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Metabolomic analysis provides novel chemotaxonomic characteristics for phenotypic cultivars of tree peony†

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Metabolomic analysis is an important molecular phenotyping method for understanding plant ecotypic variations. Here, we systematically characterized the metabolomic variations associated with five Chinese cultivars of tree peony (*Paeonia suffruticosa* Andrews) using high performance liquid chromatography-mass spectrometry (HPLC-MS) and multivariate data analysis. Our results indicated that the metabolite profile of the root bark in tree peony was largely dominated by 5 primary metabolites and 41 secondary ones including 7 phenolics, 7 flavonoids, 16 monoterpene glycosides and 11 acetophenones. The distribution of these secondary metabolites varied in the different tree peony cultivars. Some secondary metabolites, such as galloyl glucoses, procyanidins, mudanpiosides and acetophenones, will become the novel and potential chemotaxonomic markers to differentiate tree peony cultivars when the conventional classification methods are not practicable. These results demonstrated that HPLC-MS based metabolomics was an effective tool for the classification of phenotypic cultivars and provided novel and potential chemotaxonomic characteristics of tree peony.

1. Introduction

Tree peony (*Paeonia suffruticosa* Andrews), a woody deciduous shrub, belongs to the section Moutan DC. in the genus Paeonia of the Paeoniaceae family. Tree peonies are well-known garden flowers and very important medicinal plants with about 2000 cultivars in the world. Most of the cultivars with various flower colors have been produced by conventional breeding, especially in China. As a consequence, delimitations of so many cultivars remain a challenge due to the limited morphological markers, the existence of synonyms and homonyms and severe depopulation of maternal lineages.

The traditional classification of tree peony cultivars relied on geography and morphology such as flower colors and forms. For example, the cultivars of tree peony were classified into four groups in China, including cultivars of Zhongyuan (in the Central Plains of China), Xibei (in Northwestern China), Jiangnan (in the Southern region along the Yangtze river) and Xinan (in Southwestern China).[1] A classification of Zhongyuan tree peony cultivars was described by flower color into nine groups, including yellow, red, indigo, white, black, green, purple, pink, and double color flowered cultivars.[2] Similarly, tree peony cultivars in Japan were also classified into six groups by flower color.[3] In most cases, it is difficult and even impossible to discriminate two closely related tree peony cultivars when they are not flowering.

As an alternative method, chemotaxonomy may allow one to utilize secondary metabolites to distinguish tree peony at the species level. Tree peony cultivars were classified into different phenotypes using chromatography according to the chemical structures, compositions and contents of anthocyanins in association with petal coloration;[4,5] petal flavonoid compositions of 39 tree peony cultivars from Xibei (northwest China) were investigated using HPLC in order to obtain the chemotaxonomic relationship among tree peony species;[6] five anthocyanins together with three flavones and three flavonol aglycones were also used as taxonomic markers to classify different tree peony species from China.[7] However, these methods are limited in their ability to fully characterize phenotypic cultivars in that only a small number of markers such as anthocyanins and flavonoids are routinely measured.

In recent studies, some newly developed DNA markers were used as an independent source of taxonomic characteristics. Single nucleotide polymorphism detected from the hyper-variable regions of the chloroplast genome was employed to separate tree peony cultivars into different maternal lineages which can be expressed briefly by a nucleotide molecular formula.[8] Sequence-related amplified polymorphism (SRAP) was used to examine genetic relatedness and genetic diversity in 16 tree peony cultivars having different flower colors.[9] These approaches provided novel evidence and higher resolution ability which are helpful in building an effective classification

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system for evaluation, conservation and utilization of the tree peony germplasm resources; nevertheless, most of the database sequences of tree peony cultivars have either been misidentified or remain unidentified at the species level.

Molecular phenotyping is an important approach for plant functional genomic studies, especially when only subtle or no obvious phenotypic variations are observable at the morphological level.[10] In such cases, analysis of plant metabolite composition and its variations associated with the phenotypes (namely metabolomic analysis) becomes a particularly useful biological approach for phenotypic characterization at the molecular level. This is because metabolomics concerns with the metabolome of the integrated biological systems and dynamic responses to the alteration of endogenous and/or exogenous factors. In fact, metabolomic approaches have already been established as a powerful tool to apply in the metabolic classification of Trichoderma,[11] South American Ilex,[12] Pinus[13] and *Salvia miltiorrhiza* Bunge.[14] However, metabolomic analysis remains challenging for plant molecular phenotypes due to the complexity of plant metabolite composition and limitations of the analytical method.

With the birth of metabolomics, in the past twenty years, HPLC-MS coupled with multivariate data analysis has been applied to analyze metabolite profiles and successfully detect variations in the compositions of phytomedicines, foods and biofluids.[15–17] HPLC-MS based metabolomics has become an indispensable tool to meet the increasing demand for complex sample analysis due to its rapid compound separation, precise mass determination, and high sensitivity and selectivity. In this study, five phenotypic cultivars of tree peony (*Paeonia suffruticosa* Andrews) having different flower colors were selected as excellent challenging examples for plant molecular phenotypes with a complex metabonome. The metabolite compositions of tree peony cultivars were characterized using HPLC-MS in conjunction with multivariate data analysis. The aims of this study were (1) to explore the feasibility of HPLC-MS methods for the identification and classification of tree peony cultivars with different molecular phenotypes and (2) to provide the potential taxonomic characteristics of the different tree peony cultivars from the same growing location.

2. Materials and methods

2.1 Chemicals

HPLC-grade acetonitrile and methanol from Fisher Scientific Products of the United States were used for analysis. Analytical-grade ammonium acetate was obtained from Tianjin Hengxing Reagent Company (Tianjin, PR China). Water was purified with a Milli-Q academic water purification system (Millipore, Bedford, MA, U.S.A.).

2.2 Sample collection and extraction

Five representative cultivars of tree peony (*Paeonia suffruticosa*, Zhongyuan group) having different color flowers at the age of 3 were collected from the Heze Xinjun Peony Garden in Shandong province, China, in October 2012. Their floral colors were quantified by the Royal Horticultural Society Color Chart (RHSCC). The five cultivars, including 'Xiangyu' with white flowers (RHSCC = 155D), 'Wujinyaohui' with blackish purple flowers (RHSCC = 187D), 'Yaohuang' (RHSCC = 8D) with light yellow flowers, 'Yingrihong' with red flowers (RHSCC = 52C) and 'Roufurong' with deep pink flowers (RHSCC = 52D), were identified by Professor Minfeng Fang. 5 plants of each cultivar were gathered and their main roots (diameter 8–9 mm) were carefully separated. The roots were peeled and the internal cores were eliminated according to the Chinese Pharmacopoeia (2010 edition, vol. 1, pp. 160–161). The remaining materials, called 'Mudanpi' in Chinese, were immediately dried at 40 °C for 24 h.

