# Metabolomics reveals the importance of metabolites in Mussaenda pubescens for antioxidant properties and quality traits

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# Abstract

### Background

*Mussaenda pubescens* is an important source of medicinal plant that has been used as medicine or dried instead of tea. However, there are few studies on the thorough and dynamic assessment of metabolites presented in *Mussaenda pubescens*.

**Methods** In this study, UHPLC-MS/MS approach and biochemical analysis were used to investigate the substance changes of leaves at different developmental stages.

**Result** A total of 957 metabolites were identified, among which 202, 54 and 254 metabolites showed differential accumulation in three comparisons. The up-regulated metabolites were the major factors driving the function and quality of *Mussaenda pubescens*. The main pathways involved “Flavone and flavonol biosynthesis”, “Phenylalanine metabolism”, “ABC transporters”, “Folate biosynthesis”, and “Fructose and mannose metabolism”. Phenolics, flavonoids, terpenoids and anthocyanin were the primary contributors to differential antioxidant activities of bud, tender, and mature leaves.

**Conclusions** These results provide a novel insight into formation mechanism of metabolites and the development of *Mussaenda pubescens*tea.


# Background

*Mussaenda pubescens* (Mp), a member of the *Rubiaceae* family [1], is a vine shrub, which is distributed in shady slopes, valleys and shrubs of southwestern China. The stems and leaves of Mp with a sweet and cool taste have been used for medicine or dried instead of tea in China's ethnic minority areas [2]. The natural compounds of plants have been of particular importance on account of their applications in chemical, food and pharmaceutical industries [3]. Medicinal plants are being widely used in the treatment of diseases and infections throughout history as traditional healing remedies owing to their broad therapeutic spectrum and minimal or no side effects. Flavonoids, triterpenes, saponines and iridoids isolated from genus *Mussaenda* are important sources of medicinal natural substances [4], of which the abundance and wide availability of the iridoids make this plant a potential target for researching this drug’s activity [5]. The extracts of Mp showed immunopromotive, anti-RSV activity, inhibition of *β*-hematin formation [6] and osteoclast formation [7]. In addition, Mp was able to detoxify the *Gelsemium elegans* [8] and significantly improve the analgesic effect [9]. The whole plants of Mp have been applied to traditional Chinese medicine treatment (TCM) for dysentery, laryngopharyngitis and acute gastroenteritis [10] as a diuretic, antiphlogistic, diaphoretic, antipyreticm, detoxify poisons and contraceptive agent.

A large amount of secondary metabolites also known as natural products are synthesized and accumulated in plants, which provide an abundant source for modern pharmacy. Especially, TCM provides valuable potentiality for discovering natural products with bioactive and developing new modern pharmaceuticals [11]. Mp with the largest number of compounds was found in *Mussaenda*, which has

been applied in TCM. Triterpenoid, triterpenes, iridoid glycosides saponins and organic acids were reported to be the essential ingredients in Mp on the basis of the phytochemical research [12]. Numerous researches have concentrated on the identification of bioactive components in Mp leaves. Many components involved in saponins (such as mussaendosides D, E, H, S, I, J), monoterpenes (such as mussaenins A, B, C), and triterpenoid saponins (such as mussaendosides U, V, R, S [13], G, K, P, Q, F), cycloartane saponin (such as mussaendoside X, O, G [7], M, N), phenolic glycoside (such as mussaendoside L) [14], shanzhiside methylester, barlerin, (6S, 9R)-roseoside and coniferin [5] were isolated from the hydrophilic fractions of aerial parts of Mp. The main aromatic constituents in the leaves of Mp are monoterpenoids and sesquiterpenes involved in *β*-ionone, linalool, and limonene with 7.2%, 5.6% and 3.6%, respectively [12]. In addition, majority of the components identified from the Mp leaves used in previous studies were limited to four categories, such as flavonoids, polyphenols, organic acids and saponins triterpenic. Nevertheless, few research has reported a comprehensive overview of the chemical ingredients and functional characteristics of Mp. Furthermore, the dynamic study of metabolites at different growth stages of Mp leaves is largely unknown.

In current research, the species and relative contents of metabolomic profiles in Mp leaves at different developmental stages were analyzed using a widely targeted metabolome approach, which can elucidate the dynamic changes of metabolites in Mp leaves, and determine the nutrient composition in qualitative and quantitative analysis. Furthermore, comparative research was performed to identify the functional ingredients involved in total phenolics, flavonoids, terpenoids and anthocyanin related to antioxidant activities and health benefits in Mp, and provided available information for the development and utilization of Mp germplasm resources.

## Materials and methods

# Plant materials

The plants transplanted from Guilan Shui Nationality Township (Duyun, Guizhou province, China, 26°12'N, 107°30'E) have been raised on the campus of Qiannan Normal University for Nationalities (QNUN), Duyun, Guizhou province in China (107°51'N, 26°25'E), and the voucher specimen (QNSY202106003) was deposited in the Botanical Museum of QNUN. Fresh leaves that grow normally without the pests and pathogens under favorable lighting conditions were selected in September 2021, which were divided into three different stages according to their growth status, including the bud leaf (Stage 1, MpBud), tender leaf (Stage 2, MpTen) and mature leaf (Stage 3, MpMat) (Fig. 1). Leaf samples were separately gathered at the three distinctive development stages mentioned above, and then immediately frozen in liquid nitrogen and stored at -80℃ until further metabolome and biochemical analysis. There were three biological replicates for leaf samples at each stage, and each replicate of 15 leaves was used as a mixture sample with a total weight of at least 3g.

# Metabolome analysis

# Preparation and extraction of plant samples

The samples were quickly freeze-dried and crushed at 30 Hz for 1.5 min by using a mixer mill (MM 400, Retsch) with zirconia beads. 50 mg of powder was accurately weighed and then extracted with 0.50 mL methanol/water/hydrochloric acid (799:200:1, V/V/V). The extracts were subjected to vortex and sonicate for 10 min, respectively. The solution was subsequently centrifuged at 4 ℃ and 12,000 × g for 3 min to collect the supernatants, followed by filtration (PTFE, 0.22 μm) (ANPEL, Shanghai, China) before UPLC-MS/MS analysis performed by Wuhan MetWare Biotechnology Co., Ltd. (Accessed on 9 November 2021).

# UPLC and ESI-Q TRAP-MS/MS conditions

Analysis of extract was performed using an UPLC-ESI-MS/MS system (UPLC, SHIMADZU Nexera X2, https://www.shimadzu.com.cn/, accessed on 9 November 2021; MS, Applied Biosystems 4500 Q TRAP, https://www.thermofisher.cn/cn/zh/home/brands/applied-biosystems.html, accessed on 9 November 2021) with the analytical conditions following the method described by Zhou et al [15]. The effluent was alternately connected to ESI-triple quadrupole-linear ion trap (QTRAP)-MS. The ion spray voltage (IS) was set to 5500 V and − 4500 V in positive and negative ion modes. And other ESI source operation parameters were same as the protocol [15].

# Qualitative and quantitative analysis

The public metabolite database and self-built MWDB were used to perform qualitative and quantitative analysis of metabolites by mass spectrometry. The substances detected in the samples were displayed by MRM metabolite detection multi-peak diagram. The peak area of each chromatographic peak manifests the relative content of the corresponding metabolites.

# PCA, HCA and OPLS-DA analysis

Principal component analysis (PCA) was performed using statistics function prcomp in R to measure the separation trends of metabolite between groups, indicating whether there were differences in metabolites among groups or not. To further identify the differences among all samples, hierarchical cluster analysis (HCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were performed by the R-package. The prediction parameters of OPLS-DA involve $R^2X$, $R^2Y$ and $Q^2$, where $R^2X$ and $R^2Y$ express the explanation ratio of the model to X and Y matrices, respectively; $Q^2$ indicates the model's predictability. A permutation test (200 permutations) was performed by R package MetaboAnalystR to avoid overfitting.

