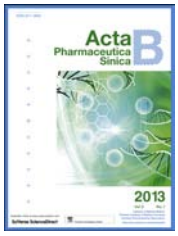
Acta Pharmaceutica Sinica B 2013;3(3):193–204

Institute of Materia Medica, Chinese Academy of Medical Sciences
Chinese Pharmaceutical Association

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



ORIGINAL ARTICLE

Chemical profiling and histochemical analysis of *Bupleurum marginatum* roots from different growing areas of Hubei province

Zhitao Liang[a], Jian Zhang[b], Guangyi Yang[c], Hubiao Chen[a], Zhongzhen Zhao[a,*]

[a]*School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China*
[b]*College of Pharmaceutical Science, Soochow University, Suzhou, 215006 China*
[c]*Taihe Hospital Affiliated with Hubei University of Medicine, Shiyan, 442000 China*

Received 13 January 2013; revised 21 February 2013; accepted 18 March 2013

KEY WORDS

Bupleurum marginatum;
Chemical profiling;
Laser microdissection;
LC–MS;
Germplasm resources

Abstract Bupleuri Radix has been widely used in traditional Chinese medicine. In the current herbal market, the species *Bupleurum marginatum* Wall. ex DC. is the main source of Bupleuri Radix. Although Bupleuri Radix from the roots of *B. marginatum* grown wild in the North West of Hubei province has higher quality compared with those from other regions according to the previous investigations, the exhaustive exploitation driven by increasing demand has drastically reduced the wild resource. As a result, germplasm evaluation and quality resource exploration are important for the sustainable utilization and cultivation of *B. marginatum*. A preliminary study indicated differences in the tissue structure of *B. marginatum* grown in different areas of North Western Hubei province. In the current study, various tissues of the roots of *B. marginatum* grown in different areas of North Western Hubei were subjected to laser microdissection and analyzed by microscopy and ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC–Q-TOF-MS). The results show that wild plants

*Corresponding author. Tel.: +852 34112424; fax: +852 34112461.
E-mail address: zzzhao@hkbu.edu.hk (Zhongzhen Zhao).

Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

ELSEVIER Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.apsb.2013.04.002

from Maqiao Town, Baokang County contain the most saikosaponins distributed mainly in cork, cortex and phloem. This study provides key chemical information for evaluating the quality of *B. marginatum* roots.

1. Introduction

Bupleuri Radix (Chinese thorowax root, known as 'Chaihu' in Chinese) is commonly used in traditional Chinese medicine (TCM) for the treatment of fevers and colds, malaria, cholecystitis, hepatitis, pancreatitis and menstrual disorders. It is often found in clinical prescriptions and patent medicines including Xiao–Chaihu–Tang, Xiao–Yao–Wan, Jia–Wei–Xiao–Yao–Wan and Chai–Ling–Tang. Modern studies have indicated that the roots of *Bupleurum marginatum* contain large amounts of saikosaponins a, c and d[1] of which a and d are the main active components to which the clinical efficacy of Bupleuri Radix is attributed[2].

In the herbal market, the species *Bupleurum chinense* DC., *Bupleurum scorzonerifolium* Willd. and *B. marginatum* Wall. ex DC. are the main sources of commercial Bupleuri Radix[3]. Although the former two are recorded as sources of Bupleuri Radix in Chinese Pharmacopoeia and are widely cultivated, the widespread demand for the herb has tended to far outstrip the supply[4]. This is particularly the case for *B. marginatum* which usually grows wild on hillside meadows in Hubei, Yunnan, Sichuan, Guizhou and Guangxi provinces. As with other important medicinal herbs, the conservation, germplasm evaluation, quality resource exploration and large-scale cultivation of wild resources have become critical for their sustainable utilization.

It is well known that the Bupleuri Radix produced in the North West of Hubei province is of higher quality than products from other regions[5,6]. It is assumed that this is due to favorable features of the geography and climate of the area. To investigate the distribution and usage of *Bupleurum* species in North Western Hubei, a systematic field survey was carried out[5]. The results indicate that *B. marginatum* is the main species of *Bupleurum* in the region and that its roots are the actual commodity sold and used as Bupleuri Radix in the region. In a preliminary study, we found that the microscopic features of transverse sections of *B. marginatum* from different growing areas were different, a fact that could be important in selecting sources for cultivation of this important medicinal plant.

A medicinal plant usually contains a complex mixture of chemical components, the production and distribution of which is directly related to the species and the environmental conditions under which it is grown. In recent years, the technique of liquid chromatography–mass spectrometry (LC–MS) has been widely applied to profile the chemical composition of herbal medicines[7,8]. Additionally, laser microdissection (LMD) has been used to facilitate tissue- and cell-specific metabolite profiling of plants[9,10]. Recently, we applied this combination of techniques to analyze tissue-specific metabolites in the stems of *Sinomenium acutum* (Thunb.) Rehd. et Wils.[11]. The objective of the present study was to analyze and compare the chemical profiles of roots of *B. marginatum* from North Western Hubei in order to enhance the quality evaluation of *B. marginatum*.

2. Materials and methods

2.1. Materials

Four batches of dried roots (Samples 1–4) and one batch of fresh roots (Sample 5) of *B. marginatum* grown in North Western Hubei province (Table 1) were collected. Samples 1, 2, 3 and 5 were grown in different areas but samples 3 and 4 were cultivated and wild plants respectively from the same area. The diameter of dried roots selected for study was about 0.7 cm and the batch of fresh roots was separated into roots of three approximate sizes *viz* 0.3, 0.6 and 1.0 cm. All samples were authenticated by Dr. Guangyi Yang and deposited in the Bank of China (Hong Kong) Chinese Medicines Centre of Hong Kong Baptist University.

2.2. Chemicals and reagents

Pure samples (>98% by HPLC) of saikosaponins a, c and d were isolated in our laboratory[1]. HPLC grade acetonitrile and methanol were from E. Merck (Darmstadt, Germany). HPLC grade formic acid (purity 96%) was purchased from Tedia (USA). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Table 1 Sources of the samples of *Bupleurum marginatum* Wall. ex DC. in North Western Hubei Province.

No.	Source	Growing condition	Collection time
1	Shangjin town, Yunxi county	Wild, altitude 500–1000 m, hillside meadow	Mar 16, 2011
2	Guandu town, Zhushan county	Wild, altitude 350–1100 m, under forest or hillside meadow	Aug 13, 2011
3	Maqiao town, Baokang county	Cultivation, flat ground	Sep 14, 2011
4	Maqiao town, Baokang county	Wild, altitude 500–800 m, hillside meadow	Aug 23, 2011
5	Qingfeng town, Fang county	Wild, altitude 800–1000 m, hillside meadow	Oct 26, 2011

Table 2 Characteristics of the chemical compounds in the roots of *Bupleurum marginatum* Wall. ex DC. as determined by UHPLC–Q-TOF/MS.

