructed by plotting  
231 the peak areas (y axis) of the analytes versus the concentration (x axis, ng/ml). Satisfactory  
232 linearity for the analysis of each component was obtained with correlation coefficients of  
233 determination ( $R^2$ ), all greater than 0.9946. Repeatability was investigated by analyzing three  
234 individual samples on the same day; the relative standard deviation (RSD) of the results was  
235 8.40% for saikosaponin a, 8.11% for saikosaponin c, and 7.34% for saikosaponin d. Stability  
236 testing was performed on a sample solution at time intervals of 0, 2, 4, 8, 12, 24 and 48h. The  
237 results showed that the RSD of saikosaponins a, c and d was 4.47%, 5.91% and 6.90% (n=7),

238 respectively. The average recovery was 77.81% for saikosaponin a, 79.5% for saikosaponin c  
239 and 75.15% for saikosaponin d, within RSDs of 14.7%, 9.40%, and 12.2% for each analyte,  
240 respectively. The LODs of these analytes, calculated by a signal-to-noise (S/N) of 3, were 6.00,  
241 10.0, and 11.0 ng/mL for saikosaponins a, c and d, respectively; while the LOQs of these  
242 analytes, calculated by the S/N of 10, were 73.65, 79.86, and 177.26 ng/mL, respectively.

243 When the tissue was dissected by LMD, a few tissue parts will be burned to induce the loss  
244 of tissue which influences the microdissected areas of each tissue. Sometimes, the clefts in cortex  
245 varied in different regions of transverse section. These factors influence the values of RSD in the  
246 test of repeatability and recovery. In our previous study on the determination of alkaloids in  
247 microdissected tissues and cells of the stem of *Sinomenium acutum*, the recovery ranges were  
248 also vast from 69.99 to 134.39% with RSDs from 9.00% to 11.08% [7]. Thus, considering the  
249 tiny sampling for analysis and the above factors, the present method validation for determination  
250 of saikosaponins in various tissues is acceptable.

251 For quantitative analysis of herbal samples, it is hard to get the “blank samples” without  
252 detected components and with similar sample background for evaluating the matrix effect. In the  
253 present study, an internal standard substance ginsenoside Rb<sub>1</sub> with the same concentration was  
254 added in the analyzed sample solutions for investigating the matrix effect. The result showed that  
255 the RSD of detected peak area of ginsenoside Rb<sub>1</sub> was 2.64%, which indicated that the matrix  
256 effect can be neglected.

257

258 *3.4. Comparison of the contents of saikosaponins a, c and d in various tissues*

259

260 The contents of saikosaponins a, c and d in cork, cortex, phloem and xylem from various  
261 samples were determined using UHPLC-QTOF-MS (Table 5). Sample solutions of laser  
262 microdissected tissues were diluted within linearity before injection. The contents of  
263 saikosaponins a, c and d in various tissues from three *Bupleurum* species varied greatly. For  
264 example, saikosaponins a, c and d couldn't be detected in the phloem and xylem from sample  
265 CH-11 and the cork contained much higher contents of saikosaponins a, c and d than those of  
266 cortex. In detail, the cork from sample CH-11 contained saikosaponins a, c and d as 403.53 ng,  
267 208.10 ng and 269.93 ng in each unit area of about 10000  $\mu\text{m}^2$  while the cortex contained 43.82  
268 ng, 23.21 ng and 27.18 ng per unit area, respectively. For the same tissue from different samples,  
269 the contents of saikosaponins a, c and d also varied greatly. For example, for cork tissue from  
270 three *Bupleurum* species, saikosaponins a, c and d were detected in amounts varying from  
271 235.44ng to 1028.96 ng, 129.58 ng to 1567.82 ng and 138.11 ng to 1091.51 ng per unit area,  
272 respectively.

273 The amount of each saikosaponin in various tissues is graphed in Fig. 4. Generally, the  
274 contents of saikosaponins a, c and d in the cork and cortex were much higher than in phloem and  
275 xylem. For *B. scorzonerifolium* and *B. falcatum*, the cork contained more of saikosaponins a, c  
276 and d than did the cortex; while for *B. chinense*, the cortex contained higher contents of  
277 saikosaponins a, c and d than did the cork. Notably, the main site of accumulation of  
278 saikosaponins in *B. chinense* was different than in *B. scorzonerifolium* and *B. falcatum*. In other  
279 words, saikosaponins are stored in the cortex in *B. chinense* and in the cork in *B.*  
280 *scorzonerifolium* and *B. falcatum*. Overall, in the four tissues investigated here, the total content  
281 of saikosaponins a, c and d was higher in *B. chinense* and *B. falcatum* than in *B. scorzonerifolium*.



282 Specially, the sample of *B. chinense* from the Lingqiu County produces plants with the most  
283 saikosaponins.

284

285 *3.5. Different accumulated and stored sites of saikosaponins in Bupleurum species*

286

287 Samples CH-11 and CH-12 of *B. scorzonerifolium* were cultivated for two years and three  
288 years, respectively. Samples CH-13, CH-15 and CH-17 of *B. chinense* were collected from the  
289 same cultivation place with the same age yet were genetically different. Two representative  
290 samples from CH-TL of *B. falcatum* were also analyzed. All the different sample sources were  
291 collected for analyzing the accumulated site of saikosaponins in various tissues. The herbal  
292 samples of *B. scorzonerifolium* and *B. chinense* were collected from the same cultivation site;  
293 thus, their growing conditions were quite similar. The classic plant taxonomy based on  
294 morphological features of the plants has demonstrated that *B. scorzonerifolium* and *B. falcatum*  
295 have a close relationship [21, 22]. The recent studies on cell type-specific transcriptome profiles  
296 have demonstrated that complex gene regulatory networks occur at the cellular level and that cell  
297 type-specific expression of many proteins is involved in secondary metabolism, many of which  
298 were masked during whole-organ analysis [23]. From above, it can be deduced that the roots of *B.*  
299 *scorzonerifolium* and *B. falcatum* may have the similar cell type-specific transcriptional profiles.  
300 The site of saikosaponin synthesis may be the cork in *B. scorzonerifolium* and *B. falcatum* but  
301 the cortex in *B. chinense*. Thus, the sites where saikosaponins are accumulated in the roots of *B.*  
302 *scorzonerifolium* and *B. falcatum* are the same but differ from *B. chinense*. In further study, cell

303 type-specific transcriptional profiling of various tissues from *B. scorzonerifolium*, *B. falcatum*  
304 and *B. chinense* will be carried out for further revealing the difference.