The dried 'Mudanpi' materials were ground and strained through a 2 mm sieve. In all cases, the raw materials (1.0 g) were extracted in a flask with 25% (v/v) aqueous methanol (50 mL) by vortexing for 30 s followed by continuous ultrasonication in an ice water bath for 30 min. The stock solutions were centrifuged at 8000 rpm for 10 min. The supernatants were stored at 4 °C in a refrigerator and filtered through a 0.45 μm membrane filter before HPLC-MS analysis.

2.3 HPLC-MS measurements

HPLC-MS analysis of 25 samples was performed using a G6520 LC/MS Q-TOF system (Agilent Technologies, USA.) with an Agilent-1200 HPLC system coupled to a quadrupole time-of-flight tandem mass spectrometer *via* an electrospray ionization source (ESI). Analytical HPLC was performed using a system including a G1312A binary pump, a G1322A vacuum degasser, a G1329 auto sample injector and a G1316A column oven. The chromatographic separation was conducted on an Agilent Extend-C18 column (150 mm × 4.6 mm, 5 μm) at 30 °C with an injection volume of 20 μL. The elution was performed using water containing 2 mM ammonium acetate (solvent A) and acetonitrile (solvent B) with a step gradient lasting 45 min at a flow rate of 0.6 mL min^{-1}. Solvent B was linearly increased from 15% at 0 min to 28% at 13 min, maintained at 28% until 22 min, increased from 28% to 58% from 22 min through 37 min and then maintained at 58% until 45 min. Approximately 10% of the eluent was directed to MS using a splitter valve. The ESI source in negative ionization mode was operated with a nebulizer pressure of 40 psi, while the drying gas was delivered at a flow rate of 10 L min^{-1} at 350 °C. The capillary voltage, skimmer voltage and fragmentor voltage were set at 4000 V, 65 V and 170 V, respectively. MS spectra at TOF mode and MS/MS spectra at targeted MS/MS mode were acquired in a scan range between m/z 100 and m/z 1000 at a rate of 1 spectrum per s. A set of collisions at energy levels of 10, 20, 30 and 40 eV were performed using targeted data-dependent acquisition. Reference solutions containing ions of m/z 112.9855 and 1033.9881 were continuously introduced into the MS system during the analysis procedure to ensure the accuracy of the measured mass.

2.4 Data analysis

The molecular feature extraction (MFE) algorithms in the Mass Hunter workstation data acquisition software (V.4.0 Qualitative

Analysis; Agilent) were used to extract molecule ions from the total ion chromatogram (TIC). The abundance of each compound, which was defined as the sum of the isotopic peaks, was calculated using the adduct ion peaks and the base peak. The MFE parameters were chosen as follows: compound filters were chosen at an absolute height of more than 10 000 counts; peak filters were set to centroid height with more than 100 counts; and the assigned charge state was limited to a maximum of 2. The calculated results were converted to '.cef' files and then imported into the Mass Profiler Professional (MPP) software (V. B 12.00, Agilent) for further analysis. Accurate masses and retention times for the peaks in each sample were calculated, and the peaks of different samples were then aligned through the mass range of *m*/*z* 100–1000, mass tolerance of 10 ppm, mass window of 0.002 Da and retention time window of 0.5 min followed by normalization of the abundance values of the peaks. The peaks appearing in at least 80% of the samples in at least one group were retained, and a data matrix (25 × 598) was established for principal component analysis (PCA) with mean-centered scaling.

The compounds in the data matrix were further filtered by one-way analysis of variance (ANOVA) and fold change (FC). The FC value of each metabolite stood for the relative level which was calculated as the ratio of the average peak intensity between two groups. The *p* value was calculated by the *t*-test. The compounds with FC > 2 and $p < 0.05$ were selected and defined as potential differential metabolites. To reduce the error caused by the statistical analysis, the potential differential metabolites exported from MPP were re-imported into the Mass Hunter software to examine the ion peak area and symmetry. The extracted ion chromatograms of the same metabolites from different samples were assembled to check whether there were obvious differences between groups. The differential molecules with good peak profiles were retained and were identified using the accurate mass and tandem mass spectra in reported papers in addition to the information in the Agilent Personal Compound Database and Library (PCDL) and online databases such as METLIN (METabolite LINk) (http://metlin.scripps.edu) and the Human Metabolome Database (HMDB) (http://www.hmdb.ca).

3. Results and discussion

3.1 HPLC-MS analysis of metabolite compositions in tree peony

Fig. 1 shows typical total ion chromatograms (TICs) of the root bark extracts derived from five tree peony cultivars, namely, 'Xiangyu', 'Wujinyaohui', 'Yaohuang', 'Yingrihong' and 'Roufurong'. The root bark extracts from the different tree peony cultivars contained the marked concentration differences for many metabolites (*e.g.*, peak 18 and peak 46), although the types of metabolites were broadly similar. The peaks were assigned to individual metabolites based on the HPLC retention time, ultraviolet-visible absorption data and the molecular ions and major fragments observed in MS or MS/MS spectra, followed by searching in the METLIN, PCDL and HMDB databases in conjunction with the reference data (Table 1). A total of 46 metabolites were characterized in this study, including 5 primary metabolites (malic acid, peak 1; citric acid, peak 2; sucrose, myoinositol, peak 4; peak 5; disaccharide, peak 6) and 41 secondary metabolites (phenolics, flavonoids, monoterpene glycosides and acetophenones). The MS or MS/MS spectra of these identified metabolites are provided in the ESI (Fig. S1†).

