Comparative Analysis of the Flavonoid Characteristics of *Camellia oleifera* Flowers in Different Plantations in Guizhou, China

Yurui Hao,[a,b] Guandi He,[a] Sanwei Yang,[a,*] Zhannan Yang,[b] and Wenxuan Quan [b,*]

Camellia oleifera is a multipurpose plant resource that contains flavonoids. To explore the differences in the characteristics and metabolic pathways of flavonoids in *Camellia oleifera* flowers from different plantations, this study compared the differences in the water content, total ash content, ethanol soluble extract, and total flavonoid content of samples from six plantations. Widely targeted flavonoid metabolomics techniques were used to analyze the metabolic characteristics of the different plantations. There were significant differences in total flavonoid content among the different plantations ($P < 0.05$), with Tianzhu County having the highest total flavonoid content (average value of 13.60 mg/g) and Liping County having the lowest total flavonoid content (average value of 2.39 mg/g). UPLC-MS/MS revealed a total of 13 categories and 758 flavonoid metabolites, among which flavonols, flavonoids, and tannins were the main flavonoid compounds. A total of 266 differentially abundant metabolites were screened *via* the OPLS-DA model, and KEGG enrichment analysis revealed that the enrichment pathways of differentially abundant metabolites between different plantations were related mainly to the biosynthesis of secondary metabolites, flavonoid biosynthesis, metabolic pathways, *etc*. This study provides a theoretical reference for the subsequent development and utilization of *Camellia oleifera* resources, especially in the utilization of *Camellia oleifera* flower resources.

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Keywords: Camellia oleifera plantation; Total flavonoids; Medicinal material indicators; Flavonoid metabolome; Metabolic pathways

*Contact information: a: College of Agriculture/Institute of New Rural Development, Guizhou University, Guiyang, China; b: Provincial Key Laboratory of Mountainous Ecological Environment, Guizhou Normal University, Guiyang, China; *Corresponding author: swyang@gzu.edu.cn; quanwenxuan@qq.com*

INTRODUCTION

Camellia oleifera Abel is an important woody oil plant in China, and its fruits accumulate large amounts of flavonoids and fatty acids (Xin *et al.* 2015; Ye *et al.* 2021). *Camellia oleifera* is cultivated mainly to extract high-nutrient edible oil, and the annual yield of *Camellia oleifera* seeds reaches 1 million tons (Zong *et al.* 2016), providing great economic benefits to farmers. *Camellia oleifera* has high medicinal value and a special aroma and is rich in nutritionally active ingredients (Yu *et al.* 2020; Geng *et al.* 2022). Most plants contain flavonoids in their bodies (Raskin *et al.* 2002). Flavonoids have antioxidant, anticancer, cardiovascular protection, and immune enhancement effects (Calis *et al.* 2020; Xiang *et al.* 2022). They also play a certain role in regulating plant root growth, promoting pollen affinity, and preventing and treating anthrax (Lan *et al.* 2017; Tan *et al.*

2019; Yang *et al.* 2022). At present, many flavonoid-related compounds, mainly flavonoids, flavonols, isoflavones, and anthocyanins, have been identified (Wu *et al.* 2018). Moreover, flavonoids also have immunomodulatory effects, assisting in the development of functional foods (Lu *et al.* 2016; Xiao *et al.* 2016; Hong *et al.* 2022).

At present, research on the medicinal value of *Camellia oleifera* flowers is relatively limited. *Camellia* leaves contain abundant flavonoids, which have potential application value in medicine, health food, and food additives (Wang *et al.* 2019). The differences in plantations affect the characteristic components of medicinal materials, and conducting research on the flavonoid components of *Camellia oleifera* flowers can help compare the quality of medicinal materials in different plantations. This study compares and analyzes the medicinal indicators and flavonoid metabolism pathways of *Camellia oleifera* flowers, thus providing a basis for their development and utilization.

EXPERIMENTAL

Research Site

Camellia oleifera flowers were obtained from six plantations: Yuping County (YP), Liping County (LP), Sansui County (SS), Songtao County (ST), Bijiang District (BJ), and Tianzhu County (TZ). Starting in mid-November 2022, random selection of *Camellia oleifera* flower samples with initial buds was conducted. From November 15 to November 20, 2022, three samples were taken from each plantation and dried in the shade.