Peak No.	Retention time (min)	$[M-H]^-$ (*m/z*)	$[M+HCOO]^-$ (*m/z*)	Identification	Sample No.				
					1	2	3	4	5
1	4.59	809.4345	855.4405	3β, 16α, 23, 28-Tetrahydroxy-olean-11, 13 (18)-dien-29-oic acid 3-*O*-β-D-glucopyranosyl-(1→3)-β-D-fucopyranoside	+	+		+	+
2	4.93	647.3829	693.3886	Unknown	+	+			
3	6.09	797.4708	843.4764	Hydroxysaikosaponin a	+	+	+	+	+
4	6.25	797.4701	843.4770	Hydroxysaikosaponin d	+	+			+
5	6.72	795.4559	841.4631	16α, 23, 28, 30-Tetrahydroxyolean-11, 13 (18)-dien-3β-yl-β-D-glucopyranol-(1→3)-β-D-fucopyranoside	+			+	+
6	6.89	795.4543	841.4610	Bupleuroside VI	+	+		+	
7	7.27		825.4651	Saikosaponin b_1 or saikosaponin b_2	+	+	+	+	+
8	7.46		971.5245	Saikosaponin c	+	+	+	+	+
9	7.57		973.5419	Saikosaponin f	+	+	+	+	+
10	8.54	779.4617	825.4663	Saikosaponin b_1 or saikosaponin b_2	+				
11	8.76	795.4556	841.4605	Bupleuroside IX	+			+	
12	8.94	811.4859	857.4920	Saikosaponin b_4	+			+	
13	10.39	779.4626	825.4698	Saikosaponin a	+	+	+	+	+
14	11.51	779.4582	825.4655	Saikosaponin b_2	+	+			
15	12.19		663.4137	Prosaikogenin F	+				
16	13.11	763.4625	809.4696	Saikosaponin e	+				
17	14.03	821.4673	867.4742	*O*-Acetyl-saikosaponin a or *O*-acetyl-saikosaponin b_2	+	+		+	
18	15.48	779.4616	825.4685	Saikosaponin d	+	+	+	+	+
19	16.23	777.4430	823.4487	Unknown	+				
20	17.20	821.4673	867.4747	*O*-acetyl-saikosaponin a	+	+	+	+	+
21	17.55	821.4685	867.4758	*O*-acetyl-saikosaponin d	+	+	+	+	+
22	17.69	863.4446		Diacetyl-saikosaponin b_2	+	+	+		+
23	17.88		663.4123	Prosaikogenin D	+				
24	18.76		661.3971	Unknown	+				
25	19.31	763.4609	809.4666	Unknown	+				
26	19.68	821.4709	867.4776	*O*-acetyl-saikosaponin d	+	+		+	+
27	21.83		909.4868	Diacetyl-saikosaponin a	+	+	+	+	+
28	22.19		909.4868	Diacetyl-saikosaponin d	+	+	+	+	+
29	23.42	293.2122		Unknown	+	+	+	+	+
30	23.72	763.4626	809.4694	Saikosaponin m	+	+	+		
31	28.94	271.2282		Unknown	+	+			
32	29.61	379.1583		Unknown	+	+	+	+	+
33	15.34	313.2384		Unknown	+				
34	17.69	265.1485		Unknown	+				
35	1.25	315.0726		Unknown		+			
36	2.41	401.1459		Unknown		+			
37	3.03	371.0991		Unknown		+			
38	3.71	413.1460		22-Stigmasterol		+			
39	3.89	383.0992		Saikochromoside A		+			
40	6.53	327.2183		Unknown		+			
41	7.90		967.5240	Rotundifolioside A		+			+
42	8.00		969.5414	Rotundifolioside B		+		+	
43	8.50	329.2336		Unknown		+			
44	11.59	821.4673	867.4732	*O*-Acetyl-saikosaponin b_2		+		+	
45	12.12	821.4696	867.4756	*O*-Acetyl-saikosaponin b_2 or *O*-acetyl-saikosaponin a		+		+	
46	15.00	311.2241		Unknown		+			
47	16.87	865.4615		Malonylsaikosaponin d		+		+	+
48	18.76	907.4715		Malonyl-acetyl-saikosaponin b_2		+			+
49	2.71	463.2194	509.2251	Unknown			+		+
50	2.84	309.1255		Unknown			+		
51	4.34	877.2921		Unknown			+		
52	6.29		987.5205	Saikosaponin n			+	+	+
53	7.89	1011.5185		Unknown			+	+	
54	8.21		841.4598	Saikosaponin t			+		
55	8.51	329.2329		Unknown			+		
56	8.99		987.5137	Saikosaponin s			+	+	
57	11.25	865.4619		Malonylsaikosaponin a			+	+	+
58	11.36	821.4681	867.4733	*O*-Acetyl-saikosaponin b_2			+	+	+

Table 2 (*continued*)

Peak No.	Retention time (min)	$[M-H]^-$ (*m/z*)	$[M+HCOO]^-$ (*m/z*)	Identification	Sample No.				
					1	2	3	4	5
59	11.60	865.4608		Malonylsaikosaponin b_1			+		+
60	12.16		867.4727	*O*-Acetyl-saikosaponin b_2			+		
61	14.55	313.2390		Unknown			+		
62	16.58	865.4581		Malonylsaikosaponin b_2			+	+	+
63	27.66	459.2978		Unknown			+		
64	28.61	435.2981		Unknown				+	
65	4.51	971.4858	1017.4917	3β, 16α, 23, 28-Tetrahydroxy-olean-11, 13 (18)-dien-30-oic acid 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)-β-D-fucopyranoside				+	
66	5.87	797.4697	843.4757	Bupleuroside XIII				+	
67	6.16	811.4483	857.4537	Saikosaponin b_3				+	
68	6.47	315.0514		1, 2, 3, 7-Tetramethoxyxanthone				+	
69	9.73		899.5005	Acetyl-saikosaponin b_3 or Acetyl-saikosaponin b_4				+	
70	9.83	941.5100	987.5169	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				+	
71	11.48		867.4763	*O*-acetyl-saikosaponin b_2				+	
72	12.50	907.4715		Malonyl-acetyl-saikosaponin a				+	+
73	12.66	907.4708		Malonyl-acetyl-saikosaponin a				+	+
74	12.85	907.4708		Malonyl-acetyl-saikosaponin a				+	+
75	13.04	907.4715		Malonyl-acetyl-saikosaponin a				+	+
76	13.26	907.4700		Malonyl-acetyl-saikosaponin a				+	+
77	13.48	909.4863		Rotundioside F or rotundifolioside J				+	
78	13.63		909.4868	Diacetyl-saikosaponin a or diacetyl-saikosaponin d				+	
79	14.16		987.5118	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				+	
80	14.98	851.4781		Acetyl-saikosaponin e or acetyl-saikosaponin m				+	
81	18.07	865.4647		Malonyl-saikosaponin d				+	
82	18.80	907.4697		Malonyl-acetyl-saikosaponin d				+	
83	20.10	863.4820	909.4886	Diacetyl-saikosaponin a or diacetyl-saikosaponin d				+	
84	21.29	949.4852		Unknown				+	+
85	21.59		909.4895	Diacetyl-saikosaponin a or diacetyl-saikosaponin d				+	
86	22.27	863.4849	909.4902	Diacetyl-saikosaponin a or diacetyl-saikosaponin d				+	
87	5.02	895.4342		Rotundifolioside I					+
88	5.33	1047.5342	1093.5414	Unknown					+
89	12.30	907.4718		Malonyl-acetyl-saikosaponin a					+
90	14.47	849.4646		Unknown					+
91	17.73	907.4687		Malonyl-acetyl-saikosaponin b_2					+
92	19.83	993.4708		Unknown					+
93	21.45	949.4766		Unknown					+

