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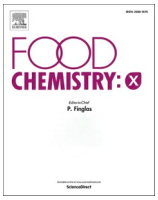

Metabolic profiling provides insights into the accumulation patterns of flavonoids and phenolic acids in different parts of *Lactuca indica* L.

Junfeng Hao, Gentu Ge, Yushan Jia, Feng Han, Muqier Zhao, Zhijun Wang *

College of Grassland Resources and Environment, Key Laboratory of Forage Cultivation, Processing and High Efficient Utilization of Ministry of Agriculture and Rural Affairs, Key Laboratory of Grassland Resources, Ministry of Education, Inner Mongolia Agricultural University, Hohhot 010019, China

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ABSTRACT

Lactuca indica L. contains high amounts of flavonoids and phenolic acids. However, there is limited information on the composition of these compounds in different parts of the plant. The present study analyzed the secondary metabolite profiles of the stem, leaf, flower, and seed of *Lactuca indica* L.cv. Mengzao (LIM) using a widely targeted metabolomic approach. A total of 576 secondary metabolites were identified, including 218, 267, 232, 286, 302, and 308 differentially accumulated metabolites (DAMs) in the stem_vs_leaf, stem_vs_flower, stem_vs_seed, leaf_vs_flower, leaf_vs_seed, and flower_vs_seed comparisons. In particular, considerable differences were detected in the flavonoids and phenolic acids, five flavonoids, five phenolic acids, one triterpenoid and one alkaloid being differentially accumulated in the four parts. Compared to the stem and flower, the leaf and seed had higher total flavonoid content and total phenolic content. Thses findings provide comprehensive insights into utilizing different parts of LIM in developing functional food products.

Introduction

The *Lactuca indica* L., a member of the Compositae family, has edible and medicinal values (Hao et al., 2023; Kim et al., 2012), and is widely grown in Korea, Russia, Indonesia, Iran, Japan, India, Afghanistan, and northern China (Li et al., 2020; Oliya et al., 2018). The delicate and nutritious leaves of this species have been widely used in salad, sushi, and soup (Oliya et al., 2018; Richard et al., 1991). Studies have demonstrated various pharmacological activities of *Lactuca indica* L., such as anticancer, antioxidant, antibacterial and enzyme inhibition properties, those are related to its bioactive compounds (e.g., flavonoids, triterpenes and quinic acid derivatives (Hao et al., 2023; Kim et al., 2007; Li et al., 2020; Gawlik-Dziki et al., 2016; Goesaert et al., 2005). However, the differences in mechanism of action of pharmacological effects, probaly due to the diversity of active contents in *Lactuca indica* L.

As an early Chinese medicine, the whole plant of *Lactuca indica* L., as well as its stem, leaf, flower, and seed, have been used medicinally (Hu et al., 2018). Like other plants of the Lettuce genus, the leaf and stem of *Lactuca indica* L. also release a milky white juice when injured. This juice is rich in bioactive compounds, such as phenols and sesquiterpene lactones, which protect the plant against herbivorous insects (Agrawal et al., 2009). Specificallly, the total concentrations of flavonoids and phenolic compounds in *Lactuca indica* L.cv. Mengzao (LIM) have been reported to be 6.69–10.22 mg/g DW and 3.39–10.69 mg/g DW, respectively (Hao, 2021). Besides, we identified 15 flavonoids and 2 phenolic acids from the whole LIM plant (Hao, 2021; Hao et al., 2021). Researchers have also detected significant differences in phenolic compounds' distribution, content, and antioxidant propertie among *Lactuca indica* L. varieties, parts, and growth stages. For example, Kim and Yoon (2014) found higher levels of polyphenols and flavonoids in wild *L. indica* than the cultivated *Lactuca indica* L. Whereas, only gallic acid has been found in the root of the cultivated *Lactuca indica* L. In addition, Rong (2020) found that the content of flavonoids in LIM first increased and then decreased with delayed fertility, the highest content of flavonids in flowering period. The phenolic compounds accumulate mainly in the above-ground parts of LIM, and the levels vary considerably among the different plant parts. Specifically, the stem has higher quercetin and apigenin than the leaf, while the flower has high concentrations of rutin and luteolin (Hao et al., 2021). Meanwhile, some species contain triterpene acetate, quinic acid derivatives, and flavonoids, with antioxidant activity and cholesterol-lowering properties (Yi et al., 2019). In summary, there are obvious differences in medicinal value between different parts and stages.

Metabolomics is a powerful technique for identifying small-molecule

* Corresponding author.
E-mail address: zhijunwang321@126.com (Z. Wang).

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metabolites in plants, food, and humans (Rosalía et al., 2019; Qian et al., 2023; Song & Tang, 2023; Song et al., 2021). Specifically, gas chromatography-mass spectrometry (GC–MS) and liquid chromatography-mass spectrometry (LC-MS) have been used to analyze the chemical composition of *Lactuca indica* L. (Deng et al., 2016; Hao et al., 2023; Li et al., 2020). However, few studies have conducted detailed detection, identification, and quantification of all chemical and nutritional components in different parts of *Lactuca indica* L. Widely targeted metabolomics is a high throughput technique used to acquire metabolome data with comprehensive coverage and high sensitivity and combines the advantages of targeted and untargeted metabolite detection; this method helps to determine the impact of tissue parts on various secondary metabolites (Shi et al., 2022; Wang et al., 2020).

Therefore, the present study explored the differences in the bioactive composition of LIM's major medicinal parts, including stem, leaf, flower, and seed. The study used a widely-targeted metabolomic approach to identify the secondary metabolites and compared the composition of the functional components (flavonoids and phenolic acids) associated with health benefits in differernt parets of LIM. The findings of this study provide a reference for utilizing LIM parts in functional foods.

Materials and methods

Materials and chemicals

LIM plants were grown in the field at the experimental site of Inner Mongolia Agricultural University (40°81′N, 111°70′E). Studies have shown that during the reproductive growth stage, the LIM root have low nutritional value, while other parts (stem, leaf, flower and seed) contain high levels of flavonoids and polyphenols (Hao et al., 2021). The stem, leaf, and flower were collected at the flowering stage, and the seed was collected at the fruiting stage. All samples were vacuum freeze-dried for 48 h in a Christ ALPHA 1-4LDplus lyophilizer (Marin Christ, Osterode, Germany) and crushed into a fine powder (100 mesh). Thesamples are shown in Fig. S1.

Standards, including rutin and gallic acid, and the Folin-Ciocalteu solution were obtained from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Formic acid, methanol, ethanol, and acetonitrile were obtained from Merck & Co., Inc. (NJ, USA).

Determination of total flavonoid and phenolic acid content

The LIM sample (stem, leaf, flower, or seed) was extracted using the method proposed by Hao et al. (2018) with a slight modifications. Approximately 0.5 g of tissue power was placed in a corked conical flask, mixed with 20 mL of 60 % ethanol, extracted by ultrasonication for 30 min (500 W, 70 °C), fixed volume to scale mark. The supernatant was then filtered through a 0.45 μm filter membrane and stored at 4 °C. There were three times for each test.