A number of phenolic compounds from *Paeonia* species have been previously observed.[18–24] This study indicated that 3 phenolic acids, including gallic acid, methyl gallate and hydroxybenzoic acid, were detected in the five tree peony cultivars (Fig. 2). The MS/MS pattern of gallic acid showed $[M - H]^-$ at *m*/*z* 169 (peak 3) and $[M - H - 44]^-$ at *m*/*z* 125. The ion $[M - H]^-$ at *m*/*z* 137 (peak 14) and product ions at *m*/*z* 93 and 65, presumably originating from successive loss of CO_2 and CO, were shown in the MS/MS spectrum of hydroxybenzoic acid. The ion $[M - H]^-$ at *m*/*z* 183 (peak 17), combined with neutral losses of 15 Da for CH_3 and 44 Da for a CO_2 group, was assigned to methyl gallate. Moreover, 4 galloyl glucosides, including glucogallin (peak 7), galloylsucrose (peak 8), mudanoside B (peak 9) and pentagalloyl glucose (peak 44), were characterized in the present study (Fig. 2). It is well documented that galloyl glucosides contain different numbers of glucose moieties and gallic acid moieties. Accordingly, galloyl glucosides consist of a common daughter ion at *m*/*z* 169 for a gallic acid moiety and a neutral loss of 162 Da for a glucose moiety.[25] At times, a neutral loss of 44 Da for a CO_2 group and a neutral loss of 170/152 Da for a galloyl group were also observed for galloyl glucosides.

Flavonoids, such as catechin glucoside at *m*/*z* 451 (peak 11), procyanidin B at *m*/*z* 577 (peak 12) and catechin at *m*/*z* 289 (peak 15), were observed in the five tree peony cultivars (Fig. 2). The MS or MS/MS spectra of these metabolites were consistent with the literature data.[25] They showed two common neutral losses of 152 Da for $C_8H_8O_3$ and 138 Da for $C_7H_6O_3$ as well as two fragment ions at *m*/*z* 137 and *m*/*z* 151 because of Retro Diels–Alders cleavage, suggesting the presence of a catechin unit. The fragmentation ion of $[M - H - 162]^-$ at *m*/*z* 289 indicated that the molecule of catechin glucoside contained a glucose moiety and a catechin moiety. The fragmented patterns of the ions $[M - H]^-$ at *m*/*z* 577, $[M - H - 152]^-$ at *m*/*z* 425, $[M - H - 289]^-$ at *m*/*z* 289, $[M - H - 152 - 152]^-$ at *m*/*z* 273 and $[M - 289 - 152]^-$ at *m*/*z* 137 demonstrated the presence of the two catechin units linked either C4–C6 or C4–C8 (B-type procyanidin); it can be assigned tentatively to procyanidin B due to the lack of sufficient configuration information. In addition to the above constituents, kaempferol dihexoside (peak 13), quercetin-3,7-di-*O*-glucoside (peak 21), isorhamnetin-3,7-di-*O*-glucoside (peak 27) and trihydroxytrimethoxyflavanone (peak 43), were also present in the tree peony cultivars. The MS data were consistent with the presence of the flavonoids in the flowers of *Paeonia suffruticosa*.[26,27]

Monoterpenes, especially monoterpene glycosides, are the major bioactive constituents of *Paeonia* species that have been reported in the literature.[22,28–32] In this work, 16 monoterpene glycosides were characterized, including oxypaeoniflorin (peak 16), galloyloxypaeoniflorin (peak 20), 4-*O*-methyl-4′-hydroxy-3′-methoxy-paeoniflorin (peak 23), paeoniflorin (peak 24), galloylpaeoniflorin (peak 31), mudanpioside H (peak 32),

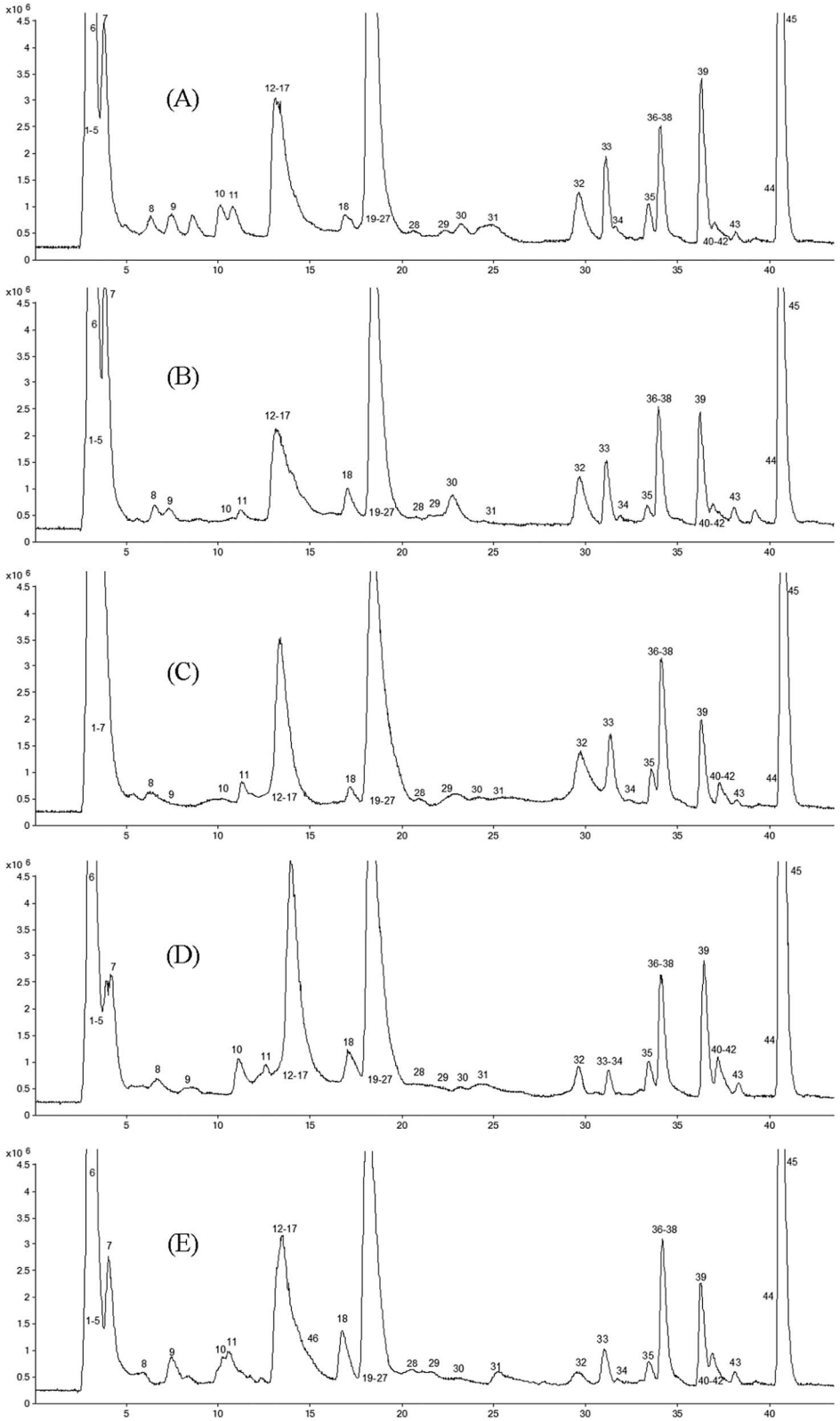

Fig. 1 Typical total ion chromatograms (TICs) of the root bark extracts from the different tree peony cultivars including 'Xiangyu' (A), 'Wujinyaohui' (B), 'Yaohuang' (C), 'Yingrihong' (D) and 'Roufurong' (E).