Determination of the Medicinal Material Indicators of *Camellia oleifera* Flowers

The muffle furnace dry ashing technique was used to determine the total ash content (TAC) of various 18 samples (Islam *et al.* 2023). The water content (WC) was measured by drying and weighing the samples in a convection oven at 105 °C ± 1 °C for 3 h (Roby *et al.* 2020). Ethanol soluble extract (ESE) of 18 samples were extracted using ethanol, and the dried flower powder was filled into a thimble, stored in a Soxhlet extractor, and extracted with ethanol. The ethanol extract was concentrated by evaporating the solvent, and the solids were weighed (Dinakaran *et al.* 2018). The total flavonoid content (TFC) was determined using the aluminum trichloride colorimetric method, and the TFC was quantified from the calibration curve based on a reference flavonoid standard (rutin) measured under the same experimental conditions and at the same wavelength (Suksathan *et al.* 2021; Zhang *et al.* 2022).

Determination of Flavonoid Metabolites Widely Targeted by *Camellia oleifera* Flowers

The samples were placed in a vacuum freeze-drying machine (Scientz-100F) and freeze-dried. The sample was ground with a grinder (Retsch MM 400, Retsch) at 30 Hz for 1.5 minutes until it became a powder. Fifty milligrams of the sample was weighed, and 1200 microliters of 70% methanol aqueous internal standard extraction solution whose temperature was -20 ℃ were added. At least 50 mg of the extraction agent was added, and the mixture was vortexed 6 times, once every 0.5 h, for 0.5 minutes each time. After centrifugation (10 000 rpm, 3 min), the upper clear liquid was aspirated, a microporous filter membrane (0.22 μm filter) was used, and the sample was stored in an injection bottle for UPLC-MS/MS analysis.

Conditions for Chromatography and Mass Spectrometry Collection

Metabolite profiling of flavonoids was performed as described in a previous study (Shi *et al*. 2021) by Metware Biotechnology Co., Ltd. (Wuhan, China). Ultra-performance liquid chromatography (UPLC) (ExionLCTMAD, https://sciex.com.cn/); tandem mass spectrometry (MS/MS); chromatographic column: Agilent SB-C18 (0.1 m × 2.1 mm, 1.8 µm). The mobile phase consisted of mobile phase A, which was water containing 0.1% formic acid, and mobile phase B, which was acetonitrile containing 0.1% formic acid. The qualitative analysis of metabolites was based on the Metware database and the public database of metabolite information. Secondary spectral information was also used for the qualitative analysis of substances. The quantitative analysis of metabolites was based on the multi reaction monitoring (MRM) mode of triple quadrupole mass spectrometry.

Screening of Differential Cumulative Metabolites

Orthogonal partial least squares discriminant analyze (OPLS-DA) of total metabolites between different groups of samples and metabolites within different groups of samples was performed to study the differences in flavonoid metabolites in *Camellia oleifera* samples from six different plantations. The screening criteria for differentially abundant metabolites were variable importance projection (VIP) > 1 and a significance threshold of $P < 0.05$.

Data Processing

Single factor analysis of variance (ANOVA) was used to analyse the differences between different plantations of medicinal herbs. Multiple bar charts were drawn using the R package (version 3.5.1). LSD was used to test the differences between different plantations, with a significance level of 0.05. The clustering diagram was generated with ComplexHeatmap 2.12.0 from R software (version 4.2.0) to normalize the data.

The R software package was used to perform principal component analysis (PCA) and OPLS-DA on filtered and processed data, to identify differentially abundant metabolites, and to perform cluster analysis on the heatmap package to characterize the accumulation patterns of different groups of metabolites. The z score was used to normalize the data. Functional annotation and enrichment analysis of metabolites whose contents significantly differed were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto 2000).

RESULTS AND DISCUSSION

Medicinal Material Indicators of *Camellia oleifera* at Different Plantations

The contents of ethanol-soluble extracts in the *Camellia oleifera* flower samples from Liping County and Tianzhu County were the highest, and there was a significant difference ($P < 0.05$) compared with that in the samples from the other four plantations. The ash content of the *Camellia oleifera* samples from Songtao County was the lowest, and there was a significant difference ($P < 0.05$) compared with that of the samples from the other five plantations. There were significant differences ($P < 0.05$) in the total flavonoid content of the *Camellia oleifera* flower samples from the 6 plantations. The plantations with the highest to lowest contents were Tianzhu County, Sansui County, Bijiang District, Songtao County, Yuping County, and Liping County. The water content of the *Camellia oleifera* flower samples from the 6 plantations ranged from low to high in

Tianzhu County, Liping County, Bijiang District, Songtao County, Sansui County, and Yuping County, with no significant differences among the samples (Fig. 1a).

An analysis of the clustering heatmaps of the four medicinal material indicators revealed that the *Camellia oleifera* flowers in Tianzhu County and Songtao County were grouped together, whereas those in the other four plantations (Bijiang District, Liping County, Sansui County, and Yuping County) were grouped together (Fig. 1b). The contents of the four indicators within the same group were similar; there were significant differences between the different groups ($P < 0.05$).