The total flavonoid content (TFC) was measured according to the previously described method with slight modifications (Yu et al., 2022). Briefly, 200 μL of the diluted LIM extract was mixed with 40 μL of $NaNO_3$ solution (5 %, w/v) and allowed to stand for 6 min; Then, 40 μL of Al $(NO_{3)3}$ solution (10 %, w/v) was added. The resulting mixture was vortexed for 20 s and incubated for another 6 min. Subsequently, 400 μL of NaOH solution (1 mol/L) was added, and the final volume of the mixture was adjusted with distilled water. After incubation for 15 min, the absorbance was measured at 510 nm using a U-2900UV/VIS spectrophotometer (Hitachi High-Tech (Shanghai) Co., Ltd., China), using the solvent as blank. The results were expressed as rutin per gram dry weight of LIM (mg/g DW). The standard was prepared in the solvent used for sample preparation and analyzed to generate the calibration curve.

The total phenolic content (TPC) was measured using the previously described Folin-Ciocalteu method with slight modifications (Ning et al., 2023). Here, 0.2 mL of the diluted LIM extract was mixed with 6 mL of distilled water and 0.5 mL Folin-Ciocalteu solution. After 5 min, 1.5 mL of Na_2CO_3 solution (10 %, w/v) was added, and the mixture was vortexed for 20 s, heated at 75℃ for 10 min, and incubated in the dark for 60 min at room temperature. The absorbance of the resultant mixture was measured at 765 nm using a U-2900UV/VIS spectrophotometer, using the solvent as blank. The results were expressed as gallic acid per gram dry weight of LIM (mg/g DW). The standard was prepared in the solvent used for sample preparation and analyzed to generate the calibration curve.

Analysis of secondary metabolites based on UPLC-MS/MS

The extraction, identification, and quantification of the metabolites of LIM were carried out by MetWare Biotechnology Ltd. (Wuhan, China) according to the reported methods (Chen et al., 2013). The LIM sample was freeze-dried under a vacuum in a lyophilizer (Scientz-100F) and ground to powder using a grinder (MM 400, Retsch, Germany) at 30 Hz for 1.5 min. Approximately 50 mg of the powder was weighed and dissolved in 1.2 mL of cold 70 % methanol at −20 °C and vortexed for 60 s every 30 min (6 times in total). After centrifuging at 12,000 rpm for 3 min, the supernatant was aspirated, and the sample was filtered through a 0.22 μm microporous membrane and stored in an injection vial. The sample was analyzed by ultra-performance liquid chromatography (UPLC) (ExionLC™ AD, Foster City, CA, USA, https://sciex.com.cn/) and tandem mass spectrometry (MS/MS) (Applied Biosystems 6500 QTRAP, Foster City, CA, USA, https://sciex.com.cn/) using an Agilent SB-C18 column (2.1 mm × 100 mm). Here, 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile were used as mobile phases A and B, respectively. The elution was carried out as follows: 95:5 V(A)/V(B) at 0 min, 5:95 V(A)/V(B) at 9.0 min, 5:95 V(A)/V(B) at 10.0 min, 95:5 V(A)/V(B) at 11.1 min, and 95:5 V(A)/V(B) at 14.0 min. The flow rate was set at 0.35 mL/min, the column temperature at 40 °C, and the injection volume at 2 μL. Then, the mass spectrometry data were collected in positive/negative electrospray ionization (ESI) mode with the ESI source at 500 °C and using an ionization voltage of 5500 V (positive ionization mode)/-4500 V (negative ionization mode); ionization gas I (GSI), gas II (GSII), curtain gas (CUG) were set at 50, 60 and 25 psi, respectively, and the collision-induced ionization parameters were set to high. QQQ scans were acquired as multiple reaction monitoring (MRM) experiments with the collision gas (nitrogen) set to medium. Further, the declustering potential (DP) and collision energy (CE) were optimized for each MRM ion pair. Finally, a specific set of MRM ion pairs was monitored for each period based on the metabolites eluted during this specific period.

The metabolites in the samples were identified by comparing the *m/z* values, retention times (RT) and fragmentation patterns to those of the standards in a self-built database (MetWare). Meanwhile, the metabolites were quantified using the MRM mode of the triple quadrupole mass spectrometry (Chen et al., 2013; Dong et al., 2018). In this mode, the quadrupole first screens for the precursor ions (parent ions) of the target substance and excludes the ions corresponding to other molecular weight substances to eliminate interferences. The precursor ions are then induced to ionize by the collision chamber and form several fragment ions, which are then filtered through the triple quadrupole to select the characteristic fragment ion required, this step eliminates non-target ion interferences and allows accurate and reproducible quantification. After obtaining the metabolite profiling data from different samples, the mass spectral peak areas of all compounds were integrated, and the mass spectral peaks of the same metabolite in different samples were processed for integral correction using the MultiQuant software (version 3.0.2). The spectral peak area was finally used to determine the relative abundance of a metabolite in each sample.

