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Research Article

A Comprehensive Study in the Aerial Parts of Lonicera Japonica Thunb Based on Metabolite Profiling and Multivariate Statistical Analysis

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Abstract

Lonicera japonica Thunb is one of the most important genera in the Caprifoliaceae family and plays a vital role in traditional Chinese medicine prescriptions. All aerial parts (leaf, flower bud, flower, and caulis) of it can be used as herbal medicines for different traditional efficacy. Plant metabolomics provides a powerful tool for better differentiation and discovery of chemical marker. In the present work, a strategy for integrating metabolic profiling and multi-step PLS-DA analysis was developed to separate the different aerial parts and reveal the chemical markers of *L. japonica*. The strategy consists of two portions. One is an ultra-fast liquid chromatography coupled with triple quadrupole-time of flight tandem mass spectrometry (UFLC-Triple QTOF MS/MS) method, which was employed to explore chemical compositions. The other is multi-step PLS-DA, which was applied to distinguish the different aerial parts and to reveal the differential characteristic metabolites. A total of 71 metabolites were identified from samples, followed by 8 candidate compounds (Lonicerin, Kaempferol-*3*-*O*-rutinoside, Loganin, Isochlorogenic acid B, Isochlorogenic acid C, Secologanic acid, Luteoloside, Astragalin) as optimal chemical markers based on VIP and *p*-value. This study not only established an efficient strategy for exploring metabolite profiling and finding chemical markers among the different aerial parts of *L. japonica*, but also laid the foundation for elucidating the differences in efficacy between Lonicerae Japonicae Flos (LJF) and Lonicerae Japonicae Caulis (LJC) at the phytochemistry level. Meanwhile, it also implied from the perspective of structure-activity relationship that leaf could be used as an alternative medicinal resource for LJF.

Keywords

Lonicera japonica Thunb; UFLC-Triple TOF-MS/MS; metabolic profiling; multivariate statistical analysis

Introduction

Lonicera japonica Thunb. is native to the countries of eastern Asia [1], it is one of the most important genera in the Caprifoliaceae family and plays a vital role in traditional Chinese medicine prescriptions. Some species and different parts of Lonicera have been historically utilized as herbal medicines against a variety of diseases, including exopathogenic wind-heat, epidemic febrile diseases, sores, swelling, carbuncles, furuncles, erysipelas, and some infectious diseases [2]. At the same time, it is also used to make tea, cosmetics and healthy beverage all over the world.

Lonicerae Japonicae Flos (LJF) and Lonicerae japonicae Caulis (LJC) both come from *Lonicera japonica* Thunb, but the medicinal parts are different. LJF is derived from the dried buds and flowers [3], while LJC is originated from dried stems [4]. LJF germinates in ovoid leaves, and then blooming white and yellow flowers, which is sweetly scented in the early summer and traditionally harvested at that time. One of the semi-evergreen entangled woody vines, LJC is harvested in autumn and winter. The efficacy of LJF and LJC recorded is different in Chinese Pharmacopoeia. However, the reasons for these differences are still unclear. It is universally acknowledged that chemical composition is the basis of the pharmacological activity of medicinal materials and different chemical compositions have a different bearings on the clinical efficacy. For a long time, it is still a bottleneck in view of the elusive component-effect correlation of Traditional Chinese Medicines (TCMs). There are also many factors that may lead to chemical compositions differences, such as harvest season [5,6], processed methods [7], biological and abiotic factors [8-10], etc. Therefore, the systematic study of chemical composition is necessary. This is also a precondition for identifying chemical markers that differentiate between LJF and LJC, and it may reflect different properties of them.

Metabolomic approach [11,12] focuses on the analysis of holistic metabolites in the biological system, which has been

widely applied in natural product characterization, the analysis of biological metabolites with low molecular weight, food quality evaluation and so forth. However, untargeted metabolomics research on analyzing the chemical composition of *L. japonica* (different parts and different harvesting time of flower) has not been reported to date. In such classic works as 'Ming Yi Bie Lu' listed the leaf of *L. japonica* as a top-grade herb, described: "...like the stems, the winter is not withered", "December, dried". The young leaves are also used as spring greens in Korea [13]. And the yield is about 10 times that of the flower [14]. Nevertheless, the leaf is usually discarded because it is a non-medicinal part of *L. japonica*, resulting in underutilization and a great loss of potential resources. With the increasing demand for LJF, the leaf has attracted considerable attention. Some pharmacological studies also have demonstrated that the leaf possesses many biological functions, such as antibacterial [15], anti-AIV [16], antioxidant [17], and hepatoprotective effects.

In this work, we developed a strategy integrating metabolic profiling and multi-step PLS-DA analysis to separate the different aerial parts (including Leaf, Flower Bud, Flower, and Caulis) and discover chemical markers of them. Hence, an effective method was proposed to profile the primary and secondary metabolites in leaf, flower bud, flower, and caulis *via* ultra-fast liquid chromatography coupled with triple quadrupole-time of flight tandem mass spectrometry (UFLC-Triple TOF-MS/MS). Then, based on these data of metabolite profiling, multi-step PLS-DA were applied to discriminate these samples, and further to reveal the differential compositions among them. The untargeted metabolomics study demonstrated that the chemical composition in flower bud, flower, caulis and leaf of *L. japonica* had marked difference. A total of 71 metabolites were identified from *L. japonica* samples. Then, in accordance with a series of comparisons and permutations, Lonicerin, Kaempferol-*3*-*O*-rutinoside, Loganin, Isochlorogenic acid B, Isochlorogenic acid C, Secologanic acid, Luteoloside, Astragalin could be selected as optimal chemical markers based on VIP and *p*-value to distinguish the aerial parts in practice. Last but not least, the leaf exhibited the most similar chemical composition with flower bud and flower, which implied that the leaf could be used as an alternative medicine resource for LJF. Our work is not only beneficial to reduce the waste of resources, but also provides a reference for comprehensive exploitation and utilization of *L. japonica* resources.

Results

Identification of chemical constituents

In our study, chemical compounds of samples were analyzed and identified using ESI-MS/MS, and the best analytical selectivity and sensitivity were obtained in the negative ionization mode. The total chromatograms were presented in figure 1.

According to the database that we built, reference standards, and related literature, 71 metabolites were identified, including 4 alkaloids, 24 organic acids, 15 iridoids, 23 flavonoids, and 5 saponins. Most of the compounds were identified through comparation with the retention time and characteristic fragment ions of standard substances; some compounds were obtained based on the data that reported in relative literature. The details of identified compounds from leaf, flower bud, flower, and caulis were summarized in Table 1; the structures of these compounds were provided in figure 2.

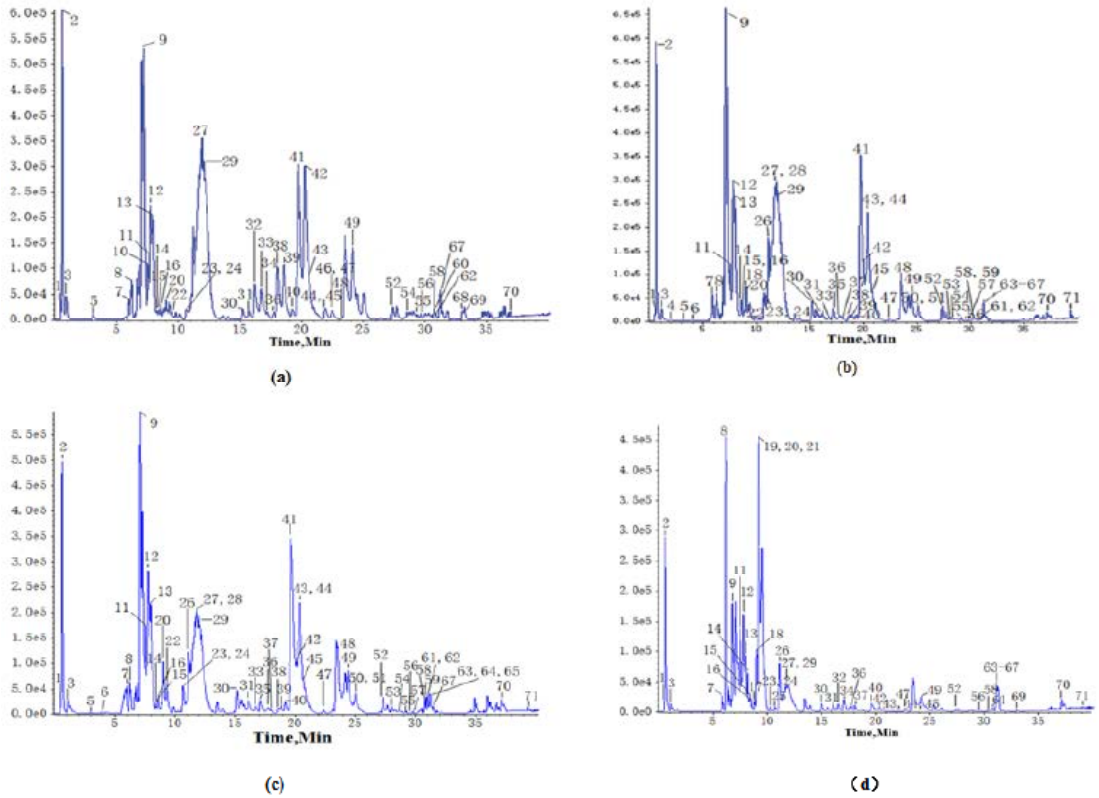
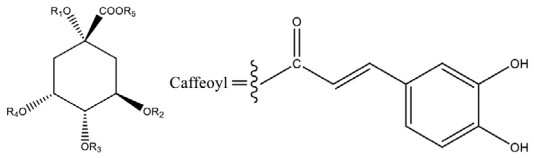
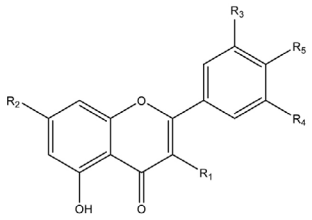