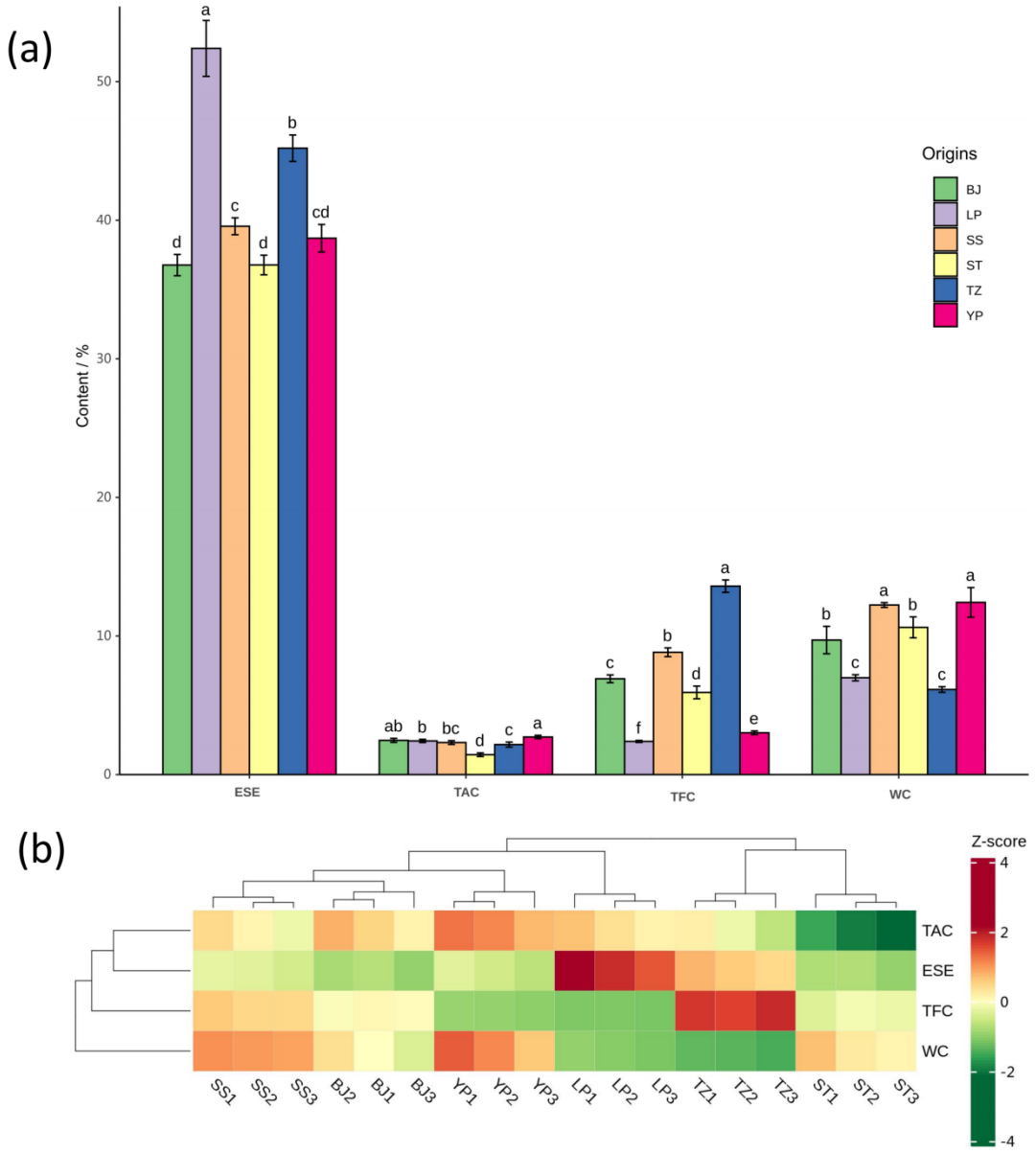

Fig. 1. Multiple bar charts of different medicinal material indicators (a) and cluster heatmaps of different medicinal material indicators (b) in different plantations of *Camellia oleifera*

Analysis of Flavonoid Metabolites in Different Plantations

On the basis of the UPLC-MS/MS detection platform, a wide target metabolite analysis was conducted on flavonoids in *Camellia oleifera* flower samples from six

plantations. A total of 758 flavonoid metabolites were identified in 13 categories, and flavonols, flavones, tannins, and flavanols were the main metabolites. According to the hierarchical clustering heatmap analysis, the samples from the 6 plantations were divided into 2 groups, with TZ, SS, and LP as one group and ST, BJ, and YP as one group. There was a significant difference between the two groups, indicating a significant difference in the accumulation of flavonoids in the *Camellia oleifera* flower samples (Fig. 2).