Statistical analysis

The hierarchical clustering analysis (HCA), principal component

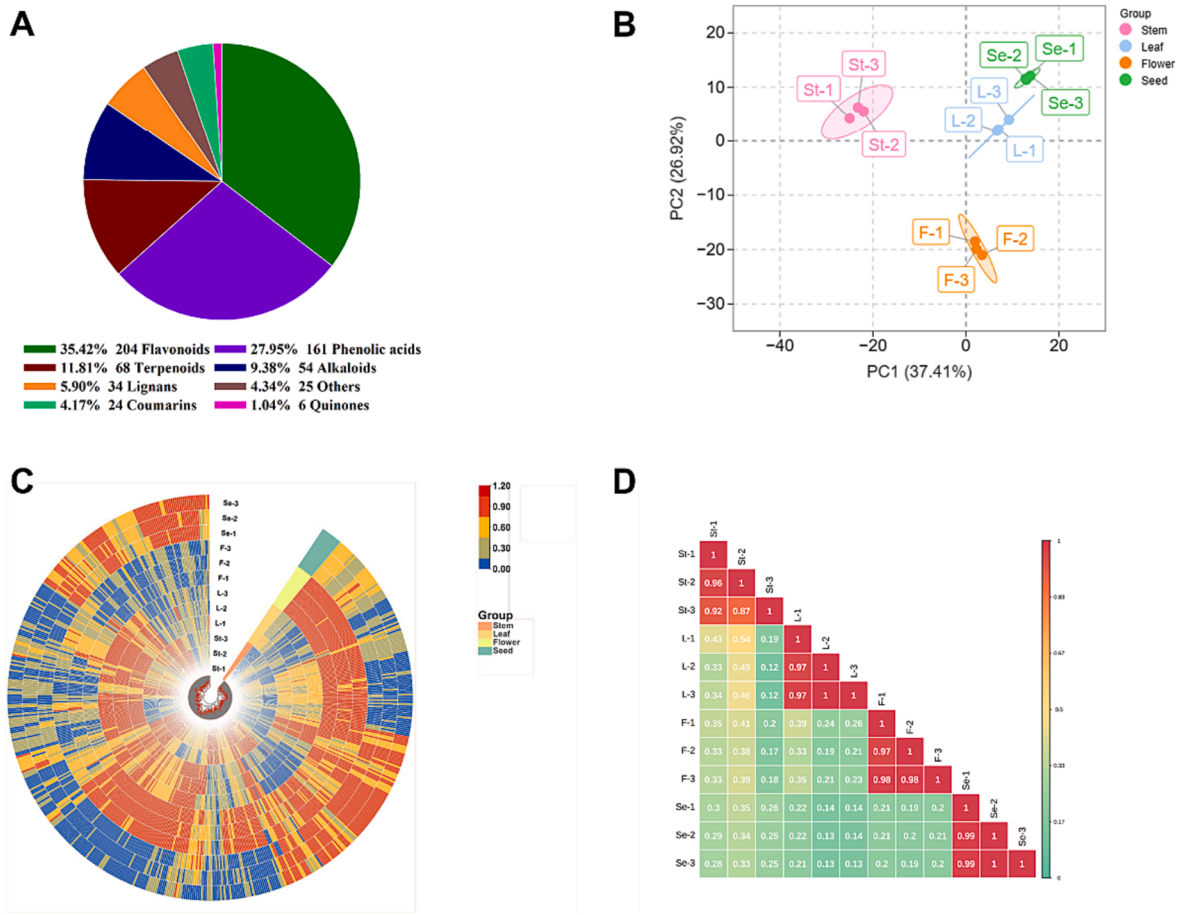

Fig. 1. (A) The classification of 576 metabolites in the stem, leaf, flower, and seed of *Lactuca indica* L. cv. Mengzao. (B) PCA score plot of LIM samples based on the relative abundance of secondary metabolites. (C) Hierarchical clustering of 576 differentially accumulated secondary metabolites. From center to edge, the data are sorted by name. Blue indicates relatively low intensity and red indicates relatively high intensity. The red boxes indicate the clusters studied in depth below. (D) Correlation map of samples of different parts of *Lactuca indica* L. cv. Mengzao. The vertical and diagonal lines represent the names of different samples. Different colors represent different Pearson correlation coefficients, with red shades representing stronger positive correlations, green shades representing weaker correlations, and blue shades representing stronger negative correlations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

analysis (PCA), and orthogonal partial least squares-discriminant analysis (OPLS-DA) were conducted with the R software (https://www.r-project.org/). The pathways enriched by the differential metabolites in different parts of LIM were annotated using Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Origin 2021 (Origin Lab®, MA, USA, https://www.originlab.com/) was used for drawing the graphic. SPSS 24.0 software (SPSS Inc., Chicago, IL, USA) was used to conduct one-way ANOVA, and Duncan's test was used to determine the significant differences between the treatment groups ($P \leq 0.05$).

Results and discussion

Determination of total flavonoids and total phenolics in LIM

The whole plant of LIM has abundant flavonoids and phenolic compounds (Hao et al., 2021), the present study explored the differences in the total flavonoids and total phenolics of LIM's major medicinal parts, including stem, leaf, flower, and seed. As shown in Fig. S2, the seed and leaf of LIM contained the highest total flavonoid contents, 100.92 mg/g DW and 98.25 mg/g DW, respectively. Compared to other parts, the LIM stem had the lowest content of total flavonoids and total phenolics, while the leaf contained the highest total phenolic content (56.98 mg/g DW). The total flavonoid content detected in the LIM leaf in this study was higher than that reported by the *Lactuca indica* leaf (Rong, 2020), the *Lactuca indica* (Hao et al., 2023), and the *Lactuca tatarica* (L.) C. A. Mey (Hou et al., 2019). This difference in the flavonoids may be due to the differences in cultivation, time of harvest, conditions of the region, and type of variety (Halim et al., 2022). Besides, in our study, LIM was harvested during flowering (August), when the high temperatures and strong light cause plants to photosynthesize intensively, accumulate more carbon, and promote secondary metabolite production (Schmidt et al., 2010), leading to the accumulation of flavonoids in the leaves.

Metabolomic analysis of LIM

In order to explore the differences in the metabolite composition of LIM's major medicinal parts, including stem, leaf, flower, and seed. Here, overlay display analysis was performed using total ion flow plots of quality control (QC) samples to test the reproducibility of secondary metabolite extraction and detection. The total ion chromatogram (TIC) plots of the QC samples and overlay analysis of sample multi-peak detection plots in positive and negative ion modes demonstrated high reliability and reproducibility of the LIM samples data (Fig. S3).

Further, after quality filtration, a total of 576 secondary metabolites were detected and quantified, including 204 flavonoids, 161 phenolic acids, 68 terpenoids, 54 alkaloids, 58 lignans and coumarins, 6 quinones, and 25 others (Table S1 and Fig. 1A). The PCA showed that PC1