Figure 1. *Representative UPLC/ESI-QTOF-MS BPI (Base peak intensity) profiles of samples ((a) Leaf (b) Flower bud (c) Flower (d) Caulis) in the negative*

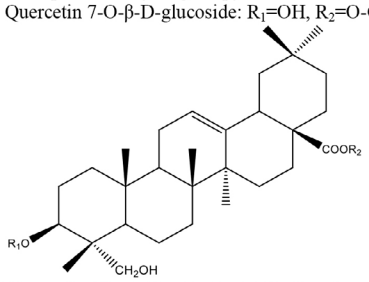


Quinic acid: R_1=H, R_2=H, R_3=H, R_4=H, R_5=H
Chlorogenic acid: R_1=H, R_2=Caffeoyl, R_3=H, R4=H, R_5=H
Neochlorogenic acid: R_1=H, R_2=H, R3=H, R_4=Caffeoyl, R_5=H
Cryptochlorogenic acid: R_1=H, R_2=H, R_3=Caffeoyl, R_4=H, R_5=H
1-O-caffeoylquinic acid: R_1=H, R_2=H, R_3=H, R_4=H, R_5=Caffeoyl
1,3-O-dicaffeoylquinic acid: R_1=H, R_2=Caffeoyl, R_3=H, R_4=H, R_5=Caffeoyl
1,4-O-dicaffeoylquinic acid: R_1=H, R_2=H, R_3=Caffeoyl, R_4=H, R_5=Caffeoyl
1,5-O-dicaffeoylquinic acid: R_1=H, R_2=H, R_3=H, R_4=Caffeoyl, R_5=Caffeoyl
3,5-O-dicaffeoylquinic acid: R_1=H, R_2=Caffcoyl, R_3=H, R_4=Caffeoyl, R_5=H
3,4-O-dicaffeoylquinic acid: R_1=H, R_2=Caffeoyl, R_3=Caffeoyl, R_4=H, R_5=H
4,5-O-dicaffeoylquinic acid: R_1=H, R_2=H, R_3=Caffeoyl, R_4=Caffeoyl, R_5=H
3-O-caffeoylquinic acid methyl ester: R_1=H, R_2=Caffeoyl, R_3=H, R_4=H, R_5=CH_3
4,5-O-dicaffeoylquinic acid methyl ester: R_1=H, R_2=H, R_3=Caffeoyl, R_4=Caffeoyl, R_5=CH_3
3,4,5-tricaffeoylquinic acid: R_1=H, R_2=Caffeoyl, R_3= Caffeoyl, R_4= Caffeoyl, R_5=H

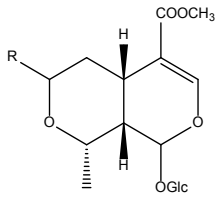


Genistein: R_1=H, R_2=OH, R_3=OH, R_4=H, R_5=H
Prunetin: R_1=H, R_2=OCH_3, R_3=OH, R_4=H, R_5=H
Diosmetin: R_1=H, R_2=OH, R_3=OCH_3, R_4=H, R_5=OH
flavoyadorinin-B: R_1=H, R_2=OCH_3, R_3=OCH_3, R_4=H, R_5=O-Glc

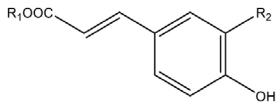
Rutin: R_1=O-rutinose, R_2=OII, R_3=OII, R_4=II, R_5=OII
Isoquercetin: R_1=O-Glc, R_2=OH, R_3=OH, R_4=H, R_5=OH
Hyperoside: R_1=O-Gal, R_2=OH, R_3=OH, R_4=H, R_5=OH
Luteoloside: R_1=H, R_2=Glc, R_3=H, R_4=OH, R_5=OH
Luteolin: R_1=H, R_2=OH, R_3=OH, R_4=H, R_5=OH
Luteolin-5-O-β-D- glucopyranoside: R_1=II, R_2=O-Glc, R_3=II, R_4=OII, R_5=OII
Tricin: R_1=H, R_2=H, R_3=OCH_3, R_4=OCH_3, R_5=OH
Lonicerin: R_1=II, R_2=L-mannose and D-Glc, R3=OII, R_4=II, R_5=OII
Rhoifolin: R_1=H, R_2= L-mannose and D-Glc, R_3=H, R_4=H, R_5=OH
Apigenin: R_1=H, R_2=OH, R_3=H, R_4=H, R_5=OH
Apigenin7-O-rutinoside: R_1=O-Glc-Rha, R_2=II, R_3=II, R_4=II, R_5=OII
Kaempferol-3-rutinoside: R_1=O-Gal, R_2=OH, R_3=H, R_4=H, R_5=OH
Kaempferol: R_1=OH, R_2=OH, R_3=H, R_4=H, R_5=OH
Astragalin: R_1=O-Glc, R_2=OH, R_3=H, R_4=H, R_5=OH
Quercetin: R_1=OH, R_2=OH, R_3=OH, R_4=H, R_5=OH
Chrysoeriol: R_1=H, R_2=OH, R_3=OCH_3, R_4=H, R_5=OH
Hesperidin: R_1=H, R_2=O-Glc(6-1)Rha, R_3=OCH_3, R_4=H, R_5=OH
Kaempferol-3-O-β-D-glucopyranoside: R_1=O- Glc, R_2=OH, R_3=H, R_4=H, R_5=OH
Quercetin 7-O-β-D-glucoside: R_1=OH, R_2=O-Glc, R_3=H, R_4=OH, R_5=OH



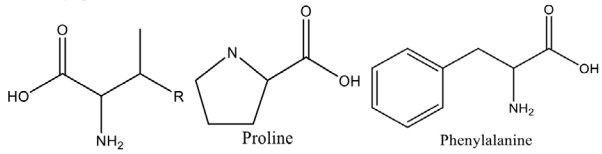
Macranthoidin A: R_1=Glc(1→3)Rha(1→2)Ara, R_2=Glc(1→6)Glc
Macranthoidin B: R_1=Glc(1→4)Glc(1→3)Rha(1→2)Ara, R_2=Glc(1→6)Glc
Dipsacoside B: R_1=Rha(1→2)Ara, R_2=Glc(1→6)Glc
Akebia saponin D: R_1=Ara(2→1)Glc, R_2= Glc(1→6)Glc
Hederagenin: R_1=H, R_2=H



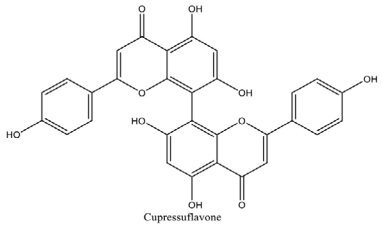
Morroniside: R=OH
Kingiside: R=O

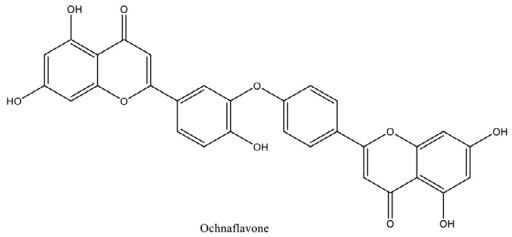


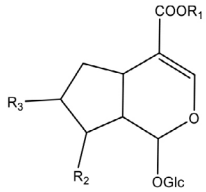
Caffeic acid: R_1=H, R_2=OH
Protocatechuic acid: R_1=H, R_2=OH
Ethyl caffeate: R_1=CH_3, R_2=OH
Caffeic acid methyl ester: R_1=CH_3, R_2=OH
Hydroxycinnamic acid: R_1=H, R_2=H
Ferulic acid: R_1=H, R_2=OCH_3
5-(p-Coumaryl)quinic acid: R_1=Glc, R_2=H
Ferulicoylquinic acid: R_1=Glc, R_2=OCH_3



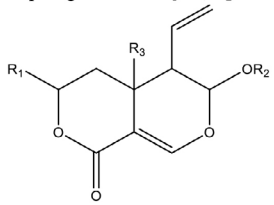
Valine: R=H
Isoleucine: R=CH_3



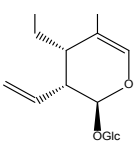


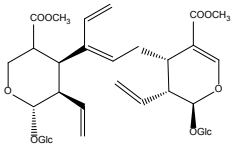


Loganin: R_1=CH_3, R_2=CH_3, R_3=OH
Loganin acid: R_1=H, R_2=CH_3, R_3=OH
7-epi-loganin: R_1=CH_3, R_2=β-CH_3, R_3=OH
8-epi-loganin: R_1=CH_3, R_2=α-CH_3, R_3=OII
8-epi-loganin acid: R_1=H, R_2=α-CH_3, R_3=OH