# Differential metabolites analysis

The screening for differential metabolites was determined by variable importance in projection (VIP) values (VIP ≥ 1) based on the results in OPLS-DA and $\log_2$FC (fold change) ≥ 2 or ≤ 0.5 [16]. The obtained metabolites were annotated using KEGG compound database (http://www.kegg.jp/kegg/compound/, accessed on 9 November 2021), and then mapped to KEGG pathway database (http://www.kegg.jp/kegg/pathway.html, accessed on 9 November 2021).

# Chemical components and antioxidant analysis

## Total phenolics, flavonoids, terpenoids and anthocyanin

The determination of total phenolics was performed referred to the methods described by Afonso et al [17]. 20 μL of extract solution was mixed with phenol reagent of Folin Ciocalteu and 7.5% $Na_2CO_3$ (100/80) in a 96-well microplate. The microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland) was incubated in the dark condition at 45℃ for 15 min. After that, the absorbance value was adjusted to 765 nm. A standard curve for various concentrations of gallic acid was performed, with the amount of total phenolics content was represented as mg/g DW.

The total flavonoids [17] were determined using the $NaNO_2$-$AlCl_3$-NaOH method by biochemicals kit (NMKD0120, Norminkoda Biotechnology Co., Ltd. Wuhan, China). 25 μL of extract solution was blended with distilled water (100 μL) and 5% $NaNO_2$ (10 μL) in a 96-well microplate. The microplate reader was subsequently incubated in the dark condition at room temperature. Five minutes later, 10% $AlCl_3$ (15 μL) and NaOH (50 μL) were added into the mixture, respectively, and finally mixed with 50 μL of distilled water. The absorbance value was determined at the wavelength of 510 nm against a blank. A standard curve for various concentrations of catechin was performed, which value was represented as mg/g DW.

0.25 g of dry powder was accurately weighed and then soaked in the NaCl solution for 6 h at a ratio of 1:18. After distillation for 8 h, the volatile oil layer was collected and dried overnight with an appropriate amount of $Na_2SO_4$ to obtain a volatile oil. 1 mL of volatile oil was blended with 9 mL of absolute ethanol and subsequently shacked well. After that, 1 mL of the extraction was distilled to a final volume of 5 mL with absolute ethanol. The extract solution (1 mL) was mixed with 5% vanillin glacial-acetic acid (3 mL), and subsequently with 3.5 mL of perchloric acid. Then, the solution was kept in 71℃ water bath for 28 min, and rapidly cooled it to room temperature. After cooling, 1.5 mL of glacial acetic acid was added to bring the final volume to 8 mL and then shacked well. The absorbance value was adjusted at the wavelength of 600 nm against a blank, and the total terpenoids content was expressed was mg/g DW.

For total anthocyanin content, extractions were performed according to pH differential method described by Lee et al [18]. About 0.1 g of powder was added into 1 mL of mixture with 8.5% aqueous formic acid and acetonitrile/methanol mixture (85/15) at a ratio of 9:1. The solution was vibrated at 75℃ for 25 min, and then was adjusted to a final volume of 1 mL with the mixture mentioned above. After that, the solution was centrifuged for 10 min at 12,000 × g to collect supernatant. The absorbance of extract solution diluted with aqueous buffer (pH 1.0) and sodium acetate buffer (pH 4.5) was recorded at the wavelength of 530nm and 700nm, respectively.

# Antioxidant activity

The biochemicals kit (NMKD0109, NMKD0110, NMKD0111, Norminkoda Biotechnology Co., Ltd. Wuhan, China) measured the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging ability, Ferric Reducing

Activity Power (FRAP) and 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) radical scavenging activity in accordance with the method described by Afonso et al [17].

For DPPH radical-scavenging activity, 0.1 g of plant powder was mixed with 80% methanol to obtain the diluted phenolic concentrations (0, 5, 10, 15, 20 and 25 μg/mL). 100 μL of solution was added into the 0.15 mL of DPPH methanolic solution and then was standardized to a final volume of 1 mL with 80% methanol. After centrifugation for 10 min at 12,000 × g and room temperature, the supernatant was collected for detection. The absorbance value was recorded at 517nm, which data was expressed as mean values ± standard deviation (SD).

For FRAP assay, 10 mM of 2,4,6-Tri(2-pyridyl)-S-triazine (TPTZ) was added into the 40 mM of HCl and then was mixed with 20 mM of $FeCl_3 \cdot 6H_2O$ and 10 times the volume of acetate buffer (pH 3.6). After that, 5 μL of the extract solution was blended with 170 μL of $Fe^{3+}$-TPTZ solution and 25 μL of distilled water. The absorbance value was recorded at 590 nm and FRAP was expressed as μmol Trolox/g DW.

For ABTS method, 7 mM of ABTS (pH 7.4) and 2.5 mM of potassium persulfate were mixed to acquire the ABTS radical solution. Afterwards, it was diluted with ethanol to obtain the absorbance value at the wavelength of 414 nm. 10 μL of extract solution was added into 190 μL of ABTS radical solution, and subsequently incubated in the dark condition at 23℃ for 10 min. The absorbance value was recorded at 414 nm, and ABTS was expressed as μmol Trolox/g DW.

## Results and discussion

# Metabolomic profiling of Mp leaves at different development stages

In present study, samples collected from bud, tender and mature leaves of Mp at different developmental stages were analyzed by metabolomics. According to the primary classification of substances (Table S1), a total of 957 metabolites were identified from the leaves of Mp, including the most abundant compounds, 185 phenolic acids, 155 lipids and 109 terpenoids. In addition, 85 organic acids, 81 flavonoids, 78 amino acids and their derivatives, 66 nucleotides and their derivatives, 33 lignans and coumarins, and 31 alkaloids were also detected. The overlay analysis of the QC-TIC diagram (Fig. S1A-B) and the sample multi-peak detection diagram (Fig. S1C-D) showed that the data recorded in this study had a good repeatability and reliability. Additionally, the three biological replicates of each sample exhibited relatively higher correlation coefficients ranged from 0.89 to 0.99 (Fig. S2), which demonstrated that the samples possessed good homogeneity.

# PCA and OPLS-DA for Mp

In the PCA plot, leaves at three different maturity stages were apparent separated, and the three biological replicates of each group were closely clustered together, with 51.96% and 14.84% variation explained by PC1 and PC2, respectively, indicating that the entire analysis was repeatable and reliable, and there were

significant differences among the three groups (Fig. 2A). A total of 957 substances were identified from all the leaves, as showed in the heatmap, which manifested the visible hierarchical clustering between groups. The content of substances in the bud leaves showed obvious changes compared with that in the tender and mature leaves, among which, around half of samples whose content was relatively higher, while the other examples was lower (Fig. 2B). According to the abundance of the first-level classification (Fig. 2C), the contents of lipids and phenolic acids in the leaves of Mp were the highest, whose levels in the bud leaves were relatively higher than that in the tender leaves and mature leaves. The total amount of organic acids, terpenoids and alkaloids was also relatively abundant in the bud leaves, with example substances including choline, and 2*α*,3*β*,9*α*,23,24-pentahydroxyolean-12-en-28-oic acid 28-*O*-glucoside,. However, the total amount of nucleotides, amino acids, lignans, coumarins and flavonoids accumulated significantly with the maturation of leaves. The corresponding compounds of alkaloids, lipids, nucleotides, and terpenoids, including choline, stearic acid, adenosine, and 2*α*,3*β*,9*α*,23,24-pentahydroxyolean-12-en-28-oic acid 28-*O*-glucoside, showed the highest abundance in tender leaves; while, in addition to the three abovementioned substances, adenosine was also most abundance in mature leaves. According to the abundance of secondary classification of substances, phenolic acids, free fatty lipids and organic acids with the highest content were observed in the bud leaves (Fig. 2D). It was also found that the total amount of phenolic acids, saccharides and alcohols, and other substances was relatively higher in tender leaves, while, the content of free fatty lipids, nucleotides and their derivatives, amino acids and their derivatives, coumarins, and flavonols showed the higher abundance in the mature leaves. These results indicated that growth and development strongly influenced the metabolite profiling of Mp leaves at different development stages.