305

### 306 3.6. Guidelines for quality evaluation of Radix Bupleuri by morphological features

307

308 Mapping of the active components in the root tissues of three officially used *Bupleurum*  
309 species can correlate the morphological features and active components of Radix Bupleuri.  
310 Morphological features of Radix Bupleuri include shape, size, color, and texture, both external  
311 and internal (e.g., cross-sections). The cross-section is a key point for morphological features of  
312 Radix Bupleuri as it reveals tissue structures and arrangement. For example, the root of *B.*  
313 *scorzonerifolium*, in the surface, cork showing as outside bark; cambium showing as a ring;  
314 radiated structures form from rays showing as radiated striations; the bark narrow as the cortex  
315 and phloem narrow; the wood part wide due to a broad xylem, occupying more than half of the  
316 radius of the root; and so on (Fig. 5). The present study has revealed that saikosaponins mainly  
317 accumulate in cork and cortex. Therefore, Radix Bupleuri with thinner main root and more  
318 lateral roots would contain higher active components, thus be of better quality in clinical  
319 application. Recently, an HPLC method for determination of saikosaponins a and d in the roots  
320 of *B. chinense* also showed that the lateral roots contained higher contents of saikosaponin a and  
321 d than those of the main root [24]. Farmers will be able to adjust their cultivation techniques and  
322 harvest time to produce the most potent herbs. When processing Radix Bupleuri, the lateral roots  
323 should be kept for medicinal use.

324

#### 325 4. Conclusions

326

327 In the present study, an approach by the combination of LMD and UHPLC-QTOF-MS was  
328 established to profile the chemicals in tissues of the roots of *B. scorzonerifolium*, *B. falcatum* and  
329 *B. chinense*. The exact tissues were separated by LMD and analyzed using UHPLC-QTOF-MS  
330 with characterization and determination of saikosaponins. The study showed that the cork and  
331 cortex from all three species contained more types of saikosaponins and higher contents of  
332 saikosaponins a, c and d than did the phloem and xylem. The interesting result is that cork is the  
333 site of the highest accumulation of saikosaponins in *B. scorzonerifolium* and *B. falcatum*; while  
334 cortex is the site of highest concentration of saikosaponins in *B. chinense* among the four tissues  
335 studied here. Results revealed the relationship between saikosaponins and herbal tissues, which  
336 indicated that Radix Bupleuri with thinner main root and more lateral roots would contain higher  
337 active components, thus be of better quality in clinical application. The established relationship  
338 also provides the valuable information for the cultivation, harvest and processing of *Bupleurum*  
339 spp. for production of Bupleuri Radix.

340

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347

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#### 404 **Figure Legends**

405 **Fig. 1.** Microscopic characteristics of the roots of *Bupleurum. scorzonerifolium* Willd. (CH-12,  
406 A), *B. chinese* DC. (CH-15, B) and *B. falcatum* L. (CH-TL1, C). i: Under normal light

407 microscope; ii: Under fluorescence mode with dichromatic mirror. CK: cork; CT: cortex; PH:  
408 phloem; XY: xylem; OC: oil canals.

409 **Fig. 2.** The representative total ions current (TIC) chromatograms of microdissected tissues  
410 from *B. chinense* (CH-13). The peak No. referred to Table 2. GS: ginsenoside Rb<sub>1</sub>.

411 **Fig. 3.** The chemical structures of saikosaponin a, c and d.

412 **Fig. 4.** The changing trend of saikosaponin a (Ssa), c (Ssc) and d (Ssd) in various tissues of  
413 *Bupleurum* species.

414 **Fig. 5.** The cross-section of the root of *Bupleurum scorzonerifolium* revealing the relationship  
415 between morphological features and tissue structures.

416

#### 417 **Table Legends**

418 **Table 1** Sample information for the analysis.

419 **Table 2** Characteristics of secondary metabolites of various tissues from the roots of *Bupleurum*  
420 *scorzonerifolium*, *B. chinense* and *B. falcatum* by UHPLC-Q-TOF/MS.

421 **Table 3** The distribution of second metabolites in various tissues from different herbal samples.

422 **Table 4** Method validation data of the detected chemicals.

423 **Table 5** Contents of saikosaponin a (Ssa), c (Ssc) and d (Ssd) in the laser dissected tissues from  
424 *Bupleurum scorzonerifolium*, *B. chinense* and *B. falcatum*.