2.3. Sample preparation for microscopy

Tissue was taken from dried herbal samples as described by Ng et al.[12]. Fresh herbal samples were directly sectioned with a cryostat (Thermo Shandon As620 Cryotome, UK) and tissue slices (thickness approximately 40 μm) were placed on non-fluorescent PET microscope steel frame slides (76 mm × 26 mm, 1.4 μm thick, Leica Microsystems, Germany). Slides were then mounted on a Leica LMD 7000 system (Leica, Benshein, Germany) and investigated in fluorescence mode with a dichromatic mirror. Microdissection was conducted using a DPSS 349 nm laser beam with aperture 6, speed 5 and power 50–60 under a Leica LMD-BGR fluorescence filter system at 10× magnification. Tissue parts with area around 4×10^6 μm^2 were dissected separately under the fluorescence inspection mode. Microdissected tissues were allowed to fall into the caps of 500 μL microcentrifuge tubes (Leica, Germany) by gravity after which they were transferred to the bottom of the tubes by centrifugation (Centrifuge 5415R, Eppendorf, Hamburg, Germany) at 10,000 rpm for 5 min. Aliquots (100 μL) of methanol were added to each microcentrifuge tube and sonicated for 30 min (CREST 1875HTAG ultrasonic processor, USA). Tubes were centrifuged again for 10 min at 10,000 rpm and 4 °C after which 90 μL aliquots of supernatant were transferred to the glass inserts of 1.5 mL brown HPLC vials (Grace, HK) with plastic bottom springs (400 μL, Grace, HK) and stored at 4 °C pending analysis. A blank was prepared by adding 100 μL methanol to a microcentrifuge tube containing a blank 4×10^6 μm^2 PET membrane.

Sectioned tissue slices of each sample were scraped from a glass slide after drying in air, collected in a 1.5 mL microcentrifuge tube and treated as above except that 200 μL methanol was used as the extraction solvent.

2.4. UHPLC–MS analysis

Stock solutions of saikosaponins a, c and d were prepared individually in methanol. Working solutions were prepared by diluting the stock solutions with methanol to give final concentrations of 36, 10 and

36 μg/mL for saikosaponins a, c and d, respectively. UHPLC–MS analysis was performed on an UHPLC coupled to an Agilent 6540 ultra-high definition accurate mass quadrupole time-of-flight spectrometer (UHPLC–Q-TOF/MS, Agilent Technologies, USA). Separation was obtained by gradient elution on a C18 analytical column (100 mm × 2.1 mm, I.D. 1.7 μm, ACQUITY UPLC® BEH, Waters, USA) preceded by a C18 pre-column (5 mm × 2.1 mm, I.D. 1.7 μm, VanGuardTM BEH, Waters, USA) at room temperature (20 °C). The mobile phase was (A) water and (B) acetonitrile both containing 0.1% (*v*/*v*) formic acid and delivered at 0.35 mL/min according to the following linear gradient: 0–5 min, 10–35% B; 5–25 min, 35–55% B; 25–28 min, 55–85% B; 28–30 min, 85–100% B. The injection volume was 4 μL. Mass spectra were acquired in the negative ionization mode by scanning from *m*/*z* 100–1700. Optimized MS parameters were as follows: dry gas (N_2) temperature 300 °C, dry gas flow rate 5 L/min; nebulizer pressure 30 psi; Vcap 3000; nozzle voltage 500 V; fragmentor voltage 200 V.