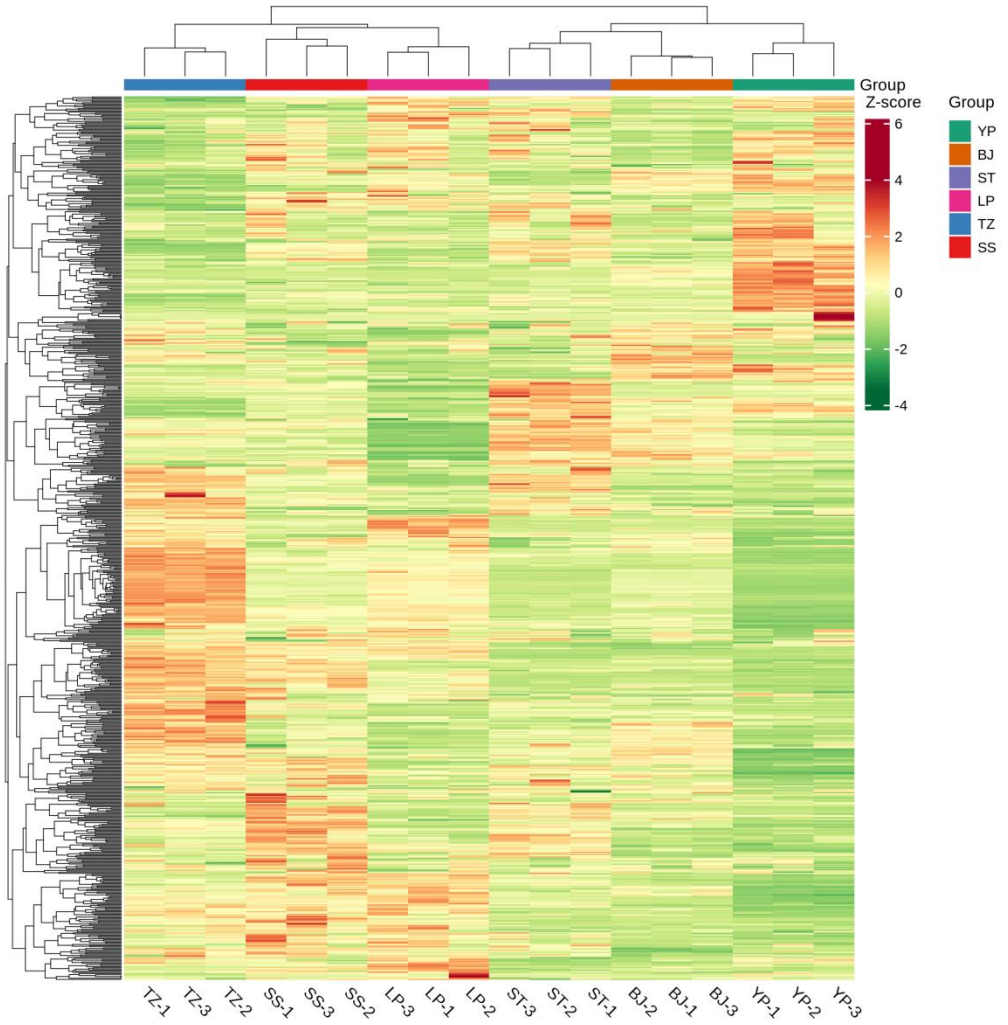

Fig. 2. Cluster analysis of total flavonoid metabolites in *Camellia oleifera* samples from different plantations

PCA and OPLS-DA of Flavonoid Metabolites

The PCA results revealed that three samples within the six planting groups clustered well, whereas there was a clear separation trend between the groups. The cumulative contribution rate of the first two principal components was 49.50%, with principal component 1 (PC1) contributing 33.36% and principal component 2 (PC2) contributing 16.14%. The intergroup samples tended to separate, and the biological reproducibility within the group was good. The PCA results reflected the overall differences in flavonoid metabolites among the six plantation samples (Fig. 3a). On the basis of the OPLS-DA results, the discrimination effect between sample groups from different plantations was significant (Fig. 3b). The OPLS-DA results show that the

horizontal axis represents the predicted principal component with a contribution rate of 11.50%, and the vertical axis represents the orthogonal principal component with a contribution rate of 32.10%.