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439 **Table 1**

440 Sample information for the analysis.

Species	Sample No.	Source	Date of collection
<i>Bupleurum scorzonerifolium</i> Willd.	CH-11	Species from Mingshui county, Hei Long Jiang Province, cultivated for two years in Taigu county , Shan Xi Province	2010.09.13
	CH-12	Species from Mingshui county, Hei Long Jiang Province, cultivated for three years in Taigu county , Shan Xi Province	2010.09.13
<i>Bupleurum chinense</i> DC.	CH-13	Species from Lingqiu county, Shan Xi Province, cultivated for three years in Taigu county , Shan Xi Province	2010.09.13
	CH-15	Species from Zuoquan county, Shan Xi Province, cultivated for three years in Taigu county , Shan Xi Province	2010.09.13
	CH-17	Species from Lingchuan county, Shan Xi Province, cultivated for three years in Taigu county , Shan Xi Province	2010.09.13
<i>Bupleurum falcatum</i> L.	CH-TL	Plantation of Shenzhen Tsumura Medicine Co., LTD	2010.06.15

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456 **Table 2**457 Characteristics of secondary metabolites of various tissues from the roots of *Bupleurum scorzoniferolium*, *B. chinense* and *B. falcatum* by  
458 UHPLC-Q-TOF/MS.

Peak No.	Retention Time (min)	Molecular formula	[M-H] <sup>-</sup> ( <i>m/z</i> ) (Mass accuracy, ppm)	[M+HCOO] <sup>-</sup> ( <i>m/z</i> ) (Mass accuracy, ppm)	Identification
1*	2.80	C <sub>21</sub> H <sub>36</sub> O <sub>11</sub>	463.2187 (0.2)	509.2244 (0.4)	Unknown
2	6.82	C <sub>42</sub> H <sub>68</sub> O <sub>14</sub>	795.4546 (1.0)	841.4603	Bupleuroside VI
3	7.52	C <sub>48</sub> H <sub>78</sub> O <sub>17</sub>		971.5245 (2.4)	<b>Saikosaponin c</b>
4	7.63	C <sub>48</sub> H <sub>80</sub> O <sub>17</sub>		973.5380 (0.2)	Saikosaponin f
5	8.28	C <sub>42</sub> H <sub>68</sub> O <sub>14</sub>	795.4566 (3.0)	841.4605 (1.2)	Bupleuroside VI isomer
6	10.52	C <sub>42</sub> H <sub>68</sub> O <sub>13</sub>	779.46 (1.3)	825.4678 (3.6)	<b>Saikosaponin a</b>
7	11.60	C <sub>44</sub> H <sub>70</sub> O <sub>14</sub>		867.4740 (-0.8)	O-acetyl-saikosaponin a or O-acetyl-saikosaponin b <sub>1</sub>
8	11.72	C <sub>44</sub> H <sub>70</sub> O <sub>14</sub>	821.4702 (0.9)	867.4748 (0)	O-acetyl-saikosaponin a or O-acetyl-saikosaponin b <sub>1</sub>
9	12.25	C <sub>42</sub> H <sub>68</sub> O <sub>13</sub>	779.4588 (-0.1)	825.4678 (3.6)	Saikosaponin b <sub>2</sub>
10	12.73	C <sub>47</sub> H <sub>76</sub> O <sub>16</sub>	895.5048 (-1.3)	941.5130 (1.5)	Rotundioside G
11	13.22	C <sub>42</sub> H <sub>68</sub> O <sub>12</sub>		809.4705 (1.2)	Saikosaponin e
12	14.12	C <sub>44</sub> H <sub>70</sub> O <sub>14</sub>	821.4690 (-0.3)	867.4748 (0)	O-acetyl-saikosaponin a or O-acetyl-saikosaponin b <sub>1</sub>
13	14.76	C <sub>17</sub> H <sub>22</sub> N <sub>9</sub> O <sub>13</sub>	558.1188 (0.2)		Unknown
14	15.20	C <sub>47</sub> H <sub>76</sub> O <sub>17</sub>	911.5015 (0.5)	957.5080 (1.5)	Rotundifolioside V
15	15.58	C <sub>42</sub> H <sub>68</sub> O <sub>13</sub>	779.4589 (0.2)	825.4651 (0.9)	<b>Saikosaponin d</b>
16	16.95	C <sub>45</sub> H <sub>70</sub> O <sub>16</sub>	865.4586 (-0.5)		Malonylsaikosaponin d
17	17.30	C <sub>44</sub> H <sub>70</sub> O <sub>14</sub>	821.4700 (0.7)	867.4737 (-1.1)	O-acetyl-saikosaponin d or O-acetyl-saikosaponin b <sub>2</sub>
18	19.81	C <sub>44</sub> H <sub>70</sub> O <sub>14</sub>	821.4699 (0.6)	867.4764 (1.6)	O-acetyl-saikosaponin d or O-acetyl-saikosaponin b <sub>2</sub>
19	29.52	C <sub>24</sub> H <sub>29</sub> O <sub>7</sub>	427.1784 (5.7)		Unknown
20	29.69	C <sub>23</sub> H <sub>24</sub> O <sub>5</sub>	379.1588 (3.7)		Unknown
21	15.63	C <sub>18</sub> H <sub>34</sub> O <sub>4</sub>	313.2379 (-0.5)		Unknown
22	22.15	C <sub>18</sub> H <sub>32</sub> O <sub>3</sub>	295.2278 (0.1)		Unknown
23	22.51	C <sub>18</sub> H <sub>33</sub> O <sub>3</sub>	295.2278 (0.1)		Unknown