benzoyloxypaeoniflorin (peak 34), paeobrin (peak 35), suffruyabioside B (peak 38), benzoylpaeoniflorin (peak 39) and mudanpioside-A (peak 41), -B (peak 40), -C (peak 36), -E (peak 22), -F (peak 10) and -J (peak 37). The chemical structures of these monoterpene glycosides contain a common pinane skeleton and mono-dimer glucose moieties, and certain

Table 1 Characterization of the dominant metabolites from the root bark of tree peony

No.	RT/min	Metabolites	Formula	UV/nm	$[M-H]^-$	MS/MS	Mass error[a]/ppm	Database	Ref.
1	2.65	Malic acid	$C_4H_6O_5$	210	133.0137		4.08	METLIN, PCDL, HMDB	
2	2.76	Citric acid	$C_6H_8O_7$	210	191.0195		1.18	METLIN, PCDL, HMDB	
3	2.81	Gallic acid	$C_7H_6O_5$	220, 271	169.0133	125, 97, 81, 69	5.57	METLIN, PCDL, HMDB	
4	2.92	Myoinositol	$C_6H_{12}O_6$		179.0551	161, 141, 87, 71, 59, 55	5.62	METLIN, HMDB	
5	3.01	Sucrose	$C_{12}H_{22}O_{11}$		341.1096	179, 119, 89, 59	−1.94	METLIN, PCDL, HMDB	
6	3.23	Disaccharide	$C_{12}H_{22}O_{10}$		325.1148	193, 161, 101, 89, 71, 59	−2.39	METLIN, HMDB	
7	3.79	Glucogallin	$C_{13}H_{16}O_{10}$	275	331.0680	271, 211, 169, 125	−2.80	METLIN, PCDL	25
8	6.41	Galloylsucrose	$C_{19}H_{26}O_{15}$	275	493.1206	331, 313, 179, 169, 125, 89, 59	−1.43	METLIN	25
9	7.52	Mudanoside B	$C_{18}H_{24}O_{14}$	218, 275	463.1088	403, 373, 343, 301, 241, 169	1.14		27
10	10.13	Mudanpioside F	$C_{16}H_{24}O_8$	252	343.1402	181, 151	−1.04		31
11	10.87	Catechin glucoside	$C_{21}H_{24}O_{11}$	256	451.1240	289, 245, 151, 137, 109	1.29	METLIN, PCDL	25
12	13.25	Procyanidin B*	$C_{30}H_{26}O_{12}$	280	577.1356	425, 289, 273, 137, 125	−0.78	METLIN, PCDL	25
13	13.34	Kaempferol dihexoside*	$C_{27}H_{30}O_{16}$	266, 346	609.1461		0.01	METLIN, PCDL	26
14	13.51	Hydroxybenzoic acid	$C_7H_6O_3$	260	137.0233	93, 65	8.10	METLIN, PCDL	27
15	13.67	Catechin	$C_{15}H_{14}O_6$	256	289.0724	245, 221, 151, 137, 125	−2.20	METLIN, PCDL	27
16	13.79	Oxypaeoniflorin	$C_{23}H_{28}O_{12}$	258	495.1506	281, 165, 137	0.40	PCDL	27
17	14.22	Methyl gallate	$C_8H_8O_5$	215, 272	183.0302	168, 124	−1.65	METLIN, PCDL	27 and 28
18	16.87	Paeonolide	$C_{20}H_{28}O_{12}$	224, 266, 295	459.1511	293, 233, 165	−0.65	METLIN, PCDL	27
19	17.98	Suffruticoside E	$C_{26}H_{38}O_{17}$	215, 268	621.2054	455, 293, 165, 161, 150, 131, 89, 59	−2.86		18
20	18.14	Galloyloxypaeoniflorin	$C_{30}H_{32}O_{16}$	215, 267	647.1612	491, 399, 313, 271, 211, 169, 137	0.86		18
21	18.25	Quercetin 3,7-di-*O*-glucoside*	$C_{27}H_{30}O_{17}$	256, 353	625.1410		0.04	PCDL	26
22	18.43	Mudanpioside E	$C_{24}H_{30}O_{13}$	221, 265, 291	525.1613	495, 363, 313, 167, 165, 123	0.12		27 and 31
23	18.68	4-*O*-Methyl-4′-hydroxy-3′-methoxy-paeoniflorin*	$C_{25}H_{32}O_{13}$	228, 283	539.1775		−0.90		29
24	18.83	Paeoniflorin	$C_{23}H_{28}O_{11}$	230	479.1572	449, 327, 165, 121, 77	−2.74	METLIN, PCDL	27
25	18.99	Suffruticoside B	$C_{27}H_{32}O_{16}$	219, 272	611.1616	445, 343, 313, 301, 283, 169, 165	0.26	PCDL	18
26	19.12	Hydroxyacetophenone	$C_8H_8O_2$	220, 275	135.0446	120, 92, 65	4.06	METLIN, PCDL	33
27	19.18	Isorhamnetin 3,7-di-*O*-glucoside*	$C_{28}H_{32}O_{17}$	252, 352	639.1567		−0.04	PCDL	26
28	20.45	Suffruticoside A	$C_{27}H_{32}O_{16}$	219, 272	611.1619	445, 343, 301, 283, 169, 165	−0.23	PCDL	18
29	22.31	Suffruticoside D	$C_{27}H_{32}O_{16}$	219, 272	611.1671	445, 343, 313, 301, 283, 169, 165, 125, 89, 59	−8.73	PCDL	18
30	23.22	Suffruticoside C	$C_{27}H_{32}O_{16}$	219, 272	611.1611	445, 343, 301, 283, 169, 165, 125, 89, 59	1.08	PCDL	18
31	25.05	Galloylpaeoniflorin	$C_{30}H_{32}O_{15}$	218, 274	631.1685	479, 313, 169, 151, 121	−2.62		33
32	29.71	Mudanpioside H	$C_{30}H_{32}O_{14}$	258	615.1710	585, 477, 447, 431, 281, 239, 137	1.51		27
33	31.18	Dihydroxyacetophenone	$C_8H_8O_3$	212, 275	151.0396	135, 108, 91	3.08	METLIN, PCDL	35
34	31.45	Benzoyloxypaeoniflorin	$C_{30}H_{32}O_{13}$	210, 259	599.1782	551, 447, 431, 281, 137, 121	−1.97	PCDL	27
35	33.58	Paeobrin	$C_{23}H_{28}O_{10}$	227, 272	463.1616	301, 179, 121	−1.36		30
36	34.05	Mudanpioside C	$C_{30}H_{32}O_{13}$	231, 258	599.1777	477, 281, 165, 137, 121	−1.14		27 and 31
37	34.11	Mudanpioside J	$C_{31}H_{34}O_{14}$	297, 222	629.1875	599, 477, 315, 167, 165, 151, 137	0.13		27
38	34.16	Suffruyabioside B	$C_{36}H_{42}O_{17}$	226, 258	745.2359	715, 623, 501, 461, 121	−1.31		21
39	36.42	Benzoylpaeoniflorin	$C_{30}H_{32}O_{12}$	230	583.1827	535, 431, 165, 121	−1.03	PCDL	27
40	37.02	Mudanpioside B	$C_{31}H_{34}O_{14}$	258	629.1858	583, 553, 535, 431, 165, 137	2.82		27 and 31
41	37.11	Mudanpioside A	$C_{31}H_{34}O_{13}$	233, 257	613.1927	461, 431, 165, 151, 121	−0.06		27 and 31
42	37.37	Hydroxypaeonol	$C_9H_{10}O_4$	212, 275	181.0508	166, 138, 123, 95, 83, 65	−0.92	METLIN	35