Sweroside: R_1=H, R_2=Glc, R_3=H
Secologanic acid: R_1=OH, R_2=Glc, R_3=H
Swertiamarin: R_1=II, R_2=Glc, R_3=OII
7-O-ethyl sweroside: R_1=OCH_2CH_3, R_2=Glc, R_3=H
Vogeloside: R_1=OCH_3, R_2=Glc, R_3=H





Centauroside

Secologanin: R_1=CH_3, R_2=CHO
Secoxyloganin: R_1=CH_3, R_2=COOH

Citation: Cai Z, Liao H, Wang C, Chen J, Tan M, et al. (2019)) A Comprehensive Study in the Aerial Parts of Lonicera Japonica Thunb Based on Metabolite Profiling and Multivariate Statistical Analysis. OSP J Bio Che 1. JBC-1-102

Figure 2. *Chemical structures of identified compounds from aerial parts of Lonicera japonica Thunb.*

Table 1. Identification of 71 metabolites in the aerial parts of *Lonicera japonica* Thunb. by UFLC-Triple TOF-MS/MS

No.	RT (min)	Molecular formula	Proposal ions	ppm	MS/MS fragment	Identification	Classification	Aerial parts				Reference
								Leaf	Flower bud	Flower	Caulis	
1	0.49	$C_5H_9NO_2$	$[M-H]^-$	4.5	114.9905$[M-H]^-$,56.9982$[M-H-C_3H_3O_2-CH_3]^-$	Proline*	alkaloid	+	+	+	+	
2	0.61	$C_7H_{12}O_6$	$[M-H]^-$	0.2	191.05615$^-$,173,154	Quinic acid*	organic acid	+	+	+	+	22
3	0.95	$C_5H_{11}NO_2$	$[M-H]^-$	5.4	101.0608$[M-H-NH_3]^-$,85.0295$[M-H-NH_3-CH_4]^-$,57.0710$[M-H-C_2H_4O_2]^-$	Valine*	alkaloid	+	+	+	+	
4	2.09	$C_6H_{13}NO_2$	$[M-H]^-$	3.8	130.874$[M-H]^-$,98.0611$[M-H_2O-CH_3]^-$	Isoleucine*	alkaloid	-	+	-	-	
5	3.32	$C_9H_{11}NO_2$	$[M-H]^-$	2.8	146.0611$[M-H-H_2O]^-$,103.0553$[M-H-COO-NH_3]^-$, 77.0397$[M-H-COO-NH_3-C_2H_2]^-$	Phenylalanine*	alkaloid	-	+	+	-	23
6	4.1	$C_7H_6O_4$	$[M-H]^-$	2.4	109.0295$[M-H-COO]^-$, 91.0189$[M-H-COO-H_2O]^-$	Protocatechuic acid*	organic acid	-	+	+	-	24
7	6.09	$C_{16}H_{24}O_{10}$	$[M-H]^-$	3.5	213.0759$[M-H-Glc]^-$,167.0447$[M-H-Glc-H_2O-CO]^-$	8-epi-loganin acid	iridoid	-	+	+	+	22,25,29
8	6.41	$C_{16}H_{18}O_9$	$[M-H]^-$	3.1	191.0569$[M-H-CA]^-$,179.0351,135.0456	1-*O*-caffeoylquinic acid*	organic acid	+	+	+	+	
9	7.5	$C_{16}H_{18}O_9$	$[M-H]^-$	-1.1	191.0568$[M-H-CA]^-$,127.0398$[[M-H-CA-2H_2O-CO]^-$	Chlorogenic acid*	organic acid	+	+	+	+	26,27
10	7.49	$C_{17}H_{26}O_{11}$	$[M+HCOO]^-$	4.1	451.1476$[M-H+HCOOH]^-$,243.0899$[M-H-Glc]^-$,191.0573,119.0368,105.0323,101.0263	Morroniside*	iridoid	+	-	-	-	
11	7.1	$C_{16}H_{24}O_{10}$	$[M-H]^-$	3.8	329.1492$[M-H-H_2O-CO]^-$,213.0759$[M-H-Glc]^-$,169.0871$[M-H-Glc-CO_2]^-$,151.0765$[M-H-Glc-CO_2-H_2O]^-$	Loganic acid*	iridoid	+	+	+	+	22,29
12	7.79	$C_{16}H_{18}O_9$	$[M-H]^-$	3.1	191.0569$[M-H-CA]^-$,179.0351,135.0456	Neochlorogenic acid*	organic acid	+	+	+	+	26,27
13	7.9	$C_{16}H_{18}O_9$	$[M-H]^-$	-1.1	191.0568$[M-H-CA]^-$,127.0398[M-H-CA-$2H_2O-CO]^-$	Cryptochlorogenic acid*	organic acid	+	+	+	+	26,27
14	8.1	$C_9H_8O_4$	$[M-H]^-$	2.9	135.0460$[M-H-CO_2]^-$	Caffeic acid*	organic acid	+	+	+	+	27
15	8.37	$C_{16}H_{22}O_{10}$	$[M-H]^-$	2.8	193.0523$[M-H-Glc-H_2O]^-$,149.0617$[M-H-Glc-H_2O-CO_2]^-$, 119.0356,101.0250	Swertiamarin	iridoid	+	+	+	+	23
16	8.37	$C_{16}H_{22}O_{10}$	$[M-H]^-$	2.9	193.0523$[M-H-Glc-H_2O]^-$,149.0617$[M-H-Glc-CO_2-H_2O]^-$, 141.0189,123.0464,119.0356, 105.0344,101.0250	Secologanic acid*	iridoid	+	+	+	+	22,29
17	8.95	$C_{11}H_{12}O_4$	$[M+HCOO]^-$	2.1	161.0329$[M-H-C_2H_5O]^-$,135.0468,133.0282$[M-H-C_2H_5O-CO]^-$	Ethyl caffeate	organic acid	-	-	-	+	27
18	9.03	$C_{17}H_{24}O_{10}$	$[M-H]^-$	-3.6	341.1093$[M-H-H_2O-CO]^-$,179.0544$[M-H-Glc-H_2O-CO]^-$, 161.0444,149.0440,131.0336, 119.0354,101.0247	Secologanin*	iridoid	+	+	-	-	
19	9.2	$C_{17}H_{26}O_{10}$	$[M+HCOO]^-$	3.4	227.0932$[M-H-Glc]^-$,209.0987$[M-H-Glc-H_2O]^-$,191.0563$[M-H-Glc-2H_2O]^-$,153.0697,149.0785,129.0559	7-epi-loganin	iridoid	-	-	-	+	25,29
20	9.23	$C_{18}H_{26}O_{10}$	$[M-H]^-$	2.8	175.0412$[M-H-Glc-CH_3CH_2OH-H_2O]^-$	7-*O*-ethyl sweroside	iridoid	+	+	+	+	28
21	9.23	$C_{17}H_26O_{10}$	$[M+HCOO]^-$	4.6	227.0911$[M-H-Glc]^-$,209.0809$[M-H-Glc-H_2O]^-$,191.0562$[M-H-Glc-2H_2O]^-$,133.0303,101.0250	8-epi-loganin	iridoid	-	-	-	+	
22	9.32	$C_{16}H_{18}O_8$	$[M-H]^-$	3.2	191.0575$[QA-H]^-$,173.0442$[QA-H-H_2O]^-$,163.0391$[M-H-QA]^-$,127.0387$[QA-H-2H_2O-CO]^-$, 117.0323$[PA-H_2O-CO]^-$	5-(p-Coumaryl) quinic acid	organic acid	+	+	+	+	23
23	10.78	$C_{17}H_{20}O_9$	$[M-H]^-$	4.7	191.0571$[QA-H]^-$,173.0469$[M-H-CA-CH_3OH]^-$,127.0401$[M-H-CA-CH_3OH-H_2O-CO]^-$	3-*O*-caffeoylquinic acid methyl ester	organic acid	+	+	+	+	23
24	10.78	$C_{17}H_{20}O_9$	$[M-H]^-$	4.7	191.0571$[QA-H]^-$,173.0469$[QA-H-H_2O]^-$,127.0401$[QA-H-2H_2O-CO]^-$,117.0360$[M-H-QA-OCH_3-CO]^-$	3-*O*-feruloylquinic acid	organic acid	+	+	+	+	23