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

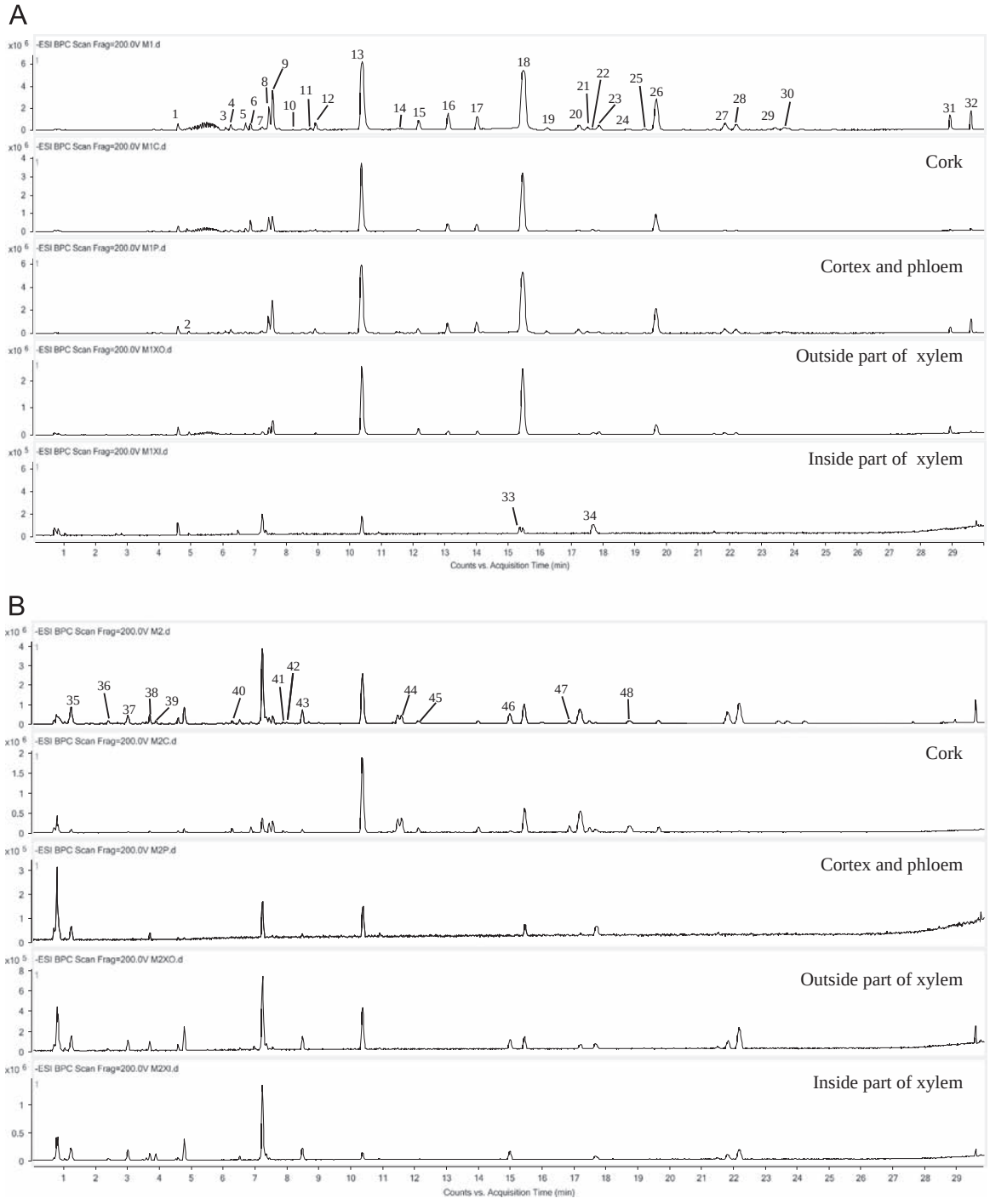


Figure 1 LC–MS base peak chromatograms of whole transverse sections and various tissues of *Bupleurum marginatum* Wall. ex DC roots. A–D refer to samples 1–4 respectively. The peak numbers refer to Table 2.

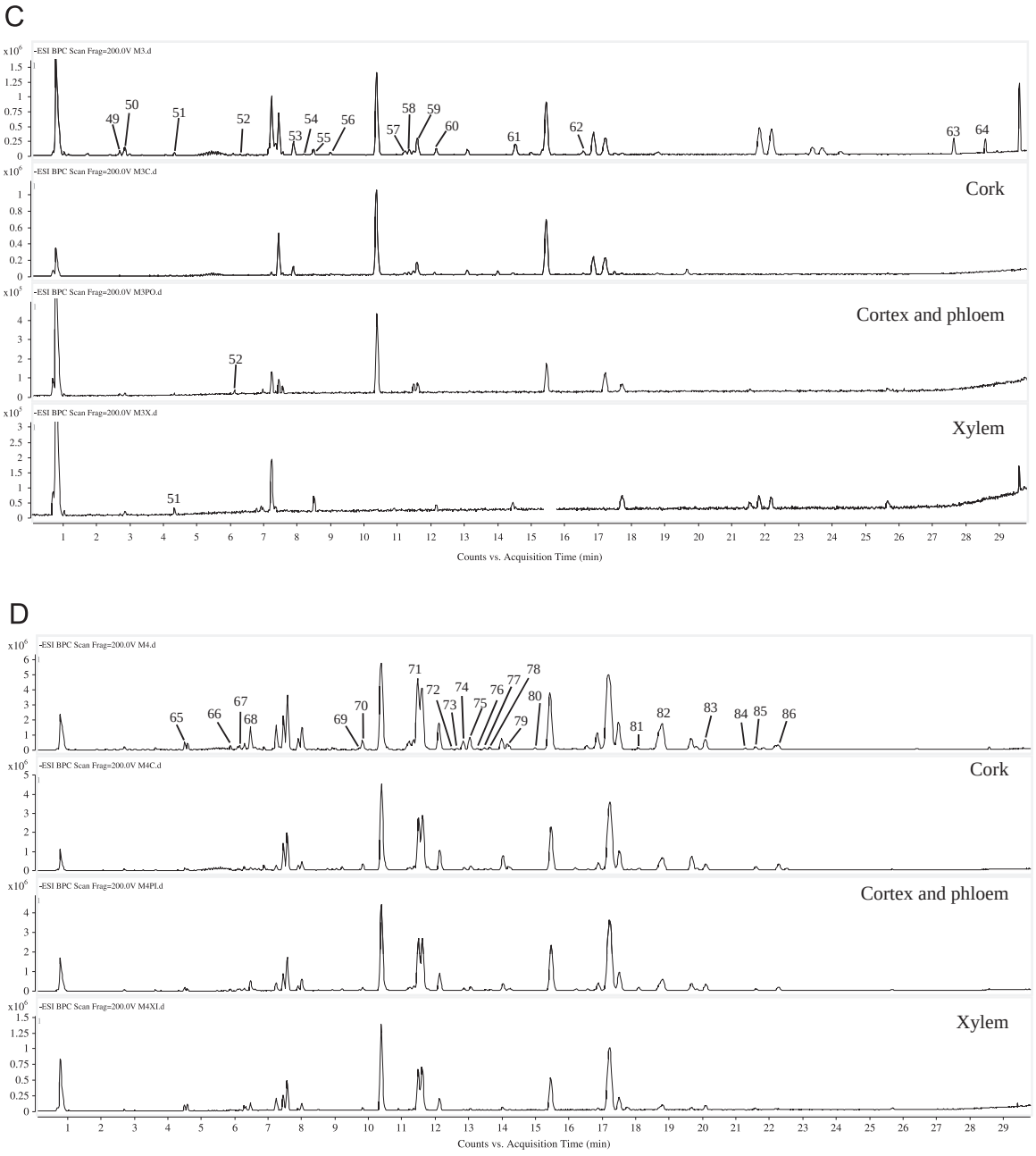


Figure 1 Continued.

Principal component analysis to analyze differences between the five herbal samples was carried out using SPSS PASW statistics 18.

3. Results and discussion

3.1. Chemical profiling

In the LC–MS analysis of tissue slices of the five samples of the roots of *B. marginatum* collected from different areas in the North West of Hubei province, a total of 93 well-separated chromatographic peaks were observed of which ten peaks were common to all samples (Table 2, Figs. 1 and 2). Of these ten peaks, peaks 8, 13 and 18 were identified as saikosaponins c, a and d, respectively, by comparison of molecular weights and chromatographic retention times with those of reference standards. Other peaks were tentatively identified by comparing mass data with those for compounds reported in the literature[13–16]. The other seven common peaks were tentatively identified as: 3, hydroxysaikosaponin a; 7, saikosaponins b_1 or b_2; 9, saikosaponin f; 20, *O*-acetyl-saikosaponin a; 21, *O*-acetyl-saikosaponin d; 27, diacetyl-saikosaponin a; and 28, diacetyl-saikosaponin d. Detailed information related to all 93 peaks is shown in Table 2. The chemical structures of major saikosaponins in the herbal samples are shown in Fig. 3 and Table 3.