Table 1 (*Contd.*)

No.	RT/min	Metabolites	Formula	UV/nm	$[M-H]^-$	MS/MS	Mass error[a]/ppm	Database	Ref.
43	38.16	Trihydroxytrimethoxy-flavanone*	$C_{18}H_{18}O_8$	270, 330	361.0929	181, 166	−0.02	METLIN	
44	40.39	Pentagalloyl glucose	$C_{41}H_{32}O_{26}$	215, 279	469.0518	769, 617, 393, 317, 241, 169, 125	0.03		27
45	40.87	Paeonol	$C_9H_{10}O_3$	228, 274, 310	165.0571	150, 135, 122, 91	−8.32	METLIN, PCDL	27
46	14.33	Apiopaeonoside	$C_{20}H_{28}O_{12}$	222, 266, 293	459.1518	293, 165	−2.17	PCDL	27

[a] Obtained as (theoretical mass − experimental mass)/theoretical mass; * the tentative assignment.

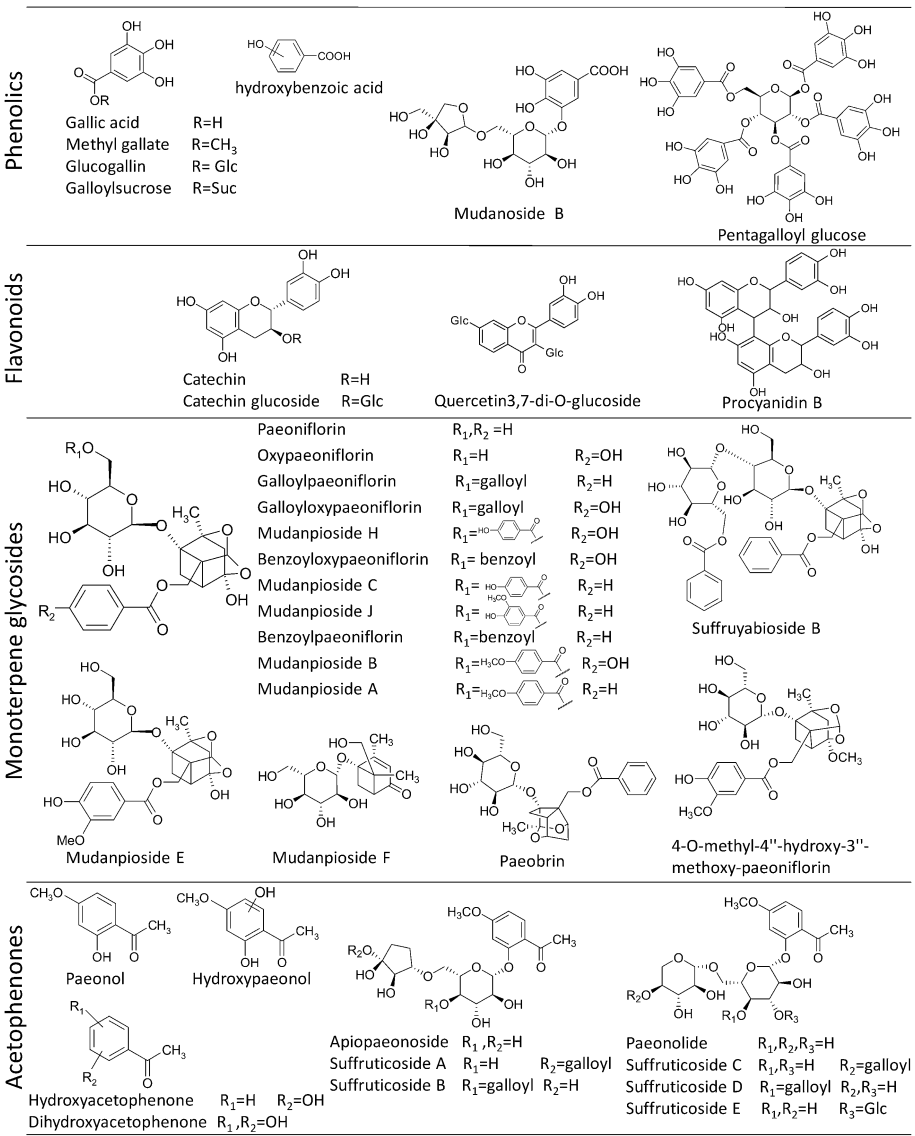

Fig. 2 Chemical structures of the major metabolites in the root bark extracts of tree peony.