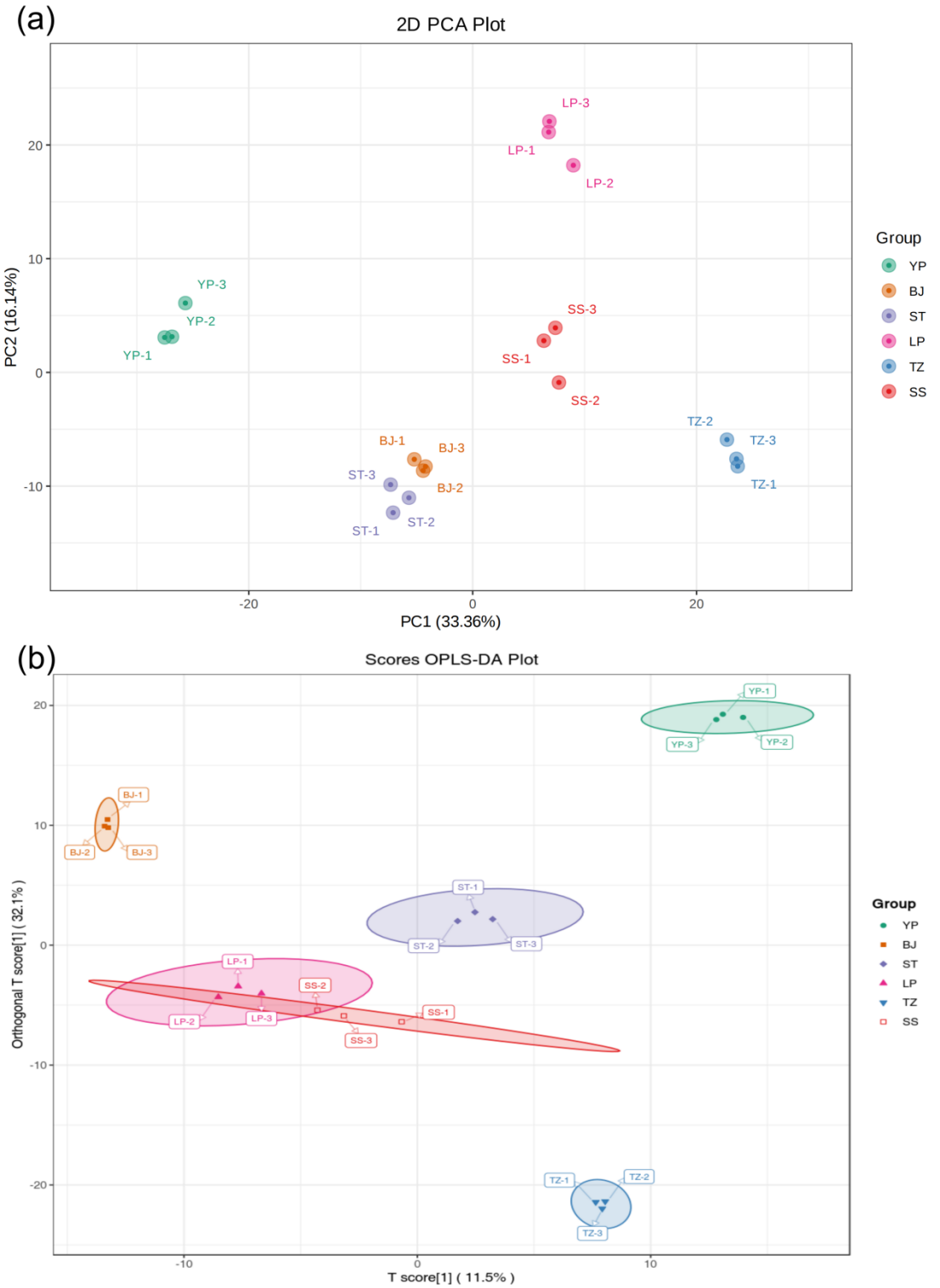

Fig. 3. 2D PCA (a) and OPLS-DA (b) score charts of flavonoid metabolites in *Camellia oleifera* flower samples from different plantations

Analysis of Differentially Abundant Metabolites among the Flavonoid Metabolites

The screening criteria were a P value < 0.05 and a VIP > 1. A total of 266 differentially abundant metabolites were identified from the samples from the 6 plantations, accounting for approximately 35.09% of the total flavonoid metabolites identified. Interestingly, only one identified proanthocyanidin was not a differentially abundant metabolite, and the substance with the lowest content in most samples was 6,8-diprenylnaringenin (except for YP). Quercetin-3-O-arabinoside, morin-3-O-xyloside, and morin-3-O-lesuvoside exhibited increased accumulation in the ST, BJ, SS, TZ, and LP groups. In the YP group, quercetin-3-O-arabinoside, morin-3-O-luteolin, and kaempferol-3-O-neohesperidin exhibited increased accumulation. Notably, the four substances mentioned above are flavanols (Fig. 4a).