Additionally, OPLS-DA (orthogonal partial least squares-discriminant analysis) was used to screen the variables responsible for differences among these three groups [19]. In the current study, the OPLS-DA model was used to compare metabolites of the samples in the comparisons to evaluate the differences in MpBud vs. MpTen ($R^2X = 0.691$, $R^2Y = 1$, $Q^2 = 0.969$), MpTen vs. MpMat ($R^2X = R^2X = 0.576$, $R^2Y = 1$, $Q^2 = 0.922$), and MpBud vs. MpMat (0.736, $R^2Y = 1$, $Q^2 = 0.979$) (Fig. 3). The $Q^2$ values of all the comparisons were greater than 0.9, indicating that these models were stable and credible. OPLS-DA score plots exhibited that these three stages were separated from each other, demonstrating there were significant differences in metabolic phenotypes of the Mp leaves at different developmental stages. These results indicated that mature stage was the most essential process in the evolution of metabolites, followed by bud stage, with tender stage produced the least impact.

## Differentially accumulated metabolites screening, functional annotation and enrichment analysis among the Mp leaves at different developmental stages

To elucidate the impact of each stage on the metabolites present at the different developmental stages of Mp leaves, the crucial differences among these processes were investigated. A total of 317 Differentially accumulated metabolites (DAMs) were screened, of which 202 DAMs were found in MpBud vs. MpTen (Fig. 4A, 74 up-regulation and 128 down-regulation), 54 DAMs were observed in MpTen vs, MpMat

(Fig. 4B, 27 up-regulation, and 27 down-regulation), and 254 DAMs were recorded in MpBud vs. MpMat (Fig. 4C, 107 up-regulation and 147 down-regulation).

The Venn diagram exhibited that there were both common and unique metabolites among the comparisons (Fig. 4D, Table S1). Particularly, MpBud vs. MpTen and MpTen vs. MpMat possess 29 common substances (i.e., 3 phenolic acids, 10 flavonoids, 4 terpenoids, 1 lipid, 2 amino acids and derivatives, 3 alkaloids, 5 lignans and coumarins, and 1 other metabolite), which were simultaneously affected by the growth and development of Mp leaves. 149 common metabolites were also observed in MpBud vs. MpTen and MpBud vs. MpMat (i.e., 26 phenolic acids, 12 flavonoids, 22 terpenoids, 7 lipids, 11 organic acids, 15 amino acids and derivatives, 8 alkaloids, 16 nucleotides and derivatives, 6 lignans and coumarins, and 26 others), which illustrated that these common metabolites accumulated more under the bud to tender stages, especially phenolic acids, flavonoids, terpenoids; while 27 common metabolites were also found in MpTen vs. MpMat and MpBud vs. MpMat, which were less affected by the growth and development of Mp leaves under the tender to mature stages.

The 90 metabolites that were unique to MpBud vs. MpMat were only affected by growth and development of the leaves. 36 metabolites (i.e., 24,30-Dihydroxy-12(13)-enolupinol, 3*β*,19*α*-Dihydroxyolean-12-en-28-oic acid, and 2*α*,3*β*,19*α*-Trihydroxyurs-12-en-23,28-dioic acid-28-*O*-glucoside) were only biosynthesized and accumulated under bud to tender stages, and these components maybe participate in the leaf color change of Mp; while ten metabolites (i.e., 4-Hydroxybenzoic acid, 5,6-Dihydroxyindole-5-*O*-*β*-glucoside, and Kaempferol-3-*O*-sophoroside) were only biosynthesized and accumulated under tender to mature stages (Fig. 4D). We proposed that the DAMs found in MpBud vs. MpTen and MpTen vs. MpMat may contribute to biosynthesis of functional and nutrition ingredients, and the leaf color change in Mp.

Multiple comparative analysis showed that among the comparisons (MpBud vs. MpTen, MpTen vs. MpMat, and MpBud vs. MpMat) shared only 12 different metabolites such as Benzamide, 2-Phenylethanol, Pinocembrin-7-*O*-glucoside (Pinocembroside), Mussaenoside, and 1*β*,2*α*,3*α*,19*α*-Tetrahydroxyurs-12-en-28-oic acid (Fig. 4D). These DAMs were the most active metabolites during the growth of Mp leaves, and may be closely related to the changes of leaf color, biochemical components and functions. These results suggested that the metabolites that caused the differences among bud leaves, tender leaves, and mature leaves were greatly different. Therefore, the various functional and nutrition ingredients were not present or its content were low under the different stages (tissues or cells) during the growth and development of Mp leaves, while these components were continuously biosynthesized and accumulated with the growth and development of leaves. These results further confirmed that the unique metabolites existed in mature leaves were not only particularly critical for the transformation of the metabolites, but also more than other leaves during Mp leaves growth and development, since the photosynthesis promoted the hydrolysis, isomerization, oxidation, and other reactions of the metabolites.

From the changes of metabolite abundance (Fig. 4E), the contents of metabolites in Sub Class 1, 4, 5, 7 and 9 were higher in the bud leaves, with a total of 176 metabolites, mainly including 2-Isopropylmaleic Acid, 2-Phyllethylamine, LysoPC 18:1 (2n isomer), and L-Cyclopentylglycine; Sub Class 3 and 6 contained 118 metabolites, which almost increased with leaves maturity, mainly including Adenosine, L-Isoleucine, Quercetin-3-*O*-galactoside (Hyperin); In Sub Class 2, the content of 20 metabolites in tender leaf was significantly higher, such as Caffeine, Kaempferol-3-*O*-rutinoside-7-*O*-glucoside, Quercetin-7-*O*-rutinoside-4'-*O*-glucoside, and Mussaenoside. Compared with tender leaves, the amount of Dehydrocastus lactone, which belongs to sesquiterpenoids, decreased to 48.2%; Mussaenoside in the sesquiterpenoids increased by 2.3 times, while Penstemonoside and Deacetylasperosidic acid decreased to 49.1% and 35.8%, respectively; Triterpenoids showed a downward trend, with a decline range of 13.1%~46.0%, and the most significant decrease was observed in 1*β*, 2*α*, 3*α*, 9*α*-Tetrahydroxurs-12-en-28-oic acid and the least in 23-Hydroxybetulinic acid; In flavonoids, the glycoside of Kaempferol increased while that of Quercetin decreased, in which Kaempferol-3-*O*-rutinoside-7-*O*-glucoside increased by 15.5 times, and Quercetin-3-*O*-(6 " - *O*-acetyl) glucoside decreased to 17.8%. Compared with mature leaves, the Isohyperoside and Quercetin-3-*O*-glucoside (Isoquercitrin)* of flavonol and Scopoletin-7-*O*-glucoside (Scopolin) of coumarin increased by 2.1–2.3 times in tender leaves; The content of 2-Phenylethylamine, which belongs to alkaloids, decreased more, and the mature leaves were only 20.7% of the tender leaves, while the mussaenoside of sesterpenes and Kaempferol-3-*O*-rutinoside-7-*O*-glucoside of flavonols were only 13.1% and 5.2% of the tender leaves; In addition, the triterpene 2,3-Dihydroxy-12-ursen-28-oic acid increased by 2.1 times.