The 3 roots of sample 5 with different diameters (0.3, 0.6 and 1.0 cm) were analyzed to determine if diameter size is associated with different chemical profiles. The results indicate that the number of compounds is the same (36 peaks in all samples) but

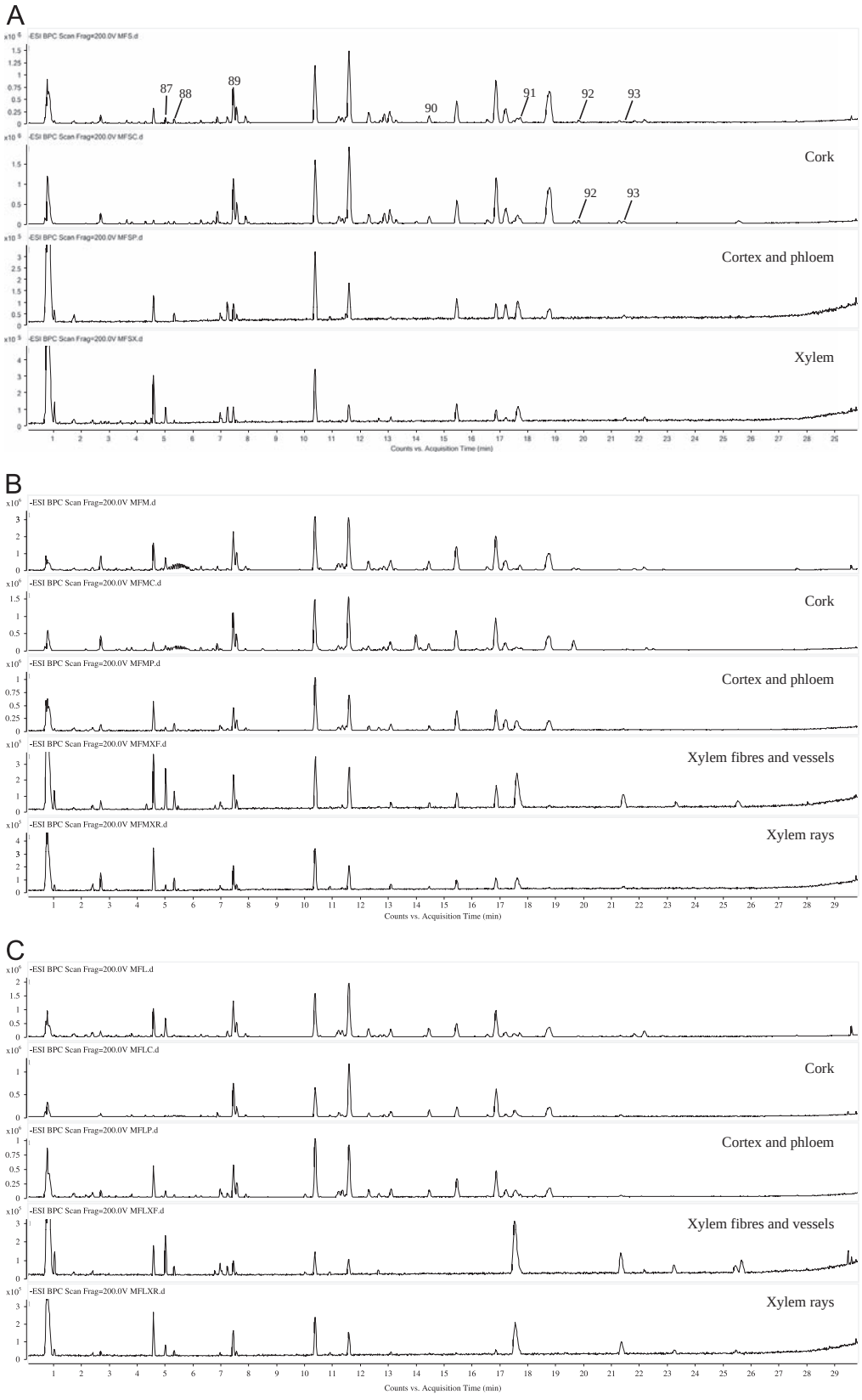


Figure 2 LC–MS base peak chromatograms of whole transverse sections and tissues of *Bupleurum marginatum* Wall. ex DC (sample 5) roots with diameters (A) 0.3 cm, (B) 0.6 cm and (C) 1.0 cm. The peak numbers refer to Table 2.

the relative amount of each compound (based on peak areas and assuming similar matrix effects) varies considerably.

The results also show distinct variations in the chromatograms of the 5 samples. For example, peaks 35–40 were only found in

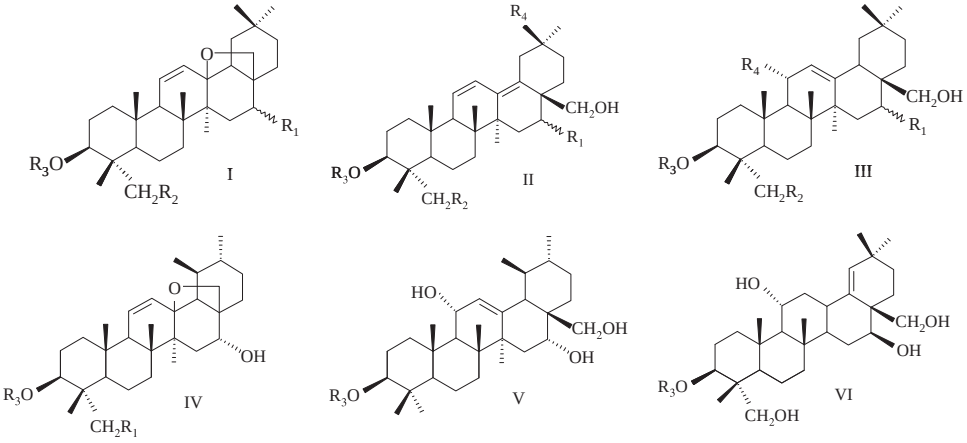


Figure 3 Chemical structures of major saikosaponins identified in the LC–MS base peak chromatograms of *Bupleurum marginatum* Wall. ex DC. See Table 2 for identification.

Table 3 Structures of the main saponins present in *Bupleurum marginatum* Wall. ex DC.