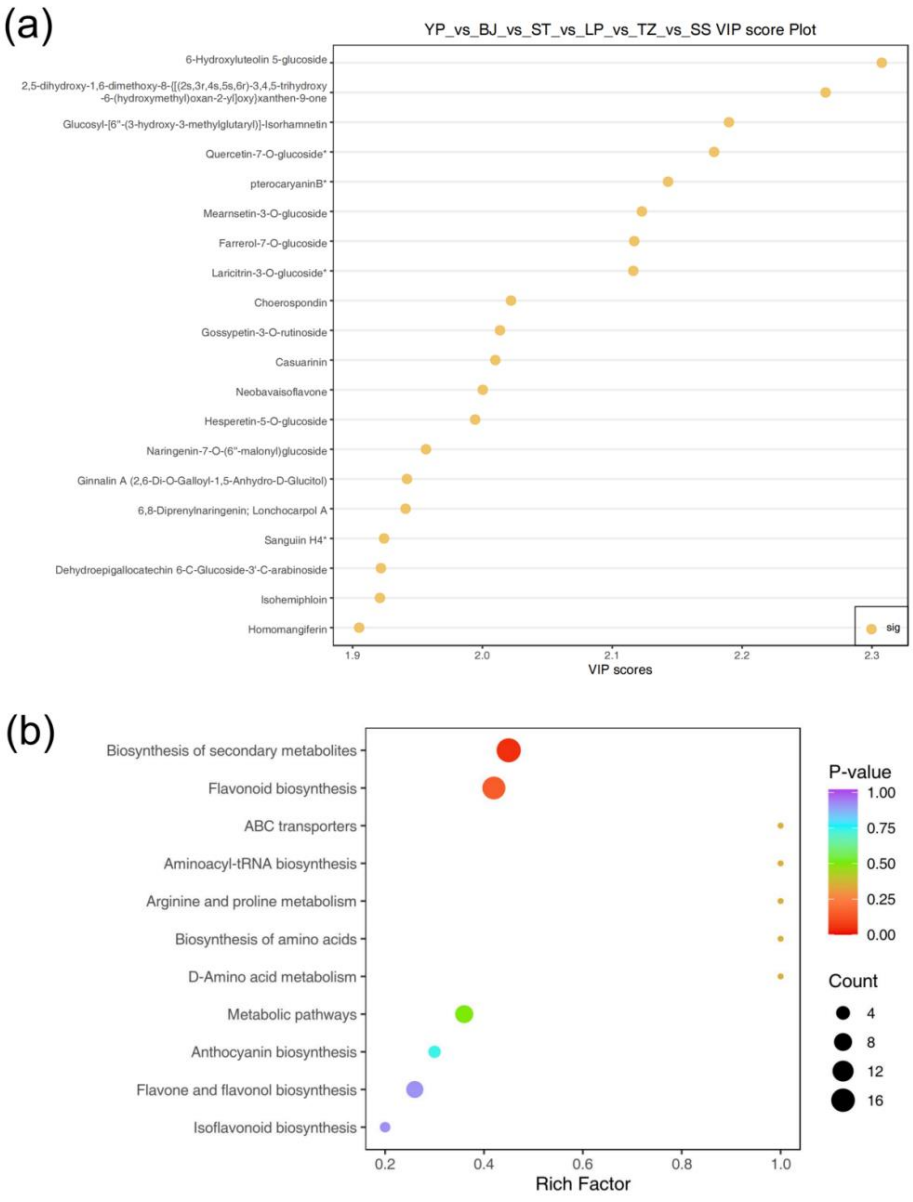

Fig. 4. VIP value diagram (a) and pathway enrichment diagram (b) of different metabolites in *Camellia oleifera* flower samples from different plantations

KEGG enrichment analysis revealed that the following KEGG pathways were enriched with differentially accumulated metabolites in *Camellia oleifera* flower samples from the six plantations: biosynthesis of secondary metabolites, flavonoid biosynthesis, metabolic pathways, flavone and flavonol biosynthesis, isoflavonoid biosynthesis, anthocyanin biosynthesis, biosynthesis of amino acids, and arginine and proline metabolism (Fig. 4b).

KEGG Metabolic Pathway Analysis of the Differentially Abundant Metabolites of Flavonoids

The KEGG metabolic pathways of 266 differentially abundant metabolites were annotated to further understand the biochemical pathways to which they belong. Among the 6 plantations, the enrichment pathways related to differential flavonoid metabolites mainly included biosynthesis of secondary metabolites, flavonoid biosynthesis, metabolic pathways, flavonoid and flavonol biosynthesis, isoflavone biosynthesis, anthocyanin biosynthesis, arginine and proline metabolism, aminoacyl-tRNA biosynthesis, ABC transporter protein, d-amino acid metabolism, and biosynthesis of amino acids (Fig. 5).