The differential metabolites for MpBud vs. MpTen, MpTen vs. MpMat, and MpBud vs. MpMat were involved in 66, 37 and 75 pathways and the major pathways were presented in bubble plots (Fig. 4, Table S2). Most noteworthy, the top ten metabolic pathways including "ABC transporters", "Purine metabolism", and "Galactose metabolism" were significantly up-regulated ($p\text{-value} < 0.05$) in MpBud vs. MpTen (Fig. 4F). The top ten metabolic pathways including “Flavone and flavonol biosynthesis”, “Nitrogen metabolism”, “Phenylalanine metabolism”, “Folate biosynthesis”, “Sphingolipid metabolism” were enriched in the comparison of MpTen vs. MpMat (Fig. 4G). Whereas, “ABC transporters”, “Nicotinate and nicotinamide metabolism”, “Fructose and mannose metabolism”, “Metabolic pathways”, “Arginine biosynthesis”, “Biosynthesis of cofactors”, “Biosynthesis of amino acids”, “Aminoacyl-tRNA biosynthesis”, “Monobactam biosynthesis”, “Indole alkaloid biosynthesis” showed a $p\text{-value} < 0.05$ in the enrichment analysis of MpBud vs. MpMat (Fig. 4H).

# Evaluation of the different metabolites during the growth and development of Mp leaves

A total of 317 different metabolites mainly involved in the 150 primary metabolites of 33 amino acids and derivatives, 21 organic acids, 16 lipids, 31 nucleotides and derivatives, and 49 others, as well as the 167 secondary metabolites involved in 48 phenolic acids, 35 flavonoids, 54 terpenoids, 14 lignans and coumarins, and 16 alkaloids (Fig. 5A), of which 36 DAMs were unique to MpBud vs. MpTen, accounting

for 11.4% of the total different metabolites (Fig. 5B), and 90 DAMs were unique to MpBud vs. MpMat, accounting for 28.4% of the total different substances (Fig. 5C).

# Secondary metabolites

Secondary metabolites involved in flavonoids, terpenoids, phenolics or alkaloids are small molecular organic compounds derived from primary metabolites such as carbohydrate substances that are not directly associated with growth and development of plant cells and organs, which function as an important health-promoting phytochemicals for functional foods or medicines as well as interact with bioenvironment and for the establishment of defense mechanism [20].

Flavonoids, the most described phenolic secondary metabolites, are widely distributed in plant, which can be classified into six categories involved in flavonols, flavanones, flavones, flavanols, isoflavones and anthocyanidins [21]. Among the 35 different flavonoids substances examined herein (Fig. S3, Table S3), 21 DAMs significantly altered involved in ten up-regulated differences (e.g., Quercetin-3-*O*-galactoside (Hyperin) and Kaempferol-4'-*O*-glucoside) and 11 down-regulated differences (e.g., Quercetin-3-*O*-Sambubioside-5-*O*-Glucoside and Kaempferol-3-*O*-rutinoside-7-*O*-rhamnoside) across the whole growth stage of Mp leaves (MpBud vs. MpMat). Additionally, a total of 25 metabolites significantly accumulated with 14 differences of Epicatechin and Apigenin-6,8-di-*C*-glucoside (Vicenin-2) or decreased with 11 differences of Luteolin-7-*O*-Sophoroside-5-*O*-arabinoside and Luteolin-7-*O*-Sophoroside-5-*O*-arabinoside as the buds growing into the tender leaves (MpBud vs. MpTen). While, 15 significantly changed with the majority of the down-regulated substances such as Kaempferol-3-*O*-rutinoside-7-*O*-glucoside and Quercetin-7-*O*-rutinoside-4'-*O*-glucoside in the maturation stage of leaves (MpTen vs. MpMat). What's more, it was found that five compounds were unique to the whole growth stage with the example of Kaempferol-3-*O*-glucoside (Astragalin) and Quercetin-4'-*O*-glucoside (Spiraeoside). Several compounds with high accumulation have been proved to have beneficial bioactivities such as kaempferol, quercetin, naringenin and their glycosides [22]. For instance, naringenin and kaempferol-3-*O*-glucosideis are flavanone and flavonoid, respectively, which possess a variety of bioactivities including antioxidant, anticancer and anti-inflammatory [23, 24]. The highly accumulated flavonoids metabolites as mentioned above were observed in the bud leaves, which confirmed that it was positively related to the antioxidant activity.

Phenolic acids possess various important medicinal compounds such as hydroxybenzoic acid, hydroxycinnamic acid and their derivatives in tea plants [25]. In this research, a total of the 48 different phenolic acid compounds were detected herein (Fig. S3, Table S4), of which 46 exhibited significant alteration across the whole growth stage with 21 up-regulated (e.g., 1-*O*-Caffeoyl-*β*-*D*-glucose*) or 25 down-regulated (e.g., Trans-4-Hydroxycinnamic Acid) DAMs. Among 26 different substances, nine significantly accumulated metabolites were observed for the example of 2-Naphtho and 11 remarkably decreased metabolites were obtained such as Benzoylmalic acid. However, only seven substances involved in five up-regulated (e.g., Ethylparaben) and two down-regulated (e.g., Benzamide) differences were found in the maturation stage (MpTen vs. MpMat). In addition, 18 DAMs were unique to the whole

growth stage of leaves (MpBud vs. MpMat), of which eight significantly up-regulated metabolites with the example of Homogentisic acid and ten remarkably down-regulated metabolites with the example of 2-Hydroxybenzaldehyde (Salicylaldehyde). The highly accumulation of these phenolic acid metabolites especially Trans-4-Hydroxycinnamic Acid Methyl Ester in the bud leaves were consistent with the results reported by Wu et al. who found that phenolic acids were higher than those of the old leaves [26].

The various terpenoid metabolites were obtained including monoterpenoids, sesquiterpenoids, triterpene and triterpene saponin (Fig. S3, Table S5). It was found that the 54 different terpenoids substances examined herein, the majority of metabolites presented a declined trend with the highest down-regulation being observed in the growth stage of the leaves (MpBud vs. MpTen), such as Ursolic acid and Morolic acid. While, eight metabolites were observed for the examples including the up-regulation of Dehydrovomifoliol and the down-regulation of Mussaenoside during the maturation stage (MpTen vs. MpMat). In addition, 11 DAMs with the example substances of Dehydrovomifoliol and Asiatic increased significantly, and 27 down-regulated metabolites were observed for Mussaenosidic acid and Mussaenoside in the whole growth stage of Mp leaves. Additionally, almost all five triterpenes (e.g., Asiatic acid), one triterpene saponin of Cadambagenic acid, and one monoterpenoid of Sweroside were remarkably up-regulated, and seven monoterpenoids with the example metabolites including Geniposidic acid and Mussaenosidic acid that were unique to this stage (MpBud vs. MpMat). A large number of terpenoids, especially Mussaenosidic acid and Mussaenoside, observed in the bud leaves were higher than those of the tender leaves or mature leaves, which confirmed that the abundant terpenoid compounds possessed antibacterial, anti-inflammatory and antioxidant effects [27, 28].

Alkaloids are considered the primary bioactive components in plant chemicals, which possess various bioactivities because of their properties [29]. Among the 16 different alkaloids substances examined herein (Fig. S3, Table S6), 14 differences exhibited remarkable up-regulation (e.g., 3-Indoleacrylic acid) or down-regulation (e.g., Betaine) during the whole growth stage (MpBud vs. MpMat). Nine DAMs involved in the three accumulated metabolites (e.g., Caffeine and *N*-Acetyl-5-hydroxytryptamine) and six decreased substances (e.g., 2-Phenylethylamine and Indole-3-cyano-2-*O*-glucoside) were observed in the growth stage of Mp leaves (MpBud vs. MpTen). In addition, only four DAMs with the three down-regulated metabolites such as Caffeine, 2-Phenylethylamine and *N*-Oleoylethanolamine were found in the maturation stage (MpTen vs. MpMat). While, almost all six substances with the example of 3-Indoleacrylic acid and 2-Glucosyl-glucosyloxy-2-phenylacetic acid amide (except for the metabolite of Betaine) that were unique to the whole growth stage showed an up-regulated trend. The health benefits of tea plant are associated with its abundant content of caffeine. Although the quantities of caffeine in the bud and tender leaves were slightly higher than that in the mature leaves, which result was consistent with a study reported by Koushik et al. who showed that the content of black tea processed by the fresh and tender buds or leaves from spring was higher than that of mature leaves from monsoon and autumn seasons [30].