monoterpene glycosides are esterified on the glucose moiety by an aromatic acid, such as benzoic acid, *p*-hydroxybenzoic acid/*p*-methoxybenzoic acid and gallic acid (Fig. 2). For these metabolites, the ion at *m*/*z* 165 was assigned to the pinane skeleton in the chemical structures; the ions at *m*/*z* $[M - H - 162]^-$ or $[M - H - 180]^-$ strongly suggested that these molecules were hexose-conjugated compounds, while the ions at *m*/*z* 121, 137, 151 and 167 indicated the presence of benzoic acid, *p*-hydroxylbenzoic acid, *p*-methoxyhydroxybenzoic acid and vanillic acid moieties, respectively. The fragmentation patterns of monoterpene glycosides using four levels of collision energy are shown in Fig. 3. The lowest collision energy (10 eV) was preferable to fragment the substituent group (R_1) linked to the glucose moiety; the 20 eV collision energy caused the loss of both the R_1 group and the benzoic acid moiety conjugated on the pinane skeleton, and the higher collision energies (30 and 40 eV) tended to simultaneously remove the R_1 group, the benzoic acid moiety and the glucose moiety. The MS/MS spectra of galloylpaeoniflorin are shown as representative examples of the fragmentation patterns at the different collision energy levels (Fig. S2†).

11 acetophenones observed in tree peony cultivars are assigned to paeonol and its derivatives, including apiopaeonoside, paeonolide, suffruticoside-A, -B, -C, -D and -E, hydroxyacetophenone, dihydroxyacetophenone and hydroxypaeonol (Fig. 2). The ion $[M - H]^-$ at *m*/*z* 165 (peak 45) and three major product ions at *m*/*z* 150, 135 and 122 were observed in the MS/MS spectrum of paeonol, the ion $[M - H - 15]^-$ at *m*/*z* 150 indicated the presence of a methyl group, and the neutral loss of 30 Da (*m*/*z* 165 to 135) could be attributed to the loss of a HCHO group originating from the methoxy radical, and the ion $[M - H - 43]^-$ at *m*/*z* 122 most likely resulted from the loss of an acetaldehyde group. The MS/MS spectra of the acetophenones, including hydroxyacetophenone at *m*/*z* 135 (peak 26), dihydroxyacetophenone at *m*/*z* 151 (peak 33) and hydroxypaeonol at *m*/*z* 181 (peak 42) showed a common neutral loss of 43 Da for an acetyl group linked to a benzene ring. The MS/MS patterns of paeonolide at *m*/*z* 459 (peak 18), suffruticoside E at *m*/*z* 621(peak 19), suffruticoside-A -B -C -D at *m*/*z* 611 (peak 28, peak 25, peak 30, peak 29, respectively), suffruyabioside B at *m*/*z* 745 (peak 38) and apiopaeonoside at *m*/*z* 459 (peak 46) showed a neutral loss of 162 Da for a glucose moiety, 132 Da for a pentose moiety and 43 Da for an acetyl group, thereby, indicating that these molecules were hexose-, pentose- and acetyl-conjugated compounds. These results were consistent with the findings in previous reports.[18,28,33–36]

3.2 Principal component analysis for the different tree peony cultivars

Fig. 4 displays a 3D PCA score plot of root bark extracts from the five tree peony cultivars. Samples from the same cultivars clustered together closely, indicating their similar metabolite compositions and excellent reproducibility of the extraction procedures and the HPLC-MS measurements. Moreover, an obvious classification was present for the samples from 'Xiangyu', 'Wujinyaohui', 'Yaohuang', 'Yingrihong' and 'Roufurong', suggesting large differences in metabolite compositions among the different cultivars. To understand the significance of the metabolites' contribution to classification, MPP and Mass Hunter software were employed to explore the differential metabolites from the different cultivars.

3.3 Metabolomic variations from the different cultivars of tree peony

The values of FC denoted the levels of these metabolites in four cultivars relative to that in 'Xiangyu'. 19 metabolites were defined as the differential metabolites from the individual cultivars according to $p < 0.05$ and FC > 2 (or $\log_2$FC > 1 in Table 2). Different kinds of metabolites such as phenolics,

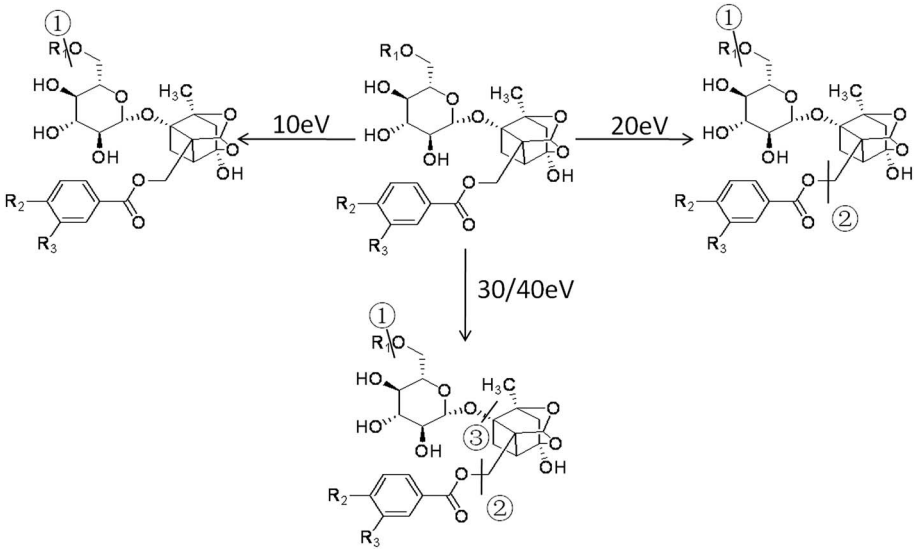

Fig. 3 Fragmentation patterns observed in the MS/MS spectra of monoterpene glycosides at different collision energies (10 eV, 20 eV, 30 eV and 40 eV).