25	11.42	$C_{10}H_{10}O_4$	$[M-H]^-$	0.15	149.0608[M-H-COO]$^-$,133.0295[M-H-COOH-CH_3]$^-$	Ferulic acid*	organic acid	-	-	-	+	
26	11.47	$C_{17}H_{26}O_{10}$	[M+HCOO]$^-$	6.1	389.1781[M-H]$^-$,227.1144[M-H-Glc]$^-$	Loganin*	iridoid	-	+	+	+	26,29
27	11.8	$C_{17}H_{24}O_{10}$	[M+HCOO]$^-$	4.7	175.0402[M-H-Glc-CH_3OH-H_2O]$^-$,151.0358[M-H-Glc-C_4H_6O]$^-$,149.0591[M-H-Glc-CH_3OH-H_2O-C_2H_2]$^-$, 119.0347,101.0251	Vogeloside	iridoid	+	+	+	+	22, 26,29
28	11.88	$C_{17}H_{24}O_{11}$	$[M-H]^-$	3.9	165.0569,149.0262[M-H-Glc-CH_3-COO-H_2O-CH_3]$^-$, 121.0311,119.0382,101.0257	Kingiside	iridoid	-	+	+	-	22
29	11.9	$C_{17}H_{24}O_{11}$	$[M-H]^-$	3.9	149.0252[M-H-Glc-H_2O-CH_3OH-C_2H_2O]$^-$,121.0308[M-H-Glc-H_2O-CH_3OH-C_2H_2O-CO]$^-$	Secoxyloganin*	iridoid	+	+	+	+	22,29
30	15.18	$C_{21}H_{20}O_{12}$	$[M-H]^-$	3.4	301.0387[M-H-Glc]$^-$,283.0131[M-H-Glc-H_2O]$^-$, 151.0054,107.0174	Hyperoside*	flavonoid	+	+	+	+	22,29
31	16.01	$C_{27}H_{30}O_{16}$	$[M-H]^-$	-1.8	301.0374[M-H-Rha-Glc]$^-$	Rutin*	flavonoid	+	+	+	+	29
32	16.56	$C_{21}H_{20}O_{12}$	$[M-H]^-$	5.8	301.0374[M-H-Glc]$^-$,271.0265[M-H-Glc-CH_2O]$^-$,151.0033	Quercetin-7-*O*-glucoside	flavonoid	+	-	-	+	
33	16.68	$C_{21}H_{20}O_{12}$	$[M-H]^-$	3.9	301.0379[M-H-Glc]$^-$,151.0044	Isoquercitrin*	flavonoid	+	+	+	-	
34	17.08	$C_{21}H_{20}O_{11}$	$[M-H]^-$	2.2	285.0418[M-H-Glc]$^-$	Luteolin-5-*O*-β-D- glucopyranoside	flavonoid	+	-	-	+	
35	17.17	$C_{21}H_{20}O_{11}$	$[M-H]^-$	3.3	285.0419[M-H-Glc]$^-$	Luteoloside*	flavonoid	-	+	+	-	
36	17.98	$C_{27}H_{30}O_{15}$	$[M-H]^-$	0.01	285.0405[M-H-Glc-Rha]$^-$	Lonicerin*	flavonoid	+	+	+	+	22,29
37	18.04	$C_{28}H_{34}O_{15}$	$[M-H]^-$	-2.8	301.036[M-H-Rha-Glc]$^-$, 271.0265,255.0304, 179.0090,151.0033	Hesperidin*	flavonoid	-	+	+	-	
38	18.54	$C_{25}H_{24}O_{12}$	$[M-H]^-$	0.6	353.0821[M-H-CA]$^-$,335.0821[M-H-CA-H_2O]$^-$,191.0567[M-H-2CA]$^-$,179.0377[CA-H]$^-$,173.0481[M-H-2CA-H_2O]$^-$,161.0268[CA-H-H_2O]$^-$,135.0613[CA-H-CO_2]$^-$	1,3-*O*-dicaffeoylquinic acid*	organic acid	+	+	+	-	22
39	19.07	$C_{27}H_{30}O_{15}$	$[M-H]^-$	4.4	593.1570,285.0419[M-H-Rha-Glc]$^-$	Kaempferol-3-*O*-rutinoside*	flavonoid	+	+	+	-	29
40	19.49	$C_{10}H_{10}O_4$	$[M-H]^-$	4.4	133.0305[M-H-CH_3-COOH]$^-$	Caffeic acid methyl ester	organic acid	+	-	+	+	33
41	20.06	$C_{25}H_{24}O_{12}$	$[M-H]^-$	-2.8	353.0924[M-H-CA]$^-$,335.0793[M-H-CA-H_2O]$^-$,191.0559[M-H-2CA]$^-$,179.0355,173.0458[M-H-2CA-H_2O]$^-$,161.0241[CA-H-H_2O]$^-$,155.0346[M-H-2CA-2H_2O]$^-$, 135.0459[CA-H-CO]$^-$	Isochlorogenic acid B*	organic acid	+	+	+	-	22, 26,
42	20.24	$C_{25}H_24O_{12}$	$[M-H]^-$	5.6	353.0910[M-H-CA]$^-$,335.0816[M-H-CA-H_2O]$^-$,191.0575[M-H-2CA]$^-$,179.0369[CA-H]$^-$,173.0472[M-H-2CA-H_2O]$^-$, 161.0257[CA-H-H_2O]-,135.0464[CA-H-CO_2]$^-$	1,5-*O*-dicaffeoylquinic acid	organic acid	+	+	+	+	23
43	20.6	$C_{21}H_{20}O_{11}$	$[M-H]^-$	2.2	285.0428[M-H-Glc]$^-$	Astragalin*	flavonoid	+	+	+	+	
44	20.62	$C_{25}H_{24}O_{12}$	$[M-H]^-$	-3.1	353.0901[M-H-CA]$^-$,191.0569[M-H-2CA]$^-$,179.0353[CA-H]$^-$, 173.0463[M-H-2CA-H_2O]$^-$,135.0457[CA-H-CO]$^-$	Isochlorogenic acid A*	organic acid	+	+	+	-	22, 26,
45	20.67	$C_{25}H_{24}O_{12}$	$[M-H]^-$	6.2	353.0905[M-H-CA]$^-$,191.0576[M-H-2CA]$^-$,179.0366[CA-H]$^-$, 173.0460[M-H-2CA-H_2O]$^-$,161.0247[CA-H-H_2O]$^-$, 135.0460[CA-H-CO]$^-$	1,4-*O*-dicaffeoylquinic acid	organic acid	+	+	+	+	22
46	22.61	$C_{27}H_{30}O_{14}$	$[M-H]^-$	-1.3	269.0490[M-H-Rha-Glc]$^-$,191.0582	Apigenin-7-*O*-rutinoside	flavonoid	+	-	-	-	23,27
47	22.68	$C_{27}H_{30}O_{14}$	$[M-H]^-$	4.5	413.0799[M-H-Rha-H_2O]$^-$,269.0462[M-H-Rha-Glc]$^-$	Rhoifolin*	flavonoid	+	+	+	+	30
48	23.09	$C_{25}H_{24}O_{12}$	$[M-H]^-$	-3.1	353.0904[M-H-CA]$^-$,191.0575[M-H-2CA]$^-$,179.0360[CA-H]$^-$, 173.0463[M-H-2CA-H_2O]$^-$,155.0364[M-H-2CA-2H_2O]$^-$, 135.0464[CA-H-CO]$^-$	Isochlorogenic acid C*	organic acid	+	+	+	-	22