24	9.92	C <sub>48</sub> H <sub>78</sub> O <sub>18</sub>	941.5098 (-1.7)	987.5169 (-0.1)	3β,16α,28,30-tetrahydroxy-olean-11, 13(18)-dien-3β-yl-β-D-glucopyranosyl-(1→6)-β-D-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside
25	14.30	C <sub>48</sub> H <sub>78</sub> O <sub>18</sub>	941.5123 (0.8)	987.5171 (0.1)	3β,16β,23-trihydroxy-olean-13, 28-epoxy-olean-11-en-3β-yl-[β-D-glucopyranosyl-(1→2)]-[β-D-glucopyranosyl-(1→3)]-β-D-fucopyranoside
26	4.64	C <sub>42</sub> H <sub>66</sub> O <sub>15</sub>	809.4369 (4.0)	855.4465 (8.1)	Saikosaponin x
27	5.12	C <sub>56</sub> H <sub>77</sub> O <sub>16</sub>	1003.5081 (-5.8)		Unknown
28	5.89	C <sub>42</sub> H <sub>70</sub> O <sub>14</sub>	797.4776 (8.3)	843.4789 (4.1)	Hydroxysaikosaponin a
29	6.12	C <sub>42</sub> H <sub>70</sub> O <sub>14</sub>	797.4757 (6.4)	843.4794 (4.6)	Hydroxysaikosaponin d
30	6.30	C <sub>48</sub> H <sub>78</sub> O <sub>18</sub>		987.5241 (7.1)	Saikosaponin n
31	8.02	C <sub>48</sub> H <sub>78</sub> O <sub>17</sub>		971.5351 (12)	Saikosaponin BK <sub>1</sub> isomer
32	11.68	C <sub>45</sub> H <sub>70</sub> O <sub>16</sub>	865.4690 (0.9)		malonylsaikosaponin b <sub>1</sub>
33	13.01	C <sub>44</sub> H <sub>70</sub> O <sub>14</sub>		867.4811 (6.3)	O-acetyl-saikosaponin a or O-acetyl-saikosaponin b <sub>1</sub>
34	14.26	C <sub>44</sub> H <sub>70</sub> O <sub>14</sub>	821.4749	867.4809 (6.1)	O-acetyl-saikosaponin a or O-acetyl-saikosaponin b <sub>1</sub>
35	16.28	C <sub>42</sub> H <sub>66</sub> O <sub>13</sub>	777.4391 (-4.0)	823.4438 (-4.7)	Unknown
36	10.98	C <sub>17</sub> H <sub>26</sub> O <sub>4</sub>	293.1794 (3.6)		Unknown
37	3.65	C <sub>53</sub> H <sub>86</sub> O <sub>24</sub>	1105.5424 (-1.2)	1151.5484 (-0.7)	Saikosaponin v
38	3.83	C <sub>47</sub> H <sub>76</sub> O <sub>19</sub>	943.4923 (1.5)	989.4968 (0.5)	Saikosaponin v1
39	4.90	C <sub>42</sub> H <sub>68</sub> O <sub>14</sub>	795.4514 (-2.2)	841.4595 (0.4)	Saikosaponin l
40	5.40	C <sub>48</sub> H <sub>78</sub> O <sub>17</sub>	925.4802 (36.4)		Saikosaponin BK <sub>1</sub>
41	5.58	C <sub>23</sub> H <sub>45</sub> O <sub>16</sub>	576.2509 (12.6)		Unknown
42	6.57	C <sub>48</sub> H <sub>78</sub> O <sub>18</sub>		987.5170 (0)	Rotundioside N
43	7.33	C <sub>47</sub> H <sub>72</sub> O <sub>19</sub>	939.4605 (1.0)		Unknown
44	17.56	C <sub>44</sub> H <sub>70</sub> O <sub>14</sub>	821.4696 (0.3)	867.4751 (0.3)	O-acetyl-saikosaponin d
45	22.34	C <sub>46</sub> H <sub>72</sub> O <sub>15</sub>	863.4769 (-2.9)	909.4861 (0.8)	diacetyl- saikosaponin d
46	21.67	C <sub>46</sub> H <sub>72</sub> O <sub>15</sub>		909.4860 (0.7)	diacetyl- saikosaponin a
47	3.88	C <sub>25</sub> H <sub>24</sub> O <sub>12</sub>	515.1186 (-0.9)		Unknown
48	4.20	C <sub>25</sub> H <sub>24</sub> O <sub>12</sub>	515.1185 (-0.8)		Unknown
49	4.36	C <sub>9</sub> H <sub>16</sub> O <sub>4</sub>	187.0973 (-0.3)		Unknown
50	7.28	C <sub>18</sub> H <sub>34</sub> O <sub>5</sub>	329.2333 (0)		Unknown
51	7.95	C <sub>51</sub> H <sub>80</sub> O <sub>20</sub>	1011.5170 (0)		Unknown
52	8.97	C <sub>43</sub> H <sub>72</sub> O <sub>14</sub>	811.4848 (-0.1)	857.4905 (-0.1)	Saikosaponin b <sub>4</sub>

53	12.22	C <sub>36</sub> H <sub>58</sub> O <sub>8</sub>		663.4106 (-0.8)	Prosaikogenin F
54	17.58	C <sub>44</sub> H <sub>70</sub> O <sub>14</sub>	821.4681 (-1.2)	867.4744 (-0.4)	O-acetyl-saikosaponin d
55	17.89	C <sub>36</sub> H <sub>58</sub> O <sub>8</sub>		663.4112 (-0.2)	Prosaikogenin D
56	4.64	C <sub>42</sub> H <sub>68</sub> O <sub>14</sub>	795.4550 (1.4)	841.4597 (0.6)	Saikosaponin l isomer
57	7.15	C <sub>42</sub> H <sub>68</sub> O <sub>14</sub>	795.4537 (0.1)	841.4602 (1.1)	Bupleuroside VI isomer
58	9.10	C <sub>50</sub> H <sub>80</sub> O <sub>18</sub>		1013.5329 (0.2)	O-acetyl-saikosaponin c
59	9.55	C <sub>42</sub> H <sub>68</sub> O <sub>13</sub>		825.4646 (0.4)	Saikosaponin b <sub>1</sub>
60	13.57	C <sub>44</sub> H <sub>70</sub> O <sub>13</sub>	805.4755 (1.1)	851.4801 (0.3)	O-acetyl-saikosaponin e

459 \* The peak numbers referred to Figure 2.