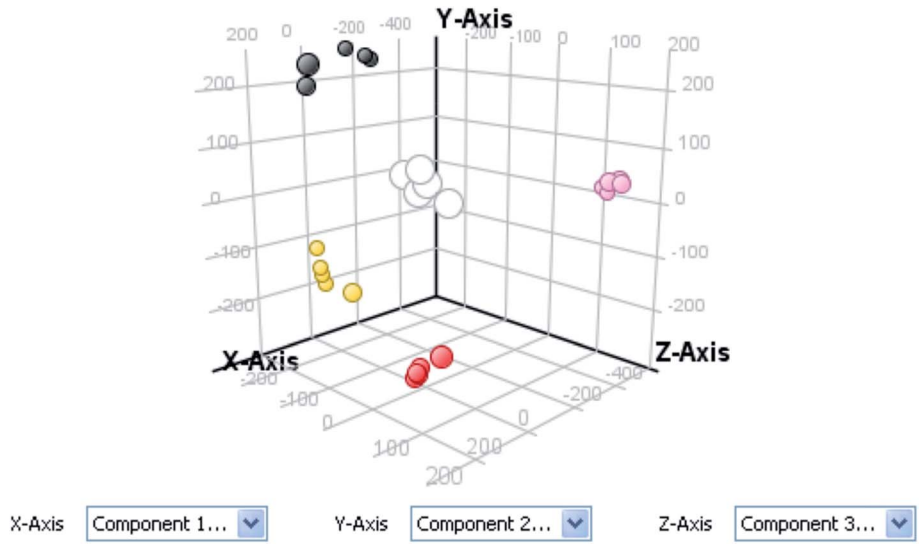

Fig. 4 The 3D PCA scores plot of the root bark extracts from five tree peony cultivars including 'Xiangyu' (white), 'Wujinyaohui' (black), 'Yaohuang' (yellow), 'Yingrihong' (red) and 'Roufurong' (pink).

Table 2 The levels of metabolites in tree peony cultivars relative to that in 'Xiangyu'

		$\log_2$FC[a]			
	Metabolites[b]	'Wujinyaohui'[c] (blackish purple)	'Yaohuang'[c] (light yellow)	'Roufurong'[c] (red)	'Yingrihong'[c] (pink red)
1. Phenolics					
	Gallic acid	−0.7	0.2	−0.1	−1.2
	Glucogallin	−2.9	−21.8	−14.7	0.8
	Methyl gallate	8.6	−17.1	9.1	−0.3
	Pentagalloyl glucose	−12.7	−11.8	0.4	7.6
	Mudanoside B	7.1	13.9	0	21.7
2. Flavonoids					
	Catechin glucoside	0	6.5	21.1	22.9
	Procyanidin B	0	0	21.7	22.1
	Catechin	−1.6	0.1	1.0	1.3
	Quercetin 3,7-di-*O*-glucoside	0	19.9	0	12.7
3. Monoterpene glycosides					
	Oxypaeoniflorin	−1.2	0.7	−0.5	1.6
	Galloyloxypaeoniflorin	−21.9	−15.1	−1.7	0.4
	Galloylpaeoniflorin	−22.9	−21.9	−1.4	−1.5
	Mudanpioside H	−21.5	0.02	−0.02	1.4
	Mudanpioside J	−18.8	−12.5	−0.07	1.6
4. Acetophenones					
	Paeonolide	0.6	1.1	1.5	0.7
	Suffruticoside B	−8.5	−21.1	0.07	−7.3
	Suffruticoside D	−15.6	−22.1	0.6	−1.0
	Hydroxypaeonol	−0.08	0.4	1.3	1.1
	Apiopaeonoside	0	0	20.8	0

[a] $\log_2$FC means $\log_2$[ratio of average peak intensity in tree peony cultivars to that in 'Xiangyu' with white flowers]. [b] The differential metabolites resulting in the classification of five tree peony cultivars according to $p < 0.05$ and FC > 2 (or $\log_2$FC > 1). [c] The tree peony with the flower color.

flavonoids, monoterpene glycosides and acetophenones were contributable to the classification of tree peony cultivars. The phenolic compositions were not identical in each sample. The slight but statistically significant decrease of gallic acid was observed in 'Yingrihong' but the levels of gallic acid were less variable in other cultivars; 'Roufurong' contained the highest

concentration of methyl gallate followed by 'Wujinyaohui', whereas 'Yaohuang' showed the lowest; 'Yingrihong' showed the highest contents of penta-*O*-galloyl glucose and mudanoside B among these five cultivars; 'Yaohuang' had the lowest content of glucogallin and methyl gallate than other cultivars. This result indicated that the accumulation of phenolic constituents may differ quite markedly in diverse cultivars of tree peony. Gallic acid metabolites are universally distributed in plants; taxonomic use has been made of the distribution of esters and derivatives of gallic acids.[37] For example, ellagitannins have been used as prominent chemotaxonomic markers due to their wide distributions in the Hamamelidae, Dilleniidae and Rosidae.[38]

As intermediates of the hydrolysable tannin biosynthesis pathway (Fig. 5A), the slight decrease of gallic acid and the increase of penta-*O*-galloyl glucose were observed in 'Yingrihong', indicating that the metabolic process of gallic acid was activated in this tree peony cultivar. However, the lower levels of glucogallin and penta-*O*-galloyl glucose in 'Yaohuang' and 'Wujinyaohui' suggested that the metabolic pathway of gallic acid was inhibited in tree peony cultivars with yellow and blackish purple flowers. The distinctive and unique features of gallic acid metabolism in higher plants demonstrated that gallic acid occurs most often in association with sugars, in particular D-glucose, resulting in galloyl-D-glucose derivatives such as β-D-glucogallin and β-penta-*O*-galloyl-D-glucose.[39] β-Penta-*O*-galloyl-D-glucose is noteworthy in plants because this intermediate appears to mark a biogenetic watershed from which other biosynthetic pathways subsequently diverge.[35] Therefore, penta-*O*-galloyl glucose, a key secondary metabolite in gallic acid metabolism, is likely to become a chemotaxonomic marker in tree peony cultivars.

Four flavonoids including quercetin 3,7-di-*O*-glucoside, procyanidin B, catechin and catechin glucoside changed in these tree peony cultivars. Quercetin 3,7-di-*O*-glucoside displayed higher concentration in 'Yaohuang' than in other cultivars, which corresponded to its higher content detected in tree peony with yellow flowers in the previous report.[27] The levels of procyanidin B, catechin and catechin glucoside show an increasing trend from 'Wujinyaohui', through 'Xiangyu', 'Yaohuang', 'Roufurong' to 'Yingrihong'. These metabolites related to anthocyanidin biosynthesis showed the highest levels in 'Yingrihong' among these cultivars, which is roughly in accordance with the previous finding that dark red cultivars contained a large amount of anthocyandins in the petals whereas pink or white cultivars contained a slight amount.[40] Procyanidin B, the dimer of catechin units or epicatechin units, is the most common class of proanthocyanidins. Based on the proanthocyanidin biosynthesis pathway (Fig. 5B), it can be inferred that the biosynthesis of procyanidins was more active in 'Yingrihhong' than other cultivars. The structure and content of anthocyandins, such as cyanidin, pelargonidin, peonidin and their glucosides, are universally analyzed, which were responsible for the coloration mechanism and the classification for the different tree peony cultivars.[4,5,41] In the current study, those routinely measured anthocyanidins were not detected in the root barks from these tree peony cultivars presumably because they were mainly distributed in the petal of tree peony. The observed metabolites including procyanidin B, catechin and catechin glucoside were associated with anthocyanidins such as cyanidin (Fig. 5B), namely, there was a connection between these identified metabolites and flower colors. This result was in agreement with the flower colors as the criteria in the conventional classification method. Therefore, procyanidin B,