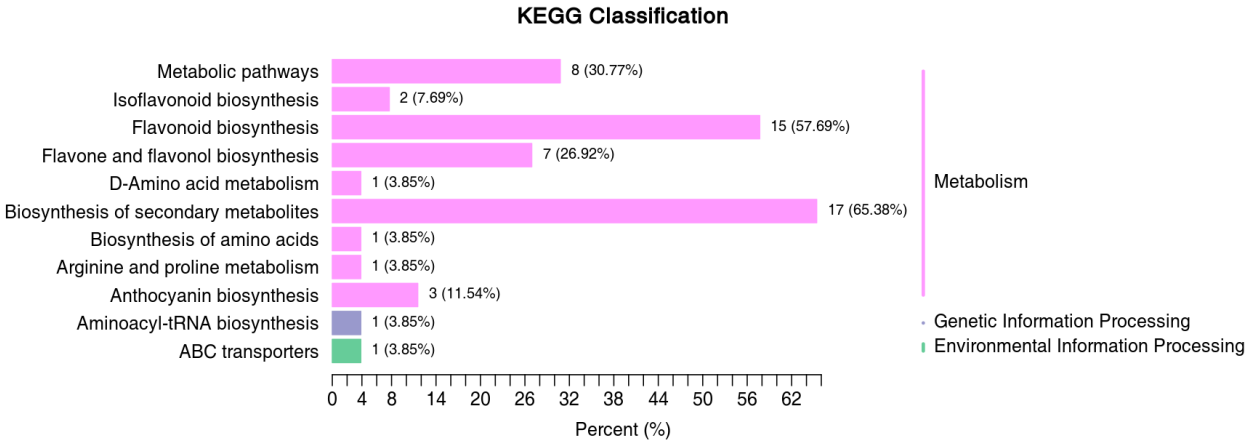

Fig. 5. KEGG classification diagram of differentially abundant flavonoid metabolite pathways

Five significantly enriched KEGG metabolic pathways were selected, pathways with fewer than 5 differentially abundant metabolites were excluded, and cluster analysis was performed on all the differentially abundant metabolites in the remaining 2 pathways (Fig. 6 a, b). In these two pathways, the *Camellia oleifera* samples from the six plantations were divided into four groups, with YP being one group, TZ being one group, BJ and LP being one group, and SS and ST being one group. Metabolites can also be divided into four groups, with the most differentially abundant metabolites accumulating in the TZ. Myricetin, philoretin, philoretin-2'-O-glucoside (philorizin), gallocatechin, epigallocatechin, leucocyanidin, leucocyanidin,3,4,5,7,3',4'-hexahydroxyflavan, catechin, afzelechin (3,5,7,4'-tetrahydroxyflavan), epicatechin, 2',4,4'-tetrahydroxychalcone, quercetin, *etc.*, all accumulate in these two pathways, but their accumulation is not consistent. It is proposed that these substances may be important among flavonoid metabolites. Surprisingly, there were more types of flavanols than flavonols, possibly because flavanols play a more important role in these two pathways.