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474 **Table 3** The distribution of second metabolites in various tissues from different herbal samples

Sample No.	Herbal Tissues / Peak No. <sup>*1</sup>			
	Cork	Cortex	Phloem	Xylem
CH-11	Peaks 1-20	Peaks 1, 3, 4, 6, 11, 13-15, 19, 20	Peaks 1, 13, 19-23	Peaks 1, 13, 19-23
CH-12	Peaks 1, 3-20, 24	Peaks 1, 3, 4, 6-8, 10-20, 24	Peaks 1, 3, 6, 13, 19, 20	Peaks 13, 19, 20
CH-13	Peaks 3, 4, 6, 9, 11-13, 15-18, 26-35, 47	Peaks 3, 4, 6, 9, 11-13, 15-18, 26-36, 47	Peaks 3, 4, 6, 11, 13, 15, 17, 18, 26, 36	Peaks 3, 4, 6, 13, 15, 26, 36
CH-15	Peaks 3, 4, 6-9, 11, 12, 15-18, 29, 30, 33-35	Peaks 3, 4, 6-9, 11, 12, 15, 17, 18, 29, 30, 33, 35, 36	Peaks 6, 15, 36	Peaks 36
CH-17	Peaks 1, 3, 4, 6-9, 11, 12, 15, 17, 18, 26, 30, 34, 37-45	Peaks 1, 3, 4, 6-9, 11, 12, 15, 17, 18, 26, 30, 34-37, 43-46	Peaks 3, 4, 6-9, 11, 15, 17, 18, 36	Peaks 3, 4, 6-8, 11, 15, 17, 18, 36
CH-TL1	Peaks 1, 3, 6-8, 11, 12, 15-18, 26, 28-30, 35-37, 39, 42, 47-55	Peaks 3, 6-8, 11, 12, 15, 17, 18, 29, 30, 36, 50, 53	Peaks 6, 50	Peak 50
CH-TL2	Peaks 2, 3, 6-9, 11, 12, 15-18, 20, 28-30, 34, 35, 37, 39, 50, 51, 54, 56-60	Peaks 2, 3, 6-9, 11-13, 15-20, 35, 50, 60	Peaks 13, 19, 20, 50	Peaks 13, 22, 23, 19, 20, 50

475 <sup>\*1</sup> The peak numbers referred to Table 2 and Figure 2.

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487 **Table 4**

488 Method validation data of the detected chemicals.

Chemicals	Calibration curve	R <sup>2</sup>	Repeatability (n=3, RSD, %)	Stability (48h, RSD, %)	Recovery (n=3, %, Mean±SD)	LODs (ng mL <sup>-1</sup> )	LOQs (ng mL <sup>-1</sup> )
Saikosaponin a	y=204.32x+1446.1	0.9983	8.40	4.47	77.81±11.44	6.00	73.65
Saikosaponin c	y=735.47x-14488	0.9946	8.11	5.91	79.50±7.47	10.00	79.86
Saikosaponin d	y=146.63x+2780.1	0.9984	7.34	6.90	75.15±9.19	11.00	177.26

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500 **Table 5**

501 Contents of saikosaponin a (Ssa), c (Ssc) and d (Ssd) in the laser dissected tissues from *Bupleurum*  
 502 *scorzonerifolium*, *B. chinense* and *B. falcatum*.

Sample No.	Tissue	Total microdissected areas ( $\mu\text{m}^2$ )	Ssa (ng)	Ssc (ng)	Ssd (ng)
CH-11	Cork	1032791	403.53	208.10	269.93
	Cortex	1183872	43.82	23.21	27.18
	Phloem	1302152	-*	-	-
	Xylem	1281726	-	-	-
CH-12	Cork	1164513	235.44	204.59	138.11
	Cortex	1190628	53.18	99.45	47.75
	Phloem	1315233	-	9.11	-
	Xylem	1364065	-	-	-
CH-13	Cork	1026844	411.26	373.31	459.29
	Cortex	1181483	758.16	535.78	962.38
	Phloem	1014707	195.24	152.07	337.46
	Xylem	1034935	28.46	24.00	21.35
CH-15	Cork	1058668	402.75	173.49	442.74
	Cortex	1071292	461.83	219.36	510.45
	Phloem	1118554	17.21	8.24	-
	Xylem	1043006	-	-	-
CH-17	Cork	1040131	367.95	129.58	352.02
	Cortex	1065687	718.87	205.42	894.51
	Phloem	1049056	118.27	25.31	173.60
	Xylem	1179142	83.33	21.63	101.97
CH-TL1	Cork	1051569	718.87	650.37	1091.51
	Cortex	1007260	316.01	206.21	336.69
	Phloem	1031779	115.24	9.90	-
	Xylem	1002570	-	-	-
CH-TL2	Cork	1150312	1028.96	567.82	981.80
	Cortex	1210182	267.77	113.90	255.92
	Phloem	1202215	-	-	-
	Xylem	1293177	-	-	-

503 \*under detection limit

504

504 Highlights

505

506 Tissue-specific metabolite profiles from different species by characterization and determination of main  
507 components