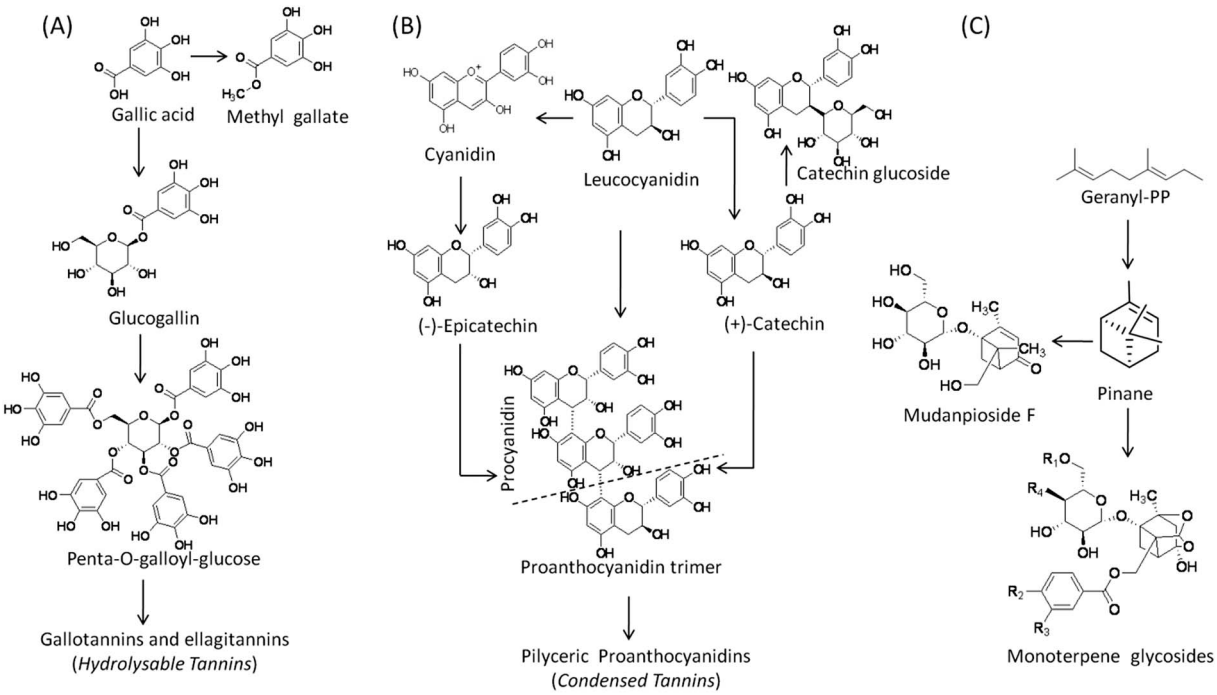

Fig. 5 The schematic plot of the metabolic pathways for hydrolysable tannins (A), condensed tannins (B) and monoterpene glycosides (C).

catechin and catechin glucoside observed in the root bark of tree peony will become the novel chemotaxonomic markers for classification of tree peony cultivars when they have no flowers.

Monoterpenes and acetophenones are major bioactive members of *Paeonia* species and are generally the key metabolites for quality assessment when tree peony is used as a medicinal plant. The levels of monoterpene glycosides and acetophenones varied in different cultivars of tree peony (Table 2). The cultivar 'Yingrihong' gave the highest values of mudanpioside H, mudanpioside J and oxypaeoniflorin whereas 'Wujinyaohui' contained the lowest levels of mudanpioside H, mudanpioside J and oxypaeoniflorin together with galloyloxypaeoniflorin and galloylpaeoniflorin. On the basis of the monoterpenoid biosynthesis pathway (Fig. 5C), the higher levels of monoterpene glycosides in 'Yingrihong', *e.g.* modanoside B, mudanpioside H and mudanpioside J, may contribute to the activated metabolic pathway from pinane to monoterpene glycosides. In addition, the higher levels of acetophenones including paeonolid, hydroxypaeonol and apiopaeonoside were observed in 'Roufurong' than in other cultivars, and the lowest levels of suffruticoside B and suffruticoside D were shown in 'Yaohuang'. To the best of our knowledge, a chemical classification of tree peony cultivars using monoterpene glycosides and acetophenones has been not proposed. Therefore, monoterpene glycosides and acetophenones might become the novel chemotaxonomic makers to differentiate these tree peony cultivars.

4. Conclusion

Metabolomic variations from different tree peony cultivars were systematically characterized using an HPLC-MS based metabolomic approach. A total of 46 metabolites were identified, including 5 primary metabolites and 41 secondary ones (7 phenolics, 7 flavonoids, 16 monoterpene glycosides and 11 acetophenones). In comparison with the 'Xiangyu' having white flowers, the 'Yingrihong' cultivar with red flowers showed the higher levels of phenolics and flavonoids including pentagalloyl glucose, mudanoside B, procyanidin B, catechin and catechin glucose, and the higher levels of monoterpene metabolites including mudanpioside H and mudanpioside J; 'Roufurong' with pink flowers contained a higher content of acetophenones including paeonolid, hydroxypaeonol and apiopaeonoside; 'Yaohuang' with yellow flowers contained a higher level of quercetin 3,7-di-*O*-glucose; 'Wujinyaohui' with blackish purple flowers lacked most of the above phenolics, flavonoids, monoterpenes glycosides and acetophenones. Some secondary metabolites, such as galloyl glucoses, procyanidins, mudanpiosides and acetophenones, will become the novel and potential chemotaxonomic markers to differentiate tree peony cultivars when the conventional classification methods are not practicable. These results demonstrated that HPLC-MS based metabolomics was an effective tool to classify phenotypic cultivars and provided the novel and potential chemotaxonomic characteristics for tree peony.

Acknowledgements

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