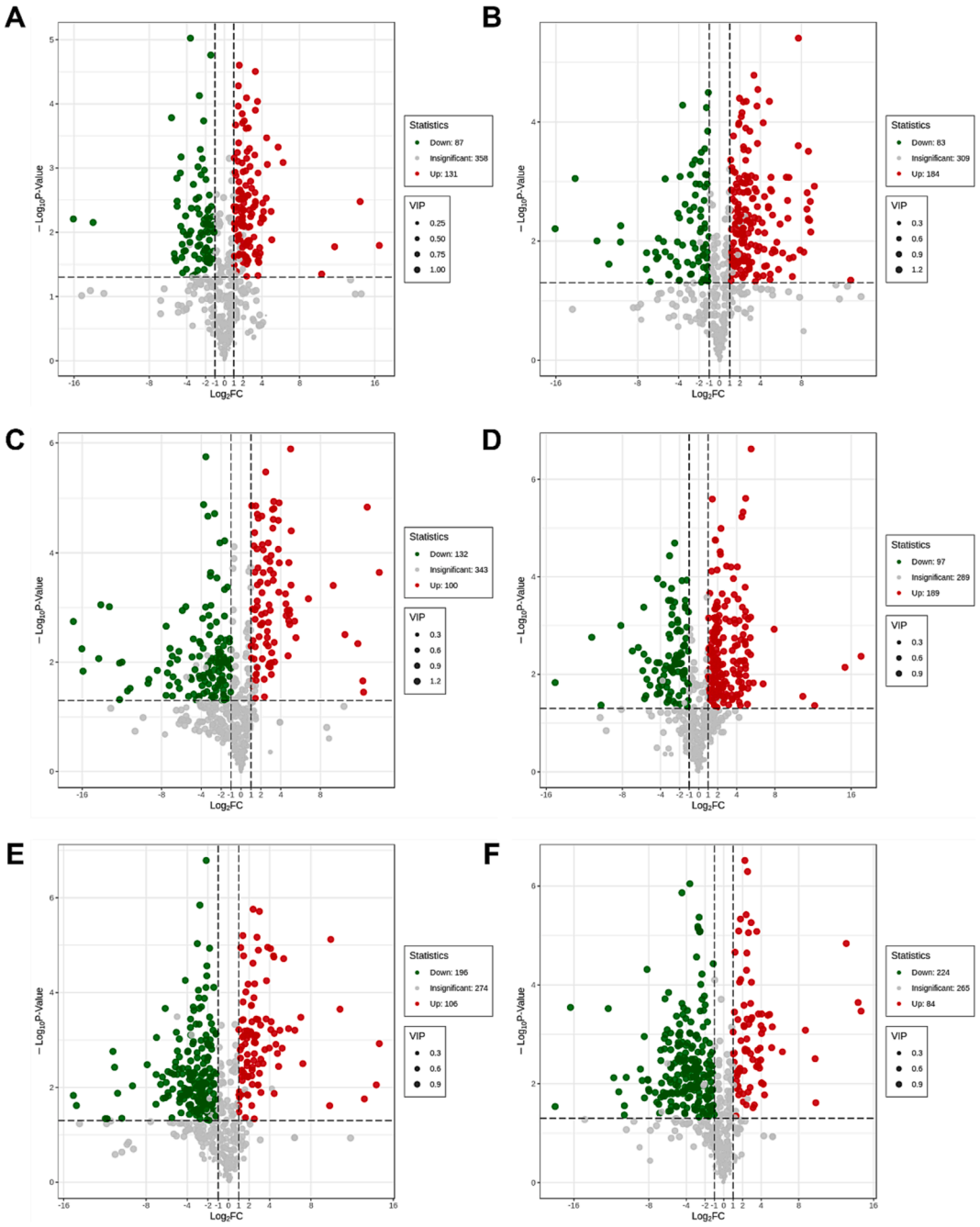

Fig. 2. Volcano plots show the differential metabolites between different parts of *Lactuca indica* L. cv. Mengzao. Red, and green dots indicate upregulated, downregulated metabolites; gray dots represent the metabolites showing no significant difference (A–F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and PC2 explained 37.41 % and 26.92 % of the total variance, respectively (Fig. 1B). Besides, the scatter plot clearly distinguished the stem, leaf, flower, and seed samples. Subsequently, the OPLS-DA model was used to compare the differentially accumulated metabsaolites (DAMs) between different parts of LIM. High predictability (Q^2) is a crucial parameter, representing the predictive capacity of the model (Qian et al., 2023). The model is considered stable when Q^2 is greater than 0.9, and the fit is strong. In this study, we compared the metabolites between stem and leaf ($Q^2 = 0.987$, $R^2X = 0.784$, and $R^2Y = 1$; Fig. S4A), stem and flower ($Q^2 = 0.989$, $R^2X = 0.820$, and $R^2Y = 1$; Fig. S4B), stem and seed ($Q^2 = 0.983$, $R^2X = 0.838$, and $R^2Y = 1$; Fig. S4C), leaf and flower ($Q^2 = 0.988$, $R^2X = 0.793$, and $R^2Y = 1$; Fig. S4D), leaf and seed ($Q^2 = 0.992$, $R^2X = 0.851$, and $R^2Y = 1$; Fig. S4E) and flower and seed ($Q^2 = 0.996$, $R^2X = 0.839$, and $R^2Y = 1$; Fig. S4F). HCA, based on the relative abundance of all the metabolites, showed that, most of the detected metabolites differed significantly in concentrations among plant parts (Fig. 1C), confirming the PCA results. Further, the correlation heat map indicated a high correlation between the replicates of different LIM samples (Fig. 1D).

A total of 204 flavonoids (73 flavones, 63 flavonols, 21 flavanones, 13 isoflavones, 10 flavanols, 9 chalcones, 6 flavanonols, 2 aurones and 5 other flavonoids) were identified (Table S1). We found that naringin, isorhoifolin, juglanin, cynaroside, cosmosiin, nicotiflorin, marein, diosmin, homoplantaginin, phlorizin, prunin, isoquercitrin, brassicin, spiraeoside, rutin, nepitrin and vicenin-2 were modified by different glycosidic bonds in LIM, and were mainly glycosylated at the 3–O, 7–O,

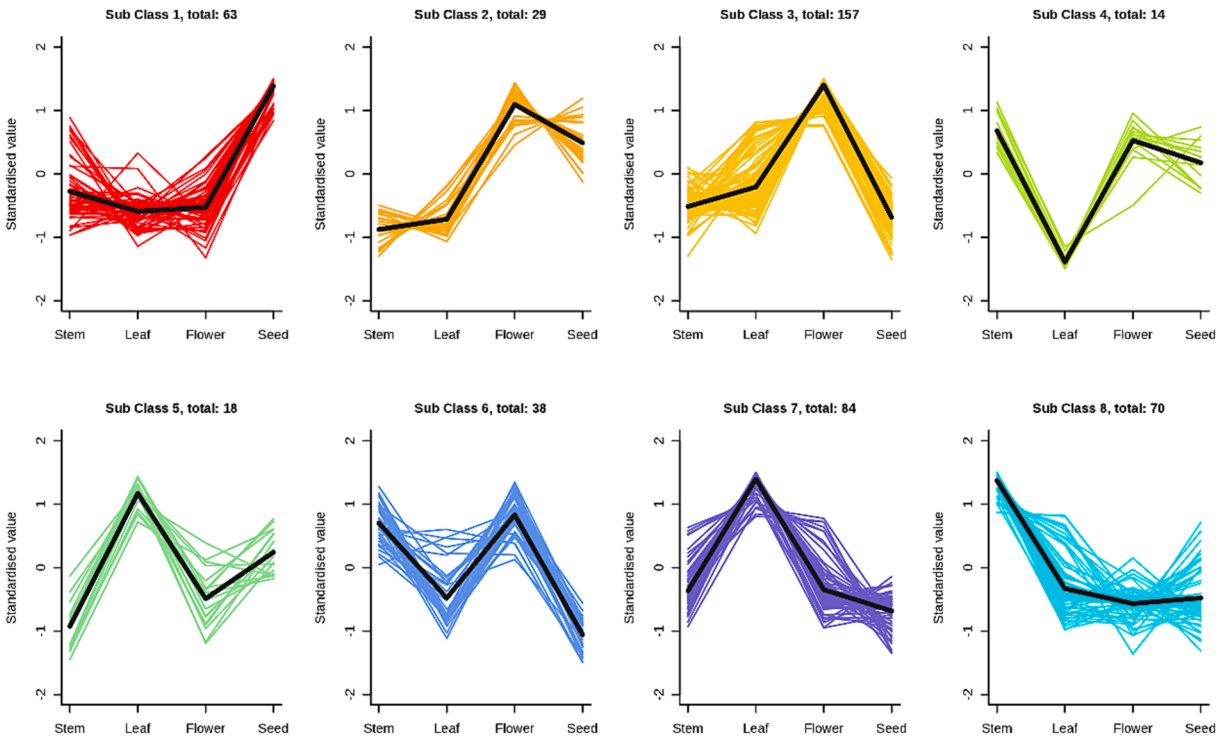

Fig. 3. K-means clustering of the differential secondary metabolites of four parts of *Lactuca indica* L. cv. Mengzao based on their accumulation patterns. The x-axis represents the different samples, and the y-axis represents the standardized content of a metabolite.

6–C, and 8–C positions. Among these, quercetin, eriodictyol, naringenin, apigenin, butin, chrysoeriol, cyanidin, luteolin, kaempferol, acacetin, diosmetin, hesperetin, isorhamnetin and hispidulin were glycosylated at the 3–O and 7–O positions by glucoside, neohesperidoside, galactoside rutinoside, or glucuronide.

Additionally, 161 phenolic acids were identified in LIM (Table S1). Like flavonoids, phenolic acids also existed as aglycones and glycosylated derivatives (Hussain et al., 2021; Qian et al., 2023). We found that erulic acid, *p*-coumaric acid, protocatechuic acid, caffeic acid, chlorogenic acid, ellagic acid, erucic acid, gallic acid, and salicylic acid were present as aglycones and glycosylated derivatives. Nevertheless, gentisoyl was present only as glycosylated derivatives.