49	24.52	$C_{34}H_{46}O_{19}$	$[M-H]^-$	0.3	725.2528[M-H-OCH_3]$^-$,595.1979[M-H-Glc]$^-$,525.1657[M-H-Glc-CH_2-C_2O_2]$^-$,179.0565	Centauroside	iridoid	+	+	+	+	28
50	24.81	$C_{25}H_{24}O_{11}$	$[M-H]^-$	4.8	353.0935[M-H-PA]$^-$,191.0560[QA-H]$^-$,179.0347[CA-H]$^-$, 173.0431[QA-H-H_2O]$^-$,163.0410[PA-H]$^-$,161.0258[CA-H-H_2O]$^-$,135.0444[CA-H-H_2O-CO]$^-$,119.0476	Coumaroyl caffeoylquinic acid	organic acid	+	+	+	-	23,25
51	24.82	$C_{25}H_{24}O_{11}$	$[M-H]^-$	5.4	353.0879[M-H-PA]$^-$,319.0844[M-H-CA]$^-$,191.0562[QA-H]$^-$, 179.0353[CA-H]$^-$,173.0427[QA-H-H_2O]$^-$,163.0407[PA-H]$^-$, 161.0242[CA-H-H_2O]$^-$,135.0444[CA-H-H_2O-CO]$^-$, 127.0399[PA-H-2H_2O]$^-$,119.0476	Coumaroyl caffeoylquinic acid isomer	organic acid	+	+	+	-	
52	27.56	$C_{23}H_{24}O11$	$[M-H]^-$	5.2	313.0715[M-H-Glc]$^-$,283.0531,279.0164,269.0429,255.030	Flavoyadorinin-B	flavonoid	-	+	+	+	
53	28.03	$C_{26}H_{26}O_{12}$	$[M-H]^-$	5.3	353.0898[M-H-$C_{10}H_8O_3$]$^-$,191.0568[M-H-$C_{10}H_8O_3$-CA]$^-$, 179.0366[CA-H]$^-$,173.0460[M-H-$C_{10}H_8O_3$-CA-H_2O]$^-$, 161.0247[CA-H-H_2O]$^-$,155.0379[M-H-$C_{10}H_8O_3$-CA-2H_2O]$^-$, 135.0460[CA-H-CO]$^-$	Feruloyl caffeoylquinic acid	organic acid	-	+	+	-	23
54	28.42	$C_{15}H_{10}O_7$	$[M-H]^-$	0.2	301.0367[M-H]$^-$,193[M-H-ringB]$^-$,151,121,107	Quercetin*	flavonoid	-	+	+	-	27,32
55	29.4	$C_{26}H_{26}O_{12}$	$[M-H]^-$	5	367.1076[M-H-CA]$^-$,349.0978[M-H-CA-H_2O]$^-$, 179.03340[CA-H]$^-$,161.0243[CA-H-H_2O]$^-$,135.0447[CA-H-CO_2]$^-$	4,5-*O*-dicaffeoylquinic acid methyl ester*	organic acid	+	+	+	-	
56	29.66	$C_{15}H_{10}O_6$	$[M-H]^-$	4.5	199.0399[M-H-H_2O-C_4H_4O]$^-$, 175.0405,151.0026,133.0300,121.0299,107.0151	Luteolin*	flavonoid	+	+	+	+	22,27,29
57	29.71	$C_{15}H_{10}O_6$	$[M-H]^-$	-1.7	285.0401,215.0382[M-H-H_2O-C_4H_4]$^-$, 175.0425,151.0027,133.0295	Kaempferol*	flavonoid	-	+	+	-	32
58	30.75	$C_{34}H_{30}O_{15}$	$[M-H]^-$	6.3	515.1256[M-H-CA]$^-$,353.0907[M-H-2CA]$^-$,335.0794[M-H-2CA-H_2O]$^-$,191.0578[QA-H]$^-$,179.0366[CA-H]$^-$,173.0470[QA-H-H_2O]$^-$,161.0261[CA-H-H_2O]$^-$,135.0454[CA-H-H_2O-C_2H_2]$^-$	3,4,5-tricaffeoylquinic acid	organic acid	+	+	+	-	22
59	30.81	$C_{59}H_{96}O_{27}$	$[M-H]^-$	2.5	911.5010[M-H-2Glc]$^-$	Macranthoidin A*	saponin	-	-	-	+	29
60	30.85	$C_{65}H_{106}O_{32}$	$[M+HCOO]^-$	-3.5	1235.6066[M-H-Glc]$^-$,911.5010[M-H-3Glc]$^-$	Macranthoidin B	saponin	+	-	-	-	29
61	30.91	$C_{53}H_{86}O_{22}$	$[M+HCOO]^-$	1	1119.548[M-H+HCOOH]$^-$,749.4482[M-H-2Glc]$^-$,323.0984	Dipsacoside B*	saponin	-	+	+	+	29
62	30.9	$C_{15}H_{10}O_5$	$[M-H]^-$	3.4	117.0357	Apigenin*	flavonoid	+	+	+	-	27,32
63	31.06	$C_{15}H_{10}O_5$	$[M-H]^-$	3.4	201.0572[M-H-C_3O_2]$^-$,159.0517[M-H-C_3O_2-C_2H_2O]$^-$,151.0089,107.0107	Genistein	flavonoid	-	+	+	+	31
64	31.09	$C_{17}H_{14}O_7$	$[M-H]^-$	-0.1	329.6663[M-H]$^-$,211	Tricin	flavonoid	-	+	+	+	22
65	31.2	$C_{16}H_{12}O_6$	$[M-H]^-$	-2.6	283.0425,255.0315[M-H-C_2H_{40}]$^-$, 227.0347,199.0434,151.0044,147.0041,133.0309,107.014	Diosmetin*	flavonoid	-	+	+	+	
66	31.2	$C_{16}H_{12}O_6$	$[M-H]^-$	6.3	283.0425,255.0315[M-H-C_2H_{40}]$^-$, 227.0347,199.0434,151.0044,147.0041,133.0309,107.014	Chrysoeriol	flavonoid	-	+	+	+	
67	31.22	$C_{16}H_{22}O_9$	$[M+HCOO]^-$	4.9	151.0809,149.0254[M-H-Glc-H_2O-CO]$^-$,125.0263[M-H-Glc-C_4H_6O]$^-$,119.0062	Sweroside*	iridoid	+	+	+	+	22,28,29
68	33.06	$C_{16}H_{12}O_5$	$[M-H]^-$	5	268.0367[M-H-CH_3]$^-$,239.0354[M-H-CH_3-CHO]$^-$, 171.0526[M-H-CH_3-CHO-C_3O_2]$^-$	Prunetin	flavonoid	+	-	-	-	31
69	33.29	$C_{30}H_{18}O_{10}$	$[M-H]^-$	6.4	519.0860[M-H-H_2O]$^-$,493.0825[M-H-H_2O-C_2H_2]$^-$, 469.0991[M-H-C_4H_4O]$^-$,427.0991[M-H-$C_6H_6O_2$]$^-$, 269.0400[M-H-$C_{15}H_9O_5$]$^-$,130.9961	Cupressuflavone/Ochanaflavone	flavonoid	+	-	-	+	
70	37.32	$C_{30}H_{48}O_4$	$[M+HCOO]^-$	6.8	517.3570[M-H+HCOOH]$^-$,471.3540,366.9864[M-H-$C_5H_{13}O_2$]$^-$	Hederagenin	saponin	+	+	+	+	23,29
71	39.22	$C_{47}H_{76}O_{18}$	$[M+HCOO]^-$	0.01	973.4995[M-H+HCOOH]$^-$,927.4959[M-H]$^-$,603.3902[M-H-2Glc]$^-$,323.0984	Akebia saponin D*	saponin	-	+	+	+	23

Note: * Compare with reference; - Not detected; + detected; QA: quinic acid; CA: caffeic acid; PA: p-coumaric acid

Identification of phenolic acids

In the negative mode, adducted ion of phenolic acid was observed as $[M-H]^-$ or $[M+HCOO]^-$. Generally, the basic structure of phenolic acids consists of one or more caffeic acid substituents bound to a portion of quinic acid. Their MS/MS spectra usually owned a basic peak at $[M-H-CA]^-$, and then lost H_2O, CO_2 or CO, which usually produced various ions for example 353 $[M-H-CA]^-$, 335 $[M-H-CA-H2O]^-$, 179, 135, 127, and so forth. Such as 1-*O*-caffeoylquinic acid, chlorogenic acid, neochlorogenic acid, and cryptochlorogenic acid showed a characteristic fragment ions at *m/z* 191, 179,135, 127, dicaffeoylquinic acids and isomers (compound 38, 41, 42, 44, 45, 48) had the same molecular ions at *m/z* 515 and the secondary fragmentation at 353, 191, 179, 173, 161, 135. The MS/MS fragmentation of compound 3,4,5-tricaffeoylquinic acid lost three caffeoyl moieties at *m/z* 515, 353, 191, which were consistent with literature reports [18].

Among these phenolic acid compounds, compound 2,6, 8, 9, 12, 13, 14, 25, 38, 41, 44, 48, 55 were identified by comparison on retention time, authentic standards and references. And other compounds were further identified by comparing fragment ions and fragmentation behaviors, which were reported in the relevant literature.

Identification of flavonoids

The fragment rules of flavonoids mainly include the following parts in the negative mode. The first is the flavonoids with a structure of 5,7-OH, which lost C_3O_2. Such as Genistein produced the fragment ion C_3O_2 at *m/z* 201. The second is the different fragmentation patterns of is flavones, flavonoids, and flavonols on the C ring. The fragment ion of *m/z* 283 $[M-H]^-$, 268 $[M-H-CH_3]^-$, 239 $[M-H-CH_3-CHO]^-$, 171 $[M-H-CH_3-CHO-C_3O_2]^-$ were obtained in the MS/MS, which were coincident with the reference. Thus, compound 68 was acknowledged as prunetin. The third is the RDA reaction pathway of flavonoids and isoflavones on aglycone C ring and the losing the fragment of C_2H_2O. Such as luteolin, apigenin often occurred RDA reaction pathway. The characteristic fragment ion of the RDA reaction pathway at *m/z* 151, 133, so compound luteolin was confirmed. In addition, hyperoside, lonicerin, astragalin, flavoyadorinin-B, and luteoloside were loss of glucose, respectively. Rhoifolin, rutin successive lost glucose and rhamnose moieties.

Among these flavonoid compounds, compound 30, 31, 33, 35, 36, 37, 39, 43, 47, 54, 56, 57, 62, 65 were identified by comparison on retention time, authentic standards and references. And other compounds were further identified by comparing fragment ions and fragmentation behaviors, which were reported in the relevant literature.