As for lignans and coumarins, 14 different substances were detected herein (Fig. S3, Table S7), of which eight metabolites significantly changes with five up-regulated differences (e.g., Daphnin) and three down-

regulated differences (e.g., 6,7-Dihydroxy-4-methylcoumarin) across the whole growth stage of Mp leaves (MpBud vs. MpMat). While five significantly accumulated metabolites involved in Daphnin and Lariciresinol-4'-*O*-glucoside, and seven remarkably decreased substances such as 6,7-Dihydroxy-4-methylcoumarin and Scopoletin (7-Hydroxy-6-methoxycoumarin) were observed as the buds growing into the tender leaves (MpBud vs. MpTen). In addition, five DAMs significantly up-regulated (e.g., Lariciresinol-4'-*O*-glucoside) or down-regulated (e.g., Epipinoresinol*) in the maturation stage (MpTen vs. MpMat). It was found that two up-regulated substances were unique to the whole growth stage of Mp leaves such as Esculin (6,7-DihydroxyCoumarin-6-glucoside) and Syringaresinol-4'-*O*-(6"-acetyl) glucoside.

# Primary metabolites

Primary metabolites involved in amino acids, organic acids, lipids or sugars are essential for maintaining the life activities of cells and function as an important energy resource and some small molecular compounds for secondary metabolism [31].

Free amino acids not only bring fresh and brisk tastes to Mp tea infusion, participating in the formation of aroma substances, but also contribute to nutritional and functional ingredients [32]. Of these 33 different amino acids and their derivatives examined herein (Fig. S4, Table S8), 30 DAMs showed significant changes across the whole growth and development of Mp leaves. 15 DAMs were significantly up-regulated as the buds grow into tender leaves, of which *L*-Arginine and *S*-Sulfo-*L*-Cysteine were most clearly affected by the growth and development of Mp leaves. While, only six metabolites significantly up-regulated or down-regulated in the mature stage of leaves, such as *L*-Serine, *L*-Glutamine, and *N*-Alpha-Acetyl-*L*-Asparagine. In addition, 12 DAMs were unique during the mature period, including nine up-regulated metabolites (e.g., ophan, *γ*-Glutamyl-*L*-valine), and three down-regulated metabolites (e.g., 5-Oxoproline). These majority of up-regulated metabolites during the growth stage might contribute to the formation of taste and aroma substances.

Organic acids function as the important intermediate products of carbohydrate catabolism, which contribute to the vinegar taste and fruity flavor of Mp tea, simultaneously restrain the bitterness and sourness [33]. It was found that 21 different organic acids examined herein (Fig. S4, Table S9), 19 substances showed significant changes during the process of buds growing into mature leaves with the majority down-regulation. A total of 11 metabolites exhibited remarkably alteration, among which nine substances down-regulated (e.g., Shikimic acid, Triethyl citrate and 2-Picolinic acid) and two compounds up-regulated (e.g., *α*-Ketoglutaric acid). While, only two metabolites significantly up-regulated (Citraconic acid) or down-regulated (*L*-Pipecolic Acid) as the tender leaves grow into the mature. The majority of the down-regulated metabolites detected in our research was consistent with Zhou et al. who reported that the content of organic acids in fresh leaves was obviously higher than that in mature leaves [34]. In addition, eight DAMs involved in three up-regulated (e.g., *γ*-Aminobutyric acid) and five down-regulated metabolites (e.g., Tartronate semialdehyde*) were unique organic acid substances during the process of maturation, which acted as a crucial role in reconciling the taste of the Mp tea, and contributed to improve the functional and nutritional components.

Lipids in fresh tea leaves are considered to be responsible for the production of flavor and aroma substances [35, 36]. A total of 16 lipids examined herein (Fig. S4, Table S10), among which 11 substances significantly up-regulated (e.g., 13-methylmyristic acid and Palmitoleic Acid) or down-regulated (e.g., LysoPC 16:1 and LysoPC 18:1). While, 12 metabolites were found to be remarkably altered with the most down-regulated differences in the growth stage of Mp leaves (MpBud vs MpTen), such as Methyl linolenate, Hydroxy ricinoleic acid and LysoPC 18:4. However, only two LysoPE 20:3 and LysoPC 18:4 were detected and both showed a downtrend. In addition, only three up-regulated or down-regulated compounds were unique to the mature stage of Mp leaves (MpBud vs MpMat) involved in (5*S*,8*R*,9*Z*,12*Z*)-5,8-Dihydroxyoctadeca-9,12-dienoate and 1-Stearidonoyl-Glycerol. The significant accumulation of majority LPC compounds, such as LysoPC 15:0, LysoPC 16:1 and LysoPC 18:1, were observed in buds and tender leaves, which was consistent with a study reported by Liu et al. who showed that PC compounds in new shoots were higher than that in mature leaves [37], indicating that highly abundance of lipid metabolites contributed to the formation of tea aroma.

# Total phenolics, flavonoids, terpenoids, anthocyanin, and antioxidant activity

Total phenolics, flavonoids, terpenoids, anthocyanin, DPPH radical scavenging activities, radical cation ABTS + scavenging activities, and ferric reducing antioxidant power (FRAP) were displayed in Fig. 6. Total phenolics, flavonoids, anthocyanin, DPPH, ABTS, and FRAP ranged from 4.27 mg/g to 6.24 mg/g, 3.66 mg/g to 5.94 mg/g, 0.82 μg/g to 24.7 μg/g, 9.93–23.59%, 108.32 to 150.87 μmol Trolox/g, and 87.05 to 140.16 μmol Trolox/g, respectively. The contents or activities of bud leaves was significantly higher than those of the other two samples, with tender leaves being the lowest, and there is no significant difference with mature leaves. However, terpenoids in the three samples ranged from 0.59 mg/g to 0.91 mg/g. The contents of mature leaves were significantly higher than those of the other two samples, with bud leaves being the lowest.

These results showed that during the growth and development of Mp leaves, the contents of total phenolics, flavonoids, and anthocyanin gradually decreased, while the contents of terpenoids increased significantly, indicating these components contributed to enhance the functional and nutritional ingredients of Mp leaves. Phenolic compounds involved in phenolics, flavonoids and anthocyanin are considered as the primary antioxidant components of diverse plants, whose contents in bud leaves were higher than those of mature leaves [38]. The relatively abundant contents of total these three components were observed in the buds, which were results that were confirmed in this study. Meanwhile, the high accumulation of anthocyanin was found in the bud leaves with the purple-red color, which was believed to be associated with the formation of leaf color [39]. The comparison of DPPH, ABTS, and FRAP values of the three samples exhibited a remarkable difference in antioxidant activities among Mp leaves, with the antioxidant activities for bud leaves in Mp being higher than that of mature leaves. This finding was positively related to the content changes of total flavonoids, phenolics and anthocyanin, which indicated that the high total phenolic compounds might contribute to the antioxidant activity [40].