Differential metabolites of LIM

Further, we screened for the metabolites with a fold change of ≥ 2 or ≤ 0.5 and VIP ≥ 1 (Qian et al., 2023) (differentially accumulated metabolites, DAMs) among the different parts of LIM, and identified 244 annotated secondary metabolites (Table S2). The DAMs in the LIM parts are shown in Table S3. The analysis identified 218 DAMs (131 upregulated, 87 downregulated) between stem and leaf (Fig. 2A), 267 (184 upregulated, 83 downregulated) between stem and flower (Fig. 2B), 232 (100 upregulated, 132 downregulated) between stem and seed (Fig. 2C), 286 (189 upregulated, 91 downregulated) between leaf and flower (Fig. 2D), 302 (106 upregulated, 196 downregulated) between leaf and seed (Fig. 2E), 308 (84 upregulated, 224 downregulated) between flower and seed (Fig. 2F).

Further, to reflect the main trends and tissue-specific expression of metabolites in different LIM parts, all DAMs were clustered into eight group (Subclass 1 to 8) based on the expression profiles using the K-means method and hierarchical clustering performed with a similar regulatory model (Fig. 3). Subclass 1 included 63 DAMs that showed the highest expression in seed than in other parts, of which phenolic acids were majority and accounted for 47.6 % of the number of metabolites. The 29 DAMs of subclass 2 and 157 DAMs of subclass 3 showed the highest expression in the flower. Among these, subclass 3 had the most flavonoids, accounting for 77.1 % of the number of metabolites. Meanwhile, the 18 DAMs of subclass 5 and 84 DAMs of subclass 7 exhibited the highest expression levels in leaf. Among them, subclass 7 had the most flavonoids and phenolic acid, accounting for 47.6 % of the number of metabolites. Beside, the 14 DAMs of subclass 4 and 38 DAMs of subclass 6 had the highest expression levels in flower and seed (Table S4).

Variation in phenolic compounds in different parts of LIM

Phenolic compounds play an important role in the stability of foods (Mutha et al., 2021). As secondary metabolites of plants, phenolic compounds can be divided into free and bound forms (Cheynier et al., 2013). *Lactuca indica* L. contains high concentrations of phenolic compounds, especially, flavonoids and phenolic acids, which exhibit significant antibacterial and antioxidant activities (Hao et al., 2023; Kim & Yoon, 2014).

Therefore, the present study further investigated the primary composition and content of flavonoids and phenolic acids in LIM stem, leaf, flower, and seed. HCA showed that the DAMs in stem_vs_leaf, stem_vs_flower, stem_vs_seed, leaf_vs_flower, leaf_vs_seed, and flower_vs_seed were mainly flavonoids and phenolic acids (Fig. S5). Based on the accumulation pattern, the flavonids were divided into five major classes (Cluster I to V) (Fig. S6a). The Cluster I, II, and III flavonoids were the highest in seed, stem, and leaf, respectively, while the cluster IV and V flavonoids were the highest in flower. Similarly, the phenolic acids were divided into seven main clusters based on accumulation (Fig. S6b). Cluster I was the highest in seed, clusters II, VI, and VII were in flower, clusters III and IV in leaf, and clusterV in stem.

We further did a pair-wise comparison of the LIM parts to understand the differences in these two major bioactives. The differences in flavonoids and phenolic acids in different parts of the LIM were investigated. A comparison between stem and leaf showed a difference ranging from 2.06 to 22381.11 fold for 74 flavonoids (58 up-regulated, 16 down-regulated) (Fig. 4A). In leaf, the top 10 upregulated flavonoids were 3,4,2′,4′,6′-pentahydroxychalcone-4′-O-glucoside (22381.11-fold), 6-

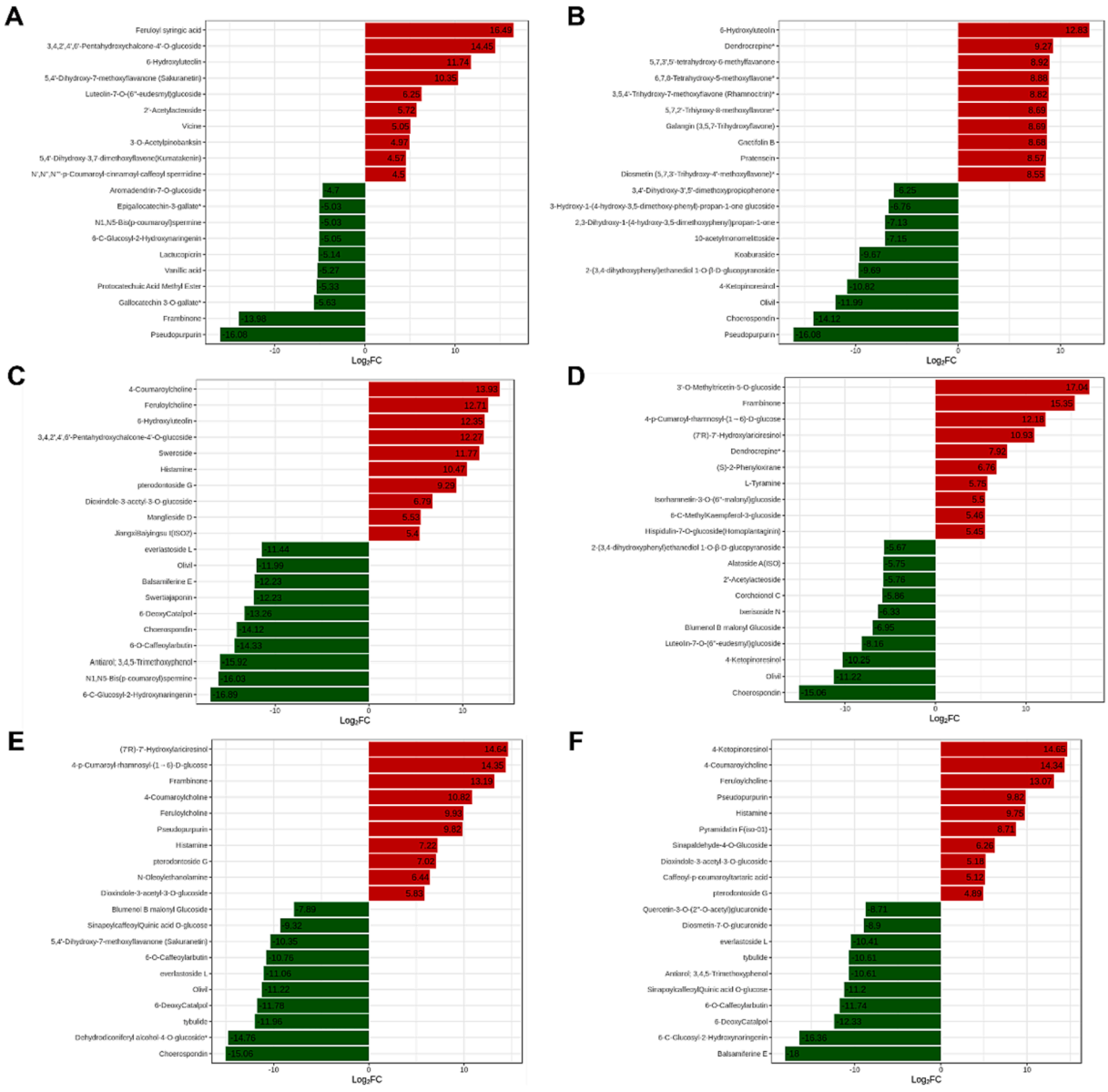

Fig. 4. Top 10 upregulated and down-regulated compounds in in stem_vs_leaf (A), stem_vs_flower (B), stem_vs_seed (C), leaf_vs_flower (D), leaf_vs_seed (E), and flower_vs_seed (F) comparison groups, respectively.