Identification of iridoid

Most of the iridoid present a relatively high abundant deprotonate molecule $[M-H]^-$ in the negative mode. Therefore, the basic peak of iridoid was $[M-H]^-$, and the adduct was $[M+HCOO]^-$. Furthermore, the characteristic fragment ions $[M-H-162]^-$ of this category of compounds were formed due to the loss of glucose, and then subsequent loss of H_2O, CO or CO_2. A total of 15 iridoids were identified from these samples with the TOF-MS/MS data and reference standards. The spectrum of compound 11 showed a fragment ion at *m/z* 329 $[M-H-H_2O-CO]^-$, 213 $[M-H-Glc]^-$,169 $[M-H-Glc-CO_2]^-$, 151 $[M-H-Glc-CO_2-H_2O]^-$, and it was identified as loganic acid. And compound 7-epi-loganin, 8-epi-loganin showed same $[M+HCOO]^-$ with loganin at *m/z* 435. Compound 16 gave several fragment ions at *m/z* 193 $[M-H-Glc-H_2O]^-$, 149 $[M-H-Glc-CO_2-H_2O]^-$, 141, 123, 119, 105, 101 in the MS/MS spectrum, and it was characterized as secologanic acid. Compound 18 displayed a highly abundant $[M-H]^-$, and its molecular formula was determined to be $C_{17}H_{24}O_{10}$, it loss of H_2O and CO at *m/z* 341, then loss of glucose generated $[M-H-Glc-H_2O-CO]^-$ at *m/z* 179, and produced several fragment ions at *m/z* 161, 149, 131, 119, 101. Compared with the standard, it was further determined as secologanin.

Among these iridoid compounds, compound 10, 11, 16, 18, 26, 29, 67 were identified by comparison on retention time, authentic standards and references. And other compounds were further identified by comparing fragment ions and fragmentation behaviors, which were reported in the relevant literature.

Identification of saponins

The basic parent nuclei of saponins consisted of aglycone and sugar. In this study, saponins responded more sensitively and had much higher abundance in negative mode. Because the mobile phase contains formic acid, ion of $[M-H+HCOOH]^-$ was appeared in most of the saponins. A typical adduct peak was *m/z* 1119 $[M-H+HCOOH]^-$ in the negative mode with 1 ppm error, and then successive loss of glucose at *m/z* 749 $[M-H-2Glc]^-$. In comparison with the reference standard, compound 61 was assigned as Dipsacoside B.

Among these 5 saponin compounds, compound 59, 61, 71 were identified by comparison on retention time and with authentic standards. And the other compounds were further identified by comparing fragment ions and fragmentation behaviors, which were reported in the relevant literature.

Multivariate statistical analysis

It is the key issue to find the potential chemical marker for distinguishing them. The PCA, multi-step PLS-DA analysis and VIP tests were performed. It could be regarded as a potential marker when the VIP-value of compounds was more than 1.

As shown in figure 3, only LJC was completely separated from the other three parts. The leaf was mingled with flower bud and the flower also mingled in the flower bud. It meant the four different parts were not entirely separated. Hence, PLS-DA was carried out to further identify metabolites that could accurately distinguish the four parts better. (Figure 4). The identification of differential metabolites between every two samples was performed using VIP values. The leaf and caulis were clearly divided into two groups in figure 4(a). Then, the same PLS-DA analysis was conducted for discriminating leaf and LJF (figure. 4(b)), caulis and LJF (figure 4(c)), flower and flower bud (figure 4(d)). They could be completely separated between every two of them. Through comprehensive analysis and comparison, compound 36, 39,

26, 41, 48, 16, 35, 43 (Lonicerin, Kaempferol-3-*O*-rutinoside, Loganin, Isochlorogenic acid B, Isochlorogenic acid C, Secologanic acid, Luteoloside, Astragalin) had greater contributions to the differentiation of the four parts, which could be considered as the potential compounds to distinguish the four different parts. Finally, compound 36, 39, 26, 41, 48, 16, 35, 43 were set as variables for a new round of PLS-DA analysis of four different parts. As shown in figure. **5** the four different parts were completely discriminated. Therefore, these compounds (Lonicerin, Kaempferol-3-*O*-rutinoside, Loganin, Isochlorogenic acid B, Isochlorogenic acid C, Secologanic acid, Luteoloside, Astragalin) were selected as optimal markers.

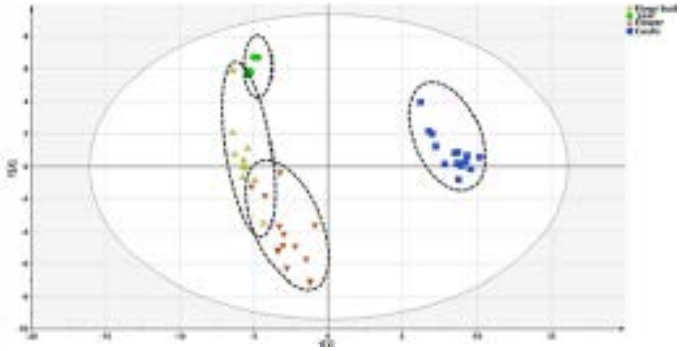


Figure 3. Principal Component Analysis (PCA) scores plot of 44 batches of samples (Green circle represent Leaf samples, Yellow triangles represent Flower bud samples, Orange inverted triangle represent Flower samples, Blue box represent Caulis samples)

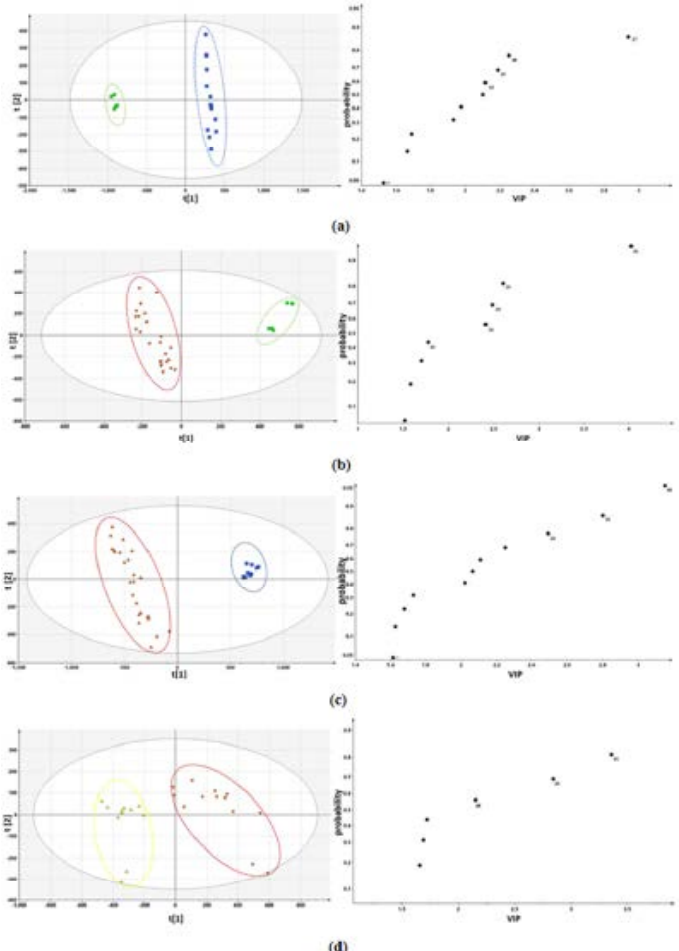


Figure 4. Partial least squares discriminant analysis (PLS-DA) scores plot and VIP plot of leaf and caulis (a), leaf and LJF (b), caulis and LJF (c), flower and flower bud 4(d).

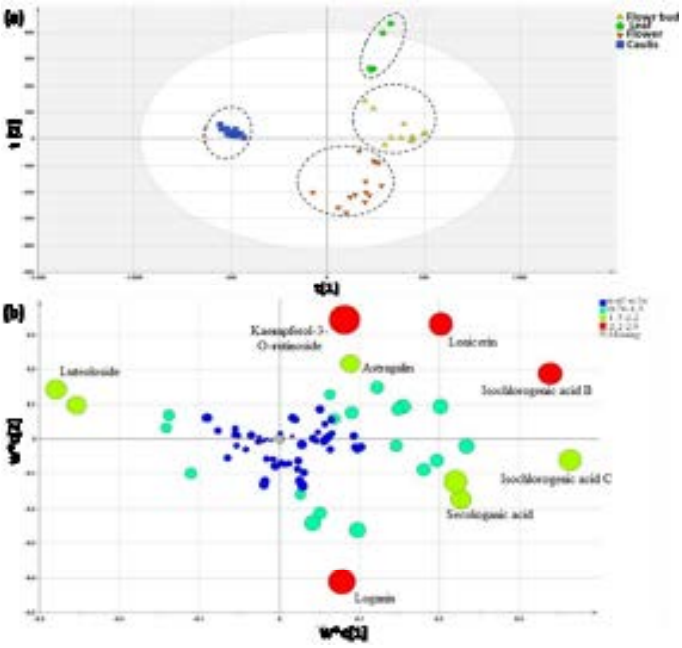


Figure 5. Partial least squares discriminant analysis (PLS-DA) scores plot(a) loading plot (b) profiles (Green circle represent Leaf samples, Yellow triangles represent Flower bud samples, Orange inverted triangle represent Flower sample, Blue box represent Caulis in Fig. (a); each circle in Fig. (b) represent one compound)

Discussion

The most common technology for metabolomics is mass spectrometry, and LC-MS is regarded as one of the most applicable and versatile methods in metabolomics, which has been widely used to investigate the metabolic profiles of plant materials. In current study, the differences in metabolic profiles among the aerial parts (including leaf, flower bud, flower, and caulis) of *L. japonica* were observed by an untargeted LC-MS method. The optimal differential metabolites were selected as chemical markers using PLS-DA analysis, VIP and *p*-value.