Compound	Structure type	R_1	R_2	R_3	R_4
Rotundioside F	I	α–OH	H	β-L-rha-(1→2)-β-D-glu-(1→2)-β-D-fuc-	Nil.
Saikosaponin a	I	β–OH	OH	β-D-glu-(1→3)-β-D-fuc-	Nil.
Saikosaponin c	I	β–OH	H	β-D-glu-(1→6)-[α-L-rha-(1→4)]-β-D-glu-	Nil.
Saikosaponin d	I	α–OH	OH	β-D-glu-(1→3)-β-D-fuc-	Nil.
Saikosaponin e	I	β–OH	H	β-D-glu-(1→3)-β-D-fuc-	Nil.
Prosaikogenin F	I	β–OH	OH	β-D-fuc-	Nil.
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	I	β–OH	OH	β-D-glu-(1→2)-[β-D-glu-(1→3)]-β-D-fuc-	Nil.
Saikosaponin b_1	II	β–OH	OH	β-D-glu-(1→3)-β-D-fuc-	CH_3
Saikosaponin b_2	II	α–OH	OH	β-D-glu-(1→3)-β-D-fuc-	CH_3
Saikosaponin m	II	H	OH	β-D-glu-(1→3)-β-D-fuc-	CH_3
Saikosaponin n	II	β–OH	OH	β-D-glu-(1→6)-[α-L-rha-(1→4)]-β-D-glu-	CH_3
Saikosaponin s	II	α–OH	OH	β-D-glu-(1→6)-[α-L-rha-(1→4)]-β-D-glu-	CH_3
Prosaikogenin D	II	α–OH	OH	β-D-fuc-	CH_3
16α, 23, 28, 30-tetrahydroxyolean-11, 13 (18)-dien-3β-yl-β-D-glucopyranol-(1→3)-β-D-fucopyranoside	II	α–OH	OH	β-D-glu-(1→3)-β-D-fuc-	CH_2OH
3β, 16α, 23, 28-tetrahydroxy-olean-11, 13 (18)-dien-30-oic acid 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)-β-D-fucopyranoside	II	α–OH	OH	β-D-glu-(1→2)-[β-D-glu-(1→3)]-β-D-fuc-	COOH
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	II	α–OH	H	β-D-glu-(1→6)-[α-L-rha-(1→4)]-β-D-glu-	CH_3
Saikosaponin b_3	III	β–OH	OH	β-D-glu-(1→3)-β-D-fuc-	OCH_3
Saikosaponin b_4	III	α–OH	OH	β-D-glu-(1→3)-β-D-fuc-	OCH_3
Saikosaponin f	III	β–OH	H	β-D-glu-(1→6)-[α-L-rha-(1→4)]-β-D-glu-	H
Saikosaponin t	III	β–OH	H	β-D-glu-(1→3)-β-D-fuc-	OCH_3
Hydroxyl saikosaponin a	III	β–OH	OH	β-D-glu-(1→3)-β-D-fuc-	OH
Hydroxyl saikosaponin c	III	β–OH	H	β-D-glu-(1→6)-[α-L-rha-(1→4)]-β-D-glu-	OH
Hydroxyl saikosaponin d	III	α–OH	OH	β-D-glu-(1→3)-β-D-fuc-	OH
Bupleuroside VI	III	β–OH	OH	β-D-glu-(1→3)-β-D-fuc-	C=O
Bupleuroside IX	III	β–OH	H	β-D-glu-(1→3)-β-D-fuc-	OCH_3

Table 3 (*continued*)

Compound	Structure type	R_1	R_2	R_3	R_4
Rotundifolioside A	IV	OH	Nil.	β-D-xyl-(1→2)-β-D-glu-(1→2)-β-D-fuc-	Nil.
Rotundifolioside J	IV	H	Nil.	α-L-rha-(1→2)-β-D-glu-(1→2)-β-D-fuc-	Nil.
Rotundifolioside I	IV	H	Nil.	β-D-xyl-(1→2)-β-D-glu-(1→2)-β-D-fuc-	Nil.
Rotundifolioside B	V	Nil.	Nil.	β-D-xyl-(1→2)-β-D-glu-(1→2)-β-D-fuc-	Nil.
Bupleuroside XIII	VI	Nil.	Nil.	β-D-glu-(1→3)-β-D-fuc-	Nil.

sample 2; peaks 50, 51, 54, 55, 60, 61 and 63 were only found in sample 3; peaks 64–71 and 77–86 were only found in sample 4 which was particularly rich in diacetyl-saikosaponins a or d; and peaks 87–93 were only found in sample 5. The fact that samples 1, 2, 3 and 5 were collected from different areas shows that the location of growth affects the chemical profile. The fact that samples 3 and 4 were from cultivated and wild plants collected at a similar time of year and that sample 5 was a fresh sample indicates that growing conditions affect the chemical profile.

In using principal component analysis to analyze differences among the 5 samples, the loading plot (Fig. 4) shows three distinct groups were separated by the two most important principal components. Thus samples 1 and 2 clustered together, samples 3 and 5 clustered together and sample 4 was separate. The results again indicate that growing area, cultivation technique and collection time affect the chemical profile.

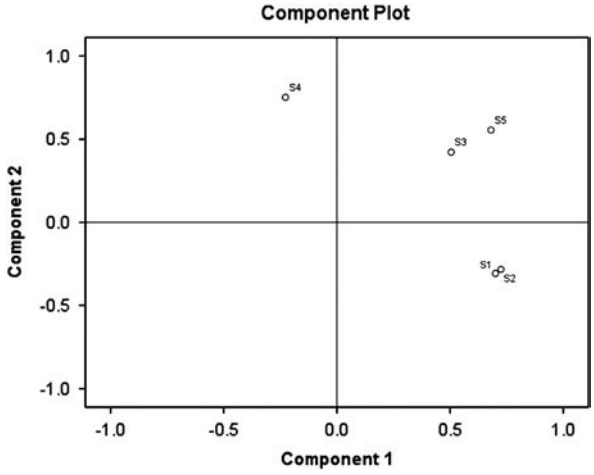


Figure 4 Component plot for components 1 and 2 of samples 1–5 derived by principal component analysis.

3.2. *Microscopic examination*

The transverse sections of the roots of *B. marginatum* showed cork, cortex, phloem, cambium and xylem (Fig. 5). Cork consisted of several layers of flat cells. The cortex and phloem were narrow with scattered oil canals whereas the xylem was broad and occupied more than half of the radius of the root. For samples 2–4, oily drops were found in the vessels. The microscopic features of primary and secondary xylem located in the outer and inner parts of xylem from samples 1 and 2 were different. In the primary xylem, there were more single or grouped vessels with few xylem fibers whereas in the secondary xylem well developed xylem fibers were found. In the xylem of transverse sections from samples 1–4, the xylem rays were not distinct. Interestingly, there were no oil canals in the phloem of fresh sample 5 (Fig. 6). In the xylem of roots of sample 5 with different diameters, the 1.0 cm diameter root contained more xylem fibers than those of the smaller roots. The xylem fibers and vessels contained more peaks than xylem rays in the roots of sample 5 with 0.6 and 1.0 cm diameter.

Using the fluorescence mode, cork showed brown or reddish-brown fluorescence while cortex and phloem showed blue fluorescence. The vessels and xylem fibers showed yellowish-blue fluorescence and xylem rays showed blue fluorescence. Our previous study established that different fluorescence characteristics of herbal tissues reflected different secondary metabolite profiles[17,18]. The various tissues were categorized according to their tissue structures and fluorescence characteristics as shown in Table 4.

3.3. *Tissue-specific chemical profiling*

As the microscopic characteristics of transverse sections from the 5 samples were different, their chemical profiles were analyzed (Table 4). Saikosaponins a, c and d were found as major constituents of cork, cortex, phloem and xylem and many compounds were present in higher amounts in cork, cortex and phloem than in xylem. In addition, more compounds were found in primary xylem with fewer fibers than in secondary xylem with many fibers. For example, in sample 1 the secondary xylem showed 11 peaks while its primary xylem showed 18 peaks. The chemical profiles of cortex and phloem from sample 5 without oil canals also contained many compounds including saikosaponins a, c and d. This suggests that oil canals do not affect the chemical profile.