# Conclusion

In summary, a UHPLC-MS/MS-based metabolome was performed to evaluate the differences in metabolites among Mp leaves at different developmental stages. The maturation process of Mp leaves possessed the greatest impact on the evolution of the metabolites. A total of 957 metabolites were identified, among which 317 DAMs were most clearly affected by the growth and development of Mp leaves. Totally, 202, 54 and 254 DAMs were found in MpBud vs. MpTen, MpTen vs. MpMat, and MpBud vs. MpMat, respectively. Metabolic pathway analysis showed significant enrichment of “Flavone and flavonol biosynthesis”, “Phenylalanine metabolism”, “ABC transporters”, “Folate biosynthesis”, “Fructose and mannose metabolism”, “Arginine biosynthesis”, and “Biosynthesis of amino acids”. Comparison of metabolites (including total phenolics, flavonoids, terpenoids, anthocyanin) and antioxidant activities among the Mp leaves showed that these compounds were the main factors for the difference of antioxidant activity among bud, tender and mature leaves. Further analysis expounded the evolutionary processes of the terpenoids, phenolic acids, flavonoids, alkaloids, amino acids and derivatives, organic acids, lipids, lignans and coumarins, and nucleotides and derivatives, wherein the up-regulation of these DAMs were the key factor driving the function and the quality of the Mp tea.

The results of this study provide new insights into the formation mechanism of the metabolites in Mp leaves, contributes to the knowledge of metabolites and functional ingredients of antioxidant activities in Mp leaves, and could help researchers to screen targeted leaves with functional properties and quality traits through comparative evaluation, which offers a new theoretical basis for the development of Mp tea.

# Declarations


### Fuding

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### Author contributions

**Caibi Zhou:** Investigation, Writing - original draft. **Li Ping:** Investigation. **Shanshan Fu:** Investigation, Formal analysis. **Yan You:** Resources, Software. **Sijian Guo:** Investigation. **Xin Mei:** Validation, Conceptualization, Methodology. **Xiaolu Zhou:** Writing - original draft, Writing - review & editing. **Chueamchaitrakun Piyaporn:** Writing - review & editing, Supervision. **Teerayoot Girdthai:** Formal analysis, Investigation, Supervision.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

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.

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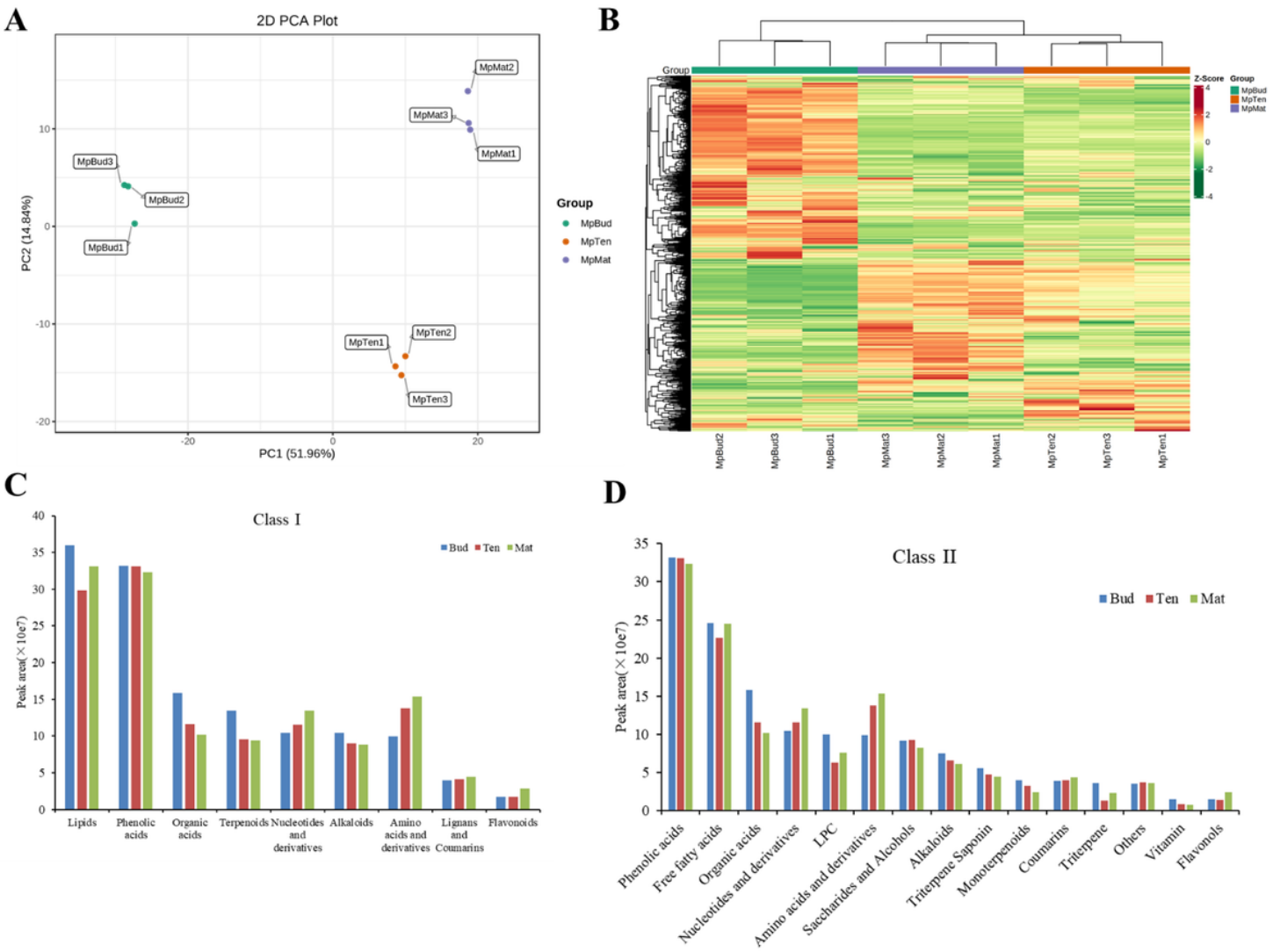
# Figures