508 Tissue-specific metabolite profiling correlates morphology with quality of Bupleuri Radix

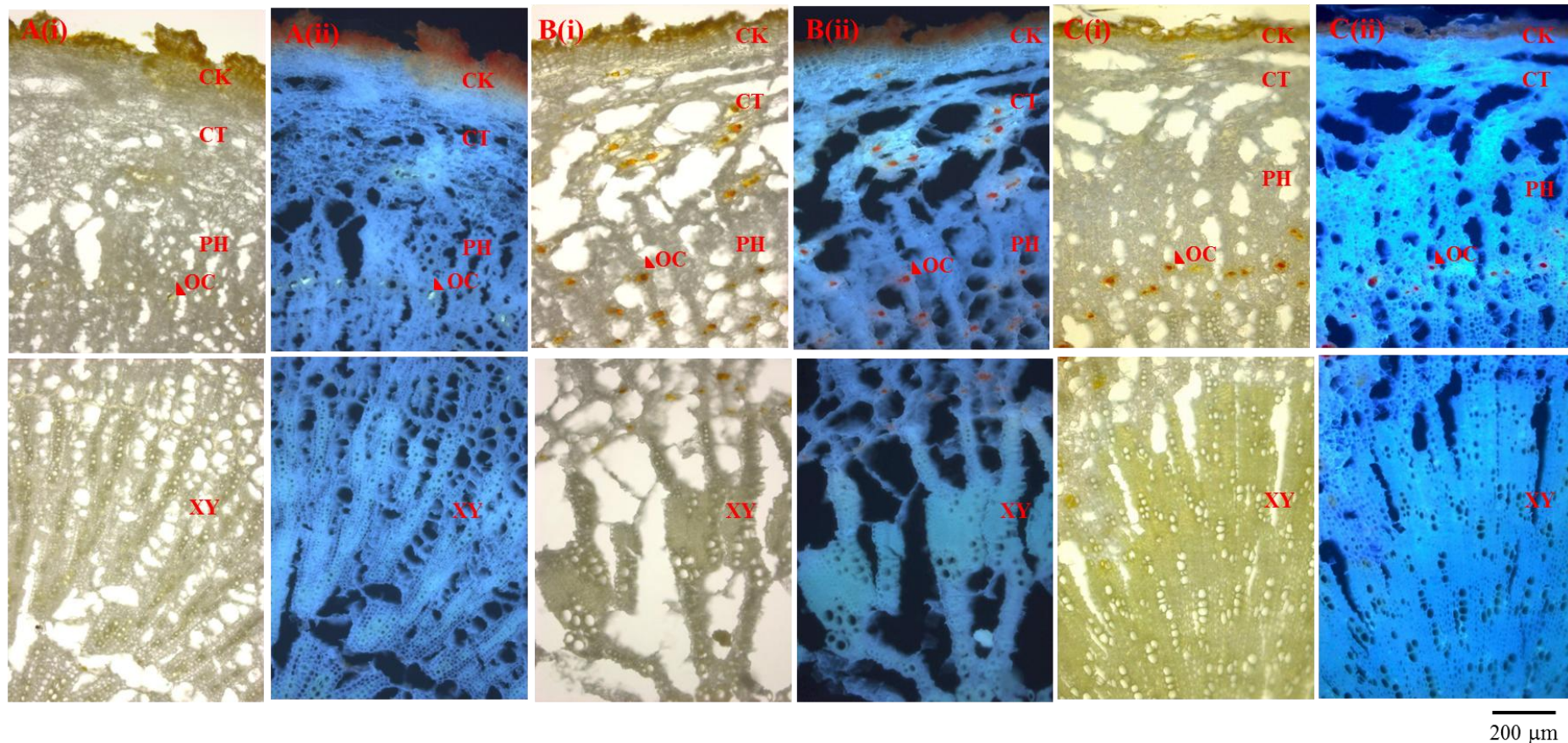
509 The correlation can be applied for evaluating the quality of Bupleuri Radix by morphological features

510

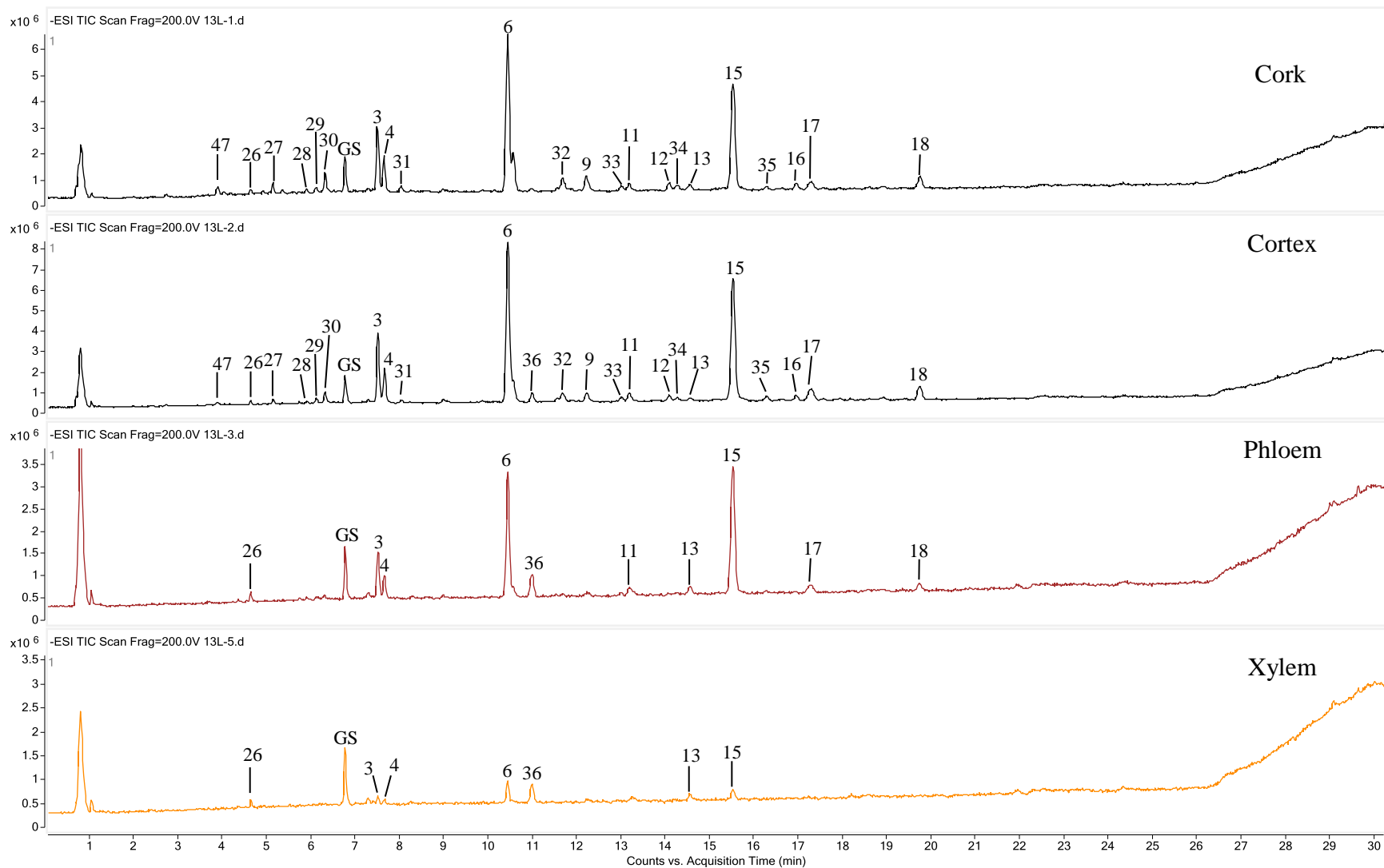
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Figure(s)