LJF and LJC were the different medicinal parts of *L. japonica*, they had been documented as independent herbal medicine in Chinese pharmacopeia (2015 Version); However, their traditional efficacy was different. The Venn plot (figure 6) and Table S1 might illustrate this phenomenon by comparing the chemical composition of different parts. Among these 71 metabolites, there were 28 mutual constituents. All the other iridoid compounds in LJC were present except kingside, and the number of iridoid compounds in LJF was less than that in caulis. In general, different compounds possess different pharmacological effects. The biological activities of loganin (one of the optimal chemical markers based on VIP and *p*-value) including neuroprotection [19,20], antithrombotic and anticoagulation, which were consistent with the efficacy of loganin (the quality marker of LJC) recorded in the Chinese Pharmacopoeia. This analysis might pave the way to elucidating the similarities and differences in the efficacy of LJF and LJC from the perspective of phytochemistry.

Chinese pharmacopeia officially record the best harvest season of LJC was autumn or winter, while the harvest time of LJF is in early summer. This indicates that the harvest time

of different TCMs is different, and the harvesting time is indeed one of the factors affecting the quality of TCMs [5,6]. In addition, the chemical composition and content are of crucial importance to the quality, and determine the curative effect in clinical. In order to further explore the effect of harvesting time on the quality of medicinal materials, we compared the chemical compositions of LJF at different harvesting periods (such as flower bud, flower). The result (figure 6) illustrated that the harvesting time had a significant effect on the chemical composition of medicinal materials. And some literatures reported that flower buds have better quality and higher medical value [34,35].

Last but not least, although leaf was not recorded in Chinese pharmacopeia, it could be seen from the figure 6 that the composition of leaves exhibited the most similarity to LJF, which was consistent with the result of previous research [21]. The common components of leaves and LJF include the main bioactivity components: phenolic acid (such as Isochlorogenic acid A, Isochlorogenic acid B, Isochlorogenic acid A), flavonoids (such as Luteolin, Kaempferol-*3-O*-rutinoside), iridoids (such as Loganin acid) and saponins (such as Hederagenin).In addition, from the perspective of structure and activity, specific components such as luteolin and kaempferol in flowers can be mutually converted and synthesized with common components such as luteolin and kaempferol-3-rutinoside. While, morroniside is contained in leaves but not in LJF. A large number of published studies has shown that morroniside possess anti-inflammatory, anti-apoptosis, angiogenesis, anti-oxidative stress, neuroprotection and anti-cancer activities [37-38]. These impliy that leaf can be used as an alternative medicinal resource for LJF. This result can provide the support for extending the range of application of *L. japonica* and reducing the waste of potential resources.

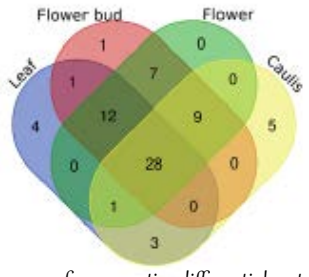


Figure 6. *Venn diagram of comparative differential metabolites in the aerial parts. The number in verlapping regions is the amount of intersection of metabolites in the different parts, and the remaining regions shows the specifically metabolites.*

Materials and Methods

Chemicals, reagents and plant materials

Acetonitrile, methanol, and formic acid (HPLC grade) was supplied by Merck (Darmstadt, Germany). All of other chemicals and reagents were of analytical grade and obtained from Yuanye Biotechnology (Shanghai Yuanye Biotechnology Co., Ltd., China). Ultrapure water was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA). The authentic standards of chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, isochlorogenic acid A, isochlorogenic acid B, 1-*O*-caffeoylquinic acid, ferulic acid, caffeic acid, loganin, Proline,Valine, Isoleucine,Phenylalanine were obtained from Shanghai Yuanye Biotechnology Co. Ltd (Shanghai,China); quinic acid, rutin, astragalin, hyperoside, isoquercitrin were purchased from the Control of Pharmaceutical and Biological Products (Beijing, China); apigenin, diosmetin, kaempferol kaempferol-*3-O*-rutinoside, sweroside, akebia saponin D were provided by Chengdu Chroma Biotechnology Co. Ltd (Sichuan, China); secoxyloganin, dipsacoside B were acquired from Nanjing Jingzhu Biotech-

Table S1. The metabolites represented by the numbers in the Venn diagram (Figure 6)

Names	Numbers	Metabolites
Caulis ∩ Flower ∩ Flower bud ∩ Leaf	28	Rutin， 5-(p-Coumaryl) quinic acid, 3-*O*-ferulicoylquinic acid, Lonicerin, Valine, Caffeic acid, Hederagenin, Proline, Neochlorogenic acid, Luteolin, Rhoifolin, Hyperoside, 1-*O*-caffeoylquinic acid, Cryptochlorogenic acid, Secologanic acid, 7-*O*-ethyl sweroside, Quinic acid, Chlorogenic acid, Vogeloside, Secoxyloganin, Loganic acid, Centauroside, Astragalin, 1,4-*O*-dicaffeoylquinic acid, Swertiamarin, Sweroside, 1,5-*O*-dicaffeoylquinic acid, 3-*O*-caffeoylquinic acid methyl ester
Flower ∩ Flower bud ∩ Leaf	12	Isoquercitrin, 1,3-*O*-dicaffeoylquinic acid, Isochlorogenic acid C, 3,4,5-tricaffeoylquinic acid, Kaempferol-3-*O*-rutinoside, Quercetin, Coumaroyl caffeoylquinic acid isomer, Apigenin, Coumaroyl caffeoylquinic acid, 4,5-*O*-dicaffeoylquinic acid methyl ester, Isochlorogenic acid A, Isochlorogenic acid B
Caulis ∩ Flower ∩ Leaf	1	Caffeic acid methyl ester
Caulis ∩ Flower ∩ Flower bud	9	Diosmetin, Chrysoeriol, Genistein, 8-epi-loganin acid, Akebia saponin D, Dipsacoside B, Flavoyadorinin-B, Loganin, Tricin
Flower bud ∩ Leaf	1	Secologanin
Caulis ∩ Leaf	3	Quercetin-7-*O*-glucoside, Cupressuflavone/Ochanaflavone, Luteolin-5-*O*-β-D-glucopyranoside
Flower ∩ Flower bud	7	Kaempferol, Kingiside, Protocatechuic acid, Luteoloside, Hesperidin, Phenylalanine, Feruloyl caffeoylquinic acid
Leaf	4	Apigenin-7-*O*-rutinoside, Prunetin, Macranthoidin B, Morroniside
Flower bud	1	Isoleucine
Caulis	5	7-epi-loganin, Ferulic acid, 8-epi-loganin, Macranthoidin A, Ethyl caffeate

nology Co. Ltd (Nanjing, China); 1,3-*O*-dicaffeoylquinic acid, isochlorogenic acid C, protocatechuic acid were received from Chengdu Prefa Technology Development Co. Ltd (Sichuan, China); 4,5-*O*-dicaffeoylquinic acid methyl ester, luteoloside, luteolin, rhoifolin lonicerin, secologanic acid, loganin acid, morroniside, macranthoidin A were offered by Liangwei Chemical Reagent Co. Ltd (Nanjing, China). The purity of each compound was more than 98% determined by HPLC analysis.

Forty-four batches of samples (including leaf, flower bud, flower, and caulis) were collected from 4 different provinces in China. Detailed information about these samples is listed in Table 2. The botanical origins of the materials were identified by Professor Xunhong Liu (Department for Authentication of Chinese Medicines, School of Pharmacy, Nanjing University of Chinese Medicine, China). Voucher specimens were deposited in the Herbarium of Pharmacy, Nanjing University of Chinese Medicine.