hydroxyluteolin (3424.75-fold), 5,4′-dihydroxy-7-methoxyflavanone (1309.66-fold), luteolin-7-O-(6′'-eudesmyl)glucoside (76.06-fold), 3-O-Acetylpinobanksin (31.32–fold), and 5,4′-dihydroxy-3,7-dimethoxy-flavone (23.82-fold), and the top 4 downregulated flavonoids were aromadendrin-7-O-glucoside (0.03-fold), epigallocatechin-3-gallate (0.03-fold), 6-C-glucosyl-2-hydroxynaringenin (0.03-fold), and gallocatechin 3-O-gallate (0.02-fold). In addition, 120 differential flavonoids were identified by comparing the stem and the flower. As shown in Fig. 4B, the top 10 upregulated DAMs in flower included nine flavonoids, with a fold change of 374.35–7260.76. In addition, 70 differential flavonoids were identified between the stem and the seed. As shown in Fig. 4C, the top 10 upregulated DAMs in seed included 6-hydroxyluteolin (5208.73-fold), and 3,4,2′,4′,6′-pentahydroxychalcone-4′-O-glucoside (4943.65-fold). The top 10 downregulated DAMs in seed included 6-C-glucosyl-2-hydroxynaringenin (8.24-fold). In addition, 117 differential flavonoids were identified between the leaf and the flower. As shown in Fig. 4D, the top 10 upregulated DAMs in flower included 3′-O-methyltricetin-5-O-glucoside (135183.91-fold), isorhamnetin-3-O-(6′'-malonyl)glucoside (45.29-fold), 6-C-methylkaempferol-3-glucoside (43.89-fold), and hispidulin-7-O-glucoside (43.60-fold), while, the top 10 downregulated DAMs included choerospondin (8.24-fold). In addition, 103 differential flavonoids were identified between the leaf and the seed, with a fold change of 0.00–24.26 (Fig. 4E). In addition, 144 differential flavonoids have been identified between the flower and the seed, with a fold change of 0.43–4.46 (Fig. 4F).

Furthermore, 67 DAMs (27 upregulated, 40 downregulated), 78 (38 upregulated, 40 downregulated), 88 (41 upregulated, 47 downregulated), 67 (39 upregulated, 28 downregulated), 90 (46 upregulated, 44 downregulated), and 84 (36 upregulated, 48 downregulated) phenolic acids were identified from the stem_vs_leaf, stem_vs_flower, stem_vs_seed, leaf_vs_flower, leaf_vs_seed, and flower_vs_seed comparis ions, respectively (Table S3). Most phenolic acids were found at higher levels in the stem than in the leaf, flower, and seed. Thses results are similar to a previous report, which detected an increased accumulation of phenolic acids in leaf compared with stem and a decreased accumulation in stem compared with root (Gao et al., 2022). These results may be related to the phenolic acid biosynthetic pathway and position in the metabolic network. In this study, LIM was harvested at late flowering, at

Table 1
Differential metabolites in the four medical parts of LIM.

No.	Compounds	Q1 (Da)	Q3 (Da)	Molecular weight (Da)	Formula	Ionization model	Class
1	Koaburaside	331.10	153.02	332.1107	$C_{14}H_{20}O_9$	$[M-H]^-$	Phenolic acids
2	2-(3,4-dihydroxyphenyl)ethanediol 1-O-β-D-glucopyranoside	331.10	153.02	332.1107	$C_{14}H_{20}O_9$	$[M-H]^-$	Phenolic acids
3	5′-Glucosyloxyjasmanic acid	387.17	207.10	388.1733	$C_{18}H_{28}O_9$	$[M-H]^-$	Phenolic acids
4	3-(3-Hydroxyphenyl)-propionic acid	167.07	123.05	166.0630	$C_9H_{10}O_3$	$[M+H]^+$	Phenolic acids
5	3,4′-Dihydroxy-3′,5′-dimethoxypropiophenone	227.09	181.05	226.0841	$C_{11}H_{14}O_5$	$[M+H]^+$	Phenolic acids
6	Apiin	565.16	271.06	564.1479	$C_{26}H_{28}O_{14}$	$[M+H]^+$	Flavonoids
7	Hispidulin-8-C-(2′′-O-xylosyl)xyloside	565.16	433.11	564.1479	$C_{26}H_{28}O_{14}$	$[M+H]^+$	Flavonoids
8	Kaempferol-3-caffeoyldiglucoside	771.18	609.14	772.1851	$C_{36}H_{36}O_{19}$	$[M-H]^-$	Flavonoids
9	Diosmetin-7-O-glucuronide	477.10	301.07	476.0955	$C_{22}H_{20}O_{12}$	$[M+H]^+$	Flavonoids
10	Apigenin-7-O-(2′′-glucosyl)arabinoside	565.16	271.60	564.1479	$C_{26}H_{28}O_{14}$	$[M+H]^+$	Flavonoids
11	Lactucopicrin	411.14	259.09	410.1366	$C_{23}H_{22}O_7$	$[M+H]^+$	Terpenoids
12	N′,N′′,N′′′-p-Coumaroyl-cinnamoyl-caffeoyl spermidine	584.28	325.00	583.2682	$C_{34}H_{37}N_3O_6$	$[M+H]^+$	Alkaloids

which stage the leaves are smaller, and the stem is lignified, the primary metabolites in the leaf get converted to phenolic acids via the benzoate biosynthetic pathway and the lauric acid biosynthetic pathway. Subsequently, phenolic acids continue to be synthesized by various enzymes in the phenol-propane metabolite pathway (Gao et al., 2022; Kuo et al., 2014). These changes in LIM probably resulted in lower phenolic acid content in the leaf than in the stem. Besides, the present study found that the flower contained higher levels of 1-caffeoylquinic acid, more than the stem and seed (2.17 and 2.23-fold), respectively. Six chlorogenic acid isomers, namely, chlorogenic acid, neochlorogenic acid, isochlorogenic acid C, isochlorogenic acid A, cryptochlorogenic acid, and isochlorogenic acid C, were slso identified in different parts of LIM.

Thus, the present study offered insights into the composition of phenolic acids in LIM and revealed that most phenolic acids were present in the conjugated form, consistent with the observations on the phenolic acids of quinoa seeds (Han et al., 2019). Meanwhile, the content of glycosylated phenolic acids contributes to antioxidant properties (Qian et al., 2023). This study found that LIM contains ferulic acid-4-O-glucoside, salicylic acid-2-O-glucoside, and dihydroferulic acid glucoside in leaf, flower, and seed, respectively. More phenolic acids, suggested that it could be a potential antioxidant resource. In addition, 4-hydroxybenzoic acid and protocatechuic acid were identified as the primary phenolic acids (Gamel & Abdel-Aal, 2012); the former was most abundant in flower and seed and the latter in stem and leaf (Table S2).