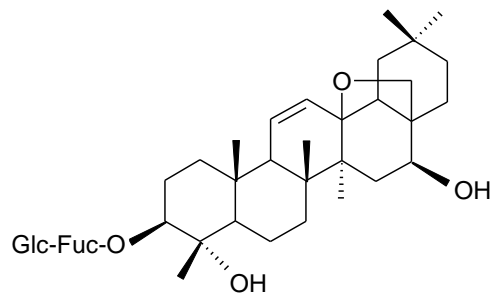


**Fig. 1.** Microscopic characteristics of the roots of *Bupleurum. scorzonerifolium* Willd.(CH-12, A), *B. chinese* DC. (CH-15, B) and *B. falcatum* L. (CH-TL1, C). i: Under normal light microscope; ii: Under fluorescence mode with dichromatic mirror. CK: cork; CT: cortex; PH: phloem; XY: xylem; OC: oil canals.

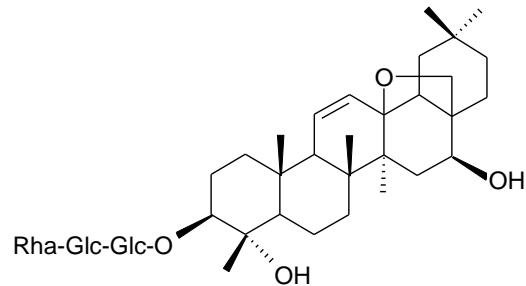


**Fig. 2.** The representative total ions current (TIC) chromatograms of microdissected tissues from *B. chinense* (CH-13). The peak No. referred to Table 2. GS: ginsenoside Rb<sub>1</sub>.