Table 2. Information on *Lonicera japonica* Thunb samples

Sample	No.	Batch No.	Habits	Origin
Leaf	S1	2.018E+09	Henan	Fengqiu
	S2	2.018E+09	Henan	Fengqiu
	S3	2.018E+09	Henan	Fengqiu
	S4	2.018E+09	Henan	Fengqiu
	S5	2.018E+10	Henan	Fengqiu
Caulis	S6	18030825	Shandong	Anhui Dichang Pharmaceutical Co., Ltd.
	S7	C16052001	Jiangsu	Zhejiang Yedong Pharmaceutical Co., Ltd.
	S8	180426	Jiangsu	Nantong Sanyue Herbal Medicine Co., Ltd.
	S9	180501	Shandong	Bozhou Beshixin traditional Chinese Medicine slice Co., Ltd.
	S10	170601	Shandong	Anhui YaoZhiyuan traditional Chinese Medicine decoction Co., Ltd.
	S11	180810	Shandong	Ningbo Mingbei Traditional Chinese Medicine Co., Ltd.
	S12	20170801	Shandong	Local collection
	S13	170501	Shandong	Local collection
	S14	20170927	Shandong	Nantong Sanyue Herbal Medicine Co., Ltd.
	S15	171020	Jiangsu	Shanghai medicine holdings Yixing Co., Ltd.
	S16	20181101	Shandong	Local collection
	S17	20181102	Shandong	Local collection
	S18	20181103	Shandong	Local collection
	S19	20181104	Shandong	Local collection
	S20	20181105	Shandong	Local collection
Flower bud	S21	180701	Shandong	Chongqing Wanli Pharmaceutical Co., Ltd.
	S22	C16011901	Henan	Zhejiang Yedong Pharmaceutical Co., Ltd.
	S23	2.018E+09	Henan	Fengqiu
	S24	20181108	Shandong	Local collection
	S25	2.018E+09	Hebei	Juluxian Goujijinyinhua market
	S26	180401	Henan	Anhui YaoZhiyuan traditional Chinese Medicine decoction Co., Ltd.
	S27	2.018E+09	Shandong	Linyi
	S28	2.018E+09	Henan	Fengqiu
	S29	2.018E+10	Henan	Fengqiu
	S30	2.018E+10	Henan	Fengqiu
	S31	2.018E+09	Henan	Fengqiu
Flower	S32	180607	Shandong	Nantong Sanyue Herbal Medicine Co., Ltd.
	S33	170802	Shandong	Bozhou Beshixin traditional Chinese Medicine slice Co., Ltd.
	S34	1708021	Shandong	Bozhou Beshixin traditional Chinese Medicine slice Co., Ltd.
	S35	2.018E+09	Shandong	Linyi
	S36	2.018E+09	Hebei	Juluxian Gouqijinyinhua market
	S37	2.018E+09	Shandong	Linyi
	S38	2.018E+10	Hebei	Juluxian Gouqijinyinhua market
	S39	2.018E+09	Shandong	Linyi
	S40	20181107	Shandong	Local herbal medicine market
	S41	2.018E+09	Henan	Fengqiu
	S42	171116	Jiangsu	Shanghai medicine holdings Yixing Co., Ltd.
	S43	2.018E+09	Hebei	Juluxian Gouqijinyinhua market
	S44	2.018E+09	Hebei	Juluxian Gouqijinyinhua market

Sample preparation

All of the samples were finely pulverized and sieved through a 50-mesh. Each dried weighed powder (1.0 g) was soaked in 40 mL of 70% methanol solution in a conical flask. After being shaken violently, the powder was extracted using ultra sonication (500 W, 40 kHz) for 45 min at room temperature; the loss of solvent was replenished by 70% methanol and later centrifuged at 12000 rpm for 10 min (8050 g). The mixture was filtered through a 0.22 μm membrane prior to UFLC-Triple TOF-MS/MS analysis. Finally, the dried samples were stored at -4 °C for further analysis.

UFLC-Triple TOF-MS/MS analysis

The UFLC system (SHIMADZUDGU Corp., Kyoto, Japan) with electronic spray ionization (ESI) source was used for sample analysis. In order to obtain a valid, optimal chromatographic condition, various parameters including X Bridge R C18 (Waters, Wexford, Ireland), Agilent ZORBAX SB C18 column (Agilent, Palo Alto, CA, USA) and Thermo Acclaim TM RSLC 120 C18 (Thermo Scientific, Waltham, MA, USA) three types of columns, and water/acetonitrile, water/methanol, 0.1% formic acid aqueous solution/acetonitrile, 0.2% formic acid aqueous solution/acetonitrile four kinds of mobile phases were considered. The result of UFLC indicated that the Agilent ZORBAX SB-C18 column and 0.2% formic acid aqueous solution (A)/acetonitrile (B)were better because of the strong hydrophilicity of organic acids. Meanwhile, the effects of flow rate, temperature, and injection volume were also investigated; In consideration of the baseline, the shape and number of chromatogram peaks, the flow rate of 0.3 mL/min, 35 °C of the column temperature and 1 μL injection volume were selected. The gradient elution was optimized and set according to the following schedule: 0–4 min: 2% B; 4–5 min: 2–10% B; 5–25 min: 10-18% B; 25–29 min: 18–25% B; 29–30 min: 25–44% B; 30–33 min: 44–48% B; 33–38 min:48–72% B; 38–41 min: 72–95% B.

Mass spectrometry detection was performed on AB SCIEX Triple TOFTM 5600 System-MS/MS (AB Sciex, Framingham, MA, USA) equipped with an electronic spray ionization (ESI) source in both negative and positive ion mode for the full scan. The full scan mass range was set to m/z 100-2000 to acquire TOF-MS data, the scanning range of m/z 50 to 1500 to acquire TOF-MS/MS. The optimized MS analysis conditions were set as follows: nebulizer gas (GS 1), 55 psi; heater gas (GS 2), 55 psi; curtain gas (CUR), 40 psi; ion spray voltage floating (ISVF), 4500 V; turbo spray temperature (TEM), 550 °C; declustering potential, -100 V; collision energy, -40 V.

A strategy for comprehensive study on the aerial parts of *Lonicera japonica* Thunb

In the present work, we developed a strategy integrating metabolic profiling and multi-step PLS-DA analysis to separate the different aerial parts and reveal the chemical markers of *L. japonica*. (figure 7) There are two main parts to this strategy; one is UFLC-Triple TOF-MS/MS method, which was employed to explore the chemical composition. The other is multi-step PLS-DA, which were applied to distinguish the aerial parts, and to reveal the differential compositions among them, and then selecting the optimal chemical markers.

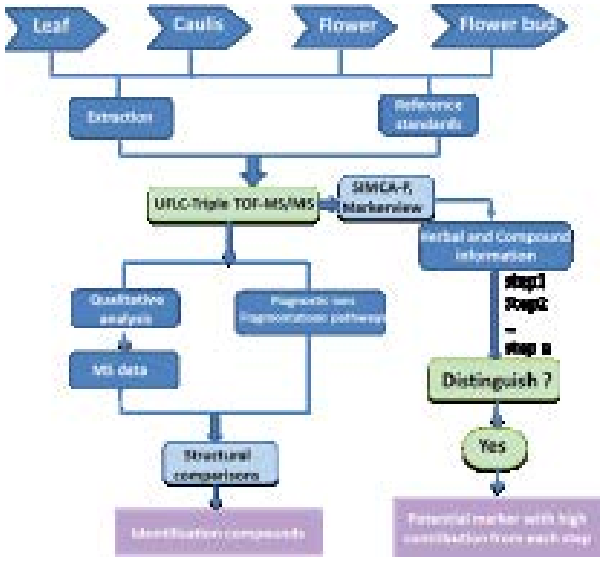


Figure 7. *A strategy for comprehensive study the aerial parts of Lonicera japonica Thunb*

Data processing

In this study, a database of chemicals from LJF and LJC was established by searching the relevant databases, including Chinese National Knowledge Infrastructure (CNKI), PubMed. Additionally, SciFinder was used to confirm the compound information (chemical names, molecular formulas and structures). The constituents of samples were identified by matching retention time, accurate mass measurement with standard substances and the databases, and rules controlling structural changes of each compound with relevant literature.

The mass spectrometry was collected by the Analyst TF 1.6 software (AB Sciex, USA), the UFLC-Triple TOF-MS/MS data was processed by PeakView1.2 (AB Sciex, USA) and MarkerView 1.2.1 software (AB Sciex, USA). Moreover, PLS-DA was performed on SIMCA-P 13.0 software (for Windows, Umetrics AB, Sweden). URL: http://bioinformatics.psb.ugent.be/webtools/Venn/, which was calculated and drawn custom Venn diagrams.

Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA)

High-dimensional and complex data can be dimensionally reduced to study the characteristics of the metabolic spectrum of samples by multivariate statistical analysis. In this study, uunsupervised Principal Component Analysis (PCA) was performed to elucidate the total metabolic differences among the samples of each group. In order to further investigate the differences among leaf, flower bud, flower, and caulis of *L. japonica*, the supervised partial least squares discriminant analysis (PLS-DA) and Variable Importance in the Projection (VIP) were carried out.

Conclusions

This study is a portion of a long-term project to explore new

strategies for quality evaluation of complex TCMs. This strategy was developed based on the establishment of chemical constituent data sets, metabolic profiling and chemical pattern recognition for leaf, flower bud, flower, and caulis of *L. japonica*. A total of 71 metabolites were identified from the aerial parts samples. Among them, Lonicerin, Kaempferol-3-*O*-rutinoside, Loganin, Isochlorogenic acid B, Isochlorogenic acid C, Secologanic acid, Luteoloside, Astragalin were selected as the optimal chemical markers. Eventually, this study laid the foundation for elucidating the differences in efficacy between LJF and LJC at the level of phytochemistry. Meanwhile, from the perspective of the structure-activity relationship, it also implied leaf could be used as an alternative medicinal resource for LJF.

Acknowledgement

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Authors' contributions

Zhichen Cai, Xunhong Liu contributed to conception and design, Zhichen Cai Chengcheng Wang, Cuihua Chen, Jiali Chen, Mengxia Tan, Yuqi Mei, Lifang Wei executed the experiments, Zhichen Cai acquisited, analysed of data and wrote the paper, Lisi Zou collected the samples.

Conflict of Interest

The authors declare that they have no conflict of interest.

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