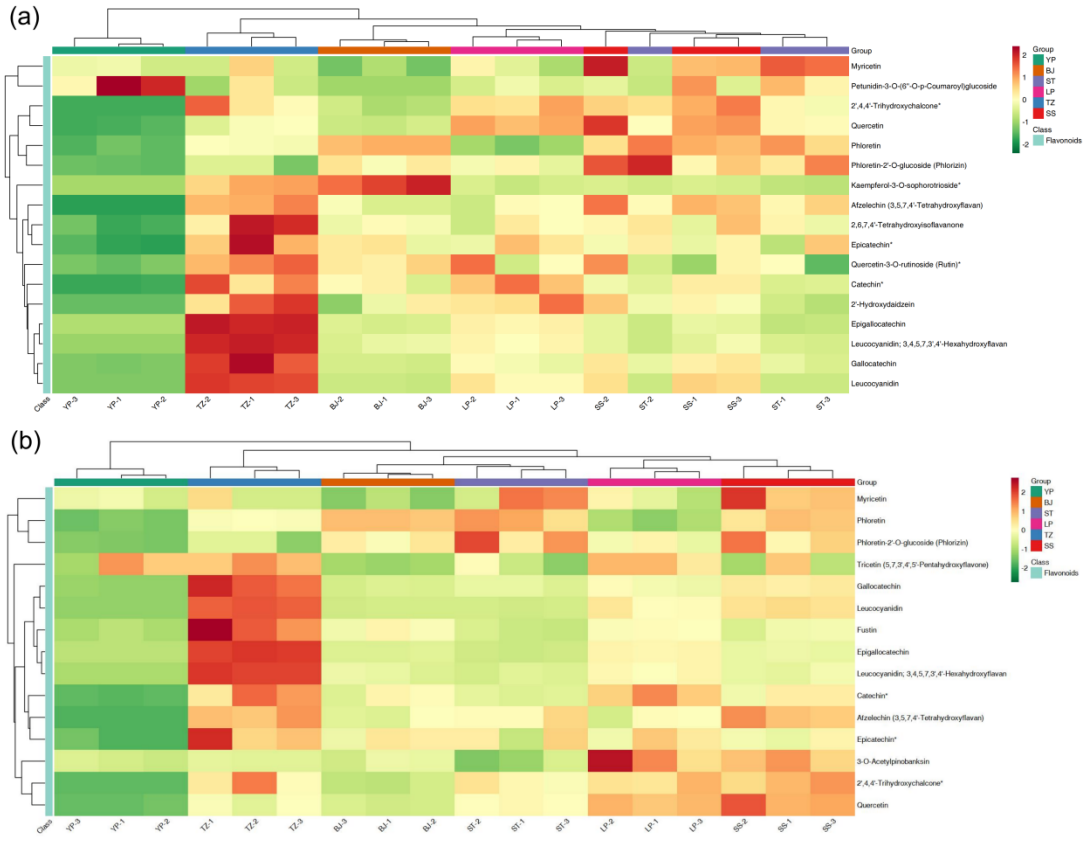

Fig. 6. Cluster heatmap of differentially abundant secondary metabolite (a) and flavonoid (b) pathways in *Camellia oleifera* samples from different plantations

Discussion

Flavonoids have attracted much attention because of their anti-atherosclerosis and antioxidant effects (Fabjan *et al.* 2003; Anandhi *et al.* 2014). In this study, there were significant differences in total flavonoid content among different plantations, with *Camellia oleifera* having a relatively high total flavonoid content, which is greater than that of traditional flavonoid medicinal plants in Thailand and Greece (Kakouri *et al.* 2023; Tiranakwit *et al.* 2023). In view of these findings, further information can be explored on flavonoids to better understand the characteristics and differences of flavonoid metabolites in *Camellia oleifera*.

This study explored the relative contents and differential flavonoid compound characteristics and metabolic pathways of *Camellia oleifera* from different plantations. Anthocyanins are present in the greatest quantities and play a role in antioxidant activity and cancer cell inhibition (Wang and Jiao, 2000; Wei *et al.* 2020). The most common types of metabolic pathway annotations were flavonols and flavanols, among which myricetin, philoretin, philoretin-2'-O-glucoside (Philorizin), gallocatechin, epigallocatechin, leucocyanidin, and leucocyanidin; 3,4,5,7,3',4'-hexahydroxyflavan, catechin, afzelechin (3,5,7,4'-tetrahydroxyflavan), epicatechin, 2',4'-tetrahydroxychalcone, and quercetin are metabolites shared by both pathways. One thing that cannot be ignored is that although fewer types of chalcones were screened, the number of metabolic pathways involved was relatively large. It is speculated that flavanols, anthocyanins, chalcones, and flavonols may be important substances that promote the effectiveness of *Camellia oleifera* flowers. Coincidentally, clustering heatmaps revealed that most of the substances accumulated in

the TZ sample. Previous studies have shown that flavanols have antioxidant properties that reduce the negative impact on vascular function by reducing mental stress (Luo *et al.* 2017; Baynham *et al.* 2021). Previous studies have noted that quercetin and its derivatives have biological functions, particularly antioxidant and anti-inflammatory activities (Tang *et al.* 2015).

In addition to the flavonoid synthesis pathway, there are two main pathways involved in arginine and proline metabolism, as well as D-amino acid metabolism. Amino acids also play a role in the stress response of *Camellia oleifera* (Bowne *et al.* 2012; Maeda and Dudareva 2012). The above results provide a theoretical basis for the effective development and rational utilization of *Camellia oleifera* and provide meaningful data for exploring the medicinal value of *Camellia oleifera* in related fields.

CONCLUSIONS

1. There were significant differences in the soluble alcohol extracts and total flavonoid contents of *Camellia oleifera* flowers from different plantations. Widely targeted metabolomics was able to identify the main components of flavonoids, with a total of 13 categories and 758 flavonoid metabolites detected, among which flavonoids, flavonols, and tannins were the main flavonoid metabolites.
2. This study identified a total of 266 differentially abundant metabolites, and the enriched KEGG pathways of these differentially abundant metabolites included mainly biosynthesis of the secondary metabolites and flavonoid biosynthesis. This study focused on the differential characteristics and metabolic pathways of flavonoids in *Camellia oleifera* flowers from different plantations, improving our understanding of the metabolic mechanism of *Camellia oleifera* flowers and providing a theoretical reference for the subsequent development and utilization of *Camellia oleifera* flower resources.

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