A previous histochemical study on *B. chinense* demonstrated that saikosaponins are mainly distributed in the pericycle and primary phloem of young roots but in the vascular cambium and secondary phloem of mature roots[19]. Another similar study indicated that saikosaponins were abundant in the cortex outside the cambium but rare in the xylem of the root[20]. The present study provides evidence that the cork, cortex and phloem of roots contain more saikosaponins than xylem. Since the morphological features of medicinal materials are linked to the structures of their inner tissues and the distribution of their chemical components[21],

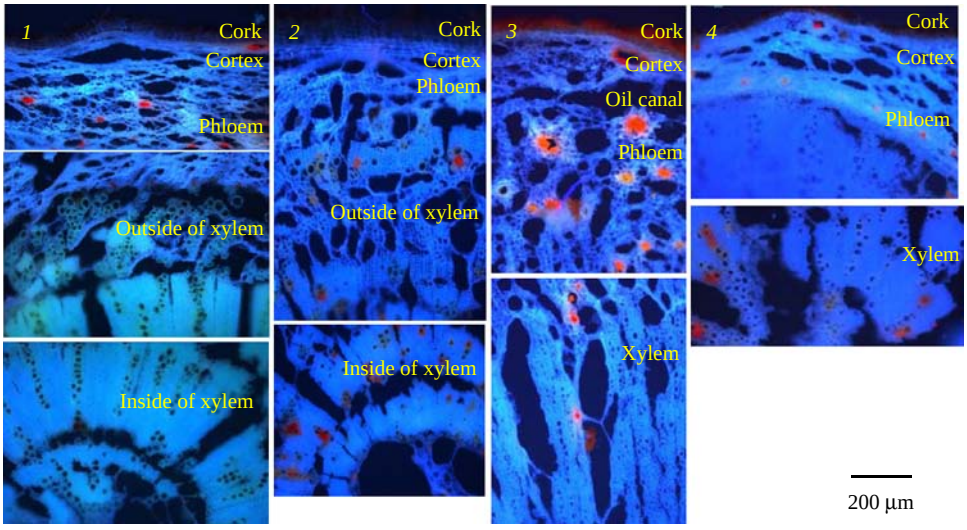


Figure 5 Microscopic characteristics of whole sectioned tissues from the roots of samples 1–4 of *Bupleurum marginatum* Wall. ex DC. investigated in fluorescence mode with dichromatic mirror.

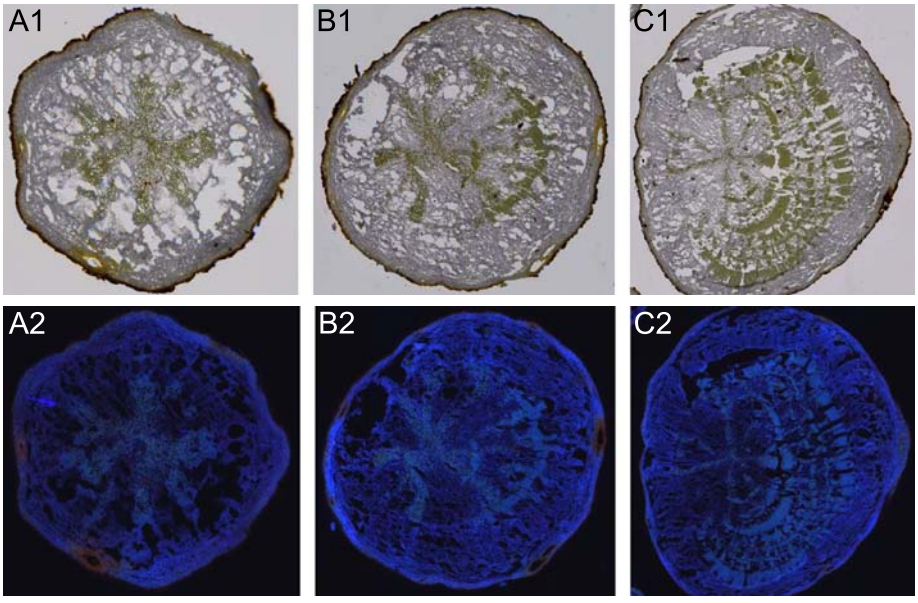


Figure 6 Microscopic characteristics of whole sectioned tissues from the roots of sample 5 of *Bupleurum marginatum* Wall. ex DC with different diameters investigated in normal light (A1–C1) and fluorescence mode with dichromatic mirror (A2–C2). A1 and A2, 0.3 cm; B1 and B2, 0.6 cm; C1 and C2, 1.0 cm.

the results indicate that the roots of *B. marginatum* with a thinner main root and more lateral roots are of better quality.

4. Conclusions

This study shows that the chemical profile and microscopic features of the roots of *B. marginatum* grown in different areas of North Western Hubei Province show considerable variability. Sample 4 from a wild plant grown near Maqiao Town, Baokang County contained more of the pharmaceutically active saikosaponins than sample 3 from a cultivated plant grown in the same area and, in fact, more than any other samples. Therefore, these plants are suitable for selection as the basis for cultivation. However, a comparative study of roots from wild and cultivated plants grown in the same area is needed to better understand the impact of cultivation on the biosynthesis of saikosaponins.

The histochemical study showed that saikosaponins were mainly present in the cork, cortex and phloem. The xylem with little xylem fibers contained more saikosaponins than that with abundant xylem fibers and the presence of oil canals was not associated with any difference in the distribution of saikosaponins.

This study shows that chemical profiling can help in the selection of good germplasm resources for subsequent cultivation of high quality *B. marginatum*, thereby alleviating pressure on shrinking wild resources, and ultimately leading to sustainable use and production of this valuable medicinal herb. Furthermore, the results indicate how the relative quality of *B. marginatum* roots can be assessed.

Table 4 Laser-microdissected tissues from various samples of *Bupleurum marginatum* Wall. ex DC.