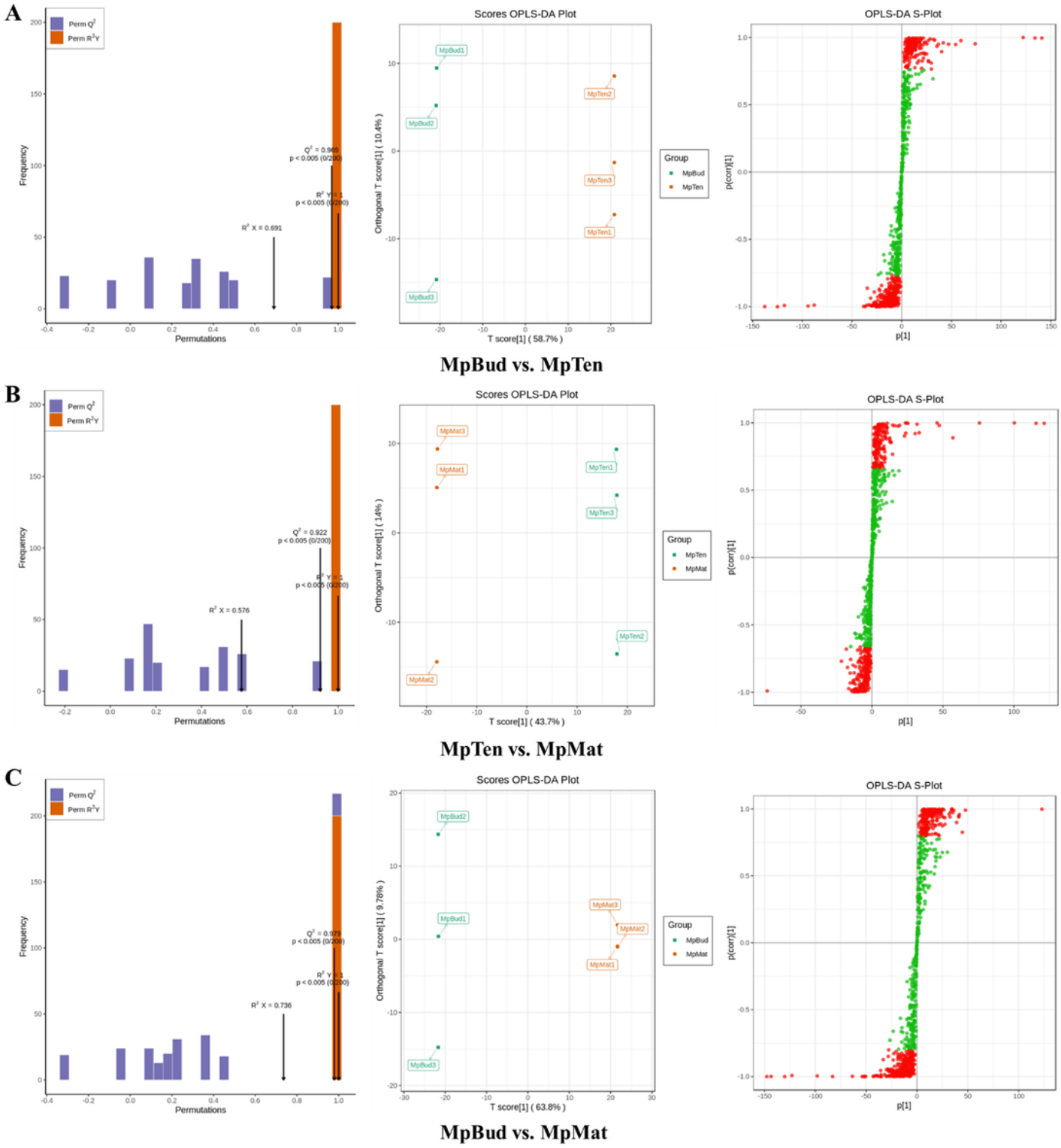
**Figure 1**

Phenotypes of the leaves surface and back of MP at the different growth stages. From left to right are MpBud, MpTen and MpMat.



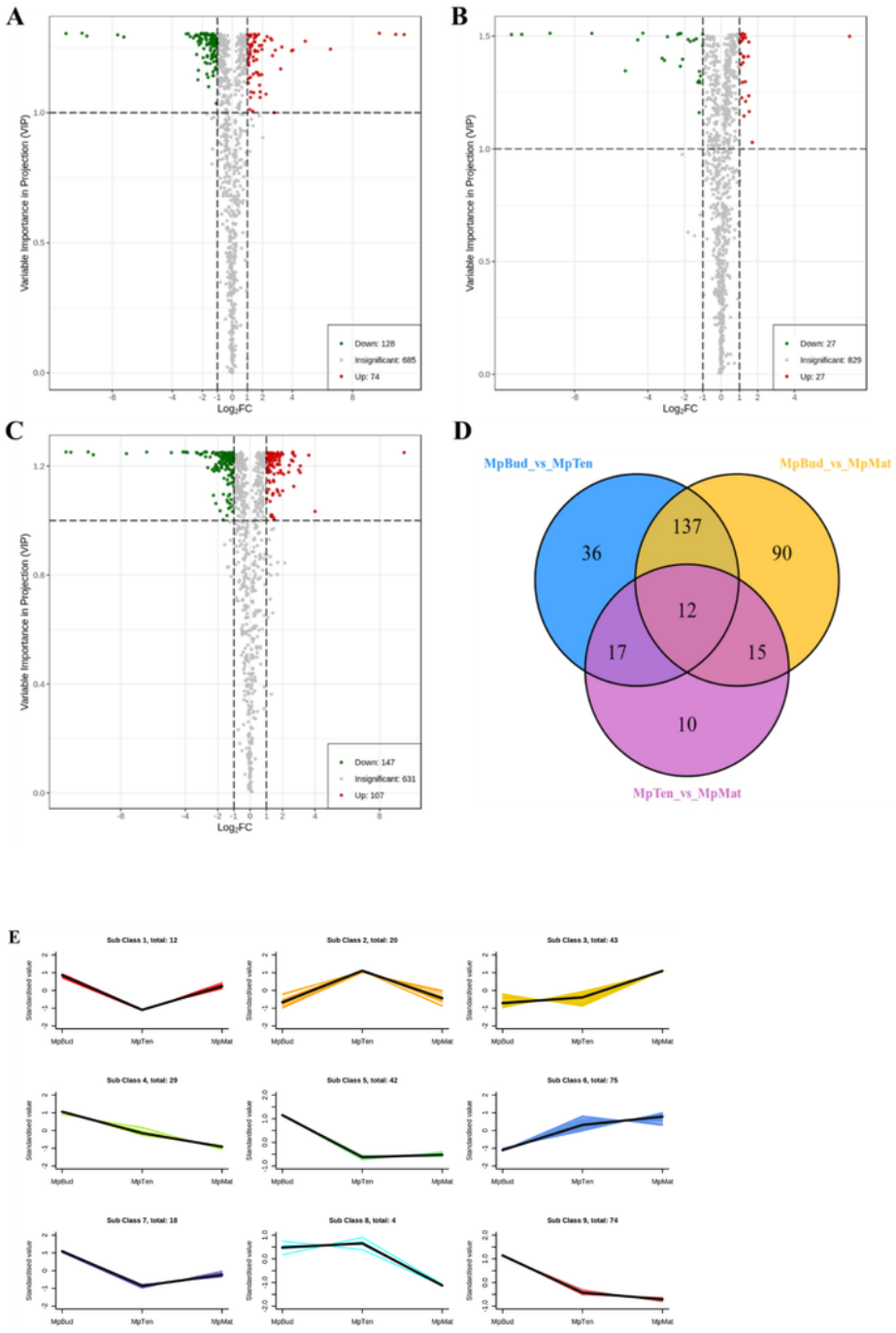

## Figure 2

Abundance and differential expression of metabolites in the leaves of Mp. (**A**) PCA analysis of metabolites, (**B**) Clustering heatmap, (**C**) Abundance of the first-level classification of metabolites, (**D**) Abundance of the second-level classification of metabolites.




## Figure 3

Orthogonal partial least squares-discriminant analysis (OPLS-DA) scores of Mp leaves in (**A**) MpBud vs. MpTen, (**B**) MpTen vs. MpMat, and (**C**) MpBud vs. MpMat. From left to right are permutation of OPLS-DA model, Scores of the OPLS-DA model, and OPLS-DA S-plot model. $R^2Y$ scores and $Q^2$ values represent the interpretation rate of the model to the Y matrix and the prediction ability of the model, respectively. When $Q^2 > 0.5$, the model can be considered an effective model, and $Q^2 > 0.9$ is an excellent model.




## Figure 4

Differential metabolites present in Mp leaves. Volcano plot of the differential metabolites in three comparisons of (**A**) MpBud vs. MpTen, (**B**) MpTen vs. MpMat, and (**C**) MpBud vs. MpMat. (**D**) Venn diagram of the differential metabolites in multiple pairwise comparison of MpBud vs. MpTen, MpTen vs. MpMat, and MpBud vs. MpMat. (**E**) Kmeans cluster of differential metabolites during the development of Mp leaves. KEGG pathways analysis in (**F**) MpBud vs. MpTen, (**G**) MpTen vs. MpMat, and (**H**) MpBud vs.

MpMat. 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 size indicates a larger impact factor. The bubble colors represent the p-values of the enrichment analysis, with darker colors showing a higher degree of enrichment.