Typically, protocatechuic acid, formed from 4-hydroxybenzoic acid by the introduction of a hydroxyl group at the 3-position of 4 HBA by 4 HBA hydroxylase (Huang et al., 2008), acts as precursors of several intricate compounds, including vanillin and anthocyanin 3-O-β-D-glucoside, and exhibits antibacterial, antiviral, and antioxidant activities (Shi et al., 2006).

Key differences in flavonoids and phenolic acids in different parts of LIM

A few non-flavonoid metabolites of plants, including phenolic acids, triterpenoids, and alkaloids, have demonstrated health-promoting effects in humans (Hu et al., 2020; Li et al., 2022; Oliya et al., 2022; Zhu et al., 2018). However, the medicinal components present in different parts of LIM remain unclear. There, the flavonoids and phenolic acids of the different parts in pairs were compared, and the analysis identified 12 compounds as the common differential metabolites between stem and leaf, stem and flower, stem and seed, leaf and flower, leaf and seed, and flower and seed (Fig. S7), including five flavonoids, five phenolic acids, one triterpenoid and one alkaloid (Table 1).

In general, plant foods are a great source of polyphenols, including flavonoids, phenolic acids and anthocyanins (Lee et al., 2013). For example, blackberry contains many bioactive compounds, including phenolic compounds, tannins, and stilbenes. Among them, tannins, anthocyanins and phenolic acids have been found to be the major compounds found in different parts of blackberry plant (Halim et al., 2022). In contrast to the phenolic compounds, apigenin derivatives were also abundant in millet grains (Chandrasekara & Shahidi, 2011). LIM has been reported to be rich in flavonoids with beneficial effects (Hao et al., 2023).

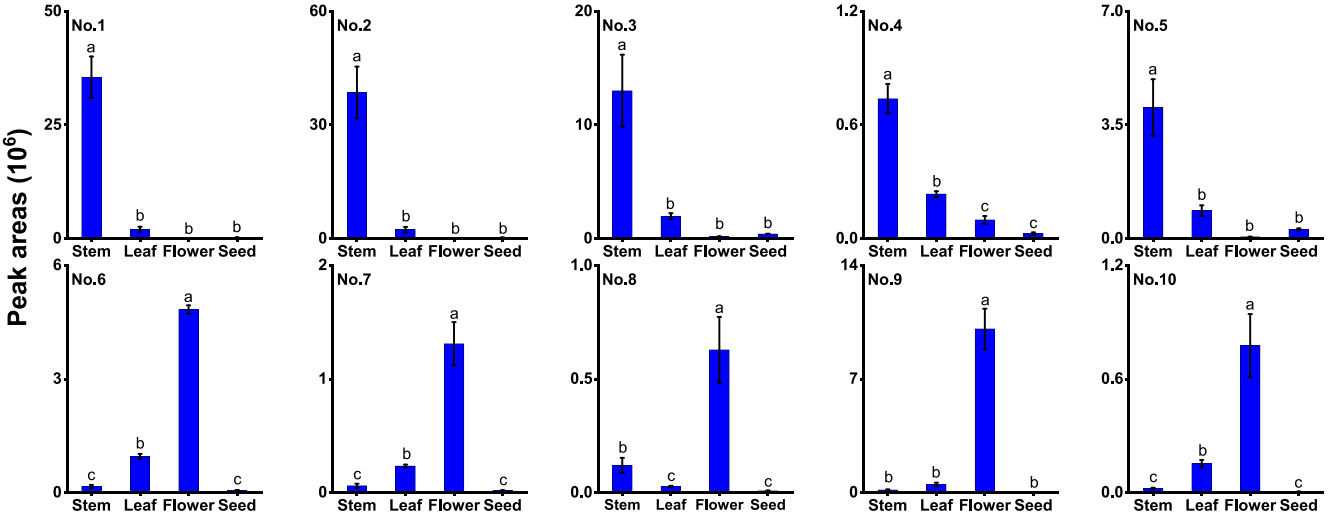

Fig. 5. Peak areas of 10 different flavonoids and phenolic acids identified in all four medical part of *Lactuca indica* L. cv. Mengzao. The compounds 1–10 are koaburaside, 2-(3,4-dihydroxyphenyl)ethanediol 1-O-β-D-glucopyranoside, 5′-glucosyloxyjasmanic acid, 3-(3-hydroxyphenyl)-propionic acid, 3,4′-dihydroxy-3′,5′-dimethoxypropiophenone, apiin, hispidulin-8-C-(2′′-O-xylosyl)xyloside, kaempferol-3-caffeoyldiglucoside, diosmetin-7-O-glucuronide, and apigenin-7-O-(2′′-glucosyl)arabinoside.