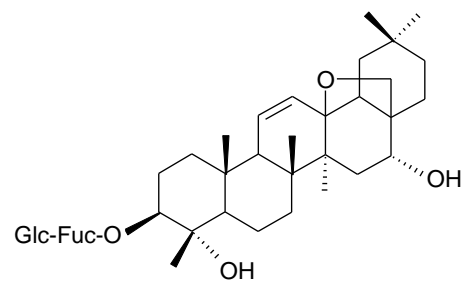




saikosaponin a  
**C<sub>42</sub>H<sub>68</sub>O<sub>13</sub>**  
MW 780.4660

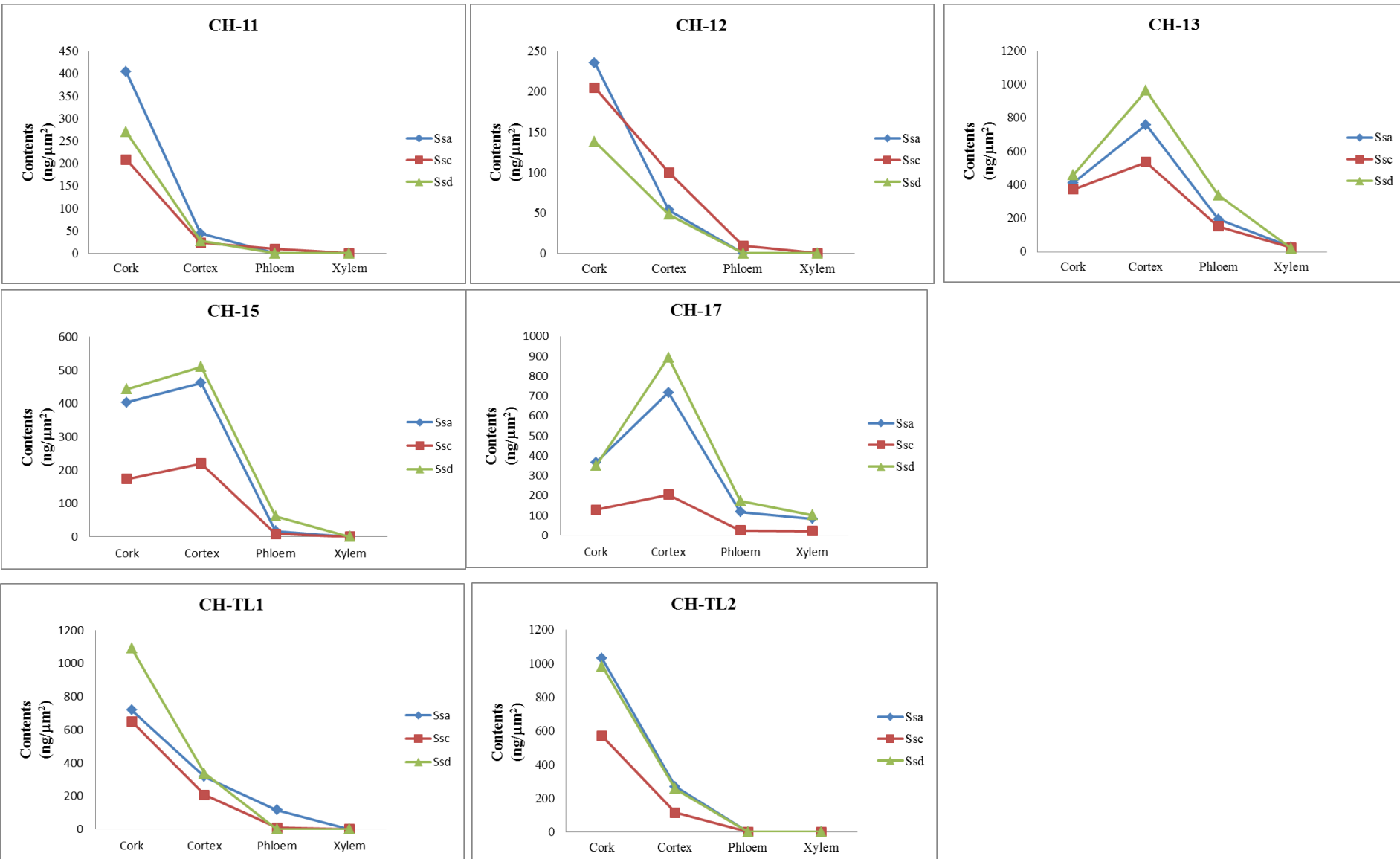


saikosaponin c  
**C<sub>48</sub>H<sub>78</sub>O<sub>17</sub>**  
MW 926.5239

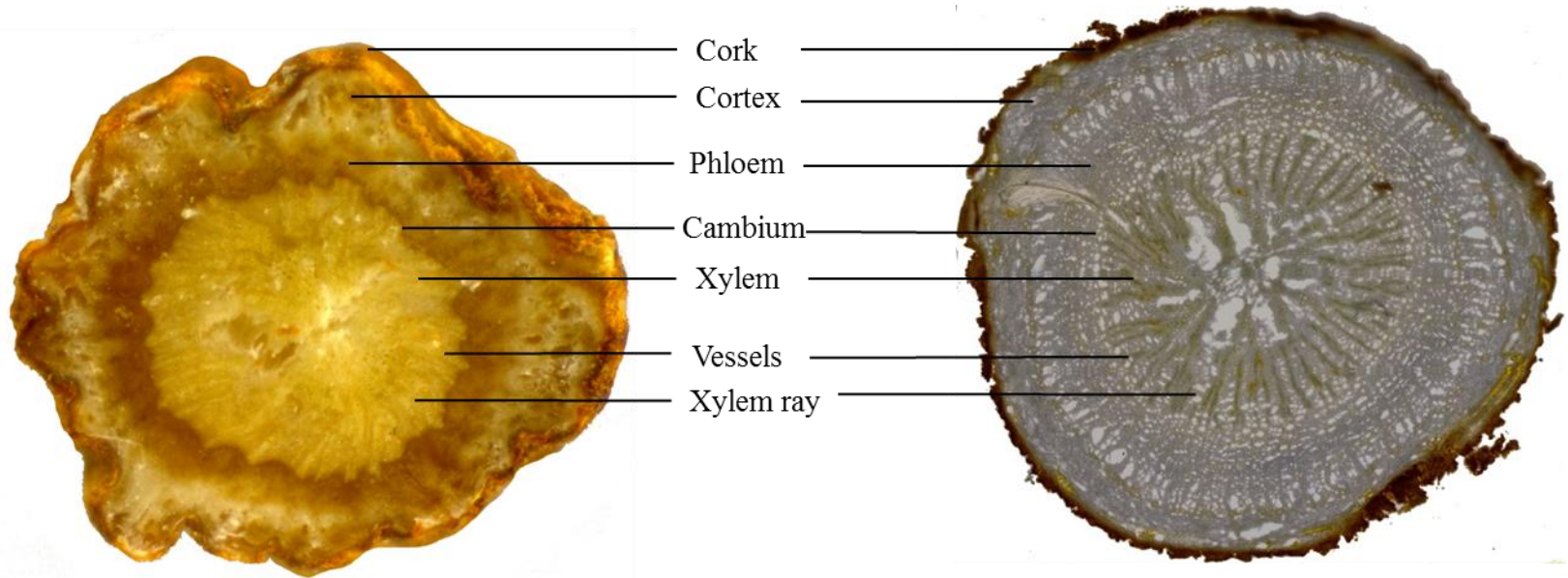


saikosaponin d  
**C<sub>42</sub>H<sub>68</sub>O<sub>13</sub>**  
MW 780.4660

**Fig. 3.** The chemical structures of saikosaponin a, c and d.



**Fig. 4.** The changing trend of saikosaponins a (Ssa), c (Ssc) and d (Ssd) in various tissues of *Bupleurum* species.



**Fig. 5.** The cross-section of the root of *Bupleurum scorzonerifolium* revealing the relationship between morphological features and tissue structures.