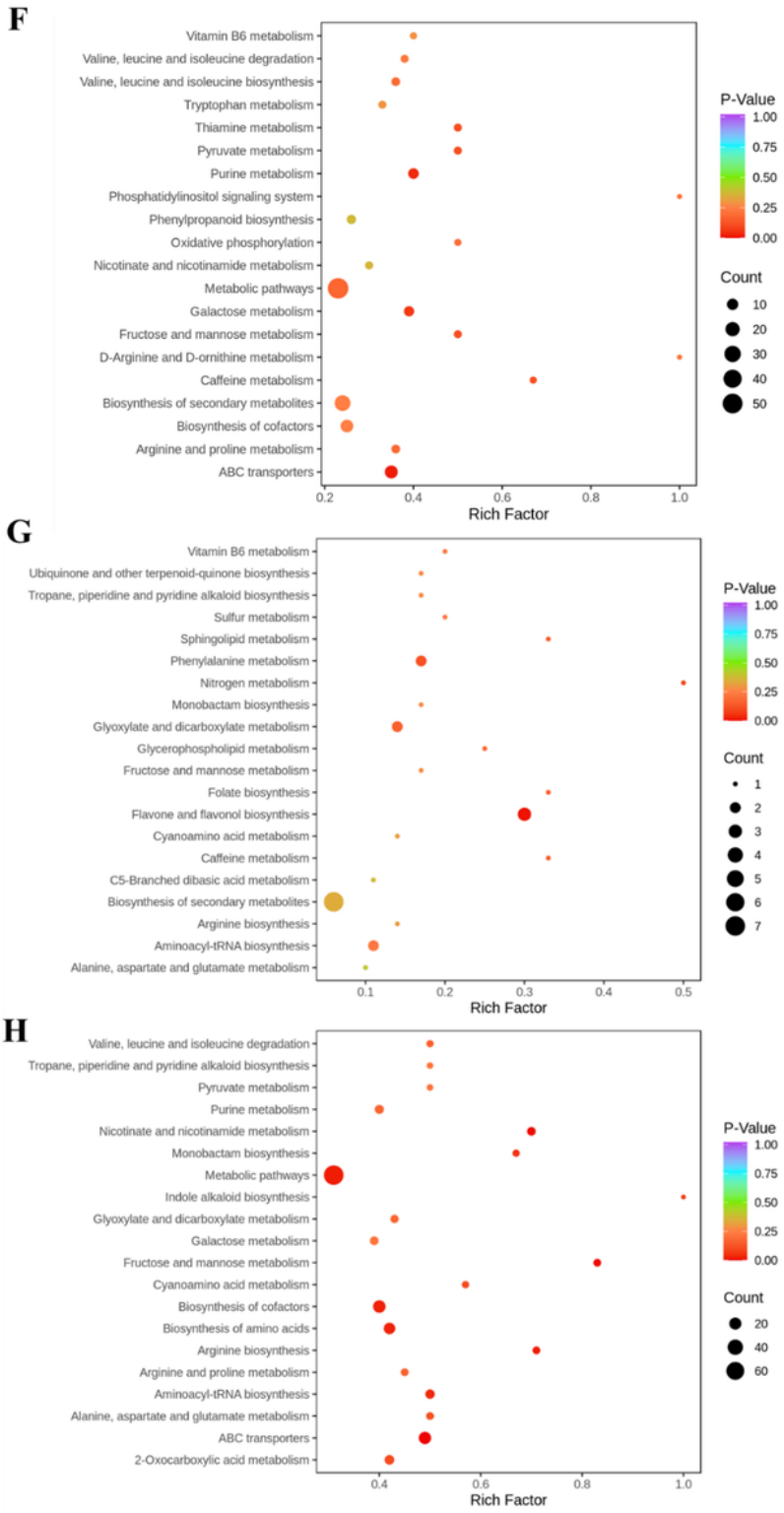



**Figure 5**

Pie chart of the number of different types in (**A**) all metabolites, (**B**) specific metabolites of MpBud vs. MpTen, and (**C**) specific metabolites of MpBud vs. MpMat.

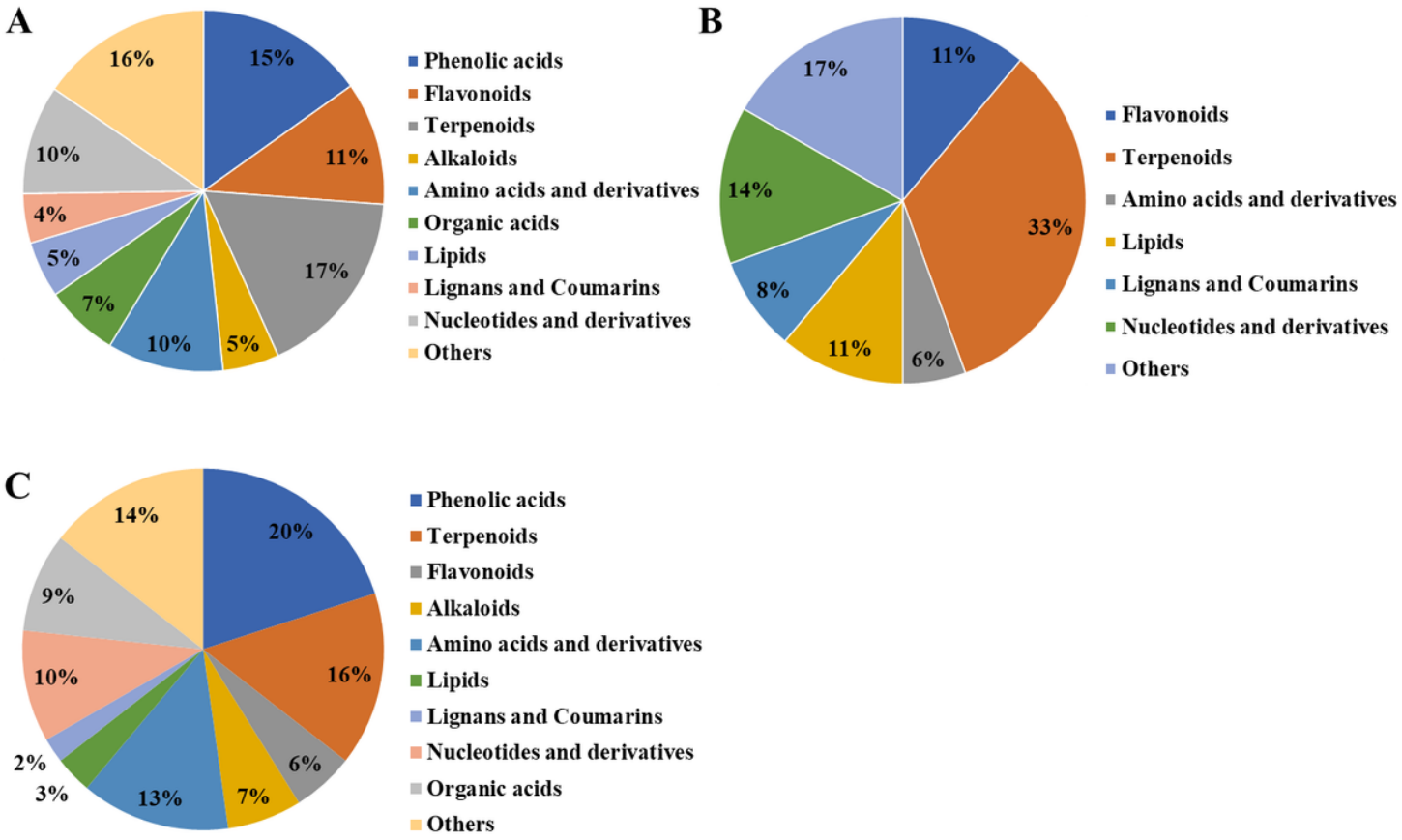



**Figure 6**

The content of total phenolics, flavonoids, terpenoids, anthocyanin, and antioxidant activities of DPPH, ABTS and FRAP assays during the whole growth stages of Mp leaves.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfigures.docx
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