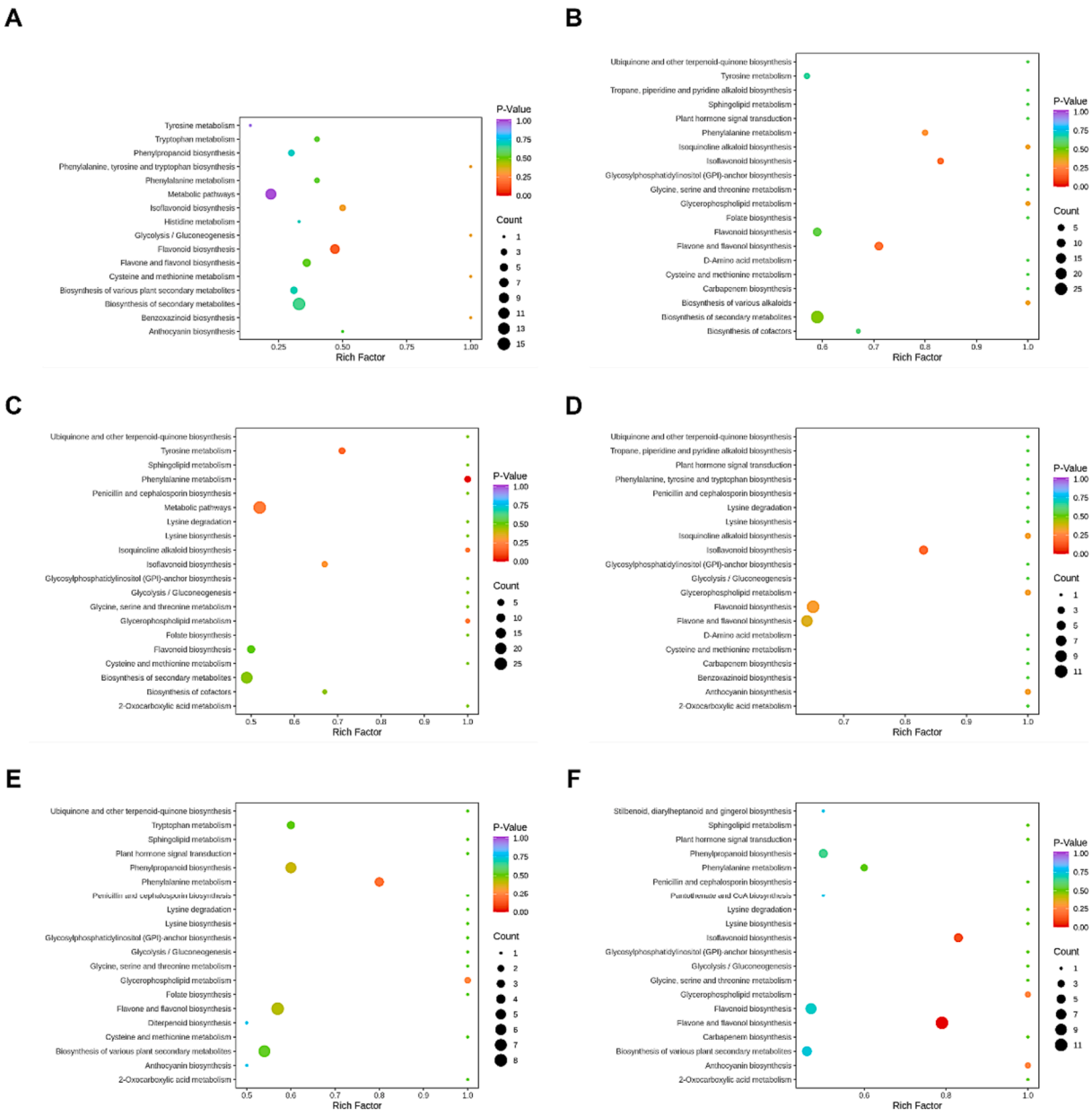

Fig. 6. Pathway analysis of the differentially accumulated secondary metabolites in the different parts of *Lactuca indica* L. cv. Mengzao. KEGG pathway enrichment of the differentially accumulated metabolites identified in the (A) stem_vs_leaf, (B) stem_vs_flower, (C) stem_vs_seed, (D) leaf_s_flower, (E) leaf_vs_seed, and (F) flower_vs_seed comparisons. Each bubble in the plot represents a metabolic pathway whose abscissa and bubble size jointly indicate the magnitude of the impact factors of the pathway; a larger bubble indicates a higher impact factor. The bubble color represents the *p*-values of the enrichment analysis, with darker colors indicating a higher degree of enrichment.

Recently, we found three major flavonoids (rutin, cynaroside, and apigenin) as well as two phenolic acids (chicoric acid and chlorogenic acid) concentrated in the leaf and flower of *Lantuca indica* (Hao, 2021). However, the exact phenolic compounds in different parts of LIM have not been fully characterized. In this study, the peak areas of compounds 1–5 (koaburaside, 2-(3,4-dihydroxyphenyl)ethanediol 1-O-β-D-glucopyranoside, 5′-glucosyloxyjasmanic acid, 3-(3-hydroxyphenyl)-propionic acid, 3,4′-dihydroxy-3′,5′-dimethoxypropiophenone) were significantly higher in the stem of LIM than in the leavesleaf, flower and seed, and all these compounds were phenolic acids (Fig. 5). Meanwhile, the peak areas of compounds 6–10 (apiin, hispidulin-8-C-(2''-O-xylosyl)xyloside, kaempferol-3-caffeoyldiglucoside, diosmetin-7-O-glucuronide, apigenin-7-O-(2''-glucosyl)arabinoside) were significantly higher in the flower than in the stem, leaf and seed, and they were all flavonoids. These differences revealed that phenolic compounds may be influenced by the variety, plant site, and environmental stress (temperature, light, and drought) (Jing et al., 2007).

Flavonoids, a subclass of flavonoids, have a double bond between C2 and C3 and a ketone group in C4; they are often modified by hydroxylation, methylation, glycosylation or alkylation (Crozier et al., 2009). In contrast, the present study yielded five key flavonoid glycosides that distinguish different parts of LIM. Generally, glycosides are the primary flavonoids in plants and they possess antioxidant, antibacterial, anti-inflammatory, and anti-tumor effects (Li et al., 2019).

Metabolic pathways enriched by the different metabolites in the different parts of LIM

The KEGG database is a valuable tool for analyzing metabolic pathways and their relationships and represents different metabolic pathways through graphs and tables (Wang et al., 2020). Finally, the

study used the KEGG database to enrich the DAMs identified in the different parts of LIM to obtain comprehensive information on their functional. A total of 16, 31, 29, 40, 32 and 30 metabolic pathways were found enriched by the DAM of stem_vs_leaf, stem_vs_flower, stem_vs_seed, leaf_vs_flower, leaf_vs_seed, and flower_vs_seed, respectively, including the biosynthesis of secondary metabolites, flavonoid biosynthesis, flavone and flavonol biosynthesis, phenylpropanoid biosynthesis, tyrosine metabolism, isoflavonoid biosynthesis, and biosynthesis of various plant secondary metabolites (Table S5 and Fig. 6). Meanwhile, the most common metabolic pathways were "metabolic pathways" (15/32, stem_vs_leaf; 27/55, stem_vs_flower; 26/45, stem_vs_seed; 24/54, leaf_vs_flower; 23/49, leaf_vs_seed; 22/48, flower_vs_seed), followed by "biosynthesis of secondary metabolites" (11/32, stem_vs_leaf; 26/55, stem_vs_flower; 22/45, stem_vs_seed), "flavonoid biosynthesis" (11/32, stem_vs_leaf; 26/55, stem_vs_flower; 22/45, stem_vs_seed; 23/54, leaf_vs_flower; 22/49, leaf_vs_seed; 21/48, flower_vs_seed), and "flavone and flavonol biosynthesis" (8/32, stem_vs_leaf. 10/55, stem_vs_flower; 8/45, stem_vs_seed; 11/54, leaf_vs_flower; 8/49, leaf_vs_seed; 11/48, flower_vs_seed). The biosynthesis of flavonoids is mainly achieved through the phenyl-propane metabolic pathway (Mierziak et al., 2014). The starting substrates 4-coumaroyl-CoA and malonyl-CoA are used to form chalcone by the action of chalcone synthase (CHS), which in turn is catalyzed by chalcone isomerase to form 4,5,7-trihydroxyflavanone, as the main product of metabolism then enters other different metabolic pathways, and leads to the formation of different flavonoids (Gao et al., 2022; Pandey and Bhushan, 2020).

Conclusion

The present study based on a widely target metabolomic approach provides insights into the metabolic profiles of LIM and the differences in the metabolites between different parts. A total of 576 secondary metabolites, including 204 flavonoids and 161 phenolic acids, were detected from the stem, leaf, flower, and seed of LIM. The seed and leaf of LIM exhibited the highest total flavonoid content, and the leaf had the highest total phenolic content. A total of 244 secondary metabolites with differences in concentrations among the different parts were further screened by multivariate statistical methods, indicating differences in the underlying metabolism. Besides, five flavonoids, five phenolic acids, one triterpenoid, and one alkaloid were identified as key metabolites by comparing the stem, leaf, flower and seed of LIM in pairs. Thus, the metabolomic analysis of this study revealed the quality of different parts of LIM, improving our understanding of the metabolic mechanisms. These findings provide a solid basis for further utilization of different parts of LIM in producing functional foods.

CRediT authorship contribution statement

Junfeng Hao: Methodology, Conceptualization, Validation, Formal analysis, Writing – original draft, Writing – review & editing. **Gentu Ge:** Conceptualization, Investigation, Supervision, Writing – review & editing, Resources, Methodology. **Yushan Jia:** Methodology, Validation, Formal analysis. **Feng Han:** Methodology, Writing – review & editing. **Muqier Zhao:** Formal analysis, Validation. **Zhijun Wang:** Methodology, Conceptualization, Validation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.101012.

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