Sample No.	Herbal tissues/total microdissected areas (μm^2)					
	Cork	Cortex and phloem	Outside part of xylem	Inside part of xylem	Xylem fibers and vessels	Xylem rays
1	2087379[a]; Peaks 1–6, 8–13, 15–21, 23, 26–28, 32	2402548; Peaks 1–4, 7–21, 23-32	4550940; Peaks 1, 2, 7–9, 12, 13, 15–18, 20, 22, 23, 26–28, 31	4533380; Peaks 1–4, 7–9, 13, 18, 33, 34	N.A.[b]	N.A.
2	4522584; Peaks 1–4, 6, 7–9, 13, 14, 17, 18, 20–22, 35, 37, 38, 40–48	1676317; Peaks 1, 7, 13, 18, 20, 22, 35, 38, 43	4606988; Peaks 1, 2, 6–9, 13, 18, 20, 22, 27, 28, 32, 35–40, 43, 46	4258390; Peaks 1, 2, 6–9, 13, 22, 27, 28, 32, 35–40, 43, 46	N.A.	N.A.
3	4120993; Peaks 3, 7–9, 13, 16, 18, 20–22, 53–62	3973354; Peaks 7–9, 13, 18, 20, 22, 49–52, 58, 59	4328102[c]; Peaks 7, 22, 27, 28, 32, 50, 51, 60, 61		N.A.	N.A.
4	4220799; Peaks 1, 3, 5–9, 11–13, 17, 18, 20, 21, 26, 28, 44, 45, 47, 52, 56, 62, 65–86	4679393; Peaks 1, 3, 5, 7–9, 11, 17,18, 20, 21, 28, 42, 44, 45, 47, 52, 53, 56, 62, 65–79, 81–83, 85	4097598[c]; Peaks 1, 7–9, 17, 18, 20, 21, 26, 32, 42, 44, 45, 47, 52, 53, 62, 65–71, 74, 75, 79, 82, 83		N.A.	N.A.
5 0.3 cm	3144679; Peaks 1, 3–5, 7–9, 18, 20–22, 26, 41, 47, 48, 52, 57–59, 72–76, 84, 87–93	5404640; Peaks 1, 5, 7–9, 13, 18, 20, 21, 32, 47, 48, 57–59, 88	N.A.	N.A.	6371445[c]; Peaks 1, 5, 7–9, 13, 18, 20, 21, 47, 59, 72, 74, 87	
0.6 cm	7439639; Peaks 1, 3–5, 8, 9, 13, 18, 20–22, 26, 27, 32, 41, 47–49, 52,57–59, 72–76, 84, 87–91	8717964; Peaks 1, 5, 7–9, 13, 18, 20, 22, 41, 47–49, 57–59, 73–75, 87–90	N.A.	N.A.	6649913; Peaks 1, 4, 5, 8, 9, 13, 18, 22, 47, 49, 59, 75,84, 87–90	5437265; Peaks 1, 5, 8, 9, 13, 18, 22, 32, 47, 49, 59, 75, 87–90
1.0 cm	2087379; Peaks 1, 3–5, 8, 9, 13,18, 20, 21, 26, 41, 47–49, 52, 57–59, 72–75, 84, 87–90	2402548; Peaks 1, 5, 7–9, 13, 18, 20–22, 26, 41, 47–49, 52, 57–59, 73–75, 84, 87–90	N.A.	N.A.	4550940; Peaks 1, 4, 5, 7–9, 13, 21, 26, 32, 59, 73, 84, 87, 88	4533380; Peaks 1, 5, 8, 9, 13, 21, 26, 32, 59, 75, 84, 87, 88

[a]Dissected areas.
[b]Not applicable.
[c]The whole xylem.

Acknowledgments

We acknowledge Mr. Alan Ho from the School of Chinese Medicine, Hong Kong Baptist University for his technical support. This work was supported by the Hong Kong Research Grants Council (GRF) (HKBU-263412).

References

1. Liang ZT, Qin MJ, Wang ZT. Study on the constituents of the roots of *Bupleurum marginatum*. *J Chin Pharm Univ* 2003;**34**:305–8.
2. Zhao ZZ, Xiao PG. *Encyclopedia on contemporary medicinal plants*, Vol. 1. Shanghai: World Publishing Corporation; 2009, p. 156 [English version].
3. Xu GJ, Xu LS, Wang ZT. *Species systematization and quality evaluation of commonly used Chinese traditional drugs*. Fuzhou: Fujiang Science and Technology Press; 1999.
4. The State Pharmacopoeia Committee. *Pharmacopoeia of the People's Republic of China*. Beijing: China Medical Science and Technology Press; 2010, p. 181.
5. Du SM, Ye F, Yang GY, Wang G, Chen KL. Investigation on *Bupleurum* resources in northwest area of Hubei province. *J Chin Med Mater* 2012;**35**:866–9.
6. Ye F, Yang GY, Du SM, Wang G, Du T, Sun RJ. Identification and quality evaluation of different varieties of *Bupleurum* under different growth conditions in Northwest Hubei. *Chin Pharmacol* 2012;**15**:1380–2.
7. Qiao X, Zhang X, Ye M, Su YF, Dong J, Han J, et al. Rapid characterization of triterpene saponins from *Conyza blinii* by liquid chromatography coupled with mass spectrometry. *Rapid Commun Mass Spectrom* 2010;**24**:3340–50.
8. Xu MJ, Wu B, Ding T, Chu JH, Li CY, Zhang J, et al. Simultaneous characterization of prenylated flavonoids and isoflavonoids in *Psoralea corylifolia* L. by liquid chromatography with diode-array detection and quadrupole time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 2012;**26**:2343–58.
9. Li SH, Schneider B, Gershenzon J. Microchemical analysis of laser-microdissected stone cells of Norway spruce by cryogenic nuclear magnetic resonance spectroscopy. *Planta* 2007;**225**:771–9.
10. Schneider B, Holscher D. Laser microdissection and cryogenic nuclear magnetic resonance spectroscopy: an alliance for cell type-specific metabolite profiling. *Planta* 2007;**225**:763–70.
11. Yi L, Liang ZT, Peng Y, Yao X, Chen HB, Zhao ZZ. Tissue-specific metabolite profiling of alkaloids in *Sinomenii Caulis* using laser microdissection and liquid chromatography–quadrupole/time of flight-mass spectrometry. *J Chromatogr A* 2012;**1248**:93–103.
12. Ng KM, Liang ZT, Lu W, Tang HW, Zhao ZZ, Che CM, et al. *In vivo* analysis and spatial profiling of phytochemicals in herbal tissue by matrix-assisted laser desorption/ionization mass spectrometry. *Anal Chem* 2007;**79**:2745–55.

13. Liang ZT, Qin MJ, Yu GD. The advance on the research of saponins of Bupleurum. *Nat Prod Res Dev* 2001;**13**:67–72.
14. Pan SL, Shun QS, Pai QM, Bao XS. *The coloured atlas of the medicinal plants from Genus Bupleurum in China*. Shanghai: Shanghai Science and Technology press; 2002.
15. Zhou JJ, Xie GR, Yan XJ. *Chemical components of source plants in traditional Chinese medicine*. Beijing: Science Press; 2009.
16. Huang HQ, Zhang X, Lin M, Shen YH, Yan SK, Zhang WD. Characterization and identification of saikosaponins in crude extracts from three *Bupleurum* species using LC–ESI-MS. *J Sep Sci* 2008;**31**:3190–201.
17. Liang ZT, Chen HB, Zhao ZZ. An experimental study on four kinds of Chinese herbal medicines containing alkaloids using fluorescence microscope and microspectrometer. *J Microsc Oxford* 2009;**233**:24–34.
18. Liang ZT, Shi YX, Chen HB, Zhao ZZ. Histochemical analysis of the root tuber of *Polygonum multiflorum* Thunb. (Fam. Polygonaceae). *Microsc Res Tech* 2011;**74**:488–95.
19. Tan LL, Cai X, Hu ZH, Ni XL. Localization and dynamic change of saikosaponin in root of *Bupleurum chinense*. *J Integrative Plant Biol* 2008;**50**:951–7.
20. Du XW, Liu MY. A histochemical study of saikosaponins. *Chin J Mater Med* 1992;**17**:261–3.
21. Zhao ZZ, Liang ZT, Guo P. Macroscopic identification of Chinese medicinal materials: traditional experiences and modern understanding. *J Ethnopharmacol* 2011;**134